ADVANCES IN APPLIED MICROBIOLOGY

VOLUME 48

ACADEMIC PRESS

Biodegradation of Nitro-Substituted Explosives by White-Rot Fungi: A Mechanistic Approach

BENOÎT VAN AKEN
Department of Civil and Environmental Engineering
University of Iowa
Iowa City, Iowa 52242

SPIROS N. AGATHOS
Unit of Bioengineering
Université Catholique de Louvain
Louvain-la-Neuve, Belgium

I. Introduction

"Perhaps the easiest way to understand the non-specific ability of these [white-rot] fungi to degrade pollutants is to consider their ecological 'niche.' White-rot fungi are those organisms that are able to degrade lignin, the structural polymer found in woody plants (Barr and Aust, 1994)."

In 1985, a well-known ligninolytic white-rot fungus, *Phanerochaete chrysosporium,* was reported to mineralize into their mineral

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 48

0065-2164/01 $35.00

components a variety of toxic pollutants recalcitrant to biodegradation by other microbes, e.g., dioxins, polychlorinated biphenyls (PCB), organochlorine compounds, and polyaromatic hydrocarbons (PAH) (Bumpus *et al.*, 1985, Aust, 1990). Since that time, an ever-increasing number of research works have been published, focusing on the exceptional bioremediation capacities of white-rot fungi and their potential use in environmental biotechnologies.

White-rot fungi are ligninolytic organisms, i.e., able to degrade lignin, a major constituent of wood. Lignin is a complex, insoluble, and randomly structured biopolymer, usually resistant to biological attack (Buswell and Odier, 1987). White-rot fungi produce powerful extracellular and *nonspecific* ligninolytic enzymes, i.e., lignin peroxidases (LiP), manganese-dependent peroxidases (MnP), and laccases, which allow them to break down a wide range of xenobiotic organic ecopollutants, including the nitro-substituted explosives 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Barr and Aust, 1994).

Best known for their explosive properties, nitroaromatic and nitroheterocyclic explosives such as TNT, RDX, and HMX are also harmful chemicals constituting a serious biological hazard for water and soils. Throughout most of the 20th century, production, use and destruction of ammunition stocks have led to an increasing contamination of military and industrial sites worldwide (Spain, 2000). TNT, RDX, and HMX are toxic for living organisms and usually recalcitrant to microbial degradation (Smock *et al.*, 1976, Won *et al.*, 1976).

Fernando and Aust (1991) reported the capacity of the white-rot fungus *Phanerochaete chrysosporium* to partially mineralize the nitro-substituted explosives TNT and RDX into CO_2 and water. Although almost every living organism is able to reduce the TNT molecule, only white-rot fungi are known to further metabolize the resulting nitrotoluidines (Bumpus and Tatarko, 1994). Fungal degradation of TNT is a two-stage process: in a first mycelium-dependent step, TNT is reduced stepwise into nitrotoluidines (Stahl and Aust, 1993a), which are supposed to be substrates for extracellular ligninolytic peroxidases, leading, in a second, oxidative step, to their partial mineralization. In contrast to TNT, whose degradation limiting step is the aromatic ring fission, a slight change in the molecular structure of the cyclic nitramines RDX or HMX lead to a fast ring cleavage, generating small nitrogen- or carbon-containing molecules harmless for the environment (Hawari, 2000). White-rot fungi have been shown to mineralize RDX and HMX, likely *via* mechanisms involving either extracellular ligninolytic enzymes or intracellular enzymes (Fernando and Aust, 1991).

Early on, ligninolytic enzymes were postulated to play a role in fungal xenobiotic degradation, but their actual involvement, as well

as their individual implication in the oxidative breakdown of TNT and its reduced metabolites remained unclear for a long time, until a few isolated research works reporting experiments with purified ligninolytic peroxidases provided some insight into the process (Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994; Hofrichter *et al.,* 1998a; Van Aken *et al.,* 1999a,b; Van Aken *et al.,* 2000b). While LiP was initially considered as the major biocatalyst involved in lignin degradation by white-rot fungi, bioremediation experiments on xenobiotic pollutants have suggested the predominance of MnP, apparently the only ligninolytic enzyme able to mineralize TNT *in vitro* (Hofrichter *et al.,* 1998a). Moreover, novel observations lead one to consider MnP as the dominant enzyme involved not only in the degradation of xenobiotic compounds but also in the ligninolysis process: i.e., the high phenolic content of natural lignin (Buswell and Odier, 1987), the inability of MnP-negative fungi to significantly degrade lignin (Hatakka, 1994), and the MnP-mediated depolymerization of lignin in the presence of reduced thiols (Forrester *et al.,* 1988) or unsaturated fatty acids (Bao *et al.,* 1994).

Some progress has also been made in the identification of the numerous metabolites in the degradation mixtures (Hawari *et al.,* 1999), as well as in the determination of the (ultimate) oxidizing agents possibly responsible for molecular breakdown: e.g., veratryl alcohol cation radicals ($VA^{\bullet+}$) (Buswell and Odier, 1987), Mn(III) chelated by organic acids (Hofrichter *et al.,* 1998a), reactive oxygen species (ROS) (Barr and Aust, 1994), carbon-centered free radicals (Urzùa *et al.,* 1998), peroxyl free radicals (Moen and Hammel, 1994), and/or thiyl free radicals (Forrester *et al.,* 1988). Despite the extensive knowledge existing about the complex mechanism of fungal ligninolysis, little information is available about the mechanism(s) underlying the biodegradation of xenobiotic pollutants by ligninolytic fungi. Thus, the basis of such a nonspecific and powerful destructive biochemical system, which is unique in the living world, continues to intrigue most scientists and may have unsuspected implications outside the strict framework of environmental biotechnologies.

Whereas lignin and xenobiotic degradation by white-rot fungi has been for a long time directly correlated with the production of ligninolytic enzymes (Bumpus and Aust, 1987), some publications have reported nonligninolytic fungal degradations of xenobiotic pollutants, including PAH (Yadav and Reddy, 1993), TNT (Spiker *et al.,* 1992), and RDX (Fernando and Aust, 1991), suggesting the involvement of intracellular enzymes, e.g., cytochrome P-450 monooxygenases, besides the extracellular ligninolytic peroxidases.

Because lignin breakdown and xenobiotic degradation by white-rot fungi are biochemically closely related, this review makes an attempt

to bring together the most essential mechanistic features underlying the fungal biotransformation of xenobiotics by focusing first on the process of lignin degradation (Section II). In the next section, the involvement of low-molecular-weight mediators and radical species, likely playing a central role in peroxidase-mediated transformations of organic molecules, is discussed (Section III). Finally, the review will cover the current knowledge on the biodegradation of nitro-substituted explosives by white-rot fungi (Section IV).

II. Biodegradation of Lignin by White-Rot Fungi

"Growing evidence indicates that the complex plant-polymer [lignin] is biodegraded by a unique enzymatic 'combustion,' i.e. a non-specific enzyme-catalyzed burning (Kirk and Farrel, 1987)."

Lignin breakdown mechanisms (i.e., ligninolysis) have been studied intensively over the last 2 decades because of interest in emerging alternative biotechnologies, such as wood biopulping, and bioremediation of environmental pollutants (Messner *et al.*, 1997). Primarily microbiological aspects of lignin degradation have been reviewed by Hall (1980), Kirk and Shimada (1985), Buswell and Odier (1987), Kirk and Farrel (1987), de Jong *et al.* (1994), Garg and Modi (1999), and Leonowicz *et al.* (1999).

A. Lignin

"Lignin biodegradation is central to the earth's carbon cycle because lignin is second only to cellulose in abundance and, perhaps more significantly, because lignin physically protects most of the world cellulose and hemicellulose from enzymatic hydrolysis (Kirk and Farrel, 1987)."

Lignin is the second (after cellulose) most abundant natural polymer on earth and the most abundant aromatic material, accounting for 20–30% of plant wood[1] (Kirk and Farrel, 1987). Wood, i.e., lignocellulose, is a partly crystalline structure in which cellulose, arranged in microfibers, is enveloped in a matrix of lignin and hemicellulose, performing a function similar to metal rods inside concrete. The structure of lignocellulose imparts solidity to cell walls, providing strength and rigidity to vascular plants (Leonowicz *et al.*, 1999).

Lignin is a water-insoluble, amorphous biopolymer characterized by a high molecular weight (600–1000 kDa) (Kirk and Farrel, 1987) and a high density of aromatic rings (Buswell and Odier, 1987). Lignin is

[1]The term "lignin" is derived from the Latin word "lignum," meaning wood (Leonowicz *et al.*, 1999).

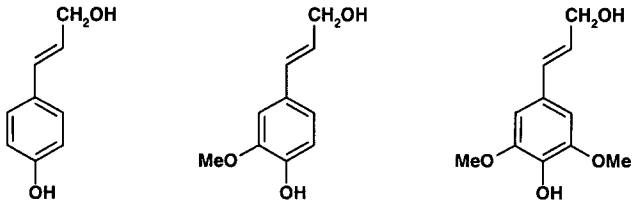

FIG. 1. Cinnamyl alcohols, precursors of lignin. Adapted from Buswell and Odier (1987).

synthesized by the random association of various phenyl propanoid subunits (cinnamyl alcohols; Fig. 1) linked by numerous carbon–carbon or ether bonds[2] (Fig. 2; Buswell and Odier, 1987, Kirk and Farrel, 1987). Lignin is therefore very resistant to biodegradation,[3] so that ligninolysis is restricted mostly to wood-rotting fungi (Hammel, 1992). The structure of lignin can be understood as a selective adaptation of vascular plants to resist against microbial degradation.

When associated with a lignin matrix, metabolizable polysaccharides of wood (i.e., cellulose and hemicellulose) are resistant to microbial breakdown (Leonowicz *et al.*, 1999). The main role of lignin biodegradation is not to provide any energy directly, but to give access to the more easily metabolizable (hemi)cellulosic material (Barr and Aust, 1994). Therefore, degradation of lignin is mainly a co-metabolic process, occurring only when a key nutrient is depleted from the growth medium (Hammel, 1992).

B. LIGNIN-DEGRADING (LIGNINOLYTIC) MICROORGANISMS

"In contrast to other [ligninolytic] fungi and bacteria, white-rot fungi are capable of completely degrading lignin to carbon dioxide and water (Cullen, 1997)."

Fungi, including yeasts and molds, and bacteria are able to modify lignin[4] (Garg and Modi, 1999). Wood-decaying fungi are classified

[2]Lignin biosynthesis arises from the random polymerization of free-radical species, generated mainly by the peroxidase- or laccase-catalyzed dehydrogenation of cinnamyl alcohols (Buswell and Odier, 1987).

[3]Lignin intermonomer linkages are resistant to the normal biochemical cleavage (i.e., hydrolysis).

[4]If higher organisms, such as xylophagic insects (e.g., termites), are known to digest lignin, this ability would be related either to their intestinal microflora or to the colonization of wood-rotting fungi (Kirk and Farrell, 1987).

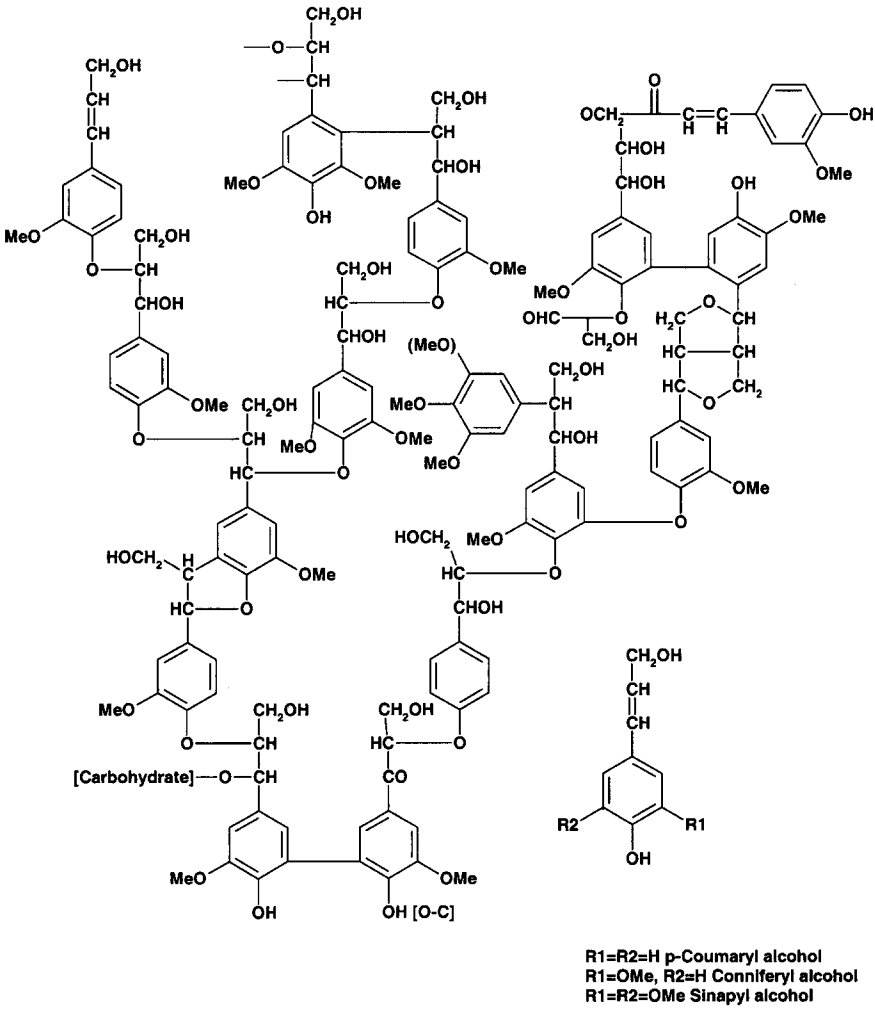

FIG. 2. Schematic structural model of lignin molecule. Adapted from Adler (1977).

into three groups: soft-rot, brown-rot, and white-rot fungi (Buswell and Odier, 1987).

1. Soft-Rot Fungi

A variety of molds (belonging to ascomycetes and Fungi Imperfecti), designated as soft-rot fungi, are able decompose all major components of wood, even though their attack on lignin is partial and restricted to certain substructures (Buswell and Odier, 1987; Garg and Modi, 1999). Their action results in a softening and a weight loss of wood tissues.

2. Brown-Rot Fungi

Brown-rot fungi, including several species of basidiomycetes, mainly decompose polysaccharides in wood (i.e., cellulose and hemicellulose), with only a limited degradation of lignin (Garg and Modi, 1999). Autooxidation of *o*-diphenolic moieties, resulting from partial ligninolysis, is responsible for the brown color of rotten wood (Buswell and Odier, 1987).

3. White-Rot Fungi

The most widespread and the most active lignin degraders belong to white-rot fungi or closely related litter-decomposing fungi (Fritsche *et al.*, 2000). They include basidiomycetes and a few ascomycetes (Kirk and Farrell, 1987). White-rot fungi are the most effective at degrading lignin and are capable of mineralizing all the major wood components to CO_2 and water. Some species of white-rot fungi may exhibit a preference for hemicellulose and lignin degradation over cellulose[5] (Buswell and Odier, 1987). The white color of white-rotten wood is likely due to the transient fibrous cellulose structure remaining after removal of the hemicellulose and lignin matrix (Garg and Modi, 1999).

4. Bacteria

Degradation of natural polymeric lignin is limited to a few aerobic bacterial strains, mainly belonging to actinomycetes (Buswell and Odier, 1987; Kirk and Farrel, 1987). Ligninolytic bacteria modify lignin only partially, releasing CO_2 from methoxyl and propyl side groups of the polymer skeleton (Garg and Modi, 1999).

C. Fungal Wood-Decaying Enzymes

"Having a versatile machinery of enzymes, the white-rot fungi are able to attack directly the 'lignin barrier.' They also use a multienzyme system including so-called 'feedback' type enzymes, allowing for simultaneous transformation of both lignin and cellulose. These enzymes may function separately or cooperatively (Leonowicz et al., 1999)."

As lignin is intimately associated with other wood components (i.e., cellulose and hemicellulose), the ligninolysis process in wood-decaying fungi is incorporated in the degradation of wood, i.e., the lignocellulosic complex. Wood-decaying enzymes of white-rot fungi can be divided into three groups (Leonowicz *et al.*, 1999):

[5]The only recognized function of fungal ligninolysis is to free the access to more metabolizable substrates, i.e., cellulose and hemicellulose!

Enzymes attacking wood constituents directly: cellulose (e.g., glucanases), hardwood (e.g., xylanases) and softwood hemicellulose (e.g., mannanases), and lignin (e.g., lignin peroxidase (LiP), manganese (-dependent) peroxidase (MnP), laccases, dioxygenases) (Sections II.D.1 and II.D.5)

Enzymes which cooperate with the first group, supplying hydrogen peroxide, but which never attack wood components directly: e.g., superoxide dismutases (SOD) and glyoxal oxidases (GLO) (Section II.E)

Feedback enzymes which play a key role in wood biodegradation, connecting the cellulolytic and the ligninolytic metabolic chains (Leonowicz *et al.,* 1999): e.g., glucose oxidase, aryl alcohol oxidases (AAO), cellobiose:quinone oxidoreductase (CBQ), and cellobiose dehydrogenase (CDH) (Section II.F)

D. Ligninolytic Enzymes

"Apart from their importance in lignin biodegradation, these enzymes [LiP, MnP, and laccases] are the focus of intense research because of their potential applications in the detoxification of a broad range of environmental pollutants ..." (Reddy, 1995)

Oxidative enzymes directly involved in lignin breakdown, so-called ligninolytic enzymes (i.e., LiP, MnP, and laccases), catalyze the one-electron oxidation of phenolic and nonphenolic compounds leading to corresponding (phenoxy) cation free radicals. Laccase oxidizes only molecules with a relatively low reduction potential ($E_0 = 780$ mV; Kersten *et al.,* 1990), whereas peroxidases LiP and MnP can oxidize molecules with a higher reduction potential ($E_0 = 1100-1500$ mV; Kersten *et al.,* 1990; Schoemaker *et al.,* 1994). According to the structure of their natural substrate (i.e., polymeric lignin), peroxidases and laccase are remarkably nonspecific and can oxidize a wide range of different molecules (Barr and Aust, 1994; Thurston, 1994).

On the basis of the ligninolytic enzymes produced, three different categories of white-rot fungi may be distinguished (Hatakka, 1994): the *LiP–MnP* group (e.g., *Penicillium chrysosporium*)—the most efficient lignin degraders-, the *LiP–laccase* group, and the *MnP–laccase* group.

To date, most studies on fungal ligninolytic enzymes have focused on *P. chrysosporium.* However, the presence of comparable peroxidase enzyme systems in a number of other white-rot fungi has been reported: e.g., *Trametes versicolor, Bjerkandera adusta, Phlebia radiata* (Kantelinen *et al.,* 1988; Niku-Paavola *et al.,*1988, Johansson and

Nyman, 1993). To date, LiP and MnP identified in other white-rot fungi have been shown to be biochemically and biophysically closely related to the corresponding peroxidases of *P. chrysosporium* (Kantelinen *et al.*, 1988; Reddy and D'Souza, 1994; Cullen, 1997).

1. Peroxidases

Peroxidases are widely distributed enzymes among living organisms. There are three superfamilies of peroxidases: *plant peroxidases, animal peroxidases, and catalases* (Dunford, 1999). The "plant" superfamily is divided into three classes: class I, *intracellular prokaryotic peroxidases* (e.g., cytochrome C peroxidases); class II, *extracellular fungal peroxidases* (e.g., LiP and MnP); and class III, *secretory plant* peroxidases (e.g., horseradish peroxidase). From an extensive comparison between their amino acid sequences, it has been proposed that heme peroxidases from bacteria, fungi, and plants are evolutionarily related (Dunford, 1999). Peroxidases oxidize several substrates, using hydrogen peroxide as an electron acceptor, while the primary function of catalases is to catalyze the dismutation of H_2O_2 into dioxygen and water (*catalatic* reaction; Eq. (1):

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \tag{1}$$

Lignin-degrading peroxidases have been reviewed extensively [see, for instance, Buswell and Odier (1987), Kirk and Farrel (1987), Hammel (1992), Gold and Alic (1993), Ander and Marzullo (1997), Cullen (1997), and Garg and Modi (1999)].

a. Lignin Peroxidase (LiP). LiP (diarylpropane peroxidase, EC 1.11.1.14) is an extracellular lignin-degrading enzyme first discovered in submerged cultures of *P. chrysosporium* (Glenn *et al.*, 1983, Tien and Kirk, 1984). LiP can depolymerize the lignin molecule and oxidizes numerous mono- or oligomers resulting from lignin degradation (Hammel and Moen, 1991; de Jong *et al.*, 1994). Veratryl alcohol (VA), a typical substrate for LiP, is oxidized to veratryl alcohol cation radical ($VA^{\bullet+}$), which may act as a mediator in lignin breakdown. As a carrier of oxidizing equivalents, $VA^{\bullet+}$ can diffuse inside the bulky lignocellulosic structure, mostly inaccessible to enzyme molecules. LiP may also be involved in the production of reactive oxygen species (ROS) (Barr *et al.*, 1992). LiP is likely a key component for the degradation of both lignin and xenobiotic pollutants.

LiP is a monomeric *N*- and likely *O*-glycosylated heteroprotein containing one ferriprotoporphyrin IX as the prosthetic group (Section II.D.2.*a*). The pH optimum is unusually low (near 3.0), since it is

inversely related to the redox potential of the enzyme (Call and Mücke, 1997). LiP is produced as a set of closely related isozymes (molecular mass ranges from 38 to 43 kDa) encoded by different genes (Reddy and D'Souza, 1994).

b. Manganese (-Dependent) Peroxidase (MnP). MnP (manganese peroxidase, EC 1.11.1.13) is also an extracellular lignin-degrading enzyme discovered in cultures of *P. chrysosporium* (Kuawahara *et al.,* 1984; Glenn and Gold, 1985; Paszczynski *et al.,* 1986). MnP mainly catalyzes the oxidation of Mn(II) to Mn(III), which, when chelated by organic acids (e.g., oxalate or malonate), is a strong oxidant able to attack various phenolic substrates, including synthetic lignin substructures and xenobiotic pollutants (Glenn *et al.,* 1986; Wariishi *et al.,* 1989a,b; de Jong *et al.,* 1994). Just like LiP-generated $VA^{\bullet+}$, Mn(III) complex is a carrier of oxidizing equivalents susceptible to diffuse inside the bulky lignocellulosic polymer.

MnP is a monomeric *N*-glycosylated heteroprotein containing one ferriprotoporphyrin IX as the prosthetic group (Section II.D.2.*a*). This enzyme is also produced as a set of closely related isozymes (molecular masses around 46 kDa) encoded by different genes (Reddy and D'Souza, 1994).

Recently, versatile peroxidases exhibiting the catalytic properties of both LiP and MnP, i.e., VA and Mn(II) oxidizing activities, have been described in several white-rot fungi, including *P. chrysosporium* (H2 isozyme) (Khindaria *et al.,* 1995; Mester and Field, 1998; Camarero *et al.,* 1999).

2. Structural Properties of Peroxidases (Class II)

This section presents the main structural features of fungal peroxidases LiP and MnP (Class II), as obtained from X-ray crystal analysis (Piontek *et al.,* 1993; Poulos *et al.,* 1993; Sundaramoorthy *et al.,* 1994). LiP and MnP from the white-rot fungus *P. chrysosporium* share 43% amino acid sequence identity, suggesting considerable structure similarity (Sundaramoorthy *et al.,* 1994).

The active site of peroxidases is a protoporphyrin IX containing a high-spin, hexacoordinate iron ion (Fig. 3; Buswell and Odier, 1987). In the resting state, the ferric ion Fe(III) is in the oxidation state +3 and is coordinated to the four pyrrole nitrogens of the heme and to a nitrogen of an axial His173[6] (so-called proximal His). The sixth coordination remains free. The catalytic process occurs through a multistep sequence:

[6]MnP numbering (Sundaramoorthy *et al.,* 1994).

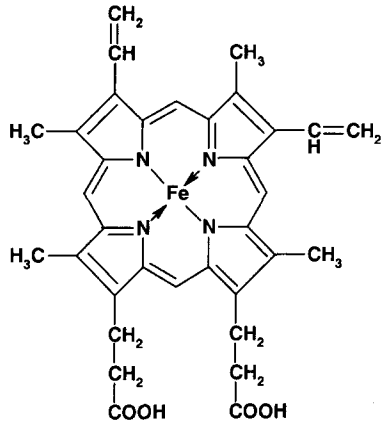

FIG. 3. Structure of ferriprotoporphyrin IX.

by reaction with H_2O_2, which is reduced to water, the protein undergoes a two-electron oxidation to give a compound I, containing an oxyferryl iron Fe(IV)=O and an organic radical cation located on the heme (porphyrin π-centered radical). Compound I oxidizes in turn one substrate molecule and is converted to compound II, where the porphyrin π radical cation is reduced to its resting state. Finally, through the oxidation of a second substrate molecule, compound II is reduced back to its resting Fe(III) state (Fig. 4; Sundaramoorthy *et al.*, 1994; Banci, 1997). The peroxidase catalytic cycle may be described as an irreversible ping-pong

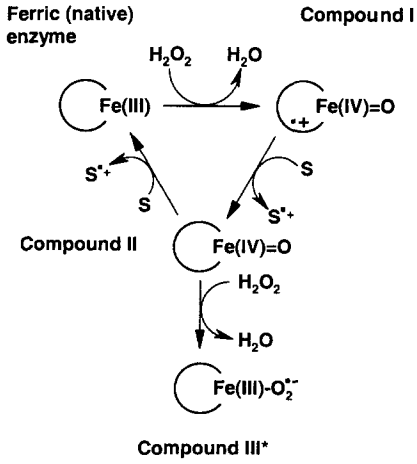

FIG. 4. Catalytic cycle of fungal peroxidases. Adapted from Tien (1987).

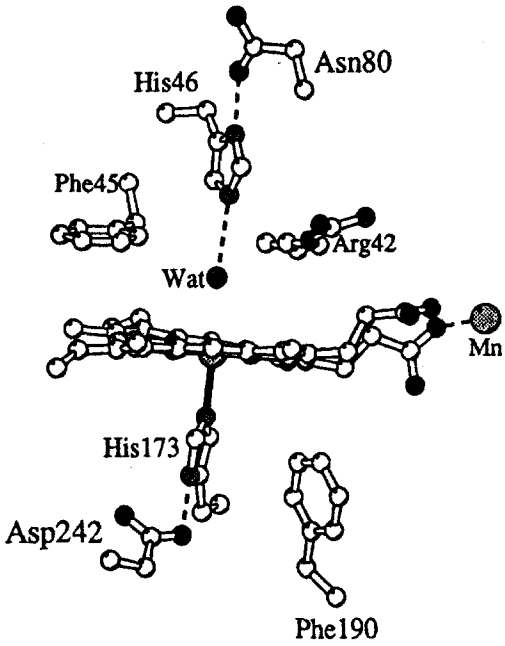

FIG. 5. Schematic structure of MnP, showing the helices as cylinders, the heme, the calcium ions, the Mn binding site, and the two *N*-acetylglucosamine residues *N*-linked to Asn^{131}. Amino acid numbering refers to MnP (Sundaramoorthy *et al.*, 1994).

mechanism[7]: one substrate (H_2O_2) binds, followed by a first product release. A modified enzyme (compound I) is the result, to which the second substrate binds, followed by a second product release. A third substrate binds the resulting modified enzyme (compound II), followed by the release of a third product (Dunford 1999). In the presence of a H_2O_2 excess and/or in the absence of a suitable substrate (e.g., VA or Mn(II)), compound II may be further oxidized to compound III*, containing a poorly reactive perferryl iron complex [$Fe(III)-O_2^{\bullet-} \leftrightarrow Fe(II)-O_2$], as observed in ferroxycatalase or oxyhaemoglobin (Fig. 4; Halliwell and Gutteridge, 1989). Compound III* is inactive and outside the peroxidase catalytic cycle (Wariishi and Gold, 1990).

a. General Structure. The enzyme possesses two different structural domains with the heme moiety sandwiched between them. The secondary structure involves 10–11 α-helices linked by loops and turns (Fig. 5; Sundaramoorthy *et al.*, 1994). Amino-acid residues supporting

[7]The conventional ping-pong mechanism is reversible. The major difference between the conventional ping-pong mechanism and the peroxidase ping-pong mechanism is that the former has a finite upper limit in rate, whereas the second has not (Dunford, 1999).

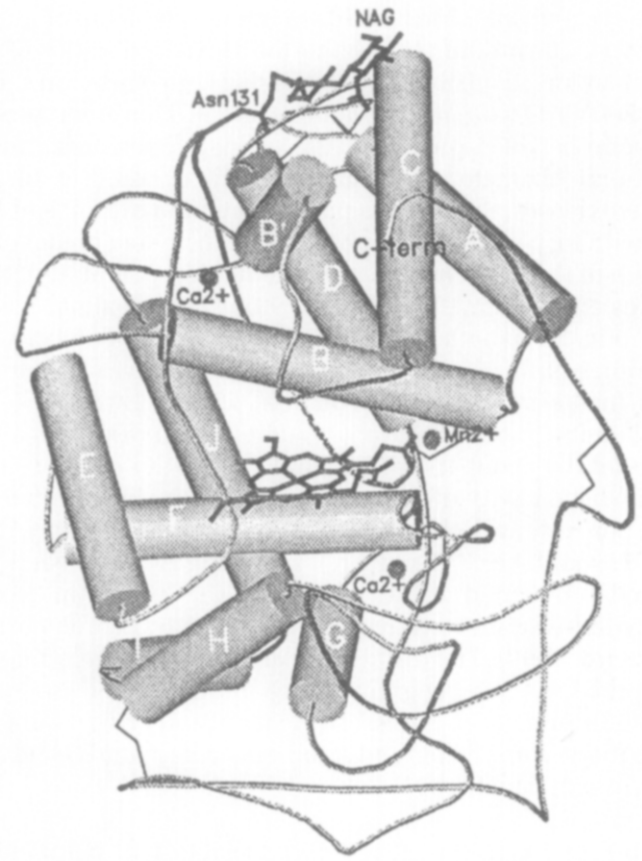

FIG. 6. The active site environment of MnP. Hydrogen bonds are represented by dashed lines. Amino acid numbering refers to MnP (Sundaramoorthy *et al.*, 1994).

the structural rigidity of the protein are highly conserved in LiP and MnP (as well as in other peroxidases): e.g., Gly and Pro residues (determining the correct backbone bending), Cys–Cys bridges (forming disulfide bonds). Two calcium ions ensure a high degree of rigidity to the active site, since they coordinate residues immediately following His^{173} (proximal His) and His^{46} (distal His) residues (Fig. 6; Sundaramoorthy *et al.*, 1994; Banci, 1997). Peroxidases exhibit high topological similarities, *a priori* in contrast to the wide range of substrates they can oxidize. In fact, the enzymes might not require significant structural changes to adapt to different substrates, since reactions might involve a long-range electron transfer from the substrate (at the protein surface) to the heme center (Banci, 1997).

b. Heme Environment. Most residues in the active site of peroxidases are conserved. The proximal His holds the fifth coordination of the heme iron and, therefore, contributes to stabilizing high oxidation states of the oxyferryl Fe(IV)=O iron in compounds I and II. This effect is reinforced by an H-bond between proximal His and Asp^{242} residues, imparting a greater anionic character to the proximal His ligand (Fig. 6). Within a class of peroxidases, the relative position and the distance of the proximal His with respect to the iron atom (and the heme plane) determine the strength of the Fe–N bond and the reduction potential of the active site (Poulos and Kraut, 1980; Banci, 1997). The proximal His belongs to helix F (Fig. 5). In both LiP and MnP, a strong H-bond between side-chain residues pulls helix F away from the heme, weakening the Fe–N bond and increasing the redox potential of the enzyme. The absence of this H-bond (e.g., in cytochrome C peroxidase) results in a lower redox potential (by about 100 mV) (Sundaramoorthy *et al.*, 1994; Banci, 1997). The distal cavity of the active center is the site of interaction of hydrogen peroxide, which involves two invariant amino-acid residues: the distal His and Arg^{42} (distal Arg). Incoming H_2O_2 transfers one proton to the deprotonated distal His, which is stabilized in this form by H-bonds with invariant residues (Figs. 6 and 7; Sundaramoorthy *et al.*, 1994; Dunford, 1999). The distal Arg stabilizes the developing negative charge on the OH^- leaving group. Once the O–O bond is cleaved, the Arg^{42} guanidinium group moves inside the distal cavity, forming an H-bond with the oxyferryl atom and stabilizing compounds I and II (Poulos and Kraut, 1980; Banci, 1997).

c. Substrate Binding Sites. The (low) specificity of peroxidases for a substrate is determined both by the reduction potential of the active site (i.e., the iron center and the heme cation radical) with respect to those of the substrates and by the presence of specific binding sites in

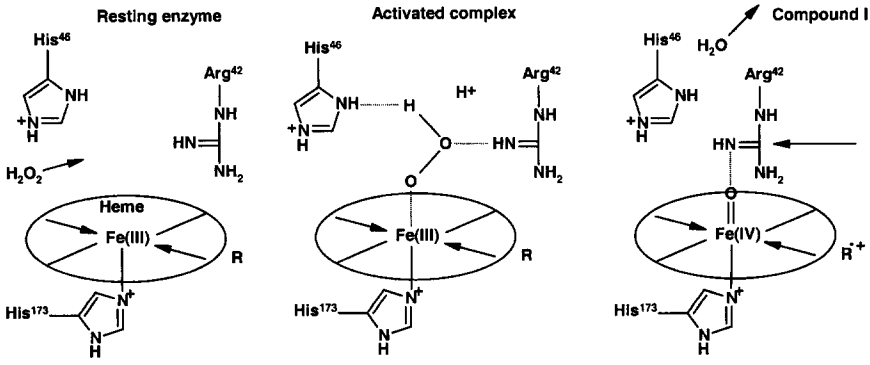

FIG. 7. Mechanism of peroxidase compound I formation. Adapted from Dunford (1999).

or on the protein structure. The specificity is modulated by a small number of amino-acid substitutions, slightly modifying the enzyme surface topology, but without significant changes of the main structure.

In MnP, the binding site of Mn(II) is located at the edge of the heme moiety. The electron could be transferred from Mn(II) to the iron atom or to the porphyrin periphery via a heme propionate, using a nearly continuous σ-bonded path (Sundaramoorthy *et al.,* 1994). Exogenous dicarboxylic acids (e.g., oxalate), known to stabilize Mn ions, are not part of the coordination environment of Mn(II) bound to the protein, suggesting that free Mn(II) is the substrate for MnP. In the presence of oxalate, a ligand displacement at the active site likely releases the oxalate molecule, since MnP binds Mn(II) more strongly than oxalate (Sundaramoorthy *et al.,* 1994, Banci, 1997).

In contrast, the VA binding site in LiP is still unknown, even though a possible place would be located near the open channel connecting the distal pocket to the enzyme surface and toward it is oriented the heme δ-*meso*-carbon (Fig. 3), so that an aromatic ring could contact the heme edge, transferring an electron to the porphyrin π-centered radical cation (Poulos *et al.,* 1993). The binding site alternatively could be located far away from the active site, a long-range electron transfer occurring through the protein molecule (Banci, 1997).

3. Molecular Genetics and Regulation of LiP and MnP Production

Heme peroxidases of *P. chrysosporium* constitute a large family of isozymes, separable by fast protein liquid chromatography (FPLC) from the extracellular fluid of ligninolytic cultures and arbitrarily designated as H1, H2, ... H10 (Kirk and Farrel, 1987). H1, H2, H6, H7, H8, and H10 exhibit a LiP activity, as assayed by the oxidation of VA to veratryl aldehyde (Tien and Kirk, 1984; Gold *et al.,* 1984). H3, H4, H5, and H9 exhibit a MnP activity, as assayed by the direct oxidation of Mn(II) to Mn(III)[8] (Glenn and Gold, 1985). Molecular biology aspects of *P. chrysosporium* have been reviewed in Gold and Alic (1993), Reddy and D'Souza (1994), Broda *et al.* (1996), Cullen and Kersten (1996), and Cullen (1997).

Extracellular ligninolytic peroxidases of most white-rot fungi are synthesized during secondary metabolism, under nitrogen and/or carbon starvation (Kirk *et al.,* 1978; Faison and Kirk, 1985). More generally, the ligninolytic system of white-rot fungi is produced in response to a stress situation, which may be induced by nutrient depletion, as well as by other environmental factors (Reddy and D'Souza, 1994). Mn(II) levels in the medium also exert a dramatic effect on the production of

[8]In addition to the VA-oxidizing activity, LiP isozyme H2 from *P. chrysosporium* has also been shown to exhibit a Mn(II)-oxidizing activity (Khindaria *et al.,* 1995). However, the initial name has been conserved for historical reasons.

peroxidases by white-rot fungi. When Mn(II) is absent, only high levels of LiP are observed; in the presence of 12 mg liter^{-1} Mn(II), both LiP and MnP are produced, while higher Mn(II) concentrations lead to the secretion of MnP alone (Bonnarme and Jeffries, 1990; Brown *et al.*, 1990). In addition, MnP expression may be enhanced by physiological levels of oxalate (Kuan *et al.*, 1993).

LiP secretion is stimulated by its favored substrate, i.e., VA, as well as by low-molecular-weight products resulting from lignin depolymerization (Faison and Kirk, 1985; Faison *et al.*, 1986).

Isolation of different deregulated mutants has shown that the nitrogen regulation of ligninolytic peroxidases is independent from the carbon regulation and that the Mn(II) regulation is independent from the nitrogen and carbon regulations (Van der Woude *et al.*, 1993). Generally speaking, production and abundance of different fungal peroxidases vary greatly with the fungal strain and the culture conditions, suggesting in turn that the expression of *lip* and *mnp* genes is regulated by environmental signals (Reddy and D'Souza, 1994; Cullen, 1997).

LiP isozymes of *P. chrysosporium* are encoded by a family of at least 10 closely related genes *lip*A, *lip*B, ... *lip*J (Gaskell and Cullen, 1993). Less is known about the *mnp* genes for which four distinct sequences have already been identified (Pease *et al.*, 1989).

4. Reactions Catalyzed by Fungal Peroxidases

Lignin is formed in plant cell walls by radical coupling reactions of different (poly)methoxy-substituted hydroxycinnamyl alcohols (Fig. 2). Numerous intramonomer linkages result, with aryl–glycerol-2–aryl ether (β-*O*-4) (50–60%) and 1,2-diaryl propane (β-1) (7%) being the most prominent (Fig. 8; Hammel, 1992).

Different experimental approaches suggest that the most important ligninolytic reaction is the oxidative cleavage of the lignin propyl side chain between α-β carbons in both β-*O*-4-aryl ether and β-1-diarylpropane substructures, as they could lead to an extensive depolymerization of natural lignin (Buswell and Odier, 1987). The first mechanistic studies of fungal ligninolysis revealed the ability of LiP, in contrast to MnP, to catalyze the cleavage of nonphenolic dimer models β-*O*-4 and β-1 (Glenn *et al.*, 1983; Tien and Kirk, 1983; Kuwahara *et al.*, 1984). MnP was thought to play a role in fungal ligninolysis, but in more typical peroxidative reactions, i.e., the oxidation of phenolic lignin substructures. Therefore, LiP-catalyzed reactions have been preferentially investigated and reviewed in the literature (Hall, 1980; Hammel *et al.*, 1985; Buswell and Odier, 1987; Kirk and Farrel, 1987; Garg and Modi, 1999). However, according to the following

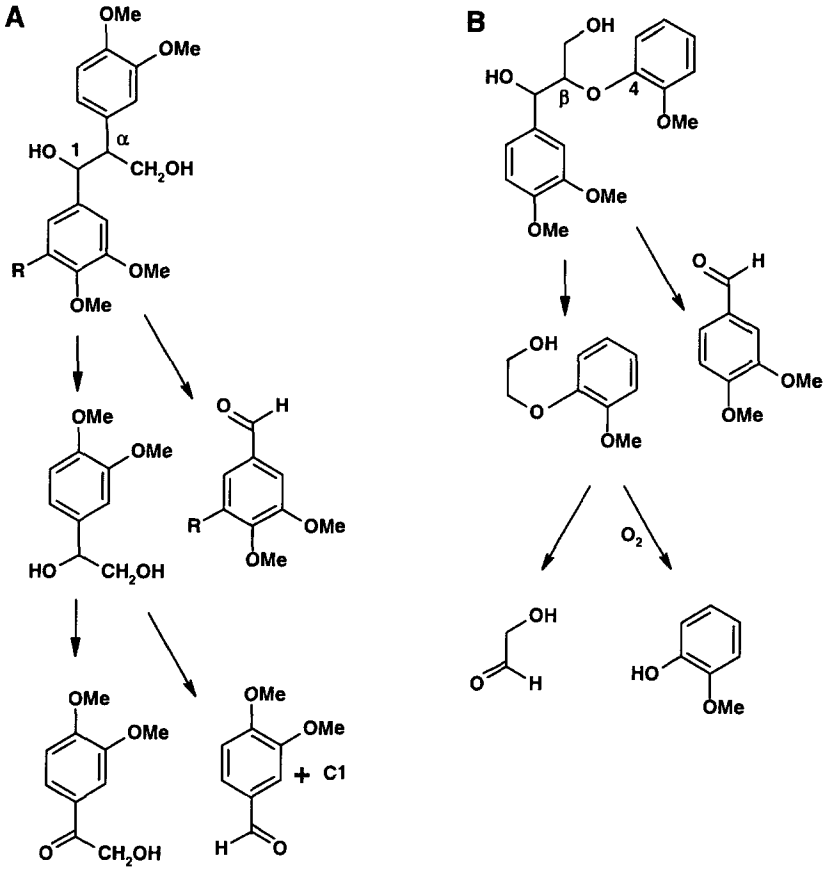

FIG. 8. (A) LiP-catalyzed cleavage of diarylpropane (*β*-**1**) dimer lignin model. (B) LiP-catalyzed cleavage of arylglycerol-*β*-aryl ether (*β*-*O*-4) dimer lignin model. Adapted from Buswell and Odier (1987).

considerations, MnP seems, at the present time, to be the decisive enzyme involved in the breakdown of both lignin and xenobiotic pollutants (Hatakka, 1994, Hofrichter *et al.*, 1998a):

- MnP catalyzes the oxidation of phenolic lignin substructures, which may lead to an extensive lignin degradation, as they account for 5–15% of natural polymeric lignin (Buswell and Odier, 1987).
- In the presence of mediators, such as reduced thiols (Forrester *et al.*, 1988) or unsaturated fatty acids (Bao *et al.*, 1994), MnP is also able to cleave nonphenolic lignin dimer models (see following).
- White-rot fungi unable to produce MnP (LiP–laccase group) are known to be poor lignin degraders, while fungi producing MnP and

possibly laccase, but no LiP (MnP–laccase group), are efficient white-rotters (Hatakka, 1994).

LiP catalyzes C_α–C_β bond cleavage of β-1 dimer models, yielding an aromatic aldehyde from the C_α and a phenylglycol product from the C_β, which undergoes a further LiP-catalyzed intradiol cleavage (Fig. 8A; Glenn *et al.*, 1983; Tien and Kirk, 1983; Gold *et al.*, 1984, Renganathan *et al.*, 1986). C_α–C_β bond cleavage of β-*O*-4 dimer models by LiP yields aromatic aldehydes (e.g., veratryl aldehyde) from the C_α moieties, while no C_β products are recovered, suggesting a further decomposition to glycolaldehyde and guaiacol (Fig. 8B; Glenn *et al.*, 1983; Tien and Kirk, 1983; Gold *et al.*, 1984; Renganathan *et al.*, 1986). C_α–C_β bond cleavage of β-*O*-4 models may alternatively result in the formation of a hydroxy-substituted benzyl radical at the C_α position and of a positively charged C_β-fragment (Lundell *et al.*, 1993). The major low-molecular-weight degradation products from partially white-rotten lignin have been identified as vanillin, syringaldazine, coniferyl aldehyde, as well as all their corresponding benzoic acids, confirming the importance of aryl side-chain cleavages (Chua *et al.*, 1982; Chen and Chang, 1985). Cleavage of the β-aryl ether bond, oxidative aromatic ring opening, and demethylation are also reactions catalyzed by LiP (Chen *et al.*, 1982; Kirk and Farrel, 1987). Besides the oxidation of phenolic substrates to phenoxy radicals, for which LiP is not different from other peroxidases, the enzyme catalyzes the oxidation of a wide range of nonphenolic aromatic molecules to give transient aryl cation radicals whose fate depends on the nature of the substrate: e.g., polyalkoxy-substituted benzenes (Kersten *et al.*, 1985) and alkoxy-substituted benzyl alcohols (Tien and Kirk, 1983; Tien *et al.*, 1986).

In contrast to LiP, MnP is unable to achieve the oxidation of nonphenolic aromatic substrates, nor the cleavage of synthetic nonphenolic lignin model compounds, nor the depolymerization of natural lignin (Forrester *et al.*, 1988). However, MnP, through the oxidation of Mn(II) to Mn(III) as well as chelated Mn(III), catalyzes the *in vitro* oxidation of several lignin-related phenolic monomers (Glenn and Gold, 1985; Glenn *et al.*, 1986, Paszczynski *et al.*, 1986) and the cleavage of phenolic diarylpropane β-1 lignin model dimers (Wariishi *et al.*, 1989a,b). In the presence of reduced thiols, such as glutathione (GSH), MnP/Mn(II) or chelated Mn(III) has been shown to oxidize nonphenolic substrates (e.g., VA) and to cleave nonphenolic β-*O*-4 lignin model compounds (Forrester *et al.*, 1988; Wariishi *et al.*, 1989c).[9] MnP also catalyzes lipid peroxidation of

[9]Reduced thiols have also been shown to strongly enhanced the MnP-catalyzed mineralization of xenobiotic pollutants (Hofrichter *et al.*, 1998a,Van Aken *et al.*, 1999a).

unsaturated fatty acids or Tween 80,[10] a system which has been shown to oxidatively break down phenolic *and* nonphenolic synthetic lignin models[11] (Bao *et al.*, 1994; Jensen *et al.*, 1996), likely through the generation of reactive acyl ($R^{\bullet}$) or peroxyl free radicals ($ROO^{\bullet}$), the major propagators of lipid peroxidation (Kapich *et al.*, 1999).

E. H_2O_2-Generating Systems

"Any biological system generating $O_2^{\bullet-}$ will produce hydrogen peroxide by the dismutation reaction unless, of course, all the $O_2^{\bullet-}$ is intercepted by some other molecule (Halliwell and Gutteridge, 1989)."

As the electron acceptor (cofactor) for peroxidases and as a Fenton ingredient, extracellular hydrogen peroxide (H_2O_2) is an essential component of fungal ligninolysis systems. White-rot fungi produce a variety of intracellular oxidases whose activity is coupled to the reduction of molecular oxygen to form hydrogen peroxide: fatty acyl-CoA oxidase (Greene and Gould, 1984), GLO (Kersten and Kirk, 1987), glucose-1-oxidase (Kelley and Reddy, 1986), glucose- or pyranose-2-oxidase (Eriksson *et al.*, 1986), methanol oxidase (Nishida and Eriksson, 1987), and aryl alcohol oxidase (AAO) (Bourbonnais and Paice, 1988).[12] These enzymes are mainly produced under ligninolytic conditions (idiophase), suggesting their involvement in the ligninolysis process.

Two other extracellular FAD-dependent enzymes produced by white-rot fungi may play a role in hydrogen peroxide production: CDH (previously cellobiose oxidase) and CBQ.[13] Both CDH and CBQ oxidize disaccharides, e.g., cellobiose, coming from the enzymatic hydrolysis of wood polysaccharides (Westermarck and Eriksson, 1974; Ayers *et al.*, 1978; Ander, 1994; Ander and Marzullo, 1997). This reaction may be coupled with the reduction of dioxygen, slowly generating hydrogen peroxide (Samejima and Eriksson, 1992).

Ligninolytic cultures of white-rot fungi may produce superoxide free radicals ($O_2^{\bullet-}$) by multiple enzymatic or nonenzymatic mechanisms (Section III.C.1.*b*). Superoxide free radicals dismutate—spontaneously or under superoxide dismutase (SOD) mediation—to hydrogen peroxide

[10]Tween 80® (polyoxyethylenesorbitan monooleate) is a source of unsaturated fatty acids.

[11]Lipid peroxidation has also been shown to promote the MnP-catalyzed degradation of xenobiotic pollutants (Moen *et al.*, 1994, Bogan and Lamar, 1995).

[12]Even though glucose-1-oxidase and pyranose-2-oxidase are usually considered as the most important H_2O_2 producers (Kelley and Reddy, 1986), no evidence exists that these oxidases are able to supply *extracellular* peroxidases with H_2O_2, as they are intracellularly located (Kirk and Farrel, 1987).

[13]It is worth noting that protease activity converts CDH into CBQ (Ander, 1994).

H_2O_2 and O_2 (Eq. (2); Halliwell and Gutteridge, 1989; Ander and Marzullo, 1997):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

The rate of the reaction is virtually zero ($k < 0.3\ M^{-1}s^{-1}$)! However, the reaction is faster when both $O_2^{\bullet -}$ and its protonated form, the hydroperoxyl radical ($HO_2^{\bullet}$), are present together ($k = 8 \times 10^7\ M^{-1}s^{-1}$), which is observed at typical pH values[14] for wood-decaying fungi (i.e., 3.0–5.0) (Eq. (3)), or when the reaction is catalyzed by iron traces (Eq. (4), which is first order in superoxide) (Halliwell and Gutteridge, 1989).

$$O_2^{\bullet -} + HO_2^{\bullet} + H^+ \longrightarrow H_2O_2 + O_2 \quad (3)$$

$$Fe^{2+} + HO_2^{\bullet} \longrightarrow Fe^{3+} + OH_2^- \quad (4)$$

$$OH_2^- + H^+ \longrightarrow H_2O_2$$

Finally, MnP may oxidize reduced substrates (e.g., GSH, NADPH, or dihydroxymaleic acid) with the coupled reduction of O_2 to H_2O_2 (Kuawahara *et al.*, 1984; Wood, 1994).

F. Cooperation of Enzymes in Degradation of Lignocellulose

"The idea of a feedback interdependence of delignification and cellulose degradation processes was postulated for the first time by Westermarck and Eriksson (1974). This hypothesis is still valid, as the report by Gottlieb et al. (1950) postulating the possibility of mycelial growth on lignin as sole carbon source has not been confirmed (Leonowicz et al., 1999)."

Depolymerization of lignin and cellulose is partly interrelated so that they operate in a synergistic way. In a scheme proposed by Leonowicz *et al.* (1986), lignin- and cellulose-degrading enzymes are secreted close to the hyphal material, where they cooperate with each other and with mediating (radical) chemical species.

Lignin depolymerization is regulated by fungal phenoxy radical and quinone reducing enzymes, such as AAO, CDH or CBQ, glucose oxidases, and GLO. Reducing phenoxy radicals and quinones, these enzymes prevent them from further polymerization and obviate the poisonous effect of quinones on enzymes and cells (Ander and Marzullo, 1997; Leonowicz *et al.*, 1999). As substrates for extracellular FAD-dependent AAO, aryl alcohols are key metabolites of the complex fungal lignocellulose degrading system. Aryl alcohols undergo oxidation by AAO, coupled with the reduction of either lignin degradation derivatives (phenoxy radicals and quinones) or dioxygen to produce hydrogen peroxide (Fig. 9; Ander and Marzullo, 1997). Aryl alcohol oxidation

[14] $O_2^- + H^+ \leftrightarrows HO_2^{\bullet}$, $pK_a = 4.8$(Halliwell and Gutteridge, 1989).

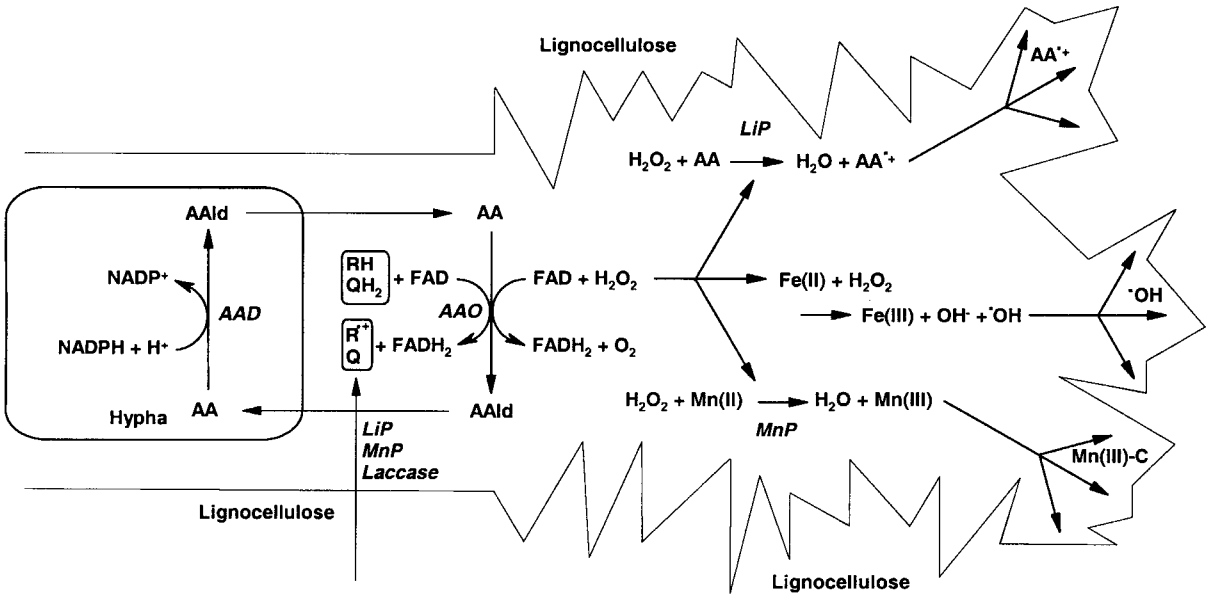

FIG. 9. Interaction of fungal hypha enzymes and veratryl alcohol oxidase with mediators and mediating radicals during transformation of lignocellulose. Adapted from Ander and Marzullo (1997). **AAO**, aryl alcohol oxidase; **AAD**, aryl alcohol dehydrogenase; **AA**; aryl alcohol; **AA**$^{\bullet+}$, aryl alcohol cation radical; **AAld**, aryl aldehyde; **R**$^{\bullet+}$, lignin derived radical; **Q**, quinone; **RH** and **QH$_2$**, reduced form of **R**$^{\bullet+}$ and **Q**; **C**, metal chelating agents (e.g., oxalate).

products (i.e., aryl aldehydes) can be recycled through their reduction by intracellular NADPH-dependent aryl alcohol dehydrogenases (AAD) (Ander and Marzullo, 1997).

Glucanase- or xylanase-dependent depolymerization of wood polysaccharides (e.g., cellulose) produces disaccharides, such as cellobiose, which may play a key role in lignocellulose degradation comparable to that of aryl alcohols. Oxidation of cellobiose by CDH or CBQ is coupled with the reduction of quinones and phenoxy radicals (Fig. 10; Leonowicz *et al.,* 1999). In addition to these reactions, CDH can reduce ferric iron and dioxygen, producing both ingredients of a Fenton reaction (Section III.C.1.*a*; Ander, 1994; Ander and Marzullo, 1997). Hydrolysis products of disaccharides by glucosidases or xylosidases may undergo enzymatic oxidation by glucose oxidase or GLO. The reaction is coupled with the reduction of quinones and phenoxy radicals (Leonowicz *et al.,* 1986; 1999).

Glucose and quinone oxidation products, i.e., gluconolactones and diphenols, are fully metabolized, respectively, through the pentose pathway and, via a dioxygenase-catalyzed ring opening, through the Krebs cycle (Leonowicz *et al.,* 1986). Alternatively, these oxidation reactions may be coupled with oxygen reduction, providing hydrogen peroxide (Section II.E). Hydrogen peroxide may be used either as electron acceptor by LiP and MnP, catalyzing the oxidation of aryl alcohols and Mn(II), respectively (Section II.D.1), or, together with reduced iron, may take part in a Fenton reaction (Section III.C.1.*a*) (Ander and Marzullo, 1997).

Quinones (Q) may also be reduced by CBQ (Ander and Marzullo, 1997), by an intracellular NAD(P)H-dependent quinone reductase (Brock *et al.,* 1995), or by a plasma membrane redox system (Stahl and Aust, 1993b) to give semiquinone radicals ($HQ^{\bullet}$) (one-electron reduction) or hydroquinones (H_2Q) (two-electron reduction), the latter being oxidized back to $HQ^{\bullet}$ through peroxidase or laccase-mediated one-electron abstraction. Semiquinone radicals $HQ^{\bullet}$ are able to reduce phenoxy radicals, preventing them from further polymerization, or ferric iron and dioxygen, generating components of the Fenton reaction (Section III.C.1.*a,b*) (Brock *et al.,* 1995).

G. Physiological Features Supporting Fungal Ligninolysis

"Understanding the factors that influence lignin biodegradation is necessary to clarify wood decay, the formation of humic substances, coal and petroleum, and to explore realistically the practical potential for utilizing lignin-degrading organisms or enzymes in lignocellulose bioconversion processes (Kirk et al., 1978)."

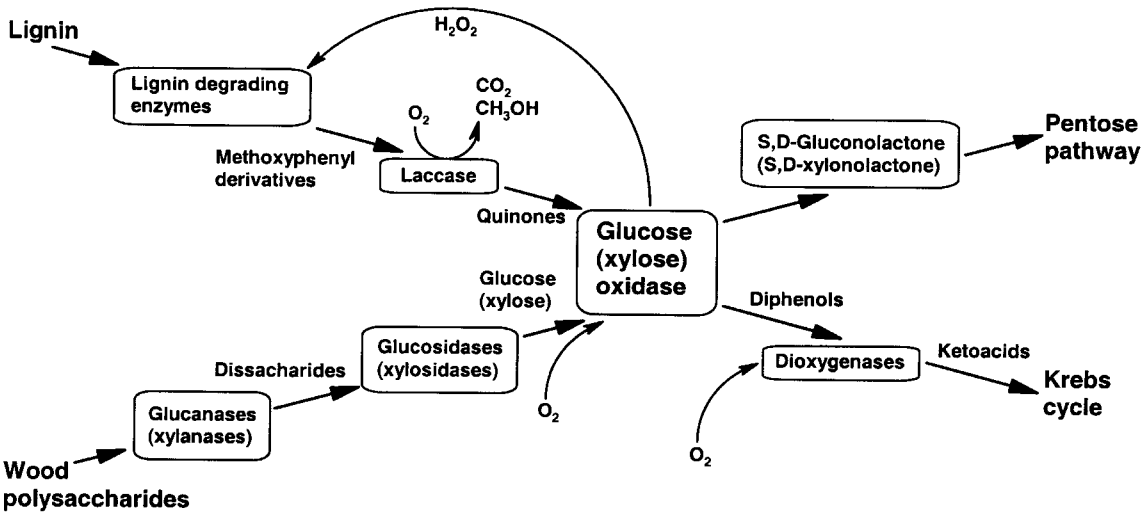

FIG. 10. Lignocellulose transformation by white-rot fungi enzymes. Adapted from Leonowicz *et al.* (1986).

Several key features of the ligninolysis process stand out (Hammel, 1992):

- The process is oxidative, as ligninolysis is stimulated by oxygen, and involves oxygen incorporation into low-molecular-weight products coming from lignin degradation.
- The process is extracellular, as lignin is water insoluble and too large to be transported intracellularly.
- Ligninolysis is a function of secondary metabolism, as it occurs almost exclusively under nutrient depletion and as lignin cannot be used as the only energy and carbon source.

Factors influencing lignin degradation by white-rot fungi were first studied by Kirk *et al.* (1978) using *P. chrysosporium* and have been reviewed by Buswell and Odier (1987), Kirk and Farrel (1987), and Garg and Modi (1999).

1. Carbon Cosubstrate

Biodegradation of lignin by white-rot fungi is a cometabolic process, i.e., requiring a primary substrate as carbon and energy source, and an expression of secondary metabolism. The basis for a growth substrate requirement has been hypothesized as follows:

- The energy recovered from lignin metabolism is too little to support growth (Jeffries *et al.*, 1981).
- Ligninolytic activity during microbial growth is very low (Kirk *et al.*, 1978).

Even though lignin is a potentially energy-rich material, it is unable to serve as the sole carbon and/or energy source, since the whole ligninolytic process has an unfavorable net energy balance (Kirk *et al.*, 1978). Ligninolytic enzymes are produced by the fungus during the idiophase, in response to a stress situation, which may be induced by different environmental triggers, e.g., carbon (or nitrogen) starvation. While glucose is the most frequently adopted substrate in laboratory experiments, a wide range of carbon sources have been found to support lignin degradation by white-rot fungi (Kirk *et al.*, 1978; Buswell and Odier, 1987).

2. Nitrogen Source

While nutrient nitrogen limitation is essential to induce the ligninolytic system in several species (Jeffries *et al.*, 1981; Kirk *et al.*, 1978), this phenomenon cannot be applied to all white-rot fungi (Buswell and Odier, 1987). The nitrogen regulation could be related to the low nitrogen levels in wood. The adverse effect of nitrogen may be explained by several factors (Garg and Modi, 1999):

High nitrogen contents increase the biomass formation and the respiration rate, which promotes a rapid depletion of the energy sources at the expense of secondary metabolism (Kirk *et al.,* 1978).

The nitrogen metabolism competes with the metabolism of lignin through the requirement for the same cofactors (Buswell and Odier, 1987).

Nitrogen regulates the synthesis of components of the ligninolytic system.

Nitrogen-based repression of the ligninolytic activity would be related to glutamate metabolism and may operate partly at the level of RNA synthesis. On the other hand, the development of ligninolytic activity under low nitrogen conditions is preceded by an increase of intracellular c-AMP level, suggesting deeper modifications in the fungus physiology which may characterize the transition between primary and secondary metabolism (Buswell and Odier, 1987).

3. Oxygen Tension

Lignin decomposition is largely an oxidative process, which is strongly enhanced by high oxygen levels (Kirk *et al.,* 1978). The absence of anaerobic mineralization of lignin could explain the formation of coal and peat deposits in the biosphere (Buswell and Odier, 1987).

The stimulatory effect of molecular oxygen on lignin biodegradation may result from several factors:

High levels of molecular oxygen increase the rate of H_2O_2 generation and are required for the production of ROS (Section III.C.1; Halliwell and Gutteridge, 1989).

Oxygen stimulates both the synthesis and the activity of ligninolytic peroxidases (Faison and Kirk, 1985).

High oxygen concentrations enhance *de novo* synthesis of the secondary metabolite VA (Lundquist and Kirk, 1978).

III. Low-Molecular-Weight Mediators and Free Radicals

"... Knowledge of the mechanism of lignin degradation is still incomplete because of lack of understanding of the nature and function of low molecular weight compounds most probably involved in lignin breakdown (Messner et al., 1997)."

Wood-rotting enzymes are large molecules unable to penetrate the bulky lignocellulosic material, suggesting that low-molecular-weight redox mediators and/or reactive (oxygen) radical species, eventually resulting from the fungal metabolism and matured by ligninolytic

enzymes, are exported inside the wood structure and act in the degradative process as "enzyme messengers" (Leonowicz *et al.*, 1999).

A. Veratryl Alcohol (VA)

"The role of VA in lignin degradation has been the subject of numerous studies and considerable debate. Recent evidence clearly shows that VA does form a cation radical upon oxidation by LiP and that it can mediate the oxidation of some substrates (Zapanta and Tien, 1997)."

VA (3,4-dimethoxybenzyl alcohol) is produced in ligninolytic cultures of *P. chrysosporium* at the same time as LiP (Lundquist and Kirk, 1978), so that VA biosynthesis and ligninolysis are closely linked (de Jong *et al.*, 1994). VA can be synthesized from the lignin degradation products vanillate or syringate, or *de novo* from L-phenylalanine (de Jong *et al.*, 1994).

In ligninolytic cultures of the white-rot fungus *P. chrysosporium*, VA is known to enhance both LiP enzymatic activity and lignin mineralization (Faison and Kirk, 1985; de Jong *et al.*, 1994). Several roles have been proposed for the intermediate metabolite VA in the ligninolysis process:

The induction of ligninolytic enzymes
The stabilization of LiP
A charge-transfer mediation
The formation of ROS
Acting as a substrate for H_2O_2-generating enzymes

Addition of VA has been shown to induce LiP production in *P. chrysosporium*. However, the VA concentration during *in vivo* ligninolysis was considerably lower than that used in induction experiments, suggesting that VA might play other roles more important than induction (de Jong *et al.*, 1994).

Incubation of LiP in the presence of an excess of H_2O_2 results in the further oxidation of compound II to give inactive compound III*, which lies outside the catalytic cycle of the enzyme (Section II.D.2.*a*; Wariishi and Gold, 1990). Substrates with high redox potential are oxidized by compound I, but not by compound II. As a consequence, the enzymatic catalytic cycle is broken: compound II is not reduced back to native LiP, but is converted to inactive compound III* (Valli *et al.*, 1990). VA could reduce compound II to native LiP, maintaining the enzyme turnover, and protect LiP against inactivation by H_2O_2 (Valli *et al.*, 1990, Wariishi and Gold, 1990). Inhibition of LiP by H_2O_2 is prevented for VA/H_2O_2 ratios greater than 200 (de Jong *et al.*, 1994). In addition, VA appears to be capable of rescuing LiP from compound III* (Valli *et al.*, 1990).

Several substrates for LiP, such as 4-methoxymandelate (Harvey *et al.,* 1992), lignin model compounds (Cui and Dolphin, 1990), 4-OHA-2,6-DNT (4-hydroxylamino-2-6-dinitrotoluene) (Bumpus and Tatarko, 1994, Michels and Gottschalk, 1994), or 2,4-DA-6-NT (2,4-diamino-6-nitrotoluene) (Van Aken *et al.,* 2000), have been shown to "inhibit" the LiP-catalyzed oxidation of VA to veratryl aldehyde. Also, oxidation of those substrates is usually stimulated in the presence of VA, suggesting a charge-transfer-mediation effect of VA (Valli *et al.,* 1990). VA is a substrate for LiP and is easily oxidized to give the reactive radical cation $VA^{\bullet+}$, whose existence was confirmed by oxygen consumption experiments (autooxidation) (Palmer *et al.,* 1987), by electron-spin resonance (ESR) (Khindaria *et al.,* 1995), and by radiation chemistry techniques (Candeias and Harvey, 1995). With substrates having a redox potential lower than VA (e.g., chloropromazine, guaiacol), $VA^{\bullet+}$ may act as a redox mediator (Harvey *et al.,* 1986, Goodwin *et al.,* 1995, Koduri and Tien, 1995). Such substrates can be oxidized directly either by LiP (Compounds I or II) or by $VA^{\bullet+}$, itself resulting from the LiP-catalyzed oxidation of VA and being reduced back to VA, preventing the formation of veratryl aldehyde (Fig. 4; Buswell and Odier, 1987; Michels and Gottschalk, 1994). This mediation model is attractive because it proposes a mechanism by which $VA^{\bullet+}$ can diffuse into the bulky lignin structure, acting as an oxidizing mediator.[15]

ROS (i.e., superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1\Delta gO_2$), and hydroxyl radical ($^{\bullet}OH$) are important in lignin degradation by white-rot fungi (Section III.C.1; Hall, 1980; Forney *et al.,* 1982, Faison and Kirk, 1983). Superoxide free radicals can be produced in fungal ligninolytic systems by several paths involving VA oxidized derivatives. $VA^{\bullet+}$ is a highly acidic species, which readily loses a proton to water, giving the veratryl alcohol radical ($VA^{\bullet}$). $VA^{\bullet}$ in turn reacts with dioxygen to form veratryl aldehyde and superoxide radical ($O_2^{\bullet-}$) (autooxidation reaction; Eq. (5); Section III.C.1.*b*; Schoemaker *et al.,* 1994):

$$VA^{\bullet} + O_2 \longrightarrow V.\,Aldehyde + O_2^{\bullet-} + H^+ \qquad (5)$$

$VA^{\bullet+}$ can oxidize hydrogen peroxide to superoxide radical (Barr and Aust, 1994; Eq. (6)):

$$VA^{+\bullet} + H_2O_2 \longrightarrow VA + O_2^{\bullet-} + 2H^+ \qquad (6)$$

$VA^{\bullet+}$ generated by LiP is able to achieve the oxidative decarboxylation of oxalate giving formyl free radicals ($COO^{\bullet-}$), which by autooxidation

[15]Pulse radiolysis experiments have shown that $VA^{\bullet+}$ could diffuse over a distance up to 7 μm in aqueous medium, and therefore could efficiently move from its production site (i.e., LiP) to or inside the lignocellulosic structure (Candeias and Harvey, 1995).

form superoxide radicals (Eqs. (7) and (8); Akamatsu *et al.*, 1990):

$$VA^{\bullet+} + HOOC - COOH \longrightarrow VA + COO^{\bullet-} + CO_2 + 2H^+ \quad (7)$$

$$COO^{\bullet-} + O_2 \longrightarrow O_2^{\bullet-} + CO_2 \quad (8)$$

While superoxide free radical is usually considered not to be an oxidant strong enough to attack organic molecules directly, it could act in lignin degradation in several other ways (Section III.C.1.*b*).

VA, together with other aryl alcohols, is a substrate for AAO, a common extracellular enzyme produced by white-rot fungi, which uses molecular oxygen as an electron acceptor, producing H_2O_2 (Bourbonnais and Paice, 1988; de Jong *et al.*, 1994).

The main function of VA in ligninolytic systems remains likely the stabilization of LiP and a charge-transfer mediation.

B. Manganese and Oxalate

"In the MnP system, mediation occurs via an Mn(III)-oxalate complex that is capable of oxidizing a wide variety of substrates. Though diffusion from enzyme may be unlikely for the VA cation radical, the chelated Mn(III) can act as a diffusible oxidant to remote areas of lignin (Zapanta and Tien, 1997)."

MnP of *P. chrysosporium*—as well as of most white-rot fungi—requires Mn(II) in order to complete its catalytic cycle (Glenn and Gold, 1985). Even though MnP compound I can oxidize various other substrates (phenols, ferrocyanide, reduced thiols), the reduction of compound II to native MnP requires Mn(II) (Wariishi *et al.*, 1989a,b).

MnP-catalyzed oxidation of Mn(II) is dependent on the presence of chelating organic acids that are (e.g., oxalate, malonate, or α-hydroxy acids) supposed to stabilize Mn(III), which tends to be disproportionate in aqueous medium, giving Mn(II) and dark MnO_2 precipitate (Glenn and Gold, 1985; Glenn *et al.*, 1986; Forrester *et al.*, 1988). Mn(III) complexes are strong oxidants able to diffuse far away from the MnP active site and capable of attacking various organic molecules, including nonphenolic lignin model compounds (Glenn *et al.*, 1986; Wariishi *et al.*, 1989a,b). Chelating acids usually increase the oxidation rate of Mn(II) by MnP compounds I and II, likely accelerating the dissociation of the enzyme–Mn(III) complex (Wariishi *et al.*, 1989a).

Oxalate is produced in ligninolytic cultures of *P. chrysosporium* at the same time as MnP (Wariishi *et al.*, 1992). Oxalate can be synthesized enzymatically through oxaloacetate hydrolysis (oxaloacetase) and glyoxalate oxidation (glyoxalate oxidase) (Akamatsu *et al.*, 1993) or produced chemically through oxidative decarboxylation of malonate by Mn(III) (Hofrichter *et al.*, 1998b).

Oxalate has been shown to play multiple roles in MnP-catalyzed degradation of lignin (Shimada *et al.,* 1994; 1997; Zapanta and Tien, 1997) and xenobiotic pollutants (Barr and Aust, 1994):

Oxalate is a metal ion chelator.
Oxalate is a source of formyl and superoxide free radicals.
Oxalate is a potent ferric iron reductant.

Besides the stabilization of Mn(III), oxalate might play additional complexing roles in lignin biodegradation, e.g., chelation of calcium ions, enlarging pore size in wood cell walls and allowing the penetration of enzyme molecules (Leonowicz *et al.,* 1999).

Oxalate also participates in the production of reactive radical (oxygen) species and hydrogen peroxide by ligninolytic cultures of white-rot fungi. Both LiP and MnP, through the mediation of $VA^{\bullet+}$ (Eq. (7); Akamatsu *et al.,* 1990; Barr *et al.,* 1992) and Mn(III), respectively (Shimada *et al.,* 1997; Urzùa *et al.,* 1998), are able to oxidatively decarboxylate oxalate generating formyl free radicals $COO^{\bullet-}$ (Eqs. (7)–(9); (Kenten and Mann, 1953; Akamatsu *et al.,* 1990; Popp *et al.,* 1990):

$$Mn^{3+} + HOOC - COOH \longrightarrow Mn^{2+} + CO_2 + COO^{\bullet-} + 2H^+ \qquad (9)$$

The latter react with molecular oxygen (autooxidation), giving superoxide free radicals $O_2^{\bullet-}$ (Eq. (8); Shimada *et al.,* 1994). In addition to a potent direct reaction with the lignin molecule, these radical species ($COO^{\bullet-}$ and $O_2^{\bullet-}$) may act on ligninolysis by several paths, including the production of Fenton reagents (Section III.C.1.*a,b*).

On the other hand, oxalate, when present, can reduce back both $VA^{\bullet+}$ and Mn(III)—two redox mediators in peroxidase-catalyzed biodegradation mechanisms, leading to a strong inhibition of the ligninolysis process (Shimada *et al.,* 1994, 1997). Moreover, the highly reactive hydroxyl radical ($^{\bullet}OH$), generated by the Fenton reaction, can be scavenged by oxalate (Barr *et al.,* 1992). Thus, oxalate decarboxylase, occurring ubiquitously in white-rot fungi, may improve lignin degradation. In short, low oxalate concentrations (1 m*M*) enhance the degradation process, likely through the generation of ROS and/or Fenton reagents, while higher concentrations (above 5 m*M*) may strongly inhibit lignin degradation by scavenging peroxidase redox mediators ($VA^{\bullet+}$ and Mn(III) and/or reactive $^{\bullet}OH$ radicals (Shimada *et al.,* 1997).

The formyl radical-supported Fenton system described here has been proposed to be a major mechanism involved in the peroxidase-catalyzed bioremediation of xenobiotic environmental pollutants (Barr and Aust, 1994).

C. Free Radicals

"The diatomic oxygen molecules in the Earth's atmosphere are themselves "free radicals" and major promoters of radical reactions in living cells (Halliwell and Gutteridge, 1989)."

Lignin-degrading enzymes (e.g., peroxidases and laccases) act mainly through one-electron abstraction (i.e., oxidation) reactions, which strongly suggests the involvement of reactive free-radical species in the ligninolysis process (Barr and Aust, 1994). Free radicals are paramagnetic and usually very reactive species (Halliwell and Gutteridge, 1989).

1. Reactive Oxygen Species (ROS)

Molecular dioxygen is toxic for all living organisms: anaerobes—from strict anaerobes to microaerophiles—and aerobes. Appearance of oxygen in the Earth's atmosphere about 2 10^9 years ago presented a major evolutionary challenge to living organisms: draw the energetic benefit of oxygen as an efficient electron acceptor and protect themselves from toxic oxygen species. The most damaging effects of oxygen, which were previously attributed to the oxygen inhibition of cellular enzymes, are known to be related to the generation of free oxygen radicals (Halliwell and Gutteridge, 1989).

Ground-state oxygen ($^3\Sigma g^- O_2$) has two unpaired electrons each located in one different antibounding orbital and is a "double" free radical (Halliwell and Gutteridge, 1987). However, unpaired electrons have parallel spin, which imposes a restriction on electron transfer to the oxygen molecule, which is therefore in its most stable state (and prevents the immediate combustion of organic matter in air!). Singlet oxygen species ($^1\Delta gO_2$, which is not a free radical, and $^1\Sigma g^+ O_2$) are generated by an input of energy to ground-state oxygen. The spin restriction on electron transfer is removed, increasing the oxidizing capability of singlet states. Accepting one single electron, ground-state oxygen is transformed into superoxide radical anion ($O_2^{\bullet -}$), which possesses henceforth only one unpaired electron and is *less* a free radical than molecular oxygen itself. Electrons accepted by dioxygen enter antibonding orbitals, weakening the O–O bond and allowing homolytic fission of H_2O_2, which yields the highly reactive hydroxyl free radical ($^\bullet OH$).

ROS, such as singlet oxygen ($^1\Delta gO_2$), superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^\bullet OH$), may play different roles in lignin biodegradation. The singlet oxygen $^1\Delta gO_2$ is a reactive species, whose implication in lignin breakdown has to be considered, even though its actual involvement has not yet been demonstrated (de Jong *et al.*, 1994). The superoxide free radical $O_2^{\bullet -}$, also involved in H_2O_2 and Fenton reagents production, is not considered to be a very reactive

species. However, the $O_2^{\bullet-}$ seems to be a key component of the fungal ligninolytic system (Section III.C.1.*b*). Hydrogen peroxide H_2O_2 is the cofactor of peroxidases and a Fenton reagent, but it does not play any direct role in lignin degradation (Section II.E). Finally, the hydroxyl free radical $^{\bullet}OH$, generated by LiP- or superoxide-assisted Fenton reaction, is the most powerful oxidizing agent in biological systems, likely participating to *in vivo* lignin depolymerization (Section III.C.1.*a*).

a. Hydroxyl Radicals and Fenton Reaction. The extremely high oxidizing power of a mixture of hydrogen peroxide H_2O_2 and ferrous iron Fe(II) was discovered more than 1 century ago by Fenton (1894). The reactive species involved in the reaction was later shown to be the hydroxyl free radical ($^{\bullet}OH$), the most powerful oxidizing agent in biological systems ($E_0 = 2180$ mV). The reactivity of $^{\bullet}OH$ is so high that it reacts with whatever organic molecule, including lignin or xenobiotic pollutants, to produce secondary less reactive radicals (Forney *et al.*, 1982; Halliwell and Gutteridge, 1989; Barr and Aust, 1994; Wood, 1994; Park *et al.*, 1997). Hydroxyl radicals are produced by the oxidation of Fe(II) by H_2O_2, in what has become known as the *Fenton reaction* (Eq. (10)):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad (10)$$

Fe(III) can be reduced to Fe(II) by superoxide free radicals $O_2^{\bullet-}$, frequently produced in biological systems (see Section III.C.1.*b*; Eq. (11)):

$$O_2^{\bullet-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \qquad (11)$$

Together, reactions (10) and (11) are nothing else than the iron-catalyzed *Haber–Weiss reaction* (Haber and Weiss, 1934), also called the *superoxide-assisted Fenton reaction* (Halliwell and Gutteridge, 1989). Dismutation of $O_2^{\bullet-}$ produces H_2O_2 (Section II.E; Eqs. (3) and (4)), and both could react together to produce reactive $^{\bullet}OH$ (Eq. (12); Haber and Weiss, 1934):

$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + {}^{\bullet}OH + OH^- \qquad (12)$$

Actually, the second-order rate constant for the Haber–Weiss reaction is virtually zero. In biological systems, the formation of $^{\bullet}OH$ can only be accounted for if the reaction is catalyzed by iron or copper traces (i.e., Fenton reaction; Halliwell and Gutteridge, 1989).

The Fenton-generated hydroxyl radical has been shown to be involved in the degradation of cellulose by brown-rot fungi (Backa *et al.*, 1992). Even though no evidence for the actual implication of $^{\bullet}OH$ in lignin degradation by white-rot fungi has been reported to date—except for an *in vitro* LiP-mediated Fenton system (Barr *et al.*, 1992)—the production

of Fenton reagents in *in vivo* peroxidase-catalyzed reactions may be considered (Wood, 1994).

Production of Fenton reagents (i.e., Fe(II) and H_2O_2) in biological systems may occur through a unique mechanism. The central reaction, represented by Eq. (11), gives either reduction of Fe(III) by $O_2^{\bullet -}$ (forward direction) or autooxidation of Fe(II) by O_2 (reverse direction). Fe(II) autooxidation takes place in the presence of strong chelators[16] (e.g., oxalate), while reduction of Fe(III) requires external reactions selectively producing superoxide radicals (Wood, 1994). H_2O_2 formation may proceed by spontaneous or SOD-mediated dismutation of $O_2^{\bullet -}$ (Section II.E).

b. Superoxide Free Radicals. Superoxide free radicals ($O_2^{\bullet -}$) are produced in ligninolytic cultures of white-rot fungi by numerous enzymatic and nonenzymatic pathways. The most important is likely the autooxidation of reactive cation free radicals generated by the one-electron peroxidase-catalyzed oxidation of different substrates (Halliwell and Gutteridge, 1989):

- Both peroxidase-generated $VA^{\bullet +}$ and Mn(III) can achieve the oxidative decarboxylation of organic acids such as oxalate, glyoxalate, and malonate, producing carbon-centered free radicals (i.e., formyl ($COO^{\bullet -}$) or acetic acid radicals ($HOOC-{}^{\bullet}CH_2$)) and, by reaction with dioxygen, superoxide radicals (Eqs. (7)–(9); Akamatsu *et al.*, 1990, Popp *et al.*, 1990; Shimada *et al.*, 1997; Hofrichter *et al.*, 1998b; Urzùa *et al.*, 1998).
- Aryl radical cations generated by LiP-mediated oxidation of aromatic substrates (e.g., dimethoxyphenylpropene) are spontaneously converted—through deprotonation and reaction with dioxygen—to peroxyl radicals ($ROO^{\bullet}$), whose decomposition generates hydroperoxyl radicals $HO_2^{\bullet}$, i.e., the protonated form of superoxide radicals (Hammel *et al.*, 1985; ten Have *et al.*, 2000).
- Enzymatic reduction of quinones (Q), coming from fungal lignin depolymerization (Section II.F), gives hydroquinones (H_2Q) (two-electron reduction) or semiquinone radicals ($HQ^{\bullet}$) (one-electron reduction), which may undergo one-electron laccase-mediated oxidation into $HQ^{\bullet}$ (Eq. (13)) or autooxidation into Q (Eq. (14)), respectively. Both reactions are coupled with the reduction of dioxygen yielding superoxide free radicals (Brock *et al.*, 1995; Leonowicz *et al.*, 1999):

$$H_2Q + O_2 \longrightarrow HQ^{\bullet} + O_2^{\bullet -} \qquad (13)$$

[16]Strong chelators of iron reduce the reduction potential of Fe(II), allowing autooxidation by O_2.

$$HQ^{\bullet} + O_2 \longrightarrow Q + O_2^{\bullet -} \quad (14)$$

LiP can oxidize NADH to form $NAD^{\bullet}$ radicals, which can reduce dioxygen to superoxide. However, NAD^{+}-reducing enzymes are intracellular while LiP is located extracellularly, making such a mechanism unlikely (Wood, 1994).

