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Review

Metal immobilisation by biofilms: Mechanisms and analytical tools

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Abstract

In biofilm environments, heavy metal and radionuclide pollutants are removed by a variety of mechanisms, including biosorption, precipitation as sulfides or phosphates and microbial reductive precipitation. Even if the elemental composition and localization of the precipitate trapped in the biofilm is well described thanks to spectroscopic and microscopic techniques, this review highlights that little is known about metal immobilisation mechanisms in microbial biofilms, i.e., mass transfer of metals, mechanisms involved in (bio)sorption and precipitation and the influence of physicochemical micro-environments within the biofilm matrix. The review shows the advantage of using a combination of different techniques to evaluate the fate of metals within microbial biofilms. By combining a variety of techniques (e.g., selective extraction, microscopy, spectroscopy and miniaturised sensors . . .), it is possible to gain high-resolution structural and chemical information of biofilms on a level of the individual cell. This approach will facilitate the characterization of the metal immobilisation sites and the metal sorption and (bio)crystallisation mechanisms in biofilms. The results provided by the combination of these techniques will allow to predict the amount of metal accumulation in biofilms as well as their chemical speciation. This review demonstrates that an interdisciplinary approach is required to study metal fate within the biofilm matrix.

1. Introduction

Most microorganisms can form biofilms and more than 99% of all microorganisms on Earth are living in such a form (Costertan et al. 1987). Biofilms allow the coexistence of microniches of different physiological requirements, enabling the simultaneous, but spatially separated occurrence of opposing redox processes (e.g. anaerobic/anoxic/aerobic) in the same biofilm environment (e.g. Lens et al. 1995; de Beer & Schramm 1999; Schramm et al. 2000). Biofilms are commonly found in natural environments as coatings around particles in soils and at water-sediment interfaces (Friese et al. 1997; Dong et al. 2000; Decho 2000; Nelson et al. 1996, 1999a; Cooke et al. 1999). In addition, surfaces in technical environments (e.g., bioreactors, food production reactors, industrial production processes, sewers) are covered by biofilms as well (Flemming 2002; Liu & Tay 2002). Thus, it is important to understand how biofilms affect geochemical processes, as compared to microorganisms growing in a planktonic form (Langley & Beveridge 1999b; Ganguli & Tripathi 2002).

The trace elements present in water will interact with biofilms through physical, chemical or biological processes (Beveridge et al. 1997; Gadd 2000). The distribution, immobilisation and remobilisation of dissolved inorganic substances, i.e., metals, depend on the sorption properties of the biofilms, determined by its pH, as well as type and concentration of the ligands present within the biofilm matrix. The redox potential (E_h) conditions in the vicinity of the cell compared to the bulk liquid are of importance as well (Dong et al. 2003; Templeton et al. 2003a; Nelson

Table 1. Overall composition of microbial biofilms (from Flemming 1995 and Sutherland 2001a).

EPS	
1	EPS (including capsules) with – cationic groups in amino sugars and proteins (e.g.: $-NH_3^+$) – anionic groups in uronic acids and proteins (e.g.: $-COO^-$; $-HPO_4^-$) – apolar groups from proteins (such as in aromatic amino acids) – groups with a high hydrogen bonding potential (such as polysaccharides)
Cell membrane	
2	Outer membrane and: – lipopolysaccharides of Gram negative cells with their lipid membrane – the lipoteichoic acids in Gram positive cells
3	Cell wall consisting of N-acetylglucosamine and N-acetylmuramic acid, offering cationic and anionic sites
4	Cytoplasmic membrane, offering a lipophilic region
5	Cytoplasm, as a water phase separated from the surrounding water
Minerals	
6	Precipitates (sulfides, carbonates, phosphates, hydroxides, ...) Free and bound metals

et al. 1999b). The relative contributions of biotic and abiotic factors to trace metal retention strongly depend on the physico-chemical characteristics of the aqueous phase, the availability of reactive mineral surface sites and the abundance of strongly complexing organic functional groups and the microenvironments of the biofilm matrix within the biofilm (Jackson et al. 1999; Templeton et al. 2001). However, due to the complexity of these interrelationships and the difficulty in probing these microenvironments, the distribution, speciation and local structure of metal sorption complexes present at the bulk-biofilm interface as well as within the biofilm matrix are poorly understood.

The aim of this review paper is to give an overview of the mechanisms involved in metal immobilisation by biofilms. In addition, a detailed description of different analytical tools that can be used to improve our knowledge on metal distribution and chemical species identification within a biofilm is given.

2. Microbial biofilm composition and metal immobilization

2.1. *Biofilm composition*

Biofilms can be described as microorganisms immobilised in a heterogeneous matrix containing extracellular polymeric substances (EPS), water, as well as sorbed substances interspersed within the interstitial voids. In comparison with bacterial cultures growing in suspension, biofilms are more robust and more resistant towards unhostile environments (i.e., Bae et al. 2000; Flemming 2002; Taizel & Parsek 2003). In general, six categories of components can be roughly distinguished in a biofilm (Table 1). Biofilms are generally composed of microbial cells and their products (extracellular polymers), with a very porous structure, in agreement with the high amount of water (95%) contained within the biofilm (Sutherland 2001a, b). Biofilms also sequester minerals, nutrient and metals from the liquid phase they are in contact with (Flemming 1995). In natural conditions, monospecies biofilms are relatively rare, and thus most biofilms are composed of mixtures of microorganisms, and may also include higher trophic groups as protozoa (ciliates and rotifers) and metazoa (snails and flies) (Wimpenny et al. 2000).

Bacteria are classified as either Gram-positive or Gram-negative as distinguished by the Gram stain (Beveridge 2001). This classification divides bacteria into two main groups that differ in their cell wall characteristics (Beveridge 1989; Sleytr & Beveridge 1999). Both cell wall types encompass a peptidoglycan layer that is rich in carboxylate groups and completely surrounds the cell (Beveridge 1989; Langley & Beveridge 1999a). The peptidoglycan layer in the Gram-positive cell wall is ca. 25 nm

thick, whereas the Gram-negative peptidoglycan layer is much thinner (ca. 7.5 nm). They differ further in that the Gram-positive peptidoglycan framework contains secondary polymers which increases the reactive nature of the cell wall by providing additional acidic functional groups: e.g., phosphoryl groups from teichoic acids and carboxyl groups from teichuronic acids (Beveridge et al. 1997). In Gram-negative bacteria, an outer membrane composed of lipopolysaccharide (LPS), protein, and phospholipid exits as the outermost layer of the cell wall. The molecular constituents of the outer membrane are phosphoryl, carboxyl and amino functional groups (Beveridge et al. 1997; Langley & Beveridge 1999a; Smith & Ferris 2001).

The biofilm matrix provides an almost infinite range of extracellular polymeric substances (EPS). The composition of bacterial EPS is complex, depending on the strain and its culture conditions. Typical constituents of EPS are mainly polysaccharides and proteins, often accompanied by nucleic acids, lipids or humic substances (Flemming 1995; Flemming & Wingender 2001). These EPS building molecules contain ionisable functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups. The charge carrying nature of these functional groups enables EPS to sequester minerals, nutrients, and toxic metals (Geesey & Jang 1989; Beech & Cheung 1995; Flemming 1995; Chen et al. 1995; Beveridge et al. 1997; Taizel & Parsek 2003). Biofilms display a number of physical and chemical properties, such as electrostatic interactions, hydrogen bonds and London dispersion forces, which are the dominating forces among macromolecules within the biofilm matrix (Mayer et al. 1999). EPS of neighbouring microbial cells may form a cross-linked network by cationic bridging and thus strengthen the structural integrity of a biofilm or granule (Yu et al. 2001; Sutherland 2001c). The role of exopolymers in biofilm production is to mediate attachment of bacteria to surfaces and to aid in the formation of the biofilm structure (O'Toole et al. 2000). Their ability to interact with other polysaccharides, macromolecules and cells, as well as with ions and low-molecular-mass solutes provide a multitude of physical (e.g., porosity) and chemical microenvironments within any biofilm (de Beer & Schramm 1999; Schramm et al. 2000).

2.2. *Mechanisms involved in the immobilisation of metals within biofilms*

Only the main mechanisms involved in the removal of metals (Figure 1) will be described in the following paragraphs.

2.2.1. *Biosorption*

Biosorption consists of several mechanisms, mainly ion exchange, chelation, adsorption, and diffusion through cell walls and membranes. Biosorption encompasses those physico-chemical mechanisms by which metal species and radionuclides are removed from an aqueous solution. This phenomenon is often attributed to the binding of metals onto the bacterial cell surface (White et al. 1995; Cox et al. 1999). In general, Gram-positive bacteria have a greater sorptive capacity due to their thicker layer of peptidoglycan, which contains numerous sorptive sites (Beveridge 1989; Beveridge et al. 1997). Despite the fact that the mechanisms of metal binding by individual cellular organelles and chemical moieties are known (Simkiss and Taylor 2001), sorption of metals to intact cells and cellular products (EPS in biofilms) is governed by a multiplicity of mechanisms and interactions, which are not always fully understood (Barkay and Schaeffer 2001).

EPS are believed to play a substantial role in the sorption of inorganic substances in biofilms. As many EPS molecules are anionic, it is possible that they assist in the biomineralisation of metal ions and could be potential agents for the concentration and deposition of fine-grained minerals (see 2.2.2.) (Geesey et al. 1989; Beveridge et al. 1997; Douglas & Beveridge 1998; Langley & Beveridge 1999a). Several studies have demonstrated the metal sorption capacity of extracellular biopolymers, which have been determined with pure bacterial cultures (Chen et al. 1995; Beech & Cheung 1995; Bridge et al. 1999; Kachlany et al. 2001; Chen et al. 2000). This occurs primarily through the formation of unidentate, bidentate and multidentate complexes of cations with anionic groups on the EPS molecules (Geesey et al. 1989).

