

# Palladium nanoparticles produced by fermentatively cultivated bacteria as catalyst for diatrizoate removal with biogenic hydrogen

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**Abstract** A new biological inspired method to produce nanopalladium is the precipitation of Pd on a bacterium, i. e., bio-Pd. This bio-Pd can be applied as catalyst in dehalogenation reactions. However, large amounts of hydrogen are required as electron donor in these reactions resulting in considerable costs. This study demonstrates how bacteria, cultivated under fermentative conditions, can be used to reductively precipitate bio-Pd catalysts and generate the electron donor hydrogen. In this way, one could avoid the costs coupled to hydrogen supply. The catalytic activities of Pd(0) nanoparticles produced by

different strains of bacteria (bio-Pd) cultivated under fermentative conditions were compared in terms of their ability to dehalogenate the recalcitrant aqueous pollutants diatrizoate and trichloroethylene. While all of the fermentative bio-Pd preparations followed first order kinetics in the dehalogenation of diatrizoate, the catalytic activity differed systematically according to hydrogen production and starting Pd(II) concentration in solution. Batch reactors with nanoparticles formed by *Citrobacter braakii* showed the highest diatrizoate dehalogenation activity with first order constants of  $0.45 \pm 0.02 \text{ h}^{-1}$  and  $5.58 \pm 0.6 \text{ h}^{-1}$  in batches with initial concentrations of 10 and 50  $\text{mg L}^{-1}$  Pd, respectively. Nanoparticles on *C. braakii*, used in a membrane bioreactor treating influent containing 20  $\text{mg L}^{-1}$  diatrizoate, were capable of dehalogenating 22  $\text{mg diatrizoate mg}^{-1}$  Pd over a period of 19 days before bio-Pd catalytic activity was exhausted. This study demonstrates the possibility to use the combination of Pd(II), a carbon source and bacteria under fermentative conditions for the abatement of environmental halogenated contaminants.

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## Introduction

There is growing interest in using microorganisms to synthesize metal nanoparticles that can be applied for catalysis or disinfection (Mandal et al. 2006; Lloyd et al. 2008; Hennebel et al. 2009a). Biologically produced nanopalladium (bio-Pd) is such a catalyst that can be used for a variety of reduction reactions. To produce bio-Pd, an electron donor is provided to promote the metabolic activity

that will reduce aqueous Pd(II) ions and generate Pd(0) nanoparticles on the cell wall, and within the periplasmic space and cytoplasm (De Windt et al. 2005). 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. 2002). This synthesis method eliminates the use of hazardous compounds, proceeds at ambient temperature and pressure, and provides a biomass scaffold to inhibit aggregation of the nanoparticles (Hennebel et al. 2009a). Two model organisms have primarily been used to study the production and application of bio-Pd: the sulfate reducing bacterium *Desulfovibrio desulfuricans* (Lloyd et al. 1998) and the metal-respiring bacterium *Shewanella oneidensis* (De Windt et al. 2005). A combination of hydrogenases with cytochrome  $c_3$  has been proposed for the Pd(II) reduction mechanism of both organisms (Lloyd et al. 1998; De Windt et al. 2005). Recently, it has been shown that bio-Pd can also be produced under fermentative conditions by *Escherichia coli* (Deplanche et al. 2010) and *Clostridium pasteurianum* (Chidambaram et al. 2010). In the case of *E. coli*, the reduction mechanism and nanoparticle formation was attributed to three hydrogenases that appear to be required to initiate the formation of Pd(0) ‘seeds’ that promote further autocatalytic reduction of Pd(II) and formate breakdown by Pd(0) (Deplanche et al. 2010).

Biogenic Pd nanoparticles have been reported to efficiently catalyze the reduction of chromate (Mabbett et al. 2002), the degradation of perchlorate and nitrate (De Windt et al. 2006), and the dehalogenation of PCBs (Baxter-Plant et al. 2003; De Windt et al. 2005), PBDEs (Harrad et al. 2007; Deplanche et al. 2009), trichloroethylene (TCE) (Hennebel et al. 2009b; Hennebel et al. 2009c), chlorophenols (Baxter-Plant et al. 2003), lindane and chlorobenzenes (Mertens et al. 2007). In these reduction and dehalogenation reactions, the Pd nanoparticles were activated by adding an external electron donor such as hydrogen or formate. Hydrogen was consistently identified as the most reactive among possible electron donors, but its use was found to be prohibitively expensive and presented significant safety and supply issues (Hennebel et al. 2009b). Several reactor types were constructed to limit the hydrogen supply (Hennebel et al. 2010) or to produce hydrogen in a sustainable way (Yong et al. 2003; Hennebel et al. 2011). Humphries et al. (2007) used hydrogen produced by *E. coli* during the fermentation of sugar waste streams. In another recent study, Pd(0) nanoparticle formation by *C. pasteurianum* BC1 was coupled with the microbial generation of hydrogen to reductively immobilize aqueous chromate using bio-hydrogen as the electron donor (Chidambaram et al. 2010).

The goal of this study was to examine the general applicability of coupling bio-Pd nanoparticle generation and bio- $H_2$  for water treatment by comparing the process

across six fermentative bacterial strains. The process was evaluated according to the capacity and rate of diatrizoate and TCE dehalogenation in both batch tests and a membrane bioreactor (MBR). The iodinated contrast media (ICM) compound diatrizoate and the chlorinated solvent TCE were used in this study to represent two important classes of recalcitrant pollutants that are not effectively removed from wastewater and groundwater using current treatment methods (Ferguson and Pietari 2000; Ternes et al. 2003).

## Materials and methods

### Bacterial growth and synthesis of Pd nanoparticles

*Clostridium butyricum* (LMG 1217), *Citrobacter braakii* (ATCC 6750), *Klebsiella pneumoniae* (DSM 2026), *Enterococcus faecium* (PhIP-M1-a), *E. coli* (ATCC 25922), and *Bacteroides vulgatus* (LMG 177672(2)) were cultivated under fermentative conditions overnight in brain heart infusion medium (BHI) at 37°C. Subsequently, 1 mL of the cultures was transferred in 30 mL medium containing 10 g L<sup>-1</sup> glucose, 1 g L<sup>-1</sup> NH<sub>4</sub>Cl, 0.2 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.02 g L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g L<sup>-1</sup> NaCl, 6 g L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, and 3 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. Oxygen was removed by replacing the headspace of the serum bottles with 100% N<sub>2</sub>, after repeated cycles of overpressure with N<sub>2</sub> and vacuum (De Windt et al. 2005). The cultures were incubated until an OD<sub>600nm</sub> of 1.6 was obtained. At this point, glucose measurements revealed that glucose was completely consumed by all six bacteria.

