Environmental Microbiology (2005) **7**(3), 314–325 doi:10.1111/j.1462-2920.2004.00696.x

Bioreductive deposition of palladium (0) nanoparticles on *Shewanella oneidensis* with catalytic activity towards reductive dechlorination of polychlorinated biphenyls

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Summary

Microbial reduction of soluble Pd(II) by cells of *Shewanella oneidensis* MR-1 and of an autoaggregating mutant (COAG) resulted in precipitation of palladium Pd(0) nanoparticles on the cell wall and inside the periplasmic space (bioPd). As a result of biosorption and subsequent bioreduction of Pd(II) with H_2, formate, lactate, pyruvate or ethanol as electron donors, recoveries higher than 90% of Pd associated with biomass could be obtained. The bioPd(0) nanoparticles thus obtained had the ability to reductively dehalogenate polychlorinated biphenyl (PCB) congeners in aqueous and sediment matrices. Bioreduction was observed in assays with concentrations up to 1000 mg Pd(II) l^{-1} with depletion of soluble Pd(II) of 77.4% and higher. More than 90% decrease of PCB 21 (2,3,4-chloro biphenyl) coupled to formation of its dechlorination products PCB 5 (2,3-chloro biphenyl) and PCB 1 (2-chloro biphenyl) was obtained at a concentration of 1 mg l^{-1} within 5 h at 28°C. Bioreductive precipitation of bioPd by *S. oneidensis* cells mixed with sediment samples contaminated with a mixture of PCB congeners, resulted in dechlorination of both highly and lightly chlorinated PCB congeners adsorbed to the contaminated sediment matrix within 48 h at 28°C. Fifty milligrams per litre of bioPd resulted in a catalytic activity that was comparable to 500 mg l^{-1} commercial Pd(0) powder. The high reactivity of 50 mg l^{-1} bioPd in the soil suspension was reflected in the reduction of the sum of seven most toxic PCBs to 27% of their initial concentration.

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Introduction

Microbiological bioreduction of precious metals involves binding of metal ions to highly reactive bacterial cell surfaces and concomitant ion reduction (Karthikeyan and Beveridge, 2002). Both dissimilatory Fe(III)-reducing bacteria and archaea have the ability to reduce and precipitate gold (Kashefi *et al*., 2001) and silver appears to react in a similar fashion (Beveridge and Murray, 1976; Korenevskii *et al*., 1999). Biosorption and recovery of precious metals could be of economic benefit. This is especially the case for palladium, because palladium supply is very limited and therefore prices are much more volatile than those of other industrial metals. Palladium belongs to the platinum group metals (PGM), together with platinum and rhodium, which are used worldwide on an increasing scale, e.g. as a result of their adoption in automotive catalytic converters to reduce gaseous emissions in vehicle exhausts (Hoffman, 1988). Lloyd and colleagues (1998) demonstrated that live cells of *Desulfovibrio desulphuricans* were able to remove Pd(II) from anaerobic cell suspensions with either pyruvate, formate or hydrogen as electron donors with concentrations up to approximately 200 mg Pd(II) l^{-1} (2.0 mM).

As a result of the increasingly high value of precious metals used in catalysis and electronics, there is a growing interest in their recovery (Yong *et al*., 2002a). Reclamation of PGM is necessary as a result of their rarity, but current chemical recovery is complicated by complex solution chemistries (Demopoulos, 1989) because Pd(II) is the only PGM with predominating divalent cation in solution (Bailar *et al*., 1973). Several process technologies exist, each having its own drawbacks: (i) PGM can be separated from other metals by elution on chelating ion exchange resins containing polyisothiourea groups (Streat and Naden, 1983; Warshansky, 1983) but the metal extraction rates and selectivity are generally low, requiring large amounts of costly extractant (Yong *et al*., 2002a); and (ii) electrochemical recovery is feasible but a large electrode area is required and recovery of the thin metal film deposit from the electrode can be difficult (Lloyd *et al*., 1998). Finally, bioreductive deposition of Pd(0) onto biomass of *D. desulphuricans* has been studied extensively (Lloyd *et al*., 1998; Yong *et al*., 2002a; Yong *et al*.,

2002b) and enhanced recovery of Pd (and other PGM) from real processing waste at the expense of H_2 suggested a single-step conversion of waste into a useful product (Yong *et al*., 2002a).

Palladium catalysts have been demonstrated to be effective for reductive dechlorination of a variety of toxic substances such as polychlorinated biphenyls (PCBs) (Grittini *et al*., 1995). In the described method, palladium is supported on iron, resulting in the bimetallic catalyst Pd/Fe. Some authors state that iron is the reagent that dechlorinates the chlorinated hydrocarbon, while the addition of palladium as a catalyst can speed up the dechlorination (Liu *et al*., 2001). This way, the role of the precious metal would be to activate hydrogen, allowing the reduction of an oxidized redox active metal that dechlorinates. This reaction scheme is analogous to reduction of nitrate (NO_3^-) by the bimetallic Pd-Cu/g-Al_2O_3 catalyst (Gauthard *et al*., 2003). Other authors, however, propose that the H_2 liberated during chemical corrosion of Fe(0) leads to the entrapment of hydrogen within the Pd(0) matrix, which concomitantly would be the route of delivery of highly reactive $H^•$ to the target PCB (Korte *et al*., 1997). As it is, the catalytic role of Pd in dechlorination of chlorinated hydrocarbons has been experimentally well established, but is still not understood.

Some bacterial cell surfaces are highly reactive towards binding of metal ions (Beveridge, 1989) and preformed nanominerals (Glasauer *et al*., 2001). In Gram negative bacteria, this reactivity arises from the presence of a wide array of ionizable groups, such as carboxylates and phosphates, present in the lipopolysaccharide matrix of the cell wall as demonstrated for *Escherichia coli* (Beveridge and Koval, 1981). In the case of catalysts like palladium, small nanoparticles represent a large specific surface area and this has large implications towards electron cloud distribution and, concomitantly, reactivity of the catalyst. *Shewanella oneidensis* is one of the most versatile of all known metal reducing species (Heidelberg *et al*., 2002) and is especially known for its ability to precipitate metal nanoparticles which are either formed *de novo* by bioreduction or adsorbed from the surrounding medium (Beveridge, 1989; Glasauer *et al*., 2001; De Windt *et al*., 2003). It has recently been shown that *S. oneidensis* MR-1, of which we possess the autoaggregating COAG mutant with altered expression of outer membrane proteins (De Windt *et al*. submitted), is capable of bioreductive precipitation of chromium and vanadium (Carpentier *et al*., 2003; Middleton *et al*., 2003). It was the objective of this study to: (i) demonstrate bioreductive precipitation of Pd(II) on *S. oneidensis* MR-1 and COAG (ii) demonstrate catalytic activity of the bioPd towards recalcitrant chlorinated compounds like PCBs, and (iii) use the COAG mutant with increased adhesive properties to easily apply the biocatalyst in sediment suspensions with sorbed PCBs. In contaminated sediments, no effective dechlorination of the adsorbed PCB congener mixture has to our knowledge been described thus far.