The role of EPS in binding aqueous metal species from wastewater was investigated by Späth et al. (1998), who evaluated sorption of Cd and Zn to the EPS and cell components of a biofilm collected from a sequencing batch biofilm reactor. Although EPS has a high density of charged functional groups, 80% of the total Cd content was sorbed to the cellular

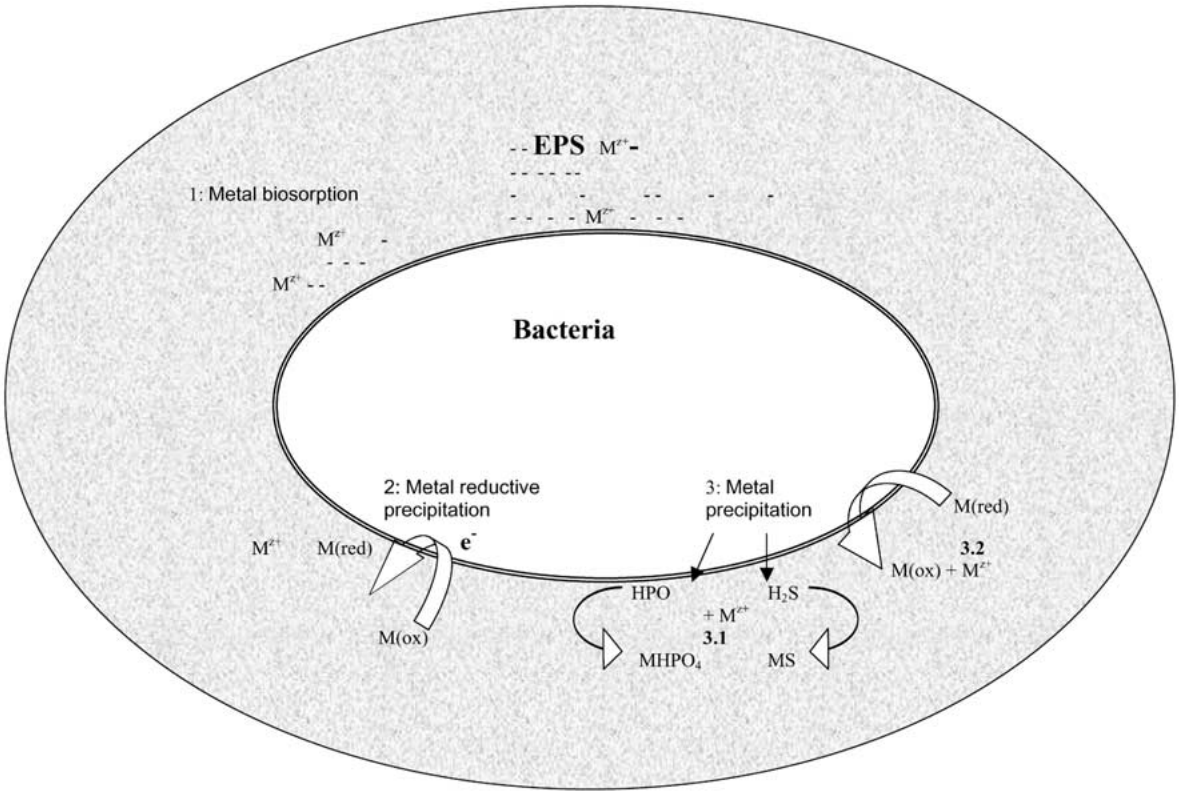


Figure 1. Schematic representation of the interaction between metals and biofilm. 1: Metal biosorption by EPS, 2: Reductive precipitation of metal by enzymatic transformation, 3: 3-1: Metal precipitation as sulfide and phosphate, 3-2 Metal precipitation as biogenic oxide (iron and manganese) and metal adsorption on biogenic oxides.

component of the biofilm. Increased Cd concentrations resulted in an even smaller percentage of that metal bound to EPS. The same trend was found for Zn. However, recent studies (Fukushi et al. 1996; Liu et al. 2001) have shown that EPS also play a crucial role in biosorption of heavy metals. The total concentrations of electrostatic binding sites in EPS of hydrogen-producing sludge and sulfate-reducing biofilm found by Liu and Fang (2002) were 20- to 30-fold those reported for bacterial cell surface, implying that EPS might be more crucial in biosorption of metals than the bacterial cell surface in wastewater treatment and bioremediation.

The strength of the binding affinity in the EPS-metal complex will depend largely on ion size/charge ratios, and a number of other factors such as EPS composition, physical gel state, pH and ionic strength. The pH of the bulk liquid can be different compared to the localized microenvironments of a biofilm due to the respiratory activities of microorganisms in the biofilm (Barkey & Scheaffer 2001, Lens et al. 1993). In general, a lower pH will result in a release of ions from a bound state, while higher pHs tend to favour their chelation (Wilson et al. 2001; Warren & Haack 2001).

Biofilms reach a quasi-steady state of growth and, in so doing, develop a macro-architecture (Stoodley et al. 2001; Wimpenny et al. 2000), which encourages efficient mass transfer and oxygen supply (de Beer & Stoodley 1995). Often, limitations in diffusion within cell clusters (pillars or pedestals and other architectural features) of the biofilm as well as chemical changes within the EPS interstices (because of microbial metabolism) lead to the development of important physico-chemical gradients (e.g., pH, E_h and oxygen) (de Beer & Stoodley 1995; Allan et al. 1999). Thus, it can be expected that the structure and spatial architecture of biofilms would have a profound influence on their metal-binding ability. The presence of other minerals surfaces (e.g., oxide, sulfide, phosphate, carbonate precipitates) may influence the sorption behaviour of biofilms as well.

2.2.2. *Precipitation and subsequent metal immobilisation*

Minerals formed in association within biofilms are commonly referred to as biominerals, e.g., sulfate, phosphate, carbonate, sulfide or silicate ions. For instance, stromatolites, made of calcium carbonate precipitates, are intimately associated with biofilms that comprise an abundant mix of cyanobacteria, bacteria and diatoms (Douglas & Beveridge 1998). Sometimes nanomineral phases can even form directly in the bacterial cytoplasm as has been described for acanthite (Ag_2S) (Klaus et al. 1999) or amorphous iron hydroxide (Glasauer et al. 2002). The tolerance of biofilms to high metal concentrations may also be due to their ability to precipitate insoluble metal salts outside the cells as sulfides, phosphates, carbonates, oxides or hydroxides (Bender et al. 1995; Douglas & Beveridge 1998).

2.2.2.1. *Sulfide precipitation.* Sulfate-reducing bacteria (SRB) are anaerobic heterotrophic bacteria utilizing a range of organic substrates as electron donor and SO_4^{2-} or other oxidized sulphur compounds as a terminal electron acceptor. Bioprecipitation of metals by microbiologically produced sulfide (SRB) is an effective way to remove and concentrate a range of metals, including Zn, Cu, Pb, Ni and Cd from wastewater. The metals precipitate as highly insoluble sulfides (entrapment, nucleation and crystallisation of insoluble sulfides), e.g. ZnS, CdS, CuS and FeS (White & Gadd, 1998, 2000; Valls & de Lorenzo 2002; Wang et al. 2001; Utgikar et al. 2002, White et al. 2003). Sulfide precipitation is an efficient means of removing toxic metals from solution, while immobilisation in a biofilm is also an affective method to reduce the mobility of the metals, which is of value in bioremediation (White et al. 1998; Kaksonen et al. 2003).

White and Gadd (1998, 2000) studied SRB biofilms grown with 200 μM Cd and sulfate in a stirred-tank bioreactor. The accumulated CdS and the protein and polysaccharide content in SRB biofilms simultaneously increased, indicating that both sulfide precipitation and entrapment by extracellular polymers occurred. The accumulation of CdS was localised in a superficial layer of the biofilm. The mechanism of uptake therefore appeared to be entrapment and/or precipitation of CdS at the biofilm surface (White & Gadd 1998; White et al. 2003). A similar study with copper showed that the mechanism of copper uptake appeared to be precipitation of Cu sulfides at the biofilm surface or in the liquid phase, followed by entrapment of precipitated Cu sulfide by the biofilm's EPS (White & Gadd 2000). In contrast, Liu and Fang (1998) showed by Scanning Electron Microscopy (SEM) that SRB cells within anaerobic granular sludge become coated by a metal sulfide layer. As this layer reduces the substrate transport to the SRB cell, metal sulfide precipitation within the granule matrix was proposed as a mechanism that forces the competition between SRB and methane producing bacteria. Utgikar et al. (2002) gave the same explanation to describe the inhibition of SRB used for the bioremediation of acid mine drainage.

Wang et al. (2001) described cadmium sulfide precipitation on the bacterial cell surface following sulfate reduction by a genetically engineered *Escherichia coli* strain. They showed that cadmium sulfide was formed at the cell surface because the cadmium sulfide formation rate is extremely fast ($k_{CdS} = 3.7 \times 10^5\ s^{-1}$) compared to the relatively slow rate of sulfide transport ($k_{diff} = 1000\ s^{-1}$) excreted at the bacterial cell surface.

2.2.2.2. *Phosphate precipitation.* Microbially enhanced chemisorption by biologically produced metal precipitates as phosphates is effective for a range of metals and radionuclides (Renninger et al. 2001; Finlay et al. 1999; Macaskie et al. 2000; Basnakova et al. 1998, see Macaskie et al. 2004 for a detailed review). A *Citrobacter* sp. is able to accumulate the uranyl ion (UO_2^{2+}) via precipitation with phosphate liberated from organic phosphate substrates by phosphatase activity. Accumulation of uranium as HUO_2PO_4 by *Citrobacter sp*. was observed in the presence of excess inorganic phosphorus (Renninger et al. 2001). Metal deposition is proposed to occur via an initial nucleation with phosphate groups localized within the lipopolysaccharide (LPS) layer. The crystals are further consolidated with additional enzymatically generated phosphate in close juxtaposition, giving high loads of LPS-bound uranyl phosphate without loss of activity and distinguishing this from simple biosorption as well as periplasmic or cellular metal accumulation mechanisms (Macaskie et al. 2000).

