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ORIGINAL PAPER

Biological control of the size and reactivity of catalytic Pd(0) produced by *Shewanella oneidensis*

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Abstract The interaction between *Shewanella oneidensis* MR-1 and the soluble metal Pd(II) during the reductive precipitation of Pd(0) determined the size and properties of the precipitated Pd(0) nanoparticles. Assessment of cell viability indicated that the bioreduction of Pd(II) was a detoxification mechanism depending on the Pd(II) concentration and on the presence and properties of the electron donor. The addition of H_2 in the headspace allowed *S. oneidensis* to resist the toxic effects of Pd(II). Interestingly, 25 mM formate was a less effective electron donor for bioreductive detoxification of Pd(II), since there was a 2 log reduction of culturable cells and a 20% decrease of viable cells within 60 min, followed by a slow recovery. When the ratio of Pd:cell dry weight (CDW) was below 5:2 at a concentration of 50 mg l^{-1} Pd(II), most of the cells remained viable. These viable cells precipitated Pd(0) crystals over a relatively larger bacterial surface area and had a particle area that was up to 100 times smaller when compared to Pd(0) crystals formed on non-viable biomass (Pd:CDW ratio of 5:2). The relatively large and densely covering Pd(0) crystals on non-viable biomass exhibited high catalytic reactivity towards hydrophobic molecules such as polychlorinated biphenyls, while the smaller and more dispersed nanocrystals on a viable bacterial carrier exhibited high catalytic reactivity towards the reductive degradation of the anionic pollutant perchlorate.

Keywords Flow cytometry · LIVE/DEAD · Toxicity · ClO_4^- · PCB

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Introduction

The sorption and precipitation onto *Shewanella* cells of biogenic mineral, de novo formed by dissimilatory reduction, or non-biogenic metal particles has been described for a number of inorganic compounds. *Shewanella putrefaciens* and *Shewanella oneidensis* are able to precipitate vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) and this can be of

relevance for metal corrosion abatement (Jorand et al. 2000; De Windt et al. 2003). Precipitation of large quantities of nanometer-scale ferrihydrite (hydrous ferric oxide), goethite (α-FeOOH), and hematite (α-Fe_2O_3) adhered to the cell surface of *S. putrefaciens* and this has been related to adherence to amorphous Fe(III) oxides during dissimilatory Fe(III)-reduction (Caccavo et al. 1997; Glasauer et al. 2001). The production of intracellular grains of iron oxide minerals during growth on two-line ferrihydrite has been described for *S. putrefaciens* (Glasauer et al. 2002). Apart from iron-containing minerals, *Shewanella* sp. can reductively precipitate a number of other elements, such as U(VI) to insoluble U(IV) oxide (Lovley et al. 1991; Caccavo et al. 1992; Truex et al. 1997) and vanadium pentoxide to a vanadyl-containing solid (Carpentier et al. 2003). All of these reactions result in the bioprecipitation of minerals, and while some interactions could lead to applications for the extraction of soluble contaminants from soil or waste streams, others contribute to our knowledge of geochemical mineral cycling. However, with the possible exception of vivianite, none of these processes results in the production of biogenic particles with added value.

Recently, when *S. oneidensis* was found to reduce Pd(II) and to bioreductively precipitate nano-scale catalytic particles of Pd(0) associated with the cell surface, termed ''bio-palladium'' (De Windt et al. 2005). Not only did this process represent an economically interesting new production method to obtain nano-scale palladium, the resulting bio-palladium was also found to be a promising catalyst towards dechlorination. Nanoscale metal particles are characterized by high surface area to volume ratios, high levels of stepped surface and high surface energies (Ichinose 1992; Averback et al. 1991), resulting in high reactivity, even at high loads of the compound to be degraded. Opposed to chemical production of highly reactive nanoparticles, which is difficult and expensive due to controlled nucleation and 'capping' with a protective layer or attachment to a support (Macaskie et al. 2005), biological production methods for nano-catalytic particles with reactivity is relatively inexpensive and rapid.

Reports of biogenic nanoscale particles that promote dechlorination reactions are not uncommon for Gram-negative bacteria, more specifically dissimilatory iron-reducing bacteria (DIRB) and sulphate-reducing bacteria (SRB) were reported to have high affinity towards the production of catalytic precipitates. Tetrachloromethane transformation on the surfaces of biogenic magnetite (Fe_3O_4) particles, produced by the DIRB *Geobacter metallireducens*, resulted in the formation of relatively benign reaction products (McCormick and Adriaens 2004), and the SRB *Desulfovibrio magneticus* produced magnetic particles intracellularly (Sakaguchi et al. 2002). Also goethite in combination with sorbed Fe(II), produced by—amongst others—DIRB *Shewanella* species, can bring about the reductive dechlorination of this compound (Kim and Picardal 1999; Amonette et al. 2000). Reductive dehalogenation of chlorinated aromatic compounds with palladium precipitated by sulphate-reducing SRB of the *Desulfovibrio* genus, was demonstrated. This biologically precipitated ''bio-palladium'' was a superior catalyst for dechlorination compared to Pd(0) prepared by reduction under H_2 alone (Yong et al. 2002; Baxter-Plant et al. 2003). Moreover, palladium-coated cells of *D. desulfuricans* ATCC 29577 could reduce 700 μM Cr(VI) within 24 h using formate or hydrogen as the electron donor, under conditions whereby cells lacking bound Pd(0) or palladium metal manufactured via chemical reduction of soluble Pd(II), did not reduce Cr(VI) (Mabbett et al. 2004).