Results

Pd(II) bioreduction assay

Fifty milligrams per litre of Pd(II) was added to bioreduction assays with several electron donors and alternate electron acceptors. Table 1 summarizes the Pd(II) reduc-

Table 1. Distribution of Pd in bioreduction assays over two fractions: un-reduced Pd(II) in solution that remains in the filtrate upon filtration over a 0.45-μm Millipore filter; and Pd(0) and Pd associated with *S. oneidensis* biomass that remains on a 0.45-μm Millipore filter upon filtration.

Support	e^- donor	Alternate e^- acceptors	C-source	Pd(II) in solution	Pd(0) and Pd associated with biomass
Autoclaved MR-1 cells	H_2	–	acetate	25.8 ± 0.7	56.1 ± 0.8
Autoclaved COAG cells	H_2	–	acetate	43.1 ± 0.4	30.4 ± 0.4
–	–	O_2	acetate	84.0 ± 1.1	0.7 ± 0.2
MR-1 cells	–	O_2	acetate	35.6 ± 0.7	49.0 ± 0.9
MR-1 cells	H_2	O_2	acetate	31.2 ± 8.3	68.8 ± 8.3
MR-1 cells	H_2	NO_3^-	acetate	0.4 ± 0.0	99.6 ± 0.0
MR-1 cells	H_2	–	acetate	0.5 ± 0.0	81.4 ± 3.3
COAG cells	H_2	–	acetate	0.4 ± 0.0	83.7 ± 1.4
Autoclaved MR-1 cells	CHOONa (*formate*)	NO_3^-, O_2	acetate	52.3 ± 1.0	37.1 ± 0.6
–	CHOONa	NO_3^-, O_2	acetate	78.9 ± 2.3	1.7 ± 0.2
MR-1 cells	CHOONa	O_2	–	0.1 ± 0.0	96.1 ± 3.7
COAG cells	CHOONa	O_2	acetate	0.3 ± 0.0	89.7 ± 1.2
MR-1 cells	CHOONa	–	acetate	0.0 ± 0.0	93.1 ± 0.5
MR-1 cells	CHOONa	NO_3^-, O_2	acetate	1.0 ± 0.5	92.8 ± 4.4
MR-1 cells	$CH_3CHOHCOONa$ (*lactate*)	O_2	–	0.1 ± 0.0	94.7 ± 2.9
MR-1 cells	$CH_3COCOONa$ (*pyruvate*)	O_2	–	10.0 ± 0.3	75.3 ± 1.4
MR-1 cells	CH_3CHOH (*ethanol*)	O_2	–	6.1 ± 0.4	76.9 ± 1.9

The treatment in each bioreduction assay is indicated, with respect to supporting biomass, electron donor for the bioreduction of Pd(II), alternate electron donors present in the assay, and possible carbon source present. Pd distribution is given as the percentage recovered from total Pd(II) added, 50 mg l^{-1}, in each fraction. All experiments were done in triplicate ($n = 3$). Results are represented as mean ± standard deviation.

tion results. In the case of H_2 as electron donor for bioreduction of Pd(II), no soluble Pd(II) was found in the filtrate when anoxic conditions were maintained. All of the recovered Pd was associated with biomass and precipitation of Pd(0) nanoparticles on the cell biomass had taken place, as observed by transmission electron microscopy (Fig. 2) and by black coloration of the biomass. The presence of 20 mg NO_3^--N l^{-1} did not inhibit the reduction process. Presence of O_2 resulted in more Pd(II) remaining in solution, however, still 68.8 ± 8.3% of the supplemented Pd(II) was found to be associated with biomass and resulted in Pd(0) precipitation. Control assays were set up in triplicate and indicated that in the case of autoclaved cells, up to 43.1 ± 0.4% of the supplemented Pd(II) was found unreduced in solution. At most, 56.1 ± 0.8% of the supplemented Pd(II) was associated with biomass. However, precipitation of nano-bioPd associated with the biomass was not observed. There was no significant difference between the behaviour of MR-1 cells compared to COAG cells, either as live cells or autoclaved cell suspensions, towards Pd(II) sorption and concomitant bioreduction.

In the case of formate as electron donor, all bioreduction assays resulted in precipitation of Pd(0) and more than 90% of the supplemented Pd(II) was recovered on the biomass (Table 1). The presence of alternate electron donors O_2 and NO_3^- did not inhibit the bioreduction and yielded no significantly different fractionation compared to anaerobic conditions. The presence of acetate as C-source had no effect on the bioreduction process and there was no difference between MR-1 and COAG cells. The control assays with autoclaved cells resulted in 37.1% of Pd on filter, whereas the chemical control assays without cells resulted in only 1.7% of Pd on filter. Because no bioreductive nanoprecipitation on autoclaved *S. oneidensis* cells could be observed, this shift in Pd distribution was probably as a result of adsorption of Pd(II) to biomass. This indicates that minor chemical Pd(II) reduction with concomitant Pd(0) precipitation took place under these conditions, comparable to the chemical Pd(0) precipitation with H_2.

Bioreduction assays with lactate, pyruvate and ethanol as electron donors indicated that under O_2 atmosphere, lactate yielded almost complete bioreductive removal of Pd(II) from solution (94.7 ± 2.9% associated with biomass on filter), whereas pyruvate and ethanol yielded slightly lower filter recoveries of 75.3 ± 1.4% and 76.9 ± 2.0%, respectively (Table 1).

To examine if these high removal efficiencies of Pd(II) from solution as a result of bioreduction by *S. oneidensis*, could also be obtained from bioreduction assays with high Pd(II) concentrations, reactions were set up in triplicate with 1000 mg Pd(II) l^{-1}. Both H_2 and formate were used as electron donors, under, respectively, anaerobic and oxygen-rich conditions. From Table 2 it appears that concentrations of 1000 mg Pd(II) l^{-1} could be bioreduced and resulted in a fraction of 77.4% and higher of cell-associated (nano-) bioPd in the bioreduction assays.

Properties of precipitate

X-ray diffraction (XRD) analysis of dried palladized cells confined the presence of Pd(0) metal associated with *S. oneidensis* cells (Fig. 1). The effect of electron donor on bioPd precipitates was studied by means of transmission electron microscopy. From Fig. 2(A and B), it can be seen that H_2 as electron donor for the Pd(II) bioreduction resulted in a lot of small Pd(0) particles associated with the cell surface and inside the periplasm, whereas formate as electron donor resulted in fewer, slightly larger precipitates associated with the cell surface and very few Pd(0) particles inside the periplasm. From Fig. 2 it can also be concluded that some cells showed no precipitation at all (Fig. 2F), and at the same time some were covered more densely by Pd(0) than others (Fig. 2D). Precipitation on pili or flagella was not observed (Fig. 2E).

Bio-nanocatalytic dechlorination of PCB 21

Polychlorinated biphenyl 21 (or 2,3,4-chloro biphenyl) was solubilized in M9 mineral medium with palladized *S. oneidensis* MR-1 or COAG cells. As electron donor for the activation of the Pd(0) catalyst, either H_2 or formate was used. A general reaction scheme for the described bio-nanocatalytic dechlorination reactions, is presented in Fig. 3. In the case of formate as electron donor, bioreduction of 500 mg l^{-1} Pd(II) resulted in palladized cells with high dechlorination rates of PCB 21. Within 1 h the concentration of PCB 21 dropped to undetectable levels

Table 2. Distribution of Pd in bioreduction assays with a high concentration (1000 mg l^{-1}) of supplemented Pd(II) over two fractions: associated with biomass, giving rise to nanopalladium particles on *S. oneidensis* cells; and as Pd(II) remaining unaltered in solution.