LiP-catalyzed oxidation of VA gives $VA^{+\bullet}$, which may form $VA^{\bullet}$ by deprotonation. $VA^{\bullet}$ can reduce dioxygen and $VA^{+\bullet}$ can oxidize H_2O_2, both reactions producing superoxide radicals $O_2^{\bullet -}$ (Eqs. (5) and (6); de Jong *et al.*, 1994).

Physicochemical studies have shown that the superoxide anion radical reacts as a reducing or oxidizing agent, but is not a very reactive species (Fridovich, 1986; Halliwell and Gutteridge, 1989). However, the existence of numerous protective mechanisms against superoxide radical in every living cell (including anaerobes) suggests that $O_2^{\bullet -}$ is a damaging and therefore a *reactive* species (cf. the "superoxide theory of oxygen toxicity"; Fridovich, 1986).

The superoxide radical $O_2^{\bullet -}$ might act directly on biological systems. In an aqueous environment—especially at the low pH existing in lignin-olytic environments (i.e., 3.0–5.0)—superoxide free radical is protonated and converted to the more reactive hydroperoxyl radical $HO_2^{\bullet}$, which is an oxidant strong enough to react with some organic molecules (Halliwell and Gutteridge, 1989). $O_2^{\bullet -}$ (or $HO_2^{\bullet}$) can inactivate enzymes: e.g., by reaction with ferric iron prosthetic groups giving a poorly reactive *perferryl* iron complex (Section II.D.2). $O_2^{\bullet -}$ or $HO_2^{\bullet}$ can also initiate radical chain reactions: e.g., fatty acids peroxidation. Finally, $O_2^{\bullet -}$, which is more reactive in hydrophobic environments, can mediate damages to biological membranes. Despite its "low" reactivity, superoxide free radical could act in lignin degradation directly, e.g., by aromatic ring opening (Kawai *et al.*, 1988) or by demethoxylation (Potthast *et al.*, 1995) of several lignin substructures.

However, in accordance with its low reactivity, $O_2^{\bullet -}$ is supposed to exert most of its degradative effects by the generation of more reactive species, such as hydroxyl radicals ($^{\bullet}OH$) or singlet oxygen ($^1\Delta gO_2$). $O_2^{\bullet -}$ can generate H_2O_2 either spontaneously or by iron- or SOD-mediated dismutation (Section II.E). Alternatively, $O_2^{\bullet -}$ dismutation may generate the highly reactive singlet oxygen $^1\Delta gO_2$, even though $O_2^{\bullet -}$ itself may be a quencher of $^1\Delta gO_2$ (Halliwell and Gutteridge, 1989). As a reductant, superoxide radical can reduce Fe(III) into Fe(II), which together with H_2O_2, constitute the ingredients of a Fenton degradative system (Section III.C.1.*a*). As an oxidant, superoxide radical can oxidize Mn(II) to Mn(III), which, when chelated by organic acids, is a strong oxidant directly involved in lignin degradation (Eq. (15); Glenn *et al.*, 1986,

Halliwell and Gutteridge, 1989; Wariishi *et al.*, 1989a,b).

$$O_2^{\bullet -} + Mn^{2+} + 2H^+ \longrightarrow Mn^{3+} + H_2O_2 \quad (15)$$

In summary, even though the superoxide anion radical may not be reactive enough to directly degrade extensively the natural lignin polymer, it likely contributes to the ligninolysis process, either directly, attacking lignin substructures (Kawai *et al.*, 1988, Potthast *et al.*, 1995), or indirectly, by the production of other reactive species (Forney *et al.*, 1982; Park *et al.*, 1997).

2. Glutathionyl Free Radicals

Reduced thiols (e.g., GSH) are easily oxidized into reactive thiyl free radicals, in turn involved in multiple potential reactions. GSH may undergo peroxidases (Eq. (16)) or transition metal-catalyzed oxidation (Eq. (17)) to produce glutathionyl free radicals (GS•) (Harman *et al.*, 1986; Ross and Moldeus, 1985):

$$GSH + Mn^{3+} \longrightarrow GS^{\bullet} + Mn^{2+} + H^+ \quad (16)$$

$$2GSH + H_2O_2 \longrightarrow 2GS^{\bullet} + 2H_2O \quad (17)$$

While less reactive than the hydroxyl radical, GS• is a highly reactive species, possibly involved in the degradation of organic molecules (e.g., lignin).

Glutathionyl radicals react easily with dioxygen to give sulphenyl hydroperoxide radicals (GSOO•) (Ross and Moldeus, 1985; O'Brien, 1988), which may react further to produce the reactive singlet oxygen $^1\Delta gO_2$ and/or hydrogen peroxide H_2O_2 (O'Brien, 1988). Glutathionyl radical may also react with another glutathione to form the glutathionyl disulfide anion radical ($GSS^{\bullet}G^-$), which may either undergo autooxidation, generating superoxide radicals (Harman *et al.*, 1986; Ross and Moldeus, 1985) or react with hydrogen peroxide to produce reactive hydroxyl radicals (O'Brien, 1988).

The presence of reduced thiols, such as GSH, has been reported to enhance the MnP- or Mn(III)-mediated oxidation of several nonphenolic aromatic substrates, as well as nonphenolic lignin model dimers (Forrester *et al.*, 1988; Wariishi *et al.*, 1989b). The stimulatory effect of thiols has been attributed to either the reactive glutathionyl radical GS• (Wariishi *et al.*, 1989c) or the superoxide radical $O_2^{\bullet -}$ (Forrester *et al.*, 1988).

Observing that horseradish peroxidase-catalyzed oxidation of veratryl alcohol required the presence of both Mn(II) and GSH, McEldoon and Dordick (1991) proposed the implication of $[Mn^{2+} - GS^{\bullet}Mn^{3+} - GS^-]$ complex as the ultimate oxidative agent involved in degradation reactions catalyzed by peroxidases in the presence of both Mn(III) and GSH.

IV. Biodegradation of Nitro-Substituted Explosives TNT, RDX, and HMX

"Munition wastes constitute a major problem for the military. In addition to their explosive properties, these compounds are biological poisons, and their accumulation in the environment has damaged many ecosystems (Bennett, 1994)."

Best known for their explosive properties, nitroaromatic and nitroheterocyclic explosives, such as TNT, RDX, and HMX, are also toxic chemicals constituting a serious biological hazard for water and soils.

Traditional treatments of toxic munition wastes (i.e., detonation, incineration (OB/OD),[17] adsorption onto activated carbon or resin, advanced photooxidation (UV/O_3)) are costly, damaging to the environment, and, in most cases, practically unfeasible because of the magnitude of the contamination. Therefore, there is a considerable interest in developing biotechnological alternatives based on microorganisms (bacteria and fungi) or plants (i.e., phytoremediation) (Bennett, 1994). Research over the last 10 years regarding physicochemical properties, biodegradation, and toxicity of nitro-substituted explosives has been reviewed in Bulusu (1990), Walsh (1990), Rosenblatt *et al.* (1991), Higson (1992), Walker and Kaplan (1992), Bennett (1994), Gorontzy *et al.* (1994), Spain (1995), Kaplan (1994), Talmage *et al.* (1999), and Spain *et al.* (2000).

A. TNT, RDX, and HMX as Environmental Pollutants

"The formation of reactive nitraryl radical intermediates, i.e. transfer of the first electron, is an endergonic reaction, which typically represents the rate-determining step in the overall reduction of nitroaromatic compounds (Haderlein et al., 2000)."

First synthesized in 1863 by stepwise polynitration of toluene, 2,4,6-trinitrotoluene (TNT) was used in the dye industry before becoming the main conventional explosive (and propellant) material employed by military forces worldwide at the beginning of the 20th century. TNT meets several valuable criteria for an explosive material: i.e., insensitive to shocks and friction, stable in the long term, nonreactive with water (Bennett, 1994).

Because of their higher stability and detonation power, nitramines, e.g., hexahydro-1,3,5-trinitro-1,3,5-triazine (hexogen or RDX)[18] and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (octagen or HMX)[19] are now the most important conventional high explosives produced and used worldwide (Gorontzy *et al.*, 1994).

[17]Open Burning/Open Detonation.
[18]British code name for Royal Demolition Explosive.
[19]Code name for High Melting Explosive.

Manufacture of nitroaromatic explosives, military operations, as well as destruction of ammunition stocks (e.g., in the framework of disarmament agreements) generate toxic wastes commonly stored in surface impoundments (i.e., pink water lagoons), ultimately leading to contamination of soils and groundwater (Isbiter *et al.*, 1984). The major U.S. explosive-polluted sites have been identified by the U.S. Army Environmental Center and consist mainly of 20th-century ammunition plants. In Germany, many explosive manufacturing facilities were destroyed at the end of World War II without regard for the environment and are now used for industry and housing. Explosive-contaminated sites have also been identified in the UK, Canada, and Australia. In the rest of the world, anecdotal information suggests that the problem is significant (Spain, 2000). Typical explosive-contaminated sites may contain up to 10,000 mg kg^{-1} TNT and 1640 mg kg^{-1} HMX in soil and up to 100 mg $liter^{-1}$ TNT in water (Jackson *et al.*, 1978; Fernando *et al.*, 1990). The U.S. Environmental Protection Agency (EPA) has determined a lifetime health advisory of 2 μg $liter^{-1}$ TNT in drinking water (Ross and Hartley, 1990) and a maximum concentration of 0.3 mg $liter^{-1}$ in surface water to protect aquatic life (Shen *et al.*, 1998). Seven nitroaromatic compounds, including TNT, RDX, and 2,4-dinitrotoluene (2,4-DNT), are listed by the EPA as priority pollutants (Keith and Telliard, 1979; Lachance *et al.*, 1999).

More than many other xenobiotic organic compounds, nitro-substituted explosives are recalcitrant to biodegradation and usually persist in the environment (Rieger and Knackmuss, 1995; Shen *et al.*, 2000). Several factors may explain this observation:

- The cyclic molecules are characterized by the presence of three or four bulky and electron-withdrawing nitro groups, introducing steric constraints and making the electronic density of the ring very low. The molecule is thus resistant to attacks by oxidative enzymes (Rieger and Knackmuss, 1995).
- Microorganisms able to mineralize nitro-substituted explosives, i.e., white-rot fungi, cannot use them as an energy or carbon source. TNT, RDX, and HMX metabolic degradation pathways do not support growth, and the mineralization of these chemicals remains a cometabolic process (Gorontzy *et al.*, 1994, Hawari *et al.*, 2000).
- Since very few naturally synthesized nitro-substituted compounds have been identified, autochthonous microflora may not possess the enzymatic apparatus required for the metabolism of nitro-substituted pollutants (Gorontzy *et al.*, 1994). However, most bacteria that have been shown to use nitroaromatic compounds as

nitrogen (and/or carbon) sources were isolated from nitroaromatic-contaminated sites, which suggests the acquisition of xenobiotic-degrading enzymes in an evolutionary process.

Nitro-substituted xenobiotic pollutants may be present at toxic or genotoxic concentrations, inhibiting the activity of potentially efficient microorganisms (Spiker *et al.*, 1992).

TNT and related nitroaromatic compounds are poorly water soluble and may be present in soil in a crystalline form which is poorly bioavailable (Fritsche *et al.*, 2000).

Because of polymerization processes or physicochemical binding to soil matrices, nitro-substituted compounds or their transformation products may not be available for biodegradation (Bruns-Nagel *et al.*, 2000).

B. Other Nitro-Substituted Compounds

"Beside the xenobiotic character of certain structural elements such as halogens, SO_3H, $H{=}N{-}R$, and NO_2, it is the electron-withdrawing character of these groups and thus the electron deficiency of the aromatic ring, which impedes electrophilic attack by oxygenases of aerobic bacteria (Rieger and Knackmuss, 1995)."

Only a few naturally occurring nitro-organic compounds have been identified to date (e.g., aureothin, chloramphenicol, 3-nitro-1-propanol, nitrosporin, pyrrolnitrin) and may be of importance in the adaptation of microorganisms for biodegradation (Gorontzy *et al.*, 1994). The large majority of nitro-substituted pollutants entering the environment are due to anthropogenic activities (Rieger and Knackmuss, 1995).

1. Explosives, Derivatives and By-Products

Besides TNT, RDX, and HMX, a variety of other toxic nitro-substituted explosives may cause biological hazards at production facilities and disposal sites (Fig. 11; Gorontzy *et al.*, 1994; Bruns-Nagel *et al.*, 2000; Spain, 2000). Nitro-substituted explosives may be classified into three groups:

Heterocyclic nitramines. Hexahydro-1,3,5-trinitro-1,3,5-triazine (hexogen or RDX), 2,2′,4,4′,6,6′-hexanitrodiphenylamine (hexyl), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (octagen or HMX), and 2,4,6-trinitrophenylmethyl-nitramine (tetryl)

Aromatic nitro-substituted compounds. 2,4,6-Trinitrotoluene (TNT), 2,4,6-trinitrophenol (picric acid), and 1,3,5-trinitrobenzene (TNB)

Aliphatic nitro-substituted compounds. Nitroglycerin (GTN), nitroguanidine, and pentaerythritoltetranitrate (PETN)

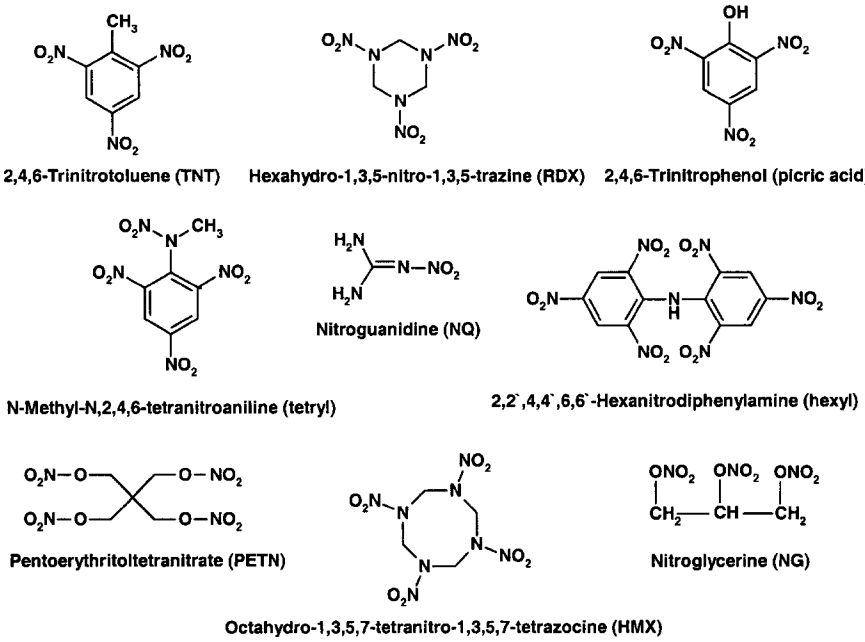

FIG. 11. Major nitro military explosives.

In addition, explosive-contaminated sites contain biotransformation derivatives of the parent chemical, e.g., TNT reduction and oxidation products (ADNTs, DANTs, 2-amino-4,6-dinitrobenzoic acid) (Spanggord *et al.,* 1982). Manufacture by-products are also commonly found in explosive-contaminated sites, e.g., dinitrotoluenes (DNTs) always associated with TNT production (Fig. 12; Spanggord *et al.,* 1982, Bruns-Nagel *et al.,* 2000). Explosive-contaminated soils often contain other pollutants: e.g., heavy metals, included in the casing of ammunition, and sodium azide or perchlorate, as part of the primary explosive (Smock *et al.,* 1976; Simini *et al.,* 1995; Sunahara *et al.,* 1999).

2. Other Nitro-Substituted Chemicals

In addition to explosives, hazardous nitro-substituted compounds are found in the industries involving pesticides, pharmaceuticals, solvents (e.g., nitrotoluene), polymers (e.g., DNTs), and dyes. Due to the chemical versatility of the nitro group, nitrophenols and nitrotoluenes are important industrial feedstocks (Nishino *et al.,* 2000). Nitroaromatic pesticides such as dinoseb, dinitrocresol, parathion, and methylparathion are intentionally released in soils and waters (Spain, 2000). Another group of nitroaromatic pollutants are synthetic fragrances included in

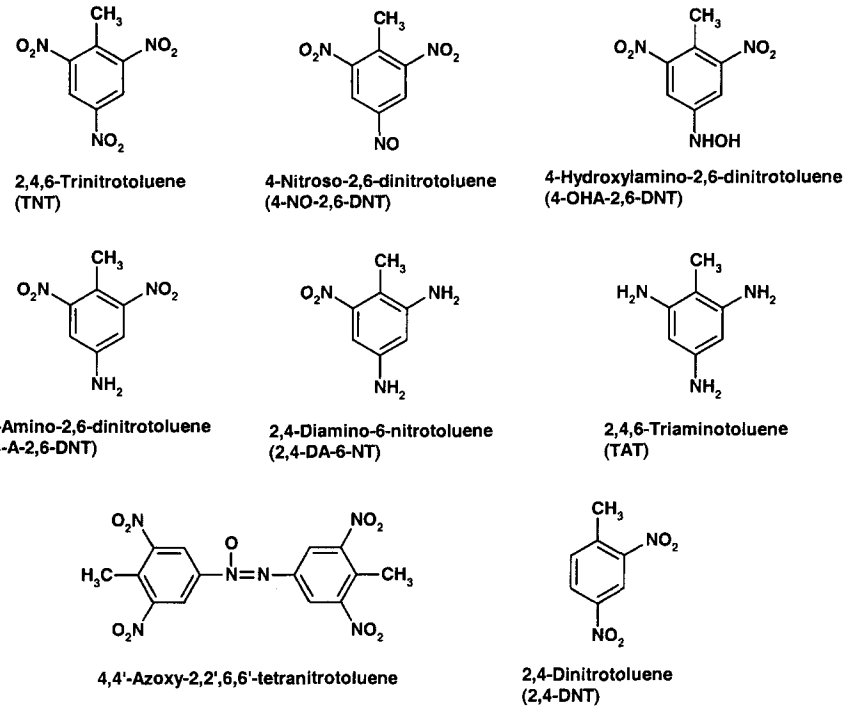

FIG. 12. TNT biotransformation derivatives and by-products.

cosmetics and lotions, such as 1-*t*-butyl-3,5-dimethyl-2,4,6-trinitrobenzene (musk xylene) and 1-*t*-butyl-3,5-dimethyl-2,6-dinitro-4-acetylbenzene (musk ketone) (Fig. 13; Bruns-Nagel *et al.*, 2000).

Although pesticides and simple nitroaromatic compounds are biodegradable by soil microflora, explosives are less easily transformed and often accumulate in soils and groundwater (Spain, 2000). However, all nitroaromatic compounds may be subject to microbial transformations. Some, such as picric acid, mono- and dinitrotoluenes, dinitrocresol, nitrobenzoates, and nitrophenols, can be used by microorganisms as sole

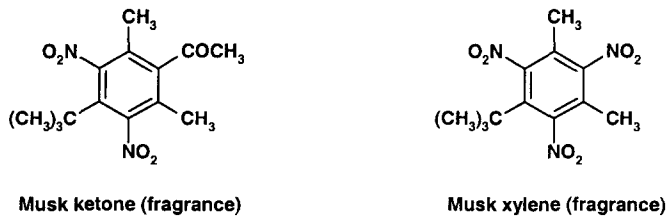

FIG. 13. Nitroaromatic musk fragrances.

carbon and energy sources. On the other hand, biodegradation of explosives such as TNT, RDX, and HMX is cometabolic, i.e., requires an additional growth substrate. Recently, phytoremediation of explosive-contaminated sites has shown considerable promise. In *situ* bioremediation seems to provide a cost-effective alternative to classical *ex situ* treatments of nitroaromatic-polluted sites, i.e., for soils, excavation followed by composting or incineration, and for water, pumping followed by carbon sorption (Spain, 2000). Moreover, while classical treatments are often quite devastating to existing ecosystems, biological processes may bring ecological benefits to the environment (Burken *et al.*, 2000).

C. Toxicity of Nitro-Substituted Explosives

"Toxicity of TNT may be caused by a one-electron reduction to form a nitro radical anion which reacts with oxygen to form superoxide radical and hydrogen peroxide and subsequently induces an oxidative stress type of cytotoxicity (Zitting et al., 1982)."

Actual mineralization of nitro-substituted explosives in soil is insignificant (Fernando *et al.*, 1990; Spiker *et al.*, 1992). Biotransformation by soil microflora results in a complex mixture of highly polar, mostly unidentified metabolites potentially harmful for the environment. The toxic effect of a complex mixture of polluting chemicals on an ecosystem may be extremely difficult to appreciate from the quantitative determination of each individual component, especially if they are not all completely identified.

1. Overview

Toxicity tests on biological systems provide a quick way to appreciate or to control the environmental relevance of a bioremediation process. Because a strong correlation exists between (geno)toxic effects of a chemical in bacteria and in mammals and because bacterial short-term genotoxicity tests are the simplest, the quickest, and the cheapest to perform, many have been proposed, the most important of which are the Ames test, the Mutatox® test, the Microtox® test, the Umu test, and the SOS chromotest (Honeycutt *et al.*, 1996). Due to their widespread use in industry and agriculture, the toxicity and metabolism of nitro-organic compounds have been widely documented (e.g., McNally, 1944; Nay *et al.*, 1974; Smock *et al.*, 1976; Won *et al.*, 1976; Yinon and Hwang, 1985; Honeycutt *et al.*, 1996; Green *et al.*, 1999; Lachance *et al.*, 1999; etc.).

The toxicity of TNT has been reported since the World War I among English ammunition workers. Symptoms include dermatitis, cyanosis, liver degeneration, and aplastic anemia (McNally, 1944). In mammals, nitroaromatic compounds can irreversibly oxidize haemoglobin

to methaemoglobin, which is unable to carry oxygen in the blood. In addition, some nitrocompounds (e.g., 1,2-dinitrophenol) are uncoupling agents of oxidative phosphorylation. In laboratory studies, TNT has been found to be toxic—acute as well as chronic toxicity—for all living organisms, including bacteria (Won *et al.*, 1976; Drzyga *et al.*, 1995), algae (Smock *et al.*, 1976; Won *et al.*, 1976), plants (Peterson *et al.*, 1996), animals (Nay *et al.*, 1974; Smock *et al.*, 1976; Honeycutt *et al.*, 1996; Green *et al.*, 1999), and humans (McNally, 1944; Yinon and Hwang, 1985; Honeycutt *et al.*, 1996). In addition, TNT is mutagenic as assayed in tests using both mammalian and prokaryotic cells (Won *et al.*, 1976; Honeycutt *et al.*, 1996; Lachance *et al.*, 1999).

The toxicity of the nitramines RDX and HMX has been determined for several organisms, including bacteria, algae, aquatic invertebrates (Sunahara *et al.*, 1999; Talmage *et al.*, 1999), earthworms (Robidoux *et al.*, 1999), and mammals (Talmage *et al.*, 1999). RDX, which was formerly used as a rat poison, is known to exhibit renal and gastrointestinal toxicity and to affect the central nervous system, leading to unconsciousness and epileptiform seizures (Harvey *et al.*, 1991). RDX is classified as a possible carcinogen by the U.S. EAP (class C) (Binks *et al.*, 1995).

2. Cytotoxicity

On the basis of cytotoxicity tests using bacterial and mammalian cells, TNT and TNB are highly toxic, RDX is slightly toxic, and HMX is nontoxic, though the toxicity may be limited by water solubility (Smock *et al.*, 1976; Won *et al.*, 1976; Drzyga *et al.*, 1995; Sunahara *et al.*, 1999; Green *et al.*, 1999). TNT reduction metabolites are less toxic than the parent compound and the order of toxicity levels follows the order of successive reductions of the nitro groups: TNT > ADNTs > DANTs > 2,4,6-triaminonitrotoluene (TAT), suggesting that microbial reduction of TNT may be linked to a detoxification process (Drzyga *et al.*, 1995; Honeycutt *et al.*, 1996; Sunahara *et al.*, 1999; Lachance *et al.*, 1999). The toxicity of TNT and of its main reduction derivatives, as assayed both by the bioluminescence Microtox® test (using the bacterium *Vibrio fischeri*) and the 96-h-growth inhibition test (using the green alga *Selenastrum capricornutum*), are presented as follows (Sunahara *et al.*, 1999): TNT: IC_{20}[20] = 0.5 μM, EC_{50}[21] = 3 μM > ADNTs: IC_{20} = 50–100 μM, EC_{50} = 13–60 μM > DANTs: IC_{20} = 120–140 μM, EC_{50} = 210–290 μM > TAT: IC_{20} = 170 μM. For the different isomers, the order of toxicity level observed in bacteria (*V. fischeri*): 4-amino-2,6-dinitrotoluene (4-A-

[20]IC_{20} (inhibition concentration) is the concentration of toxicant required to decrease by 20% bioluminescence in the Microtox® test (Sunahara *et al.*, 1999).

[21]EC_{50} (effect concentration) is the concentration of toxicant required for 50% inhibition of alga growth in the 96-h-growth inhibition test (Sunahara *et al.*, 1999).

2,6-DNT) > 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) and 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT) > 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT) differs from that observed in eukaryotes (*S. capricornutum*): 2-A-4,6-DNT > 4-A-2,6-DNT and 2,6-DA-4-NT > 2,4-DA-6-NT (Honeycutt *et al.*, 1996; Lachance *et al.*, 1999). Toxicity tests on mammalian cells (rat hepatoma and Chinese hamster ovary (CHO) cells) show a comparable order of toxicity effect: TNT > 2-A-4,6-DNT > 4-A-2,6-DNT > 2,4-DA-6-NT (Honeycutt *et al.*, 1996). In addition, the same tests reveal that three other important TNT metabolites, 4-hydroxylamino-2,6-dinitrotluene (4-OHA-2,6-DNT), 2,2′,6,6′-azoxy-4,4′-tetranitrotoluene, and 2,2′,4,4′-azoxy- 6,6′-tetranitrotoluene, exhibit a toxicity comparable to TNT (Honeycutt *et al.*, 1996). It is noteworthy that some reduced derivatives of nitro-substituted pollutants (e.g., 2,4-DNT) may be more toxic than the parent compound (Drzyzga *et al.*, 1995).

3. Mutagenicity

Very little consensus exists concerning the mutagenicity of nitro-substituted compounds. *In vitro* assays using bacterial and mammalian strains indicate that TNT is mutagenic, both with and without metabolic activation (S9)[22] (Kaplan and Kaplan, 1982; Spanggord *et al.*, 1982; Styles and Cross, 1983; Honeycutt *et al.*, 1996; Lachance *et al.*, 1999), but negative results have also been reported (Won *et al.*, 1976; Honeycutt *et al.*, 1996). TNB also exhibits a strong mutagenicity. Despite the lack of agreement in the literature, nitroheterocyclic explosives RDX and HMX are more often described as nonmutagenic (Spanggord *et al.*, 1982; Lachance *et al.*, 1999). However, even wider disagreements persist about the mutagenicity of TNT-reduced metabolites and about the effect of metabolic activation (Won *et al.*, 1976; Spanggord *et al.*, 1982; Styles and Cross 1983; Honeycutt *et al.*, 1996; Lachance *et al.*, 1999). Erratic results reported suggest that reduced metabolites of RDX, HMX, and TNT are weak mutagens detected only after a long exposure to high concentrations (i.e., *Salmonella* fluctuation test; Lachance *et al.*, 1999).

4. Field Toxicity Measurements

Laboratory results are supported by field experiments, reporting the toxicity of nitro-energetic compounds to plants, earthworms, and soil microbial communities (Simini *et al.*, 1995; Fuller and Manning, 1998).

[22]Bacteria lack the cytochrome P-450 enzyme system involved in the metabolism of toxic chemicals in higher organisms. This important metabolic effect can be imitated in bacterial assays by the addition of exogen cytochrome P-450 system, coming from arochlor 1254-induced rat liver homogenate S9 (i.e., metabolic activation). Promutagens are detected with S9 addition and direct-action mutagens are detected without S9 (Honeycutt *et al.*, 1996).

However, few studies have examined the toxicity of nitro-substituted explosives against indigenous microbes in soils, even though they constitute important bioindicators of soil pollution (Fuller and Manning, 1998).

The toxicity of a soil may be evaluated by different methods based either on a direct contact between soil and organisms (i.e., invertebrates or plants) or on the extraction (or leaching) of the contaminants from the soil, followed by chemical and toxicological analysis. As assayed by different indigenous microbial activities in soil (potential nitrification, nitrogen fixation, and dehydrogenase activity), the NOEC[23] and LOEC[24] of TNT may be as low as 0.4 and 1.0 mg kg^{-1} soil, respectively (Gong *et al.,* 1999). For higher organisms such as earthworms (*Eisenia andrei*), the nonobserved-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) of TNT have been reported to be 320 and 420 mg kg^{-1} soil, respectively (Robidoux *et al.,* 1999).

Exposure of TNT and its first reduced metabolites, ADNTs, to near ultraviolet light (NUV) results in an increase of the toxicity for several organisms, such as bacteria and marine invertebrates, likely related to the production of intermediate anion free radicals (Johnson *et al.,* 1994a,b).

5. Mechanisms of Toxicity

TNT, RDX, and HMX metabolism in animals, including humans, mainly involves a two-electron multistep reductive pathway of nitro substituents to give successively nitroso, hydroxylamino, amino, and diamino derivatives (Won *et al.,* 1976; Yinon and Hwang, 1985; Leung *et al.,* 1995).

As observed with other toxic compounds, toxicity of nitro-substituted explosives may be the result of covalent binding to cellular proteins. However, formation of the nitro-compound–protein adduct requires prior bioactivation, likely by transforming the parent pollutant to reactive nitroso intermediates, which may react with protein sulfhydryl groups to give semimercaptals. By rearrangement, the latter may form corresponding harmful sulfinamides (Leung *et al.,* 1995).

The complete reduction of a nitro group to an amino group requires the transfer of six electrons. The metabolism of nitro-substituted compounds in higher organisms can proceed by one-electron reduction mechanisms, leading to the formation of reactive nitroaryl anion radicals ($Ar\text{-}NO_2^{\bullet -}$),[25] which may undergo covalent binding with biological

[23]NOEC is the nonobserved effect concentration (Gong *et al.,* 1999).

[24]LOEC is the lowest observed effect concentration (Gong *et al.,* 1999).

[25]Theoretically, three potential free radicals can be generated: the nitro anion radical ($R\text{-}NO_2^{\bullet -}$), the hydronitroxide radical (R-NHO$^{\bullet}$), and the amino cation radical ($R\text{-}N^{\bullet}H^{2+}$). However, only $R\text{-}NO_2^{\bullet -}$ has been observed *in vivo* (Moreno and Docampo, 1985).

molecules (i.e., DNA, proteins, thiols) (Moreno and Docampo, 1985). Alternatively, Ar-$NO_2^{\bullet -}$, may react with O_2 (i.e., autooxidation), giving the parent chemical and the superoxide free radical $O_2^{\bullet -}$ (Eq. (17); Leung *et al.*, 1995; Hetherington *et al.*, 1996):

$$\text{Ar-}NO_2^{\bullet -} + O_2 \longrightarrow \text{Ar-}NO_2 + O_2^{\bullet -} \tag{17}$$

Dismutation of $O_2^{\bullet -}$ may result in ROS and lead to oxidative cell damage (Section III.C.1; Leung *et al.*, 1995; Hetherington *et al.*, 1996). When oxygen is absent, Ar-$NO_2^{\bullet -}$ undergoes spontaneous dismutation, giving the parent nitro compound and the corresponding nitroso derivative (Eq. (18); Moreno and Docampo, 1985):

$$2\text{Ar-}NO_2^{\bullet -} + 2H^+ \longrightarrow \text{Ar-}NO_2 + \text{Ar-NO} + H_2O \tag{18}$$

Both reactions (Eqs. (17) and (18)) can be seen either as a detoxification mechanism, by destruction of the nitro anion radical, or as a pathway to more toxic species, i.e., nitroso compounds or ROS (Moreno and Docampo, 1985). On the other hand, the presence of oxygen favors the one-electron nitroreduction and inhibits the two-electron nitroreduction (Leung *et al.*, 1995; Hetherington *et al.*, 1996).

D. Biodegradation of the Nitroaromatic Explosive TNT

"Whereas mono- and occasionally dinitroaromatic compounds can be attacked by oxygenases and mineralized by oxidative pathways, only initial reductive mechanisms have been described so far for productive catabolism of trinitroaromatic compounds (Lenke et al., 2000)."

Due to the strong electron-withdrawing character of nitro groups, nitroaromatic compounds, such as TNT, are electron deficient, a feature impeding electrophilic attack by oxidative enzymes. The TNT structure has to be seen as the resonance hybrid of several canonical forms (Fig. 14; Rieger and Knackmuss, 1995). Part of the π-electrons of the aromatic ring are withdrawn by the nitro groups, making the nucleus

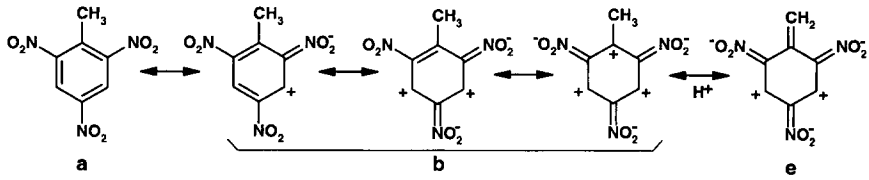

FIG. 14. Canonical forms describing the resonance hybrid of TNT, adapted from Rieger and Knackmuss (1995). (a) One form of the Kekulé structure, (b) some of the potential ionic contributions, (c) one form describing the hyperconjugative effect of the methyl group.

electrophilic. The methyl group of TNT is an electron-donating group which weakens the electron deficiency of the aromatic cycle, but makes itself less susceptible to oxygenase-catalyzed side-chain hydroxylation, by contributing a hyperconjugative form to the TNT structure (Rieger and Knackmuss, 1995). Besides such electron effects, bulky nitro groups present steric constraints impeding enzymatic attack of the molecule (Fritsche *et al.,* 2000). As a consequence, almost every living organism can reduce the nitro substituents of TNT to amino groups, mainly producing aminonitrotoluenes (ADNTs) and diaminonitrotoluenes (DANTs) (Won *et al.,* 1976). Even though the substitution of an electron-withdrawing nitro group by an electron-donor amino group increases the susceptibility of the molecule to electrophilic attack, most microorganisms are unable to further metabolize the resulting nitroanilines, which accumulate in the environment. The observation of TNT-contaminated soils 50 years after World War II indicates a very low intrinsic bioremediation capacity (Fritsche *et al.,* 2000). Ligninolytic white-rot fungi, able to metabolize and partially mineralize TNT, represent a notable exception. In addition to biological processes, some abiotic transformation of nitro-substituted compounds may occasionally occur (Gorontzy *et al.,* 1994).

1. Abiotic Transformations of TNT

In sterile soil, neither evaporation nor hydrolysis has been reported to significantly reduce the concentration of TNT (Spanggord *et al.,* 1982). However, the molecule may be degraded by photolysis when exposed to sunlight and water, giving an undefined toxic mixture of transformation products (known as pink water), including trinitroaromatic derivatives, aromatic nitroanilines, and azo or azoxy condensation products (Spanggord *et al.,* 1982). On the other hand, as a very oxidized molecule, TNT may be subject to abiotic reduction under anaerobic conditions. Numerous minerals and organic species in soil have a redox potential low enough to theoretically reduce TNT (e.g., reduced iron and sulfur species, quinone moieties, organic complexes of transition metals). However, the reduction of a nitro group to an amino group requires the stepwise transfer of six electrons. The transfer of the first electron to form the reactive nitroaryl anion radical $Ar\text{-}NO_2^{\bullet -}$ is an endergonic reaction, which requires the overcoming of a considerable energy barrier. Consequently, abiotic reduction of nitroaromatic compounds in soil, even if thermodynamically favored, may be extremely slow. The reduction process results in the formation of aromatic polyamines, which are characterized by a higher solubility, a higher mobility, and a higher tendency to bind to the soil matrix (Haderlein *et al.,* 2000).

2. Bacterial Transformation of TNT

For several decades, bacterial metabolism of polynitroaromatic compounds has been known to lead to the formation of dead-end reduced amino-nitro derivatives, which are not further mineralized (Kaplan, 1994). It is important to distinguish between *catabolic biodegradation,* whose ultimate and preferred stage implicates mineralization, and *cometabolic transformation.* Cometabolic transformation of a compound denotes its nonspecific bioconversion by enzymes involved in the catabolism of other growth substrates. On the other hand, catabolic biodegradation (mineralization)[26] of a compound indicates its complete conversion to inorganic products to yield energy and/or biomass. The bacterial mineralization process is thermodynamically self-sustaining and provides a selective advantage to the degradative organism (Nishino *et al.,* 2000). Moreover, mineralization products are harmless for the environment, whereas transformation products may be equally or even more toxic than the parent molecule (Drzyzga *et al.,* 1995).

Biodegradation of nitroaromatic pollutants using bacterial strains has been extensively reviewed in the literature (Walsh, 1990; Higson, 1992; Kaplan, 1994; Gorontzy *et al.,* 1994; Preuss and Rieger, 1995; Rieger and Knackmuss, 1995; Spain, 1995; Lenke *et al.,* 2000; Nishino *et al.,* 2000).

a. Historic. The first attempts at biodegradation of nitroaromatic explosives in activated sludge systems were unsuccessful (Ruchhoft *et al.,* 1945). Later, microbial reduction of TNT was reported in both aerobic and anaerobic systems, in the presence of an exogenous carbon source. The main transformation products in aerobiosis (*Pseudomonas* sp.) were identified as hydroxylaminodinitrotoluenes (OHADNTs), ADNTs, DANT, and azoxy condensation products (Nay *et al.,* 1974; McCormick *et al.,* 1976; Won *et al.,* 1976). Anaerobic bacteria (*Desulfovibrio* sp., *Veillonella alcalescens*) were reported to achieve a further reduction of the TNT molecule, giving TAT (McCormick *et al.,* 1976). *Pseudomonas* strains are able to use 2,4-DNT —a major component of TNT ammunition wastes—as a sole carbon source, as well as to transform TNT after growing on DNT (Spanggord *et al.,* 1991). The first indication for a possible bacterial mineralization of ^{14}C-U-ring-labeled TNT was obtained with two *Pseudomonas* strains, even though no significant release of

[26]In the bacterial world, mineralization rhymes with metabolism, i.e., the complete oxidative breakdown of an organic compound to its mineral components, providing energy and a carbon/nitrogen source. By contrast, ligninolytic white-rot fungi proceed the mineralization of lignin and of numerous xenobiotic compounds through nonspecific and mainly nonproductive (i.e., cometabolic) mechanisms sustained by the consumption of a metabolizable cosubstrate.

$^{14}CO_2$ was reported (Traxler, 1974). Naumova *et al.* (1988) described the degradation of 2,4-DA- 6-NT by *Pseudomonas fluorescens,* which would lead to a putative ring opening. More recently, a genetically engineered hybrid, resulting from the transfer of a TOL (toluene-degrading) plasmid of *Pseudomonas putida* into a TNT-tolerant *Pseudomonas* sp., designated clone A, was reported to use TNT as sole carbon and nitrogen source (Duque *et al.,* 1993).

b. Mono- and Dinitroaromatic Compounds. Aerobic bacterial catabolism of mono- and dinitroaromatic compounds may be initiated by mono- or dioxygenases, involving the elimination of nitrite(s) and generating (poly)hydroxylated aromatic intermediates. The latter may undergo a dioxygenase-catalyzed aromatic ring cleavage leading to complete metabolism of the molecule (Fig. 15, routes I and II; Spanggord *et al.,* 1991; Spain, 1995; Lenke *et al.,* 2000; Nishino *et al.,* 2000). Alternatively, mono- and dinitroaromatic compounds may undergo a reductive hydrogenation of the aromatic cycle itself, through the formation

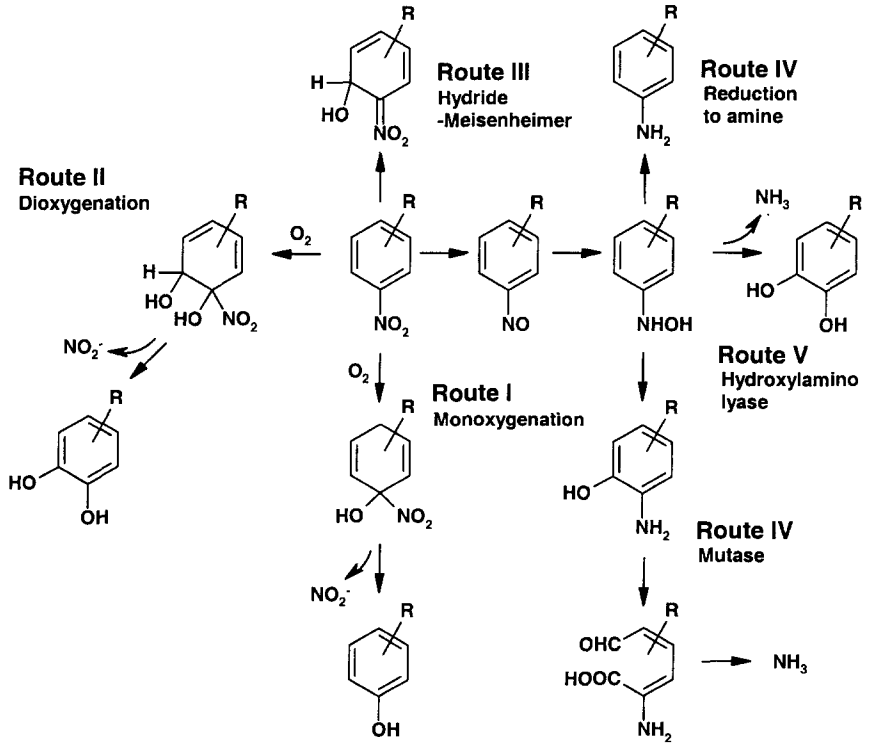

FIG. 15. Mechanisms for bacterial degradation of mono- and dinitroaromatic compounds. Adapted from Lenke *et al.* (2000).

of a hydride–Meisenheimer complex, leading to a release of nitrites and a ring opening (Fig. 15, route III; Lenke *et al.,* 1992; Lenke and Knackmuss, 1992,1996). The initial removal of a nitro group as a nitrite could confer a selective advantage to microorganisms, since it provides a nitrogen source using a single enzyme, rather than a complete catalytic sequence, required to achieve mineralization. On the other hand, the presence of electrophilic nitro groups favors the reduction of (poly)nitroaromatic compounds by microorganisms. Nearly all bacterial strains may proceed to the partial reduction of nitro groups to hydroxylamino groups, via nitroso intermediates (McCormick *et al.,* 1976). Except for TNT, further reduction into amino groups only occurs under anaerobic conditions (Fig. 15, route IV). The initial reduction, leading to hydroxylamino metabolites, has been described as a key reaction for further productive catabolic routes involving the enzymatic attack of hydroxylaminolyases or mutases (Fig. 15, routes V and VI; Schenzle *et al.,* 1999).

c. Trinitro-Substituted Aromatic Compounds. Increasing the number of electron-withdrawing nitro substituents generates high redox potential making the molecule more susceptible to an initial reduction. The trinitro-substituted explosive TNT may undergo a sequential reduction of the nitro groups to form ADNT and DANT, via nitroso and hydroxylamino intermediates (Lenke and Knackmuss, 1992). Spontaneous condensation of the latter produces binuclear azoxy compounds. However, these TNT reduction products are only poorly further metabolized under aerobic conditions. Because reduced amino groups increase the reactivity of the aromatic ring, a further oxidative metabolism should be possible, even though the majority of investigations reveal insignificant mineralization of TNT in bacterial systems (Lenke *et al.,* 2000).

Alternatively, bacterial strains can achieve a reductive hydrogenation on the aromatic cycle of picric acid and TNT, without a reduction of the nitro substituents (Lenke and Knackmuss, 1992; Lenke *et al.,* 1992; Vorbeck *et al.,* 1998). The electron deficiency of the aromatic ring favors an enzymatic NADPH-dependent reductive attack on the π-electron system (i.e., the aromatic cycle) by the transfer of a hydride anion (H^-) to form a hydride–Meisenheimer complex, which is stabilized by resonance, preventing unproductive reduction of the nitro groups (Fig. 16; Lenke and Knackmuss, 1992; Duque *et al.,* 1993; Vorbeck *et al.,* 1998). In contrast to piorate, which, after the hydride anion transfer, is susceptible to a nitrite release and enters a productive metabolic pathway, the hydride–Meisenheimer complex of TNT undergoes a second hydride transfer, giving a dihydride complex, preventing any further breakdown of the molecule (Fig. 16; Duque *et al.,* 1993; Vorbeck *et al.,* 1998).

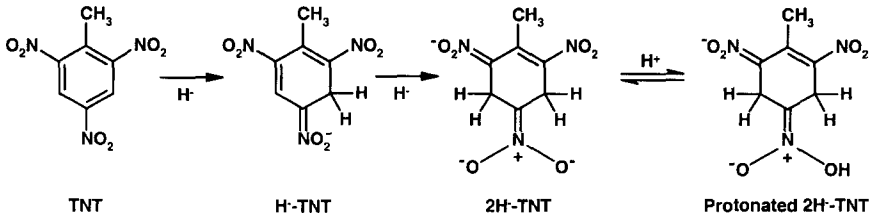

FIG. 16. (Di)hydride complexes of TNT. Adapted from Lenke *et al.* (2000).

During the reductive dinitration pathway, trinitroaromatic compounds may serve as sole source of nitrogen for bacteria.

d. Anaerobiosis. Under anaerobic, strongly reducing conditions ($E_0 < -200$ mV), all (poly)nitrotoluenes tested, including partially reduced and binuclear azoxy derivatives, are completely reduced to the corresponding (poly)amino metabolites, by both biotic and abiotic mechanisms (Rieger and Knackmuss, 1995). The three nitro groups of the TNT molecule undergo a complete reduction to give triaminonitrotoluene (TAT). Resulting reduced toluidines are only slowly further degraded by microbes and may constitute undesirable harmful dead-end products (Rieger and Knackmuss, 1995). However, TNT has been shown to be used, not only as a nitrogen source but also as a terminal electron acceptor (respiration of TNT) by a *Pseudomonas* sp. (Esteve-Nuñez *et al.,* 2000), which considerably raises the interest in anaerobic processes for cleaning up TNT-polluted environments. In addition, anaerobic bioremediation of nitroaromatic pollutants offers the potential advantages of a rapid reduction (at low redox potential) and of a limitation of the polymerization reactions, which occur mainly in the presence of oxygen (Esteve-Nuñez *et al.,* 2000).

3. Transformation of TNT by White-Rot Fungi

Being a highly oxidized molecule and because of the electron-withdrawing effect of the three nitro substituents, TNT is easily reduced by almost every living organism[27] (Won *et al.,* 1976; Fernando *et al.,* 1990; Bumpus and Tatarko, 1994). When TNT is reduced, one- or two-electron-withdrawing nitro groups (under aerobiosis) are replaced by electron-donor amino groups, facilitating the electrophilic attack by oxidative enzymes (Field *et al.,* 1993). In contrast to bacteria largely unable to metabolize TNT reduced products, white-rot fungi, producing extracellular ligninolytic enzymes, are capable of oxidatively

[27]Bacteria first reduce the *o*-nitro substituent (Naumova *et al.,* 1988), while fungi preferentially reduce the *p*-nitro substituent (Parrish, 1977).

mineralizing the aromatic cycle of TNT derivatives. However, TNT mineralization does not mean that the molecule can serve as a carbon and/or energy source. TNT metabolism remains a cometabolic process and the application of ligninolytic fungi for explosive bioremediation requires bioaugmentation or biostimulation, i.e., the addition of a growth substrate (Bumpus and Tatarko, 1994; Fernando *et al.,* 1990; Spiker *et al.,* 1992; Michels and Gottschalk, 1994; Scheibner *et al.,* 1997).

Fungal biodegradation of nitroaromatic explosives has been the subject of numerous publications, reviewed by Higson (1992), Hammel (1992), Field *et al.* (1993), Barr and Aust (1994), Bennett (1994), Paszczynski and Crawford (1995), and Reddy (1995).

a. Historic. The first report on fungal biodegradation of TNT referred to the fungus *Rhizopus nigricans,* capable of transforming almost completely 100 mg liter^{-1} of TNT (Klausmeier *et al.,* 1974). Shortly after, in a screening of 190 fungal strains belonging to nine genera, 183 strains were shown to reduce TNT to 4- hydroxylamino-2,6-dinitrotoluene (4-OHA-2,6-DNT), 4-A-2,6-DNT, and 2,4-DA-6-NT, while only seven strains were able to transform DNT (Parrish, 1977). However, despite the identification of fungi in the microbial consortia involved in composting processes (Kaplan and Kaplan, 1982), the absence of mineralization and the accumulation of reduction derivatives led to a pessimistic conclusion, which constituted the conventional wisdom for over a decade (Bennett, 1994). During the 1980s, the white-rot basidiomycete *P. chrysosporium* was shown to mineralize diverse toxic organopollutants such as polychlorinated biphenyls (PCB), pesticides (DDT), and benzo[*a*]pyrene (Bumpus *et al.,* 1985; Bumpus and Aust, 1987; Barr and Aust, 1994). Since then, the number of xenobiotic molecules reported to be transformed and partially mineralized by white-rot fungi has increased continuously (Aust, 1990; Higson, 1992; Barr and Aust, 1994; Paszczynski and Crawford, 1995; Reddy, 1995). The remarkable versatility of the biodegradation system was attributed to the ability of white-rot fungi to degrade lignin (Barr and Aust, 1994). The potential of *P. chrysosporium* for the biodegradation of TNT has been pioneered by the group of Steven D. Aust at Utah State University (Logan, UT). Under ligninolytic conditions (i.e., nitrogen-limited medium), submerged cultures of *P. chrysosporium* were able to mineralize up to 50% of the added ^{14}C-U-ring-labeled TNT, even though the mineralization rate in soil was much lower (Fernando *et al.,* 1990; Spiker *et al.,* 1992). A process for bioremediation of TNT by white-rot fungi was patented (Lebron *et al.,* 1992).

Even though ligninolytic enzymes were early postulated to play a role in fungal xenobiotic degradation, their actual involvement as well

as their individual implication in the oxidative breakdown of TNT and TNT reduced metabolites remained unclear for a long time, before a few isolated studies with purified ligninolytic peroxidases brought some insight to the process (Stahl and Aust, 1993a; Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994; Hofrichter *et al.*, 1998a; Van Aken *et al.*, 1999a,b, 2000b). Some progress has also been made in the identification of the numerous metabolites composing TNT degradation mixtures (Michel and Gottschalk, 1995; Hawari *et al.*, 1999), as well as in the determination of the (ultimate) oxidizing agents possibly involved in the process (Urzùa *et al.*, 1998; Hofrichter *et al.*, 1998b; Van Aken *et al.*, 2000a). Generally speaking, the mechanistic basis for such nonspecific and powerful destructive biochemical reactions, which is unique in the living world, continues to intrigue most scientists and might have unsuspected implications far outside the framework of environmental biotechnologies.

b. The Pathway. Mineralization of TNT by white-rot fungi involves two distinct steps. First, TNT is reduced stepwise into NODNT, OHADNT, ADNT, and DANT (Stahl and Aust, 1993a; Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994; Hofrichter *et al.*, 1998a; Van Aken *et al.*, 1999b, Hawari *et al.*, 1999). The transient reduced NODNTs and OHADNTs may spontaneously condense to form binuclear azoxy compounds (Fig. 17; Michel and Gottschalk, 1994). This first reduction step requires the intact mycelial material and involves a NADPH-dependent membrane-bound redox system associated with

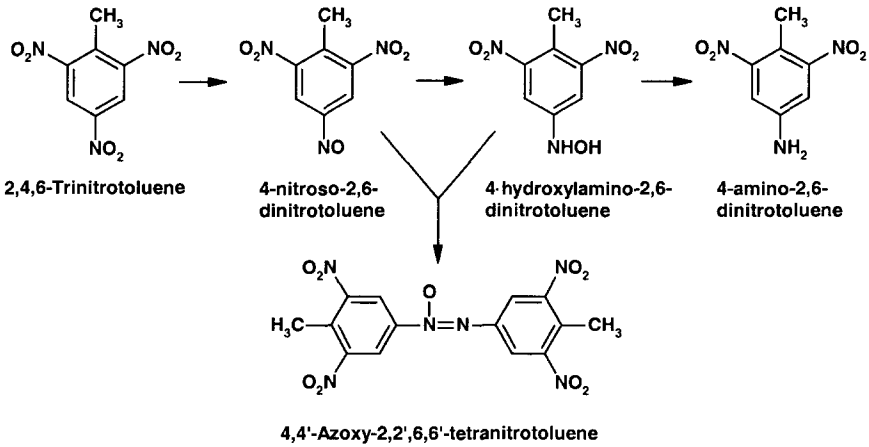

FIG. 17. Reduction of TNT and formation of azoxy compounds. Adapted from Michels and Gottschalk (1994).

a proton secretion mechanism (Stahl and Aust, 1993b), an intracellular NADPH-dependent oxidoreductase (Michels and Gottschalk, 1994), and/or a membrane-bound nitroreductase (Rieble *et al.*, 1994). Cell surface reduction systems in fungi have been hypothesized as a protection mechanism against foreign toxic compounds as well as against reactive species associated with the fungal degradation processes. Such a protective function might be particularly relevant among white-rot fungi, which produce ligninolytic extracellular enzymes, which can subsequently generate damaging (oxygen) radical species (Section III.C.1).

The extracellular oxidative enzymes of the ligninolytic system seem not to be involved in the reduction stage (Fritsche *et al.*, 2000). In a second, oxidative step, the reduction products of TNT—rendered more susceptible to oxidation reactions—are transformed and/or mineralized by the ligninolytic extracellular peroxidases (Stahl and Aust, 1993a; Michels and Gottschalk, 1994). In contrast to initial reduction of the TNT molecule, which can be achieved by almost every living organism, the further oxidative transformation of TNT reduction metabolites remains the property of white-rot fungi and is likely the rate-limiting step of the whole mineralization pathway (Michels and Gottschalk, 1994). The role played by each individual enzyme on the different TNT reduction derivatives remained for a long time unclear and incomplete. Because LiP seemed to play a predominant role in the ligninolysis process (i.e., breakdown of nonphenolic lignin model dimers),[28] more attention was first given to the bioremediation capacities of LiP (Section II.D.4; Buswell and Odier, 1987). LiP was reported to transform OHADNTs, which was also described as an inhibitor of the veratryl alcohol oxidizing activity of the enzyme (Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994). In addition, it was suggested that ADNTs do not serve as substrates for LiP (Bumpus and Tatarko, 1994), but possibly for MnP (Stahl and Aust 1993b). Finally, 2-amino-4-formamido-6-nitrotoluene, a potential intermediate in the reduction pathway of 4-A-2,6-DNT (4-amino-2,6-dinitrololuene) into 2,4-DA-6-NT (2,4-diamino -6-nitrotoluene), was reported to be a substrate for LiP (Michels and Gottschalk, 1994). Despite these partial results, LiP failed to explain the extensive TNT bioremediation achieved by white-rot fungi. Moreover, the mineralization of TNT by white-rot fungi correlates better with the MnP than with the LiP enzymatic activity, suggesting a predominant role of this peroxidase in fungal bioremediation of nitroaromatic compounds (Fritsche *et al.*, 2000). Wolfgang Fritsche and co-workers from the Friedrich Schiller University of Jena (Jena, Germany) have pioneered *in*

[28]MnP is not able to catalyze the cleavage of nonphenolic lignin model dimers (Kirk and Farrel, 1987).

vitro degradation experiments using cell-free MnP preparations, showing that MnP from the ligninolytic fungi *Stropharia rugosoannulata* and *Nematoloma frowardii* were capable of extensively mineralizing TNT and its main reduction derivatives, as well as a variety of other toxic xenobiotic pollutants (Hofrichter *et al.,* 1998a). Similar results were obtained using the white-rot fungus *P. radiata,* a basidiomycete closely related to *P. chrysosporium* (Van Aken *et al.,* 1999a). Reduced thiols, such as cysteine or glutathione (GSH), were previously shown to improve the degradation capacity of fungal MnP on both lignin synthetic substructures and natural polymeric lignin (Forrester *et al.,* 1988, Wariishi *et al.,* 1989b). Similarly, small amounts of GSH strongly enhanced the mineralization rate of nitroaromatic compounds (Hofrichter *et al.,* 1998a; Van Aken *et al.,* 1999a). Incubating the first rather stable TNT reduction derivative, i.e., 4-OH-4,6-DNT, in a cell-free MnP preparation,Van Aken *et al.* (1999b) isolated and identified for the first time 4-nitroso-2,6-dinitrotoluene (4-NO-2,6-DNT) as an oxidation product of 4-OH-4,6-DNT by MnP, suggesting the existence of futile cycles in the fungal degradation process of TNT (Bumpus and Tatarko, 1994). Small concentrations of GSH have been shown to reduce 4-OH-2,6-DNT into 4-A-2,6-DNT, which is further extensively mineralized (Van Aken *et al.,* 1999b). Besides the fraction of TNT actually mineralized into CO_2 and water, a mixture of unidentified polar aliphatic metabolites ranging from C_1 to C_3 were recovered, suggesting a nonspecific oxidative ring cleavage, so-called "enzymatic combustion" in early reports (Kirk and Farrel, 1987; Hofrichter *et al.,* 1998a; Van Aken *et al.,* 1999a,b; Fritsche *et al.,* 2000). Using *in vitro* LiP preparation from *P. chrysosporium,*Van Aken *et al.* (2000b) have reported that this enzyme is able to convert 2-A-4,6-DNT and 2,4-DA-6-NT into unidentified polar metabolites, even though no mineralization was detected. TNT itself is not significantly transformed (Van Aken *et al.,* 2000b). At the same time, Hawari *et al.* (1999) isolated from ligninolytic cultures of *P. chrysosporium* numerous TNT metabolites, which were classified into primary and secondary products. Primary products are produced prior to the ligninolytic phase and include first reduction metabolites of TNT: NODNT, OHADNTs, and ADNTs, and their transformation derivatives through Bamberger rearrangement of OHADNT (i.e., aminophenols) and through chemical condensation of NODNT and OHADNT (i.e., azoxy compounds) (Fig. 18; Hawari *et al.,* 1999). Secondary products are generated in the presence of ligninolytic peroxidases (LiP and MnP) and result mostly from the acylation of TNT reduction derivatives (i.e., acetylated and formylated ADNT, acetylated DANT, and acetylated OHADNT) (Fig. 18; Hawari *et al.,* 1999). It was suggested that the acylation of nitroanilines proceeds in a purpose of detoxification. It is noteworthy

that the acylated derivatives may be intermediates in the formation of DANT (Michels and Gottschalk, 1994). In addition, secondary products come from the stepwise reduction of azoxy dimers to azo and hydrazo derivatives (Fig. 18; Hawari *et al.*, 1999). Finally, it was suggested that the weak TNT mineralization frequently observed might result on the formation of dead-end products derouting the mineralization process.

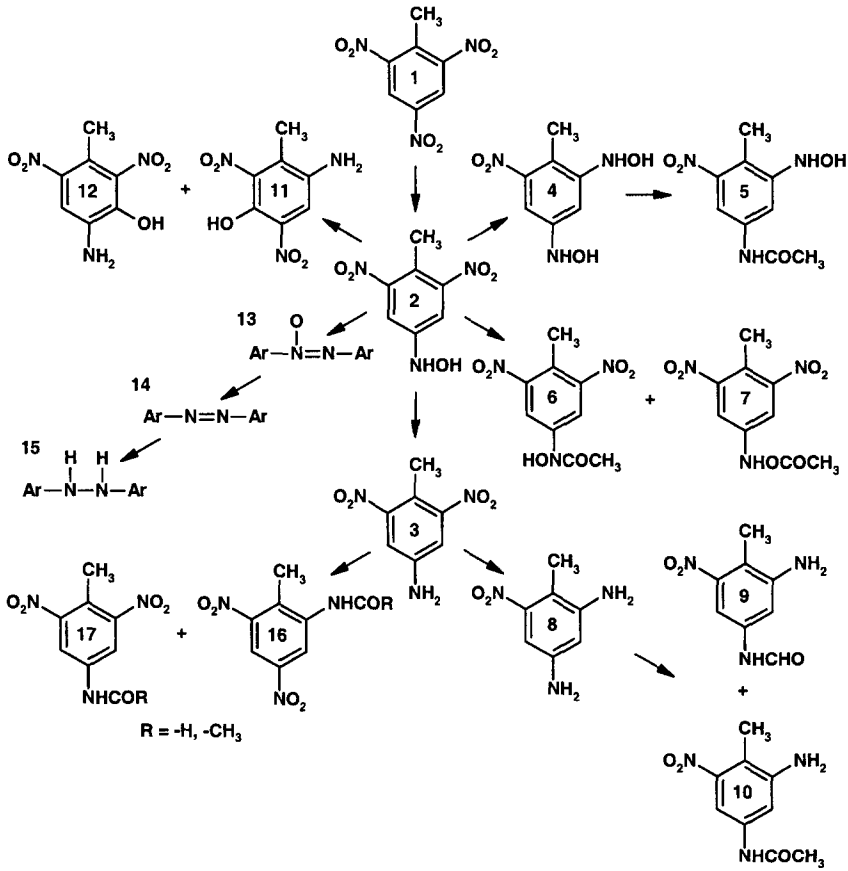

FIG. 18. Products and intermediates generated during the biodegradation of TNT by P. chrysosporium. Adapted from Hawari *et al.* (1999). (**1**) 2,4,6-trinitrotoluene, (**2**) 4-hydroxylamino-2,6-dinitrotoluene, (**3**) 4-amino-2,6-dinitrotoluene, (**4**) 2,4-dihydroxylamino-6-nitrotoluene, (**5**) 4-*N*-acetylamido-2-hydroxylamino-6-nitrotoluene (**6**) 4-*N*-acetylhydroxy-2,6-dinitrotoluene, (**7**) 4-*N*-acetoxy-2,6-dinitrotoluene, (**8**) 2,4-diamino-6-nitrotoluene, (**9**) 2-amino-4-formamido-6-nitrotoluene, (**10**) 2-amino-4-acetylamido-6-nitrotoluene, (**11**) 2-amino-5-hydroxy-4,6-dinitrotoluene, (**12**) 4-amino-3-hydroxy-2,6-dinitrotoluene, (**13**) 4,4′-azoxy-2,2′,6,6′-tetranitrotoluene, (**14**) 4,4′-azo-2,2′,6,6′-tetranitrotoluene, (**15**) 4,4′-hydrazo-2,2′,6,6′-tetranitrotoluene, (**16**) 2-*N*-acetylamido- or 2-*N*-formamido-4,6-dinitrotoluene, (**17**) 4-*N*-acetylamido- or 4-*N*-formamido-2,6-dinitrotoluene.

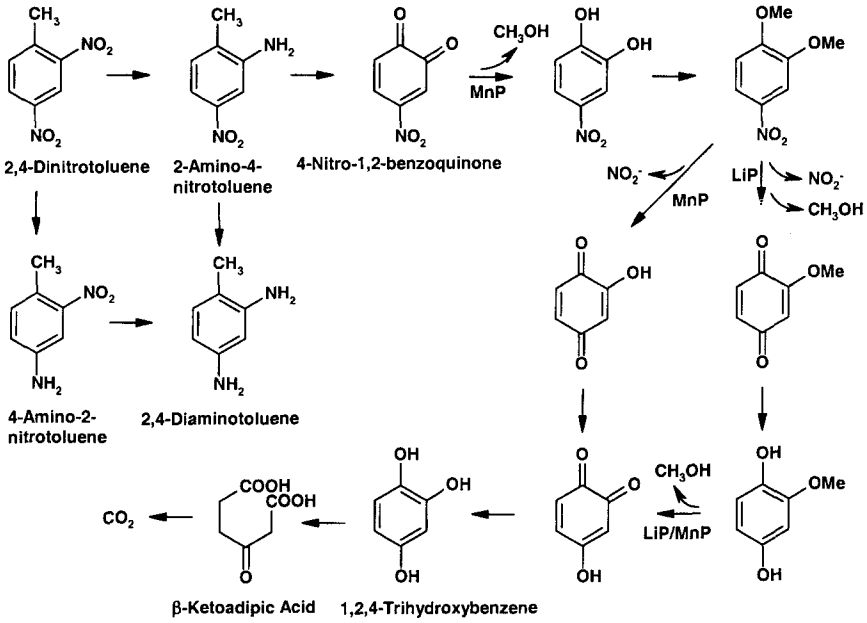

FIG. 19. Degradation pathway of 2,4-DNT by *Phanerochaete chrysosporium* (Valli *et al.*, 1992).

On the other hand, working on the biodegradation of 2,4-DNT (2,4-dinitrotoluene) by *P. chrysosporium,*Valli *et al.,* (1992) observed that MnP was the only peroxidase able to oxidize aminonitrotoluenes giving a nitrobenzoquinone. The latter undergoes a sequence of oxidations/reductions catalyzed by both LiP and MnP, and accompanied by a release of nitro and methyl groups as nitrite and methanol, respectively. The last aromatic product is a trihydroxybenzene, substrate for an intracellular dioxygenase, which catalyzes an aromatic ring cleavage to produce a metabolizable aliphatic acid (Fig. 19, Valli *et al.,* 1992).

4. Phytoremediation of TNT

Since the mid-1990s, bioremediation of nitroaromatic explosive-contaminated soils, groundwater, or surface water has entered a new field—so-called phytoremediation—involving no longer single microbes, but plants or plant–microbe systems. Since plants induce changes in soil conditions (e.g., redox potential, pH, organic matter, microbial communities, hydrodynamics), phytoremediation encompasses a range of processes beyond direct plant metabolism of pollutants and may be best described as a *plant-assisted* remediation. Phytoremediation includes phytoextraction (i.e., uptake through the roots), phytodegradation (i.e.,

biodegradation into plant tissues), phytovolatilization (i.e., volatilization through leaves), and rhizodegradation (i.e., biodegradation by rhizosphere-associated microbes) (Burken *et al.*, 2000).

Phytoremediation begins by the uptake of contaminated water by the roots (or by other organs for wetland species) and its distribution within the plant by the xylem or phloem transport system. The *green liver* model describes the fate of organic contaminants within plant tissues (Sandermann, 1994). Unlike most microbes, plants, as autotroph organisms, do not have an extensive array of catabolic pathways for using organic compounds as carbon, energy, and nitrogen sources. The metabolism of foreign compounds in plants is better considered as a detoxification mechanism.[29] Initial transformation of xenobiotic compounds in plants involves enzyme-catalyzed oxidation (hydroxylation), reduction, and hydrolysis reactions. The modified products undergo tranferase-catalyzed conjugation with an organic molecule of plant origin, leading to a reduction in toxicity. The resulting conjugates can be sequestrated (stored in plant organelles, such as vacuoles, or incorporated into biopolymers such as lignin) or excreted in the case of wetland plants (Coleman *et al.*, 1997).

TNT removal from aqueous solutions by both terrestrial and aquatic plants has been well documented (Gorge *et al.*, 1994; Thompson *et al.*, 1998; Best *et al.*, 1999; Burken *et al.*, 2000) and is an active enzymatic process different from a simple reversible sorption (Burken *et al.*, 2000). Plants are able to metabolize TNT, even though no mineralization occurs (Hughes *et al.*, 1997). Products of TNT metabolism in plants fall into four classes: ADNT, unidentified soluble products, extractable plant-associated products (which are not TNT reduced derivatives), and bound residues (Harvey *et al.*, 1991). Soluble and extractable plant-associated products account for the majority of TNT metabolites. With time, the proportion of identified nitroaromatic compounds and extractable plant-associated products decreases, while the fraction of bound residues increases, which is consistent with the green liver model (Bhadra *et al.*, 1999a).

Initial reduction of TNT in plant tissues results mainly in the formation of OHADNT and ADNT, while small amounts of DANT and azoxy compounds have been reported (Hughes *et al.*, 1997; Bhadra *et al.*, 1999a). The low level of reduction derivatives observed suggests the transient formation of reactive OHADNT, which may play a key role in the TNT metabolism (Burken *et al.*, 2000). Oxidation of TNT would follow the formation of OHADNT and involves methyl

[29]TNT is toxic for both aquatic and terrestrial plants within the range of 1 to 30 mg liter^{-1} (Burken *et al.*, 2000).

oxidations or aromatic hydroxylations (Bhadra *et al.*, 1999b). To date, enzymes involved in these initial TNT transformations have been poorly studied.

Products of the initial transformations of TNT may undergo conjugation by reaction between an amino or hydroxylamino group and one at least six-carbon unit coming from the plant (Bhadra *et al.*, 1999a). Formation of conjugates likely constitutes a gateway to bound residues. Sequestration of TNT metabolites or conjugates is characterized by a reduction of the extractability and by an increase of the molecular weight.[30] Formation of bound residues should result in a reduction of both the bioavailability and the toxicity (Burken *et al.*, 2000). However, additional research is required in order to ensure that the bound fractions do not constitute an environmental hazard if they enter the chain food or undergo later ligninolysis. Questions still exist regarding the final fate and the long-term ecological impact of potential metabolites. To date, the ecological benefits, the simplicity, and the aesthetics of phytoremediation processes have led to its strong acceptance by the public.

E. Biodegradation of Nitramines RDX and HMX

"... Nitroaromatic compounds were, in general, much more recalcitrant to biological breakdown than the nonnitrated analogs (Nishino et al., 2000)."

In contrast to TNT, whose the rate-limiting degradation step is the aromatic ring fission, while cyclic nitramines RDX or HMX undergoes a change in their molecular structure, the ring collapses easily to generate small nitrogen-containing or carbon-containing molecules harmless for the environment (Hawari, 2000). Bond dissociation energies in RDX and HMX (N–N, N–C, and C–H bonds) are relatively low (>100 kcal mol^{-1}). Once one bond of the nitramine molecule is cleaved, the remaining bonds become weaker and the entire cycle is destabilized, leading to a rapid ring fission (Bulusu, 1990). On the other hand, biotranformation of TNT gives stable aromatic metabolites that might be more toxic than the parent compound. Due to their different preferential conformations, HMX (crown-type conformation) is chemically more stable than RDX (chair conformation). Due to the crowning of atoms in the $-CH_2-N-NO_2$ group, transition states of HMX exhibit more steric hindrance than in RDX. Those differences of reactivity are in accordance with their susceptibility to both aerobic and anaerobic biodegradation. Moreover, HMX exhibits a lower water solubility (5 mg liter^{-1}) than HMX (42 mg liter^{-1}) and is therefore less amenable to biodegradation (Gorontzy *et al.*, 1994).

[30]TNT metabolites in lignin are between 1000 and 80000 Da (Burken *et al.*, 2000).

1. Abiotic Transformation of RDX and HMX

Neither volatilization nor evaporation of RDX or HMX from contaminated soil or water was reported and the half-life of RDX in the environment is about 9000 days (Gorontzy *et al.,* 1994). However, abiotic decomposition of the nitramines RDX and HMX may occur through different mechanisms.

Both RDX and HMX may undergo thermolysis, leading to a direct concerted decomposition of the nitramine cycle (Fig. 20, route Ia; Zhao *et al.,* 1988; Bulusu, 1990), one-electron transfer through photolysis or zero-valent metal reduction, producing, by release of nitrite NO_2^-, a nitrogen-centered nitryl radical (Fig. 20, route IIa; Singh *et al.,* 1996), bimolecular elimination (E2) of nitric acid HNO_3 through alkaline hydrolysis, producing a cyclohexene derivative (Fig. 20, route IIIa; Corce and Okamoto, 1979). The three mechanisms lead to a ring fission with the formation of methylenetriamine $CH_2{=}NNO_2$, which is unstable and spontaneously decomposes to nitrous oxide (N_2O) and formaldehyde (HCHO), either by rearrangement or by the transient formation of unstable nitramine NH_2NO_2, suggests that once the nitramine cycle is attacked, ring cleavage occurs easily, generating the same metabolites (Zhao *et al.,* 1988; Hawari, 2000). In addition, the ring opening following one-electron reduction (route IIa) and alkaline hydrolysis (route IIIa) generates nitrosomethylene $CH_2{=}NO$ and hydrogen cyanide HCN, respectively (Zhao *et al.,* 1988). Nitroso derivatives were frequently observed during thermolysis or zero-valent metal treatments of the RDX molecule, suggesting an initial two-electron reduction of nitro groups, leading to a ring opening (Zhao *et al.,* 1988; Singh *et al.,* 1996).

Because ring-cleavage metabolites and final products identified from the (bio)decomposition of RDX and HMX are the same, it may be assumed that both nitramines followed essentially the same catalytic pathway (Hawari, 2000).

2. Bacterial Transformation of RDX and HMX

The abiotic decomposition process described previously constitutes a paradigm for biodegradation processes of nitramines, because very little information exists at the present time about the metabolites and biotransformation pathways (Hawari, 2000).

In an early report based on bioremediation experiments of RDX in anaerobic sludge, McCormick *et al.* (1981) proposed a degradation pathway involving the stepwise reduction of the nitro substitutents, generating successively mono-, di-, and trinitroso derivatives susceptible to further reduction into corresponding hydroxylamino derivatives

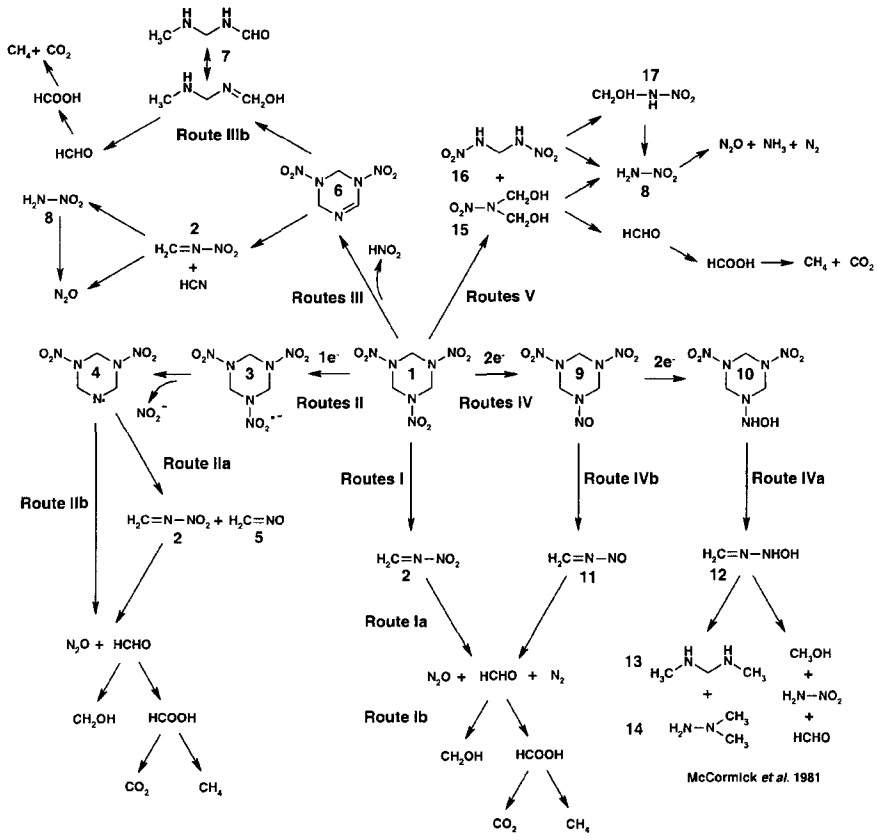

FIG. 20. Mechanisms for bacterial degradation of the cyclic nitramine RDX. Adapted from Hawari (2000). (**1**) Hexahydro-1,3,5-trinitro-1,3,5-triazine, (**2**) methylenenitramine, (**3**) hexahydro-1,3,5-trinitro-1,3,5-triazine anion radical, (**4**) 3,5-dinitro-1,3,5-triaza-cyclohex-1-nitryl radical, (**5**) nitrosomethylene, (**6**) 3,5-dinitro-1,3,5-triaza-cyclohex-1-ene, (**7**) formaminomethylnitramine, (**8**) nitramine, (**9**) hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, (**10**) hexahydro-1-hydroxylamino-3,5-dinitro-1,3,5-triazine, (**11**) methylenenitrosamine, (**12**) methylene(hydroxylamino)amine, (**13**) methyldimethylamine, (**14**) *bis*(methyl)aminoamine, (**15**) *bis*(hydroxymethyl)nitramine, (**16**) methyldinitramine, (**17**) hydroxymethylnitramine.

(Fig. 20, route IVa). The latter undergo ring cleavage and produce small metabolites similar to those observed during abiotic degradation (i.e., HCHO, CH_3OH, NH_2NH_2, and $(CH_3)_2NNH_2$). The ring fission likely occurs via the formation of methylenehydroxylamine CH_2=NNHOH, which would spontaneously decompose and/or rearrange into various low-molecular-weight products (McCormick *et al.*, 1991; Hawari *et al.*, 2000). The formation of methyldinitramine $CH_2(NHNO_2)_2$ and nitramine

was also reported.[31] The similarities between metabolites generated upon abiotic treatments and anaerobic sludge biodegradation of RDX illustrate, one more time, that any change of the initial cyclic nitramine leads to a subsequent molecule breakdown through essentially similar decomposition mechanisms.

Recent reports on microbial degradation of RDX and HMX showed that besides the anaerobic pathway described by McCormick *et al.* (1981), which remains the most detailed proposed upto the present time, other degradation routes have to be considered, both under anaerobiosis and aerobiosis. While, the McCormick pathway is restricted to stepwise two-electron reductions of the nitro groups beared by the initial nitramine cycle, generating nitroso and hydroxylamino derivatives, microbes possess a variety of catabolic enzymes (e.g., nitroreductases, cytochrome P-450 monooxygenases, hydrolases, peroxidases, laccases, etc.) susceptible to catalyze alternative attacks on the initial nitramine.

a. Anaerobic Degradation of RDX. In light of recent reports, four main degradation routes may be proposed: direct concerted decomposition (Fig. 20, route Ib), one-electron reduction (Fig. 20, route IIb), bimolecular elimination (Fig. 20, route IIIb), two-electron reduction (Fig. 20, routes IVa and IVb), and hydrolysis (Fig. 20, route V).

The cyclic molecule RDX could directly undergo a ring fission through a concerted elimination of methylenenitramines, which subsequently decompose to nitrous oxide and fromaldehyde (Fig. 20, route Ib). Acetogenic and methanogenic bacteria, often present in anaerobic sludge, may, respectively, convert formaldehyde to formic acid HCOOC and formic acid to methane CH_4 and CO_2. Alternatively, HCHO may be (abiotically) reduced to methanol CH_2OH (Hawari *et al.*, 2000).

A first denitration pathway of RDX includes enzymatic one-electron reduction of a nitro group, e.g., by nitroreductase (type II) or the cytochrome P-450 system, forming a nitro anion radical, which readily looses a nitrite NO_2^-, leaving a nitrogen-centered nitryl radical (Fig. 20, route IIb). The latter is very unstable and decomposes into N_2O and HCHO. This mechanism is important as it leads to a fission of the cyclic nitramine without the formation of harmful nitroso derivatives (Hawari, 2000).

Treatment of RDX by anaerobic sludge can lead to abiotic alkaline hydrolysis[32] (Fig. 20, routes IIIa and IIIb). RDX loses nitrous acide HNO_2 by a bimolecular elimination reaction (E2) forming a cyclohexene

[31] Suprisingly, no trace of N_2O was observed, despite chemical evidence of its formation from the decomposition of both methyldinitramine and nitramine (Hawari, 2000).

[32] Bacterial activity under anaerobiosis may increase of the pH above neutrality. Alkaline hydrolysis of RDX has been observed at pH 8.0 (Hawari, 2000).

derivative, which decomposes abiotically either into hydrogen cyanide HCN and methylenenitramine $CH_2{=}HNO_2$, subsequently converted to hydroxymethylnitramine NO_2NHCH_2OH (Fig. 20, route IIIa; Corce and Okamoto, 1979; Hawari *et al.*, 2000), or directly into NO_2NHCH_2OH and formaminomethylnitramine NO_2NHCH_2NHCHO (Fig. 20, route IIIb; Hawari *et al.*, 2000). Both NO_2NHCH_2OH and NO_2NHCH_2NHCHO may in turn decompose into nitrous oxide and formaldehyde.

Two-electron nitroreductase (type I)-catalyzed reduction of cyclic nitramines produces corresponding nitroso and hydroxylamino metabolites, which are more susceptible than RDX to undergo a concerted ring fission to yield methylenenitrosamine $CH_2{=}NNO$ and methylene(hydroxylamino)amine $CH_2{=}NNHOH$, respectively (Fig. 20, routes IVa and IVb; McCormick *et al.*, 1981; Hawari, 2000). Concerted decomposition of hydroxylamino derivatives of RDX is the pathway proposed by McCormick *et al.* (1981). Methylenenitrosamine is decomposed into formaldehyde and nitrogen N_2, through a mechanism similar to the decomposition of methylenenitramine (route IVb). Methylenehydroxylamine further decomposes according to McCormick *et al.* (1991). The enzymatic character of concerted mechanisms (routes Ib, IVa, IVb) is still in question.

Hydrolase enzymes may directly catalyze the ring fission of nitramines into methyldinitramine $CH_2(NHNO_2)_2$ and *bis*(hydroxymethyl) nitramine $NO_2N(CH_2OH)_2$, whose further decomposition produces formaldehyde and nitramine NH_2NO_2, likely via the transient formation of hydroxymethylnitramine $CH_2OHNHNO_2$ (Fig. 20, route V; Hawari *et al.*, 2000). Nitramine in turn may be (enzymatically) decomposed into nitrous oxide N_2O, amonia NH_3, and nitrogen N_2 (Singh *et al.*, 1998).

Mineralization experiments using ^{14}C-labeled nitramines in anaerobic sludge have shown extensive mineralization of RDX and of HMX (up to 59 and 46%, respectively, after 48 days of incubation) (Shen *et al.*, 1998).

b. Aerobic Degradation of RDX. Aerobic transformation of nitramines RDX and HMX have received less attention than anaerobic treatments.