The aim of this study was to (i) study the interactions and toxicity of Pd(II) to *S. oneidensis* and investigate whether the biomass can survive the ''palladization'' process; and (ii) assess whether and how the catalytic properties of the reduced Pd(0) can be enhanced by biological action. This is the first study where the interactions between Pd(II), bioreductively precipitated Pd(0) and the bacterial carrier are reported to affect the catalytic reactivity of the Pd(0) nanocatalyst. In this way, a unique way of controlling catalyst size, distribution and reactivity towards different environmental pollutants was obtained.

Materials and methods

Bacterial strains and growth conditions

Shewanella oneidensis MR-1 was obtained from the BCCM/LMG Bacterium Collection (Ghent, Belgium) under the number LMG 19005. The strain was grown aerobically in Luria-Bertani (LB) medium (Sambrook and Russell 2001) 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

S. 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 and Russell 2001). The washed cells were resuspended in 30 ml M9 medium in glass serum bottles, to a final optical density of OD_{610} = 0.5, 1.1 or 2.2, corresponding to 0.6, 1.5 or 3.0 mg cell dry weight (CDW). Serum bottles were capped with inert viton stoppers. Depending on the experimental set-up, the cell suspension was supplemented with 25 mM formate as organic electron donor or, in the case of H_2 as electron donor, the headspace was replaced with 100% Vol H_2 gas (volume headspace) after repeated cycles of overpressure with Ar and vacuum underpressure to anaerobize the suspension (De Windt et al. 2003). Finally, Pd(II) or Pd(0) was added to the desired concentration in the bioreduction assays, supplemented as Na_2PdCl_4 or black Pd(0) powder respectively (Sigma-Aldrich, Seelze, Germany). To prepare bio-palladium with Pd:CDW (cell dry weight) ratios of 1:10, 1:1 and 5:2, respectively 10 mg l^{-1}, 50 mg l^{-1} and 50 mg l^{-1} Pd(II) was added to a cell suspension with an OD_{610} of 2.0, 1.1 and 0.5. 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.

Catalytic assays

Palladised cells were obtained from Pd(II) bioreduction assays with the appropriate electron donor, as described above. Palladised 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 medium or milliQ water, respectively for PCB or perchlorate degradation, in serum bottles with viton stoppers. The suspension was supplemented with the appropriate electron donor, 25 mM formate or H_2, to activate the Pd catalytic particles. When using H_2 (100% Vol) as electron donor, cell suspensions were anaerobised as described above. The concentration of Pd metal particles in each assay, supplemented as palladised cells, was calculated based on the amount of Pd(II) dosed in the bioreduction assay, since all Pd(II) was reduced by *S. oneidensis* as described in De Windt et al. (2005). PCB 21 (2,3,4-trichlorobiphenyl) was added to catalytic assays, diluted from a methanol stock solution to a final concentration of 1 mg l^{-1}. Perchlorate (ClO_4^-) was added to a final concentration of 12 mg ClO_4-Cl l^{-1}. Consequently, the serum bottles were incubated at 28°C and shaking was applied to homogenize the suspension (100 rpm). At regular time intervals, samples were taken and PCBs were extracted by solid phase extraction (SPE), according to Nollet et al. (2002). PCB congeners were quantified by GC-ECD, Varian 3800 (Varian, Middelburg, The Netherlands), as described in De Windt et al. (2005). Chloride was determined by ion chromatography. All assays were set up in triplicate.

Live/dead analysis by flow cytometry

To detect and quantify the number of living and dead cells, samples were diluted 100 times and stained for 10 min with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Merelbeke, Belgium). Stained samples were analyzed with a Cyan™ LX flow cytometer (Dakocytomation, Heverlee, Belgium) (Boon et al. 2006). The threshold trigger was set to SSC. For each cell sample run, data for 10,000 events were collected. To calculate the proportion of living and dead cells, an overnight grown culture containing mostly of living cells was analysed and compared with a pasteurized culture, treated for 10 min at 70°C. Differences in Syto 16 and Propidium Iodide fluorescence allowed a clear

separation (Fig. 1). Both regions were used to calculate the percentage living and dead cells.

Quantification of biphenyl

Samples of 3 ml bio-nanocatalytic assay were extracted with 1 ml ethylacetate. The extraction efficiency in spiked samples was found to be 110%, probably due to volatization of the solvent during sampling; this value was corrected for when calculating the amount of biphenyl formed in the reaction assays. Extracts were analyzed by a High Pressure Liquid Chromatograph (Dionex, Belgium) equipped with a UV/VIS detector UVD 340S with the Chromeleon software package version 6.10. About 20 µl of the extract was applied on a Genesis C18 column (4,6×150 mm, 4 µm) (Jones Chromatography, UK). Elution was performed with a solvent system consisting of 0.1 (v/v)% acetic acid in water (A) and 0.1 (v/v)% acetic acid in acetonitrile (B). The flow rate was maintained at 1 ml/min and the elution program was as follows: $0 \rightarrow 5$ min: 60% B (isocratic); $5 \rightarrow 20$ min: $60 \rightarrow 90\%$ B (linear gradient); $20 \rightarrow 25$ min: $90 \rightarrow 60\%$ B (linear gradient). Biphenyl was detected at 255 nm and had a retention time of 12.02±0.13 min. A standard curve was constructed and the molar extinction coefficient (ε) of biphenyl was calculated to be 255±8 M^{-1} cm^{-1}. The lower limit of quantification (signal to noise ratio >10) was determined to be 0.2 mg l^{-1}.