### H<sub>2</sub> production by the six strains

Gaseous samples of 1 mL were withdrawn from the headspace after reaching an OD<sub>600</sub> of 1.6 and immediately analyzed by GC-FID. Subsequently, the total pressure was measured to calculate the total amount of H<sub>2</sub> in the gas phase. Glucose was completely consumed by the six bacteria and consequently from this point on, no extra hydrogen could be formed nor during the Pd reduction assays nor during the dehalogenation experiments.

### Pd reduction assays

Palladium was added to cell cultures with an OD<sub>600nm</sub> of 1.6 as a Pd(II) solution (750 mg L<sup>-1</sup> Na<sub>2</sub>PdCl<sub>4</sub>-Pd stock) to achieve a final Pd concentration of 10 or 50 mg L<sup>-1</sup>. To analyze the amount of palladium associated with the microbial biomass, the bio-Pd suspensions were centrifuged at 3,000×g for 30 min after 1 and 24 h, respectively. Subsequently, the pellet was resuspended in 10 mL distilled water. The latter suspension and the supernatant (10 mL)

were boiled with 2 mL 65% HNO<sub>3</sub> (Normapur, VWR, Belgium) and 2 mL 30% H<sub>2</sub>O<sub>2</sub> (Merck, Germany) until the solution was clear and the organic material was completely oxidized. Control experiments consisted of bacteria that were cultivated in a first step under fermentative conditions and subsequently separated from the growth medium by centrifugation for 15 min at 3,000×g. Finally, these cultures were resuspended in fresh minimal medium and 50 mg L<sup>-1</sup> Pd(II) was added immediately. The resulting Pd precipitates were harvested after 15 min of reaction by centrifugation for 30 min at 3,000×g and studied with TEM. Another set of control experiments consisted of cell-free medium to which the same amount of Pd was added to study its precipitation in the growth medium. Subsequently, the medium was centrifuged at 3,000×g for 30 min after 1 and 24 h, respectively.

Palladium concentration was measured by atomic absorption spectrometry (AAS) (Shimadzu AA-360, Japan). Experiments were performed in triplicate and the percent Pd associated with the cells was calculated by using the following formula: (Pd in pellet)/(Pd in pellet+Pd in supernatant)×100. Analysis of variance (ANOVA) was performed with S-plus 8\_0 (statistical software) to quantify the significance of the observed differences between the several strains.

#### Biocatalytic activity of bio-Pd towards dehalogenation of diatrizoate and TCE

Diatrizoate (Applichem, Darmstadt, Germany) was supplied to 30 mL cell cultures (90 mL headspace), 24 h after the Pd(II) addition to achieve a final concentration of 20 mg L<sup>-1</sup> or 100 mg L<sup>-1</sup> diatrizoate. Aqueous samples of 1 mL were withdrawn and analyzed by high performance liquid chromatography (HPLC). TCE (Sigma-Aldrich, Seelze, Germany) was added from a 10 g L<sup>-1</sup> TCE stock solution in pure methanol to achieve a final concentration of 100 mg L<sup>-1</sup> in an aqueous bio-Pd suspension. Gaseous samples of 1 mL were withdrawn from the headspace and immediately analyzed by GC-FID. In the case of both contaminants, the dehalogenation experiments occurred in the same serum flask as in which the Pd(II) was added. Hence, the total Pd concentration was the sum of the Pd(II) in the medium and the cell-associated Pd and amounted to 10 or 50 mg L<sup>-1</sup>. All experiments were done in biological triplicates and means±standard deviations were calculated.

Control experiments, in which H<sub>2</sub> was removed after 24 h of Pd(II) reduction, were conducted by replacing the headspace of the serum bottles with 100% N<sub>2</sub>, after repeated cycles of overpressure with N<sub>2</sub> and vacuum (De Windt et al. 2005). In this way, the bacteria could reduce the Pd(II) and form Pd(0) nanoparticles, but the electron donor was removed before the addition of diatrizoate and hence hydrogen could not be used in the

dehalogenation reaction. In another set of control experiments, biologically produced H<sub>2</sub> was removed in the same way but replaced with H<sub>2</sub> instead of N<sub>2</sub> during the last flushing cycle. This resulted in a H<sub>2</sub> concentration of 100% in the headspace. In a last control experiment, the serum flasks were pasteurized in a warm water bath at 80°C for 30 min.

#### Morphology of bio-Pd with TEM

Samples of bacterial suspensions with and without Pd were allowed to settle for 8 h. Subsequently, the supernatant was removed and the bacteria were fixed in 0.1 M cacodylate buffer containing 4% paraformaldehyde and 5% glutaraldehyde. After postfixation in 1% osmium tetroxide, samples were dehydrated in a series of alcohol and embedded in Epon medium (Aurion, Wageningen, the Netherlands). Ultrathin sections of 60 nm were contrasted with uranyl acetate and lead nitrate before examination or were examined without contrast and imaged with a Zeiss TEM900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at 50 kV.

#### Analytical methods

Diatrizoate was analyzed by HPLC consisting of an ASI-100 autosampler, a P580 pump and a STH585 column oven (Dionex, Sunnyvale, CA, USA) on an Agilent Zorbax SB-C18 column (150 mm×4.6 mm, 5 μm). Elution was performed isocratically at 25°C and at a flow rate of 1 mL min<sup>-1</sup> with 5% solvent A (100% acetonitrile) and 95% solvent B (0.1% formic acid). The injection volume was 50 μL and the elution program ran during 15 min. Detection of diatrizoate was performed with a UV-VIS detector at a wavelength of 237 nm. Standard solutions, ranging from 0.1 to 50 mg L<sup>-1</sup>, were prepared for calibration, starting from an individual stock solution of 5 g L<sup>-1</sup>. Calibration was performed every month and the standard curves showed good correlation in all cases ( $R^2=0.99$ ). The limit of detection (LOD) was set at 100 μg L<sup>-1</sup>, based on a signal-to-noise ( $S/N$ ) ratio>3.