Anaerobic pathway may involve the initial nitroreductase-catalyzed reduction of the nitro group either *via* a two-electron transfer (type I), generating nitroso derivatives or *via* a one-electron transfer (type II) generating nitramine anion radical. As described under anaerobiosis, two-electron reduction would lead to a complete decomposition of the molecule, through the transient formation of $CH_2{=}NNO$, into N_2 and HCHO (Fig. 20, route Ib). However, several publications concerning aerobic bacterial degradation of RDX did not observe nitroso metabolites (Binks, 1995; Jones *et al.*, 1995; Tekoah and Abeliovich, 1999), suggesting a one-electron reduction process. A one-electron reduction

product of RDX may either release a nitrite, as under anaerobiosis, forming an unstable nitrogen-centered nitryl radical or revert back to the parent nitramine by autooxidation in the presence of dioxygen, generating superoxide anion radicals $O_2^{\bullet -}$ (Fig. 20, routes IIb and IIc). Superoxide radicals are reactive species (Section III.C.1.*b*) susceptible to interaction with RDX molecules, contributing to accelerate their decomposition (Hawari *et al.*, 2000). Alternatively, nitryl radical intermediates undergo ring fission, either, as described under anaerobiosis, with the formation of $CH_2{=}NO$ and $CH_2{=}NNO_2$ (route IIa) or with the formation of $CH_2(NHNO_2)_2$ and/or NO_2NHCH_2NHCHO (route IIb, Binks *et al.*, 1995). Both $CH_2(NHNO_2)_2$ and NO_2NHCH_2NHCHO undergo further decomposition into nitrous oxide N_2O and formaldehyde HCHO, via the formation of nitramine NH_2NO_2, following pathways as described in routes IV and IIIb, respectively. Cytochrome P-450 enzyme complex might be involved in aerobic catalysis of cyclic nitramines (Tekoah and Abeliovich, 1999). Generally speaking, there is a considerable lack of information in the literature concerning catabolic enzymes (e.g., nitroreductases, cytochrome P-450 mono-oxygenases, peroxidases) that are potentially involved in anaerobic and, moreover, in aerobic degradation pathways of RDX and HMX.

A *Rhodococcus* sp. isolated from a RDX-contaminated soil was reported to achieve a 30% ^{14}C-RDX mineralization in pure culture. The only metabolite identified was nitrite, which did not accumulate in the medium, and which suggests its utilization as a nitrogen source (Jones *et al.*, 1995). In contrast, several bacterial strains were shown to entirely convert both RDX and HMX into corresponding nitroso derivatives after 9 days of incubation. Accumulation of nitroso compounds in the medium suggests that they were used as neither carbon nor nitrogen sources (Hawari, 2000). Presently only very controversial results have been obtained conceiving the aerobic bioremediation of nitramines RDX and HMX.

3. Transformation of RDX and HMX by White-Rot Fungi

The white-rot fungus *P. chrysosporium* has been shown to mineralize 67 and 76% ^{14}C-RDX in liquid cultures and soil slurries, respectively (Fernando and Aust, 1991). White-rot fungi are known to degrade numerous organic pollutants *via* mechanisms involving either extracellular ligninolytic enzymes (i.e., peroxidases and laccases; Stahl and Aust, 1993b; Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994) or intracellular enzymes (e.g., cytochrome P450 mono-oxygenase complex and nitroreductases; Fernando and Aust, 1991; Rieble *et al.*, 1994). Although no enzymatic analysers were performed, the authors postulated the implication of ligninolytic peroxidases in the

mineralization process. Degradation of TNT by white-rot fungi is known to proceed through a two-step mechanism, involving first the nitroreductase-mediated reduction of nitro groups, giving nitroso, hydroxylamino, and amino derivatives, which are substrates for extracellular ligninolytic peroxidases, the ability to mineralize them in a second oxidative step (Section IV.D.3.*b*). Sublette *et al.* (1991) reported the degradation of a mixture of TNT and RDX by *P. chrysosporium* in soil and water, but did not provide any further information about the intermediates, the metabolic pathway, or the enzymes involved in the process.

4. Phytoremediation of RDX and HMX

Only very little information is available about RDX and HMX phytoremediation. Uptake and limited transformation of RDX by both terrestrial and wetland plant species have been reported, but no metabolites have been identified to date (Rivera *et al.*, 1998; Best *et al.*, 1999; Thompson *et al.*, 1999). RDX was shown to be translocated in the leaf tissues where it is poorly further transformed (Thompson *et al.*, 1999). Uptake and phytotransformation of HMX have also been mentioned, without further information about the fate of potential metabolites (Rivera *et al.*, 1998).

Both terrestrial and wetland plants have been shown to take up and to transform TNT, RDX, and HMX. However, except for the identification of reduced TNT derivatives as transient metabolites, no additional information is available about metabolic pathways of nitro-substituted explosives by plant tissues or about the enzymes potentially involved. Questions still exist regarding the final fate and the long-term ecological impact of potential metabolites. If formation of bound residues may result in a reduction of both bioavailability and toxicity, additional research is required in order to ensure that the bound fractions do not constitute an environmental hazard if they enter the chain food or undergo later ligninolysis. To date, the ecological benefits, the simplicity, and the aesthetics of phytoremediation processes have led to its strong acceptance by the public.

F. Field Experiments

The goal of composting xenobiotic pollutants recalcitrant to biodegradation is their cometabolic transformation into less toxic compounds, which would be incorporated into humic material (humification). If other nitro-substituted explosives, such as RDX or HMX, are extensively mineralized during the composting process, no significant mineralization of TNT has been reported and the transformation products remain mostly nonidentified and/or nonextractable. However,

composting TNT-contaminated soils results in a rapid and almost complete disappearance of extractable toxic explosive (Bruns-Nagel *et al.,* 2000). Toxicological tests, essential to evaluate the environmental impact of the composting process, have revealed a strong decrease of both toxicity and genotoxicity upon composting TNT-contaminated sites (Griest *et al.,* 1993). Little information is available about long-term stability and possible release of toxic metabolites during the natural turnover of humic material (Bruns-Nagel *et al.,* 2000).

From a biochemical point of view, TNT is reduced stepwise under both aerobic and anaerobic conditions to give reduced derivatives (i.e., ADNTs, DANTs, and TAT), condensation azoxy dimers, and several acylated metabolites (Kaplan and Kaplan, 1982). The acylation of TNT derivatives are likely detoxification reactions. TNT derivatives oxidized at the methyl group were also detected (Bruns-Nagel *et al.,* 1999). The lack of stoichiometric conversion and the absence of mineralization suggest a binding of TNT reduced derivatives to the soil matrix. After a phase of physicochemical adsorption, TNT transformation products bearing amino or hydroxylamino groups can be introduced to humic material (fulvic acids, humic acids, and humines) through the normal humification process. Reactive aromatic residues of lignocellulose degradation, e.g., phenolic and quinoid structures, can react with TNT amino derivatives in a polymerization mechanism similar to natural humification. Fungal ligninolytic enzymes (i.e., peroxidases and laccases), producing reactive radical species from lignin degradation, may be involved in the process (Dawel *et al.,* 1997). Resulting humus and bound residues are slowly mineralized as a result of the natural humus turnover, so that release of bound TNT metabolites cannot be excluded (Held *et al.,* 1996).

Experiments in soils have shown a rapid reduction of TNT yielding toxic polar derivatives, which may be transported out of the site by gravitational water and therefore contaminate groundwater. Reduction of nitro-substituted compounds results in an increase of both the solubility and the cationic character of the molecules (improving bindings to the soil matrix), with an unclear global effect on bioavailability (Bruns-Nagel *et al.,* 2000). In addition, the reduction process limits the volatilization of nitroaromatic pollutants (by reduction of the vapor pressure) and favors further oxidative microbial metabolism under aerobiosis (by increase of the electron density of the aromatic ring) (Rieger and Knackmuss, 1995).

The slow mineralization rates of nitro-substituted explosives usually observed in soils (Fernando *et al.,* 1990; Spiker *et al.,* 1992) could be explained by the previously described processes (i.e., transportation of polar metabolites and binding to soil matrix), as well as by the fact

that organic matter and humic acids in soils are free-radical scavengers, which might extinguish the biodegradation reactions, likely involving free-radical mediation.

G. Mechanistic Considerations

"The formation of several TNT products by one microorganism [P. chrysosporium] can be taken as proof of the involvement of several enzymes in the biotransformation process (Hawari et al., 1999)."

From a mechanistic point of view, the peroxidases LiP and MnP catalyze one-electron oxidations resulting in the formation of reactive free radical intermediates, which could be the ultimate oxidizing agents responsible for the fungal xenobiotic degradation (Fritsche *et al.,* 2000). MnP acts through the oxidation of Mn(II) to Mn(III), which is stabilized by organic acids such as malonate or oxalate. Chelated Mn(III) is a strong oxidizing agent able to attack several organic molecules and small enough to diffuse inside polymeric lignocellulose (Glenn *et al.,* 1986; Paszczynski *et al.,* 1986; Wariishi *et al.,* 1989a,b). Mn(III) was also shown to operate the oxidative decarboxylation of organic acids (e.g., oxalate and malonate), producing carbon-centered free radicals (e.g., formyl radicals $COO^{\bullet-}$), susceptible to react with molecular oxygen (i.e., autooxidation) to yield superoxide free radicals $O_2^{\bullet-}$ (Section III.B; Urzùa *et al.,* 1998, Hofrichter *et al.,* 1998b). By (metal-catalyzed) dismutation, the superoxide radical may produce hydrogen peroxide (H_2O_2), and/or the powerful hydroxyl radical ($^{\bullet}OH$) (Sections II.E and III.C.1.*a*). Recently, carbon-centered free radicals resulting from the MnP-catalyzed oxidation of unsaturated fatty acids (i.e., linoleic acid) were shown to play a role in the degradation of both lignin and xenobiotic pollutants (Section II.D.4; Moen and Hammel, 1994; Bogan and Lamar, 1995; Kapich *et al.,* 1999). Therefore, Mn(III), carbon-centered free radicals, and/or ROS may be the ultimate oxidizing agent(s) involved in nitroaromatic breakdown. Both MnP and chelated Mn(III) catalyze the oxidation of GSH to give reactive glutathionyl free radicals $GS^{\bullet}$, whose production was shown to correlate with [^{14}C]-TNT and [^{14}C]-2-A-4,6-DNT mineralization (Van Aken *et al.,* 2000a). $GS^{\bullet}$ is a strong oxidant able to attack several organic molecules (Halliwell and Gutteridge, 1989).

Alternatively, the reactive complex [Mn(III)–GSH $\leftrightarrow$ Mn(II)–$GS^{\bullet}$] was proposed to be the ultimate oxidant of such a degradative system (McEldoon and Dordick, 1991; Hofrichter *et al.,* 1998a). Finally, while oxalate may have an inhibitory effect on the mineralization of nitroaromatic pollutants by fungal MnP, it was shown to be the only buffer—together with malonate to a much lesser extent—to support both the degradation

and the mineralization of nitroaromatic compounds by chemically generated Mn(III). The requirement for molecular oxygen in the process suggests, one more time, the involvement of superoxide free radicals.

H. Nonligninolytic Transformations

The current consensus considers a two-stage degradation/mineralization process of nitroaromatic pollutants by white-rot fungi, involving a first, mycelium-dependent reduction process, followed by an extracellular transformation of the resultant reduced metabolites by ligninolytic enzymes (Stahl and Aust, 1993b; Bumpus and Tatarko, 1994; Michels and Gottschalk, 1994). The mineralization of xenobiotic compounds by white-rot fungi has been for a long time directly correlated to their ligninolytic activities (Bumpus and Aust, 1987). However, some recent publications have reported nonligninolytic fungal degradations of xenobiotic pollutants, suggesting the implication of other intracellular—likely oxidative—enzymes, besides the intracellular and/or membrane-bound enzymes involved in the initial reduction of nitroaromatic compounds. The biodegradation of pentachlorophenol (Mileski *et al.*, 1988), 1,1,1-trichloro-2,2-*bis*-(4-chlorophenyl) ethane (DDT) (Köhler *et al.*, 1988), benzene, and other aromatic hydrocarbons (Yadav and Reddy, 1993) by *P. chrysosporium* has been shown not to be related to the development of the ligninolytic system. Recently, cytochrome P-450-dependent monooxygenases—a well-studied group of enzymes known to catalyze most detoxification reactions of xenobiotic compounds in eukaryotic organisms[33]—were shown to be involved in the biotransformation or mineralization of several xenobiotic pollutants by white-rot fungi, including phenanthrene (Sutherland *et al.*, 1991; Bezalel *et al.*, 1997), benzo[*a*]pyrene (Maspahy *et al.*, 1996b), and other PAHs (Bezalel *et al.*, 1996), pesticides like atrazine (Maspahy *et al.*, 1996a) and endosulfan (Kulleman and Matsumura, 1996), biphenyl ethers (Hundt *et al.*, 1999), and methyldibenzothiophene (Ichinose *et al.*, 1999).

While most authors have recognized early on the involvement of extracellular ligninolytic enzymes in the degradation of TNT by white-rot fungi, nonligninolytic mineralization—to a lesser extent than under ligninolytic conditions—has also been observed (Spiker *et al.*, 1992; Stahl and Aust, 1993b; Michels and Gottschalk, 1994). However, the first extensive nonligninolytic mineralization of TNT by the white-rot fungus *P. chrysosporium* was reported by Dutta *et al.* (1998). More

[33]The first fungal cytochrome P-450 was detected in *Cunninghamella bainieri* and was shown to catalyze the hydroxylation of aryl hydrocarbons (Ferris *et al.*, 1976).

recently, mineralization of TNT by the white-rot fungus *Bjerkandera adusta* has been shown not to be related to the production of ligninolytic peroxidases, but more likely to a TNT-induced microsomal cytochrome P-450 system (Eilers *et al.*, 1999). The transformation of RDX by *P. chrysosporium* has also been described as related either to ligninolytic peroxidases or to a cytochrome P-450 system (Fernando and Aust, 1991). Those observations suggest that besides extracellular ligninolytic peroxidases, white-rot fungi likely possess an intracellular cytochrome P-450-dependent enzymatic system catalyzing the oxidative transformation/mineralization of nitroaromatic compounds. Therefore, an entirely intracellular degradation pathway—involving the initial reduction of TNT, as well as the further oxidative mineralization of the resultant reduced metabolites—may exist in at least several ligninolytic fungal species.[34]

V. Conclusions

"An Heisenberg uncertainty-type principle is inescapable in environmental microbiology and must be confronted both in the examination of field site samples and in exploiting the spectrum of disciplines that contribute to our mechanistic understanding of microbiological processes (Madsen, 1998)."

P. chrysosporium remains the most widely studied model organism in both basic and applied research on degradation of lignin and xenobiotic pollutants. *P. chrysosporium* presents the advantages of a rapid growth in liquid defined media, of a high metabolic activity, and of an asexual reproduction by conidia spores (Fritsche *et al.*, 2000). However, even though it is characterized, under ligninolytic conditions, by the secretion of high levels of both ligninolytic peroxidases LiP and MnP, the production of laccase by *P. chrysosporium*—the third enzyme likely involved in lignin breakdown—is insignificant (Srinirasan *et al.*, 1995). Besides *P. chrysosporium*, other wood- and litter-decaying fungi were shown to be capable of metabolizing TNT. However, if all fungal species tested to date are able to reduce TNT to ADNT, only a few strains were shown to significantly mineralize it (Scheibner *et al.*, 1997; Fritsche *et al.*, 2000).

Despite these laboratory successes and the vast increase in the number of publications pertaining to the biodegradation of nitroaromatic explosive by white-rot fungi, questions remain: "Is it feasible to use

[34]A NADPH cytochrome P-450 reductase from rat liver microsomes was shown to catalyze the initial reduction of TNT (Leung *et al.*, 1995). Therefore, the entire fungal degradation pathway of TNT might be dependent on the cytochrome P-450 system.

white-rot fungi to clean up munition-contaminated military sites? Can laboratory procedures be modified and scaled up for field applications? Although the natural habitat of the white-rot fungus is decaying wood, is it capable of degrading contaminants in an amended soil medium (Bennett, 1994)?" Biodegradation of xenobiotic pollutants by white-rot fungi requires a close contact between contaminants and the fungal mycelium, adequate aeration, and nutrient limitation in order to induce the ligninolytic system.

At the present time, one of the main drawbacks for field applications is that fungi do not compete with the indigenous microflora. Moreover, laboratory scale experiments showed that the TNT mineralization rate is much lower in soil than in liquid medium (Fernando *et al.*, 1990; Spiker *et al.*, 1992).

Wood is not easily degraded by microbes because target polysaccharide molecules are embedded in a matrix of lignin. Fungi usually operate an "extracellular digestion" of macromolecules by the secretion of specific hydrolytic enzymes, which decompose large organic molecules (i.e., polysaccharides) into metabolizable monomers. In contrast, white-rot ligninolytic fungi produce *nonspecific* extracellular enzymes (i.e., peroxidases and laccases) able to break down lignin, opening the way for further degradation of cellulose and hemicellulose, which constitute the actual substrates. Fungal ligninolysis by itself is therefore a cometabolic and nonproductive mechanism. This unique and powerful system, which seems to be the exclusive property of white-rot fungi, likely involves reactive (radical) species harmful for biological systems. Therefore, if the ligninolytic apparatus confers a selective advantage to white-rot fungi, as a corollary, it requires a mechanism protecting the mycelial cells against oxidative enzymes and subsequent radical species. White-rot fungi possess membrane-associated reduction system(s) likely implicated in protection/detoxification purposes, but which seem(s) not to play any direct role in the ligninolysis process.

Both the oxidative ligninolytic and the reductive detoxification systems, which do not appear directly connected, are fortuitously involved in the fungal degradation of the nitro-substituted explosives. As oxidized and electron-deficient molecules, TNT, RDX, and HMX are poorly attacked by oxidative enzymes, but in contrast, are easily stepwise reduced by white-rot fungi, as well as by almost all living organisms. The further oxidative metabolism of reduced nitro-organic compounds is related exclusively to the extracellular ligninolytic system of basidiomycetes. The uniqueness of bioremediation techniques using white-rot fungi resides in the exploitation of side reactions associated to two distinct—one oxidative and one reductive—mechanisms.

Even though working under simplified laboratory conditions allows a deeper understanding of the mechanisms underlying the bioremediation process using white-rot fungi, it impedes to a certain extent the extrapolation of the results to more practical situations. Biodegradation experiments on xenobiotic pollutants conducted *in vivo,* i.e., in the presence of a fungal culture containing, besides the extracellular peroxidases, the whole ligninolytic system and the mycelial material, may lead to very different conclusions, since the unidentified transformation products could be further metabolized and mineralized by the mycelial material. Moreover, fungal biodegradation in field experiments, i.e., in a contaminated soil, may involve numerous additional factors susceptible to interfere with the degradative enzymes or to react with the intermediates. For instance, as the biotransformation of nitroaromatic compounds results in an increase of the polarity, the degradation products exhibit a higher water solubility, a lower volatility, and a higher susceptibility to bind with the components of the soil matrix or to be further metabolized by other microbes, with unclear overall consequences for environmental hazard.

Because direct and specific interactions between polymeric lignin and ligninolytic enzymes are rather improbable, the involvement of low-molecular-weight redox mediators (i.e., enzyme messengers) has been frequently postulated. Ligninolytic enzymes work by abstraction of one electron, producing reactive radical species, susceptible to further react with substrate molecules. This assumption is reinforced by the multiplicity of metabolites produced in fungal ligninolytic systems, which is a clue to a low selectivity, i.e., to a high reactivity. In addition, cationic radical species may undergo autooxidation, which is supported by the stimulatory effect of dioxygen frequently reported in fungal ligninolysis or xenobiotic biodegradation. Those considerations suggest the implication of ROS in ligninolytic enzyme-catalyzed degradation systems. Even though hydrogen peroxide H_2O_2 and superoxide free radicals $O_2^{\bullet-}$ are not species reactive enough to directly attack lignin or xenobiotic molecules, they may be indirectly involved in the degradation process, e.g., generating the very reactive $^{\bullet}OH$ through a Fenton reaction.

However, the reactive species responsible for the final breakdown of organic molecules is still in question. Focusing on the MnP-catalyzed degradation process, the first obvious species to be considered is Mn(III) chelated by a suitable organic acid (e.g., oxalate or malonate), which was shown to catalyze the oxidative decarboxylation of organic molecules. Alternatively, Mn(III), through decarboxylation of organic acids or peroxidation of fatty acids, may be involved in the production of carbon-centered acyl radicals $R^{\bullet}$, which, by reaction with dioxygen, generate peroxyl radicals ($RCOO^{\bullet}$). $RCOO^{\bullet}$ are reactive species able to

interact with lignin structures and xenobiotic pollutants. Reduced thiols such as GSH or cysteine, if present, may be oxydized by Mn(III) into a reactive thiyl radical, which is also capable of breaking down organic substrates. In addition, $O_2^{\cdot-}$ constitutes the basis of the "superoxide theory of oxygen toxicity," according to which $O_2^{\cdot-}$ would be the most damaging species responsible for oxygen toxicity in living cells, and therefore likely able to mediate the degradation of organic molecules (Fridovich, 1986).

REFERENCES

Akamatsu, Y., Ma, D. B., Higuchi, T., and Shimada, M. (1990). *FEBS Lett.* **269,** 261–263.

Akamatsu, Y., Takahashi, M., and Shinada, M. (1993). *Mat. Org.* **28,** 251–264.

Ander, P. (1994). *FEMS Microbiol. Rev.* **13,** 297–312.

Ander, P., and Marzullo, L. (1997). *J. Biotechnol.* **53,** 115–131.

Aust, S. D. (1990). *Microbial. Ecol.* **20,** 197–209.

Ayers, A. R., Ayers, S. B., and Eriksson, K. E. (1978). *Eur. J. Biochem.* **90,** 171–181.

Backa, S., Gierer, J., Reitberger, T., and Nielsson, T. (1992). *Holzforschung* **46,** 61–67.

Banci, L. (1997). *J. Biotechnol.* **53,** 253–263.

Bao, W., Fukushima, Y., Jensen, K. A., Jr., Moen, M. A., and Hammel, K. E. (1994). *FEBS Lett.* **354,** 297–300.

Barr, D. P., and Aust, S. D. (1994). *Environ. Sci. Technol.* **28,** 78A–87A.

Barr, D. P., Shah, M. M., Grover, T. A., and Aust, S. D. (1992). *Arch. Biochem. Biophys.* **298,** 480–485.

Bennett, J. W. (1994). *Int. Biodet. Biodegrad.* **34,** 21–34.

Best, E. H., Sprecher, S. L., Larson, S. L., Fridrickson, H. L., and Bader, D. F. (1999). *Chemosphere* **38,** 3383–3396.

Bezalel, L., Hadar, Y., and Cerniglia, C. E. (1996). *Appl. Environ. Microbiol.* **62,** 292–295.

Bezalel, L., Hadar, Y., and Cerniglia, C. E. (1997). *Appl. Environ. Microbiol.* **63,** 2495–2501.

Bhadra, R., Wayment, D., Hughes, J. B., and Shanks, J. V. (1999a). *Environ. Sci. Technol.* **33,** 446–452.

Bhadra, R., Wayment, D., Hughes, J. B., and Shanks, J. V. (1999b). *Environ. Sci. Technol.* **33,** 3354–3361.

Binks, P. R., Nicklin, S., and Bruce, N. C. (1995). *Appl. Environ. Microbiol.* **61,** 1318–1322.

Bogan, B. W., and Lamar, R. T. (1995). *Appl. Environ. Microbiol.* **61,** 2631–2635.

Bonnarme, P., and Jeffries, T. W. (1990). *Appl. Environ. Microbiol.* **56,** 210–217.

Bourbonnais, R., and Paice, M. G. (1988). *Biochem. J.* **255,** 445–450.

Brock, B. J., Rieble, S., and Gold, M. H. (1995). *Appl. Environ. Microbiol.* **61,** 3076–3081.

Broda, P., Birch, P., Brooks, P., and Sims, P. (1996). *Mol. Microbiol.* **19,** 923–932.

Brown, J. A., Glenn, J. K., and Gold, M. H. (1990). *J. Bacteriol.* **172,** 3125–3130.

Bruns-Nagel, D., Steinbach, K., Gemsa, D., and von Löw, E. (2000). *In* "Biodegradation of Nitroaromatic Compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 357–394. Lewis Publishers, New York.

Bulusu, S. N. (1990). "Chemistry and Physics of Energetic Materials." Kluwer Academic Publishers, Dordrecht, The Netherlands.

Bumpus, J. A., and Aust, S. D. (1987). *Appl. Environ. Microbiol.* **53,** 2001–2008.

Bumpus, J. A., and Tatarko, M. (1994). *Curr. Microbiol.* **28,** 185–190.

Bumpus, J. A., Tien, M., Wright, D., and Aust, S. D. (1985). *Science* **228,** 1434–1436.

Burken, J. G., Shanks, J. V., and Thompson, P. L. (2000). *In* "Biodegradation of Nitroaromatic Compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 239–276. Lewis Publishers, New York.

Buswell, J. A., and Odier, E. (1987). *CRC Crit. Rev. Biotechnol.* **6,** 1–60.

Call, H. P., and Mücke, I. (1997). *J. Biotechnol.* **53,** 163–202.

Camarero, S., Sarkar, S., Ruiz-Duenas, F. J., Martinez, M. J., and Martinez, A. T. (1999). *J. Biol. Chem.* **274,** 10324–10330.

Candeias, L. P., and Harvey, P. J. (1995). *J. Biol. Chem.* **270,** 16745–16748.

Chen, C. L., and Chang, H. M. (1985). *In* "Biosysnthesis and Biodegradation of Wood" (T. Higuchi, ed.), pp. 535–556. San Diego, CA.

Chen, C.-L., Chang, H.-M., and Kirk, T. K. (1982). *Holzforschung* **36,** 3–9.

Chua, M. G., Choi, S., and Kirk, T. K. (1982). *Holzforschung* **37,** 55–62.

Coleman, J. O., Blake-Kalff, M. M., and Davies, T. G. (1997). *Trends Plant. Sci.* **2,** 144–151.

Corce, M., and Okamoto, Y. (1979). *J. Org. Chem.* **44,** 2100–2103.

Cui, F., and Dolphin, D. (1990). *Holzforschung* **44,** 279–283.

Cullen, D. (1997). *J. Biotechnol.* **53,** 273–289.

Cullen, D., and Kersten, P. J. (1996). *In* "The Mycota III" (R. Bramble, and G. Marzluf, ed.), pp. 297–314. Springer-Verlag, Berlin, Germany.

Dawel, G., Kästner, M., Michels, J., Poppitz, W., Günter, W., and Fritsche, W. (1997). *Appl. Environ. Microbiol.* **63,** 2560–2565.

de Jong, E., Field, J. A., and de Bont, J. A. (1994). *FEMS Microbiol. Rev.* **13,** 153–188.

Drzyzga, O., Jannsen, S., and Blotevogel, K. H. (1995). *Ecotoxicol. Environ. Saf.* **31,** 149–152.

Dunford, H. B. (1999). "Heme Peroxidases." Wiley-VCH, New York.

Duque, E., Haidour, A., Godoy, F., and Ramos, J. L. (1993). *J. Bacteriol.* **175,** 2278–2283.

Dutta, S. K., Jackson, M. M., Hua Hou, L., Powell, D., and Tatem, H. E. (1998). *Biorem. J.* **2,** 97–103.

Eilers, A., Rüngeling, E., Stündl, U. M., and Gottschalk, G. (1999). *Appl. Microbiol. Biotechnol.* **53,** 75–80.

Eriksson, K.-E., Pettersson, B., Volc, J., and Musilek, V. (1986). *Appl. Microbiol. Biotechnol.* **23,** 257–262.

Esteve-Nuñez, A., Lucchesi, G., Philipp, B., Schink, B., and Ramos, J. L. (2000). *J. Bacteriol.* **182,** 1352–1355.

Faison, B., and Kirk, T. K. (1983). *Appl. Environ. Microbiol.* **46,** 1140–1145.

Faison, B., and Kirk, T. K. (1985). *Appl. Environ. Microbiol.* **49,** 299–304.

Faison, B. D., Kirk, T. K., and Farrel, R. L. (1986). *Appl. Environ. Microbiol.* **52,** 251–254.

Fenton, H. J. (1894). *J. Chem. Soc.* **65,** 899–910.

Fernando, T., and Aust, S. D. (1991). *Ind. Eng. Chem.* **486,** 214–232.

Fernando, T., Bumpus, J. A., and Aust, S. D. (1990). *Appl. Environ. Microbiol.* **56,** 1666–1671.

Field, J. A., de Jong, E., Feijoo-Costa, G., and de Bont, J. A. (1993). *TiB Tech.* **11,** 44–49.

Forney, L. J., Reddy, C. A., Tien, M., and Aust, S. D. (1982). *J. Biol. Chem.* **257,** 11455–11462.

Forrester, I. T., Grabski, A. C., Burgess, R. R., and Leatham, G. F. (1988). *Biochem. Biophys. Res. Commun.* **157,** 992–999.

Fridovich, I. (1986). *Arch. Biochem. Biophys.* **247,** 1–11.

Fritsche, W., Scheibner, K., Herre, A., and Hofrichter, M. (2000). *In* "Biodegradation of Nitroaromatic Compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 213–238. Lewis Publishers, New York.

Fuller, M. E., and Manning, J. F. Jr. (1998). *Environ. Toxicol. Chem.* **17,** 2185–2195.

Garg, S. K., and Modi, D. R. (1999). *Crit. Rev. Biotechnol.* **19,** 85–112.
Gaskell, J., and Cullen, D. (1993). *J. Biotech.* **30,** 109–114.
Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986). *Arch. Biochem. Biophys.* **251,** 688–696.
Glenn, J. K., and Gold, M. H. (1985). *Arch. Biochem. Biophys.* **242,** 329–341.
Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983). *Biochem. Biophys. Res. Commun.* **114,** 1077–1083.
Gold, M. H., and Alic, M. (1993). *Microbiol. Rev.* **57,** 605–622.
Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984). *Arch. Biochem. Biophys.* **234,** 353–362.
Gong, P., Siciliano, S. D., Greer, C. W., Paquet, L., Hawari, J., and Sunahara, G. I. (1999). *Environ. Toxicol. Chem.* **18,** 2681–2688.
Goodwin, D. C., Aust, S. D., and Grover, T. A. (1995). *Biochemistry* **34,** 5060–5065.
Gorge, E., Brandt, S., and Werner, D. (1994). *Environ. Sci. Pollut. Res.* **1,** 229–233.
Gorontzy, T., Drzyzga, O., Kahl, M. W., Bruns-Nagel, D., Breitung, J., von Loew, E., and Blotevogel, K. H. (1994). *Crit. Rev. Microbiol.* **20,** 265–284.
Gottlieb, S., Day, W. C., and Pelczar, M. J. Jr. (1950). *Phytopathology* **40,** 926–935.
Green, A., Moore, D., and Farrar, D. (1999). *Environ. Toxicol. Chem.* **18,** 1783–1790.
Greene, R. V., and Gould, J. M. (1984). *Biochem. Biophys. Res. Commun.* **111,** 437–443.
Griest, W. H., Stewart, A. J., Tyndall, R. L., Caton, J. E., Ho, C. H., Aronside, K. S., Caldwell, W. M., and Tan, E. (1993). *Environ. Toxicol. Chem.* **12,** 1105–1116.
Haber, F., and Weiss, J. (1934). *Proc. R. Soc. London (A)* **147,** 332–351.
Haderlein, S. B., Hofstetter, T. B., and Schwarzenbach, R. P. (2000). *In* "Biodegradation of Nitroaromatic Compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 311–356. Lewis Publishers, New York.
Hall, P. L. (1980). *Enzyme Microbiol. Technol.* **2,** 170–176.
Halliwell, B., and Gutteridge, J. M. C. (1989). "Free Radicals in Biology and Medicine," 2nd ed., Clarendon Press, Oxford, UK.
Hammel, K. E. (1992). *In* "Metal in Biological Systems" (vol 28). (H. Siegel, and A. Seiegel, eds.), Vol. 28, pp. 41–60. Marcel-Dekker, New York.
Hammel, K. E., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985). *J. Biol. Chem.* **260,** 8348–8353.
Harman, L. S., Carver, D. K., Schreiber, J., and Mason, R. P. (1986). *J. Biol. Chem.* **261.**
Harvey, P. J., Floris, R., Lundell, T., Palmer, J. M., Schoemaker, H. E., and Wever, R. (1992). *Biochem. Soc. Trans.* **20,** 345–349.
Harvey, P. J., Schoemaker, H. E., and Palmer, J. M. (1986). *FEBS Lett.* **195,** 242–246.
Harvey, S. D., Fellows, R. J., Cataldo, D. A., and Bean, R. M. (1991). *Environ. Toxicol. Chem.* **10,** 845–855.
Hatakka, A. I. (1994). *FEMS Microbiol. Rev.* **13,** 125–135.
Hawari, J. (2000). *In* "Biodegradation of Nitroaromatic Compounds and Explosives." (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 277–310. Lewis Publishers, New York.
Hawari, J., Halasz, A., Beaudet, S., Ampleman, G., Paquet, L., and Thiboutot, S. (1999). *Appl. Environ. Microbiol.* **65,** 2977–2986.
Hawari, J., Halasz, A., Sheremata, T., Beaudet, S., Groom, C., Paquet, L., Rhofir, C., Ampleman, G., and Thiboutot, S. (2000). *Appl. Env. Microbiol.* **66,** 2652–2657.
Held, T., Draude, G., Schmidt, F. R. J., Brokamp, A., and Reis, K. H. (1996). *Environ. Technol.* **18,** 479–487.
Hetherington, L. H., Livingstone, D. R., and Walker, C. H. (1996). *Comp. Biochem. Physiol.* **113C,** 231–239.

Higson, F. K. (1992). *Adv. Appl. Microbiol.* **37,** 1–19.
Hofrichter, M., Scheibner, K., Schneegass, I., and Fritsche, W. (1998a). *Appl. Environ. Microbiol.* **64,** 399–404.
Hofrichter, M., Ziegenhagen, D., Vares, T., Friedrich, M., Jäger, M. G., Fritsche, W., and Hatakka, A. I. (1998b). *FEBS Lett.* **434,** 362–366.
Honeycutt, M. E., Jarvis, A. S., and McFarland, V. A. (1996). *Ecotoxicol. Environ. Saf.* **35,** 282–287.
Hughes, J. B., Shanks, J. V., Vanderford, M., Lauritzen, J., and Bhadra, R. (1997). *Environ. Sci. Technol.* **31,** 266–271.
Hundt, K., Jonas, U., Hammer, E., and Schauer, F. (1999). *Biodegradation* **10,** 279–286.
Ichinose, H., Wariichi, H., and Tanaka, H. (1999). *Biotechnol. Prog.* **15,** 706–714.
Isbister, J. D., Anspach, G. L., Kitchens, J. F., and Doyle, R. C. (1984). *Microbiologica* **7,** 47–75.
Jackson, M., Green, J. M., Hash, R. L., Lindsen, D. C., and Tatyrec, F. A. (1978). Report **ARLCD-77013.** US Army Armamant Research and Development Command, Dover, NJ.
Jeffries, T. W., Choi, S., and Kirk, T. K. (1981). *Appl. Environ. Microbiol.* **42,** 290–296.
Johansson, T., and Nyman, P. O. (1993). *Arch. Biochem. Biophys.* **300,** 49–56.
Johnson, L. R., Davenport, R., Balbach, H., and Schaeffer, D. J. (1994a). *Ecotoxicol. Environ. Saf.* **27,** 34–49.
Johnson, L. R., Davenport, R., Balbach, H., and Schaeffer, D. J. (1994b). *Ecotoxicol. Environ. Saf.* **27,** 23–33.
Jones, A. M., Labelle, S., Paquet, L., Hawari, J., Rho, D., Samson, R., Greer, C. W., Lavigne, J., Thiboutot, S., Apleman, G., and Laverdu, R. (1995). *In* "Environmental biotechnology: Principles and Applications." Kluwer Academic Publishers, Dordrecht, The Netherlands.
Kantelinen, A., Waldner, R., Niku-Paavola, M.-L., and Leisola, M. S. (1988). *Appl. Microbiol. Biotechnol.* **28.**
Kapich, A. M., Hofrichter, M., Vares, T., and Hatakka, A. I. (1999). *Biochem. Biophys. Res. Commun.* **259,** 212–219.
Kaplan, D. L. (1994). *In* "Organic Energetic Compounds" (M. P. Nova, ed.), pp. 1–39. Science Publishers, New York.
Kaplan, D. L., and Kaplan, A. M. (1982). *Bull. Environ. Contam. Toxicol.* **28,** 33–38.
Kawai, S., Umezawa, T., Shimada, M., and Higuchi, T. (1988). *FEBS Lett.* **236,** 309–311.
Keith, L. H., and Telliard, W. A. (1979). *Environ. Sci. Technol.* **13,** 416–423.
Kelley, R. L., and Reddy, A. C. (1986). *J. Bacteriol.* **166,** 269–274.
Kenten, R. H., and Mann, P. J. (1953). *Biochem. J.* **53,** 498–505.
Kersten, P. J., and Kirk, T. K. (1987). *J. Bacteriol.* **169,** 2195–2201.
Kersten, P. J., Stephens, S. K., and Kirk, T. K. (1990). *In* "Biotechnology in the Pulp and Paper Manufacture" (T. K. Kirk, and H.-M. Chang, eds.), pp. 457–463. Butterworth-Heinemann, Stoneham, MA.
Kersten, P. J., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985). *J. Biol. Chem.* **260,** 2609–2612.
Khindaria, A., Barr, D. P., and Aust, S. D. (1995). *Biochemistry* **34,** 7773–7779.
Kim, H. Y., and Song, H. G. (2000). *J. Microbiol.* **38,** 250–254.
Kirk, T. K., and Farrell, R. L. (1987). *Annu. Rev. Microbiol.* **41,** 465–505.
Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978). *Arch. Microbiol.* **117,** 227–285.
Kirk, T. K., and Shimada, M. (1985). *In* "Biosynthesis and Biodegradation of Wood Components" (T. Higuchi, ed.), pp. 579–605. Academic Press, Orlando, FL.

Klausmeier, R. E., Osmon, J. L., and Walls, D. R. (1974). *Dev. Ind. Microbiol.* **15,** 309–317.
Koduri, R. S., and Tien, M. (1995). *J. Biol. Chem.* **270,** 22254–22258.
Köhler, A., Jäger, A., Willershausen, H., and Graf, H. (1988). *Appl. Microbiol. Biotechnol.* **29,** 618–620.
Kuan, I. C., Johnson, K. A., and Tien, M. (1993). *J. Biol. Chem.* **268,** 20064–20070.
Kuawahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984). *FEBS Lett.* **169,** 247–250.
Kulleman, S. W., and Matsumura, F. (1996). *Appl. Environ. Microbiol.* **62,** 593–600.
Lachance, B., Robidoux, P. Y., Hawari, J., Ampleman, G., Thiboutot, S., and Sunahara, G. I. (1999). *Mutat. Res.* **444,** 25–39.
Lebron, C. A., Karr, L. A., Fernando, T., and Aust, S. D. (1992). U.S. Pat. 5085998.
Lenke, H., Achtnich, C., and Knackmuss, H. J. (2000). *In* "Biodegradation of Nitroaromatic compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 91–126. Lewis Publishers, New York.
Lenke, H., and Knackmuss, H. J. (1992). *Appl. Environ. Microbiol.* **58,** 2933–2937.
Lenke, H., and Knackmuss, H. J. (1996). *Appl. Environ. Microbiol.* **62,** 784–790.
Lenke, H., Pieper, D., Bruhn, C., and Knackmuss, H. J. (1992). *Appl. Environ. Microbiol.* **58,** 2928–2932.
Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N.-S., Hofrichter, M., and Rogalski, J. (1999). *Fung. Gen. Biol.* **27,** 175–185.
Leonowicz, A., Rogalski, J., Szczodrak, J., and Fiedurek, J. (1986). *In* "Proc. 3rd Int. Conf. Biotechnol. Pulp Paper Ind.," 16–19 June, pp. 160–162. Stockholm, Sweden.
Leung, K. H., Yao, M., Stearns, R., and Chiu, S. H. (1995). *Chem. Biol. Interact.* **97,** 37–51.
Lewis, T. A., Ederer, M. M., Crawford, R. L., and Crawford, D. L. (1997). *J. Ind. Microbiol. Biotechnol.* **18,** 89–96.
Lundell, T., Schoemaker, H., Hatakka, A. I., and Brunow, G. (1993). *Holzforschung* **47,** 219–224.
Lundquist, K., and Kirk, T. K. (1978). *Phytochemistry* **17,** 1676.
Maspahy, S., Henis, Y., and Levanon, D. (1996a). *Appl. Environ. Microbiol.* **62,** 3587–3593.
Maspahy, S., Levanon, D., Henis, Y., Venkateswarlu, K., and Kelly, S. L. (1996b). *FEMS Lett.* **135,** 51–55.
McCormick, N. G., Cornel, J. H., and Kaplan, A. M. (1981). *Appl. Environ. Microbiol.* **42,** 817–823.
McCormick, N. G., Feeherry, F. E., and Levinson, H. S. (1976). *Appl. Environ. Microbiol.* **31,** 949–958.
McEldoon, J. P., and Dordick, J. S. (1991).
McNally, W. D. (1944). *Indust. Med.* **13,** 491–494.
Messner, K., Srebotnik, E., and Fietcher, A. (1997). *J. Biotechnol.* **53,** 91–92.
Mester, T., and Field, J. A. (1998). *J. Biol. Chem.* **273,** 15412–15417.
Michels, J., and Gottschalk, G. (1994). *Appl. Environ. Microbiol.* **60,** 187–194.
Mileski, G. J., Bumpus, J. A., Jurek, M. A., and Aust, S. D. (1988). *Appl. Environ. Microbiol.* **54,** 2885–2889.
Moen, M. A., and Hammel, K. E. (1994). *Appl. Environ. Microbiol.* **60,** 1956–1961.
Moreno, S. N., and Docampo, R. (1985). *Environ. Health Persp.* **64,** 199–208.
Naumova, R. P., Selivanovskaya, S. Y., and Mingatina, F. A. (1988). *Microbiology* **57,** 169–173.
Nay, M. W., Randall, C. W., and King, P. H. (1974). *J. Water Pollut. Control Fed.* **46,** 485–497.
Niku-Paavola, M. L., Karhunen, E., Salola, P., and Raunio, V. (1988). *Biochem. J.* **254,** 877–884.
Nishida, A., and Eriksson, K. E. (1987). *Biotechnol. Appl. Biochem.* **9,** 325–338.

Nishino, S. F., Spain, J. C., and He, Z. (2000). *In* "Biodegradation of Nitroaromatic compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 7–62. Lewis Publishers, New York.

O'Brien, P. J. (1988). *Free Rad. Biol. Med.* **4,** 169–183.

Palmer, J. M., Harvey, P. J., and Schoemaker, H. E. (1987). *Phil. Trans. R. Soc. London* **A321,** 495–505.

Park, J. S. B., Wood, P. M., Davies, M. J., Gilbert, B. C., and Whiwood, A. C. (1997). *Free Rad. Res.* **27,** 447–458.

Parrish, F. W. (1977). *Appl. Environ. Microbiol.* **34,** 232–233.

Paszczynski, A., and Crawford, R. (1995). *Biotechnol. Prog.* **11,** 368–379.

Paszczynski, A., Huynh, V. B., and Crawford, R. (1986). *Arch. Biochem. Biophys.* **244,** 750–765.

Pease, E. A., Andrawis, A., and Tien, M. (1989). *J. Biol. Chem.* **264,** 13531–13535.

Peterson, M. M., Horst, G. L., Shea, P. J., Comfort, S. D., and Peterson, R. K. (1996). *Environ. Poll.* **93,** 57–62.

Piontek, K., Glumoff, T., and Winterhalter, K. (1993). *FEBS Lett.* **315,** 119–124.

Popp, J. L., Kalyanaraman, B., and Kirk, T. K. (1990). *Biochemistry* **29,** 10475–10480.

Potthast, A., Rosenau, T., Chen, C.-L., and Gratzl, J. S. (1995). *J. Org. Chem.* **60,** 4320–4321.

Poulos, T. L., Edwards, S. L., Wariishi, H., and Gold, M. H. (1993). *J. Biol. Chem.* **268,** 4429–4440.

Poulos, T. L., and Kraut, J. (1980). *J. Biol. Chem.* **255,** 8199–8205.

Preuss, A., and Rieger, P. G. (1995). *In* "Biodegradation of Nitroaromatic Compounds" (J. C. Spain, ed.), pp. 69–85. Plenum Press, New York.

Reddy, A. C. (1995). *Curr. Op. Biotechnol.* **6,** 320–328.

Reddy, A. C., and D'Souza, T. M. (1994). *FEMS Microbiol. Rev.* **13,** 137–152.

Renganathan, V., Miki, K., and Gold, M. H. (1986). *Arch. Biochem. Biophys.* **246,** 155–161.

Rieble, S., Joshi, D. K., and Gold, M. H. (1994). *Biochem. Biophys. Res. Commun.* **205,** 298–304.

Rieger, P.-G., and Knackmuss, H.-J. (1995). *In* "Biodegradation of Nitroaromatic Compounds" (J. C. Spain, ed.), pp. 1–18. Plenum Press, New York.

Rivera, R., Medina, V., Larson, S. L., and McCutcheon, S. C. (1998). *J. Soil. Contam.* **7,** 511–529.

Robidoux, P. Y., Hawari, J., Thiboutot, S., Ampleman, G., and Sunahara, G. I. (1999). *Ecotoxicol. Environ. Saf.* **44,** 311–321.

Rosenblatt, D. H., Burrows, S. P., Mitchell, W. R., and Parmer, D. L. (1991). *In* "The Handbook of Environmental Chemistry" (O. Hutzinger, ed.), Vol. 3, Part G, pp. 195–234. Springer-Verlag, Berlin-Heidelberg, RFA.

Ross, R. H., and Hartley, W. R. (1990). *Regul. Toxicol. Pharmacol.* **11,** 114–117.

Ross, D., and Moldeus, P. (1985). *Environ. Health Persp.* **64,** 253–257.

Ruchhoft, C. C., Lebosquet, M., and Meckler, W. G. (1945). *Ind. Eng. Chem.* **37,** 937–943.

Samejima, M., and Eriksson, K. E. (1992). *Eur. J. Biochem.* **207,** 103–107.

Sanderman, J. H. (1994). *Pharmacogenetics* **4,** 225–241.

Scheibner, K., Hofrichter, M., Herre, A., Michels, J., and Fritsche, W. (1997). *Appl. Microbiol. Biotechnol.* **47,** 452–457.

Schenzle, A., Lenke, H., Spain, J. C., and Knackmuss, H.-J. (1999). *J. Bacteriol.* **181,** 1444–1450.

Schoemaker, H. E., Lundell, T. K., Hatakka, A. I., and Piontek, K. (1994). *FEMS Microbiol. Rev.* **13,** 321–332.

Shen, C. F., Guiot, S. R., Thiboutot, S., Ampleman, G., and Hawari, J. (1998). *Biodegradation* **8,** 339–347.

Shen, C. F., Hawari, J., Ampleman, G., Thiboutot, S., and Guiot, S. R. (2000). *Biorem. J.* **4,** 29–37.

Sheremata, T. W., and Hawari, J. (2000). *Environ. Sci. Technol.* **34,** 3384–3388.

Shimada, M., Akamtsu, Y., Tokimatsu, T., Mii, K., and Hattori, T. (1997). *J. Biotechnol.* **53,** 103–113.

Shimada, M., Ma, D.-B., Akamatsu, Y., and Hattori, T. (1994). *FEMS Microbiol. Rev.* **13,** 285–296.

Simini, M., Wentsel, R. S., Checkai, R. T., Phillips, C. T., Chester, N. A., Major, M. A., and Amos, J. C. (1995). *Environ. Toxicol. Chem.* **14,** 623–630.

Singh, J., Comfort, S. D., and Shea, P. J. (1988). *J. Environ. Qual.* **27,** 1240–1245.

Smock, L. A., Stoneburner, D. L., and Clark, J. R. (1976). *Water Res.* **10,** 537–543.

Spain, J. C. (1995). *In* "Biodegradation of Nitroaromatic Compounds" (J. C. Spain, ed.), pp. 19–35. Plenum Press, New York.

Spain, J. C. (1995). "Biodegradation of Nitroaromatic Compounds" Plenum Press, New York.

Spain, J. C. (2000). *In* "Biodegradation of Nitroaromatic Compounds and Explosives" (J. C. Spain, J. B. Hughes, and H. J. Knackmuss, eds.), pp. 1–6. Lewis Publishers, New York.

Spain, J. C., Hughes, J. B., and Knackmuss, H. J. (2000). "Biodegradation of Nitroaromatic Compounds and Explosives." Lewis Publishers, New York.

Spanggord, R. J., Gibson, B. W., Keck, R. G., and Thomas, D. W. (1982). *Environ. Sci. Technol.* **16,** 229–232.

Spanggord, R. J., Spain, J. C., Nishino, S. F., and Mortelmans, K. E. (1991). *Appl. Environ. Microbiol.* **57,** 3200–3205.

Spiker, J. K., Crawford, D. L., and Crawford, R. L. (1992). *Appl. Environ. Microbiol.* **58,** 3199–3202.

Stahl, J. D., and Aust, S. D. (1993a). *Biochem. Biophys. Res. Commun.* **192,** 471–476.

Stahl, J. D., and Aust, S. D. (1993b). *Biochem. Biophys. Res. Commun.* **192,** 477–482.

Styles, J. A., and Cross, M. F. (1983). *Cancer Lett.* **20,** 103–108.

Sublette, K. L., Cho, P., Maule, A., Schwartz, S., and Pak, D. (1991). *In* "Proc. Int. Environmental Pollution," 1-ICEP.1, pp. 756–763. Interscience Enterprises Ltd.

Sublette, K. L., Ganpathy, E. V., and Schwartz, S. (1992). *Appl. Biochem. Biotechnol.* **34/35,** 709–723.

Sunahara, G. I., Dodard, S., Sarrazin, M., Paquet, L., Ampleman, G., Thiboutot, S., Hawari, J., and Renoux, A. Y. (1998). *Ecotoxicol. Environ. Saf.* **39,** 185–194.

Sunahara, G. I., Dodard, S., Sarrazin, M., Paquet, L., Hawari, J., Greer, C. W., Ampleman, G., Thiboutot, S., and Renoux, A. Y. (1999). *Ecotoxicol. Environ. Saf.* **43,** 138–148.

Sundaramoorthy, M., Kishi, K., Gold, M. H., and Poulos, T. L. (1994). *J. Biol. Chem.* **269,** 32759–32767.

Sutherland, J. B., Selby, A. L., Freeman, J. P., Evans, F. E., and Cerniglia, C. E. (1991). *Appl. Environ. Microbiol.* **57,** 3310–3316.

Talmage, S. S., Opresko, D. M., Maxwell, C. J., Welsh, C. J., Cretella, F. M., Reno, P. H., and Daniel, F. B. (1999). *Rev. Environ. Contam. Toxicol.* **161,** 1–156.

Tekoah, Y., and Abeliovich, N. A. (1999). *Int. Symp. Biodegradation Nitroaromatic Compounds Explosives,* September 8–9, Leesburg, VA, p. 7.

ten Have, R., Franssen, M. C., and Field, J. A. (2000). *Biochem. J.* **347,** 585–591.

Thompson, D. N., Hames, B. R., Reddy, C. A., and Grethlein, H. E. (1998). *Appl. Biochem. Biotechnol.* **70–72,** 967–982.

Thompson, P. L., Ramer, L. A., and Schnoor, J. L. (1999). *Env. Toxicol. Chem.* **18,** 279–284.

Thurston, C. F. (1994). *Microbiology* **140,** 19–26.

Tien, M., and Kirk, T. K. (1984). *Proc Natl Acad Sci USA* **81,** 2280–2284.

Tien, M., Kirk, T. K., Bull, C., and Fee, J. A. (1986). *J. Biol. Chem.* **261,** 1687–1693.
Traxler, R. W. (1974). "Biodegradation of Trinitrotoluene (α-TNT) and its Production Isomers," ADA 029346. Department of Plant Pathology-Enthomology, University of Rhode Island, Kingston, RI.
Urzúa, U., Kersten, P. J., and Vicuna, R. (1998). *Appl. Environ. Microbiol.* **64,** 68–73.
Valli, K., Brock, B. J., Joshi, D. K., and Gold, M. H. (1992). *Appl. Environ. Microbiol.* **58,** 221–228.
Valli, K., Wariishi, H., and Gold, M. H. (1990). *Biochemistry* **29,** 8535–8539.
Van Aken, B., Cameron, M. D., Stahl, J. D., Plumat, A., Naveau, H., Aust, S. D., and Agathos, S. N. (2000a). *Appl. Microbiol. Biotechnol. In Press*
Van Aken, B., Godefroid, L. M., Peres, C. M., Naveau, H., and Agathos, S. N. (1999b). *J. Biotechnol.* **68,** 159–169.
Van Aken, B., Hofrichter, M., Scheibner, K., Hatakka, A. I., Naveau, H., and Agathos, S. N. (1999a). Biodegradation **10,** 83–91.
Van Aken, B., Stahl, J. D., Naveau, H., Agathos, S. N., and Aust, S. D. (2000b). *Bioremediation J.* **4,** 135–145.
Van der Woude, M. W., Boominathan, K., and Reddy, C. A. (1993). *Arch. Microbiol.* **160,** 1–4.
Vorbeck, C. H., Lenke, H., Fischer, P., Spain, J. C., and Knackmuss, H.-J. (1998). *Appl. Environ. Microbiol.* **64,** 246–252.
Walker, J. E., and Kaplan, D. L. (1992). Biodegradation **3,** 369–385.
Walsh, M. E. (1990). *Environmental Transformation Products of Nitroaromatics and Nitroamines: Litterature Review and Recommendations for Analytical Method Development* [Special Report] **90–2.** US Army Corps of Engineers. Cold Regions Research and Engineering Laboratory.
Wariishi, H., Dunford, H. B., McDonald, I. D., and Gold, M. H. (1989a). *J. Biol. Chem.* **264,** 3335–3340.
Wariishi, H., and Gold, M. H. (1990). *J. Biol. Chem.* **265,** 2070–2077.
Wariishi, H., Valli, K., and Gold, M. H. (1989b). *Biochemistry* **28,** 6017–6023.
Wariishi, H., Valli, K., and Gold, M. H. (1992). *J. Biol. Chem.* **267,** 23688–23695.
Wariishi, H., Valli, K., Renganathan, V., and Gold, M. H. (1989c). *J. Biol. Chem.* **264,** 14185–14191.
Westermark, U., and Eriksson, K. E. (1974). *Acta. Chem. Scand.* **B28,** 209–214.
Won, W. D., DiSalvo, L. H., and Ng, J. (1976). *Appl. Environ. Microbiol.* **31,** 576–580.
Wood, P. M. (1994). *FEMS Microbiol. Rev.* **13,** 313–320.
Yadav, J. S., and Reddy, C. A. (1993). *Appl. Environ. Microbiol.* **59,** 756–762.
Yinon, J., and Hwang, D. G. (1985). *Toxicol. Lett.* **26,** 205–209.
Zapanta, L. S., and Tien, M. (1997). *J. Biotechnol.* **53,** 93–102.
Zhao, X., Hintsa, E. J., and Lee, Y. T. (1988). *J. Chem. Phys.* **88,** 801–810.
Zitting, A., Szumanka, G., Nickels, J., and Savolainen, H. (1982). *Arch. Toxicol.* **51,** 53–64.

Microbial Degradation of Pollutants in Pulp Mill Effluents

PRATIMA BAJPAI

Thapar Centre for Industrial Research & Development
Bhadson Road, Post Box No. 68, Patiala-147 001, India

I. Background

A vast pulp and paper industry exists throughout the world to cater an ever-increasing demand for a wide variety of paper products. Global consumption of paper and board products was over 268 million tons in 1994 which, by all estimates, will continue to increase in future (Bijur, 1997).

The pulp and paper industry generates about 175 m^3 of wastewater per ton of paper produced. Of the different wastewaters generated by the pulp and paper industry, bleach plant effluents are considered to be the most polluting. Production of 1 ton of pulp contributes about 100 kg of color-imparting substances and 2–4 kg of organochlorine compounds to the bleach plant effluents. Pollutants such as chlorinated phenolics and dioxins are toxic, nonbiodegradable, and have the tendency to contaminate food chains through bioaccumulation. The dioxins are known for their extreme toxicity and are believed to be carcinogenic.

Bleach effluents are both colored and toxic. They contain chlorinated and nonchlorinated products of lignin and extractives of wood. Because of their ability to block light transmission, the productivity of aquatic ecosystems gets affected when these effluents are discharged. Color also affects the downstream uses (municipal and industrial) of water. It makes water treatment difficult and costly. The chlorinated organic compounds and the lignin derivatives of the bleach effluents are recalcitrant with subsequent bioaccumulation along the food chains.

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 48

0065-2164/01 $35.00

The low-molecular-weight fraction of bleach effluent contains potentially problematic (toxic) compounds. These have the ability to penetrate cell membranes and a tendency to bioaccumulate. Low-molecular-weight chlorinated organic compounds significantly affect the biology of aquatic ecosystems. Disappearance of benthic invertebrates, high incidence of fish diseases, and mutagenic effects on the aquatic fauna are some of the consequences of the disposal of bleach effluents into surface waters.

Increasing awareness of environmental consequences of bleach effluent has lead to stringent environmental regulations. Prior to 1985, there were prescribed limits for only conventional parameters such as chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS) etc. But now, most nations have imposed limits on total organochlorine (TOCl) or adsorbable organic halides (AOX) of the effluents. In some nations, limits have also been set on individual chlorinated organic compounds of bleach effluents viz. 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD), and 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TCDF).

In response to environmental concerns and environmental regulations, the pulp and paper industry has reacted by making process modifications based on existing and new proven technologies. For a bleached kraft mill, a number of alternative technologies are available. From these, it is possible to select a combination that can meet the present or future effluent discharge limits. Initially, the effluent requirements varied from country to country for reasons of differing national priorities and this has led to a diverse range of technological responses. However, as a result of recent concern regarding dioxins and polychlorinated organic materials, and as a result of more stringent regulations, the industry tends to evaluate and to avail itself with the multitude of wide-ranging options available. This review describes the environmental impact of bleach plant effluents, environmental regulations, and measures to reduce the pollution load by internal process modification and external treatment of bleach plant effluents.

II. Pulp Bleaching

In kraft pulping, about 90–95% of wood lignin gets solubilized during the cooking process. The remaining 5–10% of lignin is mainly responsible for the brown color of the kraft pulp and unbleached paper. The basic aim of bleaching is to remove the residual lignin from the pulp as selectively as possible, without degrading the pulp carbohydrate, especially cellulose, which would decrease the strength of the pulp. Pulp bleaching is carried out in a series of steps employing bleach chemicals such as Cl_2, ClO_2, O_3, O_2, H_2O_2, etc. Normally, chlorination is the first bleaching stage

where unbleached pulp is treated with elemental chlorine at a pH of 0.5–1.5 and consistency of 3–4%. Chlorine reacts with lignin by substitution of H-atoms with Cl-atoms, oxidation of lignin fragments to carboxylic acid groups, and to a small extent addition of Cl_2 across carbon–carbon double bonds. Only a small amount of lignin is removed in this stage. The extraction stage involves extraction of degraded lignin compounds with NaOH solution which, otherwise, would increase the chemical usage in subsequent bleaching stages. The alkali displaces chlorine and makes the lignin soluble. Bleaching with hypochlorite solution, usually in the form of sodium or calcium salts (NaClO, CaClO) is called the H-stage. This stage is carried out at 4–8% consistency, 35–45°C for 1–5 h, at a pH of 10. Hypochlorite is more selective than elemental chlorine and extracts lignin as it is depolymerized. Bleaching with chlorine dioxide is called D-stage. ClO_2 is relatively expensive but highly selective for lignin. This makes it very useful in the latter stages of bleaching where lignin is present in very low concentrations. P-stage involves bleaching with H_2O_2 and is not very common for chemical pulps. However, when it is used to bleach chemical pulps, it appears as the last stage of the sequence such as CEHP or CEHDP (C—Chlorination, E—Alkali extraction, H—Hypochlorite, D—Chlorine dioxide, P—Hydrogen peroxide). Oxygen bleaching involves delignification of pulp using oxygen under pressure (550–700 kPa) and in the presence of NaOH (3–5% on pulp).

III. Bleach Effluents

During bleaching, the wood components, mainly lignin, get degraded, heavily modified, chlorinated, and finally, dissolved in the effluent (Dence and Reeve, 1996). As a result, the effluent from the bleaching process is dark brown due to the presence of chromophoric polymeric lignin derivatives. The amount of chlorinated organics produced during the pulp bleaching, varies with wood species, kappa number of the pulp, bleaching sequence, and conditions employed. Typically, color, BOD_5, COD, and TOCl (AOX) in the effluent from the bleaching of softwood kraft pulp by the conventional sequence are in the range of 150–200, 8–17, 50–70, and 3–5 kg/ton of pulp bleached, respectively (Springer *et al.*, 1994). The pollution loads from a hardwood kraft pulp bleach plant are, generally, lower than those from a softwood pulp bleaching plant.

Of the total chlorine used in the bleach plant, about 90% form common salt (NaCl), and 10% or less gets bound to the organic material removed from the pulp. This organically bound chlorine is also termed AOX. A physicochemical classification of this chlorinated organic material, present in spent liquors from conventionally pulped and bleached softwood kraft pulp, is shown in Fig. 1 (Axegard, 1993; Gergov, *et al.*,

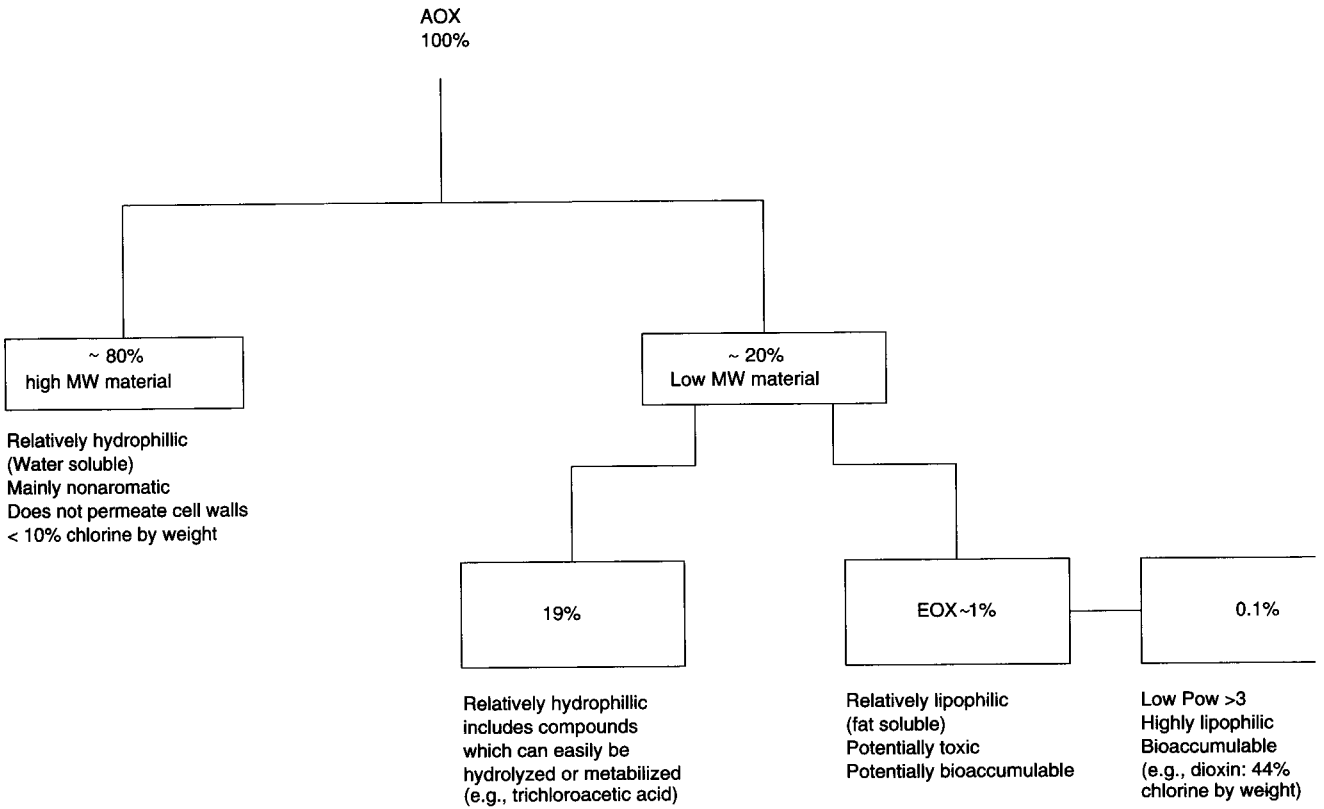

FIG. 1. The character of adsorbable organic halides (AOX) in the effluent from conventionally pulped and bleached kraft pulp.

1988, Lindstrom and Mohamed, 1988). The figure illustrates that as much as 80% or more of the organically bound chlorine corresponds to high-molecular-weight (MW > 1000) chlorinated lignin material, commonly referred to as chlorolignin. The exact chemical nature of the chlorolignin is not clearly understood, but is assumed to include mainly chlorine-substituted polycarboxylic acid polymers, originating from the oxidative degradation of residual lignin, and is devoid of aromatic structure.

About 20% of the organically bound chlorine corresponds to relatively low-molecular-weight material. This fraction is expected to contain potentially problematic compounds (toxic to aquatic organisms) due to their ability to penetrate cell membranes or their tendency to bioaccumulate in the fatty tissues of higher organisms. Some of the major components of this low-molecular-weight fraction have been found to consist of relatively water-soluble substances such as chlorinated acetic acids or chlorinated acetone (Gergov *et al.*, 1988, Lindstrom and Mohamed, 1988), which are easily broken down before or during biotreatment and are, therefore, of little environmental significance.

The fraction of AOX which is extractable by a nonpolar organic solvent (n-hexane), and referred to as EOX, accounts for about 1–3% of the TOCl. This fraction contains lipophilic (fat-soluble) neutral organic compounds primarily of low molecular weight and, therefore, of greater environmental significance than the remaining 99% of the AOX material.

About 456 different compounds have been identified in the effluents from conventional bleach plants. About 330 of those are chlorinated organic compounds, which include chlorinated phenolics, dioxins, hydrocarbons, and resin acids (Mckague and Carlberg, 1996).

IV. Environmental Impact of Bleach Kraft Mill Effluent

Bleach kraft mill effluent is a complex mixture of chlorinated and nonchlorinated products of lignin and/or extractives of wood that imparts dark color to the effluent. Colored effluent may result in the following detrimental effects upon the receiving water body:

1. Color, derived from lignin, is an indicator of the presence of potentially inhibiting compounds.
2. Color reduces the visual appeal and recreational value of the water.
3. It affects downstream municipal and industrial water uses, and increases the cost and difficulty of pretreatment for industrial processes.

4. It retards sunlight transmission, thus reducing the productivity of the aquatic community by interfering with photosynthesis.
5. Color-imparting substances form complexes with metal ions such as iron or copper, and form tar-like residues. These residues may have direct inhibitory effects on some of the lower organisms in the food chain.
6. Color bodies exert long-term BOD (20–100 days) that cannot be measured in terms of 5–day BOD.

Bleached kraft mill effluent can effect the biological quality of the receiving water. Disappearance of benthic invertebrates, such as mussels, and high incidences of fish diseases are some of the effects (Sundelin, 1988; Sodergren *et al.,* 1993). Bleached kraft and bleached sulfite mill effluents have been demonstrated to impair the functions of liver, enzyme systems, and metabolic cycles in the exposed fish. Furthermore, such exposures have been demonstrated to increase the incidence of spinal deformities and reduced gonad development.

A major part of the organically bound chlorine (80%) is believed to be heterogeneous material of relatively high-molecular-weight compounds. These compounds apparently contribute little to the effluent BOD and acute toxicity. Their major contribution is toward color, COD, and chronic toxicity. Ecological/natural processes, such as sedimentation, biodegradation, and bioaccumulation, are apparently correlated with the molecular size and hydrophobicity of the compounds. Highly polar and high-molecular-mass constituents are responsible for the toxicity of the bleach effluents during early life stages of marine animals and plants (Higachi *et al.,* 1992). Chlorocymenes and chlorocymenenes in the bleach effluent have been reported to bioaccumulate in fish and mussels (Suntio *et al.,* 1998).

Chlorinated dioxins, which are present in very low concentrations in the bleach plant effluent (usually in ppt levels), account for a 10 billionth of the total AOX discharged. About 210 different dioxins, belonging to the two families, namely, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), have been reported in the bleach effluents. 2,3,7,8-TCDF and 2,3,7,8-TCDD are especially toxic, carcinogenic, and bioaccumulable. Dioxins are almost insoluble in water. They tend to enter the food chains and accumulate in high concentrations in predators, such as fish-eating birds (McCubbin, 1989, McCubbin *et al.,* 1990). Adverse effects of dioxins have been observed in almost all species tested. According to an Environmental Protection Agency (EPA) report (Anonymous, 1994), human beings lie somewhere in the middle of the sensitivity range (from extremely responsive to extremely resistant) for dioxins. Even in trace amounts,

TABLE I

DISCHARGE LIMITS FOR CONVENTIONAL PARAMETERS[a]

	Toxicity	BOD (kg/TP)	COD (kg/TP)	TSS[b] (kg/TP)
Canada	$LC_{50} \geq 100\%$	7.5–1.5	—	11.25
United States	Chronic and acute[c]	5.5–12.5	—	11.9–20.1
Finland	—	6.8–3.4	65	5–15
Sweden	—	7.5–17	39–107	0.3–5.8

[a] Based on Axegard *et al.* (1993).

[b] Because filters of different coarseness are used for TSS by various countries, the values in this column are not comparable.

[c] Varies by province or state.

dioxins may cause a wide range of adverse health conditions, such as disruption of regulatory hormones, reproductive and immune system disorders, and abnormal fetal development (Bajpai and Bajpai, 1996).

V. Environmental Regulations

Traditional concerns over oxygen deficiency, fiber deposition, and the health of the fish community in receiving waters have led to the setting of discharge limits for the so-called conventional parameters, such as BOD, COD, TSS, and acute toxicity to fish (Table I).

More recently, there has been a shift in emphasis toward concerns about the discharge of persistent and bioaccumulable compounds which have potential, at lower than lethal concentrations, for chronic (long-term) adverse biological effects. Such concerns have focused attention on mills producing chemical pulps bleached with chlorine compounds. This has led, in turn, to the introduction of regulatory measures ranging from limits of overall discharge of chlorinated organic materials such as AOX to specific polychlorinated organic compounds such as 2,3,7,8-TCDDs and 2,3,7,8-TCDFs.

The Canadian Environmental Protection Act prohibits release of final effluent that contains any measurable concentrations of 2,3,7,8-TCDDs (>15 ppq) and 2,3,7,8-TCDFs (>50 ppq) (Canada Gazette, 1992). Many countries prescribe 1 kg/ton of pulp as the standard for AOX discharge. This limit is imposed on sulfite pulp mills in countries like Austria, Germany, and Norway, probably because control of AOX discharge is relatively easier in these mills which use softwood as raw material. In certain regions of Sweden, even kraft mills have limits as low as 0.3 kg/ton of pulp (Rennel, 1995). Some established or planned regulations are shown in Table II.

TABLE II

AOX/TOCL DISCHARGE LIMITS[a]

Country	AOX/TOCl (kg/ton)	Remark
Australia	1.0	For new mills
Austria	0.5–1.0	
Canada		
Alberta	0.29	
British Columbia	0–0.5	
Ontario	1.5	
Quebec	0.5–1.5	Lower limits for hardwood pulps
Finland	1.0–2.0	
Germany	1.0	
India	2.0 (TOCl)	
Japan	1.5	
Norway	1.0–2.0	Lower limits for hardwood pulps
Sweden	0.3–0.5	Lower limits for hardwood pulps
United States	0.623	Monthly average
	0.512	Annual average
	0.951	Daily maximum
	Nondetect	Dioxin and 12 chlorinated phenolics
	31.9 pg/l	TCDF (daily maximum)
	4.14 g/ton	Chloroform

[a] Based on Bajpai *et al.* (1999).

The permitted levels for AOX in many more countries are likely to decrease to less than 1 kg/ton of pulp in the next few years. According to a decision taken in 1992 by PARCOM (Paris Convention for the Prevention of Marine Pollution) for land-based sources and rivers, 1 kg/ton is the limit agreed for AOX since 1995. This limit applies to effluents from all types of chemically bleached pulps and has been accepted by European countries (Belgium, Denmark, France, Germany, Ireland, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, and UK).

The so-called "cluster rule," first proposed in December 1993, was promulgated by the Environmental Prótection Agency (EPA) on November 14, 1997, after some modifications (Anonymous, 1997). Limits proposed by the US-EPA, for various parameters, are shown in Table III. The regulated chlorinated phenols include trichlorosyringol; 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 3,4,5-trichlorocatechol, 3,4,5-trichloroguaiacol, 3,4,6-trichlorocatechol, 3,4,6-trichloroguaiacol, 4,5,6-trichloroguaiacol, tetrachlorocatechol, tetrachloroguaiacol, 2,3,4,6-tetracholorophenol, pentachlorophenol (Vice and Carrol, 1998).

The Ministry of Environment and Forests, Government of India, has categorized the pulp and paper industry as one of the 20 most polluting

TABLE III

LIMITS FOR EXISTING BLEACHED PAPER GRADE KRAFT AND SODA MILLS IN THE UNITED STATES[a]

Parameter	Daily maximum	Monthly average
2,3,7,8-TCDD (pg[b]/liter)	<ML[c]	n.a.[d]
2,3,7,8-TCDF (pg[b]/liter)	31.9	n.a.[d]
Chlorinated phenolics (μg/liter)	<ML	n.a.[d]
Chloroform (g/K kg)[e]	6.92	4.14
COD (kg/K kg)	Reserved	Reserved
AOX (kg/K kg)	0.951	0.623
Color, acetone, MEK, methylene chloride	No limit	No limit

[a] Based on Vice and Carrol (1998).
[b] Picogram.
[c] ML—minimum level. The level at which the analytical signal gives recognizable signals and an acceptable calibration point.
[d] n.a.—not applicable.
[e] 1 K kg = 1 metric ton.

industries and has proposed standards (Table IV) for the disposal of pulp and paper mill effluents. Some of the State Pollution Control Boards in India have already introduced TOCl, at 2 kg/ton of paper, as a controlling factor.

VI. Measures To Reduce Pollution Load

In a conventional bleached kraft mill, with low liquor losses in the recovery cycle, the bleach plant effluent represents, by far, the largest source of COD, BOD, and color, and is practically the only source

TABLE IV

EFFLUENT STANDARDS FOR INDIAN PULP AND PAPER INDUSTRY[a]

	Discharge limits				
Mill type	BOD_5 (mg/liter)	COD (mg/liter)	TSS (mg/liter)	TOCl (kg/ton)	Flow[b] (m^3/ton)
Large pulp and paper	30	350	50–100	2	200 (100)
Newsprint/rayon grade	30	350	50–100	2	150
Small pulp and paper					
Agro based	30	—	100	—	200 (50)
Waste paper based	30	—	100	—	75 (50)

[a] Personal communication, Mall (1996).
[b] The values in the parentheses are applicable to the mills established after January 1992.

of AOX (Aprahamian, 1990). The following sections review the control measures adopted to reduce the organochlorines in bleach plant effluents.

A. Internal Process Modifications

The content of chlorinated organic compounds in bleach plant effluents can be reduced by (a) lignin removal before chlorination, (b) modification of conventional bleaching, and (c) recovery of bleach plant effluent.

In order to minimize the formation of chlorinated organic material, and to ensure compliance with various regulations on AOX and dioxin limits, the producers of the bleached kraft pulp have made substantial changes in the pulp manufacturing technology (McDonough, 1995). As the quantity of chlorine necessary to bleach the pulp is a function of lignin content of the pulp, a lower lignin content before the chlorination stage which contributes toward reduction in chlorine requirements. Several methods have been tried to reduce the lignin content of the pulp. These include extended delignification, oxygen delignification, enzyme pretreatment, and fungal pretreatment.

The key to reducing the bleach plant pollutant loading is to extend the delignification of the brown stock pulp as far as possible, before applying chlorine species (McDonough, 1995, Malinen and Fuhrmann, 1995; Shin and Mera, 1994). Extended cooking removes 35–40% more lignin than conventional cooking. The limiting factors for extending the delignification are the pulp viscosity and its quality. Oxygen delignification in the prebleaching stages decreases pulp lignin content and hence, the pollution load (BOD, COD, color, AOX, chlorinated phenolics, and toxicity) from the bleach plant effluent (Prasad *et al.,* 1996).

Oxygen bleaching was the first step taken to achieve the zero discharge bleach plant in Sweden. Today, all Swedish kraft pulp mills have installed the oxygen bleaching process. In North America, about 46% of the mills have installed an oxygen delignification system (Eklund, 1995; Pryke 1995).

Pretreatment of pulp with enzymes such as xylanases and ligninolytic enzymes prior to bleaching helps in improving final brightness of the pulp, and in reducing or eliminating the use of chlorine and chlorine compounds (Eklund, 1995; Grant, 1995; Tolan *et al.,* 1995). The reduction in chemical charges can translate into significant cost savings, where high levels of ClO_2 and H_2O_2 are used. Reduction in the use of chlorine-based chemicals clearly reduces the formation of chlorinated organic compounds and their release into the effluent and the pulp (Tolan and Foody, 1995).

Pretreatment with fungi has been shown to replace up to 72% of the chemicals needed to bleach the kraft pulp (Fujita *et al.,* 1991). Only a few white-rot fungi have been tested for their ability to delignify kraft pulps. In Japan, a 5-day fungal (F) treatment of hardwood kraft pulp with strain IZU-154 replaced the CE_1DE_2D (C—Chlorination, E_1—First alkali extraction, D—Chlorine dioxide, E_2—Second alkali extraction) sequence with the FCED (F—Fungal treatment, C—Chlorination, E—Alkali extraction, D—Chlorine dioxide) sequence, resulting in 72% chlorine saving (Fujita *et al.,* 1991). Nishida *et al.* (1995) investigated the bio-bleaching of hardwood unbleached kraft pulp by *Phanerochaete chrysosporium* and *Trametes versicolor,* and established a positive correlation between the decrease in kappa number (measure of lignin content in the pulp) and increase in the brightness of the fungal treated pulp. Very few researchers have measured the impact of fungal bleaching on the effluent quality. Fujita *et al.* (1991) reported 50 and 80% reductions in COD and color loading, respectively, in FCED bleaching sequence. Despite the emphasis on fungal bleaching as a means to reduce the use of chlorine and associated formation of chlorinated organics, the effect upon chlorinated organics has not been reported.

The substitution of ClO_2 for Cl_2 reduces the formation of AOX by 80%, which may be a reasonable solution to satisfy the existing AOX regulation. Further reduction in AOX discharge by the addition of dimethylsulfoxide along with ClO_2 substitution was reported (Lachenal *et al.,* 1996). Parthasarathy *et al.* (1994) showed that even with 70% substitution at the first stage, 2,3,7,8-TCDD and 2,3,7,8-TCDF concentrations in the effluent were not detectable. Switching to 100% ClO_2 substitution would result in AOX reduction of as much as 75%. AOX from the bleach plant was reduced to less than 1.8 kg/ton of pulp. There was a substantial decrease of chloroform in the untreated effluent. After the treatment, chloroform concentration was found to be below the detection limit.

Total chlorine-free (TCF) bleaching has been studied extensively over the last 5 years. The use of oxygen-based chemicals (O_3, O_2, H_2O_2) in lieu of chlorine-containing bleaching agents not only decreases the amount of chlorinated organic material in bleaching effluent but also results in an effluent which is almost free from corrosive components (Ristolainen and Alen, 1996). Oxygen bleaching is now an established bleaching technique for both elemental chlorine-free (ECF) and TCF sequences. Being a cheap, nontoxic, renewable, and widely available reagent, oxygen is an excellent alternative to conventional inorganic chlorinated chemicals.

Mapple *et al.* (1994a,b) have reviewed bleach filtrate recovery directed toward bleach plant closure (zero-discharge). Laboratory studies

indicated that effluent volume, color, AOX, and BOD could be reduced by 50, 90, 85, and 70%, respectively. Evans *et al.* (1994) showed that, for the existing mills, it would be economically beneficial to eliminate the use of gaseous chlorine and hypochlorite to minimize the chlorine input, and to evaporate and incinerate the bleach plant waste separately.

Although the implementation of recovery of the bleach plant effluent is considered technically possible today, there are a number of areas where additional developmental work has to be carried out to reduce the risks that are involved in implementing these new concepts. These areas include product quality development, management of nonprocess elements, reduction of solid waste generation and air emissions, management of process upset conditions, and mill chemical balances (Mannisto *et al.*, 1994).

B. External Treatment of Bleach Plant Effluent

Process modifications and allied solutions can reduce the pollution load but not to the extent that the waste generation is totally eliminated. The wastes generated will still require treatment, in order to meet the prescribed effluent standards, before disposal into the environment. The technologies that can be used to treat bleach plant effluent (end-of-pipe remedies) include—physicochemical processes, electrochemical processes, enzymatic treatment, and biological treatment processes.

1. Physicochemical Processes

A variety of physicochemical methods have been tried for the treatment of bleach plant effluent. These include—coagulation, flocculation, settling (Dilek and Goekcay, 1994; Stephenson and Duff 1996a,b; Ganjidoust *et al.*, 1996), adsorption on active surfaces such as fly ash (Nancy *et al.*, 1996), and membrane techniques (Yao *et al.*, 1994; Pejot and Pelayo, 1993).

A wide variety of coagulants have been tested for their effectiveness in the removal of colour from bleach plant effluents. Coagulation with alum dosage of 100 mg/liter has been reported to reduce 80% color and 50% COD (Dilek and Goekcay, 1994). Through the use of a mixture of polyethylene and modified starches, Milstein *et al.* (1991) reported 75, 59, and 80% removal of AOX, COD, and color, respectively. Chloride and sulfate salts of iron and aluminium were effective in treating bleach kraft mill effluents (Stephenson and Duff, 1996a,b). Removal efficiencies of 88 and 98% were observed for total organic carbon (TOC) and turbidity, respectively. Toxicity was also markedly reduced. With chitosin as a coagulant, 90 and 70% reduction of color and TOC, respectively, were reported (Ganjidoust *et al.*, 1996).