Transmission electron microscopy

To prepare thin sections, pellets of bacteria were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde and 2% formaldehyde, and embedded in 3% low melting agarose (Difco Laboratories, Detroit, Michigan, USA). These samples were postfixed in 1% osmium tetroxide. Between and after fixation steps, samples were washed with distilled water. Afterwards, samples were dehydrated in increasing concentrations of ethanol and, finally, in anhydrous propylene oxide. After embedding in Epon-Spurr medium, the specimen blocks were trimmed with a TM60 trimming unit (Reichert-Jung A.G., Vienna, Austria) to obtain a cutting face of 0.5×1 mm^2-1×2 mm^2, and ultra-thin sections in the gold to mat silver interference colour range were cut using the Ultracut microtome (Reichert-Jung A.G., Vienna, Austria). The sections were brought on pioloform and carbon coated copper grids (200 mesh). Once this was done, thin sections were stained with 2% uranyl acetate and then with lead citrate to determine the ultrastructure of the cells. Chemicals and grids

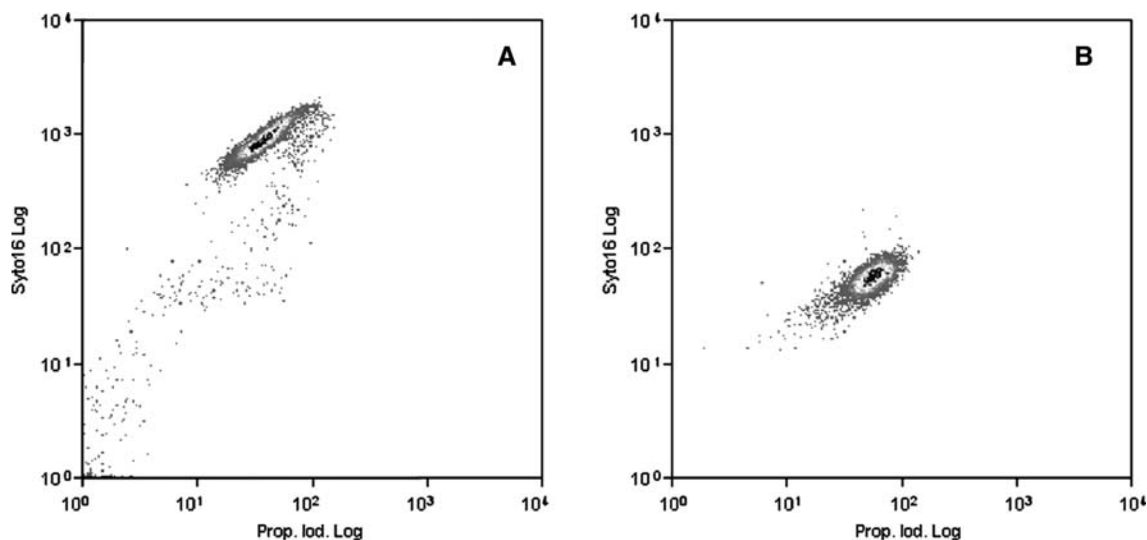


Fig. 1 Representative scatter plot of *S. oneidensis* to determine the amount of living and dead cells by LIVE/DEAD® BacLight™ Bacterial Viability staining: (**a**) overnight grown culture of living cells. (**b**) heat-treated cells (10 min at 70°C)

were obtained from Agar Scientific (Stansted, UK). Imaging was performed with a EM208S transmission electron microscope (FEI, Eindhoven, the Netherlands) operating at 80 kV.

Microbial adhesion to hydrocarbons (MATH)

The MATH protocol used was as described by Rosenberg et al. (1980). MATH tests were performed with cell suspensions at OD_{610} 0.9–1.1 in M9 mineral medium. Cell suspension aliquots (2 ml) were then transferred to clean, round-bottom test tubes and 0.3 ml heptane (UCB, Leuven, Belgium) was added. After the mix was homogenized for 2 min, the hydrocarbon phase was allowed to separate completely and the aqueous phase was removed to determine the OD_{610}. The percentage of adhesion to hydrocarbons was calculated using the following expression:

$$\text{Percentage of adhesion} = \frac{100 \times [OD_{610}(\text{initial bacterial suspension}) - OD_{610}(\text{aqueous phase})]}{OD_{610}(\text{initial bacterial suspension})}$$

Contact angle measurements

S. oneidensis cells that had been exposed to different treatments, were filtered over 0.45 μm Millipore filters (Millipore, Bedford, USA) and the filters were dried during 48 h at 30°C. The contact angle between 1 μl HPLC-grade water (Acros Organics, Geel, Belgium) and the biomass on the filter was determined by the Drop Shape Analysis (DSA) System 10 (Krüss GmbH, Hamburg, Germany) at the time of initial contact. Camera calibration and focusing were done before measurements took place. DSA software version 1.8 was used for contact angle determination with the tangent-1 method (Krüss GmbH, Hamburg, Germany). Briefly, the complete profile of a sessile drop was adapted to fit a general section equation. The derivative of this equation at the intersection point of the contour line with the baseline resulted in the slope at the 3-phase contact point and therefore the contact angle.

Determination of the sorption characteristics of bio-palladium towards PCB 21

Increasing concentrations of PCB 21 were dosed to assays with a suspension of *S. oneidensis* cells (controls) or bio-palladium. PCB 21 was added in acetone, and the solvent was evaporated before the cell suspension was added. Each assay consisted of 0.85 mg *S. oneidensis* cell dry weight (CDW) suspended in 30 ml M9 medium, and in the case of bio-palladium, the ratio of Pd:CDW was set at 5:2. Bio-palladium was prepared as described above and was washed thrice with M9 medium. No H_2 was added to the headspace, to avoid reactivity of the palladium towards PCB dechlorination. The system was allowed to reach an equilibrium during 48 h, at which time the biomass or bio-palladium was separated from the M9 medium by filtration on Millipore filters (Millipore, Bedford, USA). The filtrate was extracted by SPE and PCB 21 in the filtrate was quantified by GC-ECD. The biomass on the filters was extracted during 24 h with an acetone:hexane (1:1) solvent in glass bottles with VITON stoppers. The extractant was evaporated, hexane was added and GC analysis was done to quantify PCB 21 associated with biomass.