Cell-bound HUO_2PO_4 facilitated also Ni^{2+} removal by intercalative ion exchange into the polycrystalline lattice and promoted also zirconium removal (Basnakova et al. 1998). The potential of uranyl phosphate accumulated by bacterial biomass as a bio-inorganic ion exchanger has been enhanced

Table 2. Overview of metal reductive precipitation in biofilms

Type of biofilm	Metal reduced and comments	Reference
D. desulfuricans cells immobilized in a hollow-fiber reactor	Reduction of Tc(VII) to insoluble Tc(IV) in the periplasm of *D. desulfuricans*	Lloyd et al. 1999
Desulfovibrio desulfuricans immobilized in polyacrylamide gel	The enzymatic reduction of Cr, Mo, Se and U by immobilized cells was shown to be a practical method for removing these metals from solution in a bioreactor.	Tucker et al. 1998 Battaglia-Brunet et al. 2002
Mixed culture of sulfate-reducing bacterial biofilms	Ability of sulfate-reducing bacterial biofilms to reduce chromium (Cr(VI)) to insoluble Cr(III) partially trapped in EPS matrix	Smith and Gadd 2000
Pseudomonas aeruginosa biofilms react with and precipitate toxic soluble gold	Reduction of Au^{3+} resulted in the formation of extracellular metallic gold colloids (Au°)	Karthikeyan and Beveridge 2002

in biofilm-immobilised cells (*Citrobacter sp*.) in an air-lift bioreactor (Finlay et al. 1999).

2.2.2.3. *Entrapment and adsorption by iron and manganese oxides.* Microorganisms catalyse oxidation of Fe(II) in acidic and near neutral solutions to produce iron (Fe(III)) hydroxides (Brown et al. 1999). Iron oxide precipitates associated with biofilm EPS were identified as ferrihydrite in both pH conditions but the precipitate morphology and minor elemental composition was pH dependent (Ferris et al. 1989). Investigation of biofilm consortia from groundwater by Transmission Electronic Microscopy (TEM) showed that the accumulation of iron on the cell surfaces was stepwise and that iron precipitates were found at the cell surfaces and throughout the biofilm matrix (Brown et al. 1998). Bacterial cell walls and EPS are generally negatively charged, which allows positively-charged metal ions to be adsorbed. These metal ions may then act as nucleation sites for the deposition of more iron from solution as shown by Brown et al. (1998).

Many investigations report that the sorptive capacities of Fe/Mn oxides for metals are increased in the presence of microorganisms (Haack & Warren 2003; Nelson et al. 2002). The iron oxide present in the biofilm can adsorb zinc, lead and cadmium in the biofilm (Baldi et al. 2001). Lack et al. (2002) showed that the bio-oxidation of 10 mM Fe(II) by *Dechlorosoma* species and precipitation of Fe(III) oxides by these microorganisms resulted in rapid adsorption and removal of 55 μM uranium and 81 μM cobalt from solution. Nelson et al. (1995, 1996, 1999a, 1999b, 2002), Templeton et al. (2001) and Wilson et al. (2001) showed that the binding capacity of iron, manganese and aluminium oxide is cumulative in the presence of EPS produced by the biofilm. Nelson et al. (1999a) reported that Pb adsorption in lacustrine biofilms to Fe and Mn oxides was significantly higher than that estimated for organic materials, and that Pb binding by Al oxide was relatively negligible. Nelson et al. (2002) showed that biogenic manganese (III/IV) (hydr)oxide prepared by enzymatic oxidation of Mn(II) by the bacterium *Leptothrix discophora* SS-1 adsorbed five times mores Pb (per mole of Mn) than abiotic manganese(hydr)oxide prepared by oxidation of Mn(II) with permanganate.

2.2.2.4. *Metal reductive precipitation.* Certain heavy metals (Cr, Mo, Se, U, Pd, Tc, Au) can be immobilised in biofilms following enzymatic reduction of these elements into an insoluble lower redox state (Table 2) (Tucker et al. 1998; Lloyd et al. 2001; Ganguli & Tripathi 2002; Yong et al. 2002). The mobility of most of these elements in the environment is strongly dependent on their chemical oxidation state. Under oxidizing conditions, they occur as highly soluble and mobile ions, however, under reducing conditions they usually form insoluble phases. An electron donor as lactate or H_2 is generally required to reduce Cr(VI), Mo(VI), Se(VI) and U(VI) (Tucker et al. 1998; Battaglia-Brunet et al. 2002). A recent review gives a detailed understanding of some of these transformations at a molecular level (Lloyd 2003).

The metals are generally immobilised intracellularly or extracellularly, depending on the molecular mechanisms involved in the reduction (Lloyd 2003). For instance, Lloyd et al. (1998) showed using TEM studies in combination with energy dispersive X-ray microanalysis spectroscopy (EDS) Pd precipitation in the periplasm. Moreover, X-ray diffraction studies confirmed the reduction of Pd^{2+} to Pd(0). Molybdenum (VI) reduction by *D. desulfuricans* in the presence of sulfide resulted in the extracellular precipitation of the mineral molybdenite $MoS_{2(S)}$ (Tucker et al. 1997). Lloyd et al. (2001) show that SRB reduce Tc(VII) and this metal precipitates as TcO_2 at the periphery of the cell. Microbial reduction of toxic Se(VI) and Se(IV) to the relatively unreactive Se(0) can be used to remove Se from wastewater (Lloyd et al. 2001). Elemental selenium has not been localized on the bacterial cells (Lloyd et al. 2001) but was released in the bulk solution.

Most of these studies have focused on planktonic cells, but some recent studies have also addressed immobilised cells (Table 2). SRB biofilms have been shown to reduce Cr(VI) to Cr(III), which precipitates as insoluble hydroxides (Cr(III)) (Smith & Gadd 2000). Immobilised cells of *Bacillus* sp. (Chirwa & Wang 1997a) and *Pseudomonas fluorescens* LB 300 (Chirwa & Wang 1997b) have also been used to treat Cr(VI)-contaminated water. Immobilised cells have been applied by Lloyd et al. (1999) in order to remove Tc (VII).

3. Investigation of metal-biofilm interactions

The previous section shows that the knowledge on metal-biofilms interactions are still limited. This section presents the major and most promising tools used to investigate these interactions and thus can assist in the unravelling of heavy metal immobilisation (Table 3).

3.1. *Biofilm fractionation: Extraction techniques*

Biofilms represent heterogeneous matrices of variable composition of associated minerals and biominerals as well as organic constituents, i.e., bacterial cells and associated extracellular material of the biofilm itself. These components are differentially reactive, display different affinities for specific elements, and scavenge metals through a variety of processes. Characterising which biofilm solid phases as well as the organic components are involved in metal retention through sequential extraction can provide insight into the likely processes and associated physico-chemical controls.

3.1.1. *Fractionation of the biofilm: Extraction of EPS and metals*

Wuertz et al. (2001) have proposed an extraction protocol for EPS to determine the role played by EPS and surface cells as sorption sites for metals of the exposed biofilm. Therefore, an EPS extraction method with a crown ether (dicyclohexyl-18-crown-6 ether) was used. The extraction method was compared with the one using the ion exchange resin DOWEX 50X8 (Frølund et al. 1996). The complexing properties of isolated extracellular biopolymers may be altered during the extraction procedure. However, in contrast to other published methods, crown ether selectively binds alkaline and alkaline earth metals (e.g., Ca, Mg ...), but not heavy metals (e.g., Zn, Cu, Ni, Co ...). It thus allows the fractionation of metals bounds to EPS without interfering with the distribution of metals within the biofilm.

The method was used to determine the role of the EPS fraction of a biofilm in binding zinc. Wuertz et al. (2001) showed that EPS do not play a major role in sorption in the presence of high metal concentrations. In aerobic biofilms, 12.2 and 9.1% of, respectively, the dosed cadmium and zinc was found in the EPS. The distribution of metals within the biomass was dose dependent: the percentage of metals found in the EPS decreased with increasing metal concentration. This indicates a higher affinity of metals for cellular binding sites. Time course experiments in a rotating biofilm annular reactor revealed a gradual change in the zinc concentration associated with EPS, although the total zinc concentration in the biomass remained constant. In a similar study, Späth et al. (1998) showed that after EPS extraction more than 80% of the total cadmium and zinc content was present in the cellular fraction of a biofilm growing in a biological wastewater treatment plant. This confirms the higher affinity of bacterial cell surfaces for metal binding compared to EPS.