Support	e^- donor	Alternate e^- acceptors	C-source	Pd associated with biomass (mg l^{-1})	Pd(II) remaining in solution (mg l^{-1})
MR-1 cells	H_2	–	–	980.6 ± 70.8	0.1 ± 0.1
COAG cells	H_2	–	–	849.0 ± 39.2	0.6 ± 0.6
MR-1 cells	formate	O_2	–	815.1 ± 125.6	72.7 ± 16.6
COAG cells	formate	O_2	–	787.2 ± 42.1	23.5 ± 1.9

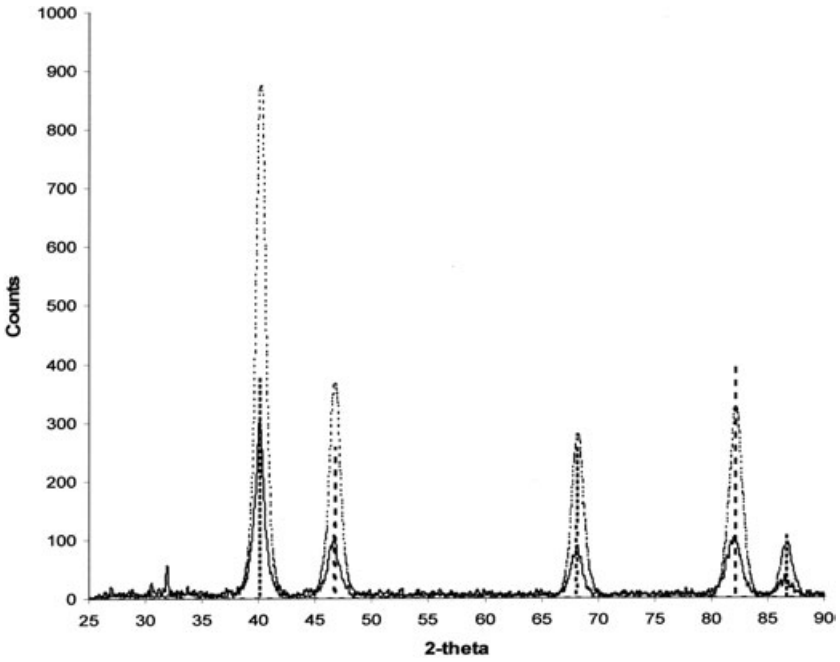


Fig. 1. X-ray diffraction (XRD) spectra of commercial Pd(0) powder (.....); bioreduced Pd(0) precipitated on *S. oneidensis* biomass or bioPd (——); and XRD spectrum of an internal standard (– –).

(Fig. 4A) with both palladized MR-1 and COAG cells. During the timespan of this experiment, only small peaks of PCB 1 and PCB 5 could be detected, but not quantified, attributed to almost immediate dechlorination. The chemical control with commercial Pd(0) powder resulted in somewhat slower dechlorination rates of PCB 21, only after 7 h did the concentration become less than 0.1 mg l^{-1}. To assess the influence of sorption on PCB 21 recovery, assays with *S. oneidensis* cells at the same cell density but not covered with bioPd were extracted with the solid phase extraction (SPE) protocol, 400 min after PCB 21 supplementation. No significant decrease of PCB 21 could be detected (results not shown), indicating that the SPE protocol could recover adsorbed PCBs from the suspended cells. At lower concentrations of bioPd (50 mg l^{-1}) and with formate as e^- donor, decrease of PCB 21 could only be detected after 48 h (data not shown).

With H_2 as an electron donor for the catalytic dechlorination process, there was catalytic activity at concentrations of 50 mg bioPd l^{-1} (Fig. 4B–D). Dechlorination rates of PCB 21 were comparable between palladized MR-1 or COAG cells and commercial Pd(0) powder at the same concentration. Dechlorination products of PCB 21, PCB 5 or 2,3-chloro biphenyl and PCB 1 or 2-chloro biphenyl, could be detected in all treatments. For palladized MR-1 and COAG cells, there was a clear shift of peaks in the chromatogram from PCB 21 over PCB 5 to PCB 1, also observable from Fig. 4. However, the chemical treatment resulted in only small amounts of both dechlorination products PCB 5 and PCB 1 at any sampling moment, indicative of increased dechlorination rates for these intermediates.

Bio-nanocatalytic dechlorination of PCB congeners from contaminated sediments

To address the issues of specificity of Pd(0) precipitation on *S. oneidensis* cells and selectivity of PCB dechlorination in a complex sediment matrix, dechlorination experiments were set up in triplicate with palladized COAG cells in suspensions of contaminated sediment. From Fig. 5(A) it can be seen that dechlorination of the PCB congeners in the sediment matrix was very fast and efficient in treatments with 500 mg l^{-1} commercial Pd(0) powder and either 50 mg l^{-1} or 500 mg l^{-1} bioPd as catalytic agent compared to 50 mg l^{-1} commercial Pd(0) powder. Chromatograms were obtained from three biological replicates ($n = 3$). The seven most toxic PCBs were sampled and analysed in each of the treatments. For all treatments it appeared that 48 h of incubation resulted in significant dechlorination of these seven congeners (Fig. 5B). No PCBs could be observed in the M9 supernatant above centrifuged soil pellets in any of the treatments upon SPE extraction, indicating that desorption of PCBs from the sediment did not take place. From these results it can be concluded that the catalytic activity of 50 mg l^{-1} bioPd is comparable to that of 500 mg l^{-1} commercial Pd(0) powder in complex sediment matrices with adsorbed PCBs at low temperatures (28°C) and within 48 h.

To address specificity of bioPd precipitation in the sediment matrix, controls with contaminated sediments and without *S. oneidensis* cells were incubated with Pd(II) at the same concentration. No catalytic effect towards PCB dechlorination could be observed and no dark coloration