Chloride measurements

The catalytic assay supernatant was analyzed for chloride content by ion chromatography after centrifugation at 5000 × *g* for 10 min and filtering through a 0.45 μm filter. The DX-600 system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump GP50, a Dionex Autosampler Model AS50 (injection volume is 100 μl), a Dionex ED50 Electrochemical Detector and a PeakNet 6 software system version 6.10. Ionpac AS9-HC (250 × 4 mm ID; 9 μm particle size; Dionex) column and Ionpac CS12-HC (250 × 4 mm ID; 8 μm particle size; Dionex) were

used for anion and cation separation respectively. The mobile phase consisted of Na_2CO_3 (9 mM) and methanesulfonic acid (20 mM) for anion and cation analysis respectively, with a flow rate of 1 ml/min.

Results

Viability of S. oneidensis cell suspension exposed to different Pd(II) concentrations and electron donors

S. oneidensis MR-1 was exposed to different concentrations of Pd(II) both with and without electron donors for Pd(II) bioreductive precipitation. A concentration of 5 mg l^{-1} Pd(II), when added to a *S. oneidensis* suspension without an electron donor, reduced the culturable fraction of cells with approximately 2 log units (Fig. 2a). However, no significant effect of this treatment could be observed by flow cytometric evaluation of a Live/Dead stained cell suspension. When 10 mg l^{-1} Pd(II) was added without any electron donor, there was a 4 log reduction of the culturable population within 300 min, and flow cytometry indicated a reduction to 60% viable cells. From Fig. 2b the protective effect of Pd(II) bioreduction in the presence of a suitable electron donor can clearly be observed. Cells exposed to 25 mg l^{-1} Pd(II) in the absence of an electron donor were no longer culturable after 10 min (data not shown) and the lethal effect of Pd(II) at this concentration could also be demonstrated by viability staining, since the percentage of live cells was reduced to almost 20% within 300 min. The addition of H_2 in the headspace allowed *S. oneidensis* to resist the toxic effects of Pd(II) and gave rise to no significant decrease of the number of culturable or viable cells as observed by both plate counting and viability staining. Interestingly, 25 mM formate proved to be a less effective electron donor for bioreductive detoxification of Pd(II), since there was a 2 log reduction of culturable cells and a 20% decrease of viable cells within 60 min. However, plate counts indicated a slow recovery of the amount of culturable cells from

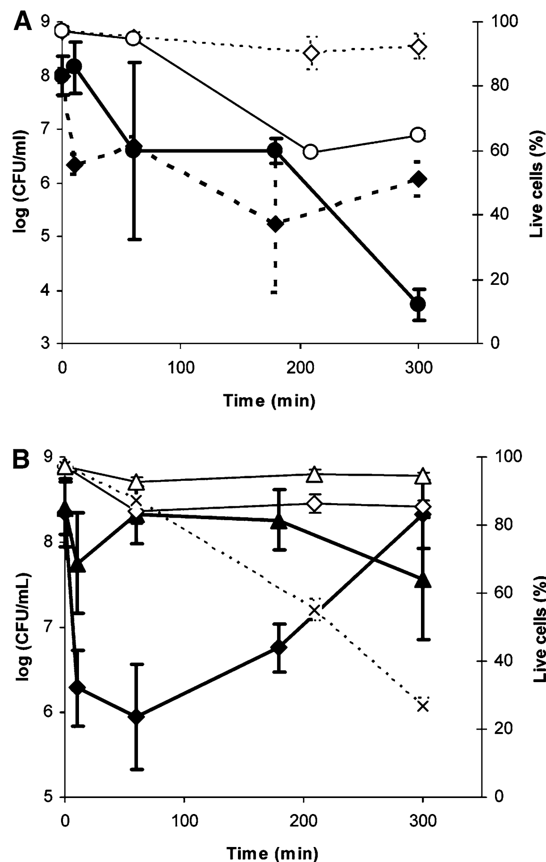


Fig. 2 (**a**) Assessment of culturability of *S. oneidensis* cells during exposure to 5 mg l^{-1} Pd(II) (♦) and to 10 mg l^{-1} Pd(II) (●); viability of *S. oneidensis* cells under the same conditions was also assessed by flow cytometry: 5 mg l^{-1} Pd(II), (◇), and 10 mg l^{-1} Pd(II), (○). No electron donor was provided during these experiments. (**b**) Assessment of culturability of *S. oneidensis* cells during exposure to 25 mg l^{-1} Pd(II) in the presence of electron donors hydrogen (◇) and formate (♦); viability of *S. oneidensis* cells under the same conditions was also assessed by flow cytometry: hydrogen, (Δ), and formate, (▲). Viability of *S. oneidensis* cells during exposure to 25 mg l^{-1} Pd(II) in the absence of any electron donor was assessed by flow cytometry as well (×). All experiments were done in triplicate and mean ± stdev are given

that point onward, to a concentration equal to the original cell number after 300 min incubation. This slow recovery was observed in biopalladium preparations with a ratio Pd:CDW of 1:10 and 1:10 as well. However, the highest Pd:CDW ratio tested, 5:2, resulted in complete loss of viability of the cell suspension and no viable or culturable cells could be maintained in this system (results not shown).

Effect of the ratio Pd:CDW on Pd particle size and surface coverage by Pd nanoparticles

The effect of three ratios of Pd:cell dry weight (CDW) on the size and distribution of palladium nanoparticles over the *S. oneidensis* cell surface was investigated by Transmission Electron Microscopy (Fig. 3). The ratio Pd:CDW was the determining factor for both the size and number of particles deposited per cell surface area (Fig. 4). Generally, a lower ratio of Pd:CDW, yielding viable, ''palladized'' *S. oneidensis* cells, resulted in particles with a smaller surface area (up to ten times smaller when comparing the 5:2 and 1:10 ratios) and significantly less particles per cell surface area (up to a hundred times less Pd surface area per μm cell wall when comparing the 5:2 and 1:10 ratios). The size and surface distribution of the nano-particles was very reproducible within one sample and between different samples of the same treatment.