Analysis of TCE was performed by GC (CP-3800; Varian Palo Alto, CA, USA) with a flame ionization detector (FID). GC conditions were: injection temperature=200°C; detector temperature=250°C; initial column temperature=35°C (hold 2 min), increase to 75°C at a rate of 5°C min<sup>-1</sup>; column pressure=153 kPa (hold 2 min), increase to 176.5 kPa at a rate of 3 kPa min<sup>-1</sup>. The column used was a Factor Four<sup>TM</sup> low bleed capillary column (VF-624 ms, 30 m x 0.25 mm ID [inner diameter], DF [film thickness]=0.25 μm, Varian). The detection limit was 0.761 μM (100 μg L<sup>-1</sup>). Analysis of hydrogen was performed by GC (Global Analyser Solutions, Interscience, Breda, The Netherlands) with a

thermal conductivity detector (TCD). The pre-column and column were a Parabond Q (2 m×0.32 mm) and a Molsieve 5A column (7 m×0.32 mm), respectively. GC conditions were: column temperature=50°C; column pressure=100 kPa and detector temperature 60°C. The detection limit amounted to 1 ppm.

X-ray diffraction (XRD) of the palladium on the biomass, 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). The wavelength CuK $\alpha$  corresponded to 1.54Å. Measurements were made between 30° and 80° 2 $\theta$  with a step size of 0.02° 2 $\theta$ .

#### Dehalogenation of diatrizoate by bio-Pd in a membrane bioreactor

A membrane bioreactor (MBR) was used to develop a new approach for the removal of diatrizoate with bio-Pd nanoparticles and bio-H<sub>2</sub> as the electron donor. A schematic representation of the MBR can be found in De Gussemé et al. (2011). Briefly, the MBR consisted of a 10-L reservoir with one plate membrane made of chlorinated polyethylene (Kubota, Japan). The pore size of the membranes and the total filtration surface amounted to 0.4  $\mu$ m and 0.1 m<sup>2</sup>, respectively. The reactor was supplied with 8 L of a 50 mg L<sup>-1</sup> bio-Pd suspension, which was previously produced in batch using *C. braakii*. No extra inoculum of *C. braakii* was supplied since the bacteria survived to a large extent the Pd addition and hence could produce H<sub>2</sub> from glucose. The influent was spiked with 20 mg L<sup>-1</sup> diatrizoate and pumped in the reactor at the bottom at a flow rate of 8 L day<sup>-1</sup>. The effluent was pumped out of the reactor at a flow rate of 8 L day<sup>-1</sup>. The influent contained 0.5 g L<sup>-1</sup> glucose, 0.1 g L<sup>-1</sup> NH<sub>4</sub>Cl, 0.05 g L<sup>-1</sup> NaCl, 0.6 g L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, and 0.3 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>. The water level and the pH were steered by a level and pH controller, respectively.

## Results

#### Reduction of Pd(II) to Pd(0) by fermentatively cultivated bacteria

The Pd(II) reduction and Pd(0) nanoparticle formation capacity of six bacteria, cultivated under fermentative conditions, were evaluated with AAS, XRD, and TEM. The amount of Pd associated with the bacterial cell mass was determined using AAS by measuring the Pd content of the pellet after centrifugation. After the addition of 50 mg L<sup>-1</sup> Pd(II) to the bacterial cell cultures, large differences in the amount of Pd associated with the biomass

were detected (Table 1). The highest Pd mass was associated with *E. faecium* and amounted to 98±1%, while the lowest amount was associated with *C. braakii* (38±4%). In the cell-free controls, most of the Pd could be recovered in the supernatant (Table 1), meaning that the Pd(II) did not significantly precipitate with medium compounds. To study the influence of the initial Pd(II) concentration, 10 mg L<sup>-1</sup> Pd(II) was added to *C. braakii* cultures. The percentage Pd recovered on *C. braakii* (38±1%) was not differing significantly from the recovered percentage applying an initial Pd(II) concentration of 50 mg L<sup>-1</sup>.

The valency of the Pd associated with the bacterial cell mass was studied with XRD. The color change of the medium after the addition of the aqueous Pd(II), from pale yellow to black, was indicative for the formation of metallic Pd particles. This was in contrast with cell-free controls, in which neither a color change nor black precipitation was observed. The presence of Pd(0) on bacterial cells was confirmed by XRD for the six bacterial species under all conditions. Representative XRD diffractograms showed the characteristic diffraction peaks of crystalline Pd(0) particles at 2 $\theta$ =40°, 47°, and 68°. The biomass-associated Pd precipitates were also studied with TEM in the cases of *C. butyricum* (Fig. 1a–c) and *C. braakii* (Fig. 1d–f). Figure 1 a, b, d, and e show bacteria that were cultivated under fermentative conditions after which 50 mg L<sup>-1</sup> Pd(II) was added to the medium. These images show that black Pd precipitates were formed on the cell wall of both bacterial species. Contrary to *C. braakii* where the majority of the cells were covered with nanoparticles, only a limited number of *C. butyricum* cells showed nanoparticles on their cell surface. In addition to the dominant type of cell-associated nanoparticles, larger irregular Pd precipitates were observed on cell debris, on whole cells and independent from biomass in TEM images of both bacteria. These precipitates are indicated by arrows in Fig. 1. Control experiments were used to determine if bacteria have the capacity to reduce Pd(II) and form nanoparticles in minimal media that is free of bio-H<sub>2</sub> and other reductants formed during glucose fermentation. In these control experiments, bacteria were cultivated under fermentative conditions, separated from the growth medium, and suspended in fresh minimal medium prior to the addition of 50 mg L<sup>-1</sup> Pd(II). TEM images from these control experiments (Fig. 1c and f) showed that Pd nanoparticles formed at the cell surface, suggesting that not only chemical processes involving H<sub>2</sub> but also biological or other chemical processes are involved in generating Pd(0) nanoparticles.