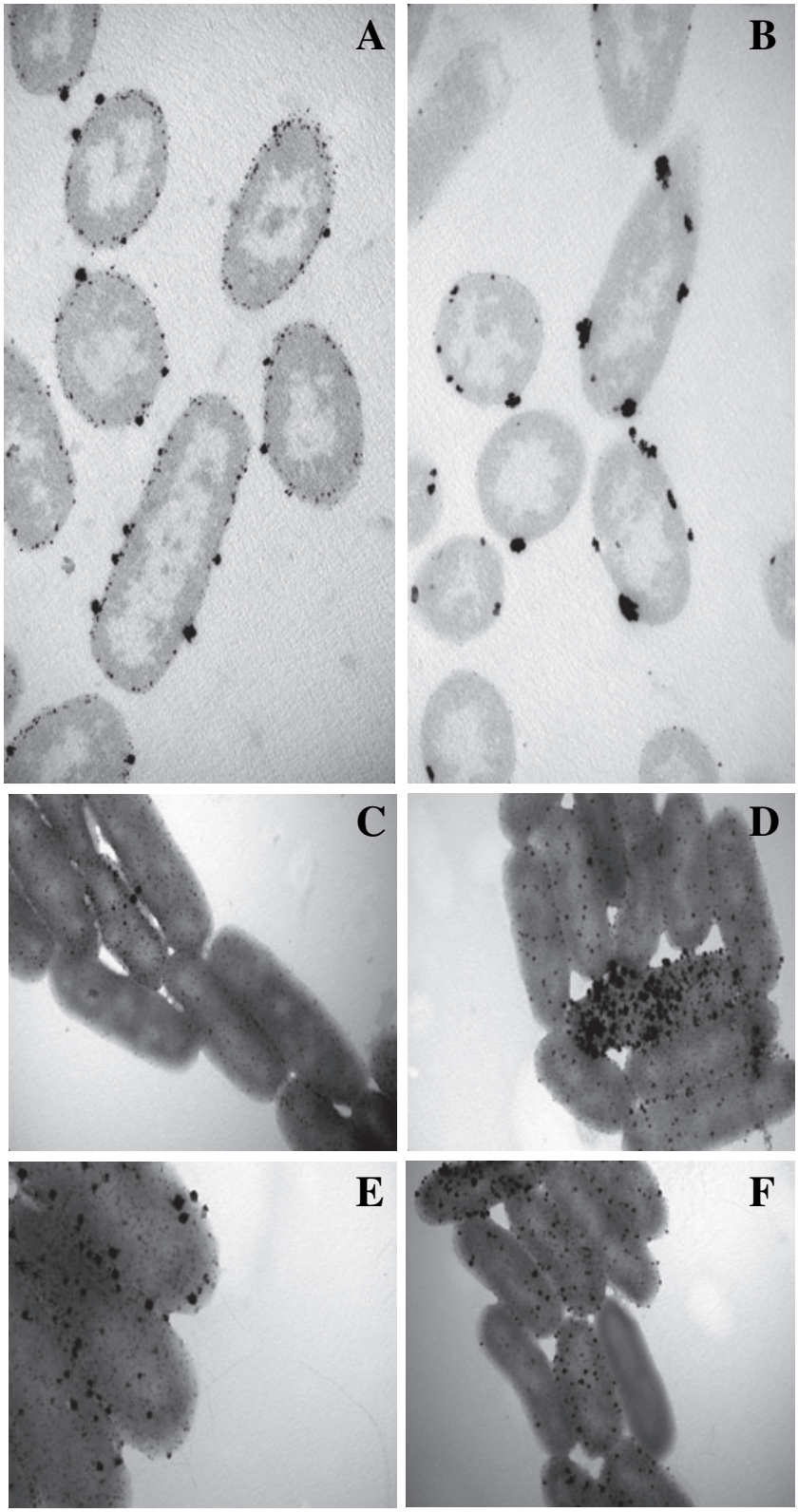


Fig. 2. Ultrathin sections of *S. oneidensis* MR-1 as viewed by transmission electron microscopy (TEM) in the case of: (A) bioreductive deposition of Pd(0) particles with H_2 as the electron donor for this process; (B) bioreductive deposition of Pd(0) particles with formate as electron donor for this process. Precipitation took place on the outer membrane and in the periplasmic space. Whole mount preparations of *S. oneidensis* MR-1 as viewed by TEM in the case of: (C) bioreductive deposition of Pd(0) particles with H_2 as the electron donor for this process; (D), (E) and (F) bioreductive deposition of Pd(0) particles with formate as electron donor for this process. From (D) and (F) it can be seen that the amount of bioPd precipitated per cell can vary with some cells showing no biodeposition at all. From (E) it can be observed that precipitation on pili or flagella did not take place.

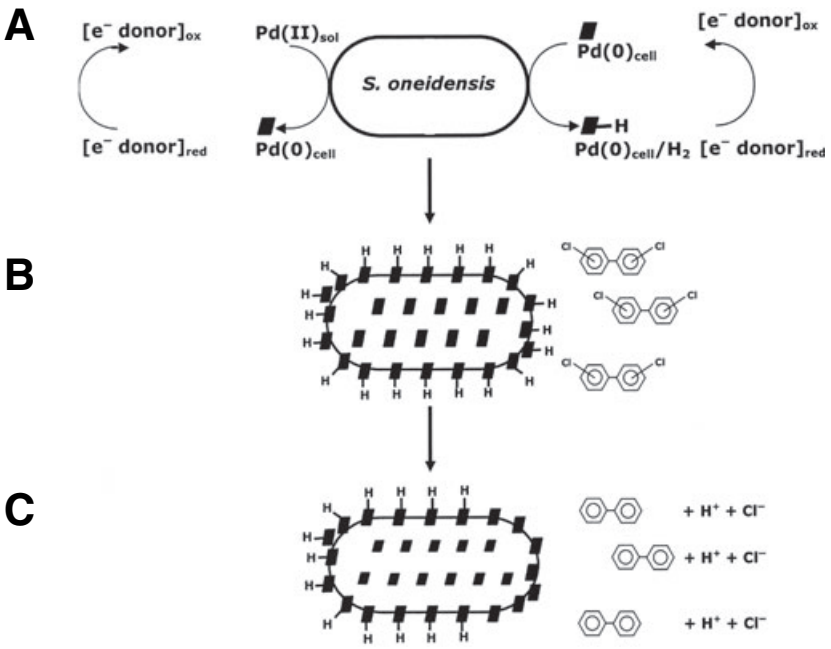


Fig. 3. Suggested general reaction scheme for dechlorination reactions by nanocrystals of Pd(0) at the *Shewanella oneidensis* cell surface.
A. *Shewanella oneidensis* cells couple the reduction of soluble Pd(II) to oxidation of several electron donors, including H_2, formate, pyruvate and ethanol. The resulting Pd(0) particles are precipitated as Pd(0), 'bio-palladium', associated with the cell surface. Reactivity of these nanocatalytic particles is obtained by charging them with H• radicals, originating from an electron donor (H_2 or formate).
B. The charged nanocatalytic particles associated with the *S. oneidensis* cell surface, are brought in contact with chlorinated compounds, like polychlorinated biphenyls (PCBs).
C. The catalytic reaction between the PCBs and the H• radicals from the bio-palladium, results in dechlorination of the chlorinated compound.

of the sediments, resulting from bioreductive Pd(0) precipitation, could be seen (results not shown).