Assessment of biomass hydrophobicity and sorption characteristics of bio-palladium towards PCB's

Contact angle measurements combined with MATH (Microbial Adhesion To HydroCarbons) tests were set up to investigate biomass hydrophobicity during different treatments. It was observed that the initial contact angle of MR-1 biomass increased from (44.4±0.9)° to (54.3±5.8)° when a hydrophobic tri-chlorinated PCB, PCB 21 or 2,3,4-trichlorobiphenyl, was spiked to the biomass suspension. The contact angle further increased when palladium was deposited on the biomass in a ratio of Pd:CDW of 5:2, to a value of (102.4±3.4)°. MATH yielded similar results: the percentage MATH of *S. oneidensis* biomass increased from (19.5±1.5)% to (40.5±1.9) % when the cells had been exposed to PCB 21. The % MATH further increased to (62.9±6.7)%

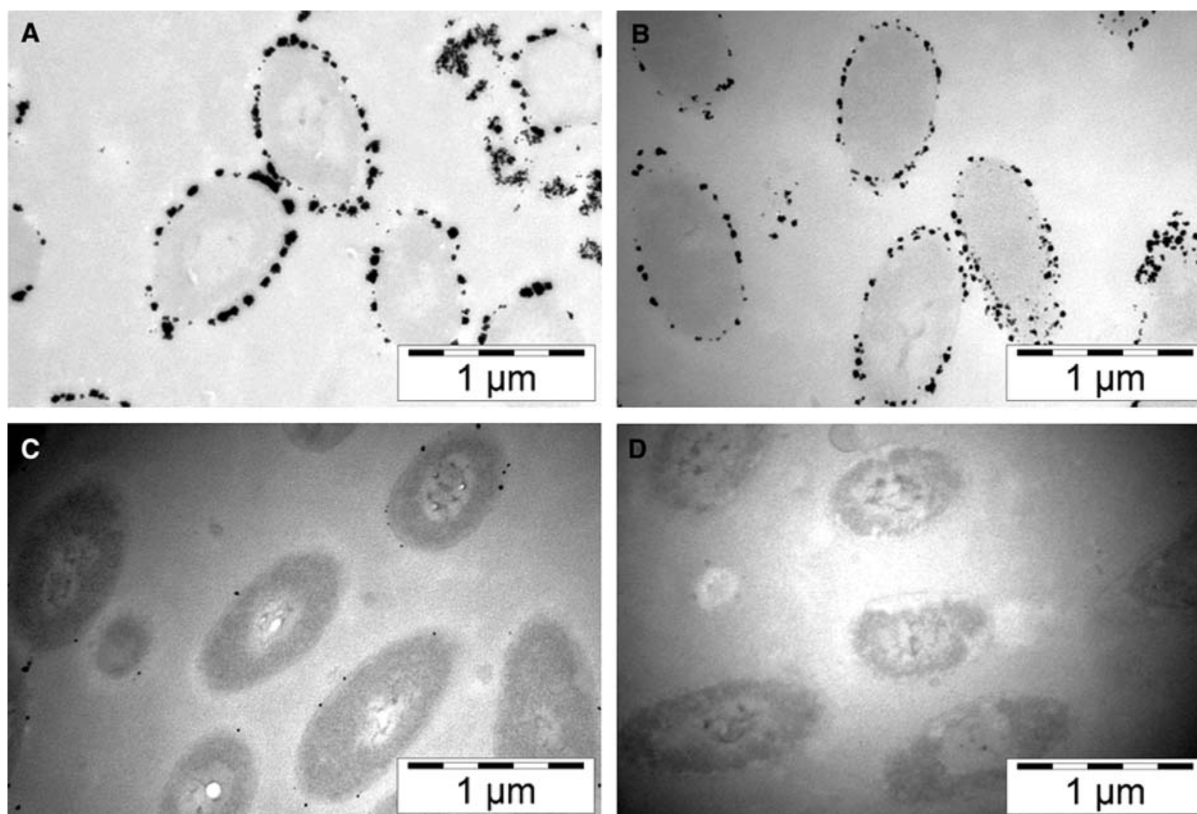


Fig. 3 Transmission electron microscopy (TEM) of palladium at the *Shewanella oneidensis* cell surface at different Pd:cell dry weight (CDW) ratios: (**a**) Pd:CDW ratio 5:2; (**b**) Pd:CDW ratio 1:1; (**c**) Pd:CDW ratio 1:10; **(d)** cell suspension without Pd