3.1.2. *Fractionation of the biofilm: Sequential extraction*

In sequential extraction procedures, chemical extractants of various types are successively applied to the biofilm, each subsequent treatment being more drastic in chemical action than the previous one. The use of selective extractants is a useful approach for deter-

Table 3. Overview of the analytical techniques used for the investigation of metal interactions within biofilms

Technique	Objectives	References
Biofilm fractionation		
– Extraction of EPS associated metals – Sequential extraction	– Accumulation inside or outside the bacterial cells – Role of EPS in metal immobilization – Distribution of metal binding strength within the biofilm	Späth et al. 1998 Wuertz et al. 2001 Stover et al. 1976 Dong et al. 2000
Localization of metals in the biofilms		
1	1	1
– Microscopic techniques (TEM, SEM, Environmental SEM, Environmental TEM)	Localization of metal within the biofilm Matrix	Ray & Little 2003 White & Gadd 2000 Yamaguchi et al. 2001 Kerthikeyan & Beveridge 2002
2	2	2
– Confocal Laser Scanning Microscopy fluorescent probes	Localization of metal penetration and bacterial species identification	Wuertz et al. 2000
3	3	3
– Nuclear Magnetic Resonance imaging	Mass transfer of metals inside the biofilm	Lens & van As 2003 Nestle & Kimmich 1996 Nott et al. 2001
Identification of metals in the biofilms		
– Microscopy coupled with EDS – XAS (EXAFS, XANES)	Identification of metal species in the biofilms	Geesey et al. 2002 Templeton et al. 2001, 2003a, 2003b
Supporting tools		
– Microelectrodes	Study of microenvironment inside the biofilm matrix: pH, redox potential, O_2, sulfide, iron and manganese	Lewandowski & Beyenal 2003 Haack & Warren 2003 Glazer et al. 2002

mining the relative significance of the mineral and organic components in controlling trace metal retention. The sequential extraction procedures described in the literature differ mainly in the extractants used, the order of extractions and operational details such as the solid-to-extractant ratio and extraction time. Sequential extraction procedures are easy to perform and consider more than one phase. This methodology, however, is not free from artefacts (Filgueiras et al. 2002). In particular, the initiation of cell lyses and appreciable destruction of cellular matter prior to treatment with hydrogen peroxide has been demonstrated by Lead et al. (1998). These changes in biological material may affect metal binding, complicating interpretation in terms of simple mineral and organic phases. Another limitation is that extractants can potentially desorb trace metals from other components that were not extracted, which would lead to an overestimation of trace metal associated with the target component. The operational nature of sequential extraction means that it is very hard to compare results obtained by different laboratories using different protocols (Filgueiras et al. 2002). However, if used with care and the limitations are taken into account, sequential extraction can be useful in the study of the metal distribution within biofilms (Haack & Warren 2003; Dong et al. 2000, 2001a, 2001b, 2002, 2003; van Hullebusch et al., in preparation).

Although developed for soil extraction, sequential extraction methods have been adopted first for anaerobic biofilms. Stover et al. (1976) adapted a chemical extraction method to categorize sludge metals into exchangeable metals, adsorbed to some solid phases, organic-bound metals, carbonates, sulfides and residual fractions (Table 4). The Stover et al.

Table 4. Operating conditions required in the Stover sequential extraction method*

Fraction	Extracting agent	Extraction Conditions	
		Shaking time[1]	Temp
1. Exchangeable	30 mL KNO_3 (1 M, pH = 7)	16 hours	20 °C
2. Sorbed	48 mL KF (0.5 M, pH = 6.5)	16 hours	20 °C
3. Organically bound	48 mL $Na_4P_2O_4$ (0.1 M)	16 hours	20 °C
4. Carbonate	48 mL EDTA (0.1 M, pH = 6.5)	2∗8 hours	20 °C
5. Sulphide	30 mL HNO_3 (1 M)	16 hours	20 °C
6. Residual	10 mL demineralised water and 10 mL *Aquaregia* (HCl/HNO_3, 3:1)	26 min.	Microwave-oven

[1]Shaking was applied at 200 rpm.
*Amount of sample 0.6 g dry weight basis.

Table 5. Operating conditions required in the modified Tessier sequential extraction method*

Fraction	Extracting agent	Extraction conditions	
		Shaking time[1]	Temp
1. Exchangeable	10 mL NH_4CH_3COO (1 M, pH = 7)	1 hour	20 °C
2. Carbonates	10 mL CH_3COOH (1 M, pH = 5.5)	1 hour	20 °C
3. Organic Matter and sulphides	5 mL H_2O_2 (30% pH = 2)	3 hours	35 °C
4. Residual	10 mL demineralised water and 10 mL *Aqua regia* (HCl/HNO_3, 3:1)	26 min	Microwave-oven

[1]Shaking was applied at 100 rpm.
*Amount of sample 1 g dry weight basis.

(1976) scheme differentiates the organic matter and sulfides fractions and thus allows the differentiation between the main fractions involved in metal sorption in anaerobic biological materials (e.g., carbonates, sulfides and organic matter). This method has been used to study metal distribution into different types of anaerobic granular sludges from UASB reactors (Ilangovan & Noyola 1993; Espinosa et al. 1995; Leighton & Forster 1998). The metal fractionation method is suitable for measuring shifts in metal distribution (Carliell & Wheatley 1997), although the characterisation of the specific metal species present is not possible.

Zandvoort et al. (2002) and Osuna et al. (2004a, in press) have used the sequential extraction methods proposed by Tessier et al. (1979) which were first used to study metal speciation in sediments. Ruiz et al. (1991) and Modak et al. (1992) modified this scheme (Table 5). The extraction scheme used with the methanogenic granular sludge differentiates four fractions following a decreasing pattern of metal lability: soluble and exchangeable, carbonates, sulfide/organic-

Table 6. Operating conditions required in the revised BCR sequential extraction method*

Fraction	Extracting agent	Extraction conditions	
		Shaking time[1]	Temp
1. Exchangeable + carbonates	40 mL CH_3COOH (0.11 M, pH = 7)	16 hours	20 °C
2. Iron and manganese oxides	40 mL NH_2OH-HCl (0,1 M, pH = 1.5)	16 hours	20 °C
3. Organic Matter and sulphides	20 mL H_2O_2 (30% pH = 2)	1 hour 2 hours	20 °C 85 °C
	and then 40 mL CH_3COONH_4 (1 M, pH = 2)	16 hours	20 °C
4. Residual	10 mL demineralised water and 10 mL *Aquaregia* (HCl/HNO_3, 3:1)	26 min.	Microwave-oven

[1] Shaking was applied at 30 rpm.
*Amount of sample 1 g dry weight basis.

bound and a residual fraction. The utilization of this scheme allows to study the fate of trace metals (Ni, Co, Fe, Mo, Se) in granular methanogenic sludge (Zandvoort et al. 2002; Osuna et al. 2004a; van Hullebusch et al., in preparation).

Dong et al. (2000, 2001a, 2001b, 2002, 2003) used a selective chemical extraction technique to reveal the relative contribution of trace metal adsorption on manganese and iron oxides and organic material in natural coatings (e.g., biofilms and associated minerals, i.e., Mn and Fe oxides) in aquatic environments. Hydroxylamine hydrochloride (0.01 M $NH_2OH.HCl$ + 0.01 M HNO_3 for 30 min. at 25 °C) was used to extract manganese oxides. Sodium dithionite (0.4 M $Na_2S_2O_4$for 40 min. at pH 6.0) was used to extract both manganese and iron oxide whereas, nitric acid (10% HNO_3) was used to remove all the materials (organic and inorganic components of the biofilm). Under the experimental conditions, the target components (i.e., iron or manganese) were extracted with efficiencies above 91%. Lead, one of the representative contaminants in aquatic systems, was shown to absorb strongly to the Mn oxides in biofilms (Dong et al. 2002, 2003).

One scheme comparable as the one proposed by the Community Bureau of Reference (BCR, now Standards, Measurements and Testing Programme, M&T, Mossop & Davidson 2003) (BCR scheme, Table 6) should be developed to harmonise the extraction schemes used in biofilm research. Given the importance of validation for accuracy assessment, additional solid materials (e.g., aerobic or anaerobic biofilm) with a certified extractable metal contents are needed for quality control.

3.1.3. *Modelling approach using solid phase biofilm reconstruction*

A modelling approach has been proposed in a number of recent studies (Nelson et al. 1996; Small et al. 1999; Wilson et al. 2001). These have established that inorganic mineral sorbents like hydrous Fe(III) and Mn (III/VI) oxides accumulating in oxic biofilms have a profound influence on the existence of multiple sorption sites on each of the individual sorbent solids that may occur within a biofilm. The sorption capacity of individual solid phase sorbents comprising the biofilm can be considered separately, as done by Dong et al. (2000, 2001a, 2001b, 2002, 2003) and Nelson et al. (1996) (see sequential extraction section). Langmuir adsorption isotherms were used to estimate the relative contributions of each surface coating constituent to metal binding of the biofilms and to model their sorption behaviour (Dong et al. 2000, 2001a, 2001b, 2002, 2003). A Langmuir model for pH-dependent adsorption of Pb to heterogeneous particulate surface mixtures including amorphous Fe oxide, biogenic Mn oxide produced by a Mn(II) oxidizing bacterium (*Leptothrix discophora* SS-1), Al oxide, the common green alga *Chlorella vulgaris*, and *Leptothrix discophora* SS-1 was developed. A linearisation of Pb

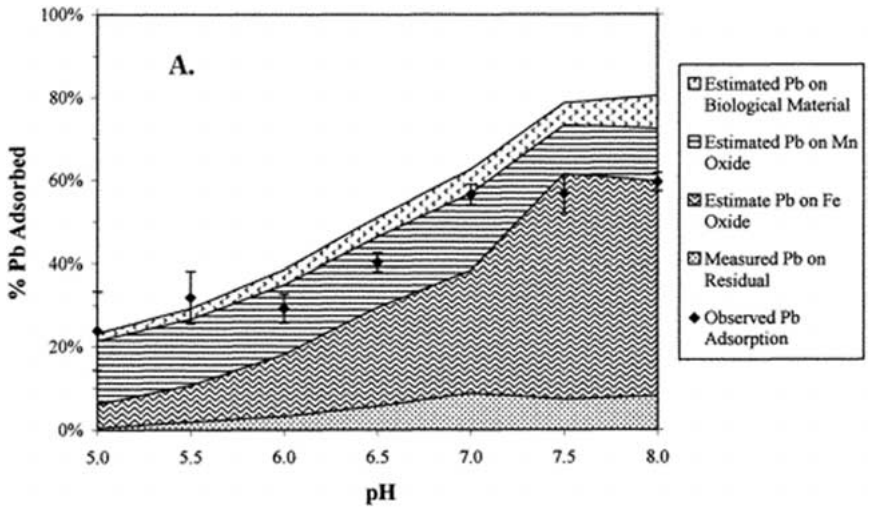


Figure 2. Observed versus predicted Pb adsorption onto natural biofilms from Cayuga Lake, NY, USA. Error bars indicate the standard deviation of observed Pb adsorption to 3 slides at each pH (from Wilson et al. 2001). That's the best we have.

adsorption data for each adsorbent was used by Wilson et al. (2001) to quantify the relationship between Pb adsorption and pH. The parameters for individual adsorbents were incorporated into an additive mathematical model to predict the total Pb adsorption in multiple-adsorbent natural surface coatings that were collected from Cayuga Lake, NY, USA. The observed Pb adsorption is consistent with the model predictions (within 1–24%) over the range of solution pH values considered (Figure 2). The concept of additivity assumes that the total amount of bound metal can be calculated based on simple addition of adsorption to individual phases. Although this approach will not adequately consider interactive effects (e.g., site masking), some prior studies have shown that it is an acceptable assumption for modelling in certain multisorbent systems. This type of protocol has been developed for studies of aerobic biofilms (Nelson et al. 1996; Wilson et al. 2001; Dong et al. 2000, 2001a, 2001b, 2002, 2003). A modified protocol, however, is required to study anaerobic biofilms, due to the different solid phases involved in the accumulation (carbonates, sulfides . . .).