#### H<sub>2</sub> production by the six strains

Since the hydrogen concentration was expected to significantly influence both the Pd(II) reduction and pollutant

**Table 1** Amount of Pd associated with the biomass of fermentative bacteria after  $t=1$  and 24 h

Species	Pd(II) concentration (mg L <sup>-1</sup> )	Percent Pd associated with biomass at $t=1$ h	Percent Pd associated with biomass at $t=24$ h	$k_{\text{obs}}$ (h <sup>-1</sup> )	$k_{\text{cat}}$ (h <sup>-1</sup> L g <sup>-1</sup> Pd)	H <sub>2</sub> production at $t=24$ h (mmol) <sup>a</sup>
<i>Citrobacter braakii</i> (G <sup>-</sup> )	50	33±4	38±4	5.6±0.6	112±12	1.31±0.15
<i>Clostridium butyricum</i> (G <sup>+</sup> )	50	76±1	84±3	3.5±0.1	70±2	0.84±0.19
<i>Escherichia coli</i> (G <sup>-</sup> )	50	60±6	55±8	nd	nd	0.64±0.23
<i>Klebsiella pneumoniae</i> (G <sup>-</sup> )	50	63±2	87±4	nd	nd	1.19±0.34
<i>Bacteroides vulgatus</i> (G <sup>-</sup> )	50	55±2	81±2	nd	nd	0.77±0.04
<i>Enterococcus faecium</i> (G <sup>+</sup> )	50	51±7	98±1	nd	nd	1.08±0.44
No bacteria <sup>a</sup>	50	14±2	10±2	nd	nd	/
<i>Citrobacter braakii</i> (G <sup>-</sup> )	10	25±4	38±1	0.45±0.03	45±3	nd
<i>Clostridium butyricum</i> (G <sup>+</sup> )	10	nd	nd	0.023±0.002	2.3±0.2	nd
<i>Escherichia coli</i> (G <sup>-</sup> )	10	nd	nd	0.016±0.001	1.6±0.1	nd
<i>Klebsiella pneumoniae</i> (G <sup>-</sup> )	10	nd	nd	0.22±0.02	22±2	nd
<i>Bacteroides vulgatus</i> (G <sup>-</sup> )	10	nd	nd	0.066±0.001	6.6±0.1	nd
<i>Enterococcus faecium</i> (G <sup>+</sup> )	10	nd	nd	0.026±0.004	2.6±0.4	nd
No bacteria <sup>b</sup>	10	11±1	10±1	nd	nd	/

Percent Pd associated with the cells was calculated by using the following formula: (Pd in pellet)/(Pd in pellet+Pd in supernatant)×100. First order rate coefficients ( $k_{\text{obs}}$ ) and concentration normalized rate coefficients ( $k_{\text{cat}}$ ) of diatrizoate removal and hydrogen (mol) produced after 24 h of bacterial growth. All experiments were performed in triplicate ( $n=3$ ), and results are presented as mean±standard deviation

<sup>a</sup> Hydrogen concentrations were measured before Pd(II) addition

<sup>b</sup> When no bacteria were present, the given percentage represents the amount recovered in the pellet due to precipitation of Pd(II) in the bacterial growth medium

nd not determined, / not applicable

dehalogenation capacity, the cumulative hydrogen production by the six bacterial strains was measured following 24 h of fermentative cultivation on glucose before the Pd addition (Table 1). The cumulative hydrogen production (mol H<sub>2</sub>) decreased in the following order: *C. braakii*>*K. pneumoniae*>*E. faecium*>*C. butyricum*>*B. vulgatus*>*E. coli*. In the case of *C. braakii*, the hydrogen production was also measured after separation of bio-Pd from the spent medium and resuspension in fresh medium. The cumulative hydrogen production amounted to 0.81±0.32 mol, which means that the cells were still viable to a large extent after Pd addition. The latter observation was also confirmed by plate counts.

#### Dehalogenation of diatrizoate with biogenic Pd(0)

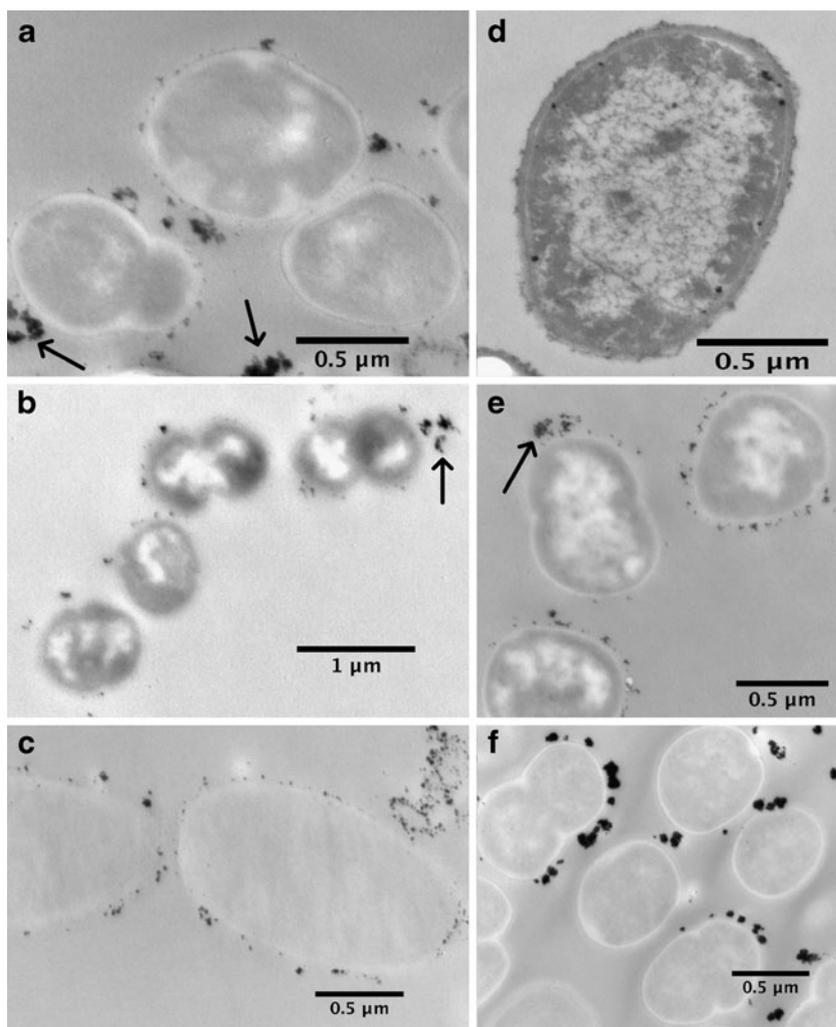
In a second stage of the research, the catalytic activity of the Pd(0) nanoparticles produced by the six bacteria was studied in the dehalogenation reaction of diatrizoate. Figure 2a shows the degradation of 20 mg L<sup>-1</sup> diatrizoate by 10 mg L<sup>-1</sup> of bio-Pd without the addition of an external electron donor. The best fit to the data indicates that the dehalogenation of diatrizoate with biogenic Pd(0) as catalyst and biogenic hydrogen as the electron donor follows first order kinetics. According to the apparent first

order rate constant  $k_{\text{obs}}$ , *C. braakii* and *K. pneumoniae* showed the highest activity with  $k_{\text{obs}}=0.45±0.02$  h<sup>-1</sup> and  $k_{\text{obs}}=0.22±0.02$  h<sup>-1</sup>, respectively, while *C. butyricum* showed almost no activity (Table 1).