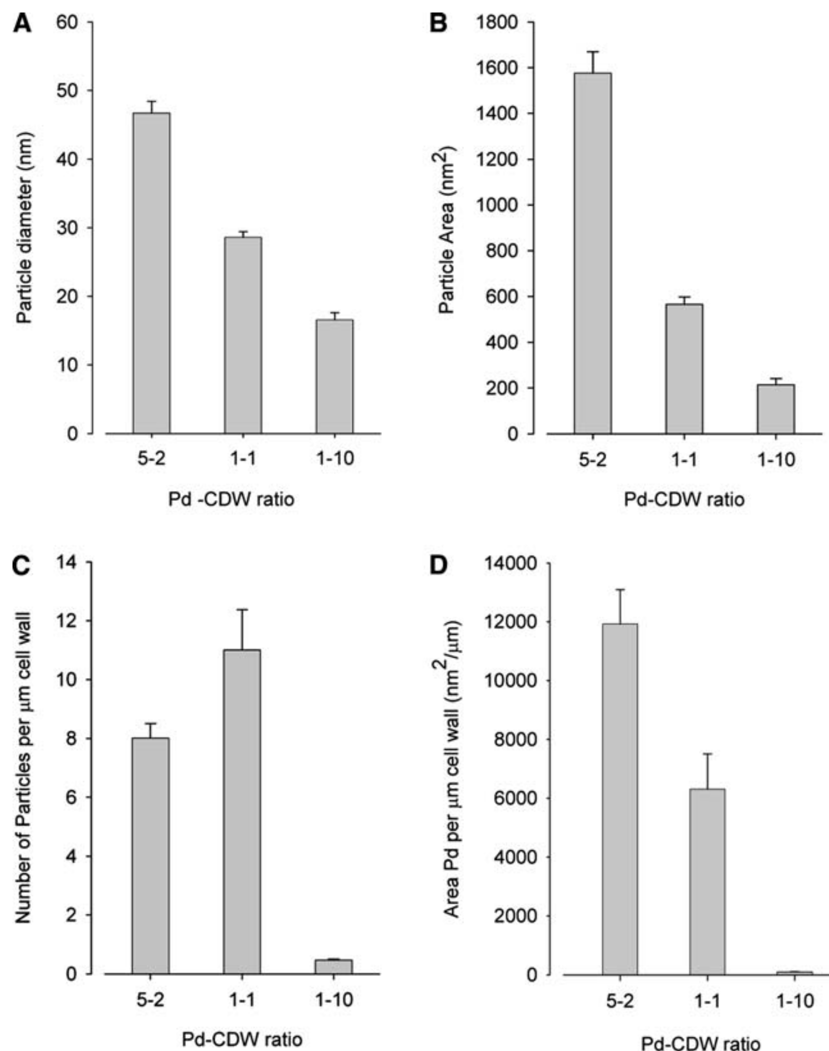


Fig. 4 Measured parameters by TEM observation of bio-palladium at different Pd:CDW ratios: (**a**) particle diameter; (**b**) particle area; (**c**) number of particles per µm cell wall; (**d**) area of Pd particles per µm cell wall

when the cells had been covered in a 5:2 Pd:CDW ratio.

The ‘‘palladized’’ biomass at a ratio Pd:CDW of 5:2 resulted in significantly more sorption of PCB 21 (2,3,4-trichlorobiphenyl) within 48 h at the higher PCB concentrations, although the contribution of the biomass to the sorption of PCBs from the aqueous environment can not be underestimated (Fig. 5).

Dechlorination of PCBs under different Pd:CDW ratios

To establish the reactivity of different Pd:CDW ratios, catalytic assays were set up. An increasing Pd:CDW ratio from 1:1 to 5:2 lead to a dechlorination rate of PCB 21 (Fig. 6a). Although the final bio-palladium concentration in both reaction systems equalled 50 mg l^{-1}, the Pd:CDW ratio governed the dechlorination kinetics. When the ratio was 1:1, PCB 21 was reduced from 1000 µg l^{-1} to 256±75 µg l^{-1} within 5 h of incubation, whereas the ratio 5:2 allowed PCB 21 reduction to a concentration lower than the detection limit within 60 min. At a Pd:CDW ratio of 1:10, PCB 21 was not significantly further degraded between 180 and 360 min, remaining at a concentration of 312±179 µg l^{-1} and 270±111 µg l^{-1} respectively (results not shown). This effect of the Pd:CDW ratio could also be observed on the formation of dechlorination intermediate PCB 5 (2,3-dichlorobiphenyl)

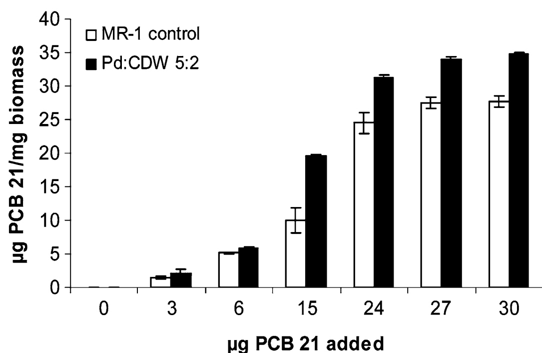


Fig. 5 Sorption of PCB 21 at different concentrations on 0.85 mg of biomass cell dry weight, both for non-treated *S. oneidensis* biomass (□) and for "palladized" biomass at a Pd:CDW ratio of 5:2 (■). All experiments were done in triplicate and mean ± stdev are given

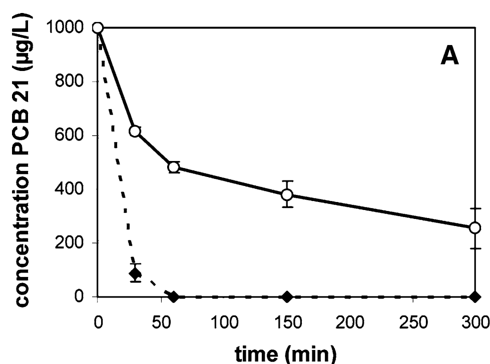
(Fig. 6b). When this ratio was 1:1, PCB 5 gradually started to accumulate in the reaction assays and after 5 h incubation, 129±16 µg l^{-1} could still be detected. Similarly, at a Pd:CDW ratio of 1:10, PCB 5 accumulated to 134±9 µg l^{-1} after 360 min (results not shown). When the ratio was increased to 5:2, PCB 5 could only be detected during the first 60 min of incubation with bio-palladium. The PCB 5 concentration was highest (140 µg l^{-1}) at 30 min, after which the concentration decreased below the detection limit within 120 min of incubation. Under these optimized reaction conditions, biphenyl accumulation could be observed at concentrations of up to 320±52 µg l^{-1} after 5 h of incubation. This amounts to 53.5% of the theoretical maximum.