3.2. *Localisation and identification of metals in the biofilm*

Generally, extraction techniques yield limited information on the nature and localisation of the inorganic solids formed in the presence of microorganisms or the inorganic material adsorbed to surface-associated microbial biomass. Thus, other analytical techniques are needed to get insight in the localisation and chemical nature of the metals in the solid phase of the biofilm.

3.2.1. *Microscopic and spectroscopic analysis of metal localisation in the biofilm*

In almost any biofilm sample, preliminary examination of the biofilm by electron microscopy reveals fine-grained metal deposits closely associated with bacterial cells (see Surman et al. 1996 for review). Scanning electron microscopy (SEM) and transmission electron microscope (TEM) provide an option for the investigation of biofilms. The advantage of electron microscopy is that it provides a very high image resolution. The level of resolution is in the order of 1 Å and 0.1 Å for SEM and TEM, respectively (Brown et al. 1998). SEM is capable of imaging complex structures, while TEM visualises cross-sectional detail of the individual microorganisms and their relationship to each other (Surman et al. 1996, Lee & Beveridge 2001; Karthikeyan & Beveridge 2002; Lawrence et al. 2003). However, it must be taken into account that the process of sample preparation includes complete dewatering. Considering that biofilms consist mainly of water, the picture obtained is necessarily an artefact. It still gives insight into the structure of the biofilm and can reveal the presence of different kinds of EPS structure and inorganic precipitates. SEM and TEM alone cannot give compositional analyses of the metal deposits nor can it provide the identity of the

Table 7. Summary of studies describing metal fate within microbial biofilms

Techniques used	Elements	Biofilm type	Remarks	References
Metal precipitates localization				
TEM	Fe	*Pseudomonas aeruginosa* biofilm	Fe (II) was removed from solution by surface complexation with negatively charged functional groups in the bacterial cell wall via nucleation and mineralisation processes	Lee & Beveridge 2001
SEM CLSM	U and Ni	*Citrobacter* biofilm	Bio-mineralisation of uranium as HUO_2PO_4 was observed in the presence of excess inorganic phosphorus. Cell-bound HUO_2PO_4 facilitated Ni^{2+} removal by intercalative ion exchange into the polycrystalline lattice	Finlay et al. 1999
TEM	Au	*Pseudomonas aeruginosa* biofilms	Formation of intracellular and extra-cellular gold colloids, but no gold crystals were observed in cells	Karthikeyan & Beveridge 2002
Metal localization and elemental identification				
TEM-EPXMA	Ni and uranylphosphate (HUO_2PO_4)	Immobilized cells of *Citrobacter sp*. In polyacrylamide gel	EPXMA Identification of the nickel Uranyl phosphate deposits on *Citrobacter sp*. surface cells	Basnakova et al. 1998
SEM and TEM-EDXA	Zn	Natural biofilms of Sulfate reducing bacteria	Formation of sphalerite (ZnS) deposits in natural biofilm of sulfate reducing bacteria	Labrenz et al. 2000
SEM-EDXA	Cu	Sulfate reducing bacterial biofilm	Precipitation of Cu sulfides at the biofilm surface and entrapment of precipitated Cu sulfides by the exopolymer enhanced biofilm	White & Gadd 2000
SEM-EDXA	Cr	Sulfate reducing bacterial biofilms	SEM-EDXA clearly showed the presence of insoluble Cr(III) precipited material	Smith & Gadd 2000
SEM-EDS XRD	Zn and Fe	Metal sulphide precipitation in sulfate reducing biofilm from UASB reactor	Metal precipitates in the UASB reactor were predominantly FeS_2, ZnS and FeS	Kaksonen et al. 2003
TEM-EDS	Cu, Fe, Au and La	*Pseudomonas aeruginosa* biofilm	The amount accumulated by bacteria was different by the mode of growth (i.e., planktonic versus biofilm growth). Cu was shown to be accumulated in a much higher quantity in biofilm compared to planktonic	Langley & Beveridge 1999
TEM-EDS SEM CLSM	Fe	Natural microbial biofilms	TEM EDS revealed the precise location of the iron precipitation (probably ferric iron as ferrihydrite and ferrous iron as siderite) on cell surfaces, including the S layers and also throughout the extra polymeric slime of the biofilm	Brown et al. 1998
SEM-EDXA	Cd	SRB biofilms (mixed sulfate reducing culture selected from natural sediment samples)	EDXA showed that accumulation of Cd in the from of CdS occurred in SRB biofilms	White & Gadd 1998

Table 7. Continued

Techniques used	Elements	Biofilm type	Remarks	References
Metal localization and metal speciation				
TEM-EDS EXAFS	Pb	*Burkholderia cepecia* biofilms	EXAFS data and TEM observations show that the enhanced Pb accumulation is due to the formation of nanoscale crystals of pyromorphite ($Pb_5(PO_4)_3(OH)$) adjacent to the outer-membrane of a fraction of the total population of *B. cepacia* cells	Templeton et al. 2003a
EXAFS	Pb	*Burkholderia cepacia* biofilms coated with goethite particles	EXAFS spectra determined the type of complexes formed for different lead concentration on goethite surface	Templeton et al. 2003b
XANES	Se	*Burkholderia cepacia* biofilms formed on - Al_2O_3 surfaces	The distribution and speciation of Se within aerobic *Burkholderia cepacia* biofilms formed on – Al_2O_3	Templeton et al. 2003c
XPS	Fe	SRB biofilms and corrosion of Steel	X ray photoelectron spectroscopy analysis suggeted that Fe released from steel was associated with sulfate reducers exopolymers such as Fe(III)	Beech et al. 1999
Environmental microscopy EC-TEM-EELS	Cr	Metal-reducing bacteria, *Shewanella oneidensis*	Measurements by EELS showed that precipitates were a reduced form of Cr in the oxidation state +3 or lower	Daulton et al. 2002

mineral phase unless additional associated equipment and expertise is used. Table 7 shows an overview of microscopic and spectrometric analysis studies of metal fate in the biofilm.

Mineralogical inspection using X-ray diffraction (XRD) has been used for many years to determine the symmetry of crystals and to measure lattice parameters with high accuracy. It has been used extensively to determine the bulk mineralogical composition of geological material (rocks, sediments, etc.). The diffraction pattern obtained from a sample is compared with that of known minerals provided by databases such as the International Center for Diffraction Data for sample identification or by theoretical interpretation (Geesey et al. 2002). Samples containing complex mixtures of crystalline phases or with a predominant amorphous phase are difficult to interpret. Remoudaki et al. (2003) were, nevertheless, able to use XRD to identify the main minerals accumulated in biofilm reactors (moving bed sand filters) used to remove heavy metals from industrial wastewater. Moreover, high-resolution TEM analysis of biofilms allows the localisation and identification of minerals by measuring the lattice fringes-spacing. Templeton et al. (2003a) identified the formation of nanoscale crystals of pyromorphite ($Pb_5(PO_4)_3(OH)$) adjacent to the outer-membrane of a fraction of the total population of *B. cepacia* cells in biofilms.

Energy-dispersive X-ray spectroscopy (EDS) can provide compositional information of a biofilm. This technique relies on the capture of the "signature" obtained when X-rays, that are emitted from the specimen as the electron beam, interact with cells. Scanning electron microscopy (SEM) with backscattered electron (BSE) imaging allows to differentiate organic-rich and metal-rich zones within a cross section of anaerobic granular sludge (Gonzalez-Gil et al. 2001). Electrons are back scattered in metal-rich zones, thus lighting up these zones (white in the image). In contrast, organic matter absorbs the electrons, resulting in black zones in the picture (Figure 4). Using SEM-BSE and TEM, Ascaso et al. (2002) were able to observe a mineral network structure adjacent to the cyanobacterial wall that might be related to calcium biomobilization processes. The SEM technique with back-scattered electron imaging (SEM-BSE) is one of the best currently available tools for this purpose, since it permits undisturbed visualization of the biofilm. The simultaneous application of the EDS microanalytical system with SEM-BSE serves to chemically characterize the mineral features (Gonzalez-Gil et al. 2001).