*C. braakii* was selected to further explore the effect of the Pd concentration on the dehalogenation kinetics. Activity experiments were repeated with a fivefold increase of initial Pd(II) concentration (50 mg L<sup>-1</sup> Pd). This resulted in an increased diatrizoate removal rate compared to the 10 mg L<sup>-1</sup> Pd(II) preparation at an equivalent cell density (Fig. 2b). The dehalogenation still followed first order kinetics with  $k_{\text{obs}}=5.58±0.60$  h<sup>-1</sup>.

In order to address the function of biogenic hydrogen, control experiments with *C. braakii*, in which the hydrogen in the headspace and the growth medium was purged by N<sub>2</sub> flushing after the Pd(0) nanoparticle formation, were conducted. The latter showed a dramatic decrease in dehalogenation rates with  $k_{\text{obs}}=0.041±0.002$  h<sup>-1</sup>. Moreover, the addition of external hydrogen could only partially restore the activity at a Pd concentration of 10 mg L<sup>-1</sup> ( $k_{\text{obs}}=0.069±0.003$  h<sup>-1</sup>). In a final control experiment, the bio-Pd suspension was pasteurized to stop all biological activity and hence address the importance of viable bacteria in the dehalogenation reaction. No dehalogenation of diatrizoate could be detected in these heat-treated controls.

**Fig. 1** TEM images of Pd nanoparticles on *Clostridium butyricum* (a, b, and c) and *Citrobacter braakii* (d, e, and f). Images a, b, d, and e show bacteria that were grown anaerobically after which 50 mg L<sup>-1</sup> aqueous Pd(II) was added. Images c and f show nanoparticle formation by bacteria that were grown anaerobically, separated from their growth medium, and resuspended in fresh minimal medium after which 50 mg L<sup>-1</sup> Pd(II) was added. Arrows indicate Pd precipitates which are not associated with the bacterial biomass



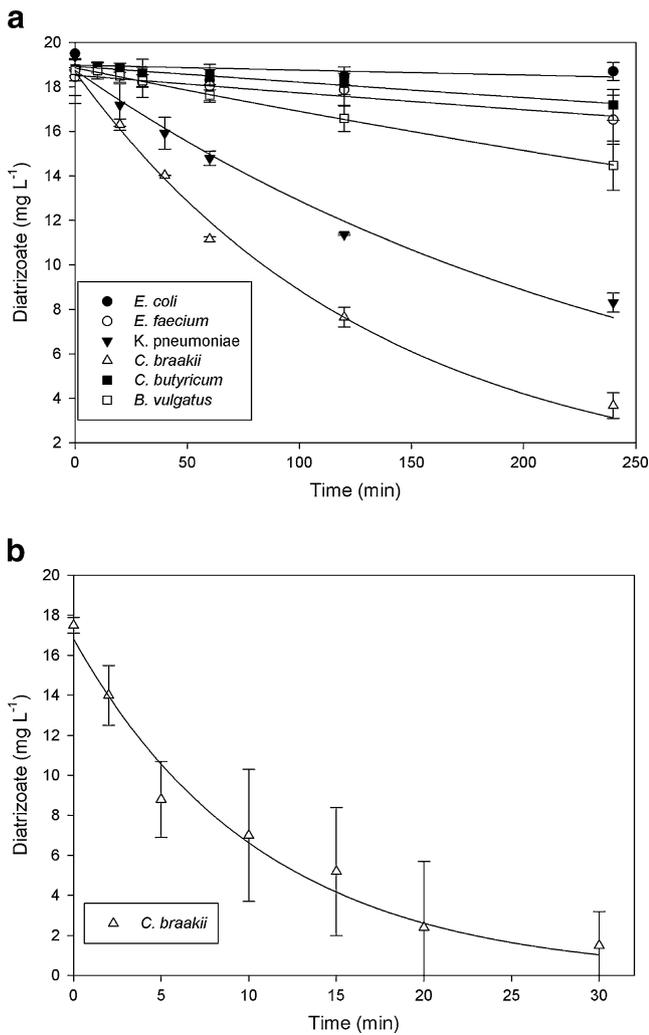
#### Repeated dehalogenation of diatrizoate and TCE with biogenic Pd(0) produced by *C. braakii*

The activity of bio-Pd produced by *C. braakii*, which showed the highest dehalogenation activity towards diatrizoate, was studied in the dechlorination reaction of TCE in order to evaluate the spectrum of activity. With 10 mg L<sup>-1</sup> Pd, no degradation of 100 mg L<sup>-1</sup> TCE could be detected. However, dosing 50 mg L<sup>-1</sup> Pd resulted in a complete removal of TCE within 24 h. Ethane was detected as the only end product pointing towards full dehalogenation. Subsequently, 50 mg L<sup>-1</sup> bio-Pd suspensions were repeatedly spiked with 100 mg L<sup>-1</sup> diatrizoate (Fig. 3a) or 100 mg L<sup>-1</sup> TCE (Fig. 3b), and the removal was measured 24 h after each addition. These experiments were conducted to study the ability to reactivate the bio-Pd with biogenic hydrogen. It can be seen for both xenobiotics that complete degradation was only achieved after the first spikes, and the concentration of both xenobiotics recovered from the medium increased with each subsequent spike. This resulted in a reduced ability to

degrade diatrizoate after 16 cycles of spiking. Dehalogenation activity stopped after 11 spiking cycles in the case of TCE. In these batch experiments, a total removal of 1.01±0.03 mg diatrizoate mg<sup>-1</sup> Pd and 0.47±0.02 mg TCE mg<sup>-1</sup> Pd was obtained without any new supply of bacterial feed.