Adsorption on active surfaces has frequently been employed for removing pollutants such as biphenyls, organochlorines, and heterocyclic organics. For example, fly ash as an adsorbing medium removes COD and color efficiently (Nancy *et al.,* 1996).

Ultrafiltration is another good method for removing colored material from bleach kraft mill effluent. Using the technique, Pejot and Pelayo (1993) have achieved 79–91% decolorization and 74–88% COD removal.Yao *et al.* (1994) have achieved 90 and 99% reduction in TOC and AOX, respectively, through the use of this technique. Brite (1994) had tried nanofiltration, combined with electrodialysis, at a pilot-scale level to treat pulp bleach effluent. He reported over 95% removal of the contaminating toxic organic halides, salts, and colorants, and the treated effluents were found suitable for process reuse.

Oxidation can accomplish the destruction of both chromophoric and toxic compounds. Oxidants that have been used or proposed include chlorine (Clark *et al.,* 1994), oxygen (Sun *et al.,* 1992), ozone (Hostachy *et al.,* 1996), and peroxide (Smith and Frailey, 1990). Ozonation was reported to remove 72% of the effluent color at a dosage of 40 ppm. Further, it was found to selectively destroy acute toxicity of chemomechanical pulp effluents (Roy-Arcand and Archibald 1991a,b). Hostachy *et al.* (1996) reported complete detoxification of bleached kraft mill effluent (BKME) at low ozone doses (0.5–1.0 kg/ton air dried pulp). Sun *et al.* (1992) removed approximately 70–80% of TOCl and 60–70% of the effluent color associated with high-molecular-weight chlorolignins by oxidation at high temperatures under alkaline conditions. Clark *et al.* (1994) reported 50–90% decolorization of the effluent with chlorine. The cost of the oxidizing agent is a significant issue. The inexpensive oxidants (chlorine and hypochlorite) are also the ones that produce unwanted organochloride by-products, especially chloroform. The other oxidizing agents are either very expensive or unstable or both. To make these processes more economical, it has been proposed to use them as a pretreatment to biological treatment. Pretreatment partially degrades the compounds that otherwise resist biological treatment into forms that are biodegradable, thus eliminating color and toxicity. Pretreatment with ozone or peroxide can increase the biodegradability of kraft mill caustic extraction stage effluents (Hilleke, 1993).

Advanced oxidation process technology is widely used for treating contaminated groundwater. Some investigators have found beneficial effects from using ozone or peroxide in combination with ultraviolet (UV) light in treating bleachery waste. Color removal efficiencies of as high as 80% were achieved in a pilot-scale treatment of bleach kraft mill effluent through oxidation, first with peroxide (480 mg/liter), and then

with UV radiation (Smith and Frailey, 1990). Smith (1990) employed UV radiation, ozone, and peroxide to treat bleach mill effluent and reduced the color to 1.5 kg/ton from an initial value of 3.5 kg/ton.

In summary, physicochemical technologies are costly and rather unreliable. Oxidation using ozone and hydrogen peroxide is expensive. The coagulation/precipitation methods of treatment produce voluminous sludges, the handling of which poses difficulty. Oxidation using chlorine species (chlorine and hypochlorite) are reported to generate secondary pollutants such as chloroform. Membrane filtration techniques require pretreatment and are capital intensive. Membrane fouling is the another problem associated with these techniques.

2. Electrochemical Processes

In electrochemical treatment, chloride in the effluent is converted by electrolysis to chlorate, hypochlorite, and chlorine. The chlorine and hypochlorite oxidize the organic compounds in the effluent and chloride is regenerated. Springer *et al.* (1994) used a bench-scale electrochemical cell in a study for investigating technical and economic feasibility of electrochemical treatment as a method for the removal of color and toxicity. Operating costs were between $0.50 and $2.32/1000 gal of effluent or $5–$23/ton of pulp. Although the electrochemical systems are effective, they are high in operating costs because much of electrochemical energy is consumed in undesirable side reactions.

3. Enzymatic Treatment

Some enzymes, particularly peroxidases and laccases, also seem to have the potential to remove color and AOX from pulp and paper mill effluents. Roy-Arcand and Archibald (1991) carried out a systematic study on direct dechlorination of chlorophenolic compounds in pulp and paper mill effluent by laccases from *T. versicolor* and found that all the major laccases secreted by *T. versicolor* could partially dechlorinate a variety of chlorophenolics.

A patented process (Call, 1991) for the decolorization and decontamination of wastewater from pulp and paper mills is based on laccase from *T. versicolor* to remove 70–90% of the lignin contained in the wastewater. The lignin is polymerized and converted into insoluble matter and then removed by flocculation and/or filtration.

Roy-Arcand and Archibald (1991) also studied effects of horseradish peroxidase (HRP) and *Phanerochaete chrysosporium* peroxidase on a mixture of five chlorophenolics (pentachlorophenol, tetrachloroguaicol, 4,5,6-trichloroguaicol, 4,5-dichloroguaicol, 2,4,6-trichlorophenol). Both peroxidase enzymes degraded the majority of substrates except pentachlorophenol. The *P. chrysosporium* peroxidase was superior to both horseradish peroxidase and laccase in degrading pentachlorophenol.

The use of enzyme-based treatments offer some distinct advantages over physical and chemical methods in that only catalytic amounts of reagents are needed. On the other hand, disadvantages include biochemical instability and difficulty in reusing of the enzyme.

Immobilization of the enzymes is required for biochemical stability and reuse of the enzymes. Carbon-immobilized laccase was used by Davis and Burns (1992) to decolorize extraction stage effluent at the rate of 115 PCU/enzyme unit/h. The removal rate increased with higher effluent concentration. Dezotti *et al.* (1995) developed a simple immobilization method where activated silica was used as a support and used it for enzymatic color removal from extraction stage effluent by lignin peroxidase (LiP) from *Chrysonilia sitophila* and by commercial horseradish peroxidase (HRP). Immobilized HRP gave 73% decolorization and LiP gave 65 and 12% reductions in COD and color, respectively. Immobilized enzymes retained activity even after 5 days of contact with the kraft mill effluent.

4. Biological Treatment

Biological wastewater treatments can reliably reduce pollution reduction from pulp and paper mill effluents. The biological processes currently employed include aerobic, anaerobic, and combination of both treatments.

a. Aerobic Biological Treatment. In aerobic biological processes, organic matter is oxidized to CO_2 and H_2O by microorganisms in the presence of oxygen. A fraction of the organic matter removed is synthesized to form new microbial cells. The microorganisms are a mixture of bacteria, algae, fungi, protozoa, etc. Bacteria are the predominant species and are primary consumers of the organics.

Factors affecting aerobic oxidation efficiency include the concentration of dissolved oxygen, pH, temperature, and nutrients. To ensure aerobic conditions, it is generally accepted that the dissolved oxygen must be maintained above 2 mg/liter. The optimum pH lies between 6.8 and 8.0, and often the pH of the pulp mill effluent requires some adjustment. Systems currently used in pulp mill effluent treatment are operated in the mesophillic temperature range (35°C). Since the temperature of the most process streams exceeds this value, most of the pulp mill effluents require cooling (from 60 to 35°C) before entering the biological treatment systems. Thermophillic operation (50–60°C) of a treatment system is, therefore, attractive and is the subject of some current research (Barr *et al.*, 1996). Nutrients include nitrogen and phosphorus as well as micronutrients (trace metals). To support aerobic bacterial growth, the nutrients with a BOD:N:P ratio of 100:5:1 by mass are considered to be suitable. Since pulp mill effluents are normally deficient in nitrogen

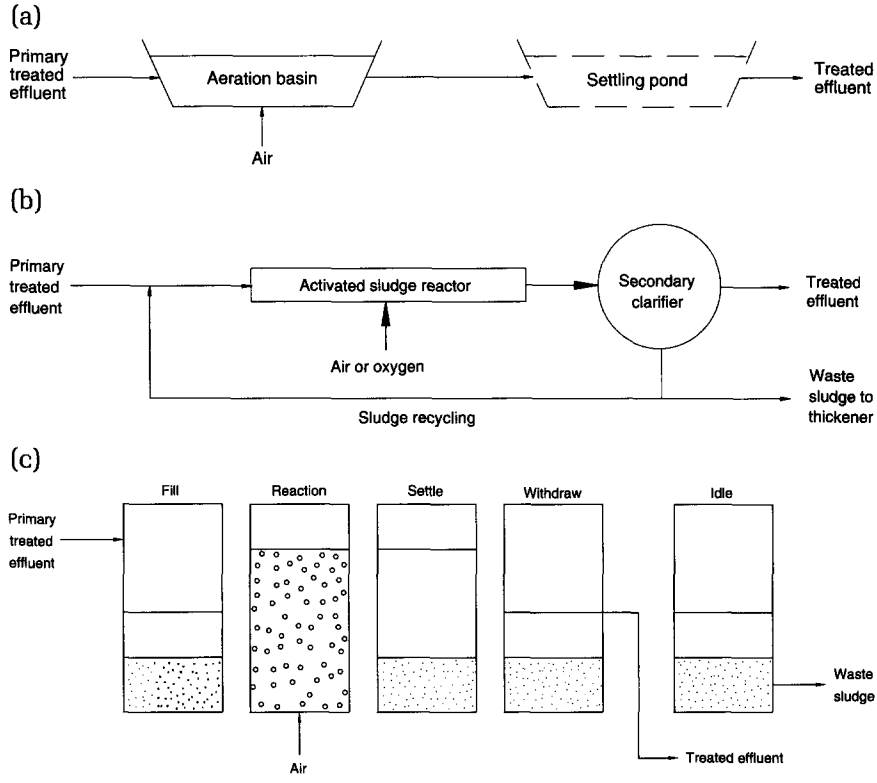

FIG. 2. (a) Aerated lagoon treatment process, (b) Conventional activated sludge treatment process, (c) Sequencing batch reactor treatment process.

and phosphorus, these two nutrients need to be added. The amounts of the trace metals in the effluent are normally considered sufficient.

The most common aerobic biological methods used in the treatment of pulp mill effluents are aerated lagoons or stabilization basins (ASB), activated sludge treatment (AST) processes, and sequencing batch reactors (SBR). (Figs. 2a–2c). Rotating biological contactors (RBCS) and trickling filters are rarely used.

AERATED LAGOON TREATMENT (ASB). The aerated lagoon (Fig. 2a) is a low-rate aerobic biological process. The oldest and simplest type of aerobic biological treatment system to construct and operate, it requires an aeration system for supplying dissolved oxygen. The wastewater is continuously fed into the aeration lagoon where biooxidation of the organic matter occurs, then directly flows out to the receiving environment. In some cases, a settling pond following the aeration basin is installed to remove the biological and other solids from the treated wastewater. A

large portion of the sludge produced settles in the lagoon or settling pond, where it subsequently undergoes autooxidation or endogenous respiration, which not only reduces the sludge production but also releases and reuses the nutrients from the sludge. Since the ASBs do not recycle the biomass, they normally require a long hydraulic retention time (HRT) (volume/volumetric flow) of 5–10 days, and the microorganism concentration in the lagoon is too (<0.2 g dry wt./liter) low.

ASBs have long been widely employed in the treatment of kraft mill effluent (Tomar and Allen, 1991; McCubbin, 1983). In recent years, ASBs have also been used for other types of pulp mill effluents including thermomechanical pulp (TMP) and chemithermomechanical pulp (CTMP) for the removal of BOD and toxicity chlorophenols, low-molecular-weight AOX, resin and fatty acids of pulp mill effluents (Liu *et al.*, 1996; Johnson and Chatterjee, 1995; Saunamaki *et al.*, 1991; Tomar and Allen, 1991; Jokela *et al.*, 1993). To produce a reliable high-quality effluent, this method generally uses a long HRT of 5–7 days, which achieves high BOD removal (85–95%) and effluent detoxification. Extensive experience in applying ASBs in the treatment of pulp mill effluent is now available. In both Canada and the United States (Wilson and Holloran, 1992; Turk, 1988), most of the early constructed secondary treatment systems in pulp and paper mills, where available land space is not limited, are aerated lagoons. In China, India, and other developing countries, lagoons are the major process for the treatment of pulp mill effluent. Removals of AOX from bleached kraft mill effluents are quite variable among systems, ranging from 15 to 60% with an average of 30% (Wilson and Holloran, 1992). The complex and variable properties of AOX compounds from different processes account for this range in the removal rate.

Experiments on recirculation of biomass in aerated lagoons have indicated that a fourfold increase in lagoon biomass could increase removal efficiency from 50 to 60% (Boman *et al.*, 1988). Significant work has been done to determine the mechanism of AOX removal in aerated lagoon (Yin *et al.*, 1989a; Bryant *et al.*, 1987, 1988). It has been postulated that AOX removal occurs by biosorption of organohalides to biomass and anaerobic dehalogenation and degradation in the benthyl layer of the lagoon with biosorption providing the transport mechanism (Bryant *et al.*, 1987, 1988; Amy *et al.*, 1988). Both high- and low-molecular-weight chlorolignins adsorb to aerobic biomass but aerobic dehalogenation has not been reported.

Conversely, it has been suggested that the majority of AOX removal in an aerated lagoon is due to aeration-enhanced hydrolytic splitting of chlorine from the organic substrate (Yin *et al.*, 1989a). It is said that milliliter suspended solid (MLSS) levels in an aerated lagoon are too

small to allow significant biosorption to sludge. Removal of resin and fatty acids in CTMP effluent is generally >95% and degradation seem to take place by three mechanisms: biooxidation by microorganisms, adsorption on to sludge, and air oxidation. Biooxidation is the main removal mechanism. Adsorption on to sludge is the primary mechanism. However, when the treatment time is very short, air oxidation plays a minor role (Liu *et al.*, 1996). Analysis of relative removals of different MW fractions in three North American ASBs was reported (Bryant *et al.*, 1990). Low-molecular-weight AOX was removed more effectively (43–63%) than high-molecular-weight AOX (4–31%). Effluent AOX removal from mills using hardwood and softwood furnishes was comparable but furnish changeovers reduced the removal performance. In a separate lab scale ASB study, degradation of hardwood-derived TOCl was greater (44–52%) than that for softwood-derived TOCl (44%) (Yin 1989).

Fulthrope and Allen (1995) studied the ability of three bacterial species to reduce AOX in bleached kraft mill effluents. *Ancylobacter aquaticus* A7 exhibited the broadest substrate range but could only affect significant AOX reduction in softwood effluents. *Methylobacterium* CP13 exhibited a limited range but was capable of removing significant amounts of AOX from both hardwood and softwood effluents. By contrast, *Pseudomonas sp.* Pl exhibited a limited substrate range and poor to negligible reductions in AOX levels from both effluent types. Mixed inocula of all the three species combined and inocula of sludge from mill treatment systems removed as much AOX from softwood effluents as did pure populations of *Methylobacterium* CP13. Rogers *et al.* (1975) treated the bleached kraft mill effluent in a bench-scale aerated lagoon for 29, 58, and 99 h and showed that toxicity; BOD, and resin acids were most consistently reduced during the 99-h treatment. Leach *et al.* (1978) reported the biodegradation of seven compounds representing the major categories of toxicants in a laboratory scale batch aerated lagoon. Resin acids which are the major source of acute toxicity were readily biodegradable but only part (less than 30%) of the load of chlorophenolic compounds was removed. Deardorff *et al.* (1994) reported that the efficiency of AOX removal through biotreatment of combined bleach plant effluent increases with increasing chlorine dioxide substitution. Biological treatment in an aerated lagoon reduced the concentration of polychlorinated phenolic compounds by 97%. Jokela *et al.* (1993) reported that aerobic lagoon systems removed 58 to 60% of the organochlorine compounds from the water phase, whereas the full-scale activated sludge plants removed 19 to 55%. Both biotreatments removed all sizes and classes of organochlorine molecules and slightly changed the relative size distribution of the compounds remaining in the water phase toward the large molecular weights. Eriksson and Kolar (1985)

TABLE V

REPORTED AERATED LAGOON AND ACTIVATED SLUDGE REMOVAL EFFICIENCIES FOR CHLOROPHENOLS[a]

Compound	Reduction range (%)	
	Aerated lagoon treatment	Activated sludge treatment
Dichlorophenols	22–63	78
Trichlorophenols	11–57	51–69
Tetrachlorophenols	22–67	86–100
Pentachlorophenols	25	50–80
Dichloroguaiacols	0–89	67–97
Trichloroguaiacols	400–81	18–97
Tetrachloroguaiacols	80	59–99
Dichlorocatechols	115–45	37
Trichlorocatechols	30–41	63–95
Tetrachlorocatechols	13–57	59–90
Monochlorovanillins	92–100	94
Dichlorovanillins	81–96	100

[a] Based on Wilson and Holloran (1992), Boman *et al.* (1988), Bryant *et al.* (1987), Gergov *et al.* (1988), Voss (1983), Lindstrom and Mohamed (1988), Gergov *et al.* (1990), Saunamaki (1989), Rempel *et al.* (1990), and McLeay (1987).

have shown that high-molecular-weight fraction in bleach plant effluents cannot be degraded in an aerated lagoon. In another study, it has been shown that chloroform is stripped during the biological treatment and COD, AOX, and high-molecular-weight material are reduced to a lesser extent (SSVL-85 Project 4, Final report).

Reduction of individual chlorinated organics across aerated basins has been reported by various authors (Boman *et al.*, 1988; Saunamaki *et al.*, 1991; Lindstrom and Mohamed, 1988). Individual removal efficiencies for various chlorophenols provided in Table V range from 30 to 89%. Information obtained from Paprican has indicated removal efficiency up to 100% for chlorovanillins (Willson and Holloran, 1992).

Areated stabilization basins provide distinct advantages over high-rate systems such as activated sludge treatment, including little or no nutrient addition required (except at initial start up) lower net settleable solids generation, lower energy consumptions due to avoidance of sludge handling and reduced aeration requirement, and better toxicity removal. Lagoons are generally able to detoxify pulp mill effluents because of the long HRT, thus ASBs have been universally accepted by pulp and paper mills where land space is not limited. Another major merit of ASBs is much lower capital and operating costs than AST processes. However, since the HRT in an ASB is long, the required land space for constructing an aeration basin is large, which can be a major

disadvantage. This has led to the introduction of activated sludge treatment systems, which require much less land.

ACTIVATED SLUDGE TREATMENT (AST). AST is a high-rate biological process adapted largely from sanitary waste treatment. In contrast to an ASB, in an AST process (Fig. 2b) there is sludge settler following the aeration basin. The function of the settler is to separate the sludge from the treated effluent so that it can be recycled to the aeration basin and bacterial concentration in the aeration basin can be maintained at a high level (2000–5000 mg/liter). The high biomass concentration increases the rate of treatment, so the required HRT for treating the same effluent is much shorter than that in an ASB; aeration basin size required is also greatly reduced. Two major AST processes used in paper mills are air and pure oxygen AST systems.

AST has been used by the pulp and paper industry when the available land space is small and/or a low treated effluent suspended solids concentration is required. ASTs have been adopted initially in the paper mills in the United States. They have also emerged in recent years in Canadian paper mills and are also common in some other countries, e.g., Finland. A number of full-scale AST systems are operated in the United States and in Canada for the treatment of various pulp mill effluents, including those from kraft, paper board, deinking, Thermomechanical pulp (TMP) and CTMP, sulfite and newsprint mill operations (Buckley, 1992; Paice *et al.*, 1996; Johnson and Chatterjee, 1995). ASTs generally reported to remove much higher quantities of AOX than aerated lagoons. Removal efficiencies ranging from 14 to 65% have been reported.

Melcer *et al.* (1995) carried out a pilot-scale investigation of activated sludge treatment of bleached kraft effluent at a Northern Ontario mill site over an 8-month period. The AS system was operated at a 1-day HRT, 25- to 30-day Solids Retention Time (SRT) and 30°C. Treated effluents were found to pass all acute and chronic toxicity tests as measured by Rainbrow trout LC_{50}, Microtox and *Ceriodaphnia* LC_{50} and IC_{25} tests. A high level of effluent quality was achieved with low concentrations of AOX (4–13 mg/liter), total chlorophenolics (0.3–0.32 mg/liter), toxicity equivalents-pentachlorophenol (TEQ-PCP) (0.4–5 mg/liter), total resin and fatty acids (0–4 mg/liter), BOD (4–12 mg/liter), and soluble COD (142–274 mg/liter) being recorded over the whole period of investigation. An 8- to 17-fold reduction in hepatic MFO enzyme activity was measured in the treated effluents over the influent wastewaters.

Valenzuela *et al.* (1997) studied the degradation of chlorophenols by *Alcaligenes eutrophus* TMP 134 in bleached kraft mill effluent. After 6 days of incubation, 2,4-dichlorophenoxyacetate (400 ppm) or 2,4,6 trichlorophenol (40 to 100 ppm) was extensively degraded (70 to 100%).

In short-term batch incubations, indigenous microorganisms were unable to degrade such compounds. Degradation of 2,4,6-trichlorophenol by strain JMP 134 was significantly lower at 200 to 400 ppm of compound. This strain was also able to degrade 2,4-dichlorophenoxyacetate, 2,4,6-trichlorophenol, 4-chlorophenol, and 2,4,5-trichlorophenol when bleached kraft mill effluent was amended with mixtures of these compounds. On the other hand, the chlorophenol concentration and the indigenous microorganisms inhibited the growth and survival of the strain in short-term incubations. In long-term (>1-month) incubations, strain JMP 134 was unable to maintain a large, stable population, but an extensive 2,4,6-trichlorophenol degradation was still observed. When combined effluents of a kraft pulp mill were treated in a lab-scale-activated sludge system, the average TOC and AOX removal efficiencies were found to be 83 and 21%, respectively (Ataberk and Gokcay, 1997). The highest AOX removal occurred at larger SRTs. Mass balance on the system revealed that the principal AOX removal mechanism was metabolization at long SRTs. About 90% of the AOX removed was metabolized. As SRT was lowered, AOX removal also decreased.

When the bleaching effluents from chlorination and extraction stages were treated in an activated sludge process, the AOX reduction was 30–40% in 8 days, with 70–80% of the total AOX reduction achieved in the first 4 days (Mortha *et al.*, 1991). The presence of high-molecular-weight material in the bleached kraft effluent improved the removal of chlorophenolic compounds. Growth experiments using microorganisms from a lab-scale-activated sludge reactor showed that high-molecular-weight material had a significant role in soluble COD and chlorophenol removal (Bullock *et al.*, 1994). Large decreases in the soluble COD and increases in the biomass were observed with the addition of high-molecular-weight materials to the low-molecular-weight fraction. The addition of mono-and dichlorinated phenolic compounds at concentrations up to 10 mg/liter had no effect on the metabolism or growth of the microorganisms in the activated sludge. While 6-chlorovanillin (6-CV), 2,4-dichlorophenol (2,4-DCP), and 4,5 dichloroguaiacol (4,5-DCG) were stable in uninoculated controls and inoculated low-molecular-weight effluent over a 160-h period, these compounds decreased significantly when low-molecular-weight material was inoculated with microorganisms. The removal rates of these compounds increased in the order: 6-CV > 4,5-DCP > 2,4-DCP. Gergov *et al.* (1988) investigated pollutant removal efficiencies in mill-scale biological treatment systems. About 48–65% AOX was removed in the activated sludge process.

The combined effects of oxygen delignification, ClO_2 substitution, and biological treatment on pollutants levels in bleach plant effluents

were examined. Biological treatment did not reduce color but reduced COD, BOD, AOX, and toxicity (Graves *et al.*, 1993). ClO_2 substitution reduced the discharge of all five pollutants with a large reduction in AOX. Oxygen delignification reduced discharges of the five pollutants, and effluents from the sequence with oxygen delignification were easier to treat by aerobic methods. Treatment of bleaching effluent in sequential activated sludge and nitrification systems revealed that dechlorination of bleaching effluent took place in both systems (Altnbas and Eroglu, 1997). In the activated sludge system, released inorganic chloride was 4.5–7 mg/liter at TOC loading rate of 0.03–0.07 mg/mg VSS/d, respectively; but it was decreased from 10 to 3 mg/liter at TOC loading rate of 0.006–0.06 mg/mg VSS/d to respectively.

Removal efficiencies for individual chlorinated organics range from 18 to 100% (Wilson and Holloran, 1992) and are presented in Table V. Liu *et al.* (1996) demonstrated that the AOX removal mechanism includes biodegradation, adsorption to biomass, and air oxidation. Among these three, biodegradation is the major mechanism. Apart from achieving high AOX removals in ASTs, high-performance COD, BOD, and TSS removal was also recorded (Goronzy *et al.*, 1996).

AOX removal efficiency was correlated to SRT and HRT (Rempel *et al.*, 1990) in pilot-scale tests of air- and oxygen-activated sludge systems. The maximum reported AOX removal efficiencies (>40%) were achieved for SRTs greater than 20 days and HRTs greater than 15 h. In a separate report on Finish activated sludge systems, the highest AOX removals (45%) in mill-scale units were reported for SRTs greater than 50 days (Salkinoja-Salonen, 1990). Varying the HRTs and SRTs indicated that HRT had more of an effect on treatment performance than SRT. Longer HRTs led to improved BOD, COD, toxicity, and AOX removal, longer SRTs did not significantly affect performance (Barr *et al.*, 1996). Paice *et al.* (1996) investigated effluents from chemimechanical pulping (CMP)/newsprint operation that was treated in two parallel laboratory-scale-activated sludge systems. Removal of BOD and resin fatty acids in excess of 90% was achieved with an HRT of 24 h. Moreover anoxic conditioning of the sludge (Liu *et al.*, 1997) and hydrolysis pretreatment of bleachery effluents (Zheng and Allen, 1997) have been recently demonstrated to enhance AOX removal by about 8 and 20–30%, respectively, in AST. As the temperature of mill effluent is high (60°C), there has been research on the use of thermophilic (50–60°C) bacteria in ASTs (Barr *et al.*, 1996; Rempel *et al.*, 1990; NCASI, 1990; Puhakka *et al.*, 1994).

SEQUENCING BATCH REACTORS (SBR). The SBR process is a fill and draw cyclic-batch-activated sludge process. The operation of each cycle normally consists of five sequential steps: fill reaction, settle, withdraw,

and idle (Fig. 2c). During the fill period, wastewater is fed to SBR under anoxic conditions (without aeration) and biosorption takes place. After completion of fill, the aerobic reaction starts with aeration. Following reaction, the biomass is allowed to settle under quiescent conditions in the reactor. Finally, about one-third of the SBR of the clarified treated effluent is withdrawn. For multi-SBR systems without sufficient wastewater an idle period may be necessary. The next cycle starts again at the fill stage. Sludge wasting occurs at the end of the settle period or during the idle period. Essentially, the SBR's batch stage can be compared to the unit operations in an AST, with the react stage corresponding to the aeration basin and the settle draw stages corresponding to the secondary clarifier and sludge recycle.

Sequencing batch reactors have the following advantages compared to conventional ASTs: (1) lower operating costs since there is no aeration for 30–40% of the total time, no sludge settler or recycling pumps are required; (2) control of filamentous bulking due to the anoxic fill; (3) ability to tolerate peak flow and shock loads; and (4) denitrification (i.e., conversion of nitrate and nitrite to nitrogen gas) during the anoxic fill and settle stages. In addition, the control and operation of a SBR are flexible.

SBRs were initially used for the treatment of small- and medium-sized municipal wastewaters. Before the 1980s, the application of SBR processes was limited mainly due to the lack of automatic control devices. With the rapid development of modern automatic control devices and computer technology in the 1980s, operation of an SBR can be easily accomplished through automatic control devices. As such, the application of SBRs for the treatment of various effluents has rapidly increased. In recent years SBRs have also been used for the treatment of pulp mill effluents and in North America there are at least nine full-scale SBR systems treating various pulp mill effluents including kraft, TMP, high-yield sulfite, deinking and fine paper mill effluents. SBRs generally produce smaller quantity of effluent the ASTs. One of the major problem is the lack of experience for both design and operation of SBR systems for the treatment of large quantities of effluents.

OTHER AEROBIC TREATMENT SYSTEMS. Other aerobic biological processes include rotary disc contractors and trickling filters. Mathys *et al.* (1993, 1997) studied the treatment of CTMP mill wastewater in laboratory-scale RBC. Application of these two processes for the treatment of pulp mill effluents is limited (Lunan *et al.*, 1995; Mathys *et al.*, 1997).

b. Anaerobic Treatment. Anaerobic biological treatment has been used for a long time to reduce the amount of organic pollutants in pulp and paper mill effluents, and extensive experience of this method is available.

Anaerobic treatment has many advantages over aerobic process. The most important advantages are

- Much lower electrical power demand especially for highly concentrated effluents
- Lower nutritional demand
- Production of energy rich biogas
- Lower sludge production
- The biological sludge may be stored during relatively long shutdown periods without a serious deterioration

Anaerobic digestion of organic matter is a multistage complex biological process that occurs in the absence of oxygen mediated by different groups of bacteria. It is often divided into four steps: hydrolysis, acid formation, acetogeneses, and methanogenesis (Fig. 3).

1. Hydrolysis: Nonsoluble organic compounds are hydrolyzed by enzymes excreted from acidifying bacteria. Since the rate of this

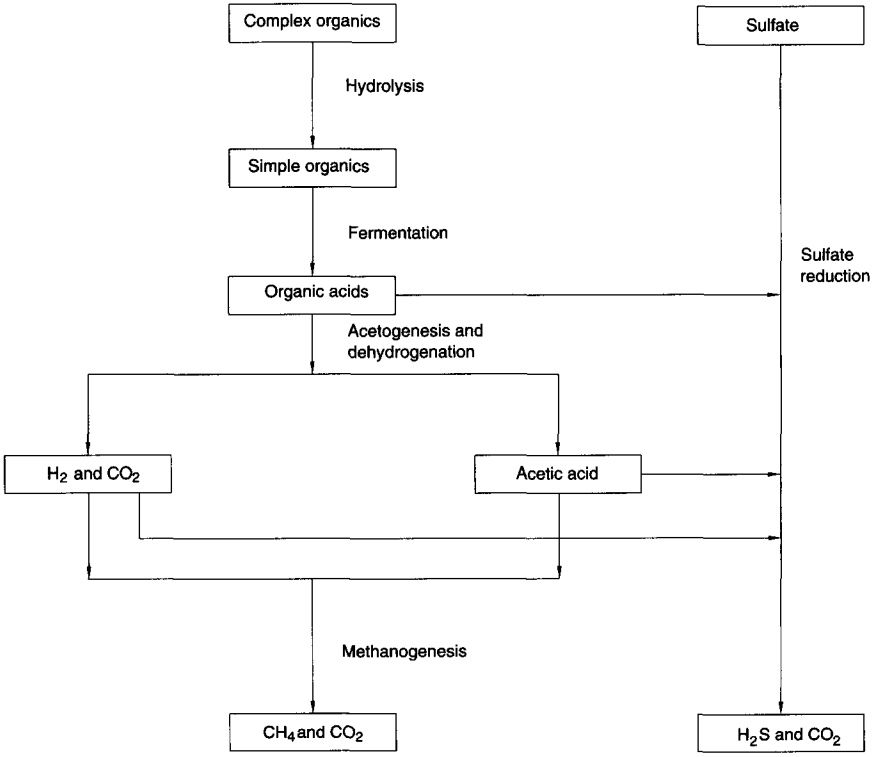

FIG. 3. Anaerobic metabolism to methane in competition with sulfate reduction.

process is rather slow, it is often regarded as the rate-controlling step for the entire anaerobic treatment.

2. Acid formation: The hydrolyzed compounds are converted into organic acids such as lactic acid, butyric acid, propionic acid, and acetic acid by acid-forming bacteria, as well as into alcohol, H_2, and CO_2.
3. Acetogenesis: Organics of the previous step are converted into acetic acid, H_2, and CO_2.
4. Methanogenesis: Methane-forming bacteria convert the products from the previous step into methane as follows:

$$\text{(a)}\ CH_3COOH \rightarrow CH_4 + CO_2$$

$$\text{(b)}\ 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

A very small fraction of the degraded fraction (<10%) of the organic matter is converted into new bacterial cells.

The principal factors affecting the rate of anaerobic digestion of a wastewater are anaerobic conditions, good mixing for intimate bacteria/substrate contact, temperature, pH and alkalinity, presence of toxic substances, nutrients, trace metals, solids retention time, volatile solids, loading rate and hydraulic retention time.

All the anaerobic treatment systems currently used in pulp mill effluent treatment are operated in mesophilic temperature range (35–38°C). The optimal pH for the growth of methanogenic bacteria is between 6.8 and 7.5, although methane production is possible in the range of 6.0–8.5. Alkalinity in an anaerobic system must be sufficient to neutralize the volatile acids produced during the process to maintain an optimum pH (6.8–7.5). Commonly, a minimum of COD:N:P ratio of 100:1:0.5 would be provided to ensure a slight excess of nitrogen and phosphorus.

The major anaerobic processes currently used for the treatment of pulp mill effluents include anaerobic lagoons, anaerobic contractor, upflow anaerobic sludge blanket (UASB), anaerobic fluidized bed, and anaerobic filter (Figs. 4a–4f).

ANAEROBIC LAGOON. The anaerobic lagoon is the oldest low-rate anaerobic treatment process. It generally consists of a large flow-through basin where SRT equals HRT. To achieve a high-treatment efficiency, the HRT is generally long from (10 to 30 days). It requires large land areas, which is the major limitation of the system (Fig. 4a).

ANAEROBIC CONTACT PROCESS. The anaerobic contact process was developed in the 1950s and was the first high-rate anaerobic treatment system (Lee *et al.*, 1993). Separation of the sludge from the settling tank is the critical factor for maintaining high biomass concentration and for operating the contact process. It is an outgrowth of anaerobic lagoon and is

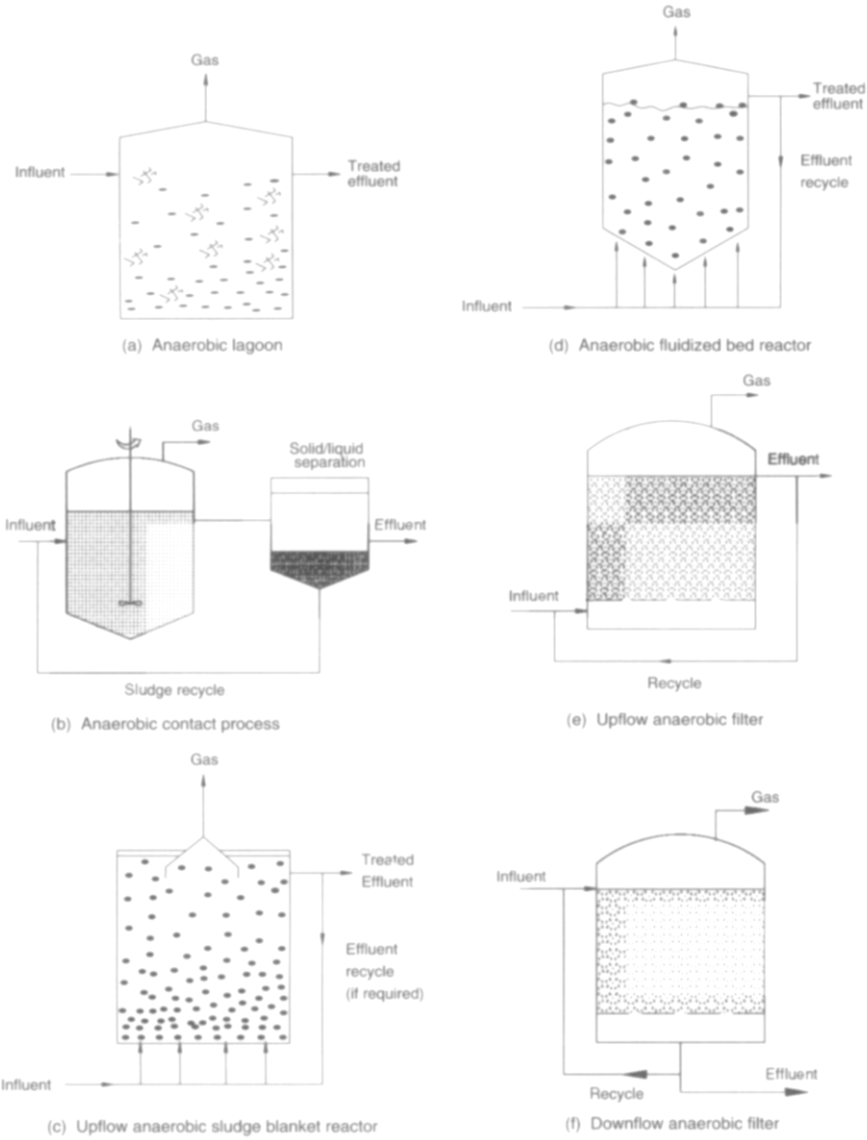

FIG. 4. Schematic of the anaerobic processes used for pulp and paper mill effluents Based on Allen and Liu (1998).

similar to the activated sludge process, consisting of a fully mixed anaerobic reactor and sludge settling tank. A portion of the sludge is returned to the contact reactor to maintain high biomass concentration (3000–10,000 mg/liter) in the reactor. Due to the recycling of sludge, the SRT can be controlled to be much longer than the HRT (Fig. 4b). Separation

of the sludge from the settling tank is the critical factor for maintaining high biomass concentration and for operating the contact process. This system is suitable for treating effluents containing a high concentration of suspended solids. It can be operated at an organic loading from 1 to 2 kg BOD/m^3/day.

UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR (UASB). The UASB reactor was developed in the Netherlands in the 1970s (Lettinga, 1980). This reactor operates entirely as a suspended growth system and consequently does not contain any packing material. It contains a gas–liquid solid separation device for the separation of biogas and treated effluent and suspended solids at the top surface of the reactor to minimize the loss of biomass (Fig. 4c). Wastewater is distributed into the bottom of the reactor and flows upward in the reactor. A dense granular sludge formation in the reactor is the critical factor in process performance, since it ensures proper settling characteristics of sludge. The SRT value is extremely high for well-adapted systems, and generally this process seems to have the potential to treat more dilute and colder effluents than the contact process. Loading rates generally range from 3.5 to 5.0 kg BOD/m^3/day and can be up to 8 kg BOD/m^3/day (Lee *et al.*, 1995). At present, most of the full-scale high-rate anaerobic systems in use in the pulp and paper industry are UASB reactors.

FLUIDIZED BED REACTOR. The effluent is distributed into the bottom of the reactor and flows upwards through a fluidized bed of microorganisms attached on a carrier. A certain amount of water usually has to be recirculated in order to keep the bed fluidized (Fig. 4d). The SRT value may be extremely high, comparable to the UASB reactor. Loading rates are in the range of 17–41 kg BOD/m^3/day (Lee *et al.*, 1995). However, operating costs of this reactor are elevated since recycling of effluent inside the reactor consumes a large amount of power.

ANAEROBIC FILTER. The anaerobic filter, also known as fixed bed or fixed film, contains a packing material, usually plastic, with a large specific area. The microorganisms grow on surface of the material and in the void space between surfaces. The effluent may pass the bed upflow or downflow (Fig. 4e and f). The loading rates range from 4 to 15 kg BOD/m^3/day.

Since the mid 1980s, the use of anaerobic technology for the treatment of pulp mill effluents has became a subject of great interest. Until then pulp and paper mill wastewaters were thought too dilute to be treated by anaerobic technology. Development of various high-rate anaerobic processes and much more concentrated pulp mill effluents (due to the extensive recycling) make the economic benefit from anaerobic treatment more significant, which in turn increases the interest in the use of this technology.

The application of the anaerobic treatment system at pulp and paper mills has become more common in the last few years. Anaerobic

technologies are already in use for many types of forest industry effluents. Currently at least 52 full-scale systems are in operation at pulp and paper industries. The upflow anaerobic sludge bed (UASB) reactor and the contact process are the most widely applied anaerobic systems. Most of the existing anaerobic full-scale plants are treating noninhibitory forest industry wastewater rich in readily biodegradable organic matter (carbohydrates and organic acids) such as recycling wastewater and mechanical pulping (TMP) effluents. On the other hand, full-scale application of anaerobic systems for chemical, semichemical, and chemithermomechanical bleaching and debarking liquors is still limited.

Thermomechanical pulping wastewaters are known to be highly biodegradable during anaerobic digestion and not toxic to methanogenic bacteria. This makes them highly suitable for anaerobic wastewater treatment (Sierra-Alvarez and Lettinga, 1991; Jurgensen *et al.,* 1985; Sierra-Alvarez *et al.,* 1990). In mesophilic anaerobic processes, loading rates up to 12–31 kg COD/m^3/day with about 60–70% COD removal efficiency have been obtained (Sierra-Alvarez *et al.,* 1991, 1990; Rintala and Vuoriranta, 1988). Under thermophillic anaerobic process conditions up to 65–75% COD removal was obtained at 55°C at a loading rate of 14–22 kg COD/m^3/day in UASB reactors (Rintala and Vuoriranta, 1988; Rintala and Lepisto, 1992).

About 60% COD removal was maintained at 50% in the UASB reactor at loading rates as high as 80 kg COD/m^3/day, which corresponds with HRT of 55 min. Kortekaas *et al.* (1998) studied anaerobic treatment of wastewaters from thermomechanical pulping of hemp. Hemp stem wood and hemp bark thermomechanical pulping wastewaters were treated in laboratory-scale UASB reactors. For both types of wastewaters, maximum COD removal of 72% was obtained at loading rates of 13–16 g COD/l/day providing 59–63% recovery of the influent COD as methane. The reactors continued to provide excellent COD removal efficiencies of 63–66% up to a loading rate of 27g COD/l/day, the highest loading rate tested. Batch toxicity assays revealed the absence of methanogenic inhibition by hemp TMP wastewaters, coinciding with the high acetolastic activity of the reactor sludge of approximately 1 g COD/g VSS/day. Due to the relatively low molecular weight of hemp TMP lignin, its removal (which was measured as UV 280 during anaerobic treatment) was markedly high and averaged 45 and 31% for the hemp stem wood and the hemp bark TMP UASB reactors, respectively. Subsequent batch aerobic post-treatment led to considerable increase of color levels and polymerization of the residual lignin to molecular weight in excess of 34 kD.

The application of anaerobic treatments for degradation and dechlorination of kraft bleach plant effluent is a novel application of a renewable

biological process. The COD removals in the anaerobic treatment of bleaching effluents have ranged from 28 to 50% (Lafond and Ferguson, 1991; Raizer Neto *et al.*, 1991; Rintala and Lepisto, 1992). Removal of AOX was improved when easily degradable cosubstrate (methanol or ethanol) was used to supplement the influent (Parker *et al.*, 1993a). Many chlorophenolic compounds, chlorinated guaiacols—catechols and chlorovanillins were removed at greater than 95% efficiency (Parker *et al.*, 1993b). Fitzsimons *et al.* (1990) investigated anaerobic dechlorination/degradation of high molecular weight in materials in bleach plant effluent in a different system. A decrease in organically bound chlorine measured as adsorbable organic halogen was found with all molecular mass fraction. The rate and extent of dechlorination and degradation of soluble AOX decreased with increasing molecular mass. As high-molecular-weight chlorolignins are not amenable to anaerobic microorganisms, dechlorination of high-molecular-weight compounds may be due to combination of energy metabolism, growth, adsorption, and hydrolysis.

Neutral sulfite semichemical (NSSC) pulping is the most widely used semichemical pulping process. Chemical recovery in semichemical pulping is not practiced in all the mills and thus there is a need to treat the spent liquor. Hall *et al.* (1986) and Wilson *et al.* (1987) demonstrated anaerobic treatability of NSSC spent liquor together with other pulping and paper mill wastewater streams. The methanogenic inhibition by NSSC spent liquor was apparently the effect of the tannins present in these wastewaters (Habets *et al.*, 1985). Formation of H_2S in the anaerobic treatment of NSSC spent liquor has been reported but not related to methanogenic toxicity. Apparently, the evaporator condensates from the NSSC production are amenable to anaerobic treatment because of their high volatile fatty acid content (Perttula *et al.*, 1991).

Unstable operations have been encountered in anaerobic treatment of pulp mill effluents, in particular with CTMP and NSSC wastewaters. The exact reasons for these operation problems are still unclear although it is believed that they may be associated with the toxins in these effluents, particularly wood extractives (resins and fattry acids). Because of the unstable operation problems, application of anaerobic treatment technology in the paper industry sector is still limited. Research is under way to develop treatment systems that combine aerobic technology with ultrafiltration processes. The sequential treatment of bleached kraft effluent in anaerobic fluidized bed and aerobic trickling filter was found to be effective in degrading the chlorinated, high- and low-molecular material (Haggblom and Salkinoja-Salonen, 1991). The treatment significantly reduced the COD, BOD, and AOX of the

wastewater. COD and BOD reduction was greatest in the aerobic process, whereas dechlorination was significant in the anaerobic process. With the combined aerobic and anaerobic treatment, over 65% reduction of AOX and over 75% reduction of chlorinated phenolics ware observed. The similar COD/AOX ratio of the wastewater before and after treatment indicates that the chlorinated material was as biodegradable as the nonchlorinated material.

Dorica and Elliott (1994) studied the treatability of bleached kraft effluent using anaerobic and a combination of aerobic and anaerobic processes. BOD reduction in the anaerobic stage varied between 31 and 53% with hardwood effluent. Similarly the AOX removal from the hardwood effluents was higher (65 and 71%) for the single- and two-stage treatments, respectively, than that for softwood effluents (34 and 40%). Chlorate was removed easily from both softwood and hardwood effluents (99 and 96%, respectively) with little difference in efficiency between the single- and two-stage anaerobic systems. At organic loadings between 0.4 and 1.0 kg COD/m^3/d, the biogas yields in the reactors were 0.16–0.37 liter per gram BOD in the feed. Biogas yield decreased with increasing BOD load for both softwood and hardwood effluents. Anaerobic plus aerobic treatment removed more than 92% of BOD and chlorate. AOX removal was 72 to 78% with hardwood effluents and 35 to 43% with softwood effluents. Most of the AOX was removed from hardwood effluents during feed preparation and storage. Parallel control treatment tests in nonbiological reactors confirmed the presence of chemical mechanisms during the treatment of hardwood effluent at 55°C. The AOX removal attributed to the anaerobic biomass ranged between 0 and 12%. The Enso-Fenox process was capable of removing 64–94% of the chlorophenol load, toxicity, mutagenicity, and chloroform in the bleaching effluent (Hakulinen, 1982).

The sequential treatment of bleached kraft effluent in an anaerobic fluidized bed and aerobic trickling filter was effective in degrading the chlorinated high- and low-molecular-weight material (Haggblom and Salkinoja-Salonen, 1991). The treatment significantly reduced the COD, BOD, and the AOX of the wastewater. COD and BOD reduction was greatest in the aerobic process, whereas dechlorination was significant in the anaerobic process. With the combined aerobic and anaerobic treatment, over 65% reduction of AOX and over 75% reduction of chlorinated phenolic compounds were observed (Table VI). The COD/AOX ratio of the wastewater was similar before and after treatment indicating that the chlorinated material was as biodegradable as the nonchlorinated material. Microbes capable of mineralizing pentachlorophenol constituted approximately 3% of the total heterotrophic microbial population in the aerobic trickling filter. Two aerobic polychlorophenol degrading

TABLE VI

REMOVAL OF POLLUTANTS BY ANAEROBIC-AEROBIC TREATMENT OF BLEACHING EFFLUENT[a]

Parameter	Reduction (%)
Chemical oxygen demand (mg O_2/liter)	61
Biochemical oxygen demand (mg O_2/liter)	78
Adsorbable organic halogens (mg Cl/liter)	68
Chlorophenolic compound	
2,3,4,6-Tetrachlorophenol	71
2,4,6-Trichlorophenol	91
2,4-Dichlorophenol	77
Tetrachloroguaiacols	84
3,4,5-Trichloroguaiacols	78
4,5,6-Trichloroguaiacols	78
4,5-Dichloroguaiacols	76
Trichlorosyringol	64

[a] Based on Haggblom and Salkinoja-Salonen (1991).

Rhodococcus strains were able to degrade polychlorinated phenols, guaiacols, and syringols in the bleaching effluent.

Pudumjee Pulp and Paper Mills in Maharashtra, India, which has a 30 tons/day bagasse pulping capacity and a paper manufacturing capacity of 50 tons/day, is running a full-scale anaerobic–aerobic plant for treatment of black liquor (Deshpande *et al.*, 1991). The process is known as Pudumjee-An-OPUR-P. The anaerobic treatment scheme includes two digesters each of 6200-m^3 capacity to treat not only the existing effluent coming from the 30 tons/day pulping operations but also to treat increased flow coming from an enhanced 50 tons/day production capacity. The anaerobic pretreatment of black liquor has reduced COD and BOD by 70 and 90%, respectively. The biogas produced is used as a fuel in boilers along with low sulfur high-speed (LSHS) oil. The anaerobic pretreatment of black liquor has reduced organic loading at aerobic treatment plants thereby reducing the electrical energy and chemical nutrient consumption.

Swedish MoDo Paper's Domsjo Sulfitfabrik is using anaerobic effluent treatment at its sulfite pulp mill and produces all the energy required at the mill (Olofsson, 1996). It also fulfills 90% of the heating requirements of the inner town of Ornskoldvik. Two bioreactors at the mill transform effluent into biogas and slime. The anaerobic unit is used to 70% capacity. A reduction of 99% has been achieved for BOD_7 and the figure for COD is 80%. There are plans to use the slime as a fertilizer.

A process based on Ultrafiltration (UF) and anaerobic and aerobic biological treatments has been proposed (Ek and Eriksson, 1987; Ek

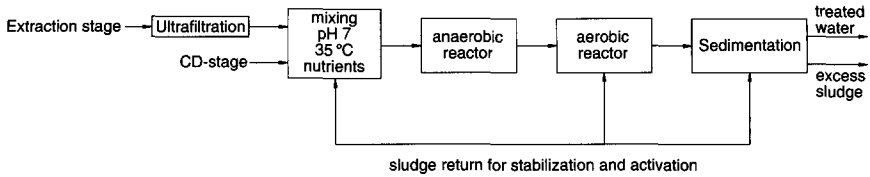

FIG. 5. Part of a system for purification of conventional waste bleach waters.

and Kolar, 1989; Eriksson, 1990). The UF was used to separate the high-molecular-weight mass, which is relatively resistant to biological degradation. Anaerobic microorganisms were believed to remove more efficiently highly chlorinated substances than aerobic microorganisms. The remaining chlorine atoms were removed by aerobic microorganisms. The combined treatments typically removed 80% of the AOX, COD, and chlorinated phenolics, and completely removed chlorate. The principle for part of the purification system is given in Fig. 5 and the results obtained using this system are compared to those normally obtained in an aerated lagoon (Table VII).

Anaerobic processes were once regarded as being too sensitive to inhibitory compounds. The recent advances in the identification of inhibitory compounds/substances in paper mill effluents as well as increasing insight over the biodegradative capacity and toxicity tolerance of anaerobic microorganisms has helped to demonstrate that anaerobic treatment of various inhibitory wastewater is feasible (Lettinga *et al.*, 1990; Rinzema and Lettinga, 1988).

The capacity of anaerobic treatments to reduce organic load depends on the presence of considerable amounts of persistent organic matter and toxic substances. The most important toxicants are sulfate and

TABLE VII

COMPARISON OF REMOVAL OF POLLUTANTS WITH ULTRAFILTRATION (UF) PLUS ANAEROBIC/AEROBIC SYSTEM AND THE AERATED LAGOON TECHNIQUE[a]

Parameter	UF plus anaerobic/aerobic predicted reductions (%)	Aerated lagoon estimated reductions (%)
BOD	95	40–55
COD	70–85	15–30
AOX	70–85	20–30
Chlorinated phenols	>90	0–30
Color	50	0
Toxicity	100	Variable
Chlorate	>99	Variable

[a] Based on Eriksson (1990), Ek and Eriksson (1987), and Ek and Kolar (1989).

sulfite (Pichon *et al.*, 1988), wood resin compounds (Sierra-Alvarez and Lettinga, 1990; McCarthy *et al.*, 1990), chlorinated phenolics (Sierra-Alvarez and Lettinga, 1991), and tannins (Field and Lettinga, 1991). These compounds are highly toxic to methanogenic bacteria at very low concentrations. In addition, a number of low-molecular-weight derivatives have been identified as methanogenic inhibitors (Sierra-Alvarez and Lettinga, 1991).

In CTMP wastewaters, resins and volatile terpenes may account for up to 10% of the wastewater COD (1000 mg/liter) (Welander and Anderson, 1985). The solids present in the CTMP effluent contributed 80–90% of the acetoclastic inhibition (Richardson *et al.*, 1991). The apparent inhibition by resin acids was overcome by diluting anaerobic reactor influent with water or aerobically treated CTMP effluent which contained less than 10% of the resin acids present in the untreated wastewater (Habets and de Vegt, 1991; MacLean *et al.*, 1990). Similarly, the inhibition by resin acids was overcome by diluting the anaerobic reactor influent with water and by aerating the wastewater to oxidize sulfite to sulfate prior to anaerobic treatment (Eeckhaut *et al.*, 1986).

The chlorinated organic compounds formed in the chlorination and alkaline extraction stages are generally considered responsible for a major portion of the methanogenic toxicity in bleaching effluents (Rintala *et al.*, 1992; Yu and Welander, 1988; Ferguson *et al.*, 1990). Anaerobic technologies can be successfully applied for reducing the organic load in the inhibitory wastewaters if dilution of the influent concentration to subtoxic levels is feasible (Ferguson and Dalentoft, 1991; Lafond and Ferguson, 1991). Dilution will prevent methanogenic inhibition and favor possible microbial adaptation to the inhibitory compounds. In practice, considerable dilution might be feasible with other noninhibitory waste streams. Kraft condensates (Edeline *et al.*, 1988) and sulfite evaporator condensates (Sarner, 1988) prior to anaerobic treatment have been shown to reduce the methanogenic toxicity.

Tannic compounds present at fairly high concentrations contribute 30–50% of the COD of the debarking wastewaters and inhibit methanogenesis (Field *et al.*, 1988, 1991). Dilution of wastewater, or polymerization of toxic tannins to high-molecular-weight compounds by auto oxidation at high pH as the only treatment (Field *et al.*, 1991), were shown to enable anaerobic treatment of debarking effluents.

c. Fungal Treatment. Fungi have been harnessed and utilized by humans for thousands of years for many diverse applications. In response to demand for innovative technologies to degrade recalcitrant materials, the nonspecific ability of fungi has been exploited to degrade many of the recalcitrant chemicals, including PCB, PCP, DDT, and

several other polycyclic hydrocarbons (Bumpus and Aust, 1995). Certain fungal species are capable of degrading complex xenobiotic chemicals (organochlorines) and sorb heavy metals from aqueous solutions (Kapoor and Viraraghavan, 1995).

Fundamental research on biological treatment of pulp mill wastewaters especially bleach effluents has been one of the important applications of fungi during the last 2 decades. White-rot fungi (*P. chrysosporium* and *T. versicolor*) are the known microbes capable of simultaneously degrading and decolorizing bleach plant effluents. White-rot fungi have been evaluated in trickling filters, fluidized bed reactors, and airlift reactors (Pellinen *et al.*, 1988; Prouty, 1990). One mycelial color removal (MyCoR) process which uses *P. chrysosporium* to metabolize lignin color bodies has crossed the bench scale and has been evaluated at pilot-scale levels (Campbell *et al.*, 1982; Jaklin-Farcher *et al.*, 1992). This process is very efficient in destroying organochlorines. However, no reactors/process studied so far have been found economically feasible. First, the energy required for lignins/chlorolignin degradation by white-rot fungi has to be derived from an added carbon source such as an easily metabolizable sugar. Moreover, the process is not self-sustaining. The white-rot fungi used do not grow well and must be replenished.

Factors affecting fungal treatment of pulp mill effluents/bleach effluents include concentration of nutrients and dissolved oxygen, pH, and temperature. Fungi, like other living organisms, require certain essential minerals for their growth. The essential mineral nutrients required can be divided into two categories, viz., macronutrients required at 10^{-3} *M* or more, and micronutrients required at 10^{-6} *M* or less. Fungal decolorization involves a series of complex reactions many of which are catalyzed by enzymes. The addition of mineral solution presumably activates the specific enzymes necessary for normal metabolism, growth, and decolorization. The fungus can tolerate a wide range of pH and temperature during decolorization compared to the growth stage. Decolorization is maximal under high oxygen concentration and the fungus requires a carbon source such as glucose or cellulose. A small addition of nitrogen is required to sustain decolorization because nitrogen is lost from the system by the extracellular enzymes secreted by the fungus.

To identify the potential of fungal strains for the treatment of bleach effluents, many researchers have screened cultures obtained from variety of sources. Fukuzumi *et al.* (1977) were probably the first to study the use of white-rot fungi for effluent treatment. The fungi were grown in Erlenmeyer flasks in a liquid medium containing nutrients, vitamins, and spent liquor from the first alkali extraction stage of pulp bleaching. Among the fungi selected from 29 species of tropical fungi and

10 species of Japanese isolates, *Tinctoporia* sp. showed the best results for decolorization of the extraction stage effluents. *Phlebia brevispora, Phlebia subserialis, Poria cinerascens,* and *Trametes versicolor* were tested by Eaton *et al.* (1982) and found to reduce the color of effluents efficiently. In another study (Livernoche *et al.*, 1983), 15 strains of white-rot fungi were screened for their ability to decolorize bleaching effluents. Five fungal strains—*T. versicolor, P. chrysosporium, Pleurotus ostreatus, Polyporus versicolor,* and one unidentified strain showed decolorizing activity. *T. versicolor* was the most efficient in shaken cultures. Galeno and Agasin (1990) evaluated several white-rot fungi collected in the south of Chile for their ability to decolorize bleaching effluents and found *Ramaira* sp. strain 158 to have the highest potential. Over 90% of the color (initial color 14,500 color units) was removed after 140 h under air with a similar rate and extent of decolorization as *P. chrysosporium* did under oxygen.

The addition of an easily metabolizable nutrient such as glucose or cellulose is required for obtaining the maximum decolorization efficiency with most of the white-rot fungal cultures. However, this increases the operational cost of the process. Moreover, if the added nutrients are not completely consumed during the decolorization stage, they could increase the BOD and COD of the effluents after fungal treatment. Esposito *et al.* (1991) and Lee *et al.* (1994) examined fungi that showed efficient decolorization of the extraction stage effluents without any addition of nutrients. Through a screening of 51 ligninolytic strains of fungi, the *Lentinus edodes* strain was shown to remove 73% of the color in 5 days without any additional carbon source. Under these conditions, *L. edodes* was more efficient than the known *P. chrysosporium* strains (Esposito *et al.*, 1991). Lee *et al.* (1994) screened fungi having high decolorization activity. The fungus KS-62 showed 70 and 80% reduction of the color after 7 and 10 days of incubation respectively. To obtain a reasonable basis for evaluation of an industrial fungal treatment, Lee *et al.* (1995) performed treatment of the extraction stage effluent with the immobilized mycelium of the fungus KS-62. This fungus showed 70% color removal (initial color 6600 PCU) without any nutrient within 1 day of incubation with four times effluent replacement; however, the color removal started to decrease at the fifth replacement with the fresh extraction stage effluent. The decolorization activity of the fungus was restored by one replacement of extraction stage effluent containing 0.5 of glucose and the high decolorization was continuously observed for four replacements in the absence of glucose. With the fungus KS-62, such decolorization activity was reportedly obtained for 29 days of total treatment period. Through screening of 100 strains at low glucose concentration, *Rhizopus oryzae*—a zygomycete,

and *Cereporiopsis subvermispora*—a wood degrading white-rot species were shown to remove 95 and 88% of the color respectively. Even in the absence of carbohydrates, significant amounts of color reductions were achieved (Nagarathnamma and Bajpai, 1999, Nagarathnamma *et al.*, 1999). Glucose was the most effective cosubstrate for decolorization by most of the white-rot fungi (Nagarathnamma *et al.*, 1999a,b; Bajpai *et al.*, 1993; Mehna *et al.*, 1995; Fukuzumi, 1980; Prasad and Joyce, 1991; Bergbauer *et al.*, 1991; Pallerla and Chambers, 1995). Belsare and Prasad (1988) showed that the decolorization efficiency of *Schizophyllum commune* could be rated in the following order: sucrose (60%), glucose (58%), cellulose (35%), and pulp (20%). With *Tinctoporia,* ethanol was also found to be very effective cosubstrate for decolorization of waste liquor (Fukuzumi, 1980). Ramaswammy (1987) observed that addition of 1% bagasse pith as a supplementary carbon source resulted in 80% color reduction in 7 days with *Schizophyllum commune.* Eaton *et al.* (1982) compared the suitabilities of three primary sludges and combined sludge with that of cellulose powder for use as a carbon source for *Phanerochaete chrysosporium* cultures. Archibald (1990) reported that *T. versicolor* removed color efficiently in the presence of inexpensive sugar refining or brewery waste. With *Rhizopus oryzae* (Nagarathnamma and Bajpai, 1999), maximum decolorization of the order of 92% was obtained with addition of glucose in 24 h. Ninety percent color reduction was measured with mycrocrystalline cellulose and lactose; 89% was measured with sucrose; and 88% was measured with carboxymethyl cellulose (CMC) and xylose. Starch and ethyl alcohol showed about 87 and 84% color reduction, respectively.

P. chrysosporium is the most studied white-rot fungus for waste treatment. The following nutritional and cultural parameters are important for lignin degradation by this fungus: (1) the presence of cometabolizable substrate, (2) high oxygen tension, (3) correct choice of buffer, (4) correct levels of certain minerals and trace elements, (5) growth limiting amounts of nutrient nitrogen.

Eaton *et al.* (1980) studied the application of this fungus for the treatment of bleaching effluents. Their report indicated that 60% decolorization of extraction stage effluent (initial color 3500 PCU) could be accomplished with *P. chrysosporium* in shake flasks. The same mycelium could be recycled up to 60 days in six successive batches. Mittar *et al.* (1992) also showed that under shaking conditions, the 7-day-old growth of the culture at 20% (v/v) inoculum concentrations resulted in maximum decolorization (70%) of the effluent along with more than 50% reduction in BOD and COD.

Sundman *et al.* (1981) studied the reactions of the chromophoric material of extraction stage effluent during the fungal treatment

without agitation. The results of these studies showed no preference toward degradation of lower-molecular-weight polymeric material over high-molecular-weight material. They noticed that the yield of high-molecular-weight material decreased to half during the fungal treatment. As the color also decreased by 80%, they concluded that chromophores were destroyed. Further, they noticed that the fungal attack led to a decrease in the content of phenolic hydroxyl groups and to an increase in oxygen content.

Joyce and Pellinen (1990) have explored ways to use white-rot fungi to decolorize and detoxify pulp and paper mill effluents. They proposed a process termed FPL–NCSU-MyCoR (Forest Products Laboratory—North Carolina State University—Mycelial color removal process) using *P. chrysosporium* for decolorization of pulp mill effluents. A fixed film MyCoR reactor is charged with growth nutrients which can include primary sludge as the carbon source and is inoculated with the fungus. The sludge will provide some of the required mineral nutrients and trace elements as well as carbon. Nitrogen-rich secondary sludge can be also used to supply the nitrogen required for growth. After the mycelium has grown over the reactor surface, it depletes the available nitrogen and becomes ligninolytic (pregrowth stage 2 to 4 days). The reactor is then ready for use. Operations for over 60 days have been achieved in bench reactors in a batch mode. This process converts 70% of the organic chlorides to inorganic chlorides in 48 h while decolorizing the effluent and reducing both COD and BOD by about half.

Huynh *et al.* (1985) used the MyCoR process for the treatment of chlorinated low-molecular-mass phenols of the extraction stage effluent. It was found that most of the chlorinated phenols and low-molecular-mass components of the effluent were removed during the fungal treatment. Pellinen *et al.* (1988) have reported that the MyCoR process can be considerably improved in terms of COD removal by simply using less glucose as the carbon source for *P. chrysosporium*. However, the decolorization was faster at high glucose concentration.Yin *et al.* (1989b) studied the kinetics of decolorization of extraction stage effluent with *P. chrysosporium* in an RBC under improved conditions. The kinetic model developed for 1- and 2-day retention times showed a characteristic pattern. The overall decolorization process can be divided into three stages viz. a rapid color reduction in the first hour of contact between the effluent and the fungus followed by a zero-order reaction and then a first-order reaction. The color removal rate on the second day of the 2-day batch treatment was less than that on the first day. The decolorization in a continuous flow reactor achieved approximately the same daily color removal rate, but the fungus had a larger working life than when in the batch reactor, thereby removing more color

over the fungal lifetime. Pellinen *et al.* (1988) studied decolorization of high-molecular-mass chlorolignin in first extraction stage effluent with white-rot fungus—*P. chrysosporium* immobilized on RBC. The AOX decreased almost by 50% during 1 day of treatment. Correlation studies suggested that decolorization and degradation of chlorolignin (as COD decrease) are metabolically connected, although these processes have different rates.

The combined treatment of extraction stage effluent with white-rot fungi and bacteria have been also reported. Yin *et al.* (1990) studied a sequential biological treatment using *P. chrysosporium* and bacteria to reduce AOX, color, and COD in conventional softwood kraft pulp bleaching effluent. In six variations of the white-rot fungus/bacterial systems studied, only the degree of fungal treatment was varied. In three of the six variations, ultrafiltration was also used to concentrate high-molecular-mass chlorolignins and to reduce effluent volume (and thus cost) prior to fungal treatment. The best sequence, using ultrafiltration/white-rot fungus/bacteria, removed 71% TOCl, 50% COD, and 65% color in the effluent. Fungal treatment enhances the ability of bacteria to degrade and dechlorinate chlorinated organics in the effluent.

The degradation of model compounds—chlorophenols and chloroguaiacols in pure water solution by fungal treatment using an RBC—has been studied by Guo *et al.* (1990). It was found that at a concentration of 30 mg/liter, 80–85% of chlorophenols and chloroguaiacols could be degraded after 3–4 h of treatment.

Prouty (1990) proposed an areated reactor in order to eliminate some of the problems associated with the RBC process. The fungal life in the aerated reactor was longer and the color removal rate was significantly higher than those of the RBC process in an air atmosphere. A preliminary economic evaluation of the RBC process indicated that the rate of decolorization and the life span of the fungus are the most critical factors (Joyce and Pellinen, 1990). Yin *et al.* (1989b) and Yin (1989) suggested that treatment of the extraction stage effluent by ultrafiltration before RBC treatment would be economically attractive. Their study also suggested that a combination of ultrafiltration and the MyCoR process could reduce the treatment cost, thereby making the process more economically feasible for industrial use.

Although the MyCoR process was efficient in removing color and AOX from bleaching effluents, it also had certain limitations. The biggest problem was the relatively short active life of the reactor. Therefore, several other bioreactors such as packed-bed and fixed-bed reactors were studied (Lankinen *et al.*, 1991; Messner *et al.*, 1990; Cammarota and Santanna, 1992). The use of a trickling filter-type bioreactor, in which

the fungus is immobilized on porous carrier material, was adopted in the MyCOPOR system (Messner *et al.,* 1990). For extraction stage effluent with an initial color between 2600 and 3700 PCU, the mean rate of color reduction was 60% during consecutive 12-h runs. The mean AOX reduction value at a color reduction of 50 to 70% in 12 h was 45 to 55%. Cammarota and Santanna (1992) developed a continuous packed-bed bioreactor in which *P. chrysosporium* was immobilized on polyurethane foam particles. The bioreactor operation at a hydraulic retention time of 5–8 days was able to promote 70% decolorization. In comparison with the MyCoR process, the fungal biomass could be maintained in this process for at least 66 days without any appreciable loss of activity.

To apply the MyCOPOR process on an industrial scale, relatively big reactors (diameter, 70 and 100 mm in diameter; volume 4 to 16 liters) were prepared and filled with polyurethane-foam cubes (1 cm^3) as carrier material. Long-term experiments were successfully carried out and it was decided to build a small pilot reactor at a large paper mill in Austria (Jaklin-Farcher *et al.,* 1992). However, many aspects related to the operating conditions must be further improved. As mentioned earlier, a disadvantage of these treatment processes is that *P. chrysosporium* required high concentrations of oxygen as well as energy sources such as glucose or cellulose, and various basal nutrients, mineral solution, and Tween 80 (Messner *et al.,* 1990). Recently, Kang *et al.* (1996) developed a submerged biofilter system in which mycelia of *P. chrysosporium* were attached to media and used to dispose wastewater from a pulp mill. Maximum reduction of BOD, COD, and lignin concentrations were 94, 91, and 90%, respectively in 12 h of hydraulic retention time.

Fukui *et al.* (1992) determined the toxicity by the microtox bacterial assay of E_P (alkaline extraction with hydrogen peroxide) effluent and ultrafiltration fractionated E_P effluent before and after fungal treatment. The overall toxicity of unfractionated effluent was reduced; however, fungal degradation of higher-molecular-weight fractions led to an increase in toxicity because of the generation of lower-molecular-weight compounds.

Matsumoto and co-workers (1985) demonstrated that RBC treatment of extraction stage effluent was effective for the removal of organically bound chlorine as well as color. Removal of AOX was determined to be 62, 43, and 45% per day for the low-molecular-weight fraction of extraction stage effluent, high-molecular-weight fraction of the same, and unfractionated extraction stage effluent, respectively. After further optimization, 49% of the high-molecular-weight AOX was transformed to inorganic chloride in 1 day and 62% in 2 days. The chloride concentration increased simultaneously with decreasing AOX including decolorization.

Another white-rot fungus—*Coriolus versicolor*—has also shown good performance. This fungus removed 60% of the color of combined bleach kraft effluents within 6 days in the presence of sucrose. Decolorization of effluent was more efficient when the concentration of sucrose and inoculum was high. When the fungus was immobilized in calcium alginate gel, it removed 80% color from the same effluent in 3 days in the presence of sucrose (Livernoche *et al.*, 1983). The decolorization process affected not only the dissolved chromophores but also the suspended solids. The solids after centrifugation of the zero time samples were dark brown while the solids after 4 days of incubation were light brown. The beads with the immobilized mycelium remained light colored throughout the experiments with no indication of accumulation of the effluent chromophores. Recycled beads were found to remove color efficiently and repeatedly in the presence of air but not under anaerobic conditions. In addition, biological reactors of the airlift type using calcium alginate beads to immobilize the fungus *C. versicolor* have been used to study the continuous decolorization of kraft mill effluents (Royer *et al.*, 1985). The effluent used contained only sucrose and no other nutrient source. An empirical kinetic model was proposed to describe the decolorization process caused by this fungus, but it did not shed any light on the chemical mechanism involved in the decolorization.

Bergbauer *et al.* (1991) showed that *C. versicolor* efficiently degraded chlorolignins from bleaching effluents. More than 50% of the chlorolignins were degraded in a 9-day incubation period, resulting in a 39% reduction in AOX and 84% decrease in effluent color. In a 3-liter laboratory fermenter, with 0.8% glucose and 12 m*M* ammonium sulfate, about 88% color reduction was achieved in 3 days. Simultaneously, the concentration of AOX dropped from 40 to 21.9 mg/liter, a 45% reduction in 2 days.

Direct use of suspended mycelium of the fungus *C. versicolor* may not be feasible because of the problem of viscosity, oxygen transfer, and recycling of the fungus. The fungus was therefore grown in the form of pellets, thus eliminating the problems with biomass recycling and making it possible to use a larger amount (Royer *et al.*, 1985). Rate of decolorization with fungal pellets was almost 10 times as high in batch culture as in continuous culture under similar conditions. The capacity for decolorization decreased markedly with increase in lignin loading (Royer *et al.*, 1985).

Bajpai *et al.* (1993) reported 93% color removal and 35% COD reduction, from first extraction stage effluent (7000 PCU) with mycelial pellets of *C. versicolor* in 48 h in batch reactor, whereas, in a continuous reactor, the same level of color and COD reduction was obtained in 38 h. No loss in decolorization ability of mycelial pellets was obtained when

the reactor was operated continuously for more than 30 days. Mehna *et al.* (1995) also used *T. versicolor* for decolorization of effluents from a pulp mill using agriresidues. With an effluent of 18,500 color units, the color reduction of 88–92% with COD reduction of 69–72% was obtained. Royer *et al.* (1991) described the use of pellets of *C. versicolor* to decolorize ultrafiltered kraft liquor under nonsterile conditions with a negligible loss of activity. The rate of decolorization was observed to be linearly related to the liquor concentration and was lower than that obtained in the MyCoR process. This could be due to lower temperature used in this work and to the use of pellets with relatively large diameters which could limit the microbial activity as compared to the free mycelium used in the MyCoR process. An effective decolorization of effluent having 400–500 color units/liter can be obtained in the presence of a simple carbon source such as glucose. In the repeated batch culture, the pellets exhibited a loss of activity dependent on the initial color concentration. Simple carbohydrates were found to be essential for effective decolorization with this fungus and a medium composed of inexpensive industrial by-products provided excellent growth and decolorization (Archibald *et al.*, 1990).

Pallerla and Chambers (1996) have shown that immobilization of *T. versicolor* in urethane prepolymers leads to significant reductions in color and chlorinated organic levels in the treatment of kraft bleach effluents. Color reduction ranging from 72 to 80% and AOX reduction ranging from 52 to 59% is possible from a continuous bioreactor at a residence time of 24 h. The highest color removal rate of 1920 PCU per day was achieved at an initial color concentration of 2700 PCU. The decolorization process was linearly dependent on the concentration of glucose cosubstrate up to a level of 0.8% by weight. The biocatalyst remained intact and stable after an extended 32-day operation.

Treatment of extraction stage effluent with ozone and the fungus *C. versicolor* has also been tried (Roy-Arcand and Archibald, 1991a,b). Both ozone treatment and biological treatment effectively destroyed effluent chromophores but the fungal process resulted in greater degradation as expressed by COD removal. Monoaromatic chlorophenolics and toxicity were removed partially by ozone and completely by *C. versicolor*. Molecular weight distributions showed roughly equal degradation of all sizes of molecules in both the treatments. The combination of a brief ozone treatment with a subsequent fungal treatment revealed a synergism between the two decolorization mechanisms on extraction stage effluent. Effluent was pretreated with ozone (110–160 mg/liter) or *C. versicolor* (24 h with 2–5 g/liter wet weight fungal biomass). The pretreatment was followed by 5-day incubation with *C. versicolor*. It was noted that partial color removal by ozone pretreatment allowed

more effective removal by the fungus than that by fungal pretreatment. After 20 h, 46–53% decolorization was observed for ozone-pretreated effluents, compared to 29% for fungal treatment alone. The contribution of ozone seemed to be most important in the first 24 h following the pretreatment. Ozone pretreatment also produced a small improvement in the bioavailability of effluent organics to the fungus. A partial replacement of chlorine by ozone in the bleach plant or a brief ozone pretreatment of extraction stage effluent should considerably reduce the low-molecular-mass toxic chlorophenolics. In addition, the use of ozone should also improve decolorization by subsequent fungal and possibly bacterial treatments.

The white-rot fungus *Tinctoporia borbonica* has been reported to decolorize the kraft waste liquor to a light yellow color (Fukuzumi, 1980). About 99% color reduction was achieved after 4 days of cultivation. Measurement of the culture filtrate by ultraviolet spectroscopy showed that the chlorine–oxylignin content also decreased with time and measurement of the culture filtrate plus mycelial extract after 14 days of cultivation showed the total removal of the chlorine–oxylignin content.

Another white-rot fungus, *Schizophyllum commune,* has also been found to decolorize and degrade lignin in pulp and paper mill effluent (Belsare and Prasad, 1988). The fungus was able to degrade lignin in the presence of an easily metabolizable carbon source. The addition of carbon and nitrogen not only improved the decolorizing efficiency of the fungus but also resulted in a reduction of the BOD and COD of the effluent. Sucrose was the best carbon source for the degradation of the lignin. A 2-day incubation period was sufficient for lignin degradation by this fungus. Under optimum conditions, this fungus reduced the color of the effluent by 90% and also reduced BOD and COD by 70 and 72% during a 2-day incubation.

Duran *et al.* (1991) reported that preradiation of the effluent, followed by fungal culture filtrate treatment, resulted in efficient decolorization. Moreover, when an effluent pre-irradiated in the presence of ZnO was treated with *L. edodes* (Esposito *et al.,* 1991), a marked enhancement of the decolorization at 48 h was obtained (Duran *et al.,* 1994). They proposed that the combined photobiological decolorization procedure appears to be an efficient decontamination method with potential for industrial effluent treatment. The white-rot fungus *C. subvermispora* has also been found to decolorize, dechlorinate, and detoxify the pulp mill effluents at low cosubstrate concentration (Nagarathnamma *et al.,* 1999). The fungus removed 91% color and 45% COD in 48 h under optimum conditions. The reductions in lignin, AOX, and EOX were 62, 32, and 36%, respectively. The color removal rate was 3185 PCU/day

TABLE VIII

EFFECT OF TREATMENT WITH *C. subvermispora* CZ-3 ON CHLOROPHENOLS AND CHLOROALDEHYDES IN THE EFFLUENT FROM EXTRACTION STAGE[a]

Compounds	Untreated effluent (mg/liter)	Treated effluent (mg/liter)	Removal[b] (%)
2-Chlorophenol	14.2	8.8	36.5
4-Chlorophenol	48.6	3.2	93.4
3-Chlorocatechol	1.8	ND[b]	100
6-Chloroguaiacol	90.1	31.6	67.0
5-Chloroguaiacol	3202.0	184.7	94.0
3,6-Dichloroguaiacol	50.0	2.0	96.0
3,6-Dichlorocatechol	4.9	Nil	100
4,5-Dichlorocatechol	50.3	Nil	100
3,4,5-Trichloroguaiacol	0.1	Nil	100
3,4,6-Trichlorocatechol	4.1	Nil	100
4,5,6-Trichloroguaiacol	8.9	2.4	73
Pentachlorophenol	2.1	Nil	100
Trichlorosyringaldehyde	2.4	1.3	45.9
Tetrachlorocatechol	64.4	Nil	100
2,6-Dichlorosyringaldehyde	46.4	16.5	64.5

[a] Based on Nagarathnamma and Bajpai (1999), and Nagarathnamma *et al.* (1999).

[b] Results are reported as mean of three measurements. ND = Not detected. Extraction stage effluent was treated with *C. subvermispora* CZ-3 (inoculum dose, 2 g dry wt./liter) in a rotary shaker at a speed of 250 rpm for 48 h at a temperature of 30°C and a pH of 4.5.

at an initial color concentration of 7000 PCU. Monomeric chlorinated aromatic compounds were removed almost completely (Table VIII) and toxicity to Zebra fish was eliminated.

A zygomycete *Rhizopus oryzae* has been reported to decolorize, dechlorinate, and detoxify extraction stage effluent at low cosubstrate concentration. Optimum conditions for treatability were determined as pH 3 to 4.5 and temperature 25 to 40°C (Nagarathnamma and Bajpai, 1999). Under optimum conditions, the fungus removed 92–95% color, 50% COD, 72% AOX, and 37% EOX, and there was complete removal of monoaromatic phenolics and toxicity. Significant reduction in chlorinated aromatic compounds was observed (Table IX) and toxicity to zebra fish was completely eliminated. The molecular weight of chlorolignins was substantially reduced after the fungal treatment. Another thermotolerant zygomycete strain *Rhizomucor pusillus* RM 7 could remove up to 71% of color and substantially reduce COD, toxicity, and AOX levels in the effluent (Christov and Steyn, 1998).

Kannan (1990) reported about 80% color removal and over 40% BOD and COD reduction with *Aspergillus niger* in 2 days. Tono *et al.* (1968) reported that *Aspergillus* sp. and *Penicillium* sp. achieved 90%

TABLE IX

EFFECT OF TREATMENT WITH *R. oryzae* ON CHLOROPHENOLS AND CHLOROALDEHYDES IN THE EFFLUENT FROM EXTRACTION STAGE[a,b]

Compounds	Untreated effluent (mg/liter)	Treated effluent (mg/liter)	Removal (%)
2-Chlorophenol	14.20	ND[b]	100
4-Chlorophenol	48.60	2.90	94
3-Chlorocatechol	1.82	Nil	100
6-Chloroguaiacol	90.12	Nil	100
5-Chloroguaiacol	3202.00	Nil	100
3,6-Dichloroguaiacol	50.00	Nil	100
3,6-Dichlorocatechol	4.92	Nil	100
4,5-Dichlorocatechol	50.27	Nil	100
3,4,5-Trichloroguaiacol	0.147	Nil	100
3,4,6-Trichlorocatechol	4.14	Nil	100
4,5,6-Trichloroguaiacol	8.89	2.49	72
Pentachlorophenol	2.11	Nil	100
Trichlorosyringaldehyde	2.38	Nil	100
Tetrachlorocatechol	64.35	27.02	58
2,6-Dichlorosyringaldehyde	46.42	Nil	100

[a] Based on Nagarathnamma and Bajpai (1999), and Nagarathnamma *et al.* (1999).

[b] Results are reported as mean of three measurements. ND = Not detected. Extraction stage effluent was treated with *R. oryzae* (inoculum dose, 2 g dry wt/liter) in rotary shaker at a speed of 250 rpm for 24 h at a temperature of 30°C and a pH of 4.5.

decolorization in 1 week's treatment at 30°C and at pH 6.8. Later Milstein *et al.* (1988) reported that these microorganisms removed appreciable levels of chlorophenols as well as chloroorganics from the bleach effluent. Gokcay and Taseli (1997) have reported over 50% AOX and color removal from softwood bleach effluents in less than 2 days of contact with *Penicillium* sp. Bergbauer *et al.* (1992) reported AOX reduction by 68% and color reduction by 90% in 5 days with the coelomycetous fungus *Stagonospora gigaspora*. Toxicity of the effluent was reduced significantly with this fungus. A few marine fungi have been also reported to decolorize the bleach plant effluents (Raghukumar *et al.*, 1996). With *Trichoderma* sp. under optimal conditions, total color and COD decreased by almost 85 and 25%, respectively after cultivation for 3 days (Prasad and Joyce, 1991).

Table X provides a comparison of the results for color and AOX reduction by a few white-rot fungi.