Reductive degradation of perchlorate by S. oneidensis and bio-palladium

The lower Pd:CDW ratio of 1:10 was highly reactive towards ClO_4^- reduction to Cl^- (Table 1). Interestingly, by increasing the Pd:CDW ratio, the reactivity towards perchlorate gradually decreased, and the lowest reactivity was observed for bio-palladium with a Pd:CDW ratio of 5:2. Biological controls were performed to assess whether these observations could be related to decreasing cell viability due to increasing Pd:CDW ratios. Cell suspensions with a cell density that was identical to that in the catalysis assays with three different Pd:CDW ratios, containing H_2 in the headspace, but without addition of palladium, were assessed for perchlorate reduction. It was found that only a small fraction of perchlorate reduction could be attributed to metabolic action (Table 1). As such, the high reactivity of bio-palladium at a Pd:CDW ratio of 1:10 towards perchlorate, was due to the properties of the chemical catalyst and not due to *S. oneidensis* metabolic activity.

Discussion

Pd(0) is widely used as an industrial reduction catalyst thanks to its ability to trap H_2 as hydrogen radicals. In this way, Pd(0) can absorb up to 900 times its own volume of H_2 (Mabbett et al.



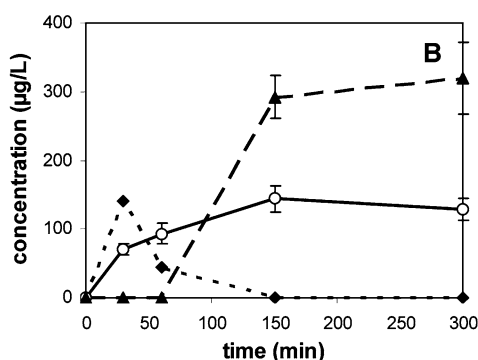


Fig. 6 (**a**) Dechlorination of 1.000 µg l^{-1} PCB 21 (2,3,4-trichloro biphenyl) with 50 mg l^{-1} bio-palladium, deposited on a *Shewanella oneidensis* cell suspension at a ratio BioPd:CDW of 1:1 (w/w) (○) and at a ratio 5:2 (w/w) (◆). (**b**) Formation of dechlorination intermediate PCB 5 (2,3-dichloro biphenyl) with 50 mg l^{-1} bio-palladium, deposited on a *S. oneidensis* cell suspension at a ratio Pd:CDW of 1:1 (w/w) (○) and at a ratio 5:2 (w/w) (◆); and of dechlorination end-product biphenyl at a ratio Pd:CDW of 5:2 (w/w) (▲).All experiments were done in triplicate and mean ± stdev are given

Table 1 Perchlorate reduction to chloride, expressed by mg Cl^{-} l^{-1} formed, as a function of the incubation time and of the different treatments

Time (h)	Bio-palladium ratio Pd:CDW			*S. oneidensis* MR-1		
	1:10	1:1	5:2	OD 2	OD 1	OD 0.5
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
48	10.49±0.09	4.27±0.20	1.31±0.03	3.10±0.60	1.25±0.50	1.10±0.50
72	10.35±0.15	4.67±0.11	1.88±0.06	3.20±0.85	0.90±0.40	0.95±0.40
120	10.85±0.36	7.31±0.05	2.62±0.12	3.00±0.60	1.25±0.45	0.80±1.25

Reactivity of bio-palladium at different Pd:CDW ratios of 1:10, 1:1 and 5:2 is expressed, as well as the reactivity of *S. oneidensis* MR-1 biomass at different cell densities, expressed as OD_{610}. All experiments were done in triplicate and mean ± stdev are given

2004; Livingstone 1973). The reactivity of catalytic particles at a nanoscale is guaranteed by their high surface area to volume ratios, high levels of stepped surface and high surface energies (Ichinose 1992; Averback et al. 1991), however they are very expensive to produce chemically. Therefore, *S. oneidensis* was used in this study to precipitate nano-palladium crystals at the cell surface by reducing Pd(II) to Pd(0). The ratio of palladium/cell dry weight (Pd:CDW) during the bioreduction process of Pd(II) by *S. oneidensis* was found to be a determining factor for the reactivity of bio-palladium towards two different types of environmental pollutants. Hydrophobic polychlorinated biphenyls, represented by PCB 21 in this study, were most efficiently dehalogenated by bio-palladium at a high Pd:CDW ratio. On the other hand, the water soluble anionic perchlorate (ClO_4^-) ion was poorly degraded by the bio-palladium catalyst at high Pd:CDW ratio. In this case, the lowest ratio of 1:10 resulted in very high perchlorate reduction activity. Interestingly, the Pd:CDW ratio strongly effected both the size and distribution of the palladium crystals formed, and the viability of the biological *S. oneidensis* carrier.

Flow cytometry in combination with fluorescent techniques was a powerful tool for assessing the loss of viability of *S. oneidensis* cells due to interactions at the cell membrane. Although the plate count approach is employed as the standard method for measuring bacterial viability, it only indicates how many cells can replicate under the conditions of growth and there is no explicit evidence that the failure of a bacterial cell to reproduce is an indication that the cell was dead at the time of sampling (Davey et al. 1999; Joux and Lebaron 2000; Ben Amor et al. 2002). Differences between the viability analysis by flow cytometry and the cultivability determination by plating were observed. Both techniques focus on different cell properties. Cells with permeabilized cell membranes will be regarded as "dead", however damaged or stressed cells with intact cell membranes could have lost their cultivatability. Moreover, flow cytometry will not detect lysed cells, so that an overestimation of the "live" cells can occur. Therefore, the log reduction as determined by plating and the % living cells as determined by flow cytometry should be considered as complementary techniques.