#### Removal of diatrizoate by biogenic Pd(0) produced by *C. braakii* in an MBR

To avoid acidification and subsequent die-off during the fermentation of glucose by *C. braakii* in the closed batch experiments, the removal of diatrizoate was tested in an MBR provided with a one-time spike of 50 mg L<sup>-1</sup> Pd(II) in order to generate and establish bio-Pd before contaminant introduction. Moreover, extra nutrients such as glucose were supplied to make a continuous production of hydrogen possible. After the first day, complete removal of 20 mg L<sup>-1</sup> was obtained in the reactor (Fig. 4). However, a gradual increase of diatrizoate in the effluent was observed after the second day and reached a maximum of 16.7 mg L<sup>-1</sup> after



**Fig. 2** Removal rate of 20 mg L<sup>-1</sup> diatrizoate by biogenic hydrogen and Pd(0) nanoparticles formed by fermentative bacterial species. **a** Removal rates of fermentative bacterial species with Pd(0) nanoparticles produced by the addition of 10 mg L<sup>-1</sup> aqueous Pd(II). **b** Removal rates of *Clostridium butyricum* and *Citrobacter braakii* with Pd(0) nanoparticles produced by the addition of 50 mg L<sup>-1</sup> aqueous Pd(II). All experiments were done in triplicate and means±standard deviations are given

8 days. After day 7, the glucose concentration of the influent was increased from 0.5 to 2 g L<sup>-1</sup> to stimulate the biogenic formation of hydrogen. This resulted in a temporary improvement of contaminant removal, obtaining a minimum of 11.7 mg L<sup>-1</sup> diatrizoate in the effluent after day 11, 4 days after increasing the glucose concentration. However, the removal efficiency decreased from day 12 to day 19, until almost no removal was observed during the last days of the experiment. During the entire experiment, 1,077 mg of diatrizoate was removed which resulted in a conversion of 2.75 mg diatrizoate mg<sup>-1</sup> Pd.

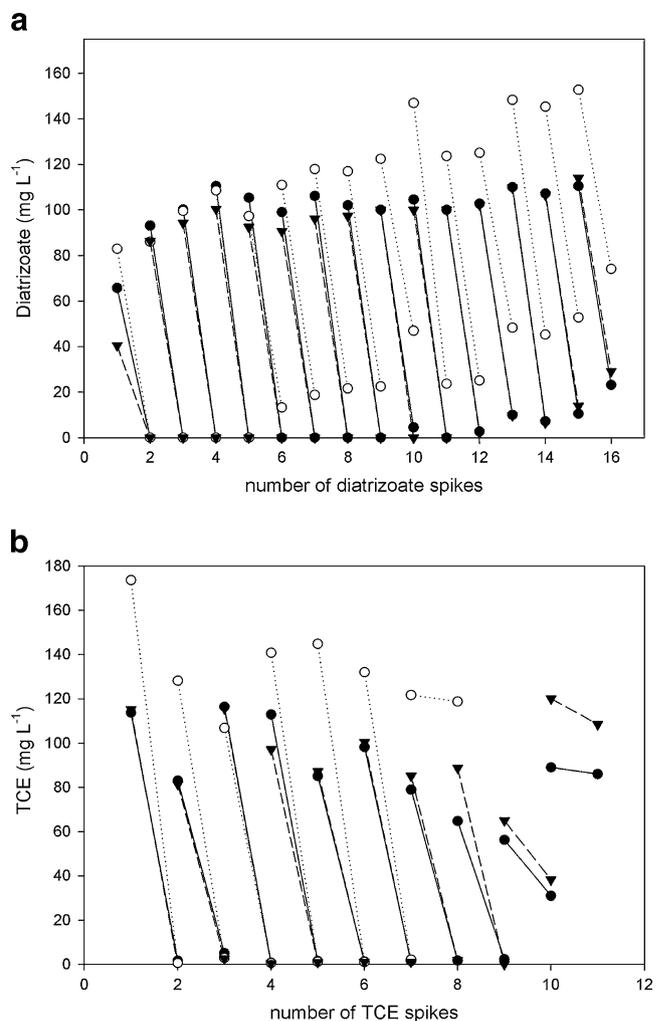
The Pd concentrations in the effluent of the MBR were also monitored in samples collected every 24 h (Fig. 4). During the first 2 days, the effluents contained peak Pd

concentrations of 3.4 and 1.1 mg L<sup>-1</sup>, respectively. During the remainder of the experiment, the Pd effluent concentration stabilized, amounting to 0.47±0.14 mg L<sup>-1</sup> on average. In total, 126 mg Pd of the initial amount of 400 mg Pd was lost over the duration of the experiment.

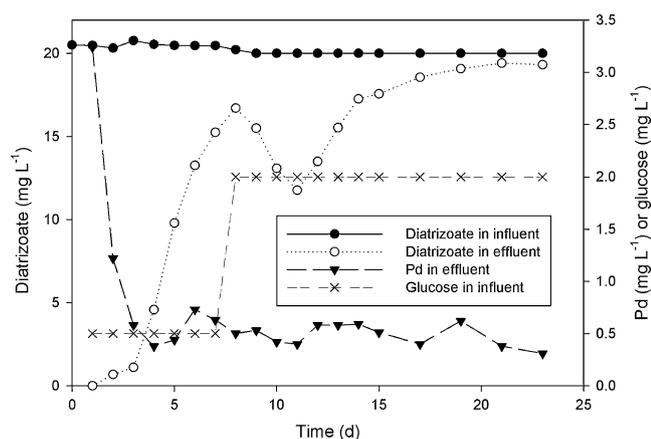
## Discussion

### Production of Pd nanoparticles by fermentatively cultivated bacteria

Recently, Pd(0) nanoparticle formation by *E. coli* (Deplanche et al. 2010) and *C. pasteurianum* BC-1 (Chidambaram et al. 2010) strains cultivated under fermentative conditions was reported. In the case of *C. pasteur-*



**Fig. 3** Removal of repeated spikes every 24 h of 100 mg L<sup>-1</sup> diatrizoate (**a**) and trichloroethylene (**b**) by biogenic hydrogen and Pd(0) nanoparticles formed by *Citrobacter braakii* (50 mg L<sup>-1</sup> aqueous Pd(II)). All experiments were performed in triplicate and are shown separately



**Fig. 4** Removal of diatrizoate in an MBR supplied with a  $50 \text{ mg L}^{-1}$  bio-Pd suspension produced by *Citrobacter braakii*. Reactor volume and HRT amounted respectively to 8 L and 24 h, respectively. Influent and effluent concentrations of diatrizoate and Pd effluent concentrations are shown as a function of time