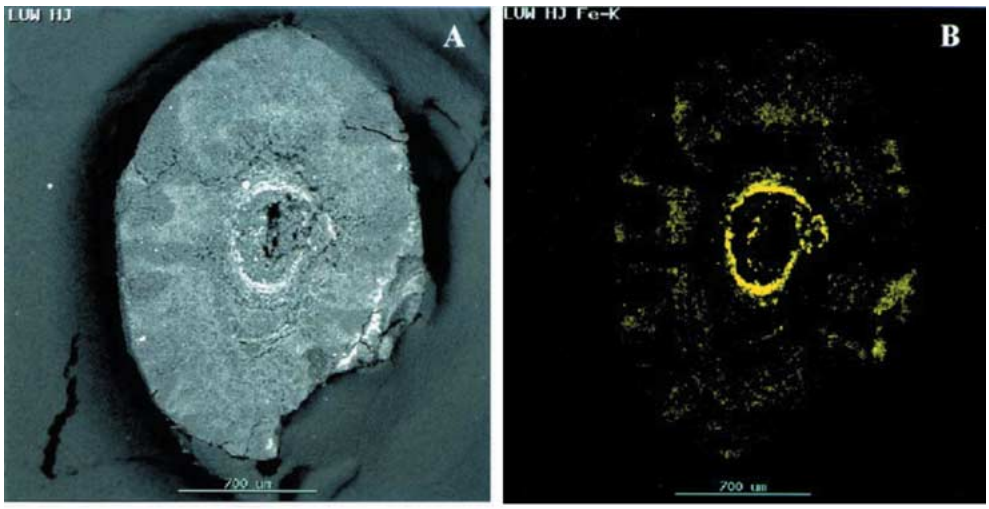


Figure 3. Analysis of a cross-sectioned aggregate grown in an expended granular sludge reactor treating brewery wastewater. (A) Back-scattered electron image from which EDX mappings of iron (B) were made. Note that the deposition of iron corresponds to the light areas observed in the back-scattered electron image (from Gonzalez et al. 2001).

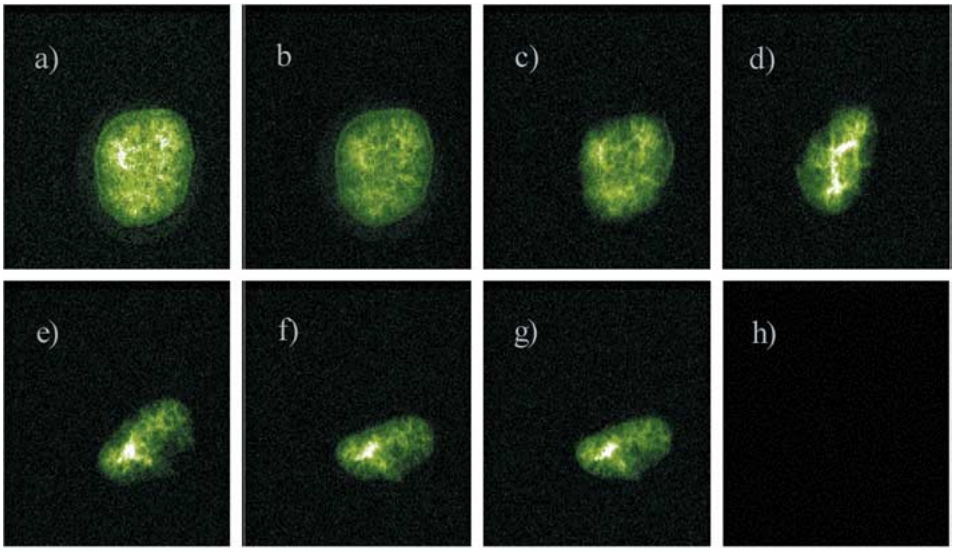


Figure 4. Temporal evolution of an individual aggregate (Eerbeek, The Netherlands) after the addition of Fe (100 mM) as manifest in fast turbo spin-echo imaging. a) prior to addition, b) addition, t = 0. Next images corresponding to c) +10 min, d) +20 min, e) +30 min, f) +40 min, g) +50 min and y h) +1 h. The effect of Fe is to reduce the signal intensity (ranging from black (zero), dark green, light green to white (max)) observed in these images. A single slice out of a complete 3D data set is presented (modified from Osuna et al. 2004b).

Modifications of SEM and TEM have been recently realized. Apart from being slow, sample preparation result for SEM and TEM in biofilm shrinkage, damage and eventually biofilm loss. Moreover, these techniques do not allow *in vivo* and *in situ* studies of intact biofilms. A new SEM Technique is now available which allows to overcome these obstacles: The Environmental Scanning Electron Microscopy (ESEM) (Ray & Little 2003; Donald 2003). ESEM allows the examination of biological materials with a minimum of manipulation, i.e., no fixation and dehydration. With the ESEM sample preparation, it could be demonstrated that the sample preparation process for classical SEM leads to a

considerable loss of material (Surman et al. 1996, Cox et al. 2002). The ESEM allows to accurately determine aerial coverage of biofilms with cells and EPS associated with surfaces. However, it is sometimes difficult to image individual cells embedded in extracellular material. ESEM used in combination with EDS allows to determine relative concentrations of elements, heavier than sodium localized in the biofilm (Cox et al. 2002). Environmental Transmission Electron Microscopy (ETEM) has the advantage that hydrated cells can be well imaged (Ray & Little 2003).

Bioaccumulation of metal precipitates by biofilm has been mainly shown by SEM, TEM, ESEM and ETEM studies and qualitative elemental identification of the precipitates associated with the biofilm was performed using EDS (Tsezos et al. 1997; Brown et al. 1998; White & Gadd 1998, 2000; Smith & Gadd 2000; Lloyd et al. 2001; Yong et al. 2002; Karthikeyan & Beveridge 2002, Table 7). This analytical approach provides further information helpful to explain differences in metal biosorption and contributed to the elucidation of underlying mechanisms. However, EDS does not provide the oxidation state of the given elements, which is important in demonstrating the accumulation of a mineral. In none of the above-mentioned studies the oxidation state of the precipitated reduction products was determined. Daulton et al. (2001, 2002) used ETEM associated electron energy loss spectroscopy (EELS) to determine the Cr oxidation state in the precipitate accumulated by the metal-reducing bacterium *Shewanella oneidensis*. They demonstrate that chemical and oxidation state information was acquired with high spatial resolution using EELS, which could provide additional data compared to conventional TEM, for example, on soluble ions lost by conventional sample preparation. All these techniques will become increasingly useful for determining crystallisation associated with the range of biogeochemical microbial transformations.

Some surface-sensitive spectroscopic techniques can resolve the oxidation state of an element: X-ray photoelectron spectroscopy (XPS) and X-ray photoelectron emission spectromicroscopy (X-PEEM) and X-ray absorption near edge structure (XANES) (Beech et al. 1999; Daulton et al. 2001; Geesey et al. 2002). X-ray absorption spectroscopy (XAS) holds great potential for answering questions concerning the geometrical details of adsorbed species. XAS is also an attractive method for determining the local structure of trace metals *in situ* and non-destructively within biofilms (see Prange and Modrow (2002) for applications). Two types of spectra are produced in this technique, an X-ray adsorption near edge structure (XANES) spectrum and an extended X-ray absorption fine structure (EXAFS) spectrum. EXAFS spectra yield interatomic distances between the element of interest and its first and second nearest neighbours to ± 0.02 Å and yields coordination numbers with these neighbours within ± 15–20%. EXAFS provides information on the average short-range order around an element of interest and has been used to characterise interactions between atoms within metal–organic complexes (bidentate, monodentate, etc.) (Geesey et al. 2002; Prange & Modrow 2002). XANES gives less quantitative information, but can also provide information concerning the local structural environment of the element of interest through comparison to spectra of well-characterised samples. With this technique, the redox state of Fe, Cr, U, Mn, Se, As etc can be determined in biofilm or bacterial samples. XANES and EXAFS allow to examine hydrated biofilms and the samples can be stored in liquid N_2 until analysis. The types of molecular scale information on surface reactions provided by these techniques start to explain mechanistically the metal-biofilm interaction seen in sorption reactions.

Templeton et al. (2003a) studied the accumulation of Pb(II) within *Burkholderia cepacia* biofilms. EXAFS measurements have been combined with macroscopic uptake data and transmission electron microscopy (TEM) results to show that Pb(II) uptake by *Burkholderia cepacia* is due to simultaneous sorption and biomineralization processes. Structural fits of Pb L(III) EXAFS data collected for heat-treated cells at near-neutral pH suggest that Pb(II) forms inner-sphere adsorption complexes with carboxyl functional groups in the biofilms. The partitioning of Pb(II) between the *Burkholderia cepacia* biofilms and iron-(oxyhydr)oxide surfaces has been measured using the EXAFS approach (Templeton et al. 2003b). Structural fitting of the EXAFS spectra at pH 6 shows that the Pb-goethite surface complexes at low lead concentration are dominated by inner-sphere bidentate, binuclear complexes bridging two adjacent singly coordinated surface oxygens. At high lead concentration, the dominant Pb(II) inner-sphere complexes on the goethite surface.

The redox state of metals accumulated in biofilm can be monitored with XANES. The distribution and speciation of Se within aerobic *Burkholderia cepacia* biofilms formed on Al_2O_3 surfaces have been

examined with XANES (Templeton et al. 2003c). Changes in the Se partitioning behaviour over time are correlated with microbially induced reduction of Se(VI) and Se(IV) to Se(0), as observed from XANES. Selenite (SeO_3^{2-}) preferentially binds to the aluminium oxide surfaces, particularly at low Se concentration, and is increasingly partitioned into the biofilms at higher Se concentration. When *B. cepacia* is metabolically active, *B. cepacia* rapidly reduces a fraction of the SeO_3^{2-} to red elemental Se(0). In contrast, selenate is preferentially partitioned into the *B. cepacia* biofilms at all Se concentration tested due to a lower affinity for binding to the aluminium oxide surface.