The enzymes lignin peroxidase, manganese peroxidase, and laccase have been implicated in the decolorization of bleaching effluents (Momohora *et al.*, 1989; Esposito *et al.*, 1991) but their roles were not critically examined until 1991. The results of Momohora *et al.* (1989)

TABLE X

COMPARISON OF SYSTEMS USED FOR THE TREATMENT OF BLEACHING EFFLUENTS WITH DIFFERENT FUNGI

Evaluation method	Operation mode	Residence time (day)	Max. color reduction (%)	Max. AOX reduction (%)	Reference
Phanerochaete chrysosporium					
Mycelium immobilized on rotating disc	Batch	1	90	—	Yin *et al.* (1989a)
Mycelium immobilized on porous material	Continuous	0:5	60	55	Messner *et al.* (1990)
Mycelium immobilized on polyurethane foam	Continuous	5–8	70	—	Cammarota and Santanna (1992)
Mycelium immobilized on net ring type	Continuous	0.5	91	—	Kang *et al.* (1996)
Trametes versicolor					
Mycelial pellets immobilized in Ca-alginate	Batch	3	80	—	Livernoche *et al.* (1983)
Mycelial petllets immobilized in Ca-alginate	Continuous	0.7	45	—	Royer *et al.* (1983)
Mycelial pellets	Batch	2	61	—	Royer *et al.* (1985)
Mycelial pellets	Continuous	0.6–1.2	50	—	Royer *et al.* (1985)
Free cells	Batch	3	88	45	Bergbauer *et al.* (1991)
Mycelial pellets	Batch	5	80	—	Archibald *et al.* (1990)
Mycelial pellets	Continuous	1	78	42	Pallerla and Chambers (1995)
Mycelial pellets immobilized in Ca-alginate beads	Continuous	1	80	40	Pallerla and Chambers (1996)
Mycelial pellets	Batch	3	88	—	Mehna *et al.* (1995)
Mycelial pellets	Continuous	1.6	93	—	Bajpai *et al.* (1993)
Rhizopus oryzae					
Mycelial pellets	Batch	2	91	—	Nagarathnamma and Bajpai (1999)
Ceriporiopsis subvermispora					
Mycelial pellets	Batch	1	95	—	Nagarathnamma *et al.* (1999)

indirectly indicated that decolorization of extraction stage effluent by *P. chrysosporium* was not catalyzed by lignin peroxidase. Lackner *et al.* (1991) concluded for the first time that MnP plays the major role in the initial breakdown and decolorization of high-molecular-weight chlorolignin in bleaching effluents with *P. chrysosporium in vivo,* by demonstrating the following:

1. *P. chrysosporium* degraded high-molecular-weight chlorolignin in bleaching effluents even though a direct contact between ligninolytic enzymes and chlorolignins was prevented by a dialysis tubing.
2. Manganese peroxidase effectively catalyzed the depolymerization of chlorolignin in the presence of Mn (11) and H_2O_2.

These researchers also investigated the biochemical mechanism of chlorolignin degradation in the MYCOPOR reactor and found that the amount of mycelium-bound manganese peroxidase correlated with decolorization rates. This explains the fact that bleaching effluents can be degraded during continuous operation of the MYCOPOR reactor for months even though the enzymes are washed out. Mycelium-bound manganese peroxidase could generate Mn (iii) which can freely diffuse into the effluent and depolymerize the chlorolignins trickling through the reactor. Michel *et al.* (1991) also investigated the role of ligninolytic enzymes of *P. chrysosporium* in decolorizing bleaching effluents. They concluded that manganese peroxidase plays an important role in effluent decolorization. Moreover, Lee *et al.* (1994) demonstrated high levels of manganese peroxidase but no lignin peroxidase activity during extraction stage effluent treatment with the fungus KS-62 which showed excellent decolorization without any additional nutrients. Because significant reduction was observed for the decolorization of a catalase added culture, they suggested that manganese peroxidase plays an important role in the decolorization of extraction stage effluent by this fungus. The role of manganese peroxidase in decolorization of bleach plant effluent has been also confirmed by Jaspers *et al.* (1994). On the other hand, Archibald and Roy (1992) reported that laccase and not peroxidase plays the primary role in effluent decolorization by *T. versicolor.* Archibald and Roy (1992) later demonstrated that *T. versicolor* laccase, in the presence of phenolic substrate, was able to generate Mn (iii) chelates similar to those produced by manganese peroxidase and which were shown by Lackner *et al.* (1991) to be responsible for the oxidation of bleaching effluent.

Manzaners *et al.* (1995) evaluated the enzymatic activities when the effluents from alkaline cooking of cereal straw were treated with *T. versicolor.* They reported that the production of laccase activity was much

higher than that obtained under the same conditions in synthetic growth media and that there was a clear relationship between the effluent concentration in the medium and laccase activity. In the decolorization medium, manganese peroxidase activity was detected when $MnSO_4$ was added to these media, although no lignin peroxidase activity was detected in any of the conditions assayed. Lankinen *et al.* (1991) treated softwood pulp bleaching effluents with carrier immobilized *Phlebia radiata* and noticed the production of large amounts of lignin peroxidase (the most characteristic lignin peroxidase isozymes in effluent media were lignin peroxidase 2 and lignin peroxidase 3) during AOX decrease and color removal.

VII. Conclusions and Future Research Needs

This review indicates that current wastewater management strategies for pulp and paper mill effluents fall into three categories: prebleaching technologies, bleaching technologies, and postbleaching technologies. No single technology can provide a cost-effective and feasible solution to treatment of pulp mill effluents. A balanced use of all the strategies may be required. Treatment before disposal of bleach effluents cannot be eliminated and it will continue to play an important role in complying with the standards prescribed. Most of the treatment technologies have not reached implementation level due to lack of technological or/and economic feasibility. Among biological treatment technologies available, fungal treatment technology appears to have an edge over the other treatment technologies. Fungal treatment technology using white-rot fungi appears to be the most promising in this regard. One of the drawbacks associated with the fungal treatment is the necessity of an easily metabolizable cosubstrate like glucose for the growth and development of ligninolytic activity. To make the fungal treatment method economically feasible, there is a need to reduce the requirement of cosubstrate or to identify a cheaper cosubstrate. Hence, efforts should be made to identify strains that show good decolorization with less or no cosubstrate or that can utilize industrial waste as a cosubstrate. Efforts should also be made to utilize the spent fungal biomass for preparing the culture medium required in the synthesis of active fungal biomass. If successful, the cost of treatment could be reduced further. As lignin degrading systems of white-rot fungus have a high oxygen requirement, use of oxygen instead of air as fluidizing media should be explored. Increasing the oxygen concentration in the culture is expected to have a dual effect: it would lead to an increased titer of the lignin degrading system and to increased stability of the existing system. A quantitative study of extracellular enzymes is also required in order to gain insight

into the possible enzymatic mechanism involved in the degradation of lignin-derived compounds present in the effluents.

Use of white-rot fungi can serve as a prelude to bacterial treatment and to enhance bacterial removal of organic chlorine and to degrade the relatively higher-molecular-weight chlorolignins. This process can be used as an alternative to internal process modifications (modified cooking, oxygen bleaching, high-level chlorine dioxide substitution, etc.) and conventional biological treatment.

Since the majority of AOX and color is in high-molecular-weight chlorolignins, the priority of research should concentrate on the fate of high-molecular-weight chlorolignins in biological treatment or in the natural environment. Since bacteria degrade significantly only those chloroorganics with molecular weights lower than 800–1000 daltons, research is needed to decrease the chlorolignin molecular weight or to remove high-molecular-weight chlorolignins before bacterial treatment is applied in order to enhance the biotreatability of bleaching effluents.

A. Abbreviations

AOX	Adsorbable organic halogens
ASB	Aerated stabilization basins
AST	Activated sludge treatment
BKME	Bleached kraft mill effluent
BOD	Biological oxygen demand
CMC	Carboxymethyl cellulose
CMP	Chemimechanical pulping
COD	Chemical oxygen demand
CTMP	Chemithermomechanical pulp/pulping
DCG	Dichloroguaiacol
DCP	Dichlorophenol
ECF	Elemental chlorine free
EPA	Environmental Protection Agency
FPL	Forest Products Laboratory
HRP	Horseradish peroxidase
HRT	Hydraulic retention time
LiP	Lignin peroxidase
LSHS	Low sulfur high speed
MLSS	Milliliter suspended solids
MyCoR	Mycelial color removal
NCSU	North Carolina State University
NSSC	Neutral sulfite semichemical
PCDD	Polychlorinated dibenzodioxins
PCDF	Polychlorinated dibenzofurans

PCP	Pentachlorophenol
RBC	Rotating biological contactors
RFA	Resins and fatty acids
SBR	Sequencing batch reactors
SRT	Solids retention time
TCDD	Tetrachlorinated dibenzodioxins
TCDF	Tetrachlorinated dibenzofurans
TCF	Totally chlorine free
TEQ	Toxicity equivalent
TMP	Thermomechanical pulp
TOC	Total organic carbon
TOCl	Total organochlorine
TSS	Total suspended solids
UASBR	Upflow anaerobic sludge blanket reactor
UF	Ultrafiltration
VSS	Volatile suspended solids
C	Chlorination
(CD)	Treatment by mixing chlorine and chlorine dioxide simultaneously (C proportion is higher than D)
D	Chlorine dioxide treatment
E	Alkaline extraction
E_1 & E_2	First and second alkaline extractions, respectively
E_p	Alkaline extraction with hydrogen peroxide
F	Fungal treatment
H	Hypochlorite treatment
P	Hydrogen peroxide treatment

References

Allen, D. G., and Liu, H. W. (1998). *In* "Encyclopedia of Environmental Analysis and Remediation" (R. A. Meyers, ed.), Vol. 6, pp. 3871–3887. Wiley Interscience, New York.

Altnbas, U., and Eroglu, V. (1997). *Fresenius Environ. Bull.* **6,** 103–108.

Amy, G. L., Bryant, C. W., Alleman, B. C., and Barrley, W. A. (1988). *J. Wat. Pollut. Cant. Fed.* **60**(8), 1445–1458.

Annergren, G., Kringstad, K. P., and Lehtiren, K. J. (1986). *EuCePa Symp. Environ. Prot. 90's,* Helsinki, Finland, May 9–22.

Annergren, G. E. (1990). *24th EuCePa Conf.,* Stockholm, Sweden.

Annergren, G. E., Osterberg, F., and Lindblad, P. (1990). *Tappi Pulping Conf.,* Toronto, Canada.

Anonymous (1994). *EPA Report Thrusts Dioxins Back into the spotLight,* ENDS Ref. No. **236,** pp. 21–24.

Anonymous (1997). *Tappi J.* **80**(2), 14.

Aprahamian, E., Jr. (1990). *Tappi Pulping Conf.,* Toronto pp. 209–215.

Archibald, F., Paice, M. G., and Jurasek, L. (1990). *Enz. Microbiol. Technol.* **12,** 846–853.

Archibald, F. S., and Roy, B. (1992). *Appl. Environ. Microbiol.* **58,** 1496–1499.
Ataberk, S., and Gokcay, C. F. (1997). *Fersenius Environ. Bull.* **6,** 147–153.
Axegard, P., Berry, R. M., Gellerstedt, G., Lindblad, P. O., Luthie, C. E., Popke, I., Voss, R. H., and Wrist, P. E. (1993). *In* "Bleaching" (H. Jameel, ed.), Vol. 2, pp. 759–770. Tappi Press, Atlanta, GA.
Bajpai, P., and Bajpai, P. K. (1996). "Organochlorine Compounds in Bleach Plant Effluents—Genesis and Control." PIRA International, Leatherhead, Surrey, UK.
Bajpai, P., Bajpai, P. K., and Kondo, R. (1999). "Biotechnology for Environmental Protection in Pulp and Paper Industry." Springer-Verlag, Germany.
Bajpai, P., Mehna, A., and Bajpai, P. K. (1993). *Proc. Biochem.* **28,** 377–384.
Barr, T. A., Taylor, T., and Duff, S. (1996). *Water Res.* **30**(4), 799–802.
Basak, T., and Gokcay, C. F. (1999). *In* "Proc. 6th IAWQ Symp. Forest Industry Wastewaters," pp. 221–228. Tampere, Finland.
Belsare, D. K., and Prasad, D. Y. (1988). *Appl. Microbiol. Biotechnol.* **28,** 301–304.
Bergbauer, M., and Eggert, C. (1992). *Biotech Lett.* **14**(9), 869–874.
Bergbauer, M., Eggert, C., and Kalnowski, G. (1992). *Biotechnol. Lett.* **14**(4), 317–322.
Bergbauer, M., Eggert, C., and Kraepelin, G. (1991). *Appl. Microbiol. Biotechnol.* **35**(1): 105–109.
Bijur, A. G. (1997). *Paperex* **97**(2/2), 5–9.
Boman, B., Ek, M., Heyman, W., and Frostell, B. (1991). *Wat. Sci. Technol.* **24**(3/4), 219–228.
Boman, B., Frostell, B., Ek, M., and Eriksson, K. E. (1988). *Nordic Pulp Paper Res. J.* **1,** 13–18.
Brite, C. E. C. (1994). *Chem. Eng.* **101,** 23–28.
Bryant, C. V., Amy, G. L., and Allemen, B. C. (1987). *J. Wat. Pollut. Control Fed.* **59**(10), 890–896.
Bryant, C. W., and Barkley, W. A. (1990). *Pacific Paper EXPO Tech. Conf. Proc. Progr. 7 Environ.* Vancouver B.C.
Bryant, C. W., Amy, G. L., Neil, R., and Ahmed, S. (1988). *Wat. Sci. Technol.* **20**(1), 73–79
Buckley, D. B. (1992). *Tappi Environ. Conf.* Tappi Press Atlanta, GA, pp. 750–810.
Bullock, J. M., Bicho, P. A., and Saddler, J. N. (1994). *In* "Proc. 1994 *Environ. Conf.,*" pp. 371–378.
Bumpus, J. A., and Aust, S. D. (1995). *Bio. Essays* **6**(4), 166–170.
Call, H. P. (1991). Patent DE 4137761.
Cammarota, M. C., and Santanna, Jr. G. L. (1992). *Environ. Technol.* **13,** 65–71.
Campbell, A. G., Gerrard, E. D., and Joyce, T. W. (1982). *In* "Proc. Tappi Res. Devel. Conf. North Carolina," Tappi Press, Atlanta, GA, pp. 209–214.
Canada Gazette (1992). Part II, Vol. 126 (11).
Christov, L. P., and Steyn, M. G. (1998). *In* "Proc. 7th Int. Conf. Biotechnol. Pulp Paper Industry," Vancouver, Canada, pp. C203–206.
Clark, T., Bruce, M., and Anderson, S. (1994). *Wat. Sci. Technol.* **29**(5/6), 421–432.
Davis, S., and Burns, R. G. (1992). *Appl. Microbiol. Biotechnol.* **37,** 474–479.
Deardorff, T. L., Willhelm, R. R., Nonni, A. J., Renard, J. J., and Phillips, R. B. (1994). *Tappi J.* **77**(8), 163–173.
Dence, C. W., and Reeve, D. W. (1996). *In* "Pulp Bleaching—Principles and Practice" (C. W. Dence, and D. W. Reeve, eds.), pp. 91–183. Tappi Press, Atlanta, GA.
Deshpande, S. H., Khanolkar, V. D., and Pudumjee, K. D. (1991). *In* "Proc. Int. Workshop on Small-Scale Chem. Recovery, High-Yield Pulping and Effluent Treatment," pp. 201–213. New Delhi, India, 16-20 September.
Dezotti, M., Innocentini-Mei, L. H., and Duran, N. (1995). *J. Biotechnol.* **43,** 161–167.
Dilek, F. B., and Goekcay, C. F. (1994). *Wat. Sci. Technol.* **29**(9), 161–163.

Dorica, J., and Elliott, A. (1994). *In* "Proc. Tappi Intl. Environ. Conf." pp. 157–163, Portland, OR, U.S.A.

Duran, N., Dezotti, M., and Rodriguez, J. (1991). *J. Photochem. Photobiol.* **62,** 269–279.

Duran, N., Esposito, E., Innocentini-Mei, L. H., and Canhos, V. P. (1994). *Biodegradation* **5**(1), 13–19.

Eaton, D. C., Chang, H. M., and Kirk, T. K. (1980). *Tappi J.* **63**(10), 103–106.

Eaton, D. C., Chang, H. M., Joyce, T. W., Jeffries, T. W., and Kirk, T. K. (1982). *Tappi J.* **65**(6), 89–92.

Edeline, F., Lambert, G., and Fatliccioni, H. (1988). *In* "Energy from Biomass—4. Proc. 3rd Contractors. Meeting" (Grassi G., ed.), pp. 463–475.

Eeckhaut, M., Alaerts, G., and Pipyn, P. (1986). *In* "PIRA Paper & Board Division Seminar. Cost-Effective Treatment of Paper Mill Effluents Using Anaerobic Technologies," 14–15 January, Leatherhead, U.K.

Ek, M., and Eriksson, K. E. (1987). *4th Int. Symp. Wood Pulping Chem.*, Paris, France.

Ek, M., and Kolar, M. C. (1989). *In* "Proc. 4th Int. Biotech. Conf. Pulp and Paper Industry," pp. 271–278, Raleigh, NC., 16–19 May.

Eklund, H. (1995). *In*"Proc. 3rd Global Conf. Paper Environ." pp. 98–100. London U.K.

Eriksson, K. E. (1990). *Water Sci. Technol.* **24,** 79–101.

Eriksson, K. E., and Kolar, M. C. (1985). *Environ. Sci. Technol.* **19**(12), 1219–1224.

Eriksson, K. E., Kolar, M. C., Ljungquist, P. O., and Kringstad., K. P. (1985). *Environ. Sci. Technol.* **19,** 1219–1224.

Esposito, E., Canhos, V. P., and Duran, N. (1991). *Biotech. Lett.* **13**(8), 571–576.

Evans, T., Sweet, W., and Monlescu, D. J. (1994). *In* "Proc. Annu. Meet. Tech. Sect. Canad. Pulp Paper Assoc." pp. 237–242. Montreal, Canada.

Ferguson, J. F., and Dalentoft, E. (1991). *Wat. Sci. Technol.* **24,** 241–250.

Ferguson, J. F., Luonsi, A., and Ritter, D. (1990). *In* "Proc. Tappi. Environ. Conf.," pp. 333–338. Atlanta, GA.

Field, J. A., and Lettinga, G. (1991). *Wat. Sci. Technol.* **24,** 127–137.

Field, J. A., Leyendeckers, M. J. H., Sierra–Alvarez, R., and Lettinga, G. (1991). *Biotechnol. Bioeng.* **37,** 247–255.

Field, J. A., Leyendeckers, M. J. H., Sierra–Alvarez, R., Lettinga, G., and Habets, L. H. A. (1988). *Wat. Sci. Technol.* **20,** 219–240.

Fitzsimons, R., Ek, M., and Eriksson, K-E. L. (1990). *Environ. Sci. Technol.* **24,** 1744–1748.

Fujita, K., Kondo, R., and Sakai, K. (1991). *Tappi J.* **74**(11), 123–127.

Fukui, H., Presnell, T. L., Joyce, T. W., and Chang, H. M. (1992). *In* "Biotechnology in Pulp and Paper Manufacture" (M. Kuwahara, and M. Shimada, ed.) pp. 75–80. Uni Publishers, Tokyo, Japan.

Fukuzumi, T. (1980). *In* "Lignin Biodegradation: Microbiology, Chemistry and Potential Applications" (T. K. Kirk, H. M. Chang, and T. Higuchi, eds.) Vol. 2, pp. 161–171. CRC Press, Boca Raton, FL.

Fukuzumi, T., Nishida, A., Aoshima, K., and Minami, K. (1977). *Mokuzai Gakkaishi* **23**(6), 290–298.

Fulthorpe, R. R., and Allen, D. G. (1995). *Appl Microbiol Biotechonol.* **42,** 782–789.

Galeno, G., and Agasin, E. (1990). *Biotechnol. Lett.* **12**(11), 869–872.

Ganjidoust, H., Tatsumi, K., Yamagishi, T., and Gohlian, R. (1996). *In* "Proc. 5th IAWQ Symp. Ind. Wastewaters," Vancouver, Canada, p. 305.

Gergov, M., Priha, M., Talka, E., Valtilla, O., Kangas, A., and Kukkonen, K. (1988). *Tappi J.* **71**(12), 175–184.

Gokcay, C. F., and Taseli, B. K. (1997). *Environ. Bull.* **6,** 220–226.

Goronzy, M., Demoulin, G., Jager, A., Srebotnik, E., and Messener, K. (1996). *In* "Proc. Sixth Int. Conf. Biotechnol. Pulp and Paper Industry," pp. 239–245, Vienna, Austria.

Grant, R. (1995). *Pulp Pap. Int.* **37**(9), 26–27.

Graves, J. W., Joyce, T. W., and Jameel, H. (1993). *Tappi J.* **76**(7), 153–159.
Guo, H. Y., Chang, H. M., Joyce, T. W., and Glasser, J. H. (1990). *In* "Biotechnology in Pulp and Paper Manufacture" (T. K. Kirk, and H. M. Chang, eds.), pp. 223–230. Butterworth-Heinemman Stoneham.
Habets, L. H. A., and de Vegt, A. L. (1991). *Wat. Sci. Technol.* **24,** 331–345.
Habets, L. H. A., Tielboard, M. H., Ferguson, A. M. D., Prong, C. F., and Chmelauskas, A. J. (1985). *Wat. Sci. Technol.* **20,** 87–97.
Haggblom, M., and Salkinoja-Salonen, M. (1991). *Water Sci. Technol. (G.B.)* **24**(3/4), 161–170.
Hakulinen, R. (1982). *Paperi Ja Puu–Paper Och Tra* **5,** 341–354.
Hall, E. R., Robson, R. D. Prong, C. F., and Chmelauskas, A. J. (1986). *In* "Proc. Tappi Environ. Conf.," pp. 207–217. Atlanta, GA.
Higachi, R., Cherr, G., Shenker, J., Macdonald, J., and Crosby, D. (1992). *Environ. Sci. Technol.* **26**(12), 2413–2420.
Hilleke, J. (1993). *In* "Industrial Environmental Control—Pulp and Paper Industry" (A. M. Springer, ed.), pp. 304–343. Tappi Press, Atlanta, GA.
Hostachy, J., Lachenal, D., and Coste, G. (1996). *In* "Proc. Tappi Int. Environ. Conf.," pp. 817–822. Orlando, FL.
Huynh, V. B., Chang, H. M., Joyce, T. W., and Kirk, T. K. (1985). *Tappi J.* **68**(7), 98–102.
Jaklin-Farcher, S., Szeker, E., Stifter, U., and Messner, K. (1992). *In* "Biotechnology in Pulp and Paper Industry" (M. Kuwahara, and M. Shimada, eds.) pp. 81–85. Tokyo, Japan.
Jaspers, C. J., Jimenez, G., and Penninck, M. J. (1994). *J. Biotechnol.* **37,** 229–236.
Johnson, T., and Chatterjee, A. (1995). Pulp Pap. *Canad.* **96**(8), 26–29.
Jokela, J. K., Laine, M., Ek, M., and Salkinoja-Salonen, M. (1993). *Environ. Sci. Technol.* **27**(3), 547–552.
Joyce, T. W., and Pellinen, J. (1990). *Tappi Env. Conf.,* Seattle, WA, April 9–11.
Jurgensen, S. J., Benjamin, M. M., and Ferguson, J. F. (1985). *In* "Proc. Tappi Environ. Conf.," Tappi Press, Atlanta, GA. pp. 83–92.
Kang, C. H., On, H. K., and Won, C. H. (1996). *In* "Biotechnology in Pulp and Paper Industry" (E. Srebotnik, and K. Messener, eds.), pp. 263–266. Facultas-Universitatsverlag, Vienna.
Kannan, K. (1990). *World J. Microbiol. Biotechnol.* **6**(2), 114–116.
Kapoor, A., and Viraraghavan, T. (1995). *Biores. Technol.* **53,** 195–206.
Kirk, T. K., Croan, S., Tein, M., Mutagh, K. E., and Farrell, R. L. (1986). *Enz. Microbial Technol.* **8,** 27–32.
Kortekaas, S., Wijngaarde, R. R., Klomp, J. W., Lettinga, G., and Field, J. A. (1998). *Wat. Res.* **32**(11), 3362–3370.
Lachenal, D., Joncourt, M. J., Froment, P., and Chirat, C. (1996). AOX during chlorine dioxide bleaching. *In* "Proc. Int. Pulp Bleaching Conf.," pp. 417–420. Washington.
Lackner, R., Srebotnik, E., and Messner, K. (1991). *Biophys. Res.* Commun. **178**(3), 1092–1098.
Lafond, R. A., and Ferguson, J. F. (1991). *In* "Proc. Tappi Environ. Conf.," pp. 797–812. Tappi Press Atlanta, GA.
Lankinen, V. P., Inkeroinen, M. M., Pellinen, J., and Hatakka, A. I. (1991). *Wat. Sci. Technol.* **24**(3/4), 189–198.
Leach, J. M., Mueller, J. C., and Walden, C. C. (1978). *Process Biochem.* **13**(1), 18–26.
Lee, J. W. (1993). *In* "Industrial Environmental Control, Pulp and Paper Industry" (A. M. Springer, ed.), pp. 405–446. Tappi Press, Atlanta, GA.
Lee, J. W., Peterson, D. L., and Stickney, A. R. (1989). "Proc. Tappi 1989 Environ. Conf.," pp. 473–496, Orlando, FL, U.S.A.

Lee, S. H., Kondo, R., and Sakai, K. (1994). *Mokuzai Gakkaishi* **40**(6), 612–619.
Lee, S. H., Kondo, R., Sakai, K., and Sonomoto, K. (1995). *Mokuzai Gakkaishi* **41**(1), 63–68.
Lettinga, G. (1980). **22,** 649–734.
Lettinga, G., Field, J. A., Sierra–Alvarez, R., Vanlier, J. B., and Rintala, J. (1990). *Wat. Sci. Technol.* **24,** 91–102.
Lindstrom, K., and Mohamed, M. (1988). *Nordic Pulp Paper Res. J.* **3,** 26–33.
Liu, H. W., Liss, S. N., and Allen, D. (1997). *Wat. Sci. Technol.* **35**(2): 77–82.
Liu, H. W., Lo, S. N., and Lavallee, H. C. (1996). *Tappi. J.* **79**(5), 145–154.
Livernoche, D., Jurasek, L., Desrochers, M., Dorica, J., and Veliky, I. A. (1981). *Biotechnol. Lett.* **3,** 701–706.
Livernoche, D., Jurasek, L., Desrochers, M., Dorica, J., and Veliky, I. A. (1983). *Biotechnol. Bioeng.* **25,** 2055–2065.
Lunan, W. E., Harden, C., and Krupa, K. (1995). *In* "Proc. Tappi Environ. Conf.," pp. 241–247. Atlanta, GA.
MacLean, B., de Vegt, A., and Droste, R. L. (1990). *Wat. Res.* **24,** 1401–1405.
Malinen, R., and Fuhrmann, A. (1995). *Pap. Puu.* **77**(3), 78–83.
Mannisto, H., Mannisto, E., and Winter, P. (1994). *In* "Proc. Tappi Int. Environ. Conf.," pp. 475–483. Portland, OR.
Manzaners, P., Fajardo, S., and Martin, C. (1995). *J. Biotechnol.* **43,** 125–143.
Mapple, G. E., Ambady, R., Caron, J. R., Stralton, S. C., and Vega Canovas, R. E. (1994a). *Tappi J.* **77**(11), 71–80.
Mapple, G. E., Ambady, R., Caron, J. R., Stralton, S. C., and Vega Canovas, R. E. (1994b). *In* "Proc. Int. Pulp *Bleaching* Conf.," pp. 253–258. Atlanta, GA.
Martin, C., and Manzanares, P. (1994). *Biores. Technol.* **47**(3), 209–214.
Mathys, R. G., Branion, R. M. R., Lo, K. V., Anderson, K. B., Leyen, P., and Louie, D. (1997). *Wat. Qual. Res. J. Canad.* **32,** 771–774.
Mathys, R. G., Branion, R. M. R., and Lo, K. V. (1993). *In* "Proc. 79th CPPA Annu. Proc.," pp. 370–374, Montreal, Canada.
Matsumoto, Y., Yin, C. F., Chang, H. M., Joyce, T. W., and Kirk, T. K. (1985). *In* "Proc. 3rd ISWPC," pp. 45–53. Vancouver.
McCarthy, P. J., Kennedy, K. J., and Droste, R. L. (1990). *Wat. Res.* **24,** 1401–1405.
McCubbin, N. (1983). *Environment Canada, Report* **EPS 6-EP-83-1**, Ottawa.
McCubbin, N. (1989). *Pulp Paper Canad.* **90**(11), 17–19.
McCubbin, N., Sprague, J. B., and Bonsor, N. (1990). *Pulp Paper Canad.* **91**(3), T112–T114.
McDonough, T. J. (1995). *Tappi J.* **78**(3), 55–62.
Mckague, A. B., and Carlberg, G. (1996). *In* "Pulp Bleaching—Principles and Practice" (C. W. Dence, and D. W. Reeve, eds.), pp. 746–820. Tappi Press. Atlanta, GA.
McLeay, D. J. (1987). *Environ. Canad. Rep.* **EPS 4/PF/1** Beauregard Press Ltd, Ottawa Ontario, Canada.
Mehna, A., Bajpai, P., and Bajpai, P. K. (1995). *Enz. Microb. Technol.* **17**(1), 18–22.
Melcer, H., Steel, P., Mckinley, A., and Cook, C. R. (1994). *In* "Proc. Tappi Int. Environ. Conf.," pp. 795–807, Portland, OR, U.S.A.
Messner, K., Ertler, G., Jaklin-Farcher, S., Boskovsky, P., Regensperger, V., and Blaha, A. (1990). *In* "Biotechnology in Pulp and Paper Manufacture" (T. K. Kirk, and H. M. Chang, eds.), pp. 245–251. Butterworth-Heinemann Newton, MA.
Michel, F. C., Dass, S. B., Grulke, E. A., and Reddy, C. A. (1991). *Appl. Environ. Microbiol.* **57**(8), 2368–2370.
Milstein, O., Haars, A., Majcherczyk, A., Trojanowski, J., Tautz, D., Zanker, H., and Huttermann, A. (1988). *Wat. Sci. Technol.* **20**(1), 161–170.
Milstein, O., Hwars, A., Krause, F., and Huettermann, (1991). *Wat. Sci Technol.* **24**(3/4), 199–206.

Mittar, D., Khanna, P. K., Marwaha, S. S., and Kennedy, J. F. (1992). *J. Chem. Technol. Biotechnol.* **53,** 81–92.

Momohara, I., Mateumoto, Y., Ishizu, A., and Chang, H. M. (1989). *Mokuzai Gakkaishi* **35**(12), 1110–1115.

Mortha, G., Mckay, L. R., Cadel, F., and Rouger, J. (1991). *6th ISWPC,* pp. 1–2.

Nagarathnamma, R., Bajpai, P., and Bajpai, P. (1999). *Process Biochem.* **34,** 939–948.

Nagarathnamma, R., and Bajpai, P. (1999). *Appl. Environ. Microbiol.* **65**(3); 1078–1082.

Nancy, S. J., Norman, J. C., and Vandenbusch, M. B. (1996). *Conserv. Recycl.* **10**(4), 279–299.

NCASI (1990). *NCASI Tech. Bull. No. 582.*

Nishida, T., Katagiri, N., and Tsustumi, Y. (1995). *In* "Biotechnology in Pulp and Paper Industry" (E. Srebotnic and K. Messner, eds.), pp. 51–54. Vienna, Austria.

Olofsson, A. (1996). *Svensk Papperstidin.* **99**(11), 33–34.

Paice, M., Elloitt, A., Young, F., and Dorica, J. (1996). *Pulp Pap. Canad.* **97**(9), 88–92.

Paice, M. G. (1995). *In* "Proc. CPPA Environ. Conf.," pp. 81–88, Halifax, Canada.

Pallerla, S., and Chambers, R. P. (1995). *J. Environ. Sci. Health* **A30**(2), 432–437.

Pallerla, S., and Chambers, R. P. (1996). *Tappi J.* **79**(5), 156–161.

Parker, W. J., Eric, R., and Farguhar, G. J. (1993a). *Wat. Environ. Res.* **65**(3), 264–270.

Parker, W. J., Eric, R., and Farguhar, G. J. (1993b). *Environ. Sci. Technol.* **27**(9), 1783–1789.

Parthasarathy, V. R., Rudie, G. F., Mendiratta, S. K., Cawifield, D. W., and Bryan., H. P. (1994). *In* "Proc. Pulping Conf.," pp. 1031–1038. San Diego, CA.

Pejot, F., and Pelayo, J. M. (1993). *In* "Proc. Tappi Environ. Conf.," pp. 389–394. Atlanta, GA.

Pellinen, J., Joyce, T. W., and Chang, H. M. (1988). *Tappi J.* **71**(9), 191–194.

Pellinen, J., Yin, C. F., Joyce, T. W., and Chang, H. M. (1988). *J. Biotechnol.* **8**(1), 67–76.

Pertulla, M., Konrusdottin, M., Pere, J., Kristjansson, J. K., and Viikari, L. (1991). *Wat. Res.* **25,** 599–604.

Pichon, M., Rouger, J., and Junet, E. (1988). *Wat. Sci. Technol.* **20,** 133–141.

Prasad, B., Kirkman, A., Jameel, H., Gratzal, J., and Magnotta, V. (1996). *Tappi J.* **78**(9), 144–152.

Prasad, D. Y., and Joyce, T. W. (1991). *Tappi J.* **74**(1), 165–169.

Prouty, A. L. (1990). *Appl. Microbiol. Biotechnol.* **32,** 490–493.

Pryke, D. (1995). *Canad. Paper Maker* **48**(4), 28–29.

Puhakka, J. A. (1994). *Wat. Sci. Technol.* **29,** 73–80.

Raghukumar, C., Chandramohan, D., Michael, F. C., Jr., and Reddy, C. A. (1996). *Biotech. Lett.* **18**(1), 105–106.

Raizer-Neto, E., Pichon, M., and Beniamin, M. M. (1991). *In* "Biotechnology in Pulp and Paper Manufacture," (T. K. Kirk, and H. M. Chang, eds.), pp. 271–278. Butterworth-Heinmann–Stoneham MA.

Ramaswammy, V. (1987). *IPPTA Convention Issue. Indian Pulp and Paper Association,* Saharanpur, India.

Rempel, W., Turk, O., and Sikes, J. E. G. (1990). *CPPA Spring Conf.,* Jasper, Alberta.

Rennel, J. (1995). *Nord. Pulp Pap. Res. J.* **10**(1), 24–32.

Richardson, D. A., Andras, E., and Kennedy, K. J. (1991). *Wat. Sci. Technol.* **24,** 103–112.

Rintala, J., and Lepisto, S. (1992). *Wat. Res.* **26,** 1297–1305.

Rintala, J., and Vuoriranta, P. (1988). *Tappi J.* **71,** 201–207.

Rintala, J., Lepisto, R., and Chand, S. (1992). *Bioresource Technol.* **42,** 17–26.

Rinzema, A., and Lettinga, G. (1988). *Biotreat. Syst.* **3,** 65–109.

Ristolainen, M., and Alen, R. (1996). *In* "Proc. Int. Pulp Bleaching Conf.," pp. 523–525. Washington.

Rogers, I. H., Davis, J. C., Kruzynski, G. M., Mahood, H. W., Servizi, J. A., and Gordon, J. W. (1975). *Tappi J.* **58**(7), 136–140.
Roy-Arcand, L., and Archibald, F. S. (1991a). *Tappi J.* **74**(9), 211–218.
Roy-Arcand, L., and Archibald, F. S. (1991b). *Enz. Microb. Technol.* **13**(3), 194–203.
Royer, G., Desrochers, M., Jurasek, L., Rouleau, D., and Mayer., R. (1985). *J. Chem. Technol. Biotechnol.* **35B**, 14–22.
Royer, G., Livernoche, D., Desrochers, M., Jurasek, L., Rouleau, D., and Mayer, R. C. (1983). *Biotech. Lett.* **5**, 321–326.
Royer, G., Yerushalmi, L., Rouleau, D., and Desrochers, M. (1991). *J. Indl. Microbiol.* **7**, 269–278.
Salkinoja–Salonen, M. (1990). *6th Colloq. Pulp and Paper Mill Effluents,* Toronto, Canada.
Sarner, E. (1988). *Wat. Sci. Technol.* **20**(1), 279–281.
Saunamaki, R. (1989). *Paperi Ja Puu* **2**, 158–164.
Saunamaki, R., Jokinen, K., Jarvinen, R., and Savolainen, M. (1991). *Wat. Sci. Technol.* **24**(3/4), 295–308.
Shin, N. H., and Mera, F. (1994). *In* "Proc. Pulping Conf.," pp. 167–175. San Diego, CA.
Sierra–Alvarez, R., Kortekaas, S., vanEckort, M., Harbrecht, J., and Lettinga, G. (1991). *Wat. Sci. Technol.* **24**, 113–125.
Sierra–Alvarez, R., and Lettinga, G. (1990). *Biol. Wastes.* **33**, 211–226.
Sierra–Alvarez, R., and Lettinga, G. (1991). *J. Chem. Technol. Biotechnol.* **50**, 443–455.
Sierra–Alvarez, R., Kato, M., and Lettinga, G. (1990). *Environ. Technol. Lett.* **11**, 891–898.
Smith, J. E., and Frailey, M. M. (1990). *In* "Proc. Tappi Environ. Conf.," pp. 101–110, Orlando, FL, U.S.A.
Smith, P. W. (1990). *In* "Proc. Tappi Environ. Conf.," pp. 769–772, Orlando, FL, U.S.A.
Sodergren, A., Adolfsson-Erici, M., Bengtsson, B. E., Jonsson, P., Lagergren, S., Rahm, R., and Wulff, F. (1993). *Environ. Prot. Agency,* **EPA 4047.** (A. Sodergren, ed.), pp. 26–46. Arlow.
Springer, A. M. (1993). *In* "Industrial Environmental Control—Pulp and Paper Industry" (A. M. Springer, ed.), pp. 7–34. Tappi Press Atlanta, GA.
Springer, A. M., Hand, V. C., and Jarvis, T. S. (1994). *In* "Proc. Tappi Int. Environ. Conf.," pp. 271–279, Portland, OR, U.S.A.
SSVL-85 Project 4 Final Rep. (1985). Stockholm, Sweden.
Stephenson, R., and Duff, S. (1996a). *Wat. Res.* **30**(4), 781–792.
Stephenson, R., and Duff, S. (1996b). *Wat. Res.* **30**(4), 793–798.
Sun, Y. B., Guo, H. Y., Joyce, T. W., and Chang, H. M. (1992). *J. Pulp Paper Sci.* **18**(2), J49–J55.
Sundelin, B. (1988). *Wat. Sci. Technol.* **20**(2), 175–177.
Sundman, G., Kirk, T. K., and Chang, H. M. (1981). *Tappi J.* **64**(9), 145–148.
Suntio, L. R., Shiu, W. Y., and Mackay, D. A. (1998). *Chemosphere* **17**(7), 1249–1299.
Tolan, J. S., and Canovas, R. V. (1992). *Pulp Paper Canad.* **93**(5), 39–42.
Tolan, J. S., and Foody, B. E. (1995). *J. Pulp Pap. Sci.* **21**(6), J191–194.
Tolan, J. S., Olson, D., and Dines, R. E. (1995). *In* "81st Annu. Meet.," pp. B91–B96. Technical Section, Montreal Quebec, Canada.
Tomar, P., and Allen, D. G. (1991). *Water Poll. Res. J. Canad.* **26**(1), 101–104.
Tono, T., Tani, Y., and Ono, K. J. (1968). *Ferment. Technol.* **46**, 569–574.
Turk, S. (1988). *Report WTC* **Bio-7-1988.** Environment Canada, Ottawa.
Valenzuela, J., Bumann, U., Cespedes, R., Padilla, L., and Gonzalez, B. (1997). *Appl Environ. Microbiol.* **63**(1), 227–232.
Vice, K., and Carrol, R. (1998). *Tappi J.* **81**(2), 91–98.

Voss, R., Wearing, J. T., and Wong, A. (1981). *In* "Advances in the Identification and Analysis of Organic Pollutants in Water," (L. H. Keith, ed.), Vol. 2, p. 1059. Ann Arbor Science, Ann Arbor.

Voss, R. H. (1983). *Environ. Sci. Technol.* **17**(9), 530–537.

Welander, T., and Anderson, P. E. (1985). *Wat. Sci. Technol.* **17,** 103–107.

Wilson, D. G., and Holloran, M. F. (1992). *Pulp Paper Canad.* **93**(12), T372–378.

Wilson, R. W., Murphy, K. L., and Frenelte, E. G. (1987). *Pulp Pap. Canad.* **88,** T4–T8.

Yao, W. Y., Kennedy, K. J., Tamchung, M. H., and John, D. (1994). *Canad. J. Chem. Eng.* **72**(6), 991–999.

Yin, C. F., Joyce, T. W., and Chang, H. M. (1989a). *Tappi Int. Symp. Wood and Pulping Chem. Raleigh, NC.* pp. 753–758. Tappi Press Atlanta, GA.

Yin, C. F., Joyce, T. W., and Chang, H. M. (1989b). *J. Biotechnol.* **10,** 67–76.

Yin, C. F., Joyce, T. W., and Chang, H. M. (1989c). *J. Biotechnol.* **10,** 77–84.

Yin, C. F. (1989). Ph.D. Thesis, North Carolina State University Raleigh, NC.

Yin, C. F., Joyce, T. W., and Chang, H. M. (1990). *In* "Biotechnology in Pulp and Paper Manufacture" (T. K. Kirk, and H. M. Chang, eds.), pp. 231–234. Butterworth-Heinemann Newton, MA.

Yu, P., and Welander, T. (1988). *In* "Proc. 5th Int. Symp. *Anaerobic Digestion,*" pp. 865–867. Bologna, Itlay.

Yu, P., and Welander, T. (1994). *Appl. Microbiol. Biotechnol.* **40,** 806–811.

Zheng, Y., and Allen, D. G. (1997). *Wat. Res.* **31,** 1595–1600.

Bioremediation Technologies for Metal-Containing Wastewaters Using Metabolically Active Microorganisms

THOMAS PÜMPEL
Leopold-Franzens-University of Innsbruck
Institute of Microbiology
Technikerstrasse 25
A-6020 Innsbruck, Austria

KISHORE M. PAKNIKAR
Agharkar Research Institute
Division of Microbial Sciences
G.G. Agarkar Road, Pune 411 004, India

I. Introduction

In today's industrial society, metals are among the most commonly used raw materials. Mining, metal refining, use of metals in manufacturing, and the final disposition of manufactured products constitute

ADVANCES IN MICROBIOLOGY, VOLUME 48

0065-2164/01 $35.00

activities resulting in metal losses. Metal wastes represent a critical loss of nonrenewable resources and pose serious health and ecological risk. International agreements and directives issued by various countries prohibit or strictly control the discharge of hazardous materials such as heavy metals into the environment.

The importance of preventing or removing contamination of the environment with heavy metals is paramount. It is widely known that such metal contamination has dangerous effects on the flora and fauna. There are many examples of human populations suffering from ingestion of heavy metals. Perhaps the most notorious example in recent times is arsenic poisoning seen in millions of people from Bangladesh and West Bengal (India) due to consumption of contaminated groundwater (Bagla and Kaiser, 1996; Dhar *et al.*, 1997). Conventional techniques for metal removal from wastewaters include chemical precipitation, ion exchange, membrane separation, evaporative distillation, liquid–liquid extraction, solvent extraction etc. These techniques are, however, becoming increasingly expensive and inefficient as stricter statutory limits for waste disposal are being introduced. Therefore, there is an urgent need for the development of cost-effective and efficient technologies that could treat metal-containing wastes including aqueous streams.

Because of the toxicity and the ubiquity of metals in the environment microbes have developed unique and sometimes bizarre ways of dealing with unwanted metals. Some microorganisms have mechanisms to sequester and immobilize metals, whereas others actually enhance metal solubility. Decades of fundamental and applied research in metal–microbe interactions have created a sound basis of understanding for the development of suitable technological applications. For example, it is known that in nature a variety of chemolithotrophic microorganisms mediate the mobilization of various metals from solids mostly by the formation of inorganic acids. This microbial activity has been applied successfully in the industry for the recovery of metals from ores. At least 25% of the copper and 33% of the gold presently produced in the world comes from such microbial leaching processes (Zechendorf, 1999). However, until recently, large-scale application of bioremediation technologies for metal-containing wastewaters was the topic of few scientists worldwide. In 1990 Jim Whitlock, one of the fathers of the revolutionary Homestake-process (Section III.A) complained about the slow progress: "It is apparent that acceptance and implementation within the industry will be with caution and at a pace somewhat slower than might be expected" (Whitlock, 1990), and the inventors of the METEX process (Section III.C) specified: "Industries that produce waste waters containing heavy metals traditionally rely on physical-chemical processes to treat these wastes. Above all companies in metal

finishing fields such as electroplating, hardening shops, printed circuit board manufacturers and other of this nature are extremely hesitant in taking to biologically based processes" (Morper and Fürst, 1991).

Within the past 15 years the pioneering full-scale plants built by Homestake, Paques, and Linde (Sections III.A–C), as well as numerous pilot and demonstration units, have confirmed the flexibility, efficiency, and economics of biotechniques in metals wastewater treatment. Nevertheless, metals bioremediation is still lagging behind in terms of commercial development but with the beginning of the new millenium there are increasing signs of faster rates of growth.

In this review we attempt to describe briefly the various mechanisms involved in microbial metal immobilization and present and evaluate technologies that have been demonstrated at commercial, pilot, and expanded-laboratory scale. It is pertinent to point out that in addition to published information, a major part of this presentation is based on extensive patent search and reliable data obtained from researchers and inventors across the globe.

II. Overview of Mechanisms Involved in Microbial Metal Immobilization

It is not the aim of this presentation to review all known processes in detail. Nevertheless, the following section briefly summarizes the operational mechanisms in the removal of metals and metalloids from aqueous solutions, forming the basis of technologies discussed subsequently.

Live microorganisms provide a broad spectrum of useful mechanisms. In natural environments these functions contribute to the global cycling of inorganic matter, and in particular lead to the formation of deposits of various minerals and ores within geological periods of time. The examples of different mineral classes (Beveridge, 1989; Schlegel, 1992), viz. limestone (calcium carbonate), gypsum (calcium sulfate), limonite and goethite (hydrated iron oxide), pyrite (iron sulfide) and iron silicates indicate some of the different mechanisms of their formation. Thompson (1996) hypothesized that such "biomineralization" processes proceed according to the following sequence:

1. Formation of microbial biofilm
2. Biosorption of soluble metals to microbial cells and exopolymers
3. Formation of metal hydroxides, oxides, carbonates (examples), and their maturation to amorphous mineral precursors
4. Stabilization of the precipitates forming a remineralization nucleation crystal template for further mineralization in the microenvironment generated by the metabolic activity of microorganisms.

Recent approaches to microbiological treatment of metal-containing wastewater attempt to make use of such biomineralization processes, mimicking the optimal conditions in various bioreactor designs. In fact, scientists even report the formation of crystals of several microns in size in fixed film reactors within just a few days of operation (Diels *et al.*, 1993; Macaskie, 1990).

Nevertheless, systematic studies on naturally occurring biomineralization processes were not the common approach adopted for the development of wastewater treatment systems presented here. Some successful developments actually emerged after careful analysis, optimization, and scale-up of accidental observations and chance discoveries.

In the systems containing mixed microbial populations and complex wastewater matrices, a complete analysis of all the metal removal processes is not possible. However, depending on the metals, their speciation, the microorganisms present and the major metabolic processes taking place, the predominant mechanisms of metal immobilization can be identified. Such mechanisms are discussed in the following sections. It may be noted that among these mechanisms, only "biosorption" and "adsorption" (Sections II.A and B) are not connected with active metabolism of the cells.

A. Biosorption

The term biosorption describes the physicochemical interaction between dissolved ionic species and binding sites in cell walls and exopolymers (Remacle, 1988). Biosorption inevitably contributes to metal immobilization in every biological system and is considered as the starting reaction for subsequent biomineralization processes. Technical applications relying on pure biosorption using dead, mostly waste, biomass have reached pilot stage, but have so far not reached the expected level of commercialization (Gadd, 2000; Kratochvil and Volesky, 1998; Tsezos, 2001; Volesky, 2001).

B. Adsorption of Suspended Solids

Adsorption (e.g., of microprecipitates) onto the cell wall and exopolymers by weak chemical bonds (e.g., van-der-Waals forces) has been shown to enhance the physical filtration efficiency of all biological systems, especially of fixed film systems like sand filters or rotating contactors. Adsorption of particulates has also been observed with fungi and was proposed as a process for the removal of suspended matter from wastewater (Paknikar *et al.*, 1999; Singleton *et al.*, 1990; Wainwright and Grayston, 1989).

C. Bioaccumulation

The enhanced active uptake and intracellular deposition of metals has been reported for several elements like copper and iron (Bazylinski *et al.*, 1993; Blakemore and Frankel, 1989), silver (Pernfuss, 1996; Klaus *et al.*, 1999), cadmium (Grupa *et al.*, 1992), calcium (Moorer *et al.*, 1993), zinc (Sakurai *et al.*, 1990), tellurium (Gharieb *et al.*, 1999) and selenium (Koren *et al.*, 1992). However, there are no reports on wastewater treatment systems based on these—mostly—detoxification reactions. It is known that many of the environmentally hazardous heavy metals and metalloids are also essential elements for living organisms; small quantities are beneficial, whereas higher concentrations often show toxic effects. Therefore, microorganisms strictly control the uptake of metals, and the normal intracellular levels are by far too low for a technical application. Hyper-accumulating *plants,* on the other hand, are already of great value in soil remediation processes (Kamnev and van der Lelie, 2000) and also their use in water treatment is under investigation (Gatliff, 1998; Raskin *et al.*, 1999; Salt *et al.*, 1998). This subject is, however, beyond the scope of the present article.

D. Excretion and Extracellular Deposition

Active uptake followed by transformation and/or excretion and extracellular deposition of metals is one of the resistance mechanisms in some microorganisms. Such types of microbial interactions have been exploited in the development of Mercury (Section III.F) and BICMER (Section IV.B) technologies.

E. Bioreduction

Extracellular, enzymatic biotransformation to a less soluble, precipitating species has been shown for several reducible metal species like Cr^{6+}, Mn^{6+}, Pd^{2+}, Se^{4+}, Se^{6+}, Tc^{7+}, Te^{6+}, U^{6+}, recently reviewed by Gadd (2000), Lovley and Coates (1997), and White and Gadd (1998b). With regard to the systems presented in this paper, bioreduction is responsible for selenium removal in the BioMats process (Section IV.A) and chromium removal in the METEX (Section III.C), Bio-Substrat (Section III.D) and ARI (Section IV.C) systems. In general, low oxygen concentration is favorable for bioreduction of metals and metalloids, but slower, aerobic processes have been identified as well (Campos *et al.*, 1995; Lovley and Coates, 1997; Mittlinger, 1998).

F. Biomineralization

In a favorable chemical microenvironment (e.g., high pH and high local concentrations of metals and anions within a biofilm) and in the presence of nucleation sites, metals can precipitate in various chemical forms. With respect to microbial wastewater treatment only inorganic precipitates have been reported so far. However, many fungi excrete oxalic acid, which forms insoluble oxalate crystals with some metals, but this mechanism has not yet been exploited for application (Sayer and Gadd, 1997; White *et al.*, 1997). Such bioprecipitation and biocrystallization processes lead to extraordinary high metal to biomass ratios (Diels *et al.*, 1993; Macaskie, 1990; Thompson, 1996). Biomineralization has been detected in most water treatment systems with active biomass, and the most common precipitation products are:

1. Hydroxides

In the neutral to alkaline pH range many metals form hydroxides of low solubility (for example, according to reaction 1). Neutralization with bases [sodium or calcium hydroxide (lime)] is widely used in the conventional, chemical treatment of the mostly acidic, metal containing waters from various sources. Many microorganisms are able to mediate such precipitation by (a) the generation of alkalinity from their metabolism (uptake of organic acids via proton symport, by sulfate reduction or cyanide degradation, for example), and (b) by cleaving metal–organic complexes, which impair normal chemical precipitation. (Many industrial wastewaters contain complexing substances like lactate, malate, gluconate, EDTA, NTA, cyanides or ammonia.) The microenvironment within a biofilm (steep concentration gradients, high local pH) particularly supports the formation of precipitates, which adsorb or remain physically entrapped in the jelly-like polymer matrix.

$$Me^{2+} + 2OH^- \rightarrow Me(OH)_2 \quad (1)$$

2. Carbonates

Addition of limestone ($CaCO_3$) or sodium bicarbonate (Na_2CO_3) is another classical method in acid wastewater treatment. In addition to the precipitation of metal hydroxides by increased pH, excess carbonate ions cause several heavy metals to precipitate as the respective metal carbonate according to simplified reaction (2). In particular for the removal of cadmium and lead, lower effluent concentrations and denser sludge can be achieved, compared to neutralization (Peters *et al.*, 1985). Diels and co-workers (see Section IV.B for details) discovered and investigated in detail a bacterial mechanism leading to the formation of

microcrystals of some heavy metal hydrogen carbonates (mainly Cu, Cd, Zn) within a biofilm. The microenvironment in the immediate vicinity of the cells becomes enriched with hydrogen carbonate ions from metabolism and with metal ions, and together with increased alkalinity the formation of crystalline metal hydrogen carbonates is favored (reaction 3).

$$Me^{2+} + CO_3^{2-} \rightarrow MeCO_3 \quad (2)$$

$$Me^{2+} + HCO_3^{-} \rightarrow MeHCO_3^{+} \quad (3)$$

3. Phosphates

Macaskie and her team worked on a bacterium overexpressing a membrane-bound phosphatase; this enzyme separates phosphate from organic donors (e.g., glycerol phosphate). The organisms are grown as biofilm on a solid support material. In the emerging milieu, insoluble hydrogen phosphates of cadmium and uranyl build up (reaction 4). Special sites on microbial exopolymers seem to act as nucleation foci initiating the growth of crystals, the weight of which by far exceeds the biomass weight (Macaskie, 1990). Up to now the process has been demonstrated successfully with real water samples on bench-scale, but for scaling-up a more economic source of phosphate has to be identified (Macaskie, 2001, pers. comm.). In the MERESAFIN sandfilter (Section III.E) a mixed culture of bacteria biocrystallized a nickel phosphate [arupite, $Ni_3(PO_4)_2$] when treating rinsing water of an electroless nickel plating line (Pümpel, unpublished data).

$$Me^{2+} + HPO_4^{2-} \rightarrow MeHPO_4 \quad (Me = Cd, UO_2) \quad (4)$$

4. Sulfides

Metal sulfides are among the least soluble solid phases, which also form in a rather low pH range of 2–5 (reaction 8). These properties also allow the removal of metals also in the presence of complexing agents often present in metal-containing wastewaters, and with precise pH adjustment the selective precipitation of some metals is possible (Peters *et al.*, 1985). Sulfate-reducing bacteria (SRB), gaining energy from the reduction of oxidized sulfur species (in most cases sulfate, SO_4^{2-}; reactions 5 and 6) or elemental sulfur (S^0; reaction 7) to sulfide (S^{2-}, HS^-, H_2S, depending on pH), are well known to drive the precipitation of metal sulfides (reaction 8) in natural, anoxic ecosystems (e.g., sediments). SRB also alkalize their environment (reactions 5 and 6), which is beneficial for the stability of the metal sulfides formed (White *et al.*, 1997). For many years SRB have been applied successfully to treat acidic, metal-containing water with various process designs like

constructed wetlands (Fortin *et al.*, 2000; Kalin *et al.*, 1993; Wildeman *et al.*, 1994), meander systems (Sterritt and Lester, 1979), and several bioreactor systems (Sections III.B–D, IV.E).

Reduction of sulfate to sulfide by SRB using molecular hydrogen (H_2; reaction 5) or organic carbon (e.g., ethanol, C_2H_5OH; reaction 6) as the electron donors (Boonstra *et al.*, 1999):

$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O \quad (5)$$

$$3SO_4^{2-} + 2C_2H_5OH \rightarrow 3HS^- + 3H_2O + 3HCO_3^- + CO_2 \quad (6)$$

Reduction of elemental sulfur to sulfide by SRB with acetate as the electron donor (Reidl *et al.*, 2000):

$$4S^0 + CH_3COO^- + 2H_2O \rightarrow 4HS^- + 2CO_2 + 3H^+ \quad (7)$$

Precipitation of divalent metal ions with hydrogen sulfide (Reidl *et al.*, 2000):

$$Me^{2+} + HS^- \rightarrow MeS + H^+ \quad (8)$$

G. Decomposition of Soluble, Organometallic Compounds

The release of metals by microbial degradation of organic complexing agents allows the metal to be deposited by one of the other mechanisms mentioned. This is one of the essential processes making the microbial treatment of complex wastewaters a story of success. Decomposition of metal cyanides is applied in the Homestake (Section III.A), BICMER (Section IV.B) and ARI (Section IV.D) processes, and decomposition of metal lactates enables the removal of nickel in the MERESAFIN (Section III.E) and BICMER (Section IV.B) systems.

H. Biosulfide Production

The bacterial production of hydrogen sulfide for the decoupled chemical precipitation of metal sulfides is a special application requiring no direct interaction between metals and microorganisms. Nevertheless, it is worth mentioning because of several advantages, discussed in Section IV.E.

III. Wastewater Treatment Systems with Full-Scale Application

A. The Homestake Rotating Biological Contactors (USA)

The Homestake Mine at Lead, South Dakota, is one of the oldest and largest underground gold mines in the western hemisphere. In the 1970s

the company constructed a tailings management facility to avoid the discharge of mill tailings to the lifeless Whitewood Creek (Whitlock, 1990). The effluent passed sandfilters (since 1979) and carbon columns (since 1980), but unable to meet discharge limits for cyanide, zinc, and copper set forth in the Clean Water Act, Homestake signed a consent decree in 1980 that committed the company to develop a water treatment technology to meet water quality standards within 4 years. Homestake's research and development efforts led to a biological system that treated mine wastewater which effectively met strict water quality standards. Homestake invested $30 million in the development and construction of a plant using the new technology. The plant began operating in 1984. The patent for the revolutionary process—which uses microorganisms to achieve water quality standards prior to mine water discharge—was donated to the South Dakota School of Mines for use elsewhere in the mining industry (Homestake Mining Company, 1999).

1. Principle

Reactor Design. Investigations of microbial cyanide degradation in the tailings impoundment suggested that an aerobic bioreactor with immobilized microorganisms would best mimic natural conditions. Based on pilot studies, rotating biological contactors (RBCs) were selected for the full-scale application.

The RBCs consist of a corrugated plastic media disk, 3.6 m in diameter and 7.6 m long; they rotate 40% submerged at 1.5 rpm. The surface area of each RBC is approximately 12.000 m^2. Supplemental aeration is provided with Roots Blowers via diffuser lines below each disk in order to keep the dissolved oxygen level above 3–4 mg/liter.

The wastewater passes a train of five RBCs, the first two for cyanide and metals removal, the other three for nitrification. Hydraulic retention time in the RBCs is 1 and 1.5 h in the first- and second-stage RBCs, respectively.

Biological Processes. Bacteria of the genus *Pseudomonas* predominate in the biofilms in the first stage of the bioreactor train (RBCs 1 and 2). They are responsible for the degradation of free and metal-complexed cyanide (reactions 9 and 10) and thiocyanate (reaction 11), and for removal of heavy metals by biosorption. Due to the slightly alkaline pH (7.5 to 8.5; Whitlock, 1990) and HCO_3^- produced within the biofilm (reactions 9–11), there is a strong likelihood that precipitation of metal hydroxides and carbonates (reactions 1–3) also contributes to metals removal, following destruction of the metal–cyanide complexes.

Microbially catalyzed reactions of stage 1 (RBCs 1 and 2) concerning cyanide degradation (Whitlock and Mudder, 1986):

$$2CN^- + 4H_2O + O_2 \rightarrow 2NH_3 + 2HCO_3^- \quad (9)$$

$$Metal_xCN_y + 4H_2O + O_2 \rightarrow \text{metal-biomass} + 2HCO_3^- \quad (10)$$

$$SCN + 2H_2O + 5/2O_2 \rightarrow SO_4^{2-} + HCO_3^- + NH_3 \quad (11)$$

In the second stage (RBCs 3 to 5) ammonia, produced in stage 1 by cyanide degradation (reactions 9 and 11), and also present in the influent, is converted to nitrate by a mixed population of nitrifying bacteria, according to the overall reaction:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+ \quad (12)$$

In addition, some polishing reactions happen in this stage, such as oxidation of residual cyanide and intermediates, and further metals removal.

Nutrition. Cyanide-degrading bacteria in the first stage need organic compounds as energy and carbon sources, phosphorous and the usually present trace elements. Nitrogen is taken from the cyanide. Autotrophic, nitrifying bacteria in the second stage grow with inorganic carbonate, provided by stage 1 (reactions 9–11).

Process Design. The wastewater first enters a mix tank, where missing nutrients are added, and then proceeds to the train of five RBCs. Excess biomass continuously sloughs off from the disks and sediments in a clarifier; flocculents (ferric chloride or polymeric flocculents) may be added to improve settling. After passing an emergency clarifier the final effluent is sand filtered (Mudder and Whitlock, 1984; Whitlock, 1987, 1989, 1990, 1992; Whitlock and Mudder, 1986; Whitlock and Smith, 1989).

2. Experiences with Full-Scale Installations

The only plant in operation, at Homestake Mine, Lead, is designed to treat up to 900 m^3/h of mine and decant water and comprises 48 RBCs (since 1984). During the first 5 years of operation a continuous improvement of the plant performance was observed, attributed to biological self-regulation and increased operator efficiency. The average removal efficiency is 99–100% for thiocyanate, 96–98% for total cyanide, 94–97% for copper, and 98–100% for ammonia conversion (Whitlock, 1989).

3. Costs

"The total cost of the treatment plant was $10 million, or approximately two-thirds of the cost of a hydrogen peroxide plant designed for the same conditions. Daily operational costs are much lower, as minute amounts of phosphoric acid are the only chemical addition" (Whitlock, 1990). In 2000 costs of operation were 0.12 $/m^3 effluent (Scheetz, 2000; pers. comm.) and 0.45 $/t of ore processed in 1990 (Whitlock, 1990).

4. Summary

Sixteen years of effective operation of the Homestake RBCs demonstrate the usefulness of biological treatment of hazardous wastewaters. All end products meet the EPA permit criteria. The effluent-receiving Whitewood Creek, for over 100 years a lifeless stream "*has now become an established trout fishery and recently yielded a state record trout*" (Whitlock, 1990).

B. The THIOPAQ® System (The Netherlands)

In the early 1990s the now commercially available system for the treatment of contaminated (ground)water was developed by Paques (the Netherlands), partly in cooperation between Shell's Sittingbourne Research Center and the Budelco zinc refinery. Sulfate reduction won the bid to build a full-scale treatment facility following a government examination of all available technologies (Barnes *et al.*, 1994; Buisman *et al.*, 1989; Buisman, 1994; Scheeren *et al.*, 1992).

1. Principle

Reactor Design. The THIOPAQ concept makes use of two biological reactors: (a) an anaerobic upflow anaerobic sludge blanket (UASB) reactor for the reduction of oxidized sulfur species, calculated for a hydraulic retention time of 2–7 h, and (b) an aerobic submerged fixed film (SFF) reactor to oxidize excess sulfide to elemental sulfur, operated at a hydraulic retention time near 0.5 h.

Biological Processes. In the anaerobic reactor, sulfate-reducing bacteria (SRB) provide H_2S (reactions 5 and 6) for the precipitation of metal sulfides (reaction 7), which can proceed in the same reactor. If toxicity of the wastewater poses a problem to microorganisms, or if separation of different heavy metals is to be be achieved, the produced H_2S can also be used for metal precipitation in separate vessels.

The THIOPAQ process also aims at the removal of oxidized sulfur species, mainly sulfate, which are present in great stoichiometric excess

to heavy metals in many industrial wastewaters. Therefore, only a small portion of the formed H_2S is removed via metal precipitation. The bulk of the H_2S proceeds to the second reactor, where it is oxidized to elemental sulfur by immobilized *Thiobacillus* species under appropriate conditions (low oxygen and/or high sulfide concentration). With low-sulfate waters (at or beyond the discharge limits) the second reactor can be omitted, and the dosage of reductant to the UASB can be adjusted to give the stoichiometric ratio of metal sulfide formation. On the other hand, if the water to be treated lacks oxidized sulfur species, elemental sulfur can be added as an inexpensive and easy-to-handle source for sulfide generation.

Nutrition. For lower-sulfate loads the SRB in the anaerobic reactor are usually grown with organic wastes or with ethanol. However, depending on the further fate of the effluent the increase of COD by incompletely metabolized carbon sources or excreted metabolites has to be considered. For higher-sulfate loads (more than ~2.5 t/d of H_2S produced) molecular hydrogen and carbon dioxide are more economic as the sources of electrons and carbon, respectively. These gases can be produced on demand by cracking methanol or by means of a natural gas reformer, both techniques producing a mixture of H_2 and CO/CO_2.

Ammonium or urea as the nitrogen sources, and phosphate have to be supplied, if missing in the feed water. Essential trace elements are usually present in contaminated waters.

Process Design. The principle involves high rate conversion of sulfur compounds and the combined recovery of heavy metals. Many process configurations are possible in order to fit specific project needs and opportunities. The most common flow scheme is described in Fig. 1: The

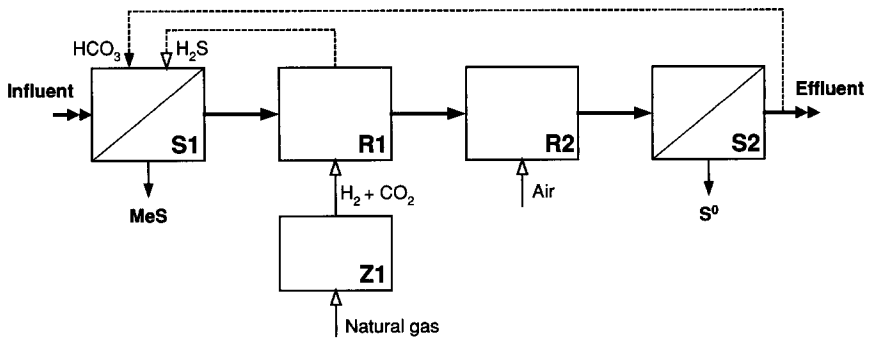

FIG. 1. Typical process flowchart of Paques' THIOPAQ® system for the removal of sulfate and metals; explanations in the text. Re-plotted from Boonstra *et al.* (1999) (with permission) from Excerpta Medica Inc.

necessary amount of H_2S, produced in the anaerobic UASB reactor **R1,** is led back to unit **S1,** where metal precipitation and sedimentation take place. A surplus of sulfide is oxidized in the aerobic SFF reactor **R2,** and the formed elemental sulfur is separated from the effluent in the tilted plate separator **S2.** On demand, the alkaline final effluent (see reactions 5 and 6 for generation of HCO_3^--alkalinity) can be partially returned to **S1** in order to increase the influent pH. In this example natural gas is converted to H_2 and CO_2 in reformer **Z1.**

With other configurations tested at pilot and demonstration scale, the formed H_2S can be used in reactors with different pH values adjusted to selectively precipitate heavy metals. Thus, sulfide precipitation makes it possible to separate copper from zinc, arsenic from copper, iron from nickel, etc. (Barnes *et al.,* 1994; Boonstra *et al.,* 1999; De Vegt *et al.,* 1998; van Lier *et al.,* 1999; Scheeren *et al.,* 1992; Janssen and Boonstra, 2000, pers. comm.).

2. Experiences with Full-Scale and Pilot Installations

Among over 400 industrial installations of anaerobic wastewater treatment, Paques has built some 30 plants to convert sulfur compounds, with the following three aiming at the recovery of metals as metal sulfides (Boonstra, 2001, pers. comm.):

- One plant treating groundwater contaminated with Zn and sulfate (400 m^3/h, since 1992)
- One plant treating wash tower acid and bleed of electrolysis circuit with Zn and sulfate (25 m^3/h, since 1999)
- One plant treating mixed process water from semiconductor production containing inorganic acids (nitric, hydrofluoric, phosphoric, sulfuric), organic substances (acetic acid, acetone, ethanol, methanol), and metals (Cr^{3+}, Cr^{6+}, Sn, Cu, Ni, MnO_4, Pb, Fe) (10 m^3/h, since 1997)

In the first two plants mentioned (located at the same site) no solid waste stream is created. Zinc sulfide is returned to the company's roaster, and elemental sulfur is used for the production of sulfuric acid. Paques claims to have enabled "*The world's first gypsum-free zinc refinery*" (Boonstra, 2000). Stipulated discharge limits are well kept with effluent concentrations of zinc <0.05 mg/l and sulfate <200 mg/liter.

The third plant listed has been inserted into an existing, conventional treatment process. The final effluent concentrations of most metals could be lowered markedly, and COD and nitrate are now degraded considerably. Possibilities for the recycling of metals from the sludge are under investigation (Reidl *et al.,* 2000).

At pilot level the THIOPAQ process also succeeded in treating acid mine drainage (AMD) at Berkeley Pit (Butte, Montana), Kennecott Utah

Copper (U.S.) and Wheal Jane Mine (Cornwall, UK) (Boonstra *et al.*, 1999; de Vegt *et al.*, 1998).

3. Costs

De Vegt *et al.*, (1998) estimate costs for the biological production of hydrogen sulfide as follows: "Taking the operational cost savings on existing lime treatment into account, the overall price for biological H_2S production is 200–500 US$/t S, which is significantly lower than alternatives." In an overall cost balance also the possibility of marketing the produced metal sulfides and elemental sulfur (for sulfuric acid production or as fertilizer) has to be looked at (Boonstra, 2001).

4. Summary

The THIOPAQ system has proven reliable in the treatment of sulfate-containing, metal-contaminated water up to a scale of 400 m^3/h for 8 years. Such waters can be found, for example, in the metal and semiconductor industry and at abandoned mine sites. Due to the low solubility of many metal sulfides the final metal effluent concentrations are very low. However, a polishing filter may have to be installed to retain small suspended solids. One of the major advantages of the whole system is the additional removal of sulfur compounds to below 200 mg/liter with the optional reuse of elemental sulfur. In contrast, conventional liming cannot reduce sulfate concentrations below 1500 mg/liter, and produces metal contaminated gypsum which usually needs to be disposed of.

C. The METEX® Anaerobic Sludge Reactor (Germany)

In the 1980s, Linde (Germany) developed the METEX process for the biological treatment of heavy metal-containing industrial wastewaters. The observed side effect of copper removal in an anaerobic vinasse treatment plant has been worked up into a process of its own (Morper, 1985).

1. Principle

Reactor Design. The METEX reactor is a cylindrical, Upflow Anaerobic Sludge Blanket (UASB) reactor, filled with anaerobic sludge from standard sewage treatment plants. Slowly moving stirrers prevent the formation of short-circuit channels through the sludge bed, but keep the desired vertical gradients. In the clarification zone above the stirrers most of the sludge is retained in the reactor. The effluent finally passes a cross-flow microfiltration unit in order to remove the finest suspended particles (flocs of microorganisms and metal precipitates)

escaping from the reactor. The hydraulic retention time in the reactor ranges between 12 and 24 h.

Biological Processes. From bottom to top subsequent zones with different metabolic activities and also different groups of microorganisms may develop in the sludge bed, depending on nutrients and on the electron acceptors available in the wastewater (e.g., aerobic, denitrifying, sulfate reducing zone). The anoxic, sulfate reducing zone is the most important one with respect to heavy metal removal in the METEX reactor, promoting the formation of highly insoluble metal sulfides (reaction 8). Further, bioprecipitation of metal carbonates (reaction 2), and biosorption/adsorption of dissolved metal species were shown to contribute to the overall metal removal process. A metal speciation analysis in the sludge of a reactor treating wastewater of a printed circuit board manufacturer, revealed the following after more than 1 year of operation: in the anoxic upper zone of the sludge bed copper was present as sulfide (80%), as carbonate (10%), adsorbed (5%), and organically bound (5%). In contrast, the fractionation of lead showed much less sulfide (30%), and much more carbonate (30%), organically bound (20%) and adsorbed lead (10%).

Another substantial chemical reaction for industrial wastewater treatment was shown to take place in the METEX reactor: hexavalent chromium (Cr^{6+}), which cannot be directly precipitated or sorbed, is readily reduced ("detoxified") to the trivalent state under the reducing conditions and in the presence of biomass. The reduced species sorbs highly to biomass and also precipitates as hydroxide.

Nutrition. In most applications the wastewaters contain all nutrients necessary for activity and growth of the microorganisms; only in a few cases one or more missing components have to be dosed. In the full-scale plants running at present the microorganisms use waste photoresist, alcohols (ethanol, isopropanol) and mineral oil, for instance, as their carbon sources, and ammonium or nitrate for nitrogen supply. Phosphates, sulfate, and the essential micro- and trace elements are usually present in industrial wastewaters.

Process Design. In a typically configured METEX plant the wastewater first passes a conventional neutralization unit with a reaction vessel, settler, and dosing station. Thus the proportion of metals accessible to the formation of metal hydroxides is precipitated and removed. The feed water for the METEX reactor then usually contains between 2 and 30 mg/liter of complexed or finely dispersed heavy metals. Excess sulfide produced in the reactor is oxidized by dosed hydrogen peroxide

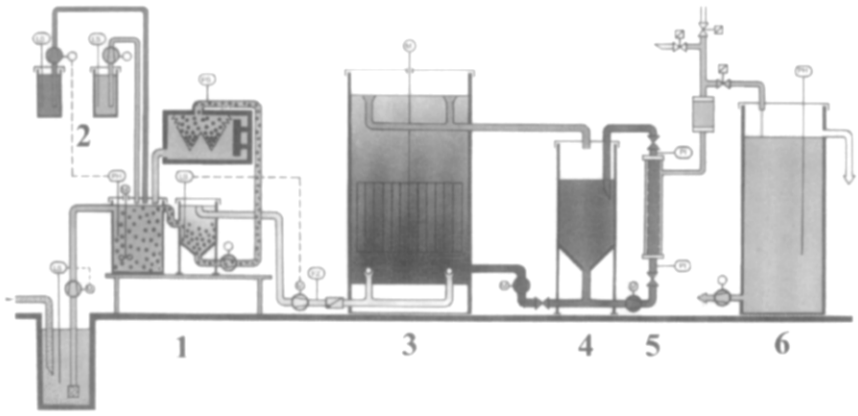

FIG. 2. Process flowchart of Linde's METEX® system: neutralization unit (1), dosing station for acid/base/nutrients (2), METEX UASB-reactor (3), circulation tank (4), membrane filtration unit (5), clean-water reservoir (6). Reproduced with permission from Morper and Fürst (1991).

(Fig. 2) (Fürst and Morper, 1993; Morper, 1985, 1986, 1997, 1999; Morper and Frydman, 1987; Morper and Fürst, 1991; Mühlbacher, 1994).

2. Experiences with Full-Scale Installations

Seven plants, erected between 1991 and 1995, are now in operation in Germany.

- Two plants in the manufacture of printed circuit boards, handling Cu, Pb, Sn, Ni, photoresist (10 resp. 14 m^3/d)
- Two plants in the manufacture of electronic microchips and hybrid integrated circuits, handling Cu, Ni, Cr^{3+}, Cr^{6+}, Au, Pd, ammonium, photoresist, carbohydrates (10 resp. 50 m^3/d)
- One plant for the treatment of a combined waste water from various metal finishing processes, handling Cu, Zn, ammonium, carbohydrates (30 m^3/d)
- One plant for the treatment of wastewater from chemical bronze staining, handling Cu, Sn, Zn, Pb (20 m^3/d)
- One plant in a large laundry, handling Cu, Zn, Pb, carbohydrates (20 m^3/d)

The German wastewater discharge limits can usually be met by all the plants. Occasionally, nickel can slightly exceed the limits. All users report stable operation of their plants and modest maintenance requirements, compared to conventional techniques. The sludge can be used for up to 8 years (or even longer) without replacement, and the metal concentrations reach dozens of grams per kilogram. One of the

electronic microchip producers estimated the actual value of the precious metals containing sludge at 6000€/t (pers. comm. with all users).

3. Costs

Investment costs of a METEX plant are in the range of those for conventional technology. The technical design is generally simpler, but the reactors are relatively large due to the long retention time needed. Users estimate that the costs for chemicals and maintenance are much less than those for other technology (pers. comm. with users; Morper, 2000, pers. comm.).

4. Summary

The METEX system is being applied successfully for the treatment of complex industrial wastewaters for 9 years, mainly in the electronics industry (manufacture of microchips, hybrid integrated circuits, and printed circuit boards). The capacities of installed plants are in the range of 4 to 50 m^3/d, with reactor sizes of 4 to 50 m^3.

D. The Bio-Substrat® Anaerobic Micro-Carrier Reactor (Germany)

1. Principle

The Bio-Substrat process (Dr. Fürst Systems and BKT-Burggräf) is similar to the METEX system described earlier. Taking advantage of the same biological and chemical processes, and using practically the same process configuration (Section III.C), the Bio-Substrat process differs from METEX in two major respects:

- The slowly stirred upflow reactor is filled with a granular micro-carrier material with high sorption capacity (zeolite).
- Natural microorganisms, which have been adapted to the particular wastewater matrix, are grown on the micro-carriers, instead of using anaerobic sludge.

Adsorption of organic and inorganic wastewater ingredients to the zeolites extends their stay in the reactor beyond the hydraulic retention time. Thus, better degradation of certain organic compounds and bioprecipitation of nickel, for instance, is achieved. As a positive side effect, the granules, which are also pumped through the cross-flow microfiltration unit, help keep the membrane clean. The reduction of sludge from publicly owned sewage treatment plants reduces the risk of growing pathogenic microorganisms in the reactor, and might increase the acceptance of a biological system in metals industry (Biosubstrat, 1999; Fürst and Burggräf, 2000; Fürst, 2001, pers. comm.).

2. Experiences with Full-Scale Installations

Within the last 3 years two full-scale plants were put into operation:

One plant treating the etching liquors from the production of shadow masks for computer and television monitors, removing Cu, Ni, Fe, Cr^{6+} and casein (150 m^3/d)
One plant in the manufacture of flexible printed circuit boards, handling Cu, developer and photoresist (5 m^3/d).

With both plants the German criteria for wastewater quality are met, also for nickel. All further comments on experiences and costs correspond with those made for the METEX process (pers. comm. with users).

3. Summary

With site-adapted microorganisms in a micro-carrier reactor the Bio-Substrat process overcomes two drawbacks of the similar METEX system: the retention time of organic and inorganic substances is increased above the hydraulic retention time, improving biodegradation and bio-precipitation reactions, and the general image and acceptance of a biological system using site-grown microorganisms is better than that of a system started with sewage sludge.

E. The MERESAFIN Sand Filter (Europe)

The biologically active moving-bed sand filter has been developed by a European consortium of 10 research and industrial partners from 1996 to 1999, funded by the European Union (Project 1).

1. Principle

Reactor Design. The core of the system is a continuously regenerating, moving-bed Astrasand® filter (Assen, 1995; Figs. 3 and 4). The feed water enters the filter via a feed pipe (**1**) and distributor (**2**) and flows in an upward direction through a sand bed (**3**) in a cylindrical vessel. Inoculated bacteria form biofilms on the sand grains, which trap trace elements from the feed water. From the bottom of the filter the laden sand (**4**) is moved upwards by an internal airlift (**5**). By attrition, biofilm fragments with heavy metals are separated from the sand grains and led out from the filter with some wash water (**7**). The sand grains keep their base biofilm and fall back to the top of the bed through a washer labyrinth (**8**). The sand circulation speed is controlled by the amount of air introduced into the airlift and by the filter bed resistance

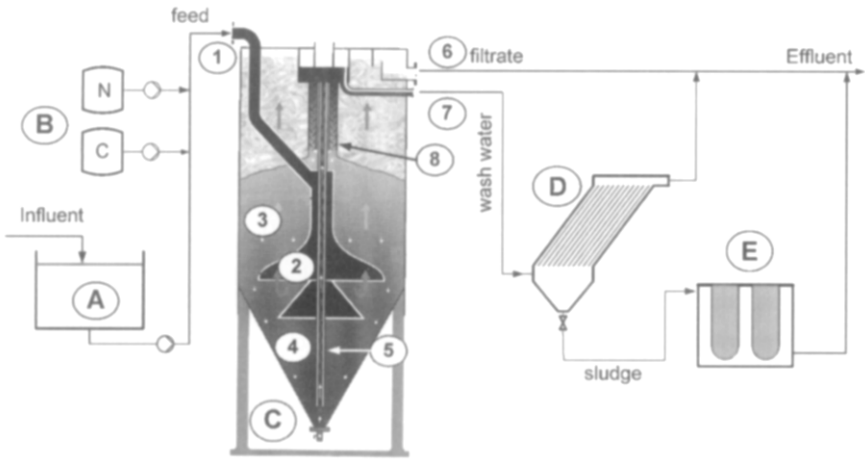

FIG. 3. Technical principle of the Astrasand moving-bed sand filter, and typical process configuration. Explanations in the text.

(the higher the pressure in the bed, the more sand is transported through the airlift). This self-regulation results in a constant bed resistance and therefore constant sand loading and filtration efficiency (Kramer and Wouters, 1993; Diels *et al.*, 1998). With a pore volume of 30%, the hydraulic retention time in the sand bed can be adjusted between 5 and 30 min.

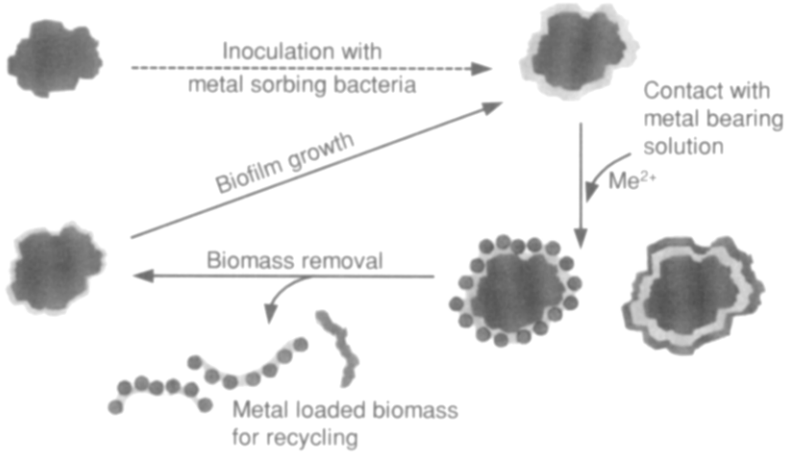

FIG. 4. The biofilm life cycle in a moving-bed sand filter. Reprinted from Diels *et al.* (1999) (with permission) from Elsevier Science.