Pd(II) is a potent enzyme inhibitor, inhibiting creatine kinase, aldolase, succinate dehydrogenase, carbonic anhydrase, alkaline phosphatase, and prolyl hydroxylase (Liu et al. 1979). In vitro studies have shown that Pd ions are capable of eliciting a series of cytotoxic effects (Kielhorn et al. 2002). Although several studies have focused on the enzymatic reduction of Pd(II) by *Desulfovibrio* and *Shewanella* bacterial biomass (Lloyd et al. 1998; Yong et al. 2002; De Windt et al. 2005), none so far have taken the Pd(II) toxicity towards bacteria under scrutiny. From our research, it was clear that Pd(II) is toxic for *S. oneidensis*, but that cells can easily recover and even grow when a suitable electron donor is present for Pd(II) bioreduction. The precipitation of Pd(0) thus results in a detoxification of the growth environment. Indeed, it is generally hypothesized that the effects of Pd metal are mediated via the presence or release of Pd(II) ions (Kielhorn et al. 2002). Interestingly, H_2

protected *S. oneidensis* cells better against the toxic effects of Pd(II) than formate, and this might suggest that the palladium bio-reduction is more efficient with H_2.

The nano-biocatalyst prepared at a 5:2 Pd:CDW ratio was characterized by a relatively high palladium particle size and the *S. oneidensis* biological carrier was found to be no longer viable under these conditions. Initial bioreduction of Pd(II) by biomass is a metabolic process that involves living bacterial cells (De Windt et al. 2005). Bioreductive precipitation of palladium in later stages of the bio-palladium formation process may be chemically catalyzed by ''auto-reduction'' (Baxter-Plant et al. 2003). In this case, bio-palladium crystals formed by metabolic action may function as reductive nucleation sites for further chemically mediated Pd(II) reduction and accumulation of palladium. This explains the larger crystal size under a high Pd:CDW ratio. At lower Pd:CDW ratios (1:1 and 1:10), the *S. oneidensis* biological carrier was still viable and culturable and the Pd crystals were smaller and more dispersed over the different cells. Moreover, the surface coverage and particle size distribution was almost identical for all cells per Pd:CDW ratio, indicating a very uniform coverage and precipitation on the different cells. This shows that the production of biopalladium can result in a batch of nanocristals with a limited variation in size.

Knowledge of the interactions between PCBs and the bio-palladium matrix was required to explain the positive effect of high Pd:CDW ratio of bio-palladium. Therefore, the result of high coverage of *S. oneidensis* cells with Pd(0) on sorption of hydrophobic PCB molecules and on hydrophobicity was examined. *S. oneidensis* biomass became more hydrophobic when it was brought into contact with hydrophobic PCB molecules, and the biomass hydrophobicity greatly increased when the biomass was palladized. Both the hydrophobicity measurements and the sorption experiments indicated high sorption of PCBs to *Shewanella* biomass. Since adsorption of the PCB molecule to the catalytic surface area is a necessary requirement for reactivity, the increased Pd(0) coverage at higher Pd:CDW ratios in part explains the increased reactivity of bio-palladium under these conditions.

Since the colloidal palladium was much more hydrophobic than the *Shewanella* biomass, it can be assumed that the sorption coefficient to palladium, K_d1, is much higher than the sorption coefficient to biomass, K_d2. The distribution of PCB molecules between the biological carrier and palladium colloids will eventually lead to complete dechlorination, even if the PCB molecules initially associated with the cell membrane. Furthermore, intracellular uptake of super-hydrophobic pollutants is prevented by steric hindrance of membrane passage, making the interaction a purely membrane-associated process (Spacie and Hamelink 1982; Shaw and Connell 1984; Geyer et al. 1987). Thus, the increasing cell surface coverage with Pd particles limited the sorption of PCB molecules to non-reactive cell surface sites, and this effectively increased the catalytic efficiency of the bio-palladium matrix.

Bio-palladium that was prepared with a 1:10 Pd:CDW ratio, exhibited the highest reactivity towards reductive degradation of perchlorate. In this case, the nano-biocatalyst was characterized by a relatively small palladium particle size and a large fraction of the *S. oneidensis* biological carrier was found to be viable under these conditions. Since it was demonstrated that biological reduction of perchlorate by *S. oneidensis* biomass under the conditions in the catalytic assays, was negligible, the effect of increasing reactivity towards this component by lowering the Pd:CDW ratio could not be related to metabolic activity. Therefore, the increased reactivity is possible related to the smaller palladium crystals spread over a larger bacterial surface area These cristals were up to 100 fold smaller when compared to bio-palladium at a Pd:CDW ratio of 5:2. This increased Pd specific surface area of these nanocrystals gave rise to increased reactivity. The effect of hydrophobic interactions that were found to be determinative for PCB dechlorination rates on bio-palladium, were in the case of the water soluble perchlorate ion of significantly less importance. Since the biomass remained viable during the complete Pd(II) reduction process at this lower Pd:CDW ratio, the fraction of Pd(II) that was chemically precipitated by the auto-reduction process on biogenic Pd nucleation sites, was relatively small. Therefore, chemically

induced accumulation of Pd(0) around the same nucleation site, leading to a larger Pd(0) crystal, occurred to a lesser extent and the nanoparticles remained small and dispersed.

Conclusions

In conclusion, by altering the ratio of Pd:CDW during bioreductive Pd(0) precipitation on *S. oneidensis* MR-1 biomass, the efficiency of the bio-palladium catalyst could be greatly enhanced for both dechlorination of PCBs and reductive degradation of perchlorate. By adjusting the ratio of Pd(II) to biomass, the size, surface distribution and reactivity of catalytic Pd(0) produced by *S. oneidensis* could be biologically controlled. This could be related to the fact that cell viability was maintained at the lower Pd:CDW ratios. By adjusting this ratio, the reactivity of bio-palladium could be optimized towards reductive degradation of different environmental pollutants. This would allow the production of palladium particles, coated on a biological carrier, with properties ‘‘on demand’’.

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