3.2.2. *Confocal laser scanning microscopy (CLSM) techniques for bacterial identification, biofilm matrices components determination and metal localisation*

The spatial distribution of organisms and their function can be determined by fluorescence *in situ* hybridisation (FISH). FISH involves applying fluorescently monolabeled DNA probes to environmental samples. These oligonucleotide probes have been designed to be complementary to, and hybridise with, target rRNA with intact biofilm cells (Zhang et al. 2003). This technique is expected to determine the genera and species of micro organisms existing in biofilms. The FISH technique has several advantages, the most obvious being a phylogenetic characterisation of the microbial consortia within a complex environmental sample. Moreover, FISH allows to determine with a high resolution both the spatial distribution and the abundance of target organisms within a biofilm. Manz et al. (1999) used the FISH technique in conjunction with Confocal Laser Scanning Microscopy (CLSM) and digital image analysis to characterise a change in the dominant bacterial groups in the biofilm community over time.

The three-dimensional reconstruction of biofilm architecture is possible with CLSM as the sample can be optically sectioned vertically and horizontally without having to slice the samples (Brown et al. 1998; Manz et al. 1999; Palmer Jr & Sternberg 1999). Lawrence et al. (1998) have extensively investigated microbial spatial relationship within biofilms using CLSM coupled with fluorescent probes. In a study of river water biofilms, Neu and Lawrence (1997) used the LIVE/DEAD stain (Molecular Probes, Eugene, OR, USA) to quantify the numbers of the viable and non viable bacteria in the biofilm community. A non specific nucleic acid stain was employed, as well as a number of lectin stains for specific carbohydrates known to be components of the EPS of certain bacteria (Staudt et al. 2003). Over time, the bacterial biomass distribution within the biofilm changed and heterogeneity with regard to certain bacteria and their associated EPS was observed. The same approach was applied by Strathmann et al. (2002) for the visualisation and biochemical characterisation of polysaccharides in biofilms of *Pseudomonas aeruginosa*. It is possible with these techniques to obtain morphological and quantitative information as well as identification of cell surface component (via labelling), identification of specific species in microbial consortia, distinction between viable and non-viable cells, and visualisation of the spatial organisation of microorganisms present in different environmental matrices.

CLSM has also been used to study the fate of metals within biofilms. The complexing agent Newport Green fluoresces upon binding with nickel, zinc or cobalt (Wuertz et al. 2000). Biofilm penetration by these fluorescent probes can be detected by CLSM. The combination of two techniques (acid stain Syto 17 to target different bacterial cells and Newport Green to target different metals, i.e., Ni, Zn or Co) allows one to determine the localisation of the metal in the biofilm when the pictures from both staining procedures are superimposed. Using the nucleic acid stain Syto 17 to detect cells (Neu & Lawrence 1997) and the complexing agent Newport Green to localize zinc (Wuertz et al. 2001), it was shown that the metal was bound primarily to cells wall and not to EPS. This trend became more obvious as the microbial population gradually changed from bacterial to algal and fungal. Clearly, the types of organisms present in a biofilm or activated sludge floc should be considered when making estimates of the sorption capacities.

3.2.3. *Nuclear Magnetic Resonance (NMR) imaging techniques for studying metal immobilisation and mass transfer in biofilms*

Nuclear magnetic resonance (NMR) spectroscopic and imaging techniques have been used in biofilm research to study metal sorption on biosorbents, the penetration of metals (Fe, Co and Ni) in biofilms as well as transport processes (diffusion and flow profiles) and hydrodynamic changes in biofilm environments (see van As & Lens 2001, and Lens & van As 2003 for review).

Direct NMR studies on heavy metal solutions at environmentally relevant concentrations are out of the

sensitivity range of present day NMR equipment for most metals. Some metals, i.e., ^{27}Al, ^{65}Zn and ^{113}Cd, however, possess magnetic properties and thus can be probed directly by NMR spectroscopy. ^{27}Al NMR has been used to investigate phosphorous precipitates (Duffy & van Loon 1995) and the architecture of zeolites (Stöcker 1996). Ogama et al. (1992) compared the binding capacity of proteins for the metals ^{65}Zn and ^{25}Mg to that for ^{43}Ca and ^{29}K by direct NMR spectroscopy. ^{113}Cd NMR spectroscopy has been used to probe metal binding sites in algae (Ke & Rayson 1990), humic acids (Chung & Moon 1994) and montmorillonite clay (Jun et al. 1996). In addition, ^{113}Cd NMR has been applied to investigate the effect of Cd pollution on enzyme systems, such as, e.g., methallothioneins (Vasak et al. 1998).

Apart from direct NMR spectroscopy of metal nuclei, metal interactions in biofilm systems can also be studied indirectly via their paramagnetic effects using relaxation time measurements. Many heavy metals of environmental relevance, i.e., Fe^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Cr^{3+}, Gd^{3+}, VO^{2+} have paramagnetic properties. These paramagnetic ions reduce the T_1 or T_2 relaxation rates of their solvent. Shortly, the two relaxation time constants T_1 and T_2 describe the rate and manner at which the nuclear spin system returns to equilibration after excitation, one time constant, the spin-lattice relaxation time T_1, describes the retrun to the equilibrirum state in the direction of the magnetic field. The second time constant, the spin-spin relaxation time T_2, characterises the return in the plane perpendicular to the applied magnetic field. Both relaxation time times depend on the physical environment (pore size and geometry, absorbing walls, packing density) as well as the chemical composition of the liquid phases (van As & Lens 2001). Thus, relaxation time measurements allow to monitor the transport of paramagnetic heavy metals within a biofilm system (Lens & Hemminga 1998; Nott et al. 2001). By binding these paramagnetic ions onto molecules of different size (e.g., dextran), these measurements also give information on the pore size of biofilms/aggregates (van As & Lens 2001).

The indirect observation of paramagnetic ions via their effect on the T_1 or T_2 relaxation rates of water molecules provides a sufficiently high sensitivity for NMR methods in general and especially for magnetic resonance imaging (MRI) as high amounts of water are present in all biofilm systems (Hoskins et al. 1999). MRI experiments allow to study the reaction-diffusion process of paramagnetic materials in a non-destructive manner with high spatial (several 10 μm) and temporal (several min) resolution (Gonzalez et al. 2001; Osuna et al. 2004b). Figure 5 gives an example of the penetration of Fe^{2+} into a UASB aggregate. At time zero, water doped with $FeCl_2$ is added to the aggregate. By applying the turbo spin echo (TSE) NMR imaging technique and rather short repetition times, only protons with short relaxation times appear as bright signals. After some time, Fe^{2+} ions penetrate into the aggregate, where they shorten the T_2 values of the water, resulting in darker signals. However, the solution concentrations of the MRI tracer must be chosen well above the quantities present in natural and bioreactor environments.

In situations where there is significant local enrichment of the metal species (such as in biosorption), it is possible to conduct experiments at much lower solution phase concentrations of the heavy metal ions. Studies on alginate based biosorbents show that there is a pronounced difference in the binding behaviour of divalent heavy metals (e.g., Cu^{2+}) compared to the predictions of the kinetic models (Nestle & Kimmich 1996). Hart et al. (2001) showed that the presence and nature of a fixed charge on the polysaccharide molecule affects the rate of cation transport in accordance with the theory of contact exchange. The presence of a negative charge on microbial exopolysaccharides may thus improve the availability of nutrient cations at the organism surface. Extending the work from relatively "clean" sorbent materials to more heterogeneous samples, which already contain paramagnetic particles, is another challenge especially in MRI studies: distortion of the images by magnetic susceptibility artefacts. Nott et al. (2001) have shown that 3D MRI can also provide the necessary contrast and spatial resolution to distinguish polyurethane foam, biofilm and precipitate from the surrounding fluid, with biosorption and bioaccumulation of Cu^{2+} providing the contrast.

3.3. *Probing local liquid phase composition in biofilms using miniaturised sensors*

Techniques to probe concentrations of chemical species in the liquid phase within biofilms must be minimally invasive in terms of disturbance of firstly, the delicate three-dimensional organization of microbial biofilm communities, secondly, the steep physicochemical gradients present over micrometer distances, and thirdly, other microenvironmental conditions such as boundary layers, diffusion and flow patterns on a