Discussion

The results of this study demonstrated strong interaction of *S. oneidensis* cells with soluble palladium salts such as Na_2PdCl_4. Suspensions of live *S. oneidensis* cells supplied with either hydrogen, formate, lactate, pyruvate or ethanol as electron donor removed Pd(II) from solution with at least 68.8% depletion of dissolved Pd(II) by overnight shaking. This resulted in a black precipitate, visually detectable after 5 min with organic electron donors, not seen with suspensions of killed (autoclaved) cells or with live cells lacking an electron donor. Bioreduction of Pd(II) by *S. oneidensis* cells resulted in recoveries higher than 90% of Pd associated with biomass when organic electron donors lactate or formate were used, regardless of the presence of O_2 or NO_3^-. Lloyd *et al*. (1998) also found that sparging of cultures with air (O_2) only had a minor effect on Pd precipitation on *D. desulphuricans*. Interestingly, the slower bioreduction with H_2 gas resulted in relatively more small particles of bioPd on the cells, as can be observed by transmission electron microscopy (TEM). Some cells were relatively more covered with bioPd than others in the same Pd(II) bioreduction assay, whereas some cells showed no precipitation at all. These results indicate that the process of Pd(0) deposition was probably not a purely chemical process, but that metabolic differences between cells could result in differences in precipitation profile. The enzyme responsible for Pd(II) reduction has not been identified in studies with *D. desulphuricans*, but the involvement of a periplasmic hydrogenase was implicated by the consumption of hydrogen as electron donor and by the inhibition of the bioreduction with 0.5 mM Cu^{2+}. A combination of hydrogenase with cytochrome c_3 activity has also been proposed (Lloyd *et al*., 1998). Since *S. oneidensis* has both [Ni/Fe] and [Fe] hydrogenases as well as cytochrome c_3 (Heidelberg *et al*., 2002), the proposed mechanism of Pd(II) reduction by *D. desulphuricans* could be valid for *S. oneidensis*. Given the efficient bioreduction of Pd(II) with formate by *S. oneidensis*, there may also be a role for the formate dehydrogenase in combination with the hydrogenase component of the formate hydrogenlyase structural unit, when formate or its precursor is supplied as electron donor. This model has already been validated for Tc(VII) reduction (Lloyd *et al*., 1997). Much like the process of gold precipitation by *Pseudomonas aeruginosa* described by Karthikeyan and Beveridge (2002), precipitation inside the periplasm was readily observed by TEM. It is likely that the participation of hydrogenase is required to initiate Pd(0) deposition on the cells (Lloyd *et al*., 1998; Baxter-Plant *et al*., 2003). The reduction of Pd(II) to Pd(0) later becomes self-sustaining via the ability of crystalline solid Pd(0) to absorb hydrogen and autocatalytically reduce more Pd(II) (Yong *et al*., 2002b). Concentrations up to 1000 mg l^{-1} could be bioreduced with H_2 or formate as electron donor. Such high concentrations have, to our knowledge, never been reported for bioprecipitation of any noble metal. These findings suggest a potential application for the binding, precipitation and possible biorecovery of palladium by *S. oneidensis*.

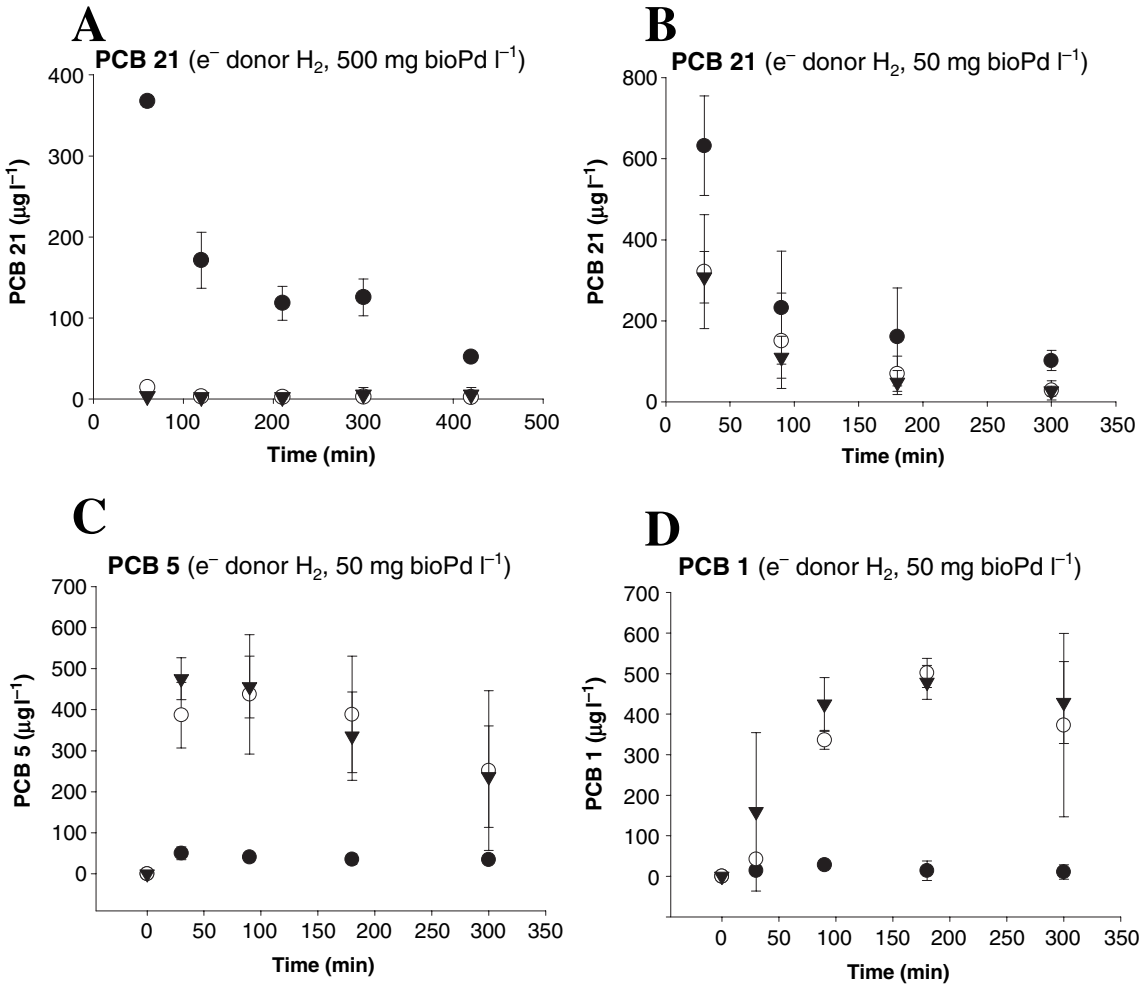
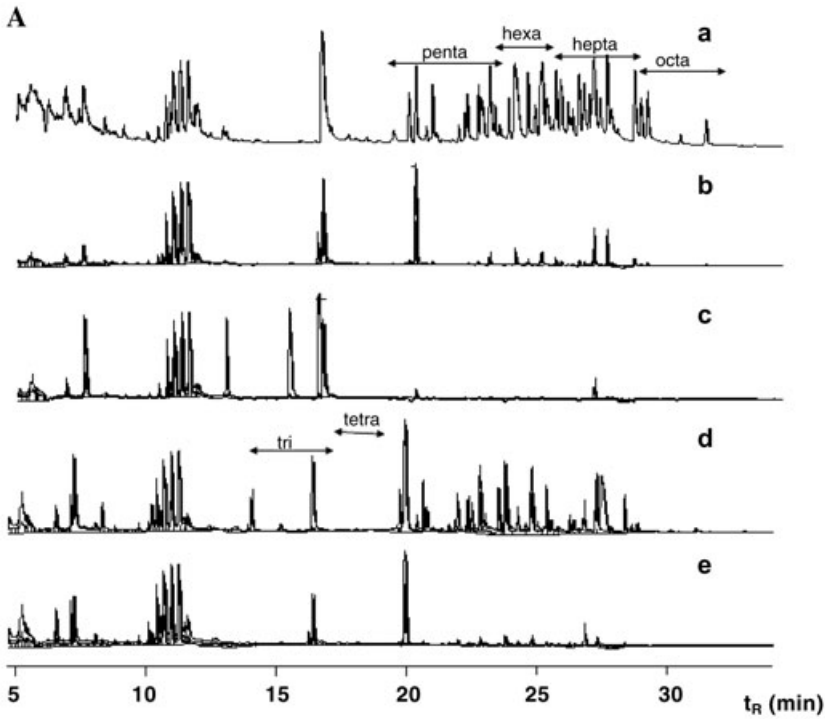