*ianum*, the co-generation of Pd(0) nanoparticles and microbially produced hydrogen (bio- $\text{H}_2$ ) was exploited to catalyze the reductive removal of chromate from an aqueous waste stream without adding an external electron donor. This treatment process advanced previous applications of bio-Pd that had first generated the Pd(0) nanoparticles before applying the biomass bound bio-Pd in a second step to catalyze dehalogenation reactions using an external source of hydrogen or formate as the electron donor (Hennebel et al. 2009a). In this paper, the unique capability of Pd production by bacteria grown under fermentative conditions was studied by selecting six bacteria, with hydrogen being a common metabolic product amongst all these strains, but differing in structural cell compounds and metabolic properties. The main differences in cell structure are characterized by the Gram staining: *C. butyricum* and *E. faecium* are Gram-positive and *K. pneumoniae*, *B. vulgatus*, *E. coli*, and *C. braakii* are Gram-negative. The metabolic pathways used during glycolysis are known to differ significantly across these bacterial strains, resulting in differences in fermentation end products and the amount of hydrogen generated (Ntaikou et al. 2010). These differences were captured by analyzing the headspace  $\text{H}_2$  after 24 h of growth. The following order of hydrogen production was observed: *C. braakii* > *K. pneumoniae* > *E. faecium* > *C. butyricum* > *B. vulgatus* > *E. coli*.

All six bacterial strains reduced Pd(II) and generated crystalline Pd(0) particles, as was determined by XRD analyses. Hence, it can be generalized that bacteria, when grown under fermentative conditions that lead to hydrogen production, form metallic Pd particles. Nanoparticle generation very likely occurs through a combination of biologically and chemically based processes, in which the bacteria

or bacterial compounds function as nucleation sites, and after which Pd(II) is reduced enzymatically, by bio- $\text{H}_2$  or by a combination of the two processes. In control experiments, in which the cells were centrifuged and resuspended in fresh minimal medium to remove excess of hydrogen, Pd(II) was reduced as well and nanoparticles associated with the cell surfaces were observed in TEM images (Fig. 2c and f). These observations can be indications that biological processes are involved in the precipitation process. However, since the bio- $\text{H}_2$  was not removed in the principal experiments, chemical reduction cannot be excluded. Previous studies attributed the reduction of inorganic contaminants by bacteria cultivated under fermentative conditions to reductive enzymes. For example, the reduction of Tc(VII) by *E. coli* was attributed to the formate dehydrogenase in combination with the hydrogenase component of the formate hydrogenlyase structural unit (Lloyd et al. 1997); the reduction of U(VI) by *Clostridium* species was attributed to a so far unknown enzymatic mechanism (Francis et al. 1994). Very recently, Deplanche et al. (2010) elucidated the Pd(II) reduction mechanism of *E. coli* and attributed this to at least three different [NiFe] hydrogenases. It is possible that these enzymes played also a crucial role in our Pd reduction experiments.

Strong differences were observed in the amount of Pd associated with the bacterial biomass from the six strains: *E. faecium* > *C. butyricum*  $\approx$  *K. pneumoniae*  $\approx$  *B. vulgatus* > *E. coli* > *C. braakii*. This can be caused by several parameters including enzyme production, cell structure and composition, Pd toxicity resistance, fermentation product type and distribution, and biogenic hydrogen production. Biogenic hydrogen production depends on the end product of glycolysis. When acetate is formed, every mole of glucose results in the formation of 4 mol of  $\text{H}_2$ . When butyrate is formed, every mole of glucose results in the formation of 2 mol of  $\text{H}_2$ . However, glucose fermentation can also lead to the generation of other products such as propionate, succinate, and lactate (mixed fermentation), and the generation of these metabolites is not accompanied by  $\text{H}_2$  generation (Ntaikou et al. 2010). Depending on the culture conditions as well as the type of microorganism, simultaneous generation of acetate and butyrate can be observed, leading thus to a hydrogen yield with values between 2 and 4. Such reactions are quite typical for some Clostridia such as *C. pasteurianum* (Daesch and Mortenso 1968) and *C. butyricum* (Jungermann et al. 1973). On the other hand, the hydrogen production via formate and acetylCoA, which results in acetate formation, is typical for enterobacteria such as *E. coli* (Nandi and Sengupta 1996).

No consistent relation between the amount of hydrogen produced and Pd(II) reduction could be observed. However, the higher production of  $\text{H}_2$  by *C. braakii* can provide a possible explanation for their low Pd-binding on their cells.

Indeed, the Pd(II) will be readily reduced and deposited on every possible nucleation site such as cell debris or exudates. The fact that Pd was strongly associated to the biomass in case of *E. faecium* and *C. butyricum* is consistent with the differences in cell wall composition. Indeed, Gram-positive bacteria have a thick cell wall made of peptidoglycan whose major compounds, i.e., polysaccharides and peptides, are known for their metal adsorption capacity. For example, the fast adsorption of Pd(II) on biomass of *Bacillus licheniformis* reached 116 mg Pd(II) g<sup>-1</sup> biomass (Lin et al. 2002). At lower Pd concentrations, no significant differences in biomass-associated Pd were detected. The low amounts of Pd were possibly complexed by medium compounds and were not available for adsorption. Detailed adsorption experiments, in the absence of hydrogen to avoid precipitation, are needed to determine if the differences in the amount of biomass-associated Pd can be solely related to differences in cell structure or to a complex combination of other factors involved. Moreover, in-depth studies of the enzymes involved in the Pd nanoparticle formation mechanisms should be performed to address the importance of enzymatical versus chemical reduction with bio-H<sub>2</sub>.