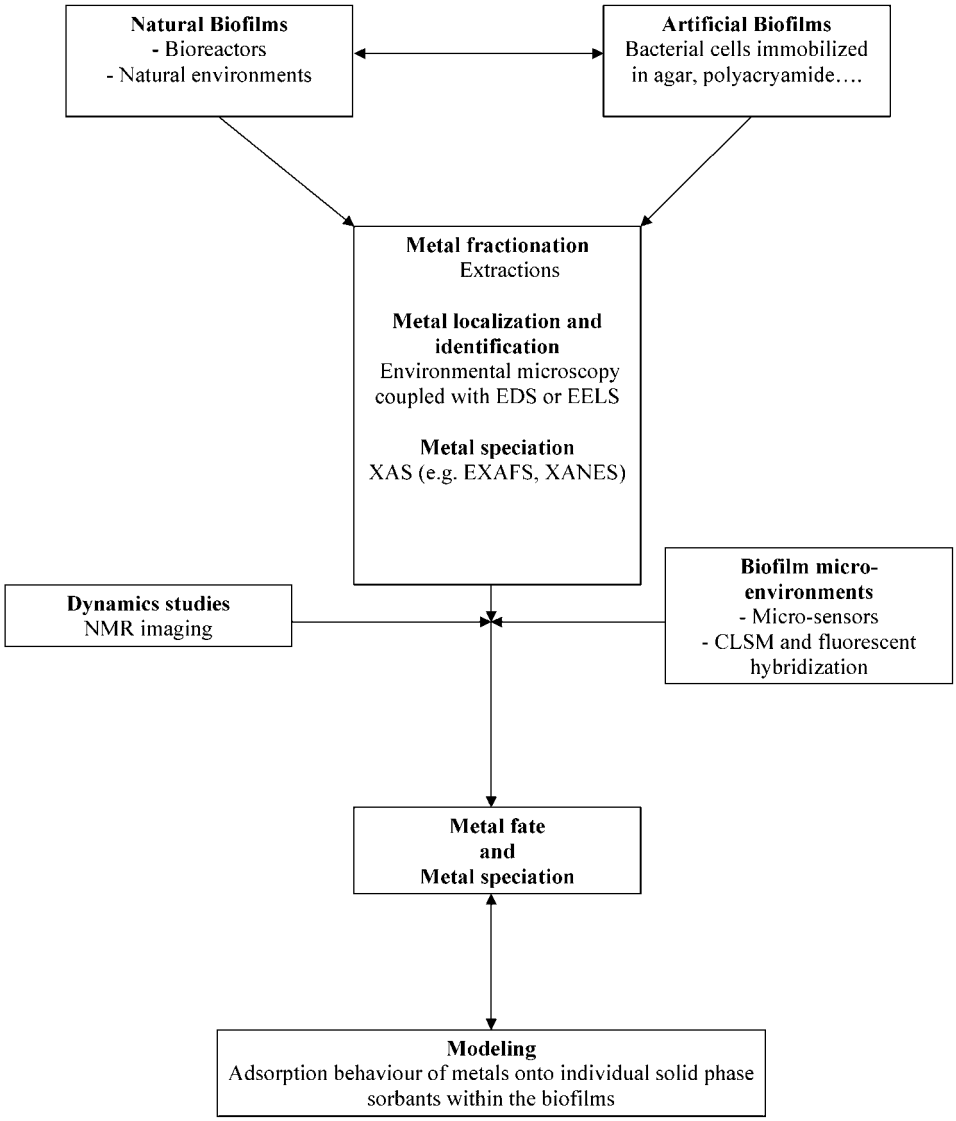


Figure 5. Proposal for a methodological study of the fate of metal within biofilms.

microscale. A good introduction to the various types and functions of microsensors currently available, i.e., potentiometric, optical, amperometric microsensors and micro-biosensors with high spatial resolution, is given by Santegoeds et al. (1998) and Lewandowski and Beyenal (2003). The high spatial resolution (<50 μm) of the microsensors is due to the small diameter of the tip (1–20 μm). Three types of microsensors are used in environmental applications: potentiometric sensors record the electrical potential at the tip generated by charge separation microsensors (Lewandowski & Beyenal 2003), amperometric sensors (H_2S, O_2) measure currents based on reduction or oxidation of a substrate inside the tip (Jeroshewski et al. 1996; Lewandowski & Beyenal 2003) and optrodes are optical fibers with a fluorescent indicator dye covering the tip (Klimant et al. 1997).

Microsensors that measure ΣH_2S, S^{2-}, H_2S, pH, redox potential and O_2 have almost become standard tools in biofilm studies (Jeroschewski et al. 1996; Allan et al. 1999; Bishop & Yu 1999; Okabe et al. 1999; Lewandowski & Beyenal 2003). Haack and Warren (2003) examined microbial biofilm dynamics using microsensors (O_2 and pH) and geochemical techniques (e.g., sequential extraction, and X-ray diffractometry to determine the bulk mineralogical

composition of air-dried homogenised biofilm samples) at both diel and seasonal time scales. They provide strong evidence for the significant role that microbial activity can play in metal geochemistry in natural environments. This study shows that two differing processes were controlling Cr and Ni/Co uptake in association with biofilm hydrous manganese oxyhydroxides (HMO) biominerals. While the mechanisms controlling Ni/Co cycling in association with HMO are likely sorption and or coprecipitation, Cr partioning is more consistent with redox associated immobilisation of Cr(III) with the HMO. All these processes appear to be contingent on the vertical migration of the oxic/anoxic boundary within the biofilm, which is itself controlled by the balance between photosynthesis and respiration on a diel basis.

Since the development of the solid state Au/Hg voltammetric (micro)electrode (Brendel & Luther 1995), it is possible to measure metals (Fe and Mn) and redox species (such as O_2 and H_2S) simultaneously. The electrodes have a (sub)millimetre vertical resolution in the biofilm ($>$100–300 μm). Direct insertion of the Au/Hg electrode into cultures (Dollhopf et al. 2000) documented the conversion of Mn(IV) to Mn(II) and Fe(III) to Fe(II) during the oxidation of organic matter. More recently, thanks to a voltammetric (micro)electrode, Glazer et al. (2002) and Luther et al. (2001) have documented the change of sulphur chemistry in salt marsh microbial mats and showed that soluble elemental sulphur, $S_2O_3^{2-}$ and polysulfides (S_x^{2-}) form in discrete layers of (sub)millimetre thickness in these environments. The electrochemical data in microbial mats correlate well with the data obtained from molecular biology (PCR amplification, and Denaturing Gradient Gel Electrophoresis (DGGE) analysis) and other methods including fluorescent microscopy with DAPI staining (Glazer et al. 2002). The microbial mat (composed of cyanobacteria, purple sulfur bacteria and sulfate reducing bacteria) described in this study is from a salt marsh panne, which produces reduced sulfur species from sediments underlying the mat. As they diffuse upward, these sulfur species can then be oxidized back to sulfate, or intermediate compounds such as elemental sulfur, polysulfides and thiosulfate, by oxygen, peroxide, iron(III), and manganese(III,IV) compounds both with and without microbial intervention. Glazer et al. (2002) describe the simultaneous *in situ* characterization of these redox species for an entire diurnal cycle using arrays of microelectrodes to document temporal, and three-dimensional spatial heterogeneity.

Microsensors are useful tools to measure *in situ* profiles of specific chemical species within biofilms at high spatial resolution (to 1 μm). The high spatial resolution of the physico-chemical information afforded by these microsensors will give insight toward a quantitative understanding of the processes involved in metal dynamics in biofilms. As there are no (bio)microsensors so far to measure microprofiles of metals, the metal dynamics need to be deducted from associated processes such as aerobic oxidation, nitrification, sulfate reduction and methanogenesis (Glazer et al. 2002; Haack & Warren 2003).

4. Future prospects

4.1. *Methodological study of the fate of metals in biofilms*

Figure 5 describes the methodology that can be applied to describe the mechanisms involved in the accumulation of metals in biofilms. Studying the fate of metals in biofilms requires minimal manipulation of this biological material before analysis to avoid any experimental artefact. For instance, examination of hydrated biofilm materials can now be realized by environmental electron microscopy (Surman et al. 1996; Ray & Little 2003). These tools can be coupled with EDS analysis or with EELS to determine the elemental composition and the oxidation state of elements, respectively (Daulton et al. 2001; Daulton et al. 2002; Ray & Little 2003). Speciation studies using sequential extraction must be validated by X-ray absorption spectroscopy (XAS). These X-ray studies permit to obtain a closer look at the actual binding sites of the biomass with the metals and clarify the role that complexing groups may play in metal binding (Kretschmer et al. 2002; Webb et al. 2003). XAS represents an ideal spectroscopic probe for investigating directly the coordination environment of metals in future biofilm studies. Figure 5 gives some ideas of what would be the ideal way to study biofilms from natural environments or biofilm cultivation systems. In order to get reproducible biofilms needed for studying metal-biofilms interactions, different kind biofilm cultivation systems can be used (see Boe-Hansen et al. 2003 for review).

4.2. *Supporting tools*

Other non-invasive techniques for the *in situ* study of biofilms are needed to elucidate the mechanism of metal binding and combine this knowledge with information on the nature of the biofilm constituents. Increasing the sensitivity and the validation of tools like NMR imaging and CLSM for metal localisation with Newport Green is required to work with trace metals so as to obtain insight into mass transfer and the dynamic behaviour as a function of the physico-chemical conditions. These tools can be used in combination with microsensors to get a good definition of the physico-chemical microenvironment within the biofilms. To relate the intra-biofilm chemistry to the biofilm structure, microsensors must be used in conjunction with other techniques commonly used in biofilms studies, such as CLSM and fluorescent *in situ* hybridisation (FISH) probes. In combination with the other measurements, microsensors provide information about local chemistries associated with the presence and distribution of various physiological groups of microorganisms, and with the physical structure of microbial aggregates in biofilms. In combination with other techniques their importance increases further. For example, one may wish to determine the distribution of SRB by applying FISH to samples from a biofilm (Wilderer et al. 2002). However, without any knowledge about the actual concentration profiles of ΣH_2S, pH, redox potential, no statements regarding the activities of these microbial populations can be made. Moreover, EDS or XAS applied to that biofilm can further document the location and chemical composition of metal accumulated within the biofilm (Figure 5). All the techniques reported in Figure 5 should be used in concert, this will allow far more accurate and detailed understanding of the biogeochemical linkages and processes controlling metal accumulation as shown in this paper.

5. Conclusions

Methodological study of the metal accumulation in biofilm requires the utilisation of geochemical techniques (sequential extraction, XAS, . . .). Studying the fate of metals in biofilms requires minimal manipulation of this biological material before analysis to avoid any experimental artefact. For instance, examination of hydrated biofilm materials can now be realized by environmental electron microscopy. XAS provides an ideal spectroscopic probe for investigating directly the speciation of metals in a biofilm. This review demonstrates that an interdisciplinary approach is required to study metal fate within the biofilm matrix, because microbial metabolic activity can create geochemical microenvironments within the cell's immediate vicinity, which differ from the bulk system conditions. Non-invasive techniques (e.g., microsensors, CLSM) for the *in situ* study of biofilms are needed to elucidate the mechanism of metal binding, which can be further linked to the information on the nature of the biofilm constituents. The combination of these techniques will allow deeper understanding of the mechanisms involved in metal accumulation in biofilms.

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