Fig. 4. A. PCB 21 dechlorination in M9 mineral medium by 500 mg l^{-1} commercial Pd(0) powder (●), 500 mg l^{-1} bioPd coated on MR-1 cells (○) and 500 mg l^{-1} bioPd coated on COAG cells (▼). For bioreduction of Pd(II) and dechlorination, the electron donor was 25 mM formate. Degradation products could be detected but were below the LOQ, because of the high rate of the catalytic reactions. All experiments were done in triplicate.
B. PCB 21 dechlorination in M9 mineral medium by 50 mg l^{-1} commercial Pd(0) powder (●), 50 mg l^{-1} bioPd coated on MR-1 cells (○) and 50 mg l^{-1} bioPd coated on COAG cells (▼). For bioreduction of Pd(II) and dechlorination, 1 atm of H_2 was the electron donor.
C. PCB 5 as a first intermediate in the dechlorination of PCB 21 as described in (B).
D. PCB 1 as the second intermediate in the dechlorination of PCB 21 as described in (b) and (c). All experiments were done in triplicate. 1,2,3-trichlorobenzene was used as an internal standard.

The precipitation of nano-scale catalytic particles was hypothesized to increase the reactivity towards recalcitrant halogenated compounds, as observed by Baxter-Plant and colleagues (2003) in a previous study. As a model compound we chose PCBs for several reasons:

1. Despite international efforts to control and regulate persistent halogenated organic pollutants, past and ongoing releases have resulted in widespread contamination of soils and sediments (Fennell *et al.*, 2004). The US Environmental Protection Agency (EPA) Toxics Release Inventory Database indicates that 1.13 million kg of PCBs were released from monitored industries in 2001 alone (US Environmental Protection Agency. Toxics Release Inventory Database, http://www.epa.gov/tri/). This contamination with PCBs is of great public concern because of bioaccumulation and potential toxicity to humans and wildlife (Erickson, 1997; Cogliano, 1998).
2. The cost of current remediation technologies, including excavation and high temperature treatments, is enormous – so much that clean up of large areas has not often been attempted.
3. Advances in technologies to remediate sediments and soils are needed (Fennell *et al.*, 2004). The use of microorganisms for *in situ* remediation represents one potential solution. Aerobic oxidative degradation of PCBs usually attacks only lightly chlorinated conge-



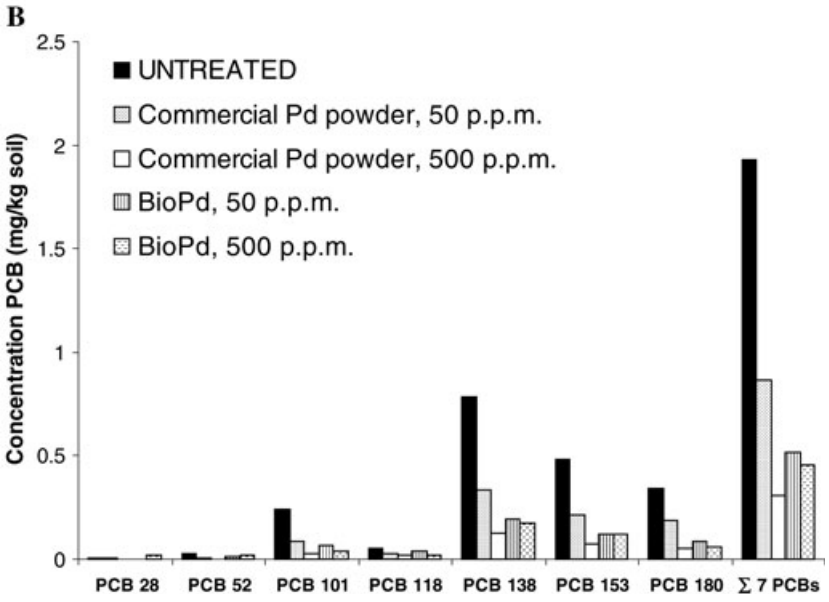


Fig. 5. A. Chromatograms of extracts from PCB-contaminated sediment suspensions (1:3 sediment v/v), untreated (a) or treated during 48 h under H_2 atmosphere with: (b) 50 mg bioPd per litre sediment suspension; (c) 500 mg bioPd per litre sediment suspension; (d) 50 mg commercial Pd powder per litre sediment suspension; (e) 500 mg commercial Pd powder per litre sediment suspension. All experiments were done in triplicate. The chromatogram of each treatment represents three replicates.
B. Concentration of seven PCB congeners in PCB-contaminated sediment suspensions (1:3 sediment v/v), before and after treatment during 48 h under H_2 atmosphere with different commercial Pd and bioPd concentrations.

ners, i.e. biphenyls with five or fewer chlorines. Anaerobic reductive dechlorination can attack a large array of highly chlorinated PCBs and reduces their toxicity, thus increasing their degradability. However, based on the published literature, there are wide variations of the rate, extent and specificity towards flanked and unflanked *meta*, *para* and *ortho* microbial dechlorination. Furthermore, based on the present results, simple inoculation of a contaminated environment with enrichment cultures appears to be neither suitable nor practicable for successful stimulations of the significant dechlorination rates required for a practical and economical process (Reviewed by Wiegel and Wu, 2000).