Biological Processes. The system was designed to promote various known microbial processes of metal immobilization in a robust biofilm reactor: physical–chemical biosorption, biologically mediated precipitation, and reduction. The filter is inoculated with a mixed population of wastewater adapted, nonpathogenic bacteria providing the desired performance (Pernfuss *et al.,* 1999). Depending on the adjusted microbial metabolism, chemical gradients (e.g., pH, carbonate, phosphate), favorable to metal precipitation, develop within the biofilm.

Nutrition. The bacteria in the filter are easily satisfied; with the wastewaters tested up to now only low concentrations of cheap nutrients (carbon and/or nitrogen source) had to be dosed. Often the already-present ingredients support sufficient growth of the microorganisms (Diels *et al.,* 1999; Pümpel *et al.,* 2001).

Process Design. The sandfilter forms the core of a water treatment system which typically consists of a buffer tank (**A**) to smooth concentration spikes, the sand filter itself (**C**), a lamella separator (**D**) to settle the laden biomass from the wash water, bag filters (**E**) or a filter press to dewater the biomass, and dosing equipment (**B**) to supply nutrients (Fig. 3).

2. Experiences with Full-Scale and Pilot Installations

Pump-and-treat polishing of a zinc-contaminated groundwater was started in 1999 (30–40 m^3/h). Around 5 mg/liter of zinc and some nitrate could be removed. Nevertheless, due to the low temperature of the groundwater the full performance of the system has not been reached and the system needs further improvement.

In pilot plants with capacities from 1 to 30 m^3/h, the removal of nickel from plating rinsing water, copper, zinc, cobalt, and nickel from nonferrous industry wastewater, and uranium from mine drainage water has been demonstrated (Project 1).

3. Costs

Including all investment (15 years of depreciation) and operation expenses into the calculation, the treatment costs are near 0.15 €/m^3 of treated water, which is markedly lower than costs using comparable conventional technology (underlying plant capacity: 200 m^3/d).

4. Summary

Using the MERESAFIN (**ME**tal **RE**moval by **SA**nd **F**ilter **IN**oculation) system, high volumes of moderately contaminated wastewaters can be treated very economically. In addition to the removal of metals, the filter biologically removes nitrate and nitrite (denitrification) and to some

extent also COD. Of course, also its original function as a mechanical filter contributes to the overall performance. Typical fields of application are the polishing of conventionally pretreated industrial wastewaters and the treatment of contaminated groundwater or leachate.

Despite low metal concentrations of a few milligrams per liter in the filter influent, the biomass contains up to 20% of heavy metals in the dry matter. Pyrometallurgical recycling of nickel, zinc, and cobalt has already been realized.

F. The Mercury Bioreduction System (Germany)

Supported by the European Community, the German Gesellschaft für Biotechnologische Forschung mbH (GBF) and partners from industry developed a bioremediation technology for mercury-contaminated wastewater (Projects 2 and 3).

1. Principle

Reactor Design. A packed-bed bioreactor is operated in upflow mode. Pumice granules of 4–6 mm in diameter, specific density of 2.4 g/cm^3, a pore volume of 80%, mainly consisting of SiO_2 (>70%) and Al_2O_3 (>10%) are used as the carrier material for biofilms. Hydraulic retention times range between 20 and 50 min, calculated with the full pore volume according to Wagner-Döbler *et al.* (2000b).

Biological Processes. The bioreactor is inoculated with a mixture of seven mercury-resistant, nonpathogenic *Pseudomonas* strains, isolated from mercury-contaminated environments. The system makes use of their well-understood mercury-resistance mechanism, encoded by the *mer*-operon (Silver *et al.,* 1989). Specialized membrane proteins bind ionic mercury (Hg^{2+}) and transport it into the cytoplasm, where it is reduced to nearly insoluble zero-valent mercury (Hg^0). The energy consumption of the mercury reducing enzyme is 1 mol NADPH/mol Hg. Elemental mercury diffuses out of the cells passively and forms small droplets captured within the biofilm (von Canstein *et al.,* 1999; Wagner-Döbler *et al.,* 2000a,b).

Nutrition. Activity and growth of the microorganisms are maintained by dosing sucrose and yeast extract in the milligram per liter range. Anoxic wastewater has to be aerated to give at least 4–5 mg/liter of dissolved oxygen. As the microorganisms work as biocatalysts and not as bioaccumulators, the dosage of nutrients can be adjusted to balance the rates of growth and mortality.

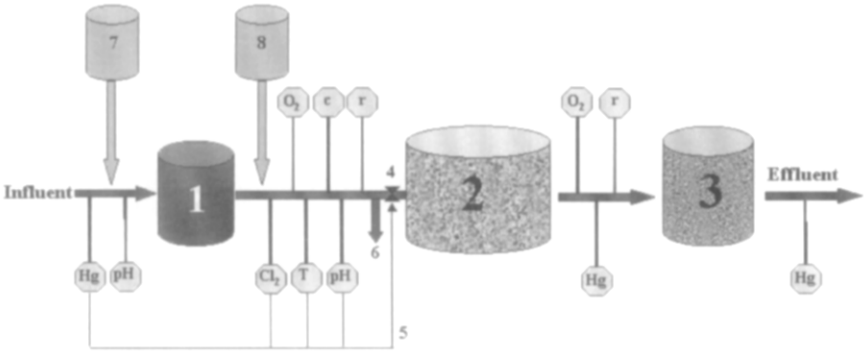

FIG. 5. Process scheme of plant for bioremediation of mercury-containing wastewater. Neutralization tank (1), packed-bed bioreactor (2), activated carbon filter (3), reactor inflow valve (4) with control (5), bypass (6), NaOH tank (7), nutrients tank (8), sensors (octagons). Reprinted with permission from Wagner-Döbler *et al.* (2000b). Copyright (2000) American Chemical Society.

Process Design. The pH of the wastewater is automatically adjusted to neutral in a neutralization tank. On demand the water is aerated, and nutrients are dosed just before the bioreactor. The effluent from the reactor then passes through an activated carbon filter, removing residual mercury. (The equilibrium concentration of dissolved mercury in pure water with a solid phase of metallic mercury is 60 μg/l, which is therefore the theoretical minimum concentration in the bioreactor effluent; in the presence of chloride or complexing substances even higher concentrations can be expected.) With time, bacteria escaping from the bioreactor colonize the activated carbon, converting the polishing filter to a polishing bioreactor (Fig. 5) (Wagner-Döbler *et al.,* 2000b; Wagner-Döbler, 2001, pers. comm.).

2. Experiences with Full-Scale Installations

In the summer of 2000, the first full-scale plant for the treatment of wastewater from a chloralkali electrolysis plant (production of chlorine from sodium and potassium chloride) was put into operation (1 m^3 bioreactor, 4 m^3/h) and works as expected from the pilot trials. Input Hg concentrations of around 5 mg/liter can be safely reduced to below 50 μg/liter keeping the relevant discharge limits (Wagner-Döbler, 2001, pers. comm.). Due to the well-balanced dosage of nutrients, the reactors can be operated for several months without clogging, before removal of biomass and elution of metallic mercury becomes necessary (Wagner-Döbler *et al.,* 2000a).

3. Costs

Running costs (energy and nutrients) of the process are calculated at 0.17 €/m^3 (Wagner-Döbler, 2001, pers. comm.).

4. Summary

Recent cases of serious mercury pollution, such as the spreading of 8 t of mercury by the bombing of a chemical plant near Beograd during the Balkan war (UNEP and UNCHS, 1999), substantiate that mercury is a problem not only of former times.

Taking advantage of a highly specific, microbial detoxification mechanism, an effective and economic bioremediation technology for mercury contaminated waters is now available. In addition to the chemical industry (e.g., production of chlorine gas by chloralkali electrolysis), also environments polluted in the past will profit from the development: Due to the lack of suitable technology the remediation of mercury polluted sites has been restricted to transfer of contaminated material to a safe landfill. A budget-priced water-treatment system opens up the possibility for on-site clean up using a controlled leaching procedure.

IV. Selected Wastewater Treatment Systems Having Reached the Pilot or Expanded-Laboratory Stage

A. The BioMat® Constructed Microbial Mats (USA)

BioMat is a "...constructed microbial mat of biological organisms that self-associate to form a complex ecosystem capable of removing contaminants from the environment. The constructed microbial mat comprises cyanobacteria and purple autotrophic bacteria and other microorganisms organized into a layered structure which is held together with slime, and has an organic nutrient source provided" (Bender and Phillips, 2000).

The initial idea behind the development of BioMat was to provide a fast-growing fish food to help feed the hungry in developing countries. However, Bender and Phillips made an accidental discovery: the microbial mats are good at bioremediation of contaminated water (Noname, 1997). Contrary to the original intention, fish should now better be prevented from eating such bioremediating mats.

Like the other active microbial systems, the BioMat is able to simultaneously sequester heavy metals and degrade organic contaminants. Controlling the activity of the oxygen-producing phototrophic bacteria by lighting intensity influences the overall oxygen concentration in the system. With low light, photosynthetic oxygen is immediately consumed

by respiration, and rather anoxic conditions develop, which favor processes like bioreduction of metals (e.g., Cr^{6+} to Cr^{3+}, Se^{4+} or Se^{6+} to Se^{0}) and dechlorination of halogenated hydrocarbons (e.g., trichlorethylene). In highly illuminated mats, ample photosynthetic oxygen drives strictly aerobic microbial metabolism, for example, nitrification of ammonia or breakdown of trinitrotoluene into small, apparently nontoxic products (Bender *et al.,* 1995; Bender, 2000, pers. comm.).

Microbial Aquatic Treatment Systems (MATS) have tested the BioMats at pilot level applying the following treatment designs (Bender *et al.,* 1995, 2000):

Floating mats on ponds. With low-flow or batch mode, large volumes of water can be treated economically (e.g., removal of manganese from acid coal mine drainage).

Mats immobilized on vertical boards have been tested for the final polishing treatment of radioactive water.

Mat microbes have also been *immobilized in silica particles* and tested for the removal of a mixture of radionuclides, including plutonium and uranium. In the words of a co-inventor of biomats, "this immobilization represents an important advance of the technology in terms of the rates of removal and ease of engineering and management" (Bender, 2000, pers. comm.).

The distinguishing feature and advantage of BioMats compared with all other systems using active microorganisms is the integration of photosynthetic bacteria into the microbial consortium, creating a self-sustainable system with minimal need for supplemental nutrition—ideal for open-air use. On the other hand, the application of active mats in a bioreactor system may be limited by rather high construction and operational costs, caused by the mat's demand for light.

B. The BICMER Membrane Reactor (Belgium)

The BICMER concept (**B**acteria **I**mmobilized **C**omposite **ME**mbrane **R**eactor) was developed by the Vlaamse instelling voor technologisch onderzoek (Vito) in the early 1990s. In the tubular membrane reactor, metal resistant bacteria (e.g., *Ralstonia metallidurans* CH34; former name: *Alcaligenes eutrophus* CH34) are immobilized in a composite Zirfon® membrane based on polysulfone and zirconium oxide. The membrane separates a small nutrient stream from the effluent to be treated, resulting in optimal microbial activity with minimal nutrient supply and leakage (Projects 4 and 5; Diels *et al.,* 1993, 1995a, 2000a).

The bioprecipitation of metal carbonates in the BICMER is based on the well-documented, plasmid-encoded metal-resistance mechanism of

CH34 (Diels *et al.*, 1995b; Dong and Mergeay, 1994) and on normal metabolic activity. The metal efflux mechanism with its countercurrent proton influx produces high metal concentrations and raises the pH at the surface of the bacteria; together with released carbon dioxide and additionally increased pH from metabolism, the ideal microenvironment for precipitation develops (reactions 1–3). Metal-induced outer membrane proteins seem to act as nucleation foci, supporting the formation of crystalline metal carbonates. Crystals grown to around 10 μm detach from the membrane and are collected in a final glass-bead filter. After induction of the process with cadmium or zinc, the removal of cadmium, zinc, copper, nickel, and silver has been shown to work (Diels *et al.*, 1995a; Projects 4 and 5).

With respect to heavy metals, BICMER systems (Fig. 6) have been tested in pilot scale (up to 18 m^2 of membrane surface, 50–300 Liter/h) to treat waters containing (Diels, 2000, pers. comm.; Vito, 2001):

Nickel lactate (nickel plating)
Copper (waste water of non-ferrous industry)
Zinc cyanide (zinc plating)
Silver cyanide (silver plating)

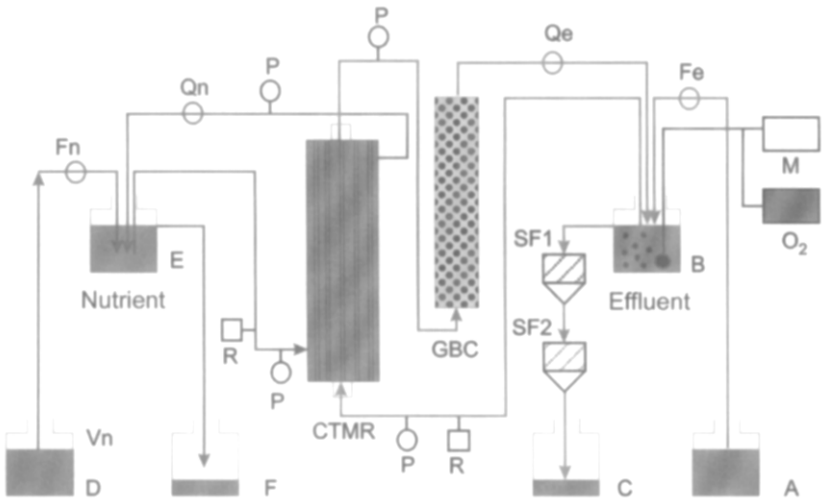

FIG. 6. BICMER concept for heavy metal removal. Effluent stock (A), effluent conditioning (B), effluent output (C), nutrient stock (D), nutrient conditioning (E), nutrient output (F), membrane module (CTMR), monitor for pH, O_2 and temperature (M), O_2 provision (O_2), manometers (P), pressure regulation (R), effluent pumps (Fe and Qe), nutrient pumps (Fn and Qn), glass-beads column (GBC), sand filters (SF1 and SF2). Courtesy of Ludo Diels, Vito.

The unique separation of waste and nutrient flow in the BICMER enables a good supply and therefore high activity of the bacteria, which is beneficial especially for the cometabolic degradation of certain organic molecules (Diels *et al.,* 2000b). The formation of crystals larger than the bacteria results in biosludge with a very high metal to biomass ratio.

C. ARI-Chromate Reduction Process (India)

Chromate compounds are used on a large scale in the cooling towers of fertilizer and other heavy industries, and also in electroplating in India, owing to their excellent corrosion-inhibiting and biocidal properties. Consequently, effluents discharged from such industries contain considerable concentrations of toxic chromate (Cr^{6+}). Agharkar Research Institute (ARI), Pune, India, developed a microbiological process for the treatment of chromate-containing wastewaters. Using a "two-stage selection" procedure a strain of *Pseudomonas mendocina* MCM B-180 was isolated which was able to reduce 2 m*M* chromate (100 mg/liter hexavalent chromium) with an efficiency >99.9% in 24 h (Rajwade and Paknikar, 1997). The strain was resistant to chromium concentrations of up to 1600 mg/liter, was tolerant to chlorides, sulfates, and cations such as iron, lead, cadmium, zinc, copper as well as biocides, viz. Quat-2-C, and methylene-bis-thiocyanate. The process, when carried out in a 20-liter continuously stirred tank reactor removed 25–100 mg chromate/l in 4.4–8 h with >99.9% efficiency in the presence of sugarcane molasses as nutrient (Bhide *et al.,* 1996). The process is able to sustain wide variations in pH (6.5–9.5) and temperature (25–40°C). Strict anaerobiosis is not required. However, the process works best under oxygen-limiting conditions (dissolved oxygen levels <2 mg/liter). The process was licensed to Global Environmental Technologies Ltd., Pune, India, for commercialization (Paknikar and Bhide, 1995; Paknikar *et al.,* 1996). A pilot plant capable of treating 40,000 liters of cooling tower effluent per hour (containing 25 mg chromium/liter) was set up at a fertilizer manufacturing industry.

The ARI process can be explained by a simple block diagram represented in Fig. 7. The chromate-bearing effluent after pH adjustment (if necessary) and nutrient supplementation enters the bioreactor containing the active chromate-reducing culture, *P. mendocina* MCM B-180. After reduction, the chromium-free effluent enters the settler. The chromic hydroxide sludge is then separated and dried. It can either be used as a land fill or can be incinerated. The water can be reused. Alternatively, the culture can be grown aerobically in a separate bioreactor in the absence of chromate which is subsequently mixed with the

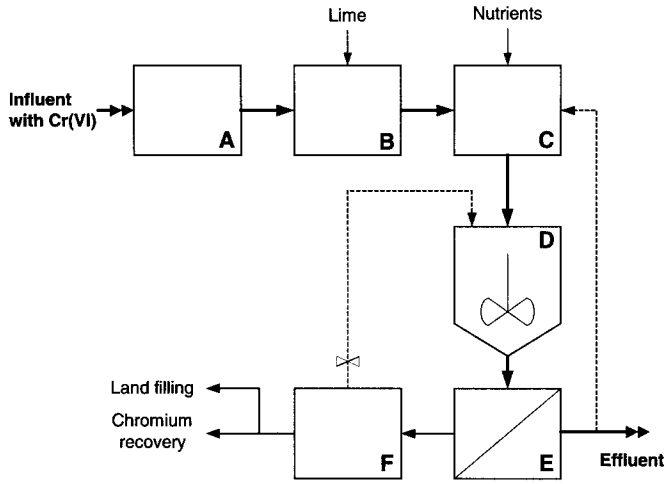

FIG. 7. ARI-chromate reduction process. Equalization tank (A), neutralization tank (B), hold tank (C), bioreactor (D), settler (E), sludge conditioning (F).

chromate-bearing effluent in another bioreactor where reduction occurs under oxygen-limiting conditions.

The process developed had several distinct advantages:

No chemical additives or aeration required
No pH adjustments for effluents generated from cooling towers required
Produced low volumes of sludge and it contained 50% metallic chromium
Easy to operate and maintain
Unaffected by the presence of contaminating metal ions and commonly used biocides
Low capital and operating costs (at least eight times less expensive than the conventional treatment methods)

With some modifications, the process can be used for bioremediation of chromate-contaminated soils (Salunkhe *et al.*, 1998).

D. ARI Process for Biodetoxification of Metal Cyanides (India)

A bacterial consortium capable of utilizing metal cyanides as a source of nitrogen was used to develop a microbiological process for the detoxification of metal cyanides, viz. copper cyanide, zinc cyanide, nickel cyanide, and silver cyanide. The treatment was carried out in 27-liter rotating biological contactors (RBCs) in continuous mode. A typical flow

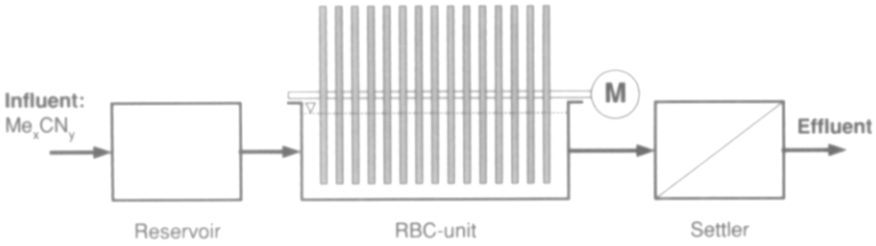

FIG. 8. Biodetoxification of metal–cyanide in a rotating biological contactor (RBC).

sheet of the process is shown in Fig. 8. The system was able to achieve >99.9% removal of metal cyanide in 10–15 h from effluents containing 5–52 mg/liter cyanide and 1–40 mg/liter metals. Sugarcane molasses was used as the source of carbon at a concentration of 0.1–0.6 ml/liter. The cyanide component of the metal–cyanide complex was completely mineralized during the process while the metals precipitated as metal hydroxides, with the exception of silver. The silver ions set free during biodegradation of silver–cyanide were efficiently adsorbed by the bacterial biomass. The RBC-treated effluents were found to be completely safe for discharge into the environment, as confirmed by chemical analysis and fish toxicity studies (Patil and Paknikar, 2000a,b). The process is ready for commercialization (Paknikar and Patil, 1998).

E. DEVELOPMENTS WITH SULFATE REDUCING BACTERIA (INTERNATIONAL)

In addition to already marketed technologies described in detail (THIOPAQ, METEX, Bio-Substrat; Sections III.B–D) the well-known potential of sulfate reducing bacteria (SRB) in the treatment of acidic, metal-containing wastewaters has led to the development of various further process designs, tested up to pilot scale. Most of them aim at the bioremediation of acid mine or rock drainage (AMD, ARD), but waste streams from other industrial processes, as, for example, from paper mills or nonferrous industry, as well as contaminated groundwaters, are also under investigation.White *et al.* (1998) also tested SRB technology in an integrated microbial process to precipitate heavy metals from acidic soil leachate, generated by the action of sulfur-oxidizing bacteria.

Reactor Designs

Because of the slow propagation of SRB, the desired biomass concentration as well as low hydraulic retention times can be achieved only with immobilized-growth bioreactors. A positive side effect of biofilms is the improvement of water clarification, as they retain fine metal

sulfide precipitates by adsorption and entrapment (White and Gadd, 1998a). Various organic and inorganic substrates have been shown to support SRB-biofilm development, such as straw (Bechard *et al.*, 1993; Estrada Rendon *et al.*, 1999), spent, acid-leached mushroom compost (Dvorak *et al.*, 1991; Hammack and Edenborn, 1992), sand (Buisman and Dijkman, 2000), zeolite (Section III.D), and pelletized ash (Du Preez and Maree, 1994). Another successful approach is the use of the carrier-free sludge blanket principle, realized in the THIOPAQ (Section III.B) and METEX (Section III.C) UASB-reactors.

Nutrition

Concerning use of energy source and substrate, SRB are a very heterogeneous group of strictly anaerobic microorganisms. Molecular hydrogen, short- and long-chain fatty acids, alcohols, and aromatic hydrocarbons may supply electrons; and these organic compounds, in addition to sugars and amino acids, as well as inorganic compounds (CO, CO_2) may provide the carbon (Schlegel, 1992). The final selection of the growth sources depends on the composition of the water to be treated (organic ingredients, e.g., short-chain acids, alcohols) and on availability of organic wastes (e.g., dairy waste products, molasses). For large SRB systems molecular hydrogen and carbon monoxide/dioxide, produced from methanol in a partial oxidation burner (Maree and Gerber, 1999; Rowley *et al.*, 1996) or from natural gas in a reformer (van Lier *et al.*, 1999), are more economic.

Process Designs

The various techniques developed may be arranged in (a) systems with the whole wastewater passing through the bioreactor, and (b) systems with a bypass-bioreactor for the production of sulfide, decoupled from the chemical metal precipitation stage. The world's first full-scale plant using SRB technology (Scheeren *et al.*, 1992; Section III.B) belongs to the first category, but Paques also tested the separated production and use of biosulfide in pilot plants for mine water treatment (Boonstra *et al.*, 1999). NTBC Research Cooperation's "Biosulphide-Process" (NTBC, 2000; Rowley, 2000, pers. comm.) and PNNL/Battelle's "Biogenic Sulfide Precipitation" (Battelle, 2001) both rely on this biological side-stream production of sulfide. In cases of moderate contamination with sulfur and metals, and with no need to degrade organic compounds (e.g., AMD-treatment), the following advantages claimed by NTBC (2000) may apply for this approach: "(1) The entire flow of water for treatment (which is often huge) is not passing through the slowest stage of the process (the bioreactors), (2) reactions in the two stages can proceed at their optimal (and different) rates, (3) the bacterial

population is not exposed to inhibitive or toxic levels of dissolved metals, and (4) a greater degree of control is possible over the extent of reactions in the two stages." Further, some metals with differing metal–sulfide stabilities can be selectively precipitated in subsequent steps (reactors) at different pH values (e.g., Cu, Zn, and Fe at pH 1.6, 3.8, and 6.2, respectively; du Preez and Maree, 1994). Nevertheless, more complex water matrices with biodegradable ingredients have stimulated the development of highly sophisticated, multireactor process designs, for example, including steps for denitrification (du Preez and Maree, 1994; Hunter and Stuart, 1999) or degradation of xenobiotic carbohydrates (Eccles, 1998; Hunter and Stuart, 1999).

V. Conclusion

There is an increased awareness of the potential dangers of environmental pollution by heavy metal compounds. With the stricter statutory limits imposed by international agencies, the conventional physical–chemical methods for the treatment of metal-containing wastewaters are proving to be expensive and also inadequate to meet the required standards. This technoeconomic impasse has led to an intensive search for new technologies. It is evident from the description of the various full-scale, pilot-scale, and expanded-laboratory-scale processes presented in this review that new and exciting prospects exist for the treatment of metal-containing wastewaters using living microorganisms. However, the practical utilization of these technologies largely depends on their cost-effectiveness as compared to existing technologies. It is clear that some of the technologies described here are competitive with existing treatments. However, the challenge extends further to the development of even cheaper technologies because in most countries, especially in the developing world, wastewater treatment has to be a low-cost proposition for its wider acceptance. Further work by scientists and technologists is needed in order to realize this goal. Finally, even if low-cost technologies are developed, the people required to conceive, structure, operate, and control these processes will have to be motivated to take on the job and build up experience with it. It is therefore essential that in the near future wastewater treatment and environmental protection become attractive business opportunities rather than statutory requirements.

ACKNOWLEDGEMENTS

For valuable input and constructive remarks on specific chapters TP would like to thank (in alphabetical order): Dr. Judith Bender (Microbial Aquatic Treatment Systems, USA), Dr. Johannes Boonstra and Dr. Guido Janssen (Paques Biosystems, The Netherlands), Dr. Ludo

Diels (Vlaamse Instelling voor technologisch Onderzoek, Belgium), Dr. Peter Fürst (Dr. Fürst Systems, Germany), Dr. Jannie Maree (CSIR, South Africa), Dr. Manfred Morper (Linde Anlagenbau, Germany), Mr. John Scheetz (Homestake Mining Company, USA) and Dr. Irene Wagner-Döbler (Gesellschaft für biotechnologische Forschung, Germany). KMP is thankful to the Director, Agharkar Research Institute, Pune, for continuous support of the research program. Both authors also thank their diploma and doctoral students and colleagues for their valuable contributions in the development of technologies described in this article.

References

Assen, H. (1995). Patent EP730895B1.

Bagla, P., and Kaiser, J. (1996). *Science* **274,** 174–175.

Barnes, L. J., Scheeren, P. J. M., and Buisman, C. J. N. (1994). *In* "Emerging Technology for Bioremediation of Metals" (J. L. Means and R. E. Hinchee, eds.), pp. 38–49. Lewis Publishers, Boca Raton, FL.

Battelle (2001). Biogenic Sulfide Precipitation, available at http://bioprocess.pnl.gov/sulfide.htm.

Bazylinski, D. A., Garratt-Reed, A. J., Abedi, A., and Frankel, R. B. (1993). *Arch. Microbiol.* **160,** 35–42.

Bechard, G., Rajan, S., and Gould, W. D. (1993). *In* "Biohydrometallurgical Technologies Vol II: Fossil Energy Materials, Bioremediation, Microbial Physiology, Proc. Int. Biohydrometallurgy Symp. IBS-93, Wyoming," pp. 277–286. The Minerals, Metals & Materials Society, Warrendale, PA.

Bender, J. (2000). Judith Bender, Microbial Aquatic Systems, Personal Communication.

Bender, J., Duff, M. C., Phillips, P., and Hill, M. (2000). *Environ. Sci. Technol.* **34,** 3235–3241.

Bender, J., Lee, R. F., and Phillips, P. (1995). *J. Ind. Microbiol.* **14,** 113–118.

Bender, J. A., and Phillips, P. C. (2000). Patent US6033559.

Beveridge, T. J. (1989). *In* "Metal-Microbe Interactions" (R. K. Poole and G. M. Gadd, eds.), Vol. 26, pp. 65–83. Special Publications of the Society for General Microbiology, IRL Press, Oxford.

Bhide, J. V., Dhakephalkar, P. K., and Paknikar, K. M. (1996). *Biotech Lett.* **18,** 667–672.

Biosubstrat (1999). Pat. AT405928B.

Blakemore, R. P., and Frankel, R. B. (1989). *In* "Metal-Microbe Interactions" (R. K. Poole and G. M. Gadd, eds.), Vol. 26, pp. 85–98. Special Publications of the Society for General Microbiology, IRL Press, Oxford.

Boonstra, J. (2001). Johannes Boonstra, Paques Bio Systems, The Netherlands, Personal Communication.

Boonstra, J. (2000). "The world's first gypsum-free zinc refinery." Paques Brochure.

Boonstra, J., van Lier, R., Janssen, G., Dijkman, H., and Buisman, C. J. N. (1999). *In* "Biohydrometallurgy and the Environment toward the Mining of the 21st Century" (R. Amils and A. Ballester, eds.), Part B, pp. 559–567. Elsevier, Amsterdam.

Buisman, C. J. (1994). Patent EP477338B1.

Buisman, C. J. N., and Dijkman, H. (2000). Pat. WO00/39035.

Buisman, C. J. N., Geraats, B. G., Ijspeert, P., and Lettinga, G. (1989). *Biotechnol. Bioeng.* **35,** 50–56.

Campos, J., Martinezpacheco, M., and Cervantes, C. (1995). *Ant. Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **68,** 203–208.

De Vegt, A., Dijkman, H., and Buisman, C. J. (1998). *In* "Sulfide Smelting '98 Current and Future Practices" (L. A. Asteljoki and R. L. Stephens, eds.), TMS, Warrendale, PA.

Dhar, R. K., Biswas, B. K., Samanta, G., Mandal, B. K., Chakrabortim, D., Roy, S., Jafar, A., Islam, A., Ara, G., Kabir, S., Khan, A. W., Ahmed, S. A., and Hadi, S. (1997). *Curr Sci.* **73,** 48–59.

Diels, L. (2000). Ludo Diels, Vlaamse instelling voor technologisch onderzoek, Belgium, Personal Communication.

Diels, L., Leysen, R., Van Roy, S., Doyen, W., and Mergeay, M. (2000a). Pat. EP579630B1.

Diels, L., Dong, Q., van der Lelie, D., Baeyens, W., and Mergeay, M. (1995b). *J. Ind. Microbiol.* **14,** 142–153.

Diels, L., Spaans, P. H., Van Roy, S., Hooyberghs, L., Wouters, H., Walter, E., Winter, J., Macaskie, L. E., Finlay, J., Pernfusse, B., and Pümpel, T. (1999). *In* "Biohydrometallurgy and the Environment toward the Mining of the 21st Century" (R. Amils and A. Ballester, eds.), Part B, pp. 607–616. Elsevier, Amsterdam.

Diels, L., Van Roy, S., Doyen, W., Mergeay, M., and Leysen, R. (1995a). *In* "Biohydrometallurgical Processing. Proc. Int. Biohydrometallurgy Symp. IBS-95, November 1995, Chile," Vol. II, pp. 201–209. University of Chile, Santiago.

Diels, L., Van Roy, S., Peys, K., Schleifenheimer, Schumann, T., M., Nitsche, K., Eckhold, K. H., and Frense, D. (2000b). *In* "Contaminated Soil 2000. Proc. 7th Int. FZK/TNO Conf. on Contaminated Soil, 18–22 September 2000, Leipzig, Germany," Vol. 2, pp. 1152–1154. Thomas Telford Publishing, London.

Diels, L., Van Roy, S., Spaans, P. H., Wouters, H., and Kramer, A. (1998). Pat. EP952120A1.

Diels, L., Van Roy, S., Taghavi, S., Doyen, W., Leysen, R., and Mergeay, M. (1993). *In* "Biohydrometallurgical Technologies Vol II: Fossil Energy Materials, Bioremediation, Microbial Physiology. Proc. Int. Biohydrometallurgy Symp. IBS-93, Wyoming." pp. 133–144. The Minerals, Metals & Materials Society, Warrendale, PA.

Dong, Q., and Mergeay, M. (1994). *Mol. Microbiol.* **14,** 185–187.

Du Preez, L. A., and Maree, J. P. (1994). *Water Sci. Technol.* **30,** 275–285.

Dvorak, D. H., Hedin, R. S., Edenborn, H. M., and McIntyre, P. E. (1991). *Biotechnol. Bioeng.* **40,** 609–616.

Eccles, H. (1998). Pat. WO98/46311.

Estrada Rendon, C. M., Amara, G., Leonard, P., Tobin, J., Roussy, J., and Degorce-Dumas, J. R. (1999). *In* "Biohydrometallurgy and the Environment toward the Mining of the 21st Century" (R. Amils and A. Ballester, eds.), Part B, pp. 577–585. Elsevier, Amsterdam.

Fortin, D., Goulet, R., and Roy, M. (2000). *Geomicrobiol. J.* **17,** 221–235.

Fürst, P. (2001). Peter Fürst, Dr. Fürst Systems, Germany, Personal Communication.

Fürst, P., and Burggräf, H. (2000). "Das Bio-Substrat Verfahren." Firmenbericht Dr. Fürst Systems.

Fürst, P., and Morper, M. (1993). "Das Linde METEX-Verfahren zur biologischen Schwermetallelimination und Abtrennung von Kohlenwasserstoffen aus industriellen Abwässern." Linde-Firmenschrift,

Gadd, G. M. (2000). *Curr. Opinion Biotechnol.* **11,** 271–279.

Gatliff, E. G. (1998). Pat. US5829191.

Gharieb, M. M., Kierans, M., and Gadd, G. M. (1999). *Mycol. Res.* **103,** 299–305.

Grupa, U., Kunz, B., and Bauer, W. (1992). *Bioengineering* **3/92,** 34–37.

Hammack, R. W., and Edenborn, H. M. (1992). *Appl. Microbiol. Biotechnol.* **37,** 674–678.

Homestake Mining Company (1999). "A History of Whitewood Creek, S. D.," available at http://www.homestake.com/health_safety/health_safety_sublevels/reports/other_reports/chron.htm.

Hunter, R. M., and Stewart, F. M. (1999). Pat. US5922204.

Janssen, G., and Boonstra, J. (2000). Guido Janssen and Johannes Boonstra, Paques Bio Systems, The Netherlands, Personal Communication.

Kalin, M., Fyson, A., and Smith, M. P. (1993). *In* "Biohydrometallurgical Technologies Vol II: Fossil Energy Materials, Bioremediation, Microbial Physiology. Proc. Int. Biohydrometallurgy Symp. IBS-93, Wyoming," pp. 319–328. The Minerals, Metals & Materials Society, Warrendale, PA.

Kamnev, A. A., and van der Lelie, D. (2000). *Biosci. Rep.* **20,** 239–258.

Klaus, T., Joerger, R., Olsson, E., and Granqvist, C. G. (1999). *Proc. Natl. Acad. Sci.* **96,** 13611–13614.

Koren, D. W., Gould, W. D., and Lortie, L. (1992). *In "Conf. Paper: Waste Proc. Recycl. Mining Metallurgical Industries,* 23–27 Aug. 1992," pp. 171–182. Canadian Institute of Mining, Metallurgy and Petroleum, Edmonton, Alberta, Canada.

Kramer, J. P., and Wouters, J. W. (1993). *J. Water SRT-Aqua* **42,** 97–104.

Kratochvil, D., and Volesky, B. (1998). *Trends Biotechnol.* **16,** 291–300.

Lovley, D. R., and Coates, J. D. (1997). *Curr. Opinion Biotechnol.* **8,** 285–289.

Macaskie, L. E. (1990). *J. Chem. Technol. Biotechnol.* **49,** 357–379.

Macaskie, L. E. (2001). Lynne E. Macaskie, University of Birmingham, School of Biological Sciences, United Kingdom, Personal Communication.

Maree, J. P., and Gerber, A. (1999). Pat. WO99/06328.

Mittlinger, C. (1998). Diploma Thesis, University of Innsbruck, Austria.

Moorer, W. R., Ten Cate, J. M., and Buijs, J. F. (1993). *J. Dental Res.* **72,** 1021–1026.

Morper, M. (1985). *Linde Rep. Sci. Technol.* **40,** 43–47.

Morper, M. (1986). *In* "Immobilization of Ions by Biosorption" (H. Eccles and S. Hunt, eds.), pp. 91–104. Ellis Horwood Publishers, London.

Morper, M. (1997). *Linde Berichte Technik Wissenschaft* **76,** 1–11.

Morper, M. (1999). *Chem. Eng.* **8/1999,** Cover Story.

Morper, M. (2000). Manfred Morper, Linde Anlagenbau, Germany, Personal Communication.

Morper, M., and Frydman, A. (1987). Pat. EP110240.

Morper, M., and Fürst, P. (1991). *Linde Rep. Sci. Technol.* **49,** 40–42.

Mudder, T. I., and Whitlock, J. L. (1984). Pat. US4440644.

Mühlbacher, R. (1994). Diploma Thesis, University of Graz, Austria.

Noname (1997). Bugs with a bite. Design News Online. November 3, 1997, available at http://www.manufacturing.net/magazine/dn/archives/1997/dn1103.97/21 news. htm.

NTBC (2000). "Sulphate reducing bacteria and their practical application in a water treatment system," available at http://www.direct.ca/ntbc/srb.htm.

Paknikar, K. M., and Bhide, J. V. (1995). Ind. Pat. Appl. 472/BOM/95.

Paknikar, K. M., Bhide, J. V., Jalgaonkar, A. D., Bhattacharjya, S. K., Dhakephalkar, P. K., Bapat, A. N., Chatterjee, D., and Pendse, S. V. (1996). Ind. Pat. Appl. 24/BOM/96.

Patil, Y. B., and Paknikar, K. M. (2000a). *Proc. Biochem.* **35,** 1139–1151.

Patil, Y. B., and Paknikar, K. M. (2000b). *Lett. Appl. Microbiol.* **30,** 33–37.

Paknikar, K. M., and Patil, Y. B. (1998). Ind. Pat. Appl. 123/BOM/98.

Paknikar, K. M., Rajwade, J. M., and Puranik, P. R. (1999). *In* "Biohydrometallurgy and the Environment toward the Mining of the 21st Century" (R. Amils and A. Ballester, eds.), Part B, pp. 725–730. Elsevier, Amsterdam.

Pernfuss, B. (1996). PhD-Thesis, University of Innsbruck, Austria.

Pernfuss, B., Ebner, C., Pümpel, T., Diels, L., Macaskie, L. E., Tsezos, M., Keszthelyi, Z., and Glombitza, F. (1999). *In* "Biohydrometallurgy and the Environment toward the Mining of the 21st Century" (R. Amils and A. Ballester, eds.), Part B, pp. 373–382. Elsevier, Amsterdam.

Peters, R. W., Ku, Y., and Bhattacharyya, D. (1985). *AIChe Symp. Ser.* ***81,*** 165–203.

Project 1 (1996–1999). European Union, Grant **BE95-1610.**

Project 2 (1997–1999). European Union, Grant **LIFE97 ENV/D/000463.**
Project 3 (1998–2000). European Union, Grant **BIO4-CT-98-0168.**
Project 4 (1990). European Union, Grant **BREU0084/C/SMA.**
Project 5 (1992–1994). European Union, Grant **BE92-5350.**
Pümpel, T., Ebner, C., Pernfuss, B., Schinner, F., Diels, L., Keszthelyi, Z., Stankovic, A., Macaskie, L., Tsezos, M., and Wouters, H. (2001). *Hydrometallurgy* **59,** 383–393.
Rajwade, J. M., and Paknikar, K. M. (1997). *In* "Proc. Int. Biohydrometallurgy Symp.-IBS'97," pp. E-ROM4.1-E-ROM4.10. Australian Mineral Foundation, Glenside, Australia.
Raskin, I., Dushenkov, S., Kapulnik, Y., and Kumar, N. P. B. A. (1999). Pat. US5876484.
Reidl, H., Janssen, G., Buisman, C., Schouten, G., Prins, R., and Kuuper, R. (2000). *Chem. Anlag. Verfahr. (CIT plus)* **6,** 42–43.
Remacle, J. ((1988).). *In* "Proc. 8th Int. Symp. Biotechnol.," pp. 1187–1197. Societe Francaise de Microbiologie, Paris.
Rowley, M. (2000). Michael Rowley, NTBC Research Cooperation, Personal Communication.
Rowley, M., Warkentin, D. D., and Piroshko, B. M. (1996). Pat. US5587079.
Sakurai, I., Kawamura, Y., Hoike, H., and Inoue, Y. (1990). *Appl. Environ. Microbiol.* **56,** 2580–2583.
Salt, D. E., Smith, R. D., and Raskin, I. (1998). *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **49,** 643–668.
Salunkhe, P. B., Dhakephalkar, P. K., and Paknikar, K. M. (1998). *Biotech. Lett.* **20,** 749–751.
Sayer, J. A., and Gadd, G. M. (1997). *Mycol. Res.* **101,** 653–661.
Scheeren, P. J. H., Koch, R. O., Buisman, C. J. N., Barnes, L. J., and Versteegh, J. H. (1992). *Trans. Inst. Min. Metal. Sect. C* **101,** 190–199.
Scheetz, J. (2000). John Scheetz, Homestake Mining Company, Personal Communication.
Schlegel, H. G. (1992). "Allgemeine Mikrobiologie." Thieme Verlag, Stuttgart.
Silver, S., Misra, T. K., and Laddaga, R. A. (1989). *In* "Metal Ions and Bacteria" (T. J. Beveridge and R. J. Doyle, eds.), pp. 121–139. Wiley, New York.
Singleton, I., Wainwright, M., and Edyvean, R. G. J. (1990). *Biorecovery* **1,** 271–289.
Sterritt, R. M., and Lester, J. N. (1979). *Minerals Environ.* **1,** 45–47.
Thompson, L. C. (1996). *In* "Managing Environmental Problems at Inactive and Abandoned Metals Mine Sites," pp. 30–53. EPA-Seminar Publication 625/R-95/007.
Tsezos, M. (2001). *Hydrometallurgy* **59,** 241–243.
UNEP and UNCHS (1999). "Balkans Task Force (BTF) Final Report. The Kosovo conflict: Consequences for the environment & human settlements," available at http://www.grid.unep.ch/btf/final/.
van Lier, R. J. M., Buisman, C. J. N., and Piret, N. L. (1999). *Global Symp. Recycl. Waste Treat. Clean Technol. REWAS'99,* 5–9 September, San Sebastian, Chile.
Vito (2001). "BICMER," available at http://www.vito.be/milieu/milieutechnologie1b.htm.
Volesky, B. (2001). *Hydrometallurgy* **59,** 203–216.
Von Canstein, H., Li, Y., Timmis, K. N., Deckwer, W. D., and Wagner-Döbler, I. (1999). *Appl. Environ. Microbiol.* **65,** 5279.
Wagner-Döbler, I. (2001). Irene Wagner-Döbler, Gesellschaft für Biotechnologische Forschung, Germany, Personal Communication.
Wagner-Döbler, I., von Canstein, H., and Li, Y. (2000a). *BioWorld* **1/00,** 2–8.
Wagner-Döbler, I., von Canstein, H., Li, Y., Timmis, K. N., and Deckwer, W. D. (2000b). *Environ. Sci. Technol.* **34,** 4628–4634.
Wainwright, M., and Grayston, S. J. (1989). *In* "Metal-Microbe Interactions" (R. K. Poole and G. M. Gadd, eds.), Volume 26, pp. 119–130. Special Publications of the Society for General Microbiology, IRL Press, Oxford.

White, C., and Gadd, G. M. (1998a). *Microbiology* **144,** 1407–1415.
White, C., and Gadd, G. M. (1998b). *In* "Extremophiles: Microbial life in Extreme Environments" (K. Horikoshi and W. D. Grant, eds.), pp. 233–254. Wiley, New York.
White, C., Sayer, J. A., and Gadd, G. M. (1997). *FEMS Microbiol. Rev.* **20,** 503–516.
White, C., Sharman, A. K., and Gadd, G. M. (1998). *Nature Biotechnol.* **16,** 572–575.
Whitlock, J. L. (1987). *In* "SME-AIME Annual Meeting, 24–27 February 1986," Preprint **87-36**, Society of Mining Engineers, Denver, CO.
Whitlock, J. (1989). *JOM J. Miner. Metals Mater. Soc.* **12/1989,** 46–47.
Whitlock, J. (1990). *Geomicrobiol. J.* **8,** 241–249.
Whitlock, J. L. (1992). Pat. US5169532.
Whitlock, J. L., and Mudder, T. I. (1986). *In* "Fundamental and Applied Biohydrometallurgy; Proc. 6th Int. Symp. Biohydrometallurgy, Vancouver 1985," pp. 327–339. Elsevier, Amsterdam.
Whitlock, J. L., and Smith, G. R. (1989). *In* "Biohydrometallurgy; Proc. 8th Int. Symp. Jackson Hole, Wyoming, 13–18 August 1989," pp. 613–625. CANMET.
Wildeman, T. R., Updegraff, D. M., Reynolds, J. S., and Bolis, J. L. (1994). *In* "Emerging Technology for Bioremediation of Metals" (J. L. Means and R. E. Hinchee, eds.), pp. 13–25. Lewis Publishers, Boca Raton, FL.
Zechendorf, B. (1999). *Trends Biotechnol.* **17,** 219–225.

The Role of Microorganisms in Ecological Risk Assessment of Hydrophobic Organic Contaminants in Soils

C. J. A. MacLeod, A. W. J. Morriss, and Kirk T. Semple

Department of Environmental Science
Institute of Environmental and Natural Sciences
Lancaster University, Lancaster, LA1 4YQ, United Kingdom

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 48

0065-2164/01 $35.00

I. Introduction

Soils throughout much of the industrialized world are contaminated with various hydrocarbon contaminants, which generally reach the soil from anthropogenic sources. Major sources of hydrocarbon contaminants found in the environment include petroleum, gas production, and timber treatment industries. Soil contamination originating from the petroleum industry occurs mainly through disposal of industrial wastes and accidental spillage. Petroleum hydrocarbon products consist of blends of distillate fractions from the processing of crude oil and include aliphatic, aromatic, and ashphaltic compounds (Table I). Petroleum products can contain a range of aromatic (benzene, dichlorobenzene, toluene, xylene, phenol, and PAHs) and aliphatic (dibromoethane, dichlorethane, heptane, hexane, isopentane, isobutane, tetrachloroethylene and trichloroethylene) priority pollutants (Calabrese *et al.*, 1988).

Coal tar is a dense, viscous nonaqueous phase liquid (NAPL) which is a major by-product of the coal gasification process, containing up to 75% PAHs by mass (Peters and Luthy, 1993). Coal–tar production and use over the last hundred years or so has resulted in contamination of the environment. There are approximately 1500 manufactured gas plant (MGP) sites in the United States alone (Hatheway, 1997), and the soils found at these sites usually contain high levels of contaminants

TABLE I

COMPOSITION OF PETROLEUM[a] (FROM ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY BY DONALD CROSBY, COPYRIGHT BY OXFORD UNIVERSITY PRESS, INC. USED BY PERMISSION OF OXFORD UNIVERSITY PRESS, INC.)

Component	Kuwait	Louisiana	Prudhoe bay	No. 2 Fuel	Bunker C oil
Low-boiling point (20–205°C), content (%)	**22.7**	**18.6**	**23.2**	—	—
Paraffins (<C11)	16.2	8.8	12.5	—	—
Naphthenes	4.1	7.7	7.4	—	—
Aromatics (C6–C11)	2.4	2.1	3.2	—	—
High boiling point (>205°C), content (%)	**77.3**	**81.4**	**76.8**	**100**	**100**
Paraffins (C11–C32)	4.7	5.2	5.8	8.1	1.7
Isoparaffin	13.2	14.0	—	22.3	5.0
Naphthenes	16.2	37.7	28.5	31.4	15.2
Aromatics (>C11)	21.9	16.5	25.0	38.2	34.2
1-2 Rings	9.7	10.5	16.9	28.1	8.6
PAH	12.2	6.0	8.1	10.1	25.6
N,S,O compounds (%)	17.9	8.4	2.9	0.0	30
Pentane-insoluble (%)	3.5	0.2	1.2	0.0	14.4

[a] NRC (1985).

TABLE II

PREDOMINANT HYDROPHOBIC ORGANIC CONTAMINANTS IN CREOSOTE (% ON WEIGHT BASIS)

Compound	Mueller *et al.* (1989)	Priddle and MacQuarrie (1994)
Naphthalene	11.05	12.5
Methylnaphthalenes	11.05	5
Dimethylnaphthalenes	6.8	5
Trimethylnaphthalenes	ND[a]	5
Biphenyl	6.8	ND
Acenaphthylene	ND	1
Acenaphthene	3.4	8.5
Fluorene	6.8	5.6
Anthracene	11.05	2.6
Phenanthrene	11.05	13
Fluoranthene	3.4	5.4
Chrysene	1.7	0.8
Pyrene	1.7	4.7
Anthraquinone	0.85	ND
Benzo[a]pyrene	0.85	0.3
Benzo[ghi]perlene	ND	ND
Pentachlorphenol	1.0	ND
Carbazole	0.5	ND
Acridine	0.25	ND

[a] ND—not determined.

including cresols, phenols, aliphatic hydrocarbons, benzenes, and PAHs (Johnston *et al.*, 1993; Berkowitz, 1988). Wood treatment activities, involving the use of creosote and anthracene oil, also represent a major cause of soil contamination. Creosote is a distillate of coal–tar and represents a complex mixture of approximately 200 different compounds, with PAHs constituting about 85% (Table II). Contamination resulting from these industries and their potential for remediation have been reviewed elsewhere (Mueller *et al.*, 1989; Pollard *et al.*, 1994).

The risk assessment of soils containing hydrocarbon contaminants is limited due to uncertainties concerning soil–contaminant interactions (Beck *et al.*, 1995; Siciliano and Roy, 1999). Soil–contaminant interactions affect the fate, transport, and bioavailability of hydrophobic organic contaminants (HOCs) in soil (Alexander, 1995). It has been recognized that biological as well as chemical information from contaminated sites may be important for risk assessment (Gaudet *et al.*, 1995; Suter, 1998). To facilitate risk assessment, a triad approach has been suggested by Chapman (1986), which involves the collection of three sources of information: (i) chemical data on the contaminants present, including an assessment of exposure to relevant ecological receptors, (ii) ecotoxicity measurements through bioassays to cover unknown

contaminants or mixture effects, and (iii) ecological field observations on relevant ecological receptors, assessed as the basis of the risk-assessment procedure.

Soil microbial tests are currently being considered for inclusion in ecological risk assessment (ERA) (Chapman, 1999). The lack of an explicit connection between soil microbial tests and assessment endpoints has been cited as the major reason for their noninclusion (Efroymson and Suter, 1999). Efroymson and Suter (1999) suggested that soil microbial tests have potentially three roles in ERAs: (i) endpoints may be properties of microbial communities, (ii) microbial responses may be used to estimate effects on vegetation, and (iii) used as surrogates in place of higher organisms.

It is intended that this review discusses the application of microbial tests for soils contaminated with hydrocarbon chemicals, in particular HOCs derived from the petroleum industry. Therefore, the aims of this review are to consider soil–HOC interactions from two perspectives: (i) understanding the interactions between pollutants and soil microflora and (ii) the application of relevant microbial methods which may be used to determine the risk associated with chemically contaminated soils.

II. Soil–Contaminant Interactions

Soil–contaminant interactions have been shown to be influenced by a number of factors including the type and concentration of contaminant and co-contaminants (Cerniglia, 1992; Alexander, 1995; Divincenzo and Sparks, 1997), soil properties (Hatzinger and Alexander, 1995; Piatt and Brusseau, 1998), and the intrinsic activity of the soil biota (Carmichael *et al.*, 1997; Cornelissen *et al.*, 1998a; Guthrie and Pfaender, 1998). When soil is contaminated by a chemical, the contaminant may become associated with one or more of the soil phases (solid, liquid, gas, or biota). Distributions of contaminants in soils depends on their chemical and physical properties, such as polarity, solubility, and/or volatility, which influence their sorption to soil components or partitioning into a given soil phase. The main processes, which determine the distribution of the contaminant are (i) volatilization, (ii) solubilization, (iii) diffusion, and (iv) sorption which are summarized in the following.

A. Volatilization

Due to the presence of an air phase, the transfer of contaminants between the water and gas phases is an important component in the unsaturated zone of soils. The vapor pressure (P_L) of a chemical provides a good indication of the extent to which a contaminant will volatilize.

However, volatilization also depends on environmental factors and the solubility of the contaminant in water. The distribution of a contaminant between gas and water phases at equilibrium is described by the Henry's Law Constant H ($Pa \cdot m^3\ mol^{-1}$), which is defined by P/C where P is partial pressure in the gas or air phase (Pc) and C is concentration in water ($mol\ m^{-3}$). Volatilization of contaminants has been reviewed in detail by Brusseau (1994).

B. Solubilization

The solubility of contaminants in water is determined by the interaction of the contaminant with water molecules. The octanol–water partition coefficient (K_{ow}) has proved to be a valuable parameter for quantifying partitioning of organic compounds between water and biota, and water and natural organic matter in soils. Since water is very polar, polar contaminants (e.g., phenols) will solubilize to a greater extent than nonpolar contaminants (e.g., PAHs). Solubilization is an important process because it can influence the amount of contaminant that will exist in the aqueous phase and be readily bioavailable. The miscibility of liquid organic contaminants (such as NAPLs) with water is important in determining the transport and fate of such contaminants. The details of these processes are discussed by Cerniglia (1984), Brusseau (1994), and Bosma *et al.* (1997).

C. Diffusion

The porous nature of soil particles means that diffusion of contaminants influences their distribution in soils. Diffusion is controlled by a concentration gradient and involves a random movement of chemicals. Several possible diffusion processes are involved in the transport of HOCs at the micro scale, including film, pore and matrix diffusion in which the contaminant may move from the bulk liquid phase into micro- and nanopores within the mineral and organic fractions of soils (Pignatello and Xing, 1996).

D. Sorption

Sorption involves the association of contaminants with the solid phase of soil and is considered to be one of the major processes influencing the fate and transport of contaminants in soils (Brusseau, 1994; Pignatello and Xing, 1996; Brusseau, 1997). The properties of a contaminant will determine the mechanism and extent of sorption to soil. Most soil particles carry a negative charge, which allows the retention

of cationic organic contaminants (e.g., atrazine), whereas organic contaminants which are negatively charged (e.g., chlorophenols) will not be retained by the same mechanisms. Organic contaminants which have low polarity and as a consequence low solubilities in water, tend to become associated with the soil organic matter (SOM). In general, the lower a chemical's water solubility the more it will sorb to soil solids, such as organic matter (Chiou *et al.*, 1979).

Adsorption occurs by several mechanisms, including van der Waals forces, charge–transfer complexation, hydrogen bonding, and hydrophobic interactions (Pignatello and Xing, 1996; Brusseau, 1997). The precise mechanisms controlling sorption/desorption kinetics are unknown. For hydrophobic contaminants in soil, it may be that both proposed diffusion and sorption mechanisms control sorption/desorption kinetics (Pignatello and Xing, 1996).

E. Ageing

As soil–contaminant contact time increases, the easily extractable and bioavailable fractions of the HOC decreases and the more recalcitrant fraction increases (Steinberg *et al.*, 1987; Pignatello, 1990). Figure 1 demonstrates the influence of contact time on the extractability of HOCs from soil. Sorption of HOCs to the soil is widely accepted to control ageing, although, other processes such as entrapment through diffusive processes into soil micropores (Steinberg *et al.*, 1987) or organic matter (Xing and Pignatello, 1997) have also been proposed as mechanisms for chemical association with soils.

Partitioning into SOM has been found to be the dominant process in soils with more than 0.1% SOM (Means *et al.*, 1980; Steinberg

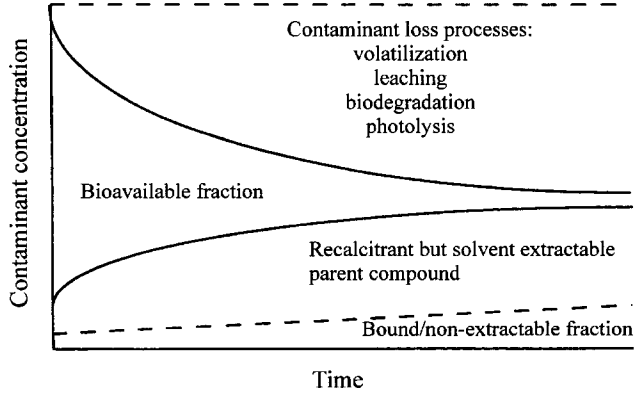

FIG. 1. Temporal changes in the fate of HOCs added to soils.

et al., 1987; Pignatello, 1990). The sorption of HOCs to SOM has been hypothesized to occur in two distinct regions: the expanded (rubbery) region in which linear, rapidly reversible partitioning can occur and the condensed (glassy) region in which nonlinear, slowly reversible sorption can occur (Pignatello, 1990; Weber *et al.*, 1992; Xing and Pignatello, 1997; Cornelissen *et al.*, 1998b). Figure 2 provides a schematic representation of HOC interactions with soil. Regions of expanded SOM are thought to control rapid desorption of HOCs from soils (Cornelissen *et al.*, 1998a). Reliable sorption kinetics are essential for correct risk assessment of hazardous contaminants, and for assessing the feasibility of bioremediation treatments at contaminated sites (Alexander, 1995; Beck *et al.*, 1995; Reid *et al.*, 2000a). Reviews on the processes inherent to ageing are provided by Alexander (1995) and Pignatello and Xing (1996). Further, Reid *et al.* (2000a) reviewed the impact of phase partitioning with respect to both solubilization and sorption, and on the biodegradation of hydrophobic pollutants. Additionally, the implications of kinetic constraints of organic contaminant desorption from soils on soil-quality limits have also been reviewed (Alexander, 1995; Beck *et al.*, 1995).

F. Bound/Solvent Nonextractable Residues

The formation of strong ionic or covalent bonds between HOCs and soil leads to irreversible sorption and the formation of bound or nonextractable residues (Khan, 1982). The formation of these residues in soil from the biodegradation and humification of organic contaminants has been observed (Roberts *et al.*, 1984; Führ, 1987) and has been suggested to be a major sink for organic contaminants, especially pesticides in soil (Bollag and Loll, 1983). A contaminant may be considered "bound" if it is not removed from the soil by a specific solvent extraction, or if a bioassay indicates that it is not bioavailable (Calderbank, 1989). The International Union of Pure and Applied Chemistry (IUPAC) proposed that bound residues were defined as "chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues" (Roberts *et al.*, 1984). Calderbank (1989) suggested that the biological availability of bound residues was more important than just their extent of formation. Two recent workshops have focused on pesticide bound residues (Führ *et al.*, 1998; Jones *et al.*, 2000). There has been a move toward the use of the term "nonextractable residues" which are operationally defined, depending on the extraction used (Northcott and Jones, 2000). Nonextractable residues have been proposed as an endpoint for the risk assessment of HOC-contaminated soils (Linz and Nakles, 1997). Bound or nonextractable residue formation

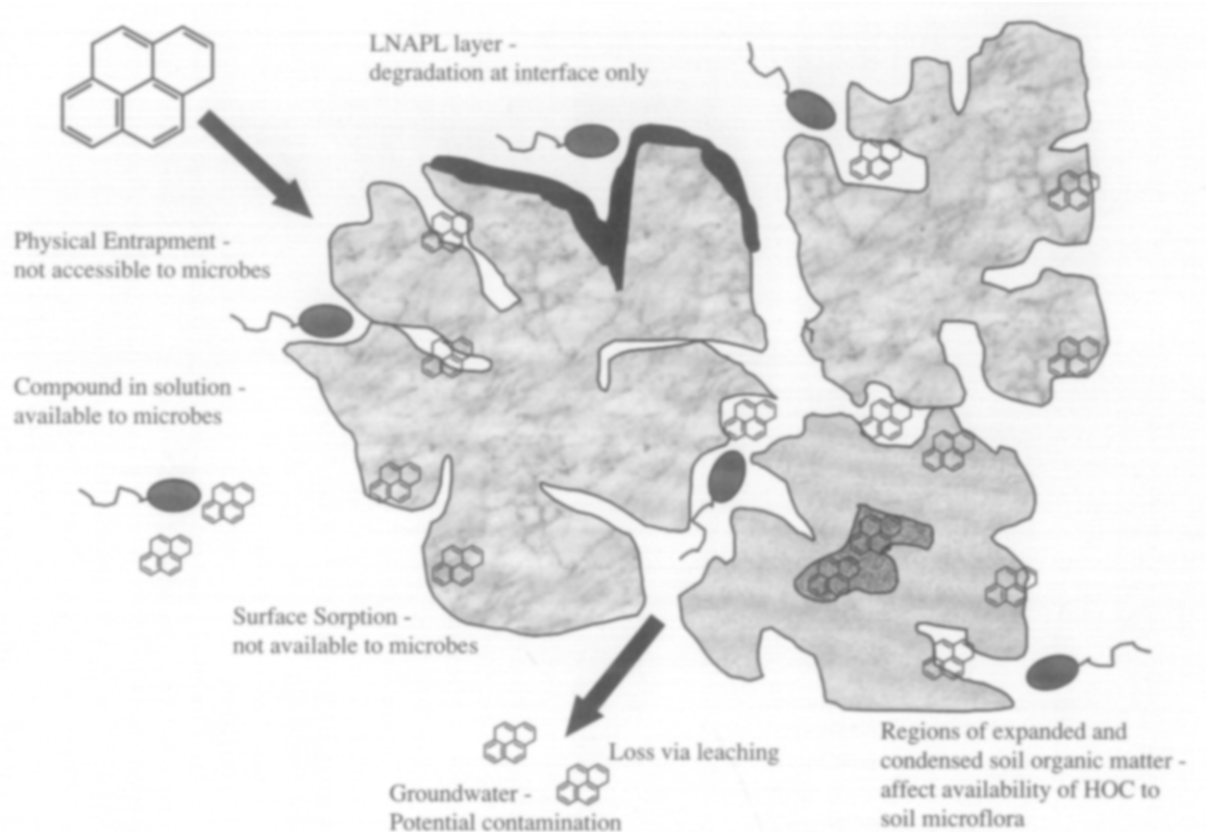

FIG. 2. Schematic representation of the interactions between HOCs and soil.

has been reviewed by Khan (1982), Calderbank (1989), and Gevao *et al.* (2000).

III. Bioavailability of Contaminants to Microbial Communities in Soil

Soil–contaminant interactions affect the bioavailability and hence biodegradation and toxicity, as well as the transport, of HOCs in soils (Alexander, 1995; Beck *et al.*, 1995; Reid *et al.*, 2000a). Slow desorption rates lead to low bioavailability and increased persistence of HOCs in the soil environment (Alexander, 1995). Bioavailability has been defined as the degree to which a compound is free to move into or onto an organism (Hamelink *et al.*, 1994). However, it has been shown that bioavailability differs between organisms and even species. For example, the extent of bioaccumulation by the earthworm (*Eisenia fetida*) and degradation by a *Pseudomonas* sp. of soil-associated phenanthrene were found to be different, though both showed a decrease in bioavailability with increased soil–phenanthrene contact time (Kelsey *et al.*, 1997; White *et al.*, 1997). Further, differences in bioavailability between species of bacteria have been reported (Guerin and Boyd, 1992). The authors found that *Pseudomonas putida* ATCC 17484 broke down 32% of the sorbed naphthalene compared to a Gram-negative isolate (NP-Alk), which only degraded 18% over 225 h. Assessment of the bioavailability of contaminants in soil is essential to understanding the risk posed by the contaminant (Alexander, 1995). However, a more precise definition of bioavailability is needed if it is to be used in the ERA process (Ferguson *et al.*, 1998). What is clear is that the bioavailability of contaminants is ultimately linked to biodegradation and toxicity in soils. These processes are discussed in the following.

A. Degradation of Contaminants by Microbial Communities in Soil

Soil microbial communities have an extensive and diverse enzymatic capacity for catalyzing pollutant transformations. The selective pressures imposed by naturally occurring compounds have created this enzymatic ability (Dagely, 1975). The chemical structure of a contaminant directly and indirectly influences how readily the substrate will be metabolized by microbial communities in soil. Biodegradation of aromatic contaminants by a variety of microorganisms has been widely studied and reviewed (Cerniglia, 1984; Leahy and Colwell, 1990; Cerniglia, 1992, Semple *et al.*, 1999). Microbial degradation and transformation are thought to be the principal processes that result in the removal of HOCs, such as PAHs, from the soil environment (Cerniglia, 1984). In certain cases, for more recalcitrant compounds, a single organism may

lack the capability to accumulate and/or metabolize a particular contaminant, but cometabolism of the recalcitrant compounds may occur in the presence of other readily utilizable substrates. The rate of microbial decomposition of HOCs in soils is a function of a number of factors, either singly or in combination:

(a) The availability of the compounds to the microorganisms that have the catabolic ability to degrade them
(b) The numbers of degrading microorganisms present in the environment
(c) The activity level of degrading microorganisms and/or
(d) The molecular structure of the contaminant

Contaminants with high water solubility will generally be degraded if the microbial community possesses the necessary catabolic ability. However, for contaminants with low solubilities in water, the rate of biodegradation will be lower as the chemicals will tend to associate more readily with the solid phases of soils (Bosma *et al.*, 1997). Microorganisms are able to utilize contaminants in the liquid phase by direct contact of cells with the organic contaminant, or with sub-micron-size droplets dispersed in the aqueous phase (Nakahara *et al.*, 1977). The biodegradation of sorbed contaminants involves two processes: (i) a physical/chemical component involving the movement of the chemical in the physical environment, in relation to the microbial population able to degrade it; and (ii) a biological component involving the metabolism of the chemical (Bosma *et al.*, 1997). For a schematic understanding of how microorganisms may acquire sorbed compounds, refer to Fig. 3. The relative importance of these mechanisms is dependent on how strongly the contaminant is sorbed and the rate of degradation of the contaminant in soil. The rate at which a sorbed HOC becomes available is influenced by the ability of the microorganisms to reduce the aqueous phase concentration and the tendency of the organisms to adhere to the sorbent (Harms and Zehnder, 1994; Calvillo and Alexander, 1996). Increased soil–PAH contact time reduces the magnitude of the rapidly desorbing phase and extent of biodegradation (Alexander, 1995; Hatzinger and Alexander, 1995; Pignatello and Xing, 1996; Cornelissen *et al.*, 1998b). Hatzinger and Alexander (1995) found that the rate of biodegradation of phenanthrene added into sterile soil declined with increasing contact time, and stated that the declining availability of these compounds needs to be taken into account when assessing the toxicity of a contaminated site.

Desorption rates of PAHs freshly added to soil have been found to be much larger than biodegradation rates (Calvillo and Alexander, 1996;

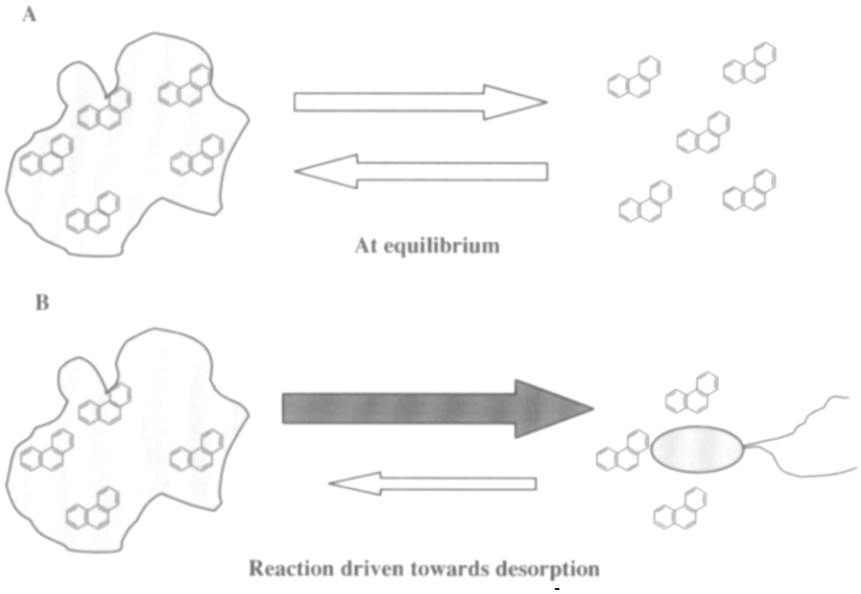

FIG. 3. Desorption behavior of HOCs in soil: (A) degrading microflora are absent and (B) degrading microflora are present.

Carmichael *et al.,* 1997; Cornelissen *et al.,* 1998b). The presence of degrading microorganisms has been shown to alter the desorption rates of contaminants from sorbed surfaces (Guerin and Boyd, 1992; Harms and Zehnder, 1994; Calvillo and Alexander, 1996). This is due to the microorganisms utilizing the contaminants which are readily available through the aqueous phase, leading to the desorption of more contaminant from the solid phase to the aqueous phase (Fig. 4) (Bosma *et al.,* 1997).

The versatility of microorganisms has been reviewed, where it was suggested that the effects of chemical contaminants on the health and diversity of microbial communities were the most important issue (Palleroni, 1995). Hydrocarbon-contaminated soils possess relatively large numbers of microorganisms compared to pristine soils, although their diversity is usually diminished and population shifts have been observed (Bossert *et al.,* 1984; Leahy and Colwell, 1990; Song and Bartha, 1990). Adapted microbial communities can be maintained by repeated contamination. Adaptation of microbial communities often occurs through the induction of enzymes involved in the biodegradation of the contaminant and an increase in the number of degrading organisms (Leahy and Colwell, 1990). Where no previous

exposure to natural or anthropogenic compounds has occurred adaptation requires a genetic change (van der Meer *et al.,* 1992). Initial contamination causes a reduction in microbial numbers, with chemical toxicity assumed to be the cause. The processes of natural attenuation, or adaptation, occur over time as only the bacteria and other soil microflora that can utilize the contaminant as a carbon source remain viable. Adaptation may occur by (i) induction and/or depression of specific enzymes, (ii) genetic variability with altered metabolic capabilities, and (iii) selected enrichment for bacteria with desired metabolic capabilities (Spain and van Veld, 1983; Leahy and Colwell, 1990).

B. Toxicity of Contaminants in Soil

The composition and quantity of aromatic hydrocarbons in petroleum products are important in the evaluation of their toxicity (Mueller *et al.,* 1989; Suter, 1997). The processing of petroleum is also important since the water-soluble fractions of refined oils are more toxic to microorganisms than crude oils (Hodson *et al.,* 1977). The increase in toxicity was suggested to be due to the increase in the aromatic content of the refined oil, particularly naphthalenes and alkylnaphthalenes (Hodson *et al.,* 1977).

There are difficulties in determining the success of remediation since most sites are contaminated with complex mixtures of contaminants, which vary in biodegradability and toxicity. Biodegradation of contaminants can be complete or partial, the latter leading to the formation of intermediate metabolites. Intermediates often have higher solubilities in water and, therefore, potentially increased toxicity, which needs to be monitored. Chemical analysis for metabolic intermediates is not always possible and, if so, is expensive. Therefore, an alternative is the increased use of ecotoxicological methodologies as part of the risk assessment of contaminated sites (Mathes and Winter, 1993). In general, abiotic and biotic transformation products (metabolites) are not included in the standard procedures used to evaluate the ecological risk of a contaminant. Only the loss of the parent compound is used to measure degradation, with the implicit assumption that degradation products do not present any risk to the environment. Since every metabolite may represent a new contaminant with its own associated risk factors such as chemical reactivity toward biological molecules, accumulative potential and persistence, it has been suggested that they are also included in the ERA process (Störmann and Jastorff, 1993).

The combined use of knowledge about general metabolism and chemical reactivity of functional groups, has lead to the elucidation of

metabolic pathways of a wide range of HOCs and the potential inclusion of metabolites in the risk assessment of contaminants (Mathes and Winter, 1993). Metabolites with the highest potential for toxicity can be found using both biochemical and toxicological information. In addition, different compounds with similar chemical structures may produce identical metabolites, leading to a reduction in the work needed to evaluate the toxicity of all metabolites. These processes of initial hazard identification can also be used to direct the next level of risk-assessment procedures (Mathes and Winter, 1993). The role of microorganisms in toxicity assessment will be discussed later.

IV. The Role of Microorganisms in Environmental Risk Assessment

A. Microorganisms as Sensitive Ecological Receptors in Soil

Microorganisms are pivotal to the sustainable health of soils, and are involved in a variety of processes performing functions necessary for overall ecosystem health, ranging from nutrient cycling to the degradation of organic contaminants (Domsch *et al.*, 1983; Bloem *et al.*, 1994; Holden and Firestone, 1997). Since >90% of global biomass and biodiversity is provided by microorganisms (Bloem *et al.*, 1994) and estimates of the diversity of microbial species range from 10^4 to 10^5 per gram of soil (Torsvik *et al.*, 1990), it is important that the activities of soil microorganisms are not disturbed by anthropogenic activities since this can lead to significant changes in the soil ecology (Parmaelee *et al.*, 1993). Microorganisms live in intimate contact with their environment and will therefore react quickly and sensitively to any changes. The sensitivity of a range of microbial test systems is shown in Table III. Impacts on microbial nutrient cycling processes in the soil by chemical stress, e.g., pesticides (Anderson, 1984), PCBs (Dusek and Tesarová, 1996) and pentachlorophenol (Salminen and Sulkava, 1997) have been demonstrated.

The influence of naturally occurring environmental stresses on microorganisms in the soil has been reviewed by Domsch *et al.* (1983). Microorganisms are exposed to changing environmental conditions, leading to stimulation and depression of microbial communities. Since natural fluctuations of microbial activities can be as large as 90% (Domsch *et al.*, 1983), the effects of HOCs on microbial communities may be masked by environmental fluctuations. Brookes (1993) commented that while any disturbance caused by anthropogenic sources may seem insignificant, any added impact on top of natural fluctuations of microbial activity will cause an overall impact on microbial processes (Brookes, 1993).

TABLE III

SENSITIVITY OF MICROBIAL TEST SYSTEMS FOR ECOLOGICAL RISK ASSESSMENT (ADAPTED FROM 'AN ECOLOGICAL CONCEPT FOR THE ASSESSMENT OF SIDE-EFFECTS OF AGROCHEMICALS ON SOIL MICROORGANISMS,' BY K. H. DOMSCH, G. JAGNOW AND T. H. ANDERSON, RESIDUE REVIEWS, VOLUME 86, P. 81, TABLE IV, 1983. COPYRIGHT NOTICE OF SPRINGER-VERLAG)

Sensitivity	Population/function	Organisms/process
High	Population	Actinomycetes
	Population	Nitrifiers
	Population	*Rhizobium*
	Function	Nitrification
	Function	Organic matter degradation
Medium	Population	Algae, bacteria, and fungi
	Function	Ammonification
	Function	Denitrification
	Function	CO_2 production
	Function	O_2 consumption
Low	Population	Azotobacter
	Population	Ammonifiers
	Population	Protein degraders
	Function	N_2 fixation

V. Microbial Tests for Evaluating Contaminant Impacts

Due to their intimate contact with the soil microenvironment, ubiquity and importance to the food web in soil (Brookes, 1993; Doelman *et al.*, 1994) microbes can be good bioindicators of soil contamination. Potential properties of microorganisms as indicators of soil contamination fall into four groups: (i) single organisms tests, (ii) measurements of microbial activities, (iii) populations, and (iv) measuring at the whole community level. However, for microbial tests to be suitable indicators of soil contamination the organisms have to be ecologically relevant (Efroymson and Suter, 1999).

The availability of a chemical to be transported to a receptor and the effect on the receptor are vital to the assessment of the relative risk of contaminants in soils and remediated residues (see Section III). Since, it has been shown that the bioavailability of contaminants decreases with increasing contact with soil (Hatzinger and Alexander, 1995), the use of toxicity tests (bioassays) provides a means to assess the bioavailability and potential impact of contaminants on various ecological receptors. Toxicity tests can be divided into two main categories: (i) those which are carried out on aqueous or soil eluent samples and (ii) those carried out on bulk soil samples. The selection of ecotoxicological tests will, in part, be determined by the exposure pathway. Aqueous and soil eluent toxicity tests are useful for estimating the potential adverse effects

contaminants may have on biological receptor through groundwater and surface water pathways. However, such toxicity tests do not indicate the potential impacts of sorbed or insoluble contaminants on biological receptors, which have direct contact with soil (Rönnpagel *et al.*, 1998).

The establishment of dose–response relationships can be difficult with both *in vitro* and *in vivo* tests, since the dose experienced by the microorganism may not be directly proportional to the concentration of the contaminant in soil, but on the soil–contaminant interactions (Alexander, 1995). Determining the microbial exposure is uncertain due to the assumption that pore water concentrations are equivalent to bioavailability. However, the link between pore water concentration and toxicity is not clear (Van Gestel *et al.*, 1996). Microorganisms are subject to surface contact and to gas intake of contaminants. Recently, it has been shown that microorganisms can utilize sorbed pollutants (Calvillo and Alexander, 1996). An *in vivo* assessment of microbial bioavailability needs to be developed to assess accurately microbial exposure to contaminants (Siciliano and Roy, 1999). However, such an assessment seems unlikely in the near future given the huge diversity of the soil environment and contaminants present within it. Published studies investigating microbial bioavailability/toxicity are generally relevant to only one type of soil or a single or small group of related contaminants. Microbial responses to toxic environmental conditions have been studied using methods for measuring microbial activity, biomass, and structure. A selection of these methodologies will now be considered in the following sections of this review.

VI. *In Vitro*-Based Bioassays

A. Liquid-Phase Bioassays

In vitro-based bioassays, including yeast (Haubenstricker *et al.*, 1990) and bacteria (Silva *et al.*, 1979), have been used in toxicity tests and have resulted in the establishment of toxicity correlations regarding energy metabolism and cell viability (Silva *et al.*, 1979). Bacterial bioluminescence has been used extensively in toxicity testing. For example Boyd *et al.* (1998) investigated the toxicity of chlorobenzenes to a *lux*-marked *Pseudomonas fluorescens* and showed that a higher degree of chlorination and symmetry of the chlorobenzenes resulted in increased toxicity to the *lux*-marked microorganism. Increasing toxicities of the chlorobenzenes were inversely proportional to their solubilities in water (Boyd *et al.*, 1998). Further, Tay *et al.* (1992) applied the Microtox® test to assess the toxicity of sediment with organic and aqueous extracts and in the solid phase. Tests with organic extracts and the solid phase showed

a higher toxicity than aqueous extracts, suggesting that the aqueous extracts contained a lower concentration of contaminants. The solvent extracts provided an estimation of particle-bound contaminants, but the use of solvents may selectively extract contaminants and the toxicity of the solvents may further distort the results (Bundy *et al.*, 1997). For these reasons, the authors used the Microtox® solid-phase test, which assesses the toxicity of bound and free contaminants without the use of solvent extractants. The authors reported a high correlation between PAHs and toxic response, though heavy metals were also present in the sediments (Tay *et al.*, 1992).

The effective use of bacterial bioluminescence is dependent upon certain conditions being met. These conditions include minimized background light, as well as selecting the correct method for measuring bioluminescence. In addition, the parameters affecting light production and microbial viability must be controlled, such as temperature, pH profile, moisture content, nutrient availability, oxygen availability, and bacterial physiology (Lapinskas, 1989; Steinberg *et al.*, 1995). Due to those limitations, liquid-phase bioassays are mainly used for toxicity testing of effluent and groundwaters.

B. Solid-Phase Bioassays

In soils contaminated with HOCs, toxicity and inhibition seem to be due to the presence of the chemicals in the solid and aqueous phases. Soil contact assays are becoming available, but still have the problem of adding an inoculum, which is likely to change the bioavailability of the contaminants (Rönnpagel *et al.*, 1998). For example, the solid phase Microtox® assay was used to assess the toxicity of sediments (Benton *et al.*, 1995). The authors found that the solid phase test was useful for testing toxicities in sediments of known particle size, and for samples collected from the same site. However, the authors observed a tendency for sediments with low actual toxicity to produce results indicating moderate toxicity. The authors suggested that this was due to differences in apparent and real toxicity caused by the increased bioavailability of adsorbed toxicants to bacteria adhered to sediment particles, and the loss of bacteria due to adsorption to fine sediment (Benton *et al.*, 1995). Salizzato *et al.* (1998) used the solid-phase Microtox® assay to determine the toxicity of sediments contaminated with heavy metals, polychlorinated biphenyls (PCBs), and PAHs. They found that the concentrations of PAHs and PCBs in most of the samples were below the sensitivity limits of the Microtox® test and attributed the toxic effects mostly to inorganic contaminants (Salizzato *et al.*, 1998).

Dorn *et al.* (1998) assessed the toxicity of light, medium, and heavy crude oils with the Microtox® solid-phase bioassay in two soil types.

The authors found that the Microtox® test was 1.3 to >77 times more sensitive than the 14 day plant seedling assay (lettuce, corn, wheat, wild oat, and rye), but 1.4 to 14 times less sensitive than the earthworm (*Eisenia foetida*) 14-day lethality assay. The light oil in the silty, low organic carbon soil (0.3% OC) was the most toxic, whereas the heavy oil spiked into the high organic carbon sandy soil (22.7% OC) exhibited the least toxicity. The authors suggested that the observed differences in toxicities were due to the lighter oils being more bioavailable compared to the heavier crude oil (Dorn *et al.*, 1998). The interaction of HOCs with the soil matrix reduces the bioavailability of the HOC to eluent-based bioassays, which has led to the development of several solid-phase assays (Rönnpagel *et al.*, 1998; Salizzato *et al.*, 1998). Solid-phase bioassays indicate the bioavailabilities of sorbed contaminants but the nature of the soil matrix used in these assays needs to be specified due to differing extents of sorption leading to confounding results (Rönnpagel *et al.*, 1998).

VII. *In Situ*-Based Bioassays

A wide range of methods is available to assess the function and structure of microbial communities *in situ*. The advantages of *in situ* assays are that the microbial community is characterized without the need to extract and cultivate which leads to inherent bias, since each extraction method selectively removes certain groups of microorganisms from the soil (Fry, 1982). Since soil is a heterogeneous matrix that determines the organisms present, impacts due to the exposure of HOCs on soil microbial processes need to be carried out in the soil environment. Community level studies in microcosms are a robust method to study the ecological impact of introduced chemicals, and the use of microcosms in ecotoxicology has been reviewed (Sheppard, 1997). Microcosms have the advantage of being able to study complex interactions under more controlled conditions than field studies (Verhoef, 1996; Sheppard, 1997). The impacts of contaminants at the system level in soil microcosms have been found to be a valid method in creating ecologically relevant and economical test systems (Van Voris *et al.*, 1985).