#### Use of Pd nanoparticles produced by fermentatively cultivated bacteria for dehalogenation of diatrizoate

In a second stage of the research, the capacity for the bio-Pd nanoparticles produced by the six bacterial strains to dehalogenate diatrizoate was tested. Efficient removal of diatrizoate was shown previously using bio-Pd produced by *S. oneidensis* and an external source of hydrogen as the electron donor (Hennebel et al. 2010). In this study, no external hydrogen was used, but instead the dehalogenation reactions depended on bio-H<sub>2</sub> produced during the fermentation. At a concentration of 10 mg L<sup>-1</sup>, especially *C. braakii* and *K. pneumoniae* showed high activities. The first order constants of diatrizoate degradation in the 10 and 50 mg L<sup>-1</sup> Pd preparations with *C. braakii* were 0.45±0.02 and 5.58±0.59 h<sup>-1</sup>, respectively. These values are comparable to rate constants observed with equivalent 10 and 50 mg L<sup>-1</sup> Pd preparations with *S. oneidensis* and an external H<sub>2</sub> source, which were 0.16±0.02 and 6.1±0.5 h<sup>-1</sup>, respectively. A large difference in activity among the six fermentative bacteria was observed: *C. braakii*>*K. pneumoniae*>*B. vulgatus*>*E. faecium*≈*C. butyricum*>*E. coli*. The activity does not correlate with the amount of bio-Pd associated with the bacterial biomass. For example, *C. braakii* showed the highest degradation rates but had generated the lowest amount of bio-Pd. Instead, the high degradation rates of *C. braakii* and *K. pneumoniae* are consistent with their efficient production of bio-H<sub>2</sub> (Table 1). Across all six bacterial strains, the trends observed in activity do generally correlate with the amount

of bio-H<sub>2</sub> generated. Hydrogen lowers the redox potential of the reaction medium and functions as an efficient electron donor in dehalogenation reactions. Hence, relatively more bio-H<sub>2</sub> should improve the bio-Pd catalyzed rate of diatrizoate degradation. Glucose fermentation processes can vary among bacterial strains, and as a result the types of end products or intermediates produced by glycolysis might impact diatrizoate degradation rates. For example, many enterobacteria produce formate (Peters et al. 1999), which has been shown to serve as an efficient electron donor in bio-Pd catalyzed dehalogenation reactions (Mertens et al. 2007).

The size distribution, morphology, and deposition loci of the bio-Pd nanoparticles might also contribute to the observed differences in the reduction rates. Indeed, differences in these characteristics of the bio-Pd nanoparticles were observed in TEM images of *C. butyricum* and *C. braakii* (Fig. 1). TEM images showed that a higher fraction of *C. braakii* cells were covered with bio-Pd nanoparticles compared to *C. butyricum*. These images also showed that relatively larger bio-Pd agglomerates decorated the *C. butyricum* cells that were covered. A systematic quantitative analysis of the complex combination of these bio-Pd characteristics is beyond the scope of this research.

Control experiments in which bio-H<sub>2</sub> gas was removed by flushing with N<sub>2</sub> and then replaced by an external source of either N<sub>2</sub> or H<sub>2</sub> showed a tenfold decrease in diatrizoate degradation rates. This observation is no evidence that one needs active bacteria during the deiodination but suggests that the bio-hydrogen, present during the Pd(0) nanoparticle formation, played a major role in the dehalogenation mechanism. Moreover, the observation that the external source of hydrogen only restores a relatively insignificant amount of the activity to the bio-Pd nanoparticles in the N<sub>2</sub> purged system, stresses the importance of co-generation of bio-H<sub>2</sub>, and bio-Pd in close proximity. It is hypothesized that during the bio-Pd generation, the nanoparticles are loaded with bio-H<sub>2</sub> as it is formed. Once the hydrogen is removed from the bio-Pd by flushing with N<sub>2</sub> or pasteurization, the loss of catalytic activity can only partially be restored by the external source of abiotic hydrogen. This cannot be due to decreased H<sub>2</sub> concentrations as after pasteurization, the total hydrogen amount did not change, and the addition of external hydrogen resulted in an even higher H<sub>2</sub> concentration.

#### Repeated dehalogenation of diatrizoate and TCE with biogenic Pd(0) produced by *C. braakii*

The dehalogenation capacity of biogenic Pd(0) produced by *C. braakii* was evaluated by applying repeated spikes of diatrizoate and TCE without providing extra glucose. Bio-Pd successfully removed repeated spikes of 100 mg L<sup>-1</sup> diatrizoate and TCE. The extent of TCE dechlorination to

ethane was comparable to the dehalogenation of TCE by bio-Pd produced by *S. oneidensis* with an external hydrogen source (Hennebel et al. 2009b). In total,  $1.01 \pm 0.03$  mg diatrizoate  $\text{mg}^{-1}$  Pd and  $0.47 \pm 0.02$  mg TCE  $\text{mg}^{-1}$  Pd could be removed after repeated spikes at  $100 \text{ mg L}^{-1}$ . To fully dehalogenate 46 mg diatrizoate and 24 mg TCE, 0.11 and 0.45 mmol of  $\text{H}_2$ , respectively, are stoichiometrically required. The amount of hydrogen produced by *C. braakii* after the addition of  $10 \text{ gL}^{-1}$  glucose was  $1.31 \pm 0.15$  mmol  $\text{H}_2$ . Hence, the produced amount of hydrogen exceeds the theoretical requirement for a complete dehalogenation of diatrizoate and TCE. However, the decreasing bio- $\text{H}_2$  concentrations might explain the increasing amount of diatrizoate and TCE remaining in the medium as the number of contaminant spikes increased. The poisoning of the Pd catalyst and the die-off of *C. braakii* due to acidification of the medium might be other factors leading to lower dehalogenation capacity. Therefore, an MBR was constructed to continuously supply glucose, ammonium, and a phosphate buffer to ensure continuous production of biogenic hydrogen and avoid bacterial die-off due to pH decreases. Nearly complete diatrizoate removal was observed during the first 4 days of MBR operation (Fig. 4). Subsequently, a gradual decline in activity was observed. Loss of Pd from the reactor and the covering of Pd nanoparticles by growing bacterial cells could have lowered the removal efficiency of the MBR. A larger dose of glucose delivered after 7 days restored some level of degradation activity, but the effect was temporary (Fig. 4). During the entire experiment,  $2.75 \text{ mg diatrizoate mg}^{-1}$  Pd was removed without adding external hydrogen. This greatly exceeded the amount of diatrizoate degraded in the batch experiments with repeated spiking. However, it remains unclear why complete removal could not be maintained over a longer period. Further research challenges include the need to completely retain the bio-Pd in the reactor while maintaining its catalytic activity. The ultimate goal would be to develop an MBR-type system in which reduced Pd species are produced and continuously regenerated by bio- $\text{H}_2$ . Conclusively, this work demonstrates the general applicability of bacteria cultivated under fermentative conditions to generate catalytic Pd(0) nanoparticles and bio- $\text{H}_2$  as hydrogen donor. These biomass-associated nanoparticles can be used to dehalogenate iodinated contrast media and chlorinated solvents without an external source of hydrogen. The suggested mechanism can be applied in MBRs to treat aqueous streams of industrial effluent and as part of pump-and-treat systems.

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