Catalysis is a relatively new approach towards fast dechlorination of PCBs under mild conditions. Recently, it was found that the activity of iron as a reagent to dechlorinate chlorinated hydrocarbons can be greatly enhanced by the addition of palladium as a catalyst (Liu *et al.*, 2001). Reductive dehalogenation of PCBs using palladized Fe(0)

surfaces had been described previously by Korte and colleagues (1997). However, these technologies are mostly suitable for dechlorination in water and as such not really applicable *in situ*. The recent work of Baxter-Plant and colleagues (2003) put forward a whole new integrated approach towards reductive dechlorination of chlorinated aromatic compounds by using palladized biomass of several *Desulfovibrio* spp. strains. In the latter study, dechlorination of PCBs within 24 h was observed, but less than 10% of all Cl-equivalents added was released as a result of catalytic activity of bioPd in the case of mono-, tri-, or tetrachloro biphenyls. With palladized *S. oneidensis* cells at a concentration of 50 mg bioPd l^{-1}, we removed 2,3,4-chloro biphenyl with efficiencies greater than 90% from suspensions in M9 with concomitant release of both its dechlorination products: 2,3-chloro biphenyl and 2-chloro biphenyl. This reaction took place with H_2 as electron donor and within 5 h at 28°C. Formate as electron donor resulted in slower reactivity towards PCB 21 at the same concentration of bioPd, but at a 10 times higher bioPd concentration, dechlorination with formate proceeded faster than with H_2. These results indicate that the electron donor for both bioreductive precipitation of Pd and activation of the catalytic Pd particles determines the rate and extent of dechlorination. As the specific surface area of a catalyst determines to a great extent its reactivity, these results could indicate that the total catalytic area of bioPd(0) produced by bioreduction with formate, is relatively small at lower concentrations of Pd(II). This is consistent with the observation that palladium bioreduction with formate as electron donor resulted in fewer and slightly larger bioPd(0) particles at the *S. oneidensis* cell surface, compared to bioPd(0) particles resulting from Pd(II) reduction with H_2. Formate as electron donor for the complete integrated approach (bioprecipitation plus dechlorination) seems feasible, but dechlorination will proceed much slower at lower concentrations of bioPd. Dechlorination with commercial Pd(0) in the chemical control assays proceeded faster than dechlorination with bioPd under H_2 atmosphere, to the extent that only minor amounts of the dechlorination products PCB 5 and PCB 1 could be detected at any sampling time, probably because of immediate reaction. The reason for this could be that, as Pd(0) powder was the only particulate material in these chemical control assays, dechlorination products PCB 1 and PCB 5 were concentrated onto the catalyst as a result of sorption. Interestingly, no differences in dechlorination kinetics were observed for palladized MR-1 cells compared to COAG cells. *Shewanella oneidensis* COAG was only recently described as a *S. oneidensis* MR-1 mutant with upregulation of some outer membrane proteins (W. De Windt, H. Gao, W. Krömer, J. Dick, J. Mast, N. Boon *et al.*, submitted). These altered phenotypic properties lead to autoaggregation and increased biofilm formation. Different *Desulfovibrio* spp. strains were described to result in different catalytic behaviour of the palladized cells, and this was linked to different 'templating' surfaces of the strains (Baxter-Plant *et al.*, 2003). However, the altered surface properties seemed to have no effect in the case of *S. oneidensis* COAG.

To assess catalytic activity of bioPd in complex sediment matrices contaminated with a mixture of PCB congeners, *S. oneidensis* cells were allowed to bioreductively precipitate Pd(0) inside the sediment matrix. The COAG strain was used for these experiments, because of its increased biofilm formation it was expected to easily colonize sediment particles. From the results of the dechlorination assays with contaminated sediment slurries, it was observed that bioPd yielded a higher rate of dechlorination than commercially available powdered Pd(0). Fifty milligrams per litre of bioPd resulted in a catalytic activity that was comparable to 500 mg l^{-1} commercial Pd(0) powder. The high reactivity of 50 mg l^{-1} bioPd in the soil suspension was reflected in the reduction of the sum of seven most toxic PCBs to 27% of their initial concentration, within 48 h and at 28°C. There was no selective effect towards dechlorination of specific PCB congeners, as the catalytic effect was observed over a wide range of highly and lightly chlorinated PCBs. These findings may contribute to the search for better alternatives for the current remediation technologies of PCBs. Further research will focus on the engineering of this technique to allow lower concentrations of bioPd and to use organic electron donors like formate, that are easy to supplement *in situ*. Also, production of H_2 in sediments, both chemically or microbiologically, could support dechlorination in combination with bioPd(0) particles *in situ* and deserves further exploration. Consequent research will focus on short- and long-term toxicity of both Pd(II) and dechlorination products as well.

Experimental procedures

Bacterial strains and growth conditions

Shewanella oneidensis MR-1 was obtained from the BCCM/LMG Bacterium Collection (Ghent, Belgium) under the number LMG 19005. *Shewanella oneidensis* COAG was obtained previously (W. De Windt, H. Gao, W. Krömer, J. Dick, J. Mast, N. Boon *et al.*, submitted). Both strains were grown aerobically in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) overnight at 28°C. Bacterial cell numbers were determined by plating dilution series in sterile physiological solution (8.5 g NaCl l^{-1}) on LB medium solidified with 15 g l^{-1} Bacto agar (Difco, Detroit, MI).

Pd(II) bioreduction assay

Shewanella oneidensis cells were harvested from an overnight LB culture in sterile 50 ml centrifuge tubes (TTP, Switzerland) by centrifuging at 3.000 *g* for 10 min and washed

three times with 50 ml M9 medium (Sambrook *et al*., 1989). The washed cells were resuspended in 30 ml M9 medium in glass serum bottles, with a concentration corresponding to a final OD_{610} of 2.0 ± 0.2. Serum bottles were capped with inert viton stoppers. Control reactors containing no cells or autoclaved cells (OD 2.0) were set up in parallel. Depending on the experimental set-up, the cell suspension was supplemented with 25 mM formate, 25 mM pyruvate, 25 mM lactate or 25 mM ethanol as organic electron donor. Bioreduction assays that excluded the presence of oxygen were flushed 10 times by 2 min of 1 atm Ar overpressure followed by 2 min of vacuum. In the case of H_2 as electron donor, the headspace was supplied with 1% v/v H_2 gas. Fifty milligrams per litre (0.8 mM) of acetate was added as a C-source during anaerobic *S. oneidensis* metabolism. The influence of alternate e^- acceptors O_2 and nitrate on Pd(II) reduction by *S. oneidensis* was tested. In the case of NO_3^-, sodium nitrate was added to a final concentration of 20 mg NO_3^-N l^{-1}. When O_2 was supplemented as alternate e^- acceptor, the headspace was filled with air. Finally, 50 mg Pd(II) l^{-1} was added to the bioreduction assay, supplemented as Na_2PdCl_4 (Sigma-Aldrich, Seelze, Germany). The serum bottles were then incubated overnight at 28°C. pH in all assays remained 7.1 ± 0.2 and all assays were set up in triplicate. After incubation, 10 ml of the Pd(II) bioreduction assay suspension was 0.45 μm filtered (Millipore, Bedford, USA). The filtrate contained soluble Pd(II) and the filter fraction biomass-associated Pd. Filters were boiled during 2 h in aqua regia to analyse Pd content in the filter cake. Aqua regia was then diluted and filtrate and solubilized filter-suspensions were analysed by Inductively Coupled Plasma (ICP). The experimental set-up is described in Table 1.

Pd analysis by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES)

Pd was measured by ICP-AES (Varian Vista MPX, Varian, Palo Alto, CA) at four wavelenghts: 229.65, 340.49, 342.12 and 360.96 nm. After calibration, final Pd concentrations were determined as the mean of the concentrations obtained at these four wavelenghts.

X-ray diffraction analysis

X-ray diffraction analysis of palladized cells, dried at 30°C, was performed with a Siemens Diffractometer D5000 with BraggBrentano optics (Siemens, Munich, Germany). X-rays were generated by a copper X-ray tube with power 1.6 kW (40 kV, 40 mA). Measurements were in between 25 and 90 degrees 2-theta with step time of 1.6 s and step size of 0.02 degrees.