A. Decomposition Bioassays

One of the most important functions of microbial communities in soil is the decomposition of organic matter and the recycling of carbon and nitrogen (Parmaelee *et al.*, 1993). Therefore, measurement of these natural processes can be used to assess the impact of contaminants on the

function of the ecosystem in question. The turnover of organic materials can be followed by the collection of CO_2 and by the use of radiochemicals, enabling the sensitive collection of substrate-specific $^{14}CO_2$. Barnhart and Vestral (1983) followed the incorporation of ^{14}C-labeled acetate into microbial lipids and microbial glucosidase activity, as measures of the impact of a range of environmental contaminants. The ^{14}C-acetate method was more sensitive in detecting the toxicity of organic contaminants, though the glucosidase methods were more sensitive to a wider range of inorganic contaminants (Barnhart and Vestral, 1983). Reid *et al.* (2000b) investigated the impact of cable insulating oil on the microbial utilization of ^{14}C-glucose. Following acute exposure (0 to 21 days soil–oil contact time), oil concentrations up to 1% promoted an increase in the extent of [1-^{14}C]glucose metabolism to $^{14}CO_2$. In contrast, higher concentrations of cable insulating oil (5 and 10%) resulted in a smaller increase in the extent of [1-^{14}C]glucose breakdown to $^{14}CO_2$. Further, chronic exposure (300-day soil–oil contact time) to oil concentrations of 1–10% continued to indicate elevated catabolism (approximately 20%), relative to the control. The authors suggested that the increase in glucose breakdown indicated that cable insulating oil is a readily available carbon source to the carbon-limited soil microflora allowing proliferation of bacteria, which when incubated with [1-^{14}C]glucose, resulted in enhanced catabolism (Reid *et al.*, 2000b). However, while carbon turnover tests may indicate the overall effects of contaminants on the respiration of unstimulated and stimulated microflora, and on the degradation of a particular contaminant, they give no indication of the impact on particular taxonomically different groups or species.

Salminen and Sulkava (1997) investigated the effect of pentachlorophenol (PCP) on soil organisms involved in decomposition, microbial community regulation, and nutrient degradation in a heterogeneously contaminated forest soil. The abundance of microbes, enchytraeids, nematodes, small oribatids, and predatory mites were reduced by addition of PCP. Microbial biomass (ATP content) and fungal biomass (ergosterol content) were lower in the patches with the highest PCP concentration compared to elsewhere in the microcosms. Salminen and Sulkava (1997) suggested that a reduction in the supply of food (microflora) for the small oribatids or direct effects of PCP caused a decrease in their numbers, indicating that the decomposer food web in the microcosm was "bottom-up" controlled. It has been previously suggested that soil decomposer food webs are mainly resource limited (Hairson *et al.*, 1960; Pimm *et al.*, 1991). Salminen and Sulkava (1997) concluded that ecological risk analysis of contaminated soil cannot be done without monitoring responses of soil communities and decomposition processes. *In situ* bioassays indicate the impact of contaminants on soil microbial

processes, but uncertainties remain relating to the processes that should be incorporated into a battery of microbial tests.

B. Changes in Microbial Biomass as a Measure of Contaminant Impact

Methods proposed to measure microbial biomass include fumigation incubation (Jenkinson and Powlson, 1976), fumigation extraction (Vance *et al.,* 1987), substrate-induced respiration (Anderson and Domsch, 1978), and a range of methods for the direct enumeration of active and total microorganisms. Dusêk and Tesarova (1996) studied the impact of PCBs on microbial biomass, soil respiration, and specific respiration rate in a grassland soil polluted with 14.0 ng PCBs˜g^{-1} of soil. In the polluted soil, the microbial biomass decreased by 23% as assessed by chloroform fumigation and the specific respiration was 14% lower than the control soil that contained 4.4 ng PCBs g^{-1} of soil. The authors then combined the field study with a laboratory investigation into the incorporation of carbon (glucose) into the biomass and found a lower extent of incorporation in the polluted soil compared to the control soil (Dusêk and Tesarova, 1996). Further, the authors found that the fumigation extraction methods were less sensitive than substrate-induced respiration in relation to differences in biomass carbon contents between the sites (Dusêk and Tesarova, 1996). More recently, Meharg *et al.* (1998) studied the response of soil microbial biomass to 1,2-dichlorobenzene additions in the presence and absence of plant residues. Addition of 10 and 50 μg 1,2-dichlorobenzene g^{-1} of soil in the absence of plant residues greatly stimulated total culturable bacteria and culturable pseudomonads in a concentration-dependent manner. Despite the increase in numbers of fluorescent pseudomonads, there was no change in the phenotypic diversity as determined by fatty acid methyl ester (FAME) characterization of isolates (Meharg *et al.,* 1998). The response of soil microbial communities to single and multiple additions of 1,2-dichlorobenzene was recently reported using microbial biomass, and estimates of the diversity of culturable bacteria using plate counts and BIOLOG™ (Thompson *et al.,* 1999). After a 22-week exposure, both the additions of 10 doses of 10 $\mu g\ g^{-1}$ and a single dose of 100 $\mu g\ g^{-1}$ resulted in significant reductions in total bacterial counts and viable fungal length. The taxonomic composition of the culturable bacteria was examined by FAME analysis, with the single-dosed soil having a lower abundance of *Arthrobacter* and *Micrcooccus* spp, while the multiple addition treatment reduced the abundance of *Pseudmonas* spp and increased the abundance of *Bacillus* spp (Thompson *et al.,* 1999). The metabolic potential of the microbial communities was assessed by BIOLOG™ profiling and the number of carbon

substrates used by the multiple-dose community (49 positives) was significantly less than that detected in the single-dose treatment (76) and control (66) (Thompson *et al.,* 1999). Finally, as a note of caution, all of the previously described methods for measuring microbial biomass are based on using calibration/correction factors derived from investigations based on adding known amounts of ^{14}C-labeled biomass. When assessing the impact of contaminants on the soil microbial biomass, the use of calibration/correction factors has been criticized (Sparling and West, 1988). These calibration/correction factors are derived from studies on soils not exposed to pesticide contamination (Anderson and Domsch, 1978) and the possibility that the addition of organic pollutants may alter the proportion and/or number of organisms that are assessed by these methods.

C. Metabolic Quotients as a Measure of Contaminant Impact

The use of respiration per unit biomass (as opposed to total respiration) may be a more accurate method to measure impacts on the activity of the microbial soil population (Killham, 1985; Bardgett *et al.,* 1995). Brookes (1993) reviewed the potential of microbiological properties as indicators of soil pollution monitoring, suggesting that a combination of microbial activity and population measurements (e.g., biomass specific respiration) might be more sensitive to soil pollution than either population or activity measurements alone, especially when combined with some form of "internal control," e.g., biomass as a percentage of soil organic matter. Further, Hund and Schenk (1994) found the respiration quotient (QR) calculated from the actual (Ra) and potential (Rp) respiration rates after addition of glucose (QR = Ra/Rp) correlated (0.997) with the PAH concentration of a contaminated site. The authors measured respiration rates by the microbial uptake of oxygen in respirometers over 1–3 days. The potential respiration rate was determined by adding a glucose solution to achieve a concentration of 1% glucose in the soil. Metabolic quotients have been shown to be sensitive indicators of contaminant impact on microbial processes (Killham, 1985; Brookes, 1993).

D. The Nitrogen Cycle as an Important Indicator of Contaminant Impact

Microbial populations involved in the nitrogen cycling present numerous opportunities as potential indicators of the impact of contaminants on soil biota. A complete discussion of the microorganisms involved in the nitrogen cycle is beyond the scope of this review. However,

Siciliano and Roy (1999) recently provided a review of the possible aspects of the nitrogen cycle that could be used in microbial tests as part of ecological risk assessment. The authors suggested that denitrification activity may be a useful test, since denitrification is generally an anaerobic process carried out by facultative anaerobes and may reflect the impact of contaminants on aerobic and anaerobic soil microbial communities (Siciliano and Roy, 1999). Denitrification activity can be determined by acetylene inhibition, which is a sensitive and relatively inexpensive method to measure denitrification in soil samples (Knowles, 1990).

Nitrification is an important process of microbial communities in soils and has been suggested as a measure of soil quality (Brookes, 1993). However, nitrification in soil is only performed by Gram-negative bacteria, whereas other processes, such as dehydrogenase activity and ammonification, are carried out by many heterotrophic Gram-positive and Gram-negative bacteria. Hund and Traunspurger (1994) investigated the changes in toxicity during bioremediation of soil contaminated with about 4500 mg PAHs kg^{-1} of soil. The authors found that after 13 months of *in situ* bioremediation, the total PAH content had decreased by 65%, though no significant degradation of five to six ring PAHs occurred (Hund and Traunspurger, 1994). The toxicity of aqueous extracts (*Pseudomonas putida, Photobacterium phosphoreum, Daphnia magna, Brachydanio rerio*) as well as introduced (*Avena sativa, Brassica rapa and Eisenia fetida*) and natural soil organisms (nematodes, microbial respiration, potential ammonium, and nitrite oxidation activity) were followed during the bioremediation of the soil. In all test systems, a correlation between decreasing toxicity and degradation of the readily biodegradable PAHs was found (Hund and Traunspurger, 1994). Of the microbial toxicity tests used, the authors suggested that microbial nitrification was more sensitive than actual microbial respiration to PAHs in the soil, though this conclusion was based on a single increase found at 10 months (Hund and Traunspurger, 1994). More recently, Chaudri *et al.* (1996) investigated the toxicity of 12 organic contaminants (including PCP and anthracene) on the indigenous population of *Rhizobium leguminosarum* biovar *trifolii*. After 5 weeks, only PCP at 200 mg kg^{-1} of soil lowered the rhizobial population. After 6 months, no rhizobia were detected in the PCP-treated soil, whereas the control soil contained 1.2×10^5 cells g^{-1} of soil. Later, Fuller and Manning (1998) investigated the impact of 2,4,6-trinitrotoluene (TNT) and other munitions on microbial communities in soils. The study found that in soils with high levels of munition contaminants, there were also high levels of organic nitrogen and NH_4-N. The authors suggested that this was due to the nitrogen in the soil organic matter not undergoing ammonification,

leading to increased ammonium concentrations because ammonium-oxidizing bacteria were the most pollutant sensitive species involved in the soil nitrogen cycle (Fuller and Manning, 1998). The inclusion of specific groups of microorganisms involved in the nitrogen cycle in the assessment of contaminated land has been strongly advocated (Giller *et al.*, 1998). As these organisms are vital to the health of soil and plants, they should be included in a battery of tests assessing the risk of contaminants in soil (Efroymson and Suter, 1999).

E. Determination of Contaminant Impacts on Microbial Community Structure

The structure of microbial communities found in soils is related to their function (Garland, 1997; Giller *et al.*, 1998). There are a range of methods that enable the direct characterization of the microbial community structure through extraction of whole cells and their biomarkers, which include multiple substrate utilization, phospholipid-fatty acids (PLFA), profiles, and nucleic acids.

1. Multiple Substrate Utilization Methods

Multiple substrate utilization methods have been recently developed allowing an insight into the functional catabolic ability of microbial communities extracted from soil (Garland and Mills, 1991). Since the late 1980s, Biolog Inc. has commercially produced microtitre plates based on redox dyes for metabolic fingerprinting of bacterial isolates. Microbial parameters determined from the analysis of Biolog™ plates are converted to a so-called community-level physiological profile (CLPP) (Garland, 1997). The CLPP provides information on the composition of the microorganisms extracted from the soil, as assessed by their function. For example, the impact of toluene and trichloroethylene (TCE) on the structure and function of microbial communities in soil microcosms was investigated by Fuller *et al.* (1997). The soil microbial populations exhibited different sole carbon source utilization patterns as revealed by BIOLOG™ Gram-negative (GN) plates when exposed to 30 or 60 μg TCE ml^{-1} plus 20 μg toluene ml^{-1}, or to toluene concentrations greater than 60 $\mu g\ ml^{-1}$ compared to control soils. These differences remained for 30 days after all the TCE and toluene had been removed from the soils.

The previously described methods provide extensive information on the metabolic profiles of extracted microorganisms. However, they are limited in their use due to their reliance on the need to extract and cultivate microorganisms, which may render the results unrepresentative of the native microflora (Konopka *et al.*, 1998). Nevertheless, their

use in monitoring impact and tolerance due to contaminants has been proposed (see Section X.B.).

2. PLFA Profiles

PLFA profiles have been found to be specific for microbial species, but vary with growth conditions and can serve as indicators of changes in community structure rather than identification or quantification of individual species (Tunlid and White, 1992). Relating changes in PLFA patterns to certain groups of microorganisms has proven difficult due to dominating PLFAs existing in a wide range of taxa (Ratledge and Wilkinson, 1988). However, the use of multivariate statistical methods, such as principal component analysis, have enabled different mixtures of soil bacteria to be separated on the basis of their fatty acid profiles (Haack *et al.,* 1994). Statistical techniques, including principle component analysis, have also been used to indicate shifts in PLFA profiles due to different environmental disturbances (Tunlid and White, 1992). Specific fatty acid biomarkers can be used as indicators of microbial community composition, e.g., eubacteria, algae, fungi, Gram-positive and Gram-negative bacteria, sphingomonads, actinomycetes and sulfate-reducing bacteria (Vestal and White, 1989; White *et al.,* 1996a,b). In addition to differentiating taxonomic groups, increases in the ratios of *trans/cis* monoenoic PLFAs in cells are indicative of the effects of sublethal/toxic stress on bacterial communities and of the growth phase of cells, respectively (Gluckert *et al.,* 1986; Heipieper *et al.,* 1992; White *et al.,* 1996a ,b). PLFA analysis of Gram-negative bacteria community structure is limited due to their PLFA profiles being dominated by monoenoic, saturated and cyclopropane fatty acids (Wilkinson, 1988), the majority of which are broadly distributed and, as such, uninformative in subdividing their community structure.

Fuller and Manning (1998) studied the effects of munition compounds including 2,4,6-trinitrotoluene (TNT) on indigenous microbial communities in a range of soils using basal respiration rates, culturable heterotrophs, and PLFA. The authors reported that high TNT and 1,3,5-trinitrobenzene (TNB) levels resulted in a slight negative correlation with basal respiration rates, culturable heterotrophs and concentrations of PLFA. Analysis of specific signature PLFAs revealed lower amounts of Gram-positive bacteria, fungi, and protozoa with increasing levels of contamination. The authors concluded that chronic exposure to munition compounds can significantly change soil microbial communities (Fuller and Manning, 1998). More recently, Tsitko *et al.* (1999) studied the effects of phenol and toluene on the cellular fatty acid composition of *Rhodococcus opacus* GM-14. The authors reported that the 10-methyl branched fatty acid content of *R. opacus* GM-14 cells increased in a

dose response following exposure to phenol or toluene. The authors suggested that the 10-methyl branched fatty acids may be involved in the adaptation of *R. opacus* GM-14 to aromatic contaminants (Tsitko *et al.*, 1999).

3. Nucleic Acid-Based Methods

The use of molecular biological techniques to study the diversity and ecology of microbial communities has been recently reviewed (Head *et al.*, 1998). *In situ* methods based on the extraction of microbial biomarkers from soil can provide valuable information on the structure and function of microbial communities in contaminated soils. Combined with activity measurements, such as nutrient cycling, they have the potential to form the basis of a battery of microbial tests for assessing the ecological risk of contaminated soils.

Nucleic acid-based methods for monitoring changes in microbial communities have many advantages over culture based methods, since only 0.01–12% of the microbial community in the soil environment being commonly cultured (Torsvik *et al.*, 1990). Relatively recently, analysis of the structure and species composition of microbial communities has expanded through the development and use of molecular biological techniques. Techniques based on 16S rDNA have been used to identify several uncultured microorganisms and to determine the genetic diversity of microbial communities (Ward *et al.*, 1990; Muyzer *et al.*, 1993). A range of PCR primers have been developed for specific bacterial genes (Wheeler *et al.*, 1996). Most of the reported 16S rDNA sequence PCR primers are designed to amplify a wide range of bacterial sequences, which can be subsequently sequenced or subjected to denaturing gradient gel electrophoresis (DGGE) for species identification (Teske *et al.*, 1996; Wheeler *et al.*, 1996).

Wilson *et al.* (1999) developed a rapid PCR-based method that identified specific microorganisms in environmental samples that were able to degrade aromatic hydrocarbons. The authors used species-specific primers sets in conjunction with a universal primer set, which acted as an internal marker allowing the quantitative estimation of certain species by competitive PCR techniques (Wilson *et al.*, 1999). Thermal gradient gel electrophoresis (TGGE) analysis of ribosomal sequences amplified directly from community DNA was used to determine changes in the structure of the microbial community following the addition of phenol to an activated sludge ecosystem (Eichner *et al.*, 1999). The Shannon index of diversity (H), was calculated for each sample to follow changes in the diversity of the communities. The addition of phenol led to a reduction in the diversity index from 1.13 to 0.22. The use of TGGE analysis with diversity index was found to be sensitive to changes in

the microbial community due to pollutant shock (Eichner *et al.*, 1999). However, 16S rDNA sequences do not allow the discrimination between species, as one TGGE/DGGE band may represent several species with identical rDNA sequences (Vallaeys *et al.*, 1997). Banding patterns are subject to bias inherent to PCR-based techniques, e.g., selectivity of DNA extraction, potential preferential amplification, and chimera formation (Wintzingerode *et al.*, 1997).

Recently, Shi *et al.* (1999) studied microbial community structure in pristine and fuel oil-contaminated aquifers. Using phylogenetic probes, the authors found a greater abundance of $\beta+\gamma$-*Proteobacteria* (B+G) relative to α-*Proteobacteria* (ALF) in the fuel-contaminated aquifer, suggesting selective pressures imposed by the fuel contamination, or shifting electron acceptor availability. In a follow-up microcosm study with pristine aquifer material amended with toluene, the dominant subclass shifted from B+G to ALF, while in the fuel oil-contaminated microcosms, 55 to 65% of the bacterial community was no longer identifiable by the phylum or subclass probes used (Shi *et al.*, 1999).

VIII. Microbial Tests for Evaluating Potential Biodegradation Activity in Soils

Traditionally, the effectiveness of a remediation process has been established by direct analysis of the hydrocarbon content of soils, providing direct evidence of contaminant removal. Methods ranging from total petroleum hydrocarbon (TPH) to gas chromatographic (GC) methods are widely used and have been reviewed by Greenberg *et al.* (1992) and Northcott and Jones (2000). However, the reliability of the information they provide is dependent on the heterogeneity of the contaminant and on the method used; these methods also tend to be expensive (Bossert and Compeau, 1995). In addition, with the realization that the total HOC content of aged contaminants are not bioavailable and with the growing use of *in situ* bioremediation and natural attenuation as cost-effective remediation strategies, measurements and indicators of biodegradation activity may be more appropriate. The success of bioremediation techniques can often be estimated by measuring the biodegradability of a contaminant. Since biodegradation is a microbially mediated process, it can be indirectly measured by assessing the microbial communities and their activity in contaminated environments. Methods based on the measurement of microbial numbers, biomass, and activity can be utilized; however, careful interpretation of their results is needed. Some of these methods will now be discussed briefly.

A. Enumeration Methods

The enumeration of organisms involved in biodegradation provides an indication of microbial growth in a particular environment and, to some extent, its potential for biodegradation. A range of classic and novel methods are available for detecting, enumerating, and identifying microorganism involved in the biodegradation process. The two most widely used methods for both laboratory and field bioremediation studies include plate counts and most probable number (MPN) determinations, using undefined and selective media (Compeau *et al.,* 1991; Beliaeff and Mary, 1993). Modifications of classic plate count methods include spray plate techniques, direct growth on vapors of volatile contaminants, and agarose overlay (Kiyohara *et al.,* 1982; Marshall and Devinny, 1986; Bogardt and Hemmingsen, 1992). Kiyohara *et al.* (1982) first suggested a method to overlay inoculated agar plates with PAHs dissolved in a volatile solvent to leave a continuous opaque coating, in which clear zones are produced by degrading colonies. Bogardt and Hemmingsen (1992) suggested the use of a more accurate enumeration method where bacteria are added to molten, cooled agarose, which contains fine particles of phenanthrene. This is poured onto the surface of an already solidified agar layer thereby keeping the concentration of solvent used low and the colonies from spreading. The authors used the previously described technique to examine the numbers of phenanthrene-degrading bacteria in diesel fuel and creosote-contaminated soil undergoing bioremediation and found substantially higher numbers than in untreated, polluted soils (Bogardt and Hemmingsen, 1992). A wide range of MPN-based methods have been used to enumerate the number of organisms involved in soil contaminant degradation (Bagy *et al.,* 1992; Haines *et al.,* 1996; Carmichael *et al.,* 1997). Moreover, traditional MPN methods have been reduced to a format based on microtitre plates to allow the screening of large numbers of samples quickly (Stieber *et al.,* 1994).

Enumeration methods have several limitations associated with their use: (i) they do not reflect *in situ* conditions, (ii) they selectivity culture only those organisms able to grow under the conditions used and (iii) may exclude some and/or the majority of organisms involved in the biodegradation process. Enumeration methods used to quantify microbial communities involved in biodegradation only provide an indication of microbial vitality and shifts in populations and an estimate of the *in situ* community. Further, numbers of microorganisms do not reflect actual microbial activity in the soil, whereas activity measurements generally provide more useful information on biodegradation activities (Bossert and Compeau, 1995).

B. Biodegradation Activity Methods

Microbial activity can be used as an indirect measure of the remediation process of hydrocarbon contaminated soils. Activity can be measured by a range of respirometric methods, e.g., O_2 consumption and CO_2 production. The measurement of the biological conversion of a contaminant to CO_2 during soil incubations is a standard means to assess biodegradation of contaminants, as well as factors that influence the process. In field-based studies, O_2 consumption and CO_2 production can also be used to assess biodegradation. A range of instrumentation and methods has been developed to follow biodegradation in the laboratory, including biometer flasks (Bartha and Pramer, 1965), and measurements of [^{14}C]-CO_2 from ^{14}C-labeled compounds (Marinucci and Bartha, 1979). These summary parameters are useful due to the relatively simple method of determination and only a few techniques and apparatus are needed to follow the biodegradation of a wide variety of chemicals (Pagga, 1997).

The use of stable and nonstable radioisotopes to assess biodegradation has been found to be more accurate and sensitive than conventional measurements of compound utilization, with the use of uniformly labeled substrates preferred, where available. HOCs labeled with ^{14}C and more recently ^{13}C have been used to follow their biodegradation in soils (Guerin and Boyd, 1992; Carmichael *et al.*, 1997; Reid *et al.*, 2000c). Complete degradation of the compound can be followed by the collection of $^{14}CO_2$ and $^{13}CO_2$. The formation of metabolites can be detected using high-performance liquid chromatography (HPLC) with a ^{14}C detector and/or by thin-layer chromatography (TLC) plates and isotopic ratio gas chromatographic mass spectral (IR-GCMS) and the fate of the ^{14}C-activity in the soil and biomass can also be assessed (Tsao and Bartha, 1999).

Recently, there has been growing interest in the use of ^{13}C- substrates, allowing the fate of the carbon in the soil and microbial community to be closely followed (Northcott and Jones, 2000). The use of radiotopes in understanding the fate of HOCs in the soil environment has been recently reviewed (Northcott and Jones, 2000). While the use of ^{13}C-labeled compounds is constrained by their availability, cost and the background signal from soil organic matter, they do not require the strict safety procedures needed for the use of ^{14}C-labeled chemicals (Northcott and Jones, 2000).

Radiolabeled substrates have been used to follow the biodegradation of contaminants. Nevertheless, the factors that influence the rate and extent of breakdown are not fully understood. For example, MacLeod and Semple (unpublished data) have found that with increasing

soil–^{14}C-pyrene contact time, concentration and rate of pyrene addition, there was a decrease in the lag times before significant decomposition. Sharabi and Bartha (1993) found that the substrate loading rate of glucose affected rates of [^{14}C]glucose utilization and breakdown of background soil organic matter. This indicates that these results need careful interpretation to assess both the rate and extent of biodegradation (Bossert and Compeau, 1995). Oxygen consumption and/or uptake rates also provide simple and rapid evaluation of biodegradation. Uptake of O_2 can be measured using manometric methods, e.g., Warburg apparatus, and electronically (Grady *et al.*, 1989). Whereas the evolution of CO_2 measures the ultimate endpoint of biodegradation, O_2 uptake reflects overall activity and includes transient uptake due to only partial oxidation of the substrate (Bossert and Compeau, 1995).

C. *Lux*-Based Methods

Microbial bioluminescence methods are attractive because of their sensitivity, speed, and ease of implementation: usually less than 2 h compared to other bioassay systems that may take several days, as well as requiring a facility for the growth and preparation of the test organisms (Steinberg *et al.*, 1995). Cellular bioluminescent response is a direct measurement of contaminant bioavailability and/or biodegradability because the contaminants must act intracellularly to induce these changes (Sayler, 1995; Sticher *et al.*, 1997).

Although the Microtox® assay has become internationally adopted as a microbial biosensor, it has been argued that it is not ecologically relevant to the soil environment because it is based on the response of the marine microorganism *Vibrio fischeri* (Stewart, 1990; Paton *et al.*, 1997; Bundy *et al.*, 1997). Further, bioluminescence is an expensive metabolic function and these physiological costs raise questions regarding the intrinsic use of *lux*-marked organisms to produce stable and specific bioluminescence in oligotrophic environments (Matrubutham *et al.*, 1997). Despite these factors, bioluminescent biosensors have been used to assess specific contaminant bioavailability and biodegradability. For example, Selifonova and Eaton (1996) constructed *Escherichia coli* HMS174 (pOS25), which produced light in the presence of inducers of the *ipb* operon. Monoalkylbenzenes, substituted benzenes and toluenes, alkanes, cycloalkanes, chlorinated solvents, and naphthalenes all acted as inducers. Complex mixtures such as gasoline, diesel fuel, jet fuels and creosote were also found to be inducers of the construct (Selifonova and Eaton, 1996). Thus, *E. coli* HMS174 (pOS25) has the potential to be used in studying the bioavailability of a wide range of HOCs in soils since, in order to induce *ipb-lux* the compounds must enter the cell and therefore must be bioavailable.

Further, Applegate *et al.* (1998) constructed *Pseudomonas putida* TVA8 a whole cell bioreporter for benzene, toluene, ethylbenzene and xylene, and aqueous solutions of JP-4 jet fuel. The construct gave a significant light response to benzene, *m*- and *p*-xylenes, phenol and water soluble JP-4 jet fuel components, but there was no bioluminescence response to *o*-xylene.

King *et al.* (1990) constructed *Pseudomonas fluorescens* HK44 (pUTK21) and demonstrated that light production was related to naphthalene exposure and degradation rate. Heitzer *et al.* (1992) further developed *P. fluorescens* HK44 and demonstrated satisfactory contaminant sensitivity enabling environmental applications to be considered. Heitzer *et al.* (1994) immobilized *P. fluorescens* HK44 onto the surface of an optical light guide and placed this into a measurement cell, producing an on-line biosensor for waste stream solutions. Specific light responses (i.e., degradation was occurring) were obtained from exposure to aqueous solutions saturated with JP-4 jet fuel or leachate from a manufactured-gas plant soil, both of which contained naphthalene. More recently Ripp *et al.* (2000) described the use of the first genetically engineered microorganism approved for field testing for bioremediation purposes in the United States. In this study, *P. fluorescens* HK44 was introduced into soil lysimeters contaminated with naphthalene, anthracene, and phenanthrene, and its growth dynamics were studied over a 2-year period. *P. fluorescens* HK44 generated bioluminescence in response to soil hydrocarbon bioavailability (Ripp *et al.*, 2000). *P. fluorescens* HK44 was able to survive inoculation into the contaminated soil environment and was recoverable after 660 days (Ripp *et al.*, 2000).

Over the last decade *lux*-based biosensors have been used to detect and degrade a wide range of HOCs in the soil environment. The recent study by Ripp *et al.* (2000) has shown encouraging results for the use of *lux*-marked microorganisms for large-scale remediation operations. However, given the present public debate concerning genetic manipulation, wide scale use of *lux*-biosensors in *in situ* applications seems unlikely.

D. Nucleic Acid-Based Methods

Nucleic acid-based methods allow the direct and sensitive detection of specific biodegradative genes, mRNA and functional types of microorganisms in environmental samples as well as for monitoring the performance of *in situ* bioremediation (Brockman, 1995). Further, DNA probes have been used to monitor the occurrence of specific degradative genotypes in environmental populations. For example, NAH7 has

been used to probe for naphthalene-degradation genes (Sayler *et al.*, 1995). The frequency of hybridization was found to correlate well with estimates of specific degradative phenotype occurrence in the microbial community (Sayler *et al.*, 1995). Sayler *et al.* (1995) suggested that colony hybridization with NAH and TOL plasmids was a good indicator of the complete aromatic degrading population in a sediment sample. However, Foght and Westlake (1991) suggested that using NAH and TOL plasmids to probe a microbial community would underestimate the occurrence of PAH-degradative genes and so a suite of probes would be needed to evaluate the PAH catabolic potential in a mixed community.

Sayler *et al.* (1995) showed that mRNA levels in PAH-contaminated soils was directly correlated ($r^2 = 0.919$) to the relative degree of contamination, as measured by either naphthalene or total PAH concentration. Wang *et al.* (1996) used PCR based on the 16S rRNA (nondegradative RNA) of *Mycobacterium* sp. PYR1 and *Mycobacterium* sp. PAH135 to measure cell concentrations in soil slurries amended with ^{14}C-pyrene. A correlation between the concentration of *Mycobacterium* sp. PYR1 and the concentration of ^{14}C-pyrene was found in the soil slurries; however, other microorganisms may have also been involved in the degradation of the pyrene molecule.

IX. Ecological Risk Assessment (ERA)

ERA estimates the probability and nature of effects of human actions on non-human organisms, populations and ecosystems. It is characterized by separation into:

- Clear formation of the problem with clear assessment endpoints
- Characterization of risk, based on both the extent and effects of the exposure
- Separation of risk assessment from risk management (Barnthouse and Suter, 1986; EPA, 1992; Suter, 1993)

Risk assessment is considered a very useful tool in environmental policy because it promises a rational and objective basis for priority setting and decision making. The application of risk assessment to contaminated sites is widely advocated by many regulators, land developers and industries (Ferguson *et al.*, 1998). Risk assessment of contaminated sites is different from other applications of risk assessment, since it is not usually a preventative approach and hence, the source-pathway-receptor model is frequently used. The use of the source-pathway-receptor model

has resulted in many countries adopting generic guideline values for dealing with contaminated sites (Ferguson *et al.*, 1998).

With the growing realization that the sustainable use of soil is very important, more effort is being put into the development of different tools for assessing the risk that contaminants pose to terrestrial ecosystems. The basis of an ERA is both an assessment of (i) exposure and (ii) effect, using a range of test strategies or quality objectives, depending on the level of protection and acceptable risk. In some countries where ERA is mandatory, it applies to all investigated sites; however, in other countries, an ERA is only mandatory for some land use classes (e.g., nature areas) (Ferguson *et al.*, 1998).

A. ERA for the Bioremediation of Contaminated Soils

Broadly defined, bioremediation is the use of biological entities (e.g., microorganisms) to remove contaminants. There are many bioremediation strategies in use and under development throughout the industrialized world, many of which are reviewed elsewhere (Wilson and Jones, 1993; Holden and Firestone, 1997; Head, 1998; Semple *et al.*, 2001).

Risk-based decision criteria for contaminated sites are being more fully developed in response to increased assessment and remediation costs and in response to the uncertainties associated with adequate protection of the environment (Linz and Nakles, 1997). Ecotoxicology tests are also being used as tools to monitor bioremediation of hydrocarbon and other wastes, both in the field and in the laboratory (Loehr and Webster, 1996; Salanitro *et al.*, 1997). The use of biologically based endpoints may help appropriately define acceptable clean-up standards for HOC-contaminated soils (Loehr and Webster, 1996; Linz and Nakles, 1997). There is an ongoing debate on defining "how clean is clean" for contaminated soils (Linz and Nakles, 1997). As a result, the establishment of environmentally acceptable endpoints (EAEs) for hydrocarbon-contaminated soils has been incorporated into a risk-based approach (Linz and Nakles, 1997). There is a growing need for site specific measurements of available contamination to enable cost-efficient tests to safeguard human and ecological health. With the growing use of bioremediation of hydrocarbon-contaminated sites as a cost-effective and low impact method of remediation, there are problems with the dichotomy of total contaminant concentration and the concentration which is considered to be bioavailable (Alexander, 1995; Reid *et al.*, 2000a). If effective bioremediation reduces the hydrocarbon concentrations to a level where they no longer cause unacceptable risk to the environment, then it is believed that this may represent an "environmentally acceptable endpoint" (Linz and Nakles, 1997). The use of nonextractable residues

as an assessment endpoint for contaminated soils has also been proposed, although there is much debate about which extraction methods best simulate biological availability (Führ *et al.*, 1987; Kelsey *et al.*, 1997; Northcott and Jones, 2000; Reid *et al.*, 2000a).

X. Choice of Assessment Endpoints

Ranges of *in vitro* and *in vivo* bioassays and microbial tests have been proposed as endpoints to measure the impacts of HOCs on soil microbial communities. Only after the relevant assessment endpoint has been chosen can the risk assessor choose an appropriate microbial test (Efroymson and Suter, 1999). Three approaches have been suggested as assessment endpoints in ERA (Ferguson *et al.*, 1998):

1. A comparison of chemical data with generic guideline values or quality criteria derived from toxicity data obtained in standardised ecotoxicology tests, as well as applying a safety factor to reviewed toxicity data and using a statistical extrapolation of toxicity data to derive a PNEC (predicted no effect concentration)
2. The use of bioassays with soil extracts or solid material from the contaminated site as a supplement to chemical analysis
3. The monitoring of biomarkers, bioconcentration, indicator species, and changes in community structure. These indicators are seldom used in current risk assessment practise, but may be employed in research projects.

Impacts on microbial communities in the soil due to contaminants often lead to impacts on plant production. Microorganisms which form symbiotic relationships with plants, e.g., mycorrhizas and rhizobia have been proposed as endpoints (Efroymson and Suter, 1999). Since the production of plants is a valued property of soils, tests on microbial processes directly linked to plant production have been recommended for inclusion as endpoints (Efroymson and Suter, 1999). Microbial tests are also used as surrogate tests for higher organisms. The Ames *Salmonella* assay and Microtox® tests are rapid and inexpensive and indicate the presence of toxicants and mutagens. Recently, a specific genotoxicity test using *Pseudomonas putida* has been developed to assess the chronic toxicity of sorbed contaminants in soils (Alexander and Alexander, 1999).

A. Level of Effect

Once an endpoint has been chosen, the magnitude of a significant effect requires definition. Efroymson and Suter (1999) point out two main difficulties: (i) how to define a negative impact on soil microbial

processes and communities and (ii) the role of community tolerance and adaptation. Once an endpoint entity (e.g., bacteria, nitrogen fixing bacteria, or the entire soil microbial community), an attribute of that entity (e.g., decomposition, N-fixation rate, biomass, or diversity) and a definition of an adverse response (e.g., an increase, a decrease) have been identified, risk assessors need to consider whether a significant extent of effect can be agreed upon (Efroymson and Suter, 1999). It has been suggested that risk managers and assessment scientists agree on a potentially significant level of effect that must be detected by the tests used during the problem formation phase of a risk assessment (Efroymson and Suter, 1999). To be able to determine the extent of a significant impact that constitutes an adverse impact, the full range of rates of a process must be elucidated.

The significance of an impact on the soil microbial community is directly linked to the duration of the impact (Fig. 4). Natural fluctuations in the rates of microbial processes, for example, due to seasonal variation in temperature, are often large. The level by which an impact exceeds the natural variation in microbial processes, at the site

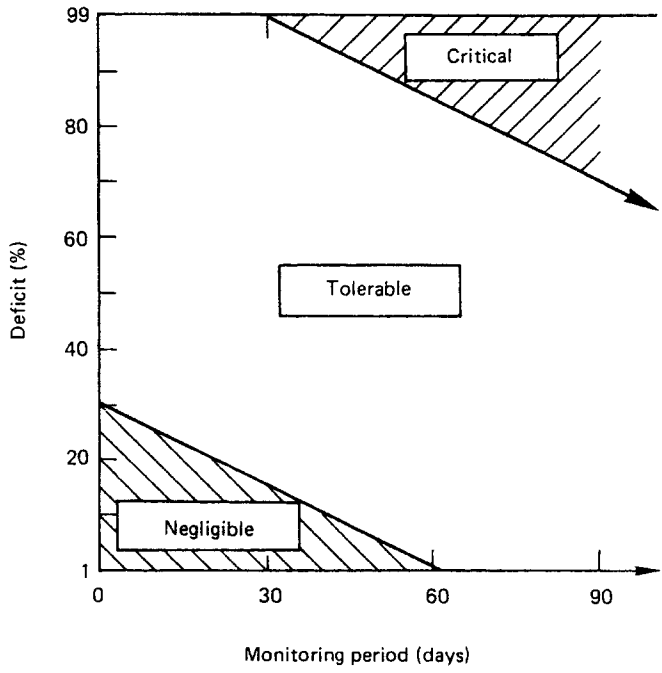

FIG. 4. Relationship between monitoring period and impact on microbial processes. (From 'An ecological concept for the assessment of side-effects of agrochemicals or soil microorganisms', by K.H. Domsch, G. Jagnow and T.H. Anderson, Residue Reviews, Volume 86, p. 80, Figure 10, 1983. Copyright notice of Springer-Verlag).

of concern, may define significance of effect of an impact. Domsch *et al.* (1983) suggested that a period of 60 days was required to assess any significant effect on soil microflora after exposure to contamination. However, it is not known if the impacts due to chemical stressors are reversible and have no lasting impact on the soil ecosystem (Brookes, 1993; Fuller *et al.*, 1997).

B. Tolerance of Soil Microbial Communities to Contaminants

Microbial communities are able to reduce the impact of contaminants on a process compared to higher organisms. These mechanisms include chemical resistance, functional redundancy, and rapid evolution (van Beelan and Doelman, 1997; Rutgers *et al.*, 1998). One method to assess the tolerance of microbial communities in the soil to contamination is pollution-induced community tolerance (PICT) (Blanck *et al.*, 1988) (Fig. 5). Toxic effects due to contaminants reduce the survival and

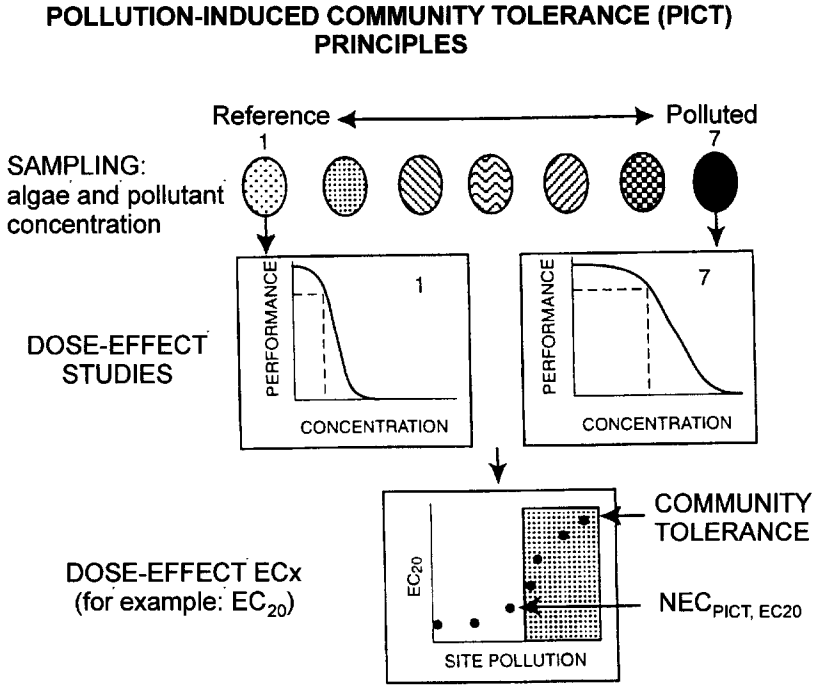

Fig. 5. The concept of pollution induced community tolerance (PICT). (From 'Effects of toxicants on population and community parameters in field conditions and their potential use in the validation of risk assessment methods', by L. Posthuma. In Ecological Risk Assessment of Contaminants in Soil, Chapter 5, p. 104, Figure 5.8, 1997. Edited by N.M. van Straalen and H. Lokke. Permission granted by Kluwer Academic/Plenum Publishers).

growth rate of the most sensitive organisms within a community, leading to an increase in the average tolerance of the community (Blanck *et al.*, 1988). In addition, tolerance of a contaminant in a community is a good indicator of the presence of that contaminant at the level to cause adverse effects. One may then be able to determine the exposure to a single contaminant in a mixture because tolerance is assessed in relation to that contaminant (Blanck *et al.*, 1988).

PICT is carried out by the addition of increasing amounts of the contaminant of interest to subsamples of a field sample and measuring the response of a microbial parameter, such as respiration (Giller *et al.*, 1998) or substrate use (Rutgers *et al.*, 1998). From this measurement, the level of pollutant that inhibits the microbial response by 50% (EC_{50}) can be determined. Tolerance is proportional to *in situ* exposure (Blanck *et al.*, 1988; Doelman *et al.*, 1994). Since tolerance is determined as an EC50, it acts as an internal standard between different communities with different basal rates. BIOLOG™ plates have also been used to measure PICT in experimental and field-contaminated sites (Rutgers *et al.*, 1998). Microorganisms are extracted from the soil and added to a series of plates with increasing levels of contamination. From the color development, EC50 values are determined for 85 to 90 different substrates (Rutgers and Breure, 1999). Moreover, it has been proposed that the presence of resistance to a contaminant is a measure of toxic effect and ecological deterioration (van Bleen and Doelman, 1997). For example, bacteria that are highly sensitive to heavy metals are able to degrade a larger range of aromatic contaminants compared to other bacteria (Doelman *et al.*, 1994). It has also been found that rhizobia in sewage sludge-amended soils which are tolerant of heavy metals are unable to fix nitrogen (Giller *et al.*, 1989). The National Institute of Public Health and the Environment in the Netherlands have suggested that resistance be included as a trait on which to base soil quality standards (van Beelen and Doelman, 1997).

XI. Current Status of Microbial Tests for Use in ERA of Organic Contamination of Soils

The use of risk assessment for contaminated sites has been reviewed (Ferguson *et al.*, 1998). The inclusion of microbial tests in the risk-assessment procedure has also been debated recently; the views of microbial ecologists and risk assessors were both represented (Chapman, 1999). In summary, Chapman (1999) suggested that "the soil microbial community needs to be included in appropriate ERAs, but that presently this is not realistically possible in most cases due to major data gaps identified by not only risk-assessment authors, but also the soil microbiologists." The development of microbial tests that measure the assessment endpoints for a wide range of contaminants in various soils

and exposure conditions is one of the largest challenges for microbial ecologists (Efroymson and Suter, 1999).

In the Netherlands and Canada, microbial processes are included in risk assessment of contaminated sites. In the Netherlands, one of the three factors that contributes to the "Intervention Value" for soil clean-up is the concentration at which 50% of microbial process are affected (Crommentuijn *et al.*, 1994). In Canada, part of the protocol for deriving Canadian Soil Quality Guidelines includes soil microbial tests. The use of microbial tests is justified under their role as supporting plant life in Appendix A of the Protocol for the Derivation of Environmental and Human Health Soil Quality Guidelines (CCME, 1996). In the United States, ecotoxicity benchmarks for microbial processes have been published, but have not implemented (Efroymson and Suter, 1999). In the UK, there is a land use-based policy for assessing and remediating contaminated land, with the use of guideline values (Ferguson *et al.*, 1998).

XII. Conclusions

This review attempts to show the importance of soil–contaminant interactions on the risks posed by HOCs in the soil environment. It is generally accepted that the longer HOCs are in contact with the soil matrix, the lower their potential for transport and availability to ecological receptors. Realistic assessments of the risk of HOCs in the soil environment need to be incorporated into the risk-assessment process (Alexander, 1995; Beck *et al.*, 1995). The use of solvent nonextractable residues as an endpoint to judge remediation of contaminated soils has been suggested (Linz and Nakles, 1997). However, both the solvent system used and the potential for remobilization of the contaminants must be clarified further.

Microorganisms play a pivotal role in the functioning of the soil ecosystem and their inclusion in the assessment of HOC-contaminated soils is needed (Siciliano and Roy, 1999). Soil microorganisms have three potential roles with respect to ecological assessment endpoints (Efroymson and Suter, 1999): (i) microbial responses may be used as surrogates for responses of higher organisms, (ii) microbial responses may be used to estimate effects on plant production, and (iii) properties of microbial communities may be endpoints. A wide range of microbial-based tests have been developed for a variety of purposes, but due to uncertainties relating to which tests should be incorporated in the ERA process, further work is needed before they can be included (Chapman, 1999). However, from reviewing the literature, the combination of activity-based bioassays with biomarker methods seems to be a promising set of tests to be included in the ERA process along with tests on higher soil organisms. With natural attenuation and bioremediation of hydrocarbon-

contaminated sites being increasingly used, ERA needs to incorporate the pivotal role of microorganisms in contaminant degradation.

References

Alexander, M. (1995). *Environ. Sci. Technol.* **29,** 2713–2717.

Alexander, R. R., and Alexander, M. (1999). *Environ. Toxicol. Chem.* **18,** 1140–1143.

Anderson, J. P. E. (1984). *Soil Biol. Biochem.* **16,** 483–489.

Anderson, J. P. E., and Domsch, K. H. (1978). *Soil Biol. Biochem.* **10,** 215–221.

Applegate, B. M., Kehrmeyer, S. R., and Sayler, G. S. (1998). *Appl. Environ. Microbiol.* **64,** 2730–2735.

Bagy, M. M. K., Shoreit, A. A. M., and Hemida, S. K. (1992). *J. Basic Microbiol.* **32,** 299–308.

Bardgett, R. D., James, L., and Leemans, D. K. (1995). *Bioresource Technol.* **52,** 175–180.

Barnhart, C. L. H., and Vestral, J. R. (1983). *Appl. Environ. Microbiol.* **46,** 970–977.

Barnthouse, L. W., and Suter, G. W. II. (1986). "User's Manual for Ecological Risk Assessment." ORNL-6251. Oak Ridge National Laboratory Oak Ridge, TN.

Bartha, R., and Pramer, D. (1965). *Soil Sci.* **100,** 68–70.

Beck, A. J., Wilson, S. C., Alcock, R. A., and Jones, K. C. (1995). *Crit. Rev. Environ. Sci. Technol.* **25,** 1–43.

Beliaeff, B., and Mary, J. Y. (1993). *Wat. Res.* **27,** 799–805.

Benton, M. J., Malott, M. L., Knight, S. S., Cooper, C. M., and Benson, W. H. (1995). *Environ. Toxicol. Chem.* **14,** 411–414.

Berkowitz, N. (1988). *In* "Polynuclear Aromatic Compounds" (L. B. Elbert, ed.), Advances in Chemistry Series Vol. 217, pp. 219–230. American Chemical Society, Washington.

Blanck, H., Wängberg, S.-Å., and Molander, S. (1988). *In* "Functional testing of Aquatic Biota for Estimating Hazards of Chemicals" (J. Crains, Jr. and J. R. Pratt, eds.), pp. 219–230. American Society for Testing and Materials Philadelphia PA.

Bloem, J., Lebbink, G. M., Zwart, K. B., Bouwman, L. A., Burgers, S. L. G. E., De Vos, J. A., and De Ruiter, P. C. (1994). *Agric. Econ. Environ.* **51,** 129–143.

Bogardt, A. H., and Hemmingsen, B. B. (1992). *Appl. Environ. Microbiol.* **58,** 2579–2582.

Bollag, J. M., and Loll, M. J. (1983). *Experientia* **39,** 1221–1231.

Bosma, T. N. P., Middeldrop, P. J. M., Schraa, G., and Zehnder, A. J. B. (1997). *Environ. Sci. Technol.* **31,** 248–252.

Bossert, I. D., and Compeau, G. C. (1995). *In* "Microbial Transformation and Degradation of Toxic Organic Chemicals" (L. Y. Young and C. E. Cerniglia, eds.), pp. 77–126. Wiley-Liss, New York.

Bossert, I., Kachel, W. M., and Bartha, R. (1984). *Appl. Environ. Microbiol.* **47,** 763–767.

Boyd, E. M., Meharg, A. A., Wright, J., and Killham, K. (1998). *Environ. Toxicol. Chem.* **11,** 2134–2140.

Brockman, F. J. (1995). *Mol. Ecol.* **4,** 567–578.

Brookes, P. C. (1993). *In* "Soil Monitoring" (R. Schulin, A., Desauces, R., Webster, B., and Von Steiger, eds.), pp. 229–253. Verlag, Basel.

Brusseau, M. L. (1994). *Rev. Geophys.* **32,** 285–314.

Brusseau, M. L. (1997). *In* "Soil Ecotoxicology" (J. Tarradellas, G. Bitton, and D. Rossel, eds.), pp. 33–53. Lewis Publishers, Boca Raton, FL.

Bundy, J. G., Wardell, J. L., Campbell, C. D., Killham, K., and Paton, G. I. (1997). *Lett. Appl. Microbiol.* **25,** 353–358.

Cairns, J. (1984). *Environ. Toxicol. Chem.* **3,** 1–3.

Calabrese, E. J., Kostecki, P. T., and Leonard, D. A. (1988). *In* "Soils Contaminated by Petroleum" (E. J. Calabrese and P. T. Kostecki, eds.), Wiley, New York.

Calderbank, A. (1989). *Rev. Environ. Contam. Toxicol.* **108,** 71–103.
Calvillo, Y. M., and Alexander, M. (1996). *Appl. Microbiol. Biotech.* **45,** 383–390.
Carmichael, L. M., Christman, R. F., and Pfaender, F. K. (1997). *Environ. Sci. Technol.* **31,** 126–132.
CCME (Canadian Council of Ministers of the Environment) (1996). A Protocol for the Derivation of Environmental and Human Health Soil Quality Guidelines. Canadian Council of Ministers of the Environment, Ottawa, Ontario. CCME-EPC-101E.
Cerniglia, C. E. (1984). *Adv. Appl. Microbiol.* **30,** 31–71.
Cerniglia, C. E. (1992). *Biodegradation* **3,** 351–368.
Chapman, P. M. (1986). *Environ. Toxicol. Chem.* **3,** 957–964.
Chapman, P. M. (1999). *Hum. Ecol. Risk Assess.* **5,** 657–660.
Chaudri, A. M., McGrath, S. P., Knight, B. P., Johnson, D. L., and Jones, K. C. (1996). *Soil Biol. Biochem.* **28,** 1483–1487.
Chiou, C. T., Peters, L. J., and Freed, V. H. (1979). *Science* **206,** 831–832.
Compeau, G. C., Mahaffrey, W. D., and Patras, L. (1991). *In* "Environmental Biotechnology for Waste Treatment" (G. S. Sayler, ed.), pp. 91–109. Plenum Press, New York.
Cornellissen, G., Rigterink, H., Ferdinandy, M. M. A., and Van Noort, P. C. M. (1998a). Rapidly desorbing fractions of PAHs in contaminated sediments as a predictor of the extent of bioremediation. *Environ. Sci. Technol.* **32,** 966–970.
Cornellissen, G., Van Noort, P. C. M., and Govers, H. A. J. (1998b). *Environ. Sci. Technol.* **32,** 3124–3131.
Crommentuijn, T., Doodeman, J. J. C., Bedaux, J. J. M., and van Gestel, C. A. M. (1994). *Environ. Toxicol. Chem.* **13,** 1781–1789.
Crosby, D. G. (1998). "Environmental Toxicology and Chemistry." Oxford University Press, New York.
Dagely, S. (1975). *In* "Essays in biochemistry" (P. N. Campbell and W. N. Aldridge, eds.), Vol. 11, pp. 81–130. Academic Press, London.
Divincenzo, J. P., and Sparks, D. L. (1997). *Environ. Sci. Technol.* **31,** 977–983.
Doelman, P., Jansen, E., Michels, M., and Van Til, M. (1994). *Biol. Fert. Soils.* **17,** 177–184.
Domsch, K. H., Jagnow, G., and Anderson, T. H. (1983). *Residue Rev.* **86,** 65–105.
Dorn, P. B., Vipond, T. E., Salanitro, J. P., and Wisniewski, H. L. (1998). *Chemosphere* **37,** 845–860.
Dusêk, L., and Tesarová, M. (1996). *Biol. Fert. Soil* **22,** 243–247.
Dyreborg, S., Arvin, E., and Broholm, K. (1996). *Biodegradation* **7,** 97–107.
Efroymson, R. A., and Suter, G. W. (1999). *Hum. Ecol. Risk Assess.* **5,** 715–727.
Eichner, C. A., Erb, R. W., Timmis, K. N., and Wagner-döbler, I. (1999). *Appl. Environ. Microbiol.* **65,** 102–109.
EPA (U.S. Environmental Protection Agency) (1992). U.S. Environmental Protection Agency, Washington, DC. **EPA/630/R-92/001.**
Ferguson, C., Darmendrail, D., Freier, K., Jensen, B. K., Jensen, J., Kasamas, H., Urzelai, A., and Vegter, J. (1998). "Risk Assessment for Contaminated Sites in Europe. Volume 1. Scientific Basis." LQM Press, Nottingham.
Foght, J. M., and Westlake, D. W. S. (1991). *Can. J. Microbiol.* **37,** 924–932.
Fry, J. C. (1982). *In* "Microbial Interactions and Communities" (A. T. Bull and J. H. Slater, eds.), pp. 103–152. Academic Press, London.
Führ, F. (1987). *In* "Pesticide Science and Biotechnology" (R. Greenhalgh and T. R. Roberts, eds.). pp. 383–389. Blackwell Science Publications UK.
Führ, F., Ophoff, H., Burauel, P., Wanner, U., and Haider, K. (1998). *In* "Pesticide Bound Residues in Soil. Report of a Workshop, September 3-4, 1996" (State Commision for the Assesment of Chemicals used in Agriculture, ed.), Wiley-VCH, DFG.
Fuller, M. E., and Manning, J. F. (1998). *Environ. Toxicol. Chem.* **17,** 2185–2195.

Fuller, M. E., Scow, K. M., Lau, S., and Ferris, H. (1997). *Soil Biol. Biochem.* **29,** 75–89.
Garland, J. L. (1997). *FEMS Microbiol. Ecol.* **24,** 289–300.
Garland, J. L., and Mills, A. L. (1991). *Appl. Environ. Microbiol.* **57,** 2351–2359.
Gaudet, C. L., Power, E. A., Milne, D. A., Nason, T. G. E., and Wong, M. P. (1995). *Hum. Ecol. Risk Assess.* **1,** 43–115.
Gevao, B., Semple, K. T., and Jones, K. C. (2000). *Environ. Poll.* **108,** 3–14.
Giller, K. E., McGrath, S. P., and Hirsch, P. R. (1989). *Soil Biol. Biochem.* **21,** 841–848.
Giller, K. E., Witter, E., and McGrath, S. P. (1998). *Soil Biol. Biochem.* **30,** 1389–1414.
Gluckert, J. B., Hood, M. A., and White, D. C. (1986). *Appl. Environ. Microbiol.* **52,** 794–801.
Grady, C. P. L., Dang, J. S., Harvey, D. M., Jobbagy, A., and Wang, X. L. (1989). *Wat. Sci. Technol.* **21,** 957–968.
Greenberg, A. E., Clesceri L. S., and Eaton, A. D. (eds.) (1992). "Standard Methods." American Public Health Association, Washington DC.
Guerin, W. F., and Boyd, S. A. (1992). *Appl. Environ. Microbiol.* **58,** 1142–1152.
Guthrie, E. A., and Pfaender, F. K. (1998). *Environ. Sci. Technol.* **32,** 501–508.
Haack, S. K., Garchow, H., Odelson, D. A., Forney, L. J., and Klug, M. J. (1994). *Appl. Environ. Microbiol.* **60,** 2483–2493.
Haines, J. R., Wrenn, B. A., Holder, E. L., Stroheimer, K. L., Herrington, R. T., and Venosa, A. D. (1996). *J. Ind. Microbiol.* **16,** 36–41.
Hairson, N., Smith, F., and Slobotkin, L. (1960). *Am. Naturalist* **94,** 421–425.
Hamelink, J. L., Landrum, P. F., Bergman, H. L., and Benson, W. H. (1994). "Bioavailability: Physical, Chemical, and Biological Interactions." Lewis Publishers, Boca Raton, FL.
Harms, H., and Zehnder, A. J. B. (1994). *Appl. Environ. Microbiol.* **60,** 2736–2745.
Hatheway, A. W. (1997). *Civil Eng.* **67,** 38–41.
Hatzinger, P. B., and Alexander, M. (1995). *Environ. Sci. Technol.* **29,** 537–545.
Haubenstricker, M. E., Meier, P. G., Mancy, K. H., and Brabec, M. J. (1990). *Bull. Environ. Contam. Toxicol.* **44,** 669–674.
Head, I. M. (1998). *Microbiology* **144,** 599–608.
Head, I. M., Saunders, J. R., and Pickup, R. W. (1998). *Microbial Ecol.* **35,** 1–21.
Heipieper, H.-J., Diefenbach, R., and Kewoloh, H. (1992). *Appl. Environ. Microbiol.* **58,** 1847–1852.
Heitzer, A., Malachowsky, K., Thonnard, J. E., Bienkowski, P. R., White, D. C., and Sayler, G. S. (1994). *Appl. Environ. Microbiol.* **60,** 1487–1494.
Heitzer, A., Webb, O. F., Thonnard, J. E., and Sayler, G. S. (1992). *Appl. Environ. Microbiol.* **58,** 1839–1846.
Hodson, R. E., Azam, F., and Lee, R. F. (1977). *Bull. Mar. Sci.* **27,** 119–126.
Holden, P. A., and Firestone, M. K. (1997). *J. Environ. Qual.* **26,** 32–40.
Hund, K., and Schenk, B. (1994). *Chemosphere* **28,** 477–490.
Hund, K., and Traunspurger, W. (1994). *Chemosphere* **29,** 371–390.
Jenkinson, D. S., and Powlson, D. S. (1976). *Soil Biol. Biochem.* **8,** 209–213.
Johnston, N., Sadler, R., Shaw, G. R., and Connell, D. W. (1993). *Chemosphere* **27,** 1151–1158.
Jones, K. C., Gevao, B., Mordant, C., Northcott, G., and Semple, K. T. (2000). *Environ. Poll.* **198,** 1–2.
Kelsey, J. W., Kottler, B. D., and Alexander, M. (1997). *Environ. Sci. Technol.* **31,** 214–217.
Khan, S. U. (1982). *Residue Rev.* **84,** 1–25.
Killham, K. (1985). *Environ. Poll.* **38,** 283–294.
King, J. M. H., DiGrazia, P. M., Applegate, B., Burlage, R., Sanseverino., Dunbar, P., Larimer, F., and Sayler, G. S. (1990). *Science* **249,** 778–781.
Kiyohara, H., Nagao, K., and Yana, K. (1982). *Appl. Environ. Microbiol.* **43,** 454–457.

Knowles, R. (1990). *In* "Denitrification in Soil and Sediments. FEMS Symposium 56" (N. Revsbech and J. Sørensen, eds.), pp. 151–166. Plenum Press, London.
Konopka, A., Oliver, L., and Turco, R. F. Jr. (1998). *Microbial Ecol.* **35,** 103–115.
Lapinskas, J. (1989). *Chem. Ind.* **23,** 784–789.
Leahy, J. G., and Colwell, R. R. (1990). *Microbiol. Rev.* **54,** 305–315.
Linz, D. G., and Nakles, D. V. (1997). "Environmentally Acceptable Endpoints in Soil. Risk-Based Approach to Contaminated Site Management on Availability of Chemicals in Soils." American Academy of Environmental Engineers, Annapolis, MD.
Loehr, R. C., and Webster, M. T. (1996). *J. Hazard Mat.* **50,** 105–128.
Macleod, C. J. A., and Semple, K. T. (2002). *Appl. Environ. Microbiol.* (Submitted).
Marinucci, A. C., and Bartha, R. (1979). *Appl. Environ. Microbiol.* **38,** 1020–1022.
Mathes, K., and Winter, G. (1993). *In* "Proc. 2nd Eur. Conf. Ecotoxicol.," Amsterdam, May 1992. *Sci. Total Environ.* 134.
Matrubutham, U., Thonnard, J. E., and Sayler, G. S. (1997). *Appl. Microbiol. Biotechno.* **47,** 604–609.
Means, J. C., Wood, S. G., Hassett, J. J., and Banwart, W. L. (1980). *Environ. Sci. Technol.* **14,** 1524–1528.
Meharg, A. A., Wyatt, C. L., Thompson, I. P., Bailey, M. J., Ellis, R. J., and Maguire, N. (1998). *Environ. Toxicol. Chem.* **17,** 1462–1468.
Mueller, J. G., Chapman, P. J., and Pritchard, P. H. (1989). *Environ. Sci. Technol.* **23,** 1197–1201.
Muyzer, G., De Waal, E. C., and Uitterlinden, A. G. (1993). *Appl. Environ. Microbiol.* **59,** 695–700.
Nakahara, T., Erickson, L. E., and Gutierrez, J. R. (1977). *Biotech. Bioeng.* **19,** 9–25.
Northcott, G. L., and Jones, K. C. (2000). *Environ. Poll.* **108,** 19–43.
NRC (1985). "Oil in the Sea." National Academy Press, Washington, DC.
Pagga, U. (1997). *Chemosphere* **35,** 2953–2972.
Palleroni, N. J. (1995). *In* " Microbial Transformation and Degradation of Toxic Organic Chemicals" (L. Y. Young and C. E. Cerniglia, eds.), pp. 3–26. Wiley-Liss, New York.
Parmaelee, R., Wentsel, R., Phillips, C., Simini, M., and Checkai, D. (1993). *Environ. Toxicol. Chem.* **12,** 1477–1486.
Paton, G. I., Palmer, G., Burton, M., Rattray, E. A. S., McGrath, S. P, Glover, L. A., and Killham, K. (1997). *Lett. Appl. Microbiol.* **24,** 296–300.
Peters, C. A., and Luthy, R. G. (1993). *Environ. Sci. Technol.* **27,** 2831–2843.
Piatt, J. J., and Brusseau, M. L. (1998). *Environ. Sci. Technol.* **32,** 1604–1608.
Pignatello, J. J. (1990). *Environ. Toxicol. Chem.* **9,** 1107–1115.
Pignatello, J. J., and Xing, B. S. (1996). *Environ. Sci. Technol.* **1,** 1–11.
Pimm, S., Lawton, J., and Cohen, J. (1991). *Nature (London),* **350,** 669–674.
Pollard, S. J. T., Hrudey, S. E., and Fedorak, P. M. (1994). *Waste Manage. Res.* **12,** 173–194.
Priddle, M. W., and MacQuarrie, K. T. B. (1994). *J. Contam. Hydro.* **15,** 27–56.
Ratledge, C., and Wilkinson, S. G. (eds.) (1988). "Microbial Lipids," Vol. 1. Academic Press, New York.
Reid, B. J., Jones, K. C., and Semple, K. T. (2000a). *Environ. Poll.* **108,** 103–112.
Reid, B. J., Lee, P. H., Macleod, C. J. A., Morriss, A. W. J., and Semple, K. T. (2000b). *FEMS Microbiol. Lett.* **182,** 367–373.
Reid, B. J., Stokes, J., Jones, K. C., and Semple, K. T. (2000c). *Environ. Sci. Technol.* **34,** 3174–3179.
Ripp, S., Nivens, D. E., Ahn, Y., Werner, C., Jarrell IV, J., Easter, J. P., Cox, C. D., Burlage, R. S., and Sayler, G. S. (2000). *Environ. Sci. Technol.* **34,** 846–853.
Roberts, T. R., Klein, W., Still, G. G., Kearney, P. C., Drescheer, N., Desnoras, J., Esser, H. O., Aharonson, N., and Vonk, J. W. (1984). *Pure Appl. Chem.* **56,** 945–956.

Rönnpagel, K., Janssen, E., and Ahlf, W. (1998). *Chemosphere* **36,** 1291–1304.
Rutgers, M., Van't Verlaat, I. M., Wind, B., Posthuma, L., and Breure, A. M. (1998). *Environ. Toxicol. Chem.* **17,** 2210–2213.
Rutgers, M., and Breure, A. M. (1999). *Hum. Ecol. Risk Assess.* **5,** 661–670.
Salizzato, M., Pavoni, B., Volpi Ghirardini, A., and Ghetti, P. F. (1998). *Chemosphere* **36,** 2949–2968.
Salminen, J. E., and Sulkava, P. O. (1997). *Environ. Poll.* **97,** 45–53.
Sanseverini, J., Applegate, B. M., King, J. M. H., and Sayler, G. S. (1993). *Appl. Environ. Microbiol.* **59,** 1931–1937.
Sayler, G. S. (1995). *In* "OECD Documents, Bioremediation." The Tokyo 1994 Workshop.
Sayler, G. S., Layton, A., Lajoie, C., Bowman, J., Tschantz, M., and Fleming, J. T. (1985). *Appl. Biochem. Biotech.* **54,** 277–290.
Selifonova, O. V., and Eaton, R. W. (1996). *Appl. Environ. Microbiol.* **62,** 778–783.
Semple, K. T., Cain, R. B., and Schmidt, S. (1999). *FEMS Microbiol. Lett.* **170,** 291–300.
Semple, K. T., Reid, B. J., and Fermor, T. R. (2001). *Environ. Poll.* **112,** 269–283.
Sharabi, N. E. D., and Bartha, R. (1993). *Appl. Environ. Microbiol.* **59,** 1201–1205.
Sheehan, P., Miller, D., Butler, G., and Bourdeau, P. (1984). "Effects of Pollutants at the Ecosystem Level." Wiley, Chichester.
Sheppard, S. C. (1997). *In* "Soil Ecotoxicology" (J. Tarradellas, G. Bitton, and D. Rossel, eds.). pp. 345–373. CRC Press, Boca Raton, FL.
Shi, Y., Zwolinski, M. D., Schreiber, M. E., Bahr, J. M., Sewell, G. W., and Hichey, W. J. (1999). *Appl. Environ. Microbiol.* **65,** 2143–2150.
Siciliano, S. D., and Roy, R. (1999). *Hum. Ecol. Risk Assess.* **5,** 671–682.
Silva, M. T., Sousa, J. C. F., Polónia, J. J., and Macedo, P. M. (1979). *J. Bacteriol.* **137,** 461–468.
Song, H. G., and Bartha, R. (1990). *Appl. Environ. Microbiol.* **56,** 646–651.
Spain, J. C., and vanVeld, P. A. (1983). *Appl. Environ. Microbiol.* **45,** 428–435.
Sparling, G. P., and West, A. W. (1988). *Soil Biol. Biochem.* **20,** 337–343.
Steinberg, S. M., Pignatello, J. J., and Sawhney, B. L. (1987). *Environ. Sci. Technol.* **21,** 1201–1208.
Steinberg, S. M., Poziomek, E. J., Engelmann, W. H., and Rogers, K. R. (1995). *Chemosphere* **30,** 2155–2197.
Stewart, G. S. A. B. (1990). *In vivo* bioluminescene: new potentials for microbiology. *Lett. Appl. Microbiol.* **10,** 1–8.
Sticher, P., Jaspers, M. C. M., Stemmler, K., Harms, H., Zehnder, A. J. B., and van der Meer, J. R. (1997). *Appl. Environ. Microbiol.* **63,** 4053–4060.
Stieber, M., Haeseler, F., Werner, P., and Frimmel, F. H. (1994). *Appl. Microbiol. Biotechnol.* **40,** 753–755.
Störmann, R., and Jastorff, B. (1993). *Sci. Total Environ.* 1655–1667.
Suter, G. W. II. (1993). "Ecological Risk Assessment." Lewis Publishers, Boca Raton, FL.
Suter, G. W. (1998). *In* "Handbook of Environmental Risk Assessment and Management" (P. Calow, ed.), pp. 177–217. Blackwell Science, Oxford.
Tay, K., Doe, K. G., Wade, S. J., Vaughan, D. A., Berringan, R. E., and Moore, M. J. (1992). *Environ. Toxicol. Chem.* **11,** 1567–1581.
Teske, A. P., Sigalevich, P., Cohen, Y., and Muyzer, G. (1996). *Appl. Environ. Microbiol.* **62,** 4210–4215.
Thompson, I. P., Bailey, M. J., Ellis, R. J., Maguire, N., and Meharg, A. A. (1999). *Soil Biol. Biochem.* **31,** 95–105.
Torstensson, L. (1997). *In* "Soil Ecotoxicology" (J. Tarradellas, G. Bitton, and D. Rossel, eds.), pp. 207–235. Lewis Publishers, Boca Raton, FL.
Torsvik, V., Goksøyr, J., and Daae, F. L. (1990). *Appl. Environ. Microbiol.* **56,** 782–787.

Tsao, C. W., and Bartha, R. (1999). *Soil Sci.* **164,** 235–241.
Tsitko, I. V., Zaitsev, G. M., Lobanok, A. G., and SalkinojaSalonen, M. S. (1999). *Appl. Environ. Microbiol.* **65,** 853–855.
Tunlid, A., and White, D. C. (1992). *In* "Soil Biochemistry" (G. Stotzky and J. M. Bollag, eds.), Vol. 7, pp. 229–262. Dekker, New York.
Vallaeys, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigeaud, A., and Soulas, G. (1997). *FEMS Microbiol. Ecol.* **58,** 1249–1258.
Van Beelen, P., and Doelman, P. (1997). *Chemosphere* **34,** 455–499.
van der Meer, J. R., Devos, W. M., Harayama, S., and Zehnder, A. J. B. (1992). *Microbiol. Rev.* **56,** 677–694.
Van Gestel, C. A. M., Adema, D. M. M., and Dirven VanBreemen, E. M. (1996). *Water Air Soil Poll.* **88,** 119–132.
Van Voris, P., Tolle, D., Arthur, M., and Chesson, J. (1985). *In* "Multispecies Toxicity Testing" (J. Cairns, Jr., ed.), pp. 117–142. Pergamon Press, New York.
Vance, E. D., Brookes, P. C., and Jenkinson, D. S. (1987). *Soil Biol. Biochem.* **19,** 703–707.
Verhoef, H. A. (1996). *Ecology* **77,** 685–690.
Vestal, J. R., and White, D. C. (1989). *BioScience* **39,** 535–541.
Wang, R. F., Luneau, A., Cao, W. W., and Cerniglia, C. E. (1996). *Environ. Sci. Technol.* **30,** 307–311.
Ward, D. M., Weller, R., and Bateson, M. M. (1990). *Nature (London)* **344,** 63–65.
Weber, W. J., McGinley, P. M., and Katz, L. E. (1992). *Environ. Sci. Technol.* **26,** 1955–1962.
Wheeler, A. E., Oerther, D. B., Larsen, N., Stahl, D. A., and Raskin, L. (1996). *Appl. Environ. Microbiol.* **62,** 3557–3559.
White, D. C., Pinkart, H. C., and Ringelberg, D. B. (1996a). *In* "Manual of Environmental Microbiology" (C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter, eds.), pp. 91–101. American Society for Microbiology, Washington, DC.
White, D. C., Stair, J. O., and Ringelberg, D. B. (1996b). *J. Ind. Microbiol.* **17,** 185–196.
White, J. C., Kelsey, J. W., Hatzinger, P. B., and Alexander, M. (1997). *Environ. Sci. Technol.* **16,** 2040–2045.
Wilkinson, S. G. (1988). *In* "Microbial Lipids" (C. Ratledge and S. G. Wilkinson, eds.), pp. 299–488. Academic Press, London.
Wilson, V. L., Tatford, B. C., Yin, X., Rajki, S. C., Walsh, M. M., and LaRock, P. (1999). *J. Microbiol. Method* **39,** 59–78.
Wilson, S. C., and Jones, K. C. (1993). *Environ. Poll.* **81,** 229–249.
Wintzingerode, F. V., Göbel, U. B., and Stackenbrandt. (1997). *FEMS Microbiol. Rev.* **21,** 213–229.
Xing, B., and Pignatello, J. J. (1997). *Environ. Sci. Technol.* **31,** 792–799.

The Developmental Biology of Fungi—A New Concept Introduced By Anton de Bary

GERHART DREWS
Albert-Ludwigs-Universität, Institute of Biology 2
Microbiology, Schaenzlestr. 1, 79104
Freiburg, Germany

I. Historical Retrospect

A. Morphological Studies in the 17th Century

The large fruiting bodies of basidiomycetes and ascomycetes, conspicuous to the unaided eye, must have been known to people since very early times. Humans have also used micro fungi, often unknowingly, for fermentation in wine, beer, and bread making. They were also adapted as food, drugs, or poison. The scientific revolution in the 16th and 17th centuries (Wilson, 1995; Shapin, 1996) and the development of the microscope as a tool for research opened the era of studies on the morphology and classification of fungi, as exemplified by Charles de L'Ecluse (Clusius, 1601) and Gaspard Bauhin (1560–1624). Bauhin was the first to discern genera and species and provided a detailed and illustrated description of 100 species of fungi in addition to 2700 species of plants (Bauhin, 1620, 1623). The fungi were separated into esculentii, noxii, and perniciosi. Joseph Pitton de Tournefort (1656–1708) presented a hierarchically ordered system with detailed description of genera (de Tournefort, 1694). Developmental stages, e.g., teleutospores of rust or mycelium of fungi, as described by Robert Hooke (1665), were not included. It was then believed that fungi originate from decaying matter. Gianbattista della Porta (1539–1615) proposed the revolutionary idea that all plants and fungi produce seeds; he described spores ("seeds") of numerous fungi, but he did not prove that spores could germinate (Porta, 1588). Marcello Malpighi (1628–1694) illustrated his own work with drawings of sporophores, sterigmata, and spores, and speculated

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 48

0065-2164/01 $35.00

whether spores were units of propagation (Malpighi, 1675, 1679). Great progress in the description of fungi was made by Pier Antonio Micheli (1679–1737). He introduced the names of many genera, such as *Mucor, Aspergillus,* and *Polyporus,* described fruiting bodies and the arrangement of spores, cultured certain molds on pieces of fruit, and with a primitive microscope followed the germination of spores up to the formation of fruiting bodies. He concluded that each fungus forms its own seeds and is reproduced only by its own kind (Micheli, 1729).

B. Progress in Cell Biology, Physiology, and Evolutionary Thoughts Stimulated Mycology in the 19th Century

In the 18th century and the beginning of the 19th century, knowledge about morphology, classification, and distribution of molds in habitats greatly increased. However, the systematics of fungi, such as that published by Christian Hendrik Persoon (1801, 1822–1828), Elias Magnus Fries (1821–1832; 1836–1838), and August Corda (1837–1854), were based exclusively on morphological data. This led to single stages of development or parts of the whole organism being described as separate species. Asci and basidia were known from studies on fruiting bodies, but their function and development as well as the role of the mycelium remained largely unknown.

The infection of plants by fungi was studied by Mathieu Tillet and Giovanni Targioni-Tozzetti. Tillet (1755) observed that smutted wheat kernels carrying spores produce smutty wheat. Targioni-Tozzetti (1767) described the infection of plants by germinating rust spores. These were the first experimental studies on infectious diseases of plants caused by fungi. In 1807, Bénédict Prévost, who was not familiar with these important observations, published his detailed studies on the germination of bunt or smut spores and the infection and development of the fungus in wheat plants. He observed that copper sulfate inhibits the germination of spores, and he demonstrated by field experiments that the disease could be controlled by soaking the wheat seeds in a solution of copper sulfate. Although this method was not widely accepted at that time, the solution was a precursor of the Bordeaux mixture, introduced by Pierre Millardet, which became in combination with the lime–sulfur solution the world's foremost preventive fungicide, used at first to fight downy mildew on grape vine leaves caused by *Plasmopara viticola* (Millardet, 1885).

Several scientists of the 17th and 18th centuries became aware that extant life forms were organized differently than those of earlier periods of the Earth's history. It was also realized that most organisms live in restricted natural habitats. The question of the origin of species was raised, and the descent of species from common ancestors was

discussed, but the static view of nature and the belief that all organisms could be traced back to creation or different forms of spontaneous generation, such as abiogenesis or heterogenesis, dominated (Farley, 1977; Mayr, 1982; Shapin, 1996). Jean Baptiste Antoine de Monet Chevalier de Lamarck (1744–1829) was one of the first to explain the multiplicity of forms of organization and their gradation from primitive to highly developed species by a process of evolution. He proposed that environmental conditions changed over long periods and that low to high complexity evolved by an inherent potential and by adaptation to the changed environmental conditions. Although he did not explain the mechanism how the acquired properties were transmitted to the next generation, his theory of evolutionary change contributed to altered perceptions about the static view of nature (Lamarck, 1809). Charles Robert Darwin (1809–1882) concluded from his own studies and from published accounts on comparative anatomy that all organisms have a common origin, and he assumed that within a species, a large and inexhaustible pool of inheritable variations exist. New species originate by natural selection from varieties in subpopulations of species living in separate habitats. New varieties optimally adapted to their surroundings survive, less-adapted varieties disappear. The principles of natural selection, evolution, and origin of species were controversially debated for decades, and strongly influenced biological thought (Darwin, 1859).

The idea of the evolutionary change in nature was accompanied by other new concepts and new experimental approaches, all of which stimulated the investigation of fungi and other thallophytes. Cells of plants were first described by Robert Hooke in 1665, but the low resolution and magnification of the available microscopes restricted studies on the cell wall and the cell as a building stone of the plant. Robert Brown (1831) observed the nucleus in stamins and cells of leaves of orchids and *Tradescantia* and described movements in the cytoplasm. Johannes E. Purkinjê (1839) was one of the first to use the term protoplasm and proposed the idea that animal and plant cells were similar. The availability of an improved composite microscope led to the birth of the cell theory in the 1840s; the theory considered the cell as an independent living entity of all organisms, with principally the same basic organization. Hugo von Mohl, Matthias J. Schleiden, and Theodor Schwann were recognized as the founders of the cell theory (Schleiden, 1842; Sachs, 1874, 1875, pp. 196–224, 336–366). Schleiden, who was not a trained botanist, fought against the "Naturphilosophie," which was an attempt to explain natural phenomena by confused speculative ideas, and he also refuted the trivial empirism of sterile systematics. In his book "Grundzüge der wissenschaftlichen Botanik" (Characteristics of Scientific Botany) Schleiden (1842) presented a broad methodical

introduction, followed by chapters which instruct on how to arrive at clear conclusions by sharp reasoning on the basis of the philosophy of Kant and Fries and for information how to use a good microscope efficiently. Schleiden was interested not only in the cell biology of plants but also in their developmental history. "It is the heuristic maxim in experimental botany to study the chemical and physical processes which occur during the modifications due to the process of development" (Schleiden, 1842, p. 103). Stimulated by Schleiden's publications, Wilhelm Hofmeister (1851, 1859, 1861), studied fecundation and embryo formation in mosses, ferns, and conifers. Through these comparative studies, Hofmeister discovered the alternation of generations in plants and the homology of respective organs in different groups of plants. Moreover he observed the division of the nucleus and of the cell.

Systematic studies on algal development were initiated by Jean-Pierre Vaucher (1803) on *Spirogyra* and *Zygnema* and extended by Gustave A.Thuret (1854, 1855; Thuret and Bornet, 1867) on brown and red algae. The sexuality of algae, the fecundation process, and the alternation of generations were discovered in *Vaucheria* by Nathanael Pringsheim (1855); in *Sphaeroplea annulina, Oedogonium,* and *Volvox globator,* by Ferdinand Cohn (1855); and in *Oedogonium* and *Bulbochaete,* by de Bary (1854a, 1856).

The fungus nucleus was first illustrated by de Bary (1866a) and shown by hematoxylin staining by Friedrich Schmitz (1879). Early botanists studied many details of the morphology and systematics of fungi, especially of those with large fruiting bodies, but fungal metabolism, development, and sexuality; the role of the different types of spores; and the physiology of infectious diseases of plants had not yet been studied thoroughly (Ainsworth, 1976). The new discoveries in cell biology, juxtaposed with the ever-present dogma of spontaneous generation, stimulated studies on spores, their germination, and their function in the development of fungi (de Bary, 1866a; Sachs, 1875; Farley, 1977).

II. Development and Sexuality of Fungi

The study of biology at the cellular level initiated a new era of thallophyte research. Organisms consisting of one or a few cells were well-suited to microscopy studies of structure and dynamics of cells and of cell differentiation. Louis René Tulasne (1815–1885) and his brother Charles Tulasne focused on the morphology, the cytology, and the developmental stages of numerous fungi, especially of Asco- and Basidiomycetes (Tulasne, 1851, 1854; Tulasne and Tulasne, 1844, 1847, 1861–1865). Their excellent characterization of these fungi set a new standard well above that of the majority of descriptions of different

species and genera available at that time. L. R. Tulasne was the first to discover that several morphological types, which had been described before as independent species, are really stages in the development of one species (pleomorphism) that follow each other in a regular succession over time (Tulasne and Tulasne 1861–1865, Vol. 1, pp. 47–78—English version).