Bio-nanocatalytic dechlorination assay of PCB 21 (2,3,4-chloro biphenyl) in mineral medium

Palladized cells were obtained from Pd(II) bioreduction assays with the appropriate electron donor depending on the experimental set-up, as described above. Palladized cells were harvested by centrifugation (3.000 *g* for 10 min), washed in M9 mineral medium and resuspended in the same volume (30 ml) of fresh M9 in serum bottles with viton stoppers. The suspension was supplemented with the appropriate e^- donor to activate the Pd catalytic particles. In the case of formate (25 mM) as electron donor, O_2 remained in the headspace. When using H_2 (1% v/v) as e^- donor, cell suspensions were made anaerobic as described above. The concentration of Pd metal particles in each assay, supplemented as palladized cells with bioPd, was calculated based on the amount of Pd(II) dosed in the bioreduction assay. Chemical controls consisted of M9 supplemented with commercial Pd(0) powder of an appropriate concentration (Sigma-Aldrich, Seelze, Germany). As a negative control, *S. oneidensis* MR-1 cells were suspended in M9 medium without Pd(II) and without Pd(0) but with addition of the electron donor. One milligram per litre of PCB 21 (2,3,4-chlorobiphenyl) was added to each dechlorination assay from a methanol stock solution. Consequently, the serum bottles were incubated at 28°C and shaking was applied to homogenize the suspension (100 r.p.m.). At regular time intervals, samples were taken and PCBs were extracted by SPE. All dechlorination assays were set up in triplicate.

Extraction of PCB from watery suspensions by SPE

Polychlorinated biphenyls were extracted from 2-ml samples by SPE according to Nollet *et al*. (2002). The cartridge used was Bond Elut C18 (Varian, Middelburg, the Netherlands), with a column volume of 3 ml, a sorbent mass of 200 mg and a particle size of 40 μm. Upon SPE, the PCBs were analysed with gas chromatograph (GC)-electron capture detector (ECD). During the extraction, 1 mg l^{-1} 1,2,3-trichlorobenzene was added to the hexane solvent as an internal control. The extraction efficiency of PCB 21 from cell suspensions with no catalytic activity was 91.8 ± 7.5%.

Bio-nanocatalytic dechlorination assay of PCB contaminated sediments

Shewanella oneidensis COAG cells were harvested from an overnight LB culture by centrifugation at 3.000 *g* for 10 min and resuspended in an equal volume of M9 medium, giving rise to a cell concentration corresponding to an OD_{610} of *c.* 1.8. Contaminated sediments were obtained from an anonymous industrial source. Sediment slurries were mixed with the cell suspension in M9 mineral medium to a 1:3 ($v_{sediment}/v_{cell\ suspension}$) ratio by vortexing. Ten mM formate was added to the sediment suspension and after homogenization, subsamples of 30 ml were distributed in serum bottles. Chemical controls consisted of sediment suspensions in M9 (1:3 v/v) without addition of suspended cells. Each assay was set up in triplicate. The headspace was replaced by 100% H_2 gas (1 atm). After overnight incubation of *S. oneidensis* COAG cells in the contaminated sediment at 28°C, 50 or 500 mg l^{-1} Pd(II) was added as Na_2PdCl_4 to yield a theoretical amount of 50 or 500 mg l^{-1} bioPd after complete bioreduction in the sediment matrix by *S. oneidensis* cells. 25 mM formate was added as the electron donor for this process. In all chemical controls, 50 or 500 mg l^{-1} commercial Pd(0) powder was added with 25 mM formate. The assays were incubated during 48 h at 28°C to allow bioreductive precipitation of bioPd and concomitant PCB dechlorination. Negative con-

trols consisted of sediment suspensions in M9 (1:3 v/v) without *S. oneidensis* COAG cells added, but with Pd(II) to yield a theoretical stoechiometric amount of 500 mg l^{-1} Pd(0) if any bioreduction would take place. After incubation, 2.5 g sediment (wet weight) was sampled from each dechlorination assay by centrifugation, and extracted as described below.

Extraction of PCB from contaminated sediment

Extraction of 2.5 g soil occurred with 10 ml acetone : hexane 1:1 (v/v) solvent during 24 h. The sediment was homogenized in this solvent mixture by vortexing. It was important that no PCB dechlorination occurred during the extraction, as Pd was present on the sediment. Although no electron donor was present during the extraction, all precautions were taken. Therefore, extraction took place by rigorous shaking (250 r.p.m.) on ice to inhibit possible catalytic processes during the extraction procedure.

To validate this extraction method, a control was set up in triplicate. In this control, Pd(0) that had been activated by overnight incubation in M9 under H_2 atmosphere, was added in a 500 mg l^{-1} concentration to a sediment suspension in M9 (1:3 v/v) just before solvent extraction with acetone:hexane. A total of 2.5 g sediment was obtained ($n = 3$) by centrifugation (5000 *g*, 10 min) and resuspended in acetone:hexane (1:1). No detectable dechlorination took place during the extraction of these sediment samples, indicating that catalytic dechlorination of the extracted PCBs did not occur in the described extraction procedure.

Cu/Florisil clean-up of PCB extracts from soil

Glass columns packed with Florisil/copper powder were used to remove interferences such as sulphur and fine granular debris from the extracted samples (Quensen *et al*., 1990). The copper was activated with conc. H_2SO_4 prior to mixing with Florisil powder in a Cu:Florisil 1:4 ratio.

Quantification of PCB congeners by GC-ECD

Polychlorinated biphenyls were analysed with a GC, Varian 3800 (Varian, Middelburg, the Netherlands) equipped with an ECD. N_2 was used as carrier gas at a flow rate of 1.3 ml min^{-1}. The extracted sample (1 μl) was injected with a split ratio of 1/10. For detection of PCB 21 (2,3,4-chloro biphenyl) and dechlorination products PCB 1 (2-chloro biphenyl) and PCB 5 (2,3-chloro biphenyl), the temperature was increased from 50°C to 170°C in steps of 30°C min^{-1}, followed by 10°C min^{-1} to 270°C. The pressure in the capillary column (CP-sil 8.3 m × 0.32 mm × 0.25 mm) was kept at 20 psi. For detection of PCB congeners from soil extracts, temperature was held for 2 min at 100°C, increased to 160°C with a gradient of 15°C min^{-1}, followed by 5°C min^{-1} to 270°C and held at 270°C for 10 min The pressure in the capillary column was 16 psi. For PCB 1, PCB 5, PCB 21, PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180, calibration curves were made. Sediment extracts were spiked with PCB 30 and PCB 209 as internal reference.

Acknowledgements

This work was supported by a grant from the Fund for Scientific Research in Flanders within scope of the FWO project with accession number G.0054.02. We thank – within Ghent University – the Department of Analytical Chemistry for ICP analyses and the Department of Geology for XRD analyses; Jan Mast (CODA) for TEM analyses; and Sylvie Seurinck, Tom Van De Wiele, Vincent Denef, Roeland Grommen and Nico Boon for critically reading the manuscript.

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