A further breakthrough on this field was achieved by Anton de Bary (1831–1888) (Fig. 1), who followed the complete developmental cycle of

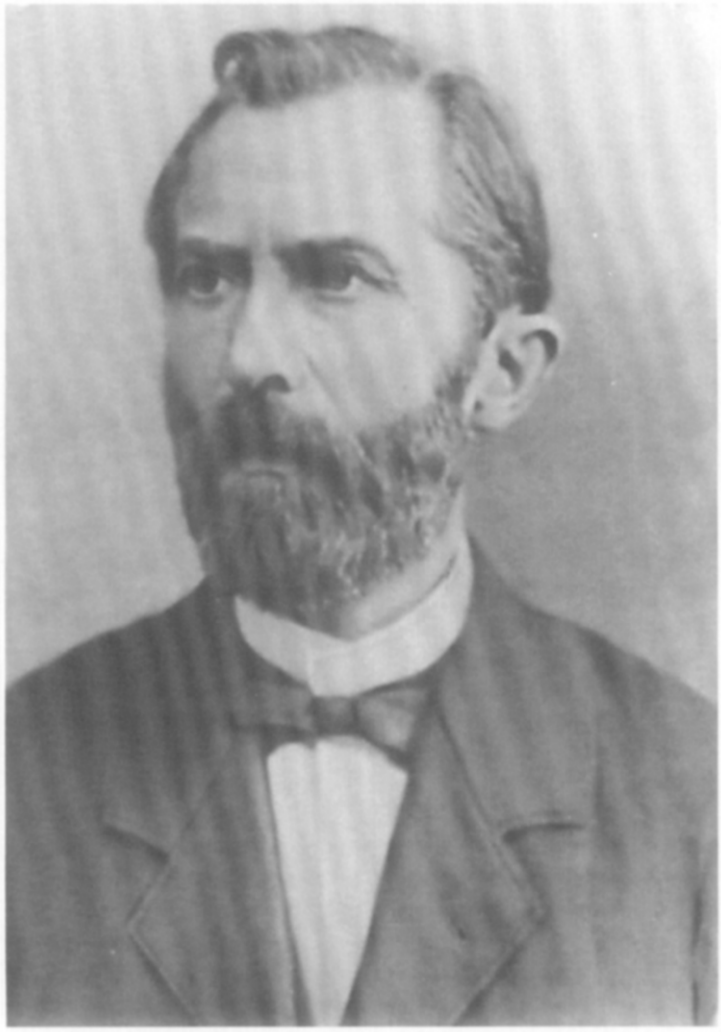

FIG. 1. Anton Heinrich de Bary.

numerous fungi. Spores were observed during germination on artificial or natural substrates through to the development of sexual and asexual units of reproduction, and the formation of fruiting bodies. He isolated clonal furgal cultures from single units of reproduction well before this method was introduced in bacteriology. In one of his early studies, de Bary cultivated a member of the Saprolegniaceae, *Achlya prolifera,* on dead insects in water. He observed the streaming of the cytoplasm in the coenocytic mycelium, determined a positive cellulose reaction of the cell walls, and followed the formation and delimitation of the club-shaped zoosporangia at the terminal cell, as well as the formation of primary and secondary zoospores (de Bary, 1852, 1860a) (Fig. 2). He observed that the encysted primary spores escape from the cyst wall,

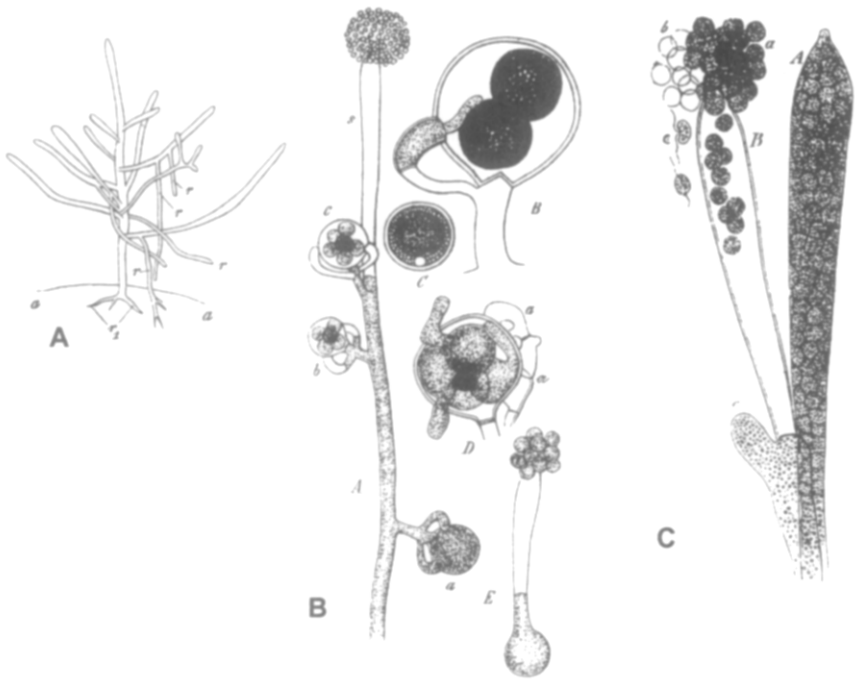

FIG. 2. *Achlya,* Saprolegniaceae, Stages of development. **(A)** *A. prolifera,* germ plant, 24 h old, height 1.5 mm, growing on a larva of a midge; *r,* rhizoids. **(B)** *A. racemosa. A,* a fruiting branch; *s,* emptied sporangium with a "Gonidienköpfchen" (conidia), most are swarmed; *a–c,* three oogonia and antheridia on short branches. *B,* oogonium with two eggs adjacent an antheridium, which has driven the fertilization tube onto the surface of one egg cell. *C,* mature oospore. *D, E*: *A. polyandra. D,* oogonium with three germinating oospores. *E,* germinating oospore, which has formed a small sporangium. **(C)** *Achlya* sp., sporangia. *A,* filled with spores. *B,* sporangium discharging spores, some of which are swarmers (*c*), slipping out of the envelope (*b*). (From de Bary, 1884, Figs. 68, 69, 70.)

move around as swarm cells after a rest period, shed the flagella, and germinate to form a new mycelium, while the larger secondary spores are formed in spherical sporangia and are not motile. de Bary described the morphological differences between *Achlya prolifera, Saprolegnia capitulifera* Braun, and *Saprolegnia ferax* Kützing. The antheridia and oogonia of Saprolegniaceae were discovered in 1857 by Pringsheim; de Bary correctly described the formation of antheridia and oogonia as a function of the growth conditions (de Bary, 1883). He postulated that the formation of sexual organs is induced by factors that are excreted from one of the partners (de Bary, 1881a, 1883). The role and chemical composition of these pheromones were experimentally determined about 60 years later by John R. Raper (1939, 1952; Mullins, 1994).

Another important aspect of de Bary's work for the taxonomy of Saprolegniaceae was the delineation of species. In an extended series of experiments starting with single spores, he investigated the constancy of species, varieties, and races. He registered the distribution of species in different habitats and analyzed his results with consideration of natural selection and evolution (de Bary 1888). The unfinished study was published posthumously by Hermann Graf zu Solms-Laubach.

Much attention was paid to members of the plant parasite family Peronosporoceae. Tulasne (1851) described a form of fructification on the surface of infected plants different from the zoosporangia. de Bary (1861) studied the sexual propagation of several species of *Peronospora*—the development of antheridia and oogonia, and fertilization (de Bary, 1881c). The process of fertilization was thought to be analogous to *fecundation* of the egg cell by the pollen tube in flowering plants. He conceded critically that these processes were not yet completely understood. Although guess work and several observations on fertilization in fungi were reported earlier by August C. J. Corda, Christian Gottfried G. Ehrenberg, and Christian Gottfried Nees von Esenbeck (Ainsworth, 1976, pp. 116–117), the article by de Bary (1861) presented one of the first proofs of the sexuality of fungi and the formation of alternate developmental stages. However, fecundation was only shown on the level of cell fusion. The role of nuclei was not recognized until 30 years later when Pierre-Augustin Dangeard (1894, 1895) described the fusion of nuclei in asci and basidia.

Aspergillus glaucus was known as a ubiquitous mold growing on many organic substrates. de Bary described the cross wall formation of regular hyphae, the thick-walled hyphae forming the conidiophores, and spherical structures containing eight spores surrounded by tightly coiled hyphae (de Bary 1854b). Later he described the formation of the ascogonium (carpogonium), the antherid (pollinodium), and copulation. It was concluded that *Aspergillus glaucus* and *Eurotium*

herbariorum are different stages of spore formation in the same species and that the yellowish perithecium is a product of a sexual process (de Bary, 1856/57, 1863a d, 1869; de Bary and Woronin, 1870).

de Bary was not only an excellent cell biologist, he was also familiar with the ecology and physiology of plants and plant diseases. de Bary's investigations on the development of gametophytes and sporophytes of *Pythium* and *Phytophthora* species included physiological studies on the host range of infected plants, the modification of plants during infection, and the influence of water on the process of disease (de Bary, 1861, 1863a). Following up on the early work of L. R. Tulasne he discovered the complex developmental cycles and the heteroecism of several rust fungi. He observed the formation of the brownish uredia and uredospores of the stem rust of wheat, caused by *Puccinia graminis;* the spores germinate immediately and are responsible for the distribution of the disease over large distances. He also described the production of the black-colored teliospores (probasidium; termed teleutospores by de Bary), which appeared later in the season and overwintered. The teliospores germinated in the spring, and the basidiospores (termed sporidie by Tulasne) infected barberry and developed a (haploid) mycelium that formed spermogonia (pycnia) on leaves and ruptured the epidermis. The receptive hyphae of the spermogonia were fertilized, and the fecundated mycelium developed an aecium and aeciospores, which infected wheat stem or leaves. The parasitic fungus on barberry (*Berberis*) was known as *Aecidium berberidis.* de Bary observed that basidiospores infected only a few species of barberry and developed spermogonia on leaves, whereas the aeciospores infected winter rye, *Triticum repens* and *Agrostis vulgaris,* but not *Berberis.* The uredospores of *P. graminis* germinated on leaves of barberry, but did not penetrate into the leaf. de Bary concluded from these observations that *Aecidium berberidis* and *P. graminis* are different developmental stages of one species, which have to change depending on the host plant since the germination of basidiospores and aeciospores and the development of mycelium are restricted to the appropriate host plants. The term heteroecious for *P. graminis* and other fungi that obligatorily change depending on the host plant for completion of their full developmental cycle, and autoecious for macrocyclic rusts, e.g., asparagus rust, which complete their life cycle on a single host, were introduced by de Bary (1865/66). His conclusions were based on careful *in vivo* and *in vitro* infection experiments using different spores and host plants.

Similarly, de Bary investigated the life cycle of *Chrysomyxa rhododendri* by a combination of extensive field studies and laboratory experiments. He observed that the fir tree *Picea excelsa* was attacked

in regions of the Alp mountains higher than 1000 m by the rust fungus *Aecidium abietum,* which forms aeciospores and spermatia on fir trees in July. This rust fungus was not found outside of the Alps, e.g., in the Black Forest or in the Voges. The aeciospores infect young sprouts of *Rhododendron ferrugineum* and *Rhododendron hirsutum* (Alp rose), which flower in June/July. The aeciospores germinate and infect the leaves of the Alp rose through the stomata. The mycelium overwinters, and uredospores and teliospores of *Chrysomyxa rhododendri* develop on *Rhododendron* in the spring. The arising basidiospores infect the budding leaves of *Picea excelsa* in June. The results proved that *Chrysomyxa rhododendri* (De Candolle) de Bary is a heteroecic fungus that develops sexual spores (the perfect state, telomorphic) on *Rhododendron* and asexual spores (imperfect, anamorphic) on *Picea excelsa. Chrysomyxa rhododendri* and *Aecidium abietum* are therefore different developmental stages of the same fungus, which was named *Chrysomyxa rhododendri* according to its teleomorphic state (de Bary, 1879a). de Bary observed that the heteroecic developmental cycle depends on the temporal and spatial coincidence of the specific spores and sensitive states of the hosts. In the same article, *Chrysomyxa ledi* (Albertini and Schweinitz), was described as a heteroecic fungus that develops aecium and spermogonium on *Picea excelsa,* and uredospores and teleutospores on *Ledum palustre* (Sumpf Porst). This species is found in northern regions in habitats of *Ledum,* while *Chrysomyxa abietes* (Wallroth) Unger is an autoecious, microcyclic rust, which develops only teleutospores on *Picea excelsa* and was believed to be a reduced form of *C. rhodendri* or *C. ledi* (de Bary, 1879a; Fischer, 1904; Gäumann, 1964).

The comprehensive work of de Bary on rust fungi paved the way for modern taxonomic, genetic, and physiological studies of this group of fungi. de Bary thus bridged the studies up to those of the Tulasne brothers and the future discoveries of the haplontic/diplontic and dikaryotic phases in the life cycle of the rust fungi (Hawksworth *et al.,* 1995, pp. 134, 135, 473–476).

de Bary's investigations on development, sexuality, and phytopathology covered a broad range of taxonomic groups (de Bary, 1871, 1878, 1881b). The microscopy studies on the development of fungi from single spores to fruiting bodies, the search for pathological modifications of host plants, and physiological experiments on the germination of spores on different host leaves initiated a new era of mycology. Other genera he studied included *Sordaria* (*Fimetaria*), *Erysiphe, Taphrina* Fr. (*Exoascus* Fuckel) *deformans* (Berk.), *Exoascus pruni* (plum pockets in young fruits of *Prunus domestica*), and representatives of the Phallales, Taphrinales, and Mucorales (de Bary, 1863b–d, 1864).

Lichens have been known to the scientific literature since the first description byMicheli (1729). Wallroth (1825/27) noticed the similarity of the colorless filaments of the lichen and the fungal hyphae and interpreted the green globular cells as reproductive organs, which he named gonidia. de Bary (1866a,b) proposed that lichens are the symbiotic product of Nostocaceae or Chroococcaceae species attacked by parasitic ascomycetes. He understood symbiosis as a close spatial coexistence (de Bary, 1879b). Max Rees, a co-worker of de Bary in Freiburg and Halle, combined the individual symbiotic partners and obtained functional lichens (Kümmel, 1998, p. 70).

de Bary believed that the plasmodial slime molds (Myxomycetes, Myxomycota) are related to the animal kingdom because of their amoeboid stages and phagotrophic nutrition. Consequently, he called them Mycetozoa (de Bary, 1860b, 1862; de Bary and Woronin, 1870). The developmental stages and fructification of *Physarum, Trichia,* and *Didymium* were described, but at that time, mitosis and meiosis could not be distinguished (de Bary, 1858). The work was supplemented by Oskar Brefeld, who was a co-worker of de Bary in Halle (Brefeld, 1869). de Bary compiled the findings of his own work and evaluated the knowledge of the time in the book "Comparative Morphology and Biology of Fungi, Mycetozoa and Bacteria" (de Bary, 1884). Fungal morphology and development were the bases of his concept on taxonomy. The role of karyogamy and meiosis in the development of fungi was later elucidated by Pierre-Augustin Dangeard (1894) and his successors.

III. Taxonomy of Fungi

The morphology of fruiting bodies has been the basis for the classification of fungi since the 17th century (Bessey, 1968). Significant progress in the classification of fungi was achieved by Christian H. Persoon (1755–1837) and Elias M. Fries (1794–1878). They collected and described numerous new species, and their classification was improved by systematic studies of spores and fruiting bodies. Unfortunately Fries did not use a microscope and was not able to describe the microscopic structure of the hymenium (Tulasne and Tulasne, 1861, pp. 48/49 of the English translation). The prominent subjects of their research were fungi with large fruiting bodies (Persoon, 1801; Fries 1836–1838). The microscopic fungi and the pathogenic fungi remained largely unknown or were superficially described. The increased knowledge of fungal development and the concept that the structures of sexual differentiation in different groups of fungi are homologous, i.e., of common evolutionary origin, inspired de Bary to determine ancestral lines and relationships for the development of a natural system of classification.

de Bary proposed a relationship between the sexual and asexual propagation of the oosporous Chlorophyceae and the Peronosporaceae (de Bary, 1881a,b). The Saprolegniae, the Peronosporeae, and the Mucorini were combined in the class Phycomycetes, a term coined by de Bary (de Bary, 1866a; Bessey, 1968, pp. 42–44). de Bary critically evaluated the confused terminology of names of fungi and their reproduction organs in use in his time. He stressed that homology, not the mode of spore formation, is the important biological criterion. de Bary proposed the "Ascomyceten-Reihe" (line of Ascomycetes), beginning with the oosporous Chlorophyceae, followed by the Peronosporaceae, Saprolegniaceae, Mucorineen (Zygomycetes), Entomophthoraceae, Ascomycetes, and Uredineen (de Bary, 1884, p. 142). He regarded the Erysipheae as the connecting link between the Peronosporaceae and the Ascomycetes. de Bary discussed, but did not believe in a polyphyletic origin of the Ascomycetes. The various asci were considered as homologous structures, whether formed sexually or by apogamy. He compared apogamous Ascomycetes with the apogamous ferns (de Bary, 1878, 1881b, 1884, pp. 128–142). The Uredineen were proposed to be related to the Ascomycetes. The Basidiomycetes were traced back to the Uredineen, the Zygomycetes and Entomophthoraceen to the Peronosporaceen. The Ustilagineen were believed to descend from the Chrytridieen via Protomyces. The relationship of Basidiomycetes and Uredineen was substantiated by the observation that some leptoforms of Teliomycetes, having only teleutospores, correspond to a stage in the development of some Tremellales.

The descendant theory and phylogeny were for de Bary the basis for all his comparative studies on systematics (de Bary, 1864, 1881a,b, 1884) beginning with his dissertation (de Bary, 1853). In the 20th century, mitosis, meiosis, and heterokaryotic dikaryons were discovered in specific stages of development and their importance for physiology and development was recognized (Blackman, 1904; Bessey, 1968). The advent of cladistic and molecular approaches strengthened the phylogenetic view of systematics. Unexpectedly and in contrast to the hypotheses of de Bary and his contemporaries, the evolutionary roots of fungi were found to be closer to Animalia than to Plantae. Fungi may have originated from protozoan ancestors before the kingdoms Animalia and Plantae split. The Chytridiomycota may have a basal position in the four phyla of the present classification (Hawksworth *et al.*, 1995, pp. 353–355).

Many competing views of classification were propagated at the time of de Bary. Brefeld traced back the higher taxa of fungi to the Zygomycetes and deduced the Ascomycetes from the line of sporangia of Myxomycetes, and the Basidiomycetes from the conidia. He refuted the

sexuality of higher fungi (Jost, 1930/31). Modern research has confirmed de Bary's concept of fungal sexuality and apogamy in some groups. At the present time, the systematics of fungi has been stabilized in the lower ranks up to the order level; above the order level, there is more diversity in usage (Hawksworth *et al.*, 1995, pp. 93, 94, 353–355).

IV. Concluding Remarks

The chemical composition and basic structure and function of the major cellular constituents, at least of model organisms, are known today. This background and molecular genetic techniques enable scientists to investigate some of the most exciting topics in biology—the development of organisms, the differentiation of tissues, and their regulation at the molecular level.

When Anton de Bary stepped into science in 1852, the morphology and anatomy of higher organisms were known while the study of the biology of the cell and of thallophytes was in its initial phase. Very early in his career, de Bary derived the concept that lower plants, especially fungi, have a complex development. He discovered the sequence of sexual and asexual propagation of fungi by following the different stages of development from the spore to the fructification organs through observation with the microscope *in vitro* and *in vivo* after inoculation of plants with spores. This experimental approach and his original concept based on Darwin's theory of evolution were the starting points for phylogenetic taxonomy. The definition of a species and subspecies and their delimitation were considered throughout his studies.

de Bary's second great achievement was his major contribution to phytopathology. He followed the whole process of the infection of plants, the germination of spores, the formation of germination tubes and haustoria in the leaf mesophyll, and the fructification of the parasites. Parasitism, symbiosis, heterospory, and heteroecy are terms he coined or redefined. His observations and experimental results were explained in the frame of a unifying concept of the phylogeny of fungi. de Bary's numerous studies on algae, lichens, ferns, and higher plants enriched the knowledge of his time.

Moreover de Bary was very modern in his style of teaching research to students. He was not authoritarian, but tried to develop self-reliance, observation skills, self-control, and critical evaluation of one's own results and conclusions. All of the work he carried out during his life was mastered by a high degree of self-discipline and sense of duty. For more detailed biographical information about de Bary's life see Drews (2000), Dudley (1888), Jost (1930–1931), Rees (1888), and Sparrow (1978).

References

Ainsworth, G. C. (1976). "Introduction to the History of Mycology," p. 359. University Press, Cambridge.

Bauhin, G. (Bauhinus) (1620). "Prodromus Theatri Botanici." Frankfurt.

Bauhin, G. (1623). "Pinax Theatri Botanici." Basle.

Bessey, E. A. (1968). "Morphology and Taxonomy of Fungi," p. 791. Hafner Publishing Company, New York.

Blackman, V. H. (1904). *Annales Botany, London* **18,** 323–373.

Brefeld, O. (1869). *Abh. Senckenberg. Naturforsch. Gesellsch.* **7,** 85–108.

Brown, R. (1831). *Trans. Linn. Soc. London* **16,** 685–745.

Clusius, C. (J.-C. de l'Écluse) (1601). "Fungorum in Pannoniis Observatorum Brevis Historia." Antwerpen, reprinted Graz 1983.

Cohn, F. (1855). *Monatsber. Königl. Akad. Wissensch.* 335–351.

Corda, A. C. J. (1837–1854). "Icones Fungorum Hucusque Cognitorum," Vol. 6, Prague.

Dangeard, P. A. (1894). *Le Botaniste* **4,** 1–2.

Dangeard, P. A. (1895). *Le Botaniste* **4,** 119–181.

Darwin, C. (1859). "The Origin of Species by Means of Natural Selection." J. M. Dent & Sons, London.

de Bary, A. (1852). *Botan. Ztg.* **10,** 473–479; 489–496; 505–511.

de Bary, A. (1853). "De Plantarum Generatione Sexuali." Schade, Berlin.

de Bary, A. (1854a). *Abh. Senckenberg. Naturforsch. Gesellsch.* **1,** 29–105.

de Bary, A. (1854b). *Botan. Ztg.* **12,** 425–434; 441–451; 465–471.

de Bary, A. (1856). *Annal. Sci. Naturel.* (*Bot.*) **VI,** 262–266.

de Bary, A. (1856/57). *Berich. Verhand. Gesellsch. Beförder. Naturwissensch.* **I,** 325–344; **II,** 215–230; **III,** 325–344.

de Bary, A. (1858). *Bot. Ztg.* **16,** 357–358; 361–364; 365–369.

de Bary, A. (1860a). *Pringsh. Jahr. Botan.* **2,** 169–192.

de Bary, A. (1860b). *Siebold Köll. Zeitsch. Wissensch. Zool.* **10,** 88–176.

de Bary, A. (1861). *Bot. Ztg.* **19,** 89–91.

de Bary, A. (1862). *Flora* **45,** 264–272; 282–287; 301–304.

de Bary, A. (1863a). "Über die Fruchtentwicklung der Ascomyceten." W. Engelmann, Leipzig.

de Bary, A. (1863b). *Ann. Sci. Nat.* (*Bot.*) **20,** 5–148.

de Bary, A. (1863c). *Flora* **46,** 401–409.

de Bary, A. (1863d). "Über die Fruchtentwicklung der Ascomyceten. Eine pflanzenphyiologische Untersuchung," p. 38. W. Engelmann, Leipzig.

de Bary, A. (1864). *Abh. Senckenb. Naturforsch. Gesellsch. Frankfurt* **5,** 137–232.

de Bary, A. (1865–1866). *Monatsber. Königl.-Preussischen Akad. Wissensch. Berlin* 15–49; 205–215.

de Bary, A. (1866a). "Morphologie und Physiologie der Pilze, Flechten und Myxomyceten." W. Engelmann, Leipzig.

de Bary, A. (1866b). *Pringsheim's Jb. Wiss. Bot.* **5,** 201–216.

de Bary, A. (1869). *Abh. Naturforsch. Gesellsch. Senckenberg.* **7,** 361–455.

de Bary, A. (1871). *Monatsber. Königl. Preuss. Akad. Wiss. Berlin* 227–239.

de Bary, A. (1878). *Bot. Ztg.* **36,** 449–487.

de Bary, A. (1879a). *Bot. Ztg.* **37,** 761–774; 777–789; 801–811; 825–830; 841–847.

de Bary, A. (1879b). *"Die Erscheinung der Symbiose. 51. Versammlung der Vereinigung deutscher Naturforscher und Ärzte in München,"* Trübner, Strassburg.

de Bary, A. (1881a). *Abh. Senckenberg. Naturforsch. Ges.* **12,** 225–370.

de Bary, A. (1881b). *Bot. Ztg.* **39,** 1–17; 33–36.

de Bary, A. (1881c). *Bot. Ztg.* **39,** 521–530; 537–544; 553–563; 569–578; 585–596; 601–609; 617–624.
de Bary, A. (1883). *Bot. Ztg.* **41,** 38–46; 54–60.
de Bary, A. (1884). "Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bacterien," p. 558. W. Engelmann, Leipzig.
de Bary, A. (1888). *Bot. Ztg.* **46,** 597–610; 613–621; 629–636; 645–653.
de Bary, A., and Woronin, M. S. (1870). *Bot. Ztg.* **28,** 790–791; 814–815; 838–839; 856–862.
de Tournefort, JP. (1694). "Éléments de Botanique ou Methode pour Connoitre les Plantes. 3," Vol. 1, pp. 438–442, 3 Tab. 327–333 (Fungi).-L'imprimerie Royale, Paris.
Drews, G. (2000). *Freiburg. Univ. Blätter* **149,** 5–25.
Dudley, W. R. (1888). *Bot. Gazette* **13,** 305–311.
Farley, J. (1977). "The Spontaneous Generation Controversy from Descartes to Oparin." John Hopkins University Press, Baltimore, MD.
Fischer, E. (1904). "Die Uredineen der Schweiz," p. 589. K. J. Wyss, Bern.
Fries, E. M. (1821–1832). "Systema Mycologicum," Vol. 3. Ernest Mauritius, Greifswald.
Fries, E. M. (1836–1838). "Synopsis Hymenomycetum." Uppsala, Lund.
Gäumann, E. (1964). "Die Pilze," p. 541, Birkhäuser, Basel.
Hawksworth, D. L., Kirk, P. M., Sutton, D. C., and Pegler, D. N. (eds.) (1995). "Ainsworth & Bisby's Dictionary of Fungi 8th ed., CAB Internat., Wallingford, Oxon, UK, New York.
Hofmeister, W. (1851). "Vergleichende Untersuchungen der Keimung, Entfaltung und Fruchtbildung höherer Kryptogamen und der Samenbildung der Coniferen," p. 179. F. Hofmeister, Leipzig.
Hofmeister, W. (1859). Math. Phys. Klasse **6,** 533–672.
Hofmeister, W. (1861). *Abh. Sächs. Akad. Wiss. Math. Phys. Klasse* **7,** 629–760.
Hooke, R. (1665). "Micrographia or Some Physiological Descriptions of Minute Bodies Made by magnifying Glasses." J. Martyn and J. Allestry, London.
Jost, L. (1930–1931). *Zeitschr. Bot.* **24,** 1–74.
Kümmel, F. (1998). 300 Jahre Botanischer Garten der M. L. Universität Halle-Wittenberg, p. 70, Mitt. Bot. Garten der Univ. Halle-Wittenberg, Fliegenkopf, Halle.
Lamarck, J.-B. de (1809). "Philosophie Zoologieque." Paris.
Malpighi, M. (1675, 1679). "Anatomia Plantarum," Vol. 2, London.
Mayr, E. (1982). "The Growth of Biological Thought," p. 974. Harvard University Press, Cambridge, MA.
Micheli, P. A. (1729). *Trans. Roy. Soc. Canad.* **9,** 1–25.
Millardet, P. M. A. (1885). *Am. Phytopathol. Soc.* 1933.
Mullins, J. T. (1994). *In* "The Mycota," (K. Esser and P. A. Lemke, eds.), pp. 413–421. Vol. I, "Growth, Differentiation and Sexuality" (J. G. H. Wessels and F. Meinhardt, eds.), p. 433. Springer, Berlin.
Persoon, C. H. (1801). "Synopsis Methodica Fungorum, Sistens Enumerationem Omnium Hucusque Detectarem Specierum," Vol. 2, Heinrich Dieterich, Göttingen. (Reprinted in 1952 by Johnson, New York.)
Persoon, C. H. (1822–1828). "Mycologia Europaea sen Completa Omnium Fungorum in Variis Europaeae Regionibus Detectorum Enumeratio, Methodo Naturali Disposita, Descriptione Succincta, Synonymia Selecta et Observationibus Criticis Additis," Vol. 9. Johann J. Palm, Erlangen.
Porta, G. D. (1588). "Phytognomica Octo Libris Contenta." Neapel.
Prevost, B. (1807). *Phytopathol. Classics,* (6) 1939.
Purkinjê, J. E. (1839). "Über die Analogie in den Strukturelementen des Pflanzlichen und Tierischen Organismus." Breslau.
Pringsheim, N. (1855). *Gesam. Abh.* **1,** 1–34. Jena 1895.

Raper, J. R. (1939). *Am. J. Bot.* **26,** 639–680.
Raper, J. R. (1952). *Bot. Rev.* **18,** 447–545.
Rees, M. (1888). *Ber. Deutsche Bot. Ges.* **6,** VIII–XXXI.
Sachs, J. (1874). "Lehrbuch der Botanik," 4th ed, p. 846. Verlag W. Engelmann, Leipzig.
Sachs, J. (1875). "Geschichte der Botanik," p. 612. W. Engelmann, Leipzig.
Schleiden, M. J. (1842). "Grundzüge der Wissenschaftlichen Botanik nebst einer methodischen Einleitung als Anleitung zum Studium der Pflanze," 2nd ed., W. Engelmann, Leipzig.
Schmitz, F. (1879). Sitzungsberichte Botanik **36,** 345–377.
Shapin, S. (1996). "The Scientific Revolution," pp. 65–165. University of Chicago Press, Chicago.
Sparrow, F. K. (1978). *Mycologia* **70,** 222–252.
Targioni-Tozzetti, G. (1767). "Alimurgo o Sia Modo di Render Meno Gravi le Carstie, Proposto per Sollievo de Poveri." Florence. (Reprinted as Reale Accademia d'Italia. Studi e Documenti 12, 1943.) English translation of Chap. 5 by L. R. Tehon, *Phytopathol Classics* (9), *Am Phytopathol. Soc.* 1952.
Thuret, G. (1854–1855). *Ann. Sci. Nat. Bot.,* Sér *IV,* **2,** 197–214; **3,** 5–28.
Thuret, G., and Bornet, E. (1867). *Ann. Sci. Nat. Bot. Ser. V,* **7,** 137–166.
Tillet, M. (1755). *Phytopathol. Classics (5). Am. Phytopathol. Soc.* 1937.
Tulasne, L. R. (1851). *Compt. Rend. Acad. Sci. Nat. Paris* **32,** 427, 470 and (1851) *Ann. Sci. Nat. Paris* **15,** 370–380.
Tulasne, L. R. (1854). *Ann. Sci. Nat.* **2,** 76–196.
Tulasne, L. R., and Tulasne, C. (1844). *Ann. Sci. Naturel.* Sér. 3, *Botanique,* **1,** 41–107.
Tulasne, L. R., and Tulasne, C. (1847). *Ann. Sci. Nat. Paris,* Sér. 3, **7,** 12–127.
Tulasne, L. R., and Tulasne, C. (1861–1865). "Selecta Fungorum Carpologia. Vol. 1: Erysiphei," "Xylariei, V-alsei, Sphaeriei," Vol. 2. "Nectriei, Phacediei, Pezizei," Vol. 3, Imprimeri Impériale, Paris. (English translation: W. B. Grove, A. H. Buller, and C. L. Shear, eds.), The Clarendon Press 1931, Oxford.
Vaucher, J.-P. (1803). "Histoire des Conferves de'eau Douce." Genève.
Wallroth, F. W. (1825–1827). "Naturgeschichte der Flechten." Vol. 2. Frankfurt.
Wilson, C. (1995). "The Invisible World," p. 280. Princeton University Press, Princeton.

Bartolomeo Gosio, 1863–1944: An Appreciation

RONALD BENTLEY
Department of Biological Sciences
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

I. Introduction

The Italian physician, Bartolomeo Gosio (1863–1944), made diverse and important contributions to microbiology at the turn of the 19th century that have had repercussions even to the present day. One such contribution was the discovery of a pure fungal metabolite with antibiotic properties. Moreover, he demonstrated that some fungi can convert inorganic forms of arsenic to a toxic, organic gas. Gosio was also an effective public health professional devoting much effort to the control of malaria and other diseases in Italy. Nevertheless, he has remained largely unknown and/or forgotten. An Italian writer has observed that Gosio is one of those persons "who brought forth fundamental contributions to medicine, and now are at risk of being forgotten even by their fellow-countrymen" (Borra, 1988). This article provides an account of his remarkable life, one that gave rise to the possibility of a Nobel Prize. Some of the reasons why he has been forgotten not only in Italy but also in the rest of the world will be explored (Fig. 1).

II. Bartolomeo Gosio—A Brief Biography

Biographical information about Gosio is not readily available. He is not listed in English language histories of biology and microbiology although he is mentioned very briefly and inaccurately (see later) in "Morton's Medical Bibliography" (Morton, 1991). Some German and Italian reference works have brief entries for him (Olpp, 1932: Fischer,

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 48

0065-2164/01 $35.00

FIG. 1. Bartolomeo Gosio.

1932; Agrifoglio, 1952; Penso, 1987), and there are two Italian obituaries (Fermi, 1944; Jerace, 1945). There is no material relating to him in the archives of the American Society for Microbiology.

Bartolomeo Gosio was born on March 17, 1863, at Magliano Alfieri (Fig. 2) in northwest Italy. His father, Giacomo Gosio, was a veterinarian, and his mother, Antonietta Troya, was a granddaughter of Vincenzo Troya (1806–1883), a distinguished educator. Magliano Alfieri was established in Roman times and has a present-day population of about 1700; the population was slightly higher in 1861 with 1825 residents (Adriano *et al.*, 1986). It is located in the Province of Cuneo in the Piedmont region, about halfway between Genoa and Turin. Gosio clearly is a "favorite son" of Magliano Alfieri where he is memorialized by a small piazza, the "Piazza Bartolomeo Gosio" (Fig. 3). A street in Rome near Ponte Milvio, the oldest Roman bridge in that city, also bears his name.

Bartolomeo's father died soon after his son had finished elementary school at Magliano Alfieri and his mother did manual labor to support further education for him at a boarding school in the nearby town of Alba. He distinguished himself not only by scholastic skills but also by playing practical jokes. He had a passion for music and it is said that in later life he made musical compositions. An organ belonging to him was still located in the church of the Episcopal Seminary at Alba as recently as 1988 (Borra, 1988).

Since there was no Lyceum in Alba, he continued studies for 3 years at the School of Medicine of the University of Turin. After transferring to the Royal University of Rome, he obtained a doctorate degree in medicine and surgery (laurea in medicina) at the age of 25 in 1888. The degree was awarded "magna cum laude and a particularly meritorious distinction." His thesis, "The Protozoa in the Morbid Processes of the Intestine," derived from work with Professor Perroncito at the Institute of Parasitology, University of Turin. Gosio's first position was in the laboratory of bacteriology and chemistry at the Health Institute (Istituo Superiore di Sanità) in Rome. In an 1893 publication he described himself as "Assistant aux Laboratoires scientifiques de la Direction de la santé publique" (Gosio, 1893a). He also studied with M. Rubner and H. Thierfelder in Berlin gaining expertise in physiological chemistry and microbiology; moreover, he became acquainted with Robert Koch gaining his praise. In 1899, through a competitive examination and according to one writer with much opposition, he was appointed as Director of the Scientific Laboratory of the Department of Public Health (Laboratori scientifici della Direzione di Sanità) in Rome, a position that he held until retirement in 1930 (Borra, 1988). At one time he was offered a position as Professor of Hygiene at the University of Sassari, but had preferred to remain in Rome.

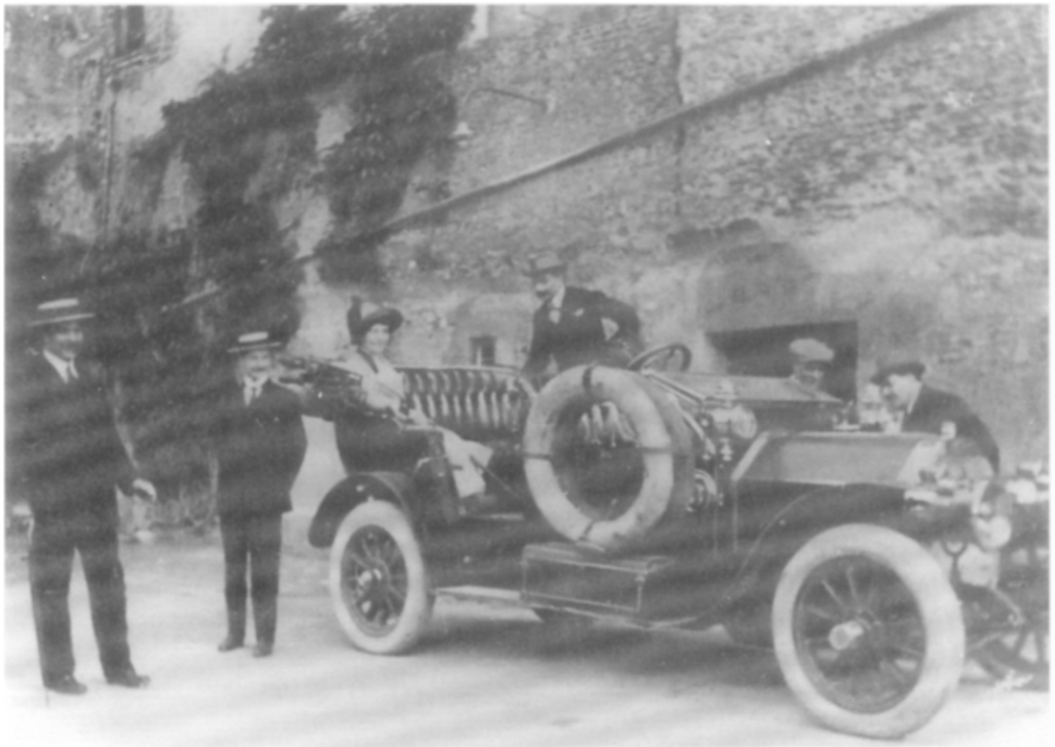

FIG. 2. Gosio arriving in Magliano Alfieri for a celebration (festeggiamenti) in 1908 in one of the first automobiles, a model T Ford, to be introduced into the village. This marked the beginning of the general use of automobiles, but the cost, 8000 lire, was still high. It is likely that the unidentified celebration was for the award of the Riberi prize; the lady in the automobile is presumably Signora Gosio.

FIG. 3. The "Piazza Bartolomeo Gosio" in Magliano Alfieri. This photograph was taken by C. Gosio in August, 1999. Although it may not be readable in the reproduction, the prominent plaque is clearly marked "Piazza Bartolomeo Gosio" in the original print.

Gosio's life covered times of great changes and encompassed the hardships of two world wars. He was "saddened and rather troubled by" the events of World War II which occurred during his last years (Borra, 1988). Italy had surrendered to the Allies on September 3, 1943, but Rome was still occupied by the Nazis at the time of his death on April

13, 1944, not being liberated until July 1944. His body was returned to Magliano Alfieri where a funeral service was held with the participation of many of the townspeople. Edoardo Borra says that the people of Magliano Alfieri felt that "like the hero in Vergil's Aenid, he embodied the sense of responsibility for the safety of others" (Borra, 1988).

III. Gosio Gas

In the latter half of the 19th century, green arsenical pigments such as Scheele's Green (copper arsenite, $CuHAsO_3$) and Schweinfurt (or Schweinfurth) Green (also named Paris Green, Venice Green, or Emerald Green, copper acetoarsenite, $3CuO.As_2O_3.Cu[OOC.CH_3]$) were widely used for an astonishing variety of decorative purposes, including foodstuffs (Bartrip, 1992, 1994; Jackson, 1996). One very extensive use of green pigments was for the coloring of wallpapers and tapestries; for example, in 1858 it was estimated that some 100 million square miles of arsenical wallpaper were present in British homes (Bartrip, 1994). Gradually, it became obvious that individuals living in rooms containing such materials were at a considerable risk. Many illnesses and fatalities were recorded and there was much controversy as to the causes. One theory held that the poisonings were due to inhalation of arsenic-containing particles. However, in 1839 the distinguished chemist, Leopold Gmelin, hypothesized the existence of a volatile arsenic compound. He had made the curious observation that a garlic odor was usually present in rooms where poisoning had occurred. That this odor is characteristic of some arsenic compounds has been known since antiquity. Since one obvious volatile compound of arsenic was arsine, AsH_3, Selmi in 1874 suggested that during growth on wallpaper and wallpaper pastes molds produced hydrogen which reduced the arsenical pigments to arsine. For many citations to this early work, see Sanger (1893a,b), Lerrigo (1932), and Challenger (1945).

It became clear that damp and moldy conditions favored the production of the gas. As early as 1891, Gosio began using the garlic odor as a microbiological assay (Gosio, 1892a–c, 1893a–c, 1901). A potato mash containing arsenic oxide was exposed to the air of a cellar and mixed cultures of fungi and bacteria capable of producing the garlic odor were isolated. Bacterial subcultures were odor-free, while several fungi produced the distinctive smell. Gosio's work was subsequently honored by naming the garlic-odored material produced by fungal volatilization of arsenic as "Gosio Gas" (Challenger *et al.,* 1933). It is probably the only gas named eponymously. One mold isolated from a carrot slice was particularly active in volatilizing arsenic; it was identified as *Penicillium brevicaule,* a fungus previously isolated from rotted paper. Gosio stated

that the gas was very toxic and that it was dangerous to approach this fungus growing in the presence of arsenic. A rat exposed to the vapor was quickly killed. In 1907, *Penicillium brevicaule* was reclassified into a new genus, *Scopulariopsis,* and it is now known formally as *Scopulariopsis brevicaulis* (Sacc.) Bainier (Raper and Thom, 1949).

In Germany, Emmerling had been unable to show arsenic volatilization by various fungi and doubted the truth of Gosio's statements (Emmerling, 1897). Gosio responded vigorously to this criticism and regretted that Emmerling had not attempted to obtain a culture of *S. brevicaulis* (then termed *P. brevicaule*) from him (Gosio, 1897). In the United States, C. R. Sanger, working at the Harvard Laboratory had investigated cases of arsenical poisoning from wallpapers beginning in 1886 and like Emmerling had been unable to demonstrate volatilization of arsenic (Sanger, 1893a,b). However, unlike Emmerling, he corresponded with Gosio, received cultures of *S. brevicaulis* and quickly confirmed that Gosio was correct.

In early work, Gosio believed that one organism, *Mucor mucedo,* did form arsine. However, analyses of the gas produced by *S. brevicaulis* revealed the presence of carbon. Work by Gosio and his colleague Biginelli, in 1901, suggested that the volatile material was diethylarsine $(C_2H_5)_2AsH$ (Gosio, 1901; Biginelli, 1900a,b). More than 3 decades later, Gosio Gas was reexamined by Frederick Challenger and his colleagues at the University of Leeds and shown to be trimethylarsine, $(CH_3)_3As$ (Challenger *et al.,* 1933; Challenger, 1945). Biomethylation of arsenic and other metalloids is today an important topic with much research in mammals as well as in microorganisms (Aposhian, 1997; Thayer, 1993).

Gosio developed a sensitive, smell-based test for the presence of arsenic using *S. brevicaulis* (Gosio, 1892b, 1893b, 1932). A suspected material was extracted with water or dilute acid and the extract was evaporated. Small quantities of the residue were added to a sterile potato slice inoculated with *S. brevicaulis.* After a few hours of incubation at 25–30°C, a garlic odor indicated the presence of arsenic. As little as 1 μg arsenic oxide in 1 g of material could be detected. In the earlier Marsh test, the material suspected to contain arsenic was reduced with zinc and acid, and the resulting arsine gas decomposed by heat to yield a black deposit. The microbiological Gosio test was qualitatively more sensitive than the Marsh test but was not adapted for quantitative use. Gosio's test was later modified by using Czapek's solution agar for the growth of *S. brevicaulis* (Smith and Cameron, 1933).

In recent years, Gosio's work on arsenic volatilization came to attention in connection with the "toxic gas" theory for "Cot Death" or "Sudden Infant Death Syndrome" (cot is the UK word for crib). It had

been proposed that *S. brevicaulis* could degrade fire retardant chemicals present in crib mattress fillings and covers with the subsequent release of toxic gases such as phosphine, arsine, or stilbene (Richardson, 1994). In New Zealand, the theory was vociferously championed with accusations of a "cover up" by various authorities (Sprott, 1996). In 1994 the Chief Medical Officer of the British Department of Health set up an independent advisory committee (The Expert Group To Investigate Cot Death Theories: Toxic Gas Hypothesis) chaired by Lady Limerick. Old data were reevaluated and Richardson's experiments were repeated with his cooperation. Organisms isolated from used mattresses were visually similar to *S. brevicaulis* as described by Richardson, but microscopic examination showed them to be bacteria. In a final report to the British Government Department of Health, The Limerick Committee found no evidence to support the toxic gas hypothesis; their report is available at the following URL: www.doh.gov.uk/limerch.htm. Nonetheless, Web material for the group, Crib Life 2000, contains a page titled "Gosio, the Chemist" promoting the toxic gas theory (www.criblife2000.com/gosio.htr). This Web site contains several important factual errors.

IV. Mycophenolic Acid

At about the same time as his work on the fungal volatilization of arsenic, Gosio also investigated pellagra, then a major problem in Italy and elsewhere (Gosio, 1893c, 1896a,b). Pellagra was concentrated in areas where corn was a major dietary staple; at that time, of course, the role of vitamins in human nutrition had not yet been discovered. Gosio hypothesized that corn spoiled by fungal action might contain toxic metabolites and that consumption of these metabolites might give rise to pellagra symptoms. He isolated a fungus from deteriorated corn and described it as *penicillium glaucum (sic)*, a name then assigned to a variety of green or blue-green molds. Later work makes it likely that his organism was actually *Penicillium brevicompactum* (Clutterbuck *et al.*, 1932).

From culture filtrates of this organism, Gosio isolated a crystalline material with phenolic properties that gave a blue to violet color with ferric chloride solutions. He did not name his material, presumably because he believed it was a known compound, *p*-hydroxyhydrocinnamic acid. However, it is now accepted that his phenol was identical to mycophenolic acid, a fungal metabolite rediscovered and characterized in 1913 from *Penicillium stoloniferum* (Alsberg and Black, 1913). The fungal strain used by these American workers had originally been isolated from spoiled corn in Italy. *P. stoloniferum* is now regarded as synonymous

with *P. brevicompactum* (Pitt, 1979). The correct structure for mycophenolic acid, (*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoic acid, was finally elucidated by Harold Raistrick and his colleagues some 60 years after its discovery by Gosio (Birkinshaw *et al.*, 1952).

Gosio had insufficient material to test whether his purified fungal metabolite produced the pellagra symptoms in animals. Nonetheless, he made the very important observation that the phenol had an antiseptic action since it inhibited the growth of the anthrax bacillus. In his words, "Sperimentati su culture pure di bacilli del carbonchio, dimostrarono notevole potere antisettico." ("Bacilli del carbonchio," carbuncle bacillus, is an old term used for anthrax.) This finding and a clear account of the chemistry of his phenol are given in his preliminary communication (Gosio, 1893c). The antibiotic action ("impedi lo sviluppo al b. del carbonchio") was discussed in more detail in later publications (Gosio, 1896a,b; Gosio and Ferrati, 1896). The later papers of 1896, rather than that of 1893, have often been cited incorrectly for the discovery of mycophenolic acid.

Curiously, mycophenolic acid was rediscovered on at least two later occasions, both as part of the post-penicillin hunt for further antibiotics and other physiologically active metabolites. In 1945, an antibiotic material present in a *Penicillium brevicompactum* strain (Wilkinson and Harris, 1943) was shown to be mycophenolic acid by Howard Florey and his colleagues at Oxford (Abraham, 1945; Florey *et al.*, 1946). Thus, only after the development of penicillin as an antibiotic was the historical significance of Gosio's observation fully recognized. At a time when the concept of antibiosis was in its infancy (the French word, "antibiose," was invented in 1890), Gosio had observed an antibiotic action by a highly purified fungal metabolite. Howard Florey and his colleagues bestowed the following accolade on Gosio's work: "mycophenolic acid enjoys the distinction of being the first antibiotic produced by a mould to be crystallised" (Florey *et al.*, 1946). Crystallinity being synonymous with purity, mycophenolic acid was the first well-characterized antibiotic ever (the qualification, "produced by a mould" is clearly not needed). Significantly, the classical paper on penicillin (Fleming, 1929) and the paper by Howard Florey and his group describing the development of penicillin (Chain *et al.*, 1940) make no reference to Gosio's work.

The second rediscovery of mycophenolic acid was in 1968 when two groups independently obtained it by the use of screening programs for antiviral activity from "*Penicillium stoloniferum*" and from a soil *Penicillium* (Williams *et al.*, 1968a,b; Ando *et al.*, 1968; Cline *et al.*, 1969). Mycophenolic acid is a truly remarkable metabolite. In addition to its

antibiotic activity, it also has antifungal, antiviral and antitumor properties, and has been used clinically in treatment of psoriasis (Jones *et al.*, 1975; Marinari *et al.*, 1977; Spatz *et al.*, 1978). More recently, it has been recognized as a powerful immunosuppressant. It was licensed in 1995 for use in kidney transplantation, and in 1998 for heart transplantation, in combination with other materials (Wu, 1994; Bentley, 2000). Mycophenolic acid is administered as a prodrug, the 2-morpholinoethyl ester, mycophenolate mofetil (brand name, CellCept). The prodrug form readily hydrolyzes in the body to the free acid. This once obscure fungal metabolite, originally investigated by an Italian physician as a possible cause of pellagra symptoms, has had a remarkable journey. It is not clear why Gosio did not continue further investigations on mycophenolic acid. Perhaps at that time it was too difficult to obtain it in significant quantities and perhaps also it became clear that it was not involved in the etiology of pellagra.

V. Other Work in Microbiology

Gosio studied the metabolism of cholera and cholera-like bacteria in Berlin in 1893, and of the bubonic plague organism in Rome in 1897. Also in Rome, he investigated the fermentation of coumarins (1907), studied the color reactions of sulfur bacteria and the diagnostic value of arbutin for dysentery bacilli (1917), and carried out studies on the diphtheria bacillus (1920, 1921). This is an incomplete survey of his lesser-known work (see later under Publications and Awards). In addition, he devised an instrument, termed in Italian, *vaccinoscopio,* a small and useful instrument permitting rapid and easy titration of bacterial vaccines. It apparently resembled a nephelometer, probably using calibrated standards of an inorganic material.

Following his work on the volatilization of arsenic, Gosio also studied the microbial decomposition of salts of selenium and tellurium with emphasis on the action of bacteria rather than fungi. Bacteria reduced these materials fairly quickly forming colored solutions or even precipitates, probably by formation of the elemental forms of selenium or tellurium (Gosio, 1904a,b). Other workers had investigated these reactions in a preliminary fashion, but Gosio extended the studies to include 181 organisms. Moreover, he adopted the color reactions accompanying the reduction of tellurium to develop a method for the determination of the "visible sterility" of preparations of sera, culture media, etc. The reductive reaction was an indicator of contamination. A small volume of a tellurite solution was added, for instance, to a serum preparation. If the preparation contained live bacteria, a distinct gray to black coloration or precipitate developed.

The reaction was most readily obtained in nutrient broth or milk, being reduced in the presence of serum. The sensitivity of the reaction could be increased by the addition of a small amount of sucrose. Gosio's definitive paper on this topic, written in German, runs to 60 pages (Gosio, 1905). The ability of various bacteria to carry out the reduction was variable so the method could not have been totally reliable and the presence of bacterial spores presented a problem. Nevertheless, in an era when sterilization procedures were quite primitive, it was a useful test. A few years later, potassium tellurite was also recommended as an indicator of microbial life by American workers (King and Davis, 1914).

Just prior to Gosio's work on selenium and tellurium reductions, the formation of a skatole-like odor from selenium compounds and a garlic-like odor from tellurium compounds had been reported with the agency of *S. brevicaulis;* moreover, the possibility that the presence of these elements could interfere with the microbiological test for arsenic was noted (Rosenheim, 1902). Three decades later, these odoriferous materials were finally identified as dimethyl selenide and dimethyl telluride, respectively (Challenger and North, 1934; Challenger, 1945). Surprisingly in view of his work on arsenic volatilization, Gosio seems to have had little interest in the formation of these volatile compounds from selenium and tellurium compounds. He made some use of these reductive properties in classifying bacteria (Gosio 1904a,b) and to some extent, selenite and tellurite containing media are still used in bacterial taxonomy in a limited number of cases (Levine, 1925; Lapage and Bascomb, 1968).

VI. Bartolomeo Gosio—A Public Health Official

In 1898, Robert Koch turned his attention to the prevention of malaria, especially in Italy, the major area for malaria in Europe (Anon, 1899; Koch, 1899). By that time, Ross's work with birds had indicated transmission of the parasite by mosquitoes. An "antimalarial campaign" was initiated in Grosseto and Koch was in Italy from August 11, to October 2, 1898, where he began to work out his methods for using quinine not only to treat those in the late stages of the disease but also prophylactically for those with latent malaria. The Italian authorities facilitated the work by assigning Gosio to the project. Koch acknowledged gracefully that Gosio deserved thanks for the efficiency and success of this expedition which had been organized in a comparatively short time (Koch, 1899). It is perhaps typical of the lack of recognition given to Gosio that his very important role in this initial malaria campaign is not mentioned by Brock (1988) in his biography of Koch, although Brock does list Koch's German colleagues. A more balanced account is presented in

the more detailed biography of Koch (Möllers, 1950) where many contacts between the two men are noted and where Gosio's contributions are acknowledged. There do not appear to be any joint publications resulting from these Koch–Gosio collaborations on malaria control.

In the early years of the 20th century, Gosio continued to work on malaria control in Italy. His laboratory in Rome was named "Laboratorio Batteriologico e Medico-micrografico" and he was the director of a permanent corps of workers focused on antimalarial campaigns in the area around the city of Grosseto and elsewhere. According to published records (Fermi, 1944; Agrifoglio, 1952) an initial report, "La Malaria di Grosseto nel 1899" was prepared for the Interior Ministry in 1900 but it has not been located by this writer. A very lengthy report, "La Campagna Antimalarica dell'Anno 1901" was also submitted (Gosio, 1902).

At that time, living conditions for the poor people in Italy were appalling. In 1910, the rural city of Andria in Southern Italy had 60,000 inhabitants "who were compelled to live in a very confined district of houses, almost without utilities, with 30,000 peasants living in real grottos, several feet below the ground, where in a few square yards of space languished up to 10 people, often together with their animals" (Borra, 1988). In one city, Gosio was described as receiving a horrifying image of a region "stricken with malaria, drought, famine, massacres, hail (that) is now stricken by cholera." The implacable foes were said to be politicians, bandits, administrators, and rich landed proprietors but the "worst factors" were to be found in Rome—presumably, the bureaucrats. It was even hinted that the Direzione Generale di Sanità had invented the cholera problem "to fabricate a merit for itself with its easy, fast cure" (Borra, 1988). It must have required considerable diplomacy and patience for Gosio to operate scientifically under those conditions.

Gosio emphasized the principle of "human reclamation," that is the use of early, energetic, and rational cure of patients to prevent or reduce relapses. Moreover his antimalarial work was extended to other parts of Italy such as Calabria and Basilicata. He was instrumental in founding the School of Malariology in Nettuno and in setting up antimalarial farming colonies (e.g., in Borghetto) as well as summer camps for children with malaria. He established several sanatoria, the first being in Rocca di Papa (south east of Rome) in 1909. One of his very last publications concerned sanatoria for children (Gosio, 1936).

Tuberculosis also attracted his interest with much research being carried out over the 12-year period, 1903–1914. This work focused on the relationship between human and bovine tuberculosis using both experimental and epidemiological approaches; it was summarized in a three-volume work (Gosio, 1912–1915). Gosio himself authored

3 chapters of this work and co-authored a fourth; the remaining 19 chapters were written by the personnel of his Laboratory under Gosio's guidance.

Another public health achievement by Gosio concerned a disease called "Haffkrankheit" (bay illness) experienced by fishermen and their families who made use of a lake inlet between Danzig and Königsberg (Borra, 1988). Beginning about 1925, many members of this population experienced severe pain in joints and muscles; 600 cases and several deaths were reported. Gosio implicated two cellulose factories using an arsenic-containing pyrite ore. Wastes from roasting the ore, presumably to obtain sulfur dioxide, were discharged into the River Haff and Gosio isolated fungi, including *Scopulariopsis brevicaulis* from mud at the bottom of the bay. Hence, he suspected poisoning by formation of volatile (and possibly other) compounds of arsenic. Measures were instituted to deal with the waste problem and the people's health improved. Gosio and his colleagues were also very active in the influenza pandemic of 1918. In particular, he attempted to determine whether the Pfeiffer bacillus (*Hemophilus influenzae*), discovered in 1892, was involved in the etiology of the disease.

His later years were also spent in the service of Public Health in Italy and throughout this long career he was completely dedicated to his responsibility for the well being of the Italian people. One writer has contrasted his official work with that of some of the bureaucrats as follows: "He is yet another example of the cultural value of the officials of the health department, who too often are considered to be bureaucrats that have fossilized among the files of their offices" (Fermi, 1944).

VII. Publications and Awards

Gosio was awarded the Riberi prize (premio Riberi) in 1908 (Bozzolo *et al.*, 1908). This prize derived from an 1861 bequest of Alessandro Riberi who died in Turin; eventually the bequest was used for an award in an international competition for the scientific discovery most useful to medicine. The prize was administered by the Royal Academy of Medicine of Turin (Reale Accademia di Medicina di Torino) and initially covered work over a time period of 3 years. However, the funds being limited, the term was changed for the 7th prize to 5 years, 1882–1886 (Bobbio, 1938). Gosio received the 11th award (11^0 Premio Riberi, 1902–1906) of 20,000 lire while he worked with the Public Health Ministry in Rome. There had been 13 contestants for the 11th award, one of whom had withdrawn (Bozzolo *et al.*, 1908). Gosio's "interesting and original studies" were described as follows and need no translation: "Sulle bioreazioni dell'arsenico, tellurio e selenio e loro applicazioni

pratiche." Robert Koch congratulated Gosio on this award in a letter from Baltimore, dated October 6, 1908. One of the other contestants was Adelchi Negri who is remembered today for his discovery of the Negri bodies—spherical inclusion bodies used as a determinant in diagnosis of rabies. Although it was suggested that Negri would be a good candidate to receive a future Riberi Prize, this did not happen. Funds being exhausted, the prize was discontinued in 1942 (Personal communication from Professor N. Riccardino).

In addition to the Riberi Prize, Gosio received several other awards and gold medals including one from the Tropical Institute in Hamburg, and one awarded at the Chemical Congress in Turin (1925). In 1928, he was honored with the Pagliani Prize as the most distinguished Public Health Hygienist in Italy. An obituary notes that he was proposed as a candidate for the Nobel Prize in 1923, presumably for medicine or physiology (Fermi, 1944).

Gosio published many papers, writing in French and German as well as Italian. One biographical source lists the titles and publication dates for some 70 papers (Agrifoglio, 1952). This listing is clearly incomplete and in any case does not indicate the journals in which the papers appeared. In addition to the previously mentioned three-volume treatise on tuberculosis, he also published at least seven other books (see Table I). The last paper indexed by Chemical Abstracts and/or Biological Abstracts under Gosio's name concerned his biological test for arsenic (Gosio, 1932). However, there were a few later papers that were not

TABLE I

SOME BOOKS PUBLISHED BY BARTOLOMEO GOSIO[a]

1893. " Analisi batteriologica e chimica di un'acqua termominerale dei Bagnoli (Napoli)." Tipografia delle Mantellate, Roma.

1894. "Analisi chimica dell'acqua termominerale." A. Tocco, Napoli.

1901. With L. Bonavia. "Manuale pratico di chimica applicata all'igiene." Roux e Viarengo, Torino.

1907. "Contributo alla diagnosi della pellagra con particolare riguardo ai suoi stadi iniziali." Tipografia delle Mantellate, Roma.

1908. "Un triennio di lotta antimalarica nelle Calabrie e Basilicata; studi e proposte." Tipografia dell'Unione cooperativa editrice, Roma.

1925. "Guida alla lotta contro la malaria: cinque lezioni per il personale ausiliario nella lotta contro la malaria." Provveditorato generale dello stato libreria, Roma. There is a record of a 3rd revised edition in 1926.

1925. "Organizzazioni antimalariche alla luce delle nuove dottrine." Provveditorato generale dello stato libreria, Roma.

[a] This listing may be incomplete since in some of the biographies it is impossible to distinguish between papers and books. However, the books listed here have been verified by library sources.

indexed by these services; one example, already noted, was his paper on sanatoria for children (Gosio, 1936).

VIII. Why Is Bartolomeo Gosio Generally Forgotten?

At the time of Gosio's work on mycophenolic acid, the political situation in Italy was unsettled. Only in 1870 had national unification been achieved and Gosio was, in fact, born during the Risorgimento (*ca.* 1815–1870). This situation at the turn of the 19th century helped to ensure that Italy did not play a major role in microbiological research, the main centers for such work being Denmark (Hansen), France (Pasteur), Germany (Koch), and The Netherlands (Beijerinck). Indeed, as already noted, Gosio left Italy to study in Germany as part of his training.

Linked to the geographical question is that of scientific journals and the languages in which they were published. Interestingly, it has been pointed out that "virtually all the work on antagonisms—apart from observations in the 1870s—was done outside English-speaking countries" (Crellin, 1980). Most of the work was published in French or German, with very little research recorded in Italian. Many of us of a certain age will remember the dreaded "foreign language requirement" for the Ph.D. degree, an examination to assure that we were prepared to read literature in a language other than English. It is safe to say that this requirement was rarely fulfilled with Italian. A further consideration is that the journal in which he published four important papers, *Rivista d'Igiene e Sanità Pubblica,* cannot be considered a "main-line" journal for microbiological discoveries. Today, it is difficult to access—copies of the 1896 work on mycophenolic acid were finally obtained by this writer through the kindness of Dr. C. Serarcangeli of the Museum of the History of Medicine in Rome. In 1957, a writer concerned about the lack of awareness of Russian publications asked the following rhetorical questions: "... what would have happened if Gibbs' phase rule papers had first appeared in a more widely read journal? Or if Gosio's 1896 paper, which noted the antibiotic properties of a *Penicillium* strain, had appeared in *Science* instead of in an Italian sanitary engineering journal?" (O'Dette, 1957). The point is well made.

One factor possibly contributing to Gosio's neglect is that his work on the antibiotic action of mycophenolic acid was not mentioned in a supposedly comprehensive book entitled "Les Associations Microbiennes. Leurs Application Thérapeutiques" (Papacostas and Gaté, 1928). This book has often been cited as providing a detailed review of the early developments in work on microbial antagonisms. Not only does it omit mention of Gosio's work on mycophenolic acid but work by another Italian investigator, Vincenzo Tiberio, dealing with early experiments

on microbial antagonisms involving fungi is also ignored (Pezzi, 1946). While Gosio is usually cited briefly in more recent historical accounts of the development of antibiotics, the important fact that he worked with a purified fungal metabolite rather than ill-defined extracts rarely is emphasized (Korzybski *et al.*, 1967; Crellin, 1980).

Gosio's eclecticism also contributes to his neglect since his research work was in disparate fields thereby tending to dilute his impact. The focused work on arsenic volatilization together with his related work on selenium and tellurium led to the Riberi Prize. However, this Italian Prize is not well known and in any case is now discontinued. Nor did Gosio exploit his discovery of mycophenolic acid.

Perhaps most problematic for Gosio' subsequent fame is that his chemical identifications were incorrect for both Gosio Gas and mycophenolic acid. In both of these cases, he was misled by incorrect analytical data. In fairness, it should be noted that "Nineteenth-century organic chemists faced tremendous difficulties" (Brock, 1993) nowhere more so than in the determination of elemental compositions of organic compounds. While Liebig's work on analysis brought some improvement by 1830, analyses for elemental content were always difficult, tedious, and time consuming. When Liebig recommended his method as a time saver, "he could only claim four hundred analyses per year with an army of research assistants" (Brock, 1993). Certainly Gosio, not located in a large department of organic chemistry and with little or no technical assistance, faced serious difficulties. For mycophenolic acid he recorded (Gosio, 1896a) a single combustion analysis requiring 150 mg of precious material (he used only 5 mg in his antibiotic study). His results suggested $C_9H_{10}O_3$ rather than the now accepted $C_{17}H_{20}O_6$:

Found:	C, 65.21; H, 6.34%
$C_9H_{10}O_3$ requires:	C, 65.06; H, 6.02%
$C_{17}H_{20}O_6$ requires:	C, 63.75; H, 6.25%

Under better conditions and with sufficient material to make repeat analyses, Gosio would have fared better.

Problems also beset the analysis of Gosio Gas by his colleague, Biginelli (1900a,b). It had been found that Gosio Gas forms a crystalline complex with mercuric chloride in hydrochloric acid; this mixture is often referred to as Biginelli's Solution. For the purpose of elemental analysis, a crystalline compound such as this complex was a much better candidate than a toxic gas. When such an elemental analysis was carried out on the mercuric chloride complex, the results, given in the following, suggested that Gosio gas was diethylarsine. However, the data are not really a good fit for the proposed structure. Moreover, they are not reconcilable with the now accepted fact that the analyzed

material was the mercuric chloride complex of trimethylarsine. Biginelli's material had not been recrystallized and was thus contaminated to some extent with other materials.

Found:	C, 6.15; H, 1, 56; Cl, 20.70; Hg, 59.35; As, 11.26%
$(C_2H_5)_2AsH.2HgCl_2$ requires:	C, 7.10; H, 1.63; Cl, 21.00; Hg, 59.17; As, 11.09%
$(CH_3)_3As.2HgCl_2$ requires:	C, 5.43, H, 1.35; Cl, 21.41; Hg, 60.5; As, 11.30%

Analytical difficulties, faced by many chemists, began to be overcome when F. Pregl introduced microanalytical techniques in 1917, so that elemental compositions could be obtained with as little as 5 mg of material. This improvement was, of course, too late to help Gosio. The fact remains that the chemical structures he postulated resulting from faulty analytical data have done his historical reputation a disservice.

When Gosio finally received praise and recognition from Florey's group, he had been dead for two years. Moreover, the tribute was casually placed in a brief discussion in a strictly medical journal (Florey *et al.,* 1946) and has received little attention. At that time there was so much excitement from the flood of work on antibiotics that it was only too easy to overlook Florey's accolade. In addition, Gosio did not die at an opportune time to receive recognition since the forces of the United States and United Kingdom were still waging their bitter war with the Axis powers with Rome remaining occupied by the Nazis. His death was not noted by the English-speaking world and the only obituaries appear to be in Italian. It would be nice to think that he became aware of the antibiotic revolution in the last years of his life, but given war-time conditions, it is, perhaps, unlikely.

Finally, it must be noted that Gosio has received some limited publicity for a discovery he did not make. While his observation of the inhibition of the growth of the anthrax bacillus by a purified fungal metabolite is certainly a landmark event in the history of medicine and microbiology, a sad misunderstanding has led him to be cited erroneously as the discoverer of penicillin itself. For instance, Penso has implied that the phenolic acid from *Penicillium glaucum* (*i.e.,* mycophenolic acid from *Penicillium brevicompactum*) was almost certainly penicillin: "Quasi certamente si trattava di penicilline cristallizzata" (Penso, 1987). Moreover, Borra has stated that the Museum of the History of Medicine (Museo di Storia della Medicina) in Rome has a glass vial with the label of the Laboratorio Batteriologico della Sanità Pubblica and in "faded ink," the words "arsina penicillare" (Borra, 1988). Borra identifies this exhibit as "The first penicillin vial !" ("Il primo flacone di penicillina !"). A photograph of this still existent vial was kindly provided to this writer by Dr. C. Serarcangeli. It shows that the writing does not appear to be faded, and that the description, as quoted by Borra, is incomplete. The

label actually reads "arsina penicillare comp. mercurico." Hence, it is clear that the material is not penicillin but actually a sample of the mercuric chloride complex of Gosio Gas. As already indicated, this mercuric chloride complex was utilized by Gosio and his colleague, P. Biginelli, in studying the chemical composition of Gosio Gas itself. In any event, the term "Arsina penicillare" never made any sense. The error appears to have originated with the donor of the vial, Professor B. Gosio, Jr., probably a grandson of Bartolomeo. The Museum Catalog edited by Professor Pazzini in 1958, described the acquisition as follows: "Glass vial containing the first penicillin extract made by Bartolomeo Gosio in 1896" ("Flaconcino di vetro contenente il primo estratto penicillare ottenuto da Bartolomeo Gosio nel 1896").

One of the very few contemporary English references to Gosio is in *Morton's Medical Bibliography* (Morton, 1991). Lamentably, this book makes a similar error by claiming that Gosio "first recorded scientific observations on the action of a penicillin." This text describes Gosio's "penicillin" as an antibacterial crystalline substance from *Penicillium glaucum;* the accompanying citation is to the work on mycophenolic acid. Similarly, the *Encyclopedia of Medical History* (McGrew, 1985) states that the "antibacterial effect of a penicillin (from *Penicillium glaucum*) was first recorded by Bartolomeo Gosio." These claims are most unfortunate misrepresentations of the actual facts. While Gosio deserves much credit for being the first to observe the antibacterial action of a purified fungal metabolite, mycophenolic acid cannot possibly be classed as "a penicillin." There is absolutely no connection between the mycophenolic acid phthalide structure containing only C, H, and O ($C_{17}H_{20}O_6$) and the penicillin structure, a thiazolidine-β-lactam containing N and S in addition to C, H, and O (e.g., for benzylpenicillin, $C_{16}H_{18}N_2O_4S$). The confusion may have arisen because both mycophenolic acid and penicillin are produced by species of *Penicillium*.

IX. Some Final Comments

While, by all accounts, Gosio enjoyed a full, rewarding, and happy life and made significant discoveries in microbiology, he has remained unsung and under appreciated among English-speaking peoples. Well trained in basic sciences such as biochemistry, chemistry, microbiology, and physiology, Gosio's primary vocation was medicine. A decisive step early in his career was his appointment as Director of the Scientific Laboratory of the Italian Department of Public Health. Concomittantly, his work turned from basic to applied research, with his talents directed to the alleviation of communicable diseases such as influenza, malaria, and tuberculosis. Even his microbiological research actually originated

in work on Public Health problems. Mycophenolic acid was discovered in attempts to understand the major problem of pellagra. Similarly, work on the fungal volatilization of arsenic derived from health considerations—many individuals, not only in Italy, were sickened by the formation of Gosio Gas from arsenic-containing wallpaper.

Although physicians have made distinguished contributions to basic science, it is hard to find examples of scientific distinction among those who have had the arduous role of being largely responsible for the Public Health of an entire nation. Gosio's microbiological discoveries came early in his career and after 1899 administrative matters and the necessity for the struggle against disease must have taken all of his energy. It is only today that his early work can be fully appreciated. Mycophenolic acid has undergone a wondrous journey to become a much used immunosuppressant for organ transplantation. In addition, the microbial volatilization of arsenic and other metalloids is now of much concern in connection with toxicity and environmental problems.

ACKNOWLEDGMENTS

The Internet provided the name and address of Cristina Gosio, Professor of Financial Mathematics, Facoltà di Economia, University of Genoa; thinking that she might be related to Bartolomeo I contacted her. Although not so related, she carried out significant research, including a visit to Magliano Alfieri, and obtained for me the publication, "Gente di Magliano" and photographed the Piazza Bartolomeo Gosio. I am truly grateful for her overwhelmingly generous response and I regret that she felt unable to co-author this paper. Signora Susanna Gosio Farano, a granddaughter of Bartolomeo who had supplied photographs for "Gente di Magliano," kindly sent me the account of Bartolomeo's life by Edoardo Borra. I am grateful to Professor Nicola Riccardino, Il Direttore della Biblioteca, Accademia di Medicina di Torino, who provided valuable information about the Riberi Prize. Dottoressa C. Serarcangeli sent me photographs of and information about the vial in the Museo di Storia della Medicina in Rome as well as material relating to Vincenzo Tiberio. I am most grateful for her assistance and I owe thanks to Dr. G. Macino for putting me in touch with her. Jeff Karr, Archivist of the American Society of Microbiology, kindly located references to Gosio in the biography of Koch by Möllers, and Joan W. Bennett provided many very helpful suggestions. Much assistance in obtaining not easily available citations came from Drynda Johnston and Ann Rogers of Langley Library, University of Pittsburgh. I thank my Pittsburgh friends, Marisa Smith and her mother, Tina Popolizio, for some translations from Italian; other translations were obtained from professional services.

REFERENCES

Abraham, E. P. (1945). *Biochem. J.* **39,** 398–408.

Adriano, A., Aimassi, M., Cardinali, V. G., and Giudice, C. (1986). "Gente di Magliano." pp. 11–13. Editore pro loco di Magliano Alfieri.

Agrifoglio, L. (1952). "Igienisti Italiani degli Ultimi Cento Anno." pp. 100–105. Hoepli, Milan.

Alsberg, C. L., and Black, O. F. (1913). "Contributions to the study of maize deterioration. Biochemical and toxicological investigations of Penicillium puberulum and Penicillium stoloniferum." U. S. D. A. Bureau of Plant Industry, Bulletin No. 270, Government Printing Office, Washington DC.

Ando, K., Suzuki, S., Tamura, G., and Arima, K. (1968). *J. Antibiot.*, **21,** 649–652.

Anonymous (1899). *Deut. Med. Woch.* **XXV,** 69–70 (2 Februar).

Aposhian, H. V. (1997). *Annu. Rev. Pharmacol. Toxicol.* **37,** 397–419.

Bartrip, P. (1992). *Med. Hist.* **36,** 53–69.

Bartrip, P. W. J. (1994). *Engl. Historical Rev.* **109,** 891–913.

Bentley, R. (2000). *Chem. Rev.* **100,** 3801–3825.

Biginelli, P. (1900a). *Atti Reale Accad. Lincei* **9,** 210–214.

Biginelli, P. (1900b). *Atti Reale Accad. Lincei* **9,** 242–249.

Birkinshaw, J. H., Raistrick, H., and Ross, D. J. (1952). *Biochem. J.* **50,** 630–634.

Bobbio, L. (1938). *Giornal. Real. Accad. Med. Torino* **Anno Cl–51,** 535–547.

Borra, E. (1988). "Alba Pompeia. Rivista Semestrale Di Studi Storici, Artistici, e Naturalistici per Alba e Territori Connessi. Nuova serie—**Anno IX**—Fascicolo II–II Semestre, 79–84."

Bozzolo, C., Fusari, R., Bajardi, D., Abba, F., and Martinotti, E. (1908). *Giornal. Real. Accad. Med. Torino* **Anno LXXI—Serie IV 14,** 45–47.

Brock, T. D. (1988). "Robert Koch. A Life in Medicine and Bacteriology." Science Tech Publishers, Madison, WI.

Brock, W. H. (1993). "The Norton History of Chemistry." pp. 194–209. Norton & Co., New York.

Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G. (1940). *Lancet,* 226–228.

Challenger, F. (1945). *Chem. Rev.* **36,** 315–361.

Challenger, F., Higginbottom, C., and Ellis, L. (1933). *J. Chem. Soc.* 95–101.

Challenger, F., and North, H. E. (1934). *J. Chem. Soc.* 68–71.

Cline, J. C., Nelson, J. D., Gerzon, K., Williams, R. H., and DeLong, D. C. (1969). *Appl. Microbiol.* **18,** 14–20.

Clutterbuck, P. W., Oxford, A. E., Raistrick, H., and Smith, G. (1932). *Biochem. J.* **26,** 1441–1458.

Crellin, J. K. (1980). *In* "The History of Antibiotics." (J. Parascandola ed.) pp. 5–13. American Institute of the History of Pharmacy, Madison WI.

Emmerling, O. (1897). *Ber. Deutsch. Chem. Ges.* **30,** 1026.

Fermi, C. (1944). *Rivista di Malariologia* **23,** 70–72.

Fischer, I. (1932). "Biographische Lexikon der hervorragenden Ärzte der letzten fünfzig Jahre." p. 519. Urban & Schwarzenberg, Berlin.

Fleming, A. (1929). *Brit. J. Exp. Path.* **10,** 226–236.

Florey, H. W., Gilliver, K., Jennings, M. A., and Sanders, A. G. (1946). *Lancet* 12 January, 46–49.

Gosio, B. (1892a). *Rivista d'Igiene e Sanità Pubbl.* **3,** 201–230.

Gosio, B. (1892b). *Rivista d'Igiene e Sanità Pubbl.* **3,** 261–273.

Gosio, B. (1892c). *Science* **19,** 104–106.

Gosio, B. (1893a). *Arch. Ital. Biol.* **18,** 253–265.

Gosio, B. (1893b). *Arch. Ital. Biol.* **18,** 298–305.

Gosio, B. (1893c). *Giornal. Real. Accad. Med. Torino* **61,** 484–487.

Gosio, B. (1896a). *Rivista d'Igiene e Sanità Pubbl.* **7** (21), 825–849.

Gosio, B. (1896b). *Rivista d'Igiene Sanità Pubbl.* **7** (22), 869–888.

Gosio, B. (1897). *Ber. Deutsch. Chem. Ges.* **30,** 1024–1026.

Gosio, B. (1901). *Arch. Ital. Biol.* **35,** 201–211.

Gosio, B. (1902). "La Campagna Antimalarica dell'Anno 1901." A report to Ministero dell'Interno, Ispettorato Generale della Sanità Pubblica. Tipografia delle Mantellate, Rome.
Gosio, B. (1904a). *Atti Accad. Lincei* **13** (1), 422–427.
Gosio, B. (1904b). *Atti Accad. Lincei* **13** (1), 642–645.
Gosio, B. (1905). *Zeit. Hyg. Infektionskrank.* **51,** 65–125.
Gosio, B. (1912–1915). "Studi sui rapporti fra tubercolosi umana e bovina. Ricerche sperimentali ed epidemiologiche." **I–III.** Tipografia delle Mantellate, Roma.
Gosio, B. (1932). *Ist. Sieroterap. Milan Boll.* **11,** 597–602.
Gosio, B. (1936). *Rivista di Malariol.* **15,** 345–357.
Gosio, B., and Ferrati, E. (1896). *Rivista D'Igiene Sanità Pubbl.* **7,** 961–981.
Jackson, W. A. (1996). *Pharmaceut. Hist.* **26,** 27–31.
Jerace, F. (1945). *Rivista Malariol.* **24,** 221–222.
Jones, E. L., Epinette, W. W., Hackney, V. C., Mendenez, L., and Frost, P. (1975). *J. Invest. Dermatol.* **65,** 537–542.
King, W. E., and Davis, L. (1914). *Am. J. Pub. Health* **4,** 917–932.
Koch, R. (1899). *Deut. Med. Woch.* **XXV,** 601–604 (14 September).
Korzybski, T., Kowszyk-Gindifer, Z., and Kurylowicz, W. (1967). "Antibiotics, Origin, Nature and Properties." Vol. 2. pp. 1203–1207. Pergamon Press, Oxford.
Lapage, S. P., and Bascomb, S. (1968). *J. Appld. Bacteriol.* **31,** 568–580.
Lerrigo, A. F. (1932). *Analyst* **57,** 155–158.
Levine, V. E. (1925). *J. Bacteriol.* **10,** 217–262.
Marinari, R., Fleischmajer, R., Schragger, A. H., and Rosenthal, A. L. (1977). *Arch. Dermatol.* 113.
McGrew, R. E. (1985). "Encyclopedia of Medical History." McGraw-Hill, New York.
Möllers, B. (1950). "Robert Koch. Persönlichkeit und Lebenswerk, 1843–1910." Schmorl & Von Seefeld, Hannover.
Morton, L. T. (1991). "Morton's Medical Bibliography; an Annotated Check-list of Texts Illustrating the History of Medicine" (J. Norman, ed.) 5th ed., Scolar Press, Aldershot.
O'Dette, R. E. (1957). *Science* **125,** 1260–1261.
Olpp, G. (1932). "Hervorragende Tropenärtze in Wort und Bild," pp. 156–157. Gmelin, Munich.
Papacostas, G., and Gaté, J. (1928). "Les Associations Microbiennes. Leurs Applications Thérapeutiques." Libraire Octave Dion, Paris.
Penso, G. (1987). "Grande Dizionario Enciclopedico UTET." p. 786, 4th ed., Vol. IX, Unione Tipografico—Editrice Torinese, Turin.
Pezzi, G. (1946). *Annal. Med. Naval. Colon.* Fasc. **III,** 3–18.
Pitt, J. I. (1979). "The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces.*" Academic Press, London.
Raper, K. B., and Thom, C. (1949). "A Manual of the *Penicillia.*" pp. 694–704. Williams & Wilkins, Baltimore,MD.
Richardson, B. A. (1994). *J. Forensic Sci. Soc.* **34,** 199–204; for erratum, see p. 284.
Rosenheim, O. (1902). *J. Chem. Soc.* 138–139.
Sanger, C. R. (1893a). *Proc. Am. Acad. Arts Sci.* **29,** 112–147.
Sanger, C. R. (1893b). *Proc. Am. Acad. Arts Sci.* **29,** 148–177.
Smith, H. R., and Cameron, E. J. (1933). *Ind. Eng. Chem. Anal. Ed.* **5,** 400–401.
Spatz, S., Rudnicka, A., and McDonald, C. J. (1978). *Brit. J. Dermatol.* **98,** 429–435.
Sprott, J. (1996). "The Cot Death Cover-up." Penguin Books, Auckland, New Zealand.
Thayer, J. S. (1993). *In* "Metal Ions in Biological Systems" (H. Sigel and A. Sigel, eds.) Vol. 29, pp. 1–36. Marcel Dekker, Inc., New York.
Wilkinson, W. H., and Harris, G. C. M. (1943). *Brit. J. Exp. Path.* **24,** 141–143.

Williams, R. H., Boeck, L. D., Cline, J. C., DeLong, D. C., Gerzon, K., Gordee, R. S., Gorman, M., Holmes, R. E., Larsen, S. H., Lively, D. H., Matthews, T. R., Nelson, J. D., Poore, G. A., Stark, W. M., and Sweeney, M. J. (1968a). *Antimicrob. Agents Chemotherap.* **8,** 229–233.

Williams, R. H., Lively, D. H., DeLong, D. C., Cline, J. C., Sweeney, M. J., Poore, G. A., and Larsen, S. H. (1968b). *J. Antibiot.* **21,** 463–464.

Wu, J. C. (1994). *Persp. Drug Disc. Design* **2,** 185–204.

SUBJECT INDEX

C

Q

R

S

CONTENTS OF PREVIOUS VOLUMES

Volume 41

Volume 42

Volume 43

Volume 44

Volume 45

Volume 46

Volume 47