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Technical Note

Removing selected steroid hormones, biocides and pharmaceuticals from water by means of biogenic manganese oxide nanoparticles *in situ* at ppb levels

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HIGHLIGHTS

- Micro-pollutants were incubated with biogenic manganese oxides *in situ*.
- A complete removal of estrone and ethinylestradiol was observed.
- *In situ* incubations appears as a practical approach considering the full scale application.
- Residual organic matter in wastewater will affect the oxidation of micro-pollutants by BioMnOx.

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ABSTRACT

The oxidation of organic micro-pollutants by biogenic manganese oxide nanoparticles (BioMnOx) has been studied with respect to possible implementation of BioMnOx in wastewater treatment. For this it would be prerequisite that microbial Mn^{2+} oxidation and BioMnOx-driven pollutant removal can occur *in situ*, i.e. in the same reactor as the removal. Here we present the *in situ* reactivity of BioMnOx produced by *Pseudomonas putida* towards a range of micro-pollutants at environmentally relevant concentrations (10 $\mu g\,L^{-1}$). We found that *in situ* formed BioMnOx completely removed the steroid hormones estrone and 17-α ethinylestradiol, while only 26% removal of diclofenac was achieved. Ibuprofen, tebuconazole, carbamazepine, carbendazim, and terbutryn were not removed under *in situ* conditions.

1. Introduction

The presence of pharmaceuticals and other bioactive organic micro-pollutants in the aquatic environment has emerged as a topic of high concern in the media and in research (Daughton and Ternes, 1999; Kümmerer, 2009). Conventional activated sludge wastewater treatment plants (WWTPs) were designed to remove biological oxygen demand (BOD) and nutrients (mg L^{-1} range). They are often not able to remove recalcitrant organic micro-pollutants such as steroid hormones, pharmaceuticals or biocides that occur at $\mu g\,L^{-1}$ concentrations. Consequently, micro-pollutants find their way into the environment via wastewater effluents and sludge (Daughton and Ternes, 1999; Ternes et al., 2004). Even though some compounds can be partially removed in conventional activated sludge WWTP (e.g. estrone and 17α-ethinylestradiol), their residues may cause adverse effects in surface waters at $\mu g\,L^{-1}$ and $ng\,L^{-1}$ levels (Routledge et al., 1998; Kidd et al., 2007). Numerous studies have demonstrated efficiency of advanced treatment processes, (advanced oxidation processes, sorption to activated carbon, as well as membrane filtration, Reungoat et al., 2010; Zimmermann et al., 2011). However, these processes also have certain drawbacks such as high costs, energy consumption and reaction by-products (Shannon et al., 2008; Hollender et al., 2009; Zimmermann et al., 2011; Reungoat et al., 2012; Vom Eyser et al., 2013).

Even though there is an apparent progress in developing new technologies targeting the removal of micro-pollutants, the challenge of finding cost effective processes remains. Thus using microbially produced biogenic manganeseoxide nanoparticles

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(BioMnOx) to remove organic micro-pollutants may be an interesting alternative (Sabirova et al., 2008; Hennebel et al., 2008; Forrez et al., 2010, 2011). Manganese oxides (MnOx) are some of the strongest oxidants naturally found in the environment (Laha and Luthy, 1990; Tebo et al., 2004; Spiro et al., 2010), and redox potentials of different MnOx vary between 500 and 600 mV (Thamdrup, 2000). Numerous studies demonstrated that MnOx are processed in biogeochemical cycles, and they participate in a wide range of redox reactions with different organic compounds (Tebo et al., 2004), including several groups of xenobiotics (de Rudder et al., 2004; Zhang and Huang, 2005; Chen and Huang, 2011). The biochemical mechanisms of manganese oxidation involve bacterial multicopper oxidases (MCOs) (van Waasbergen et al., 1996) that oxidise Mn^{2+} (dissolved) to Mn(III)oxide (particulate) via one single electron transfer (Tebo et al., 2004; Webb et al., 2004). Subsequently, Mn(III)-oxide is oxidised by the MCOs or by direct reaction with O_2 to Mn(IV)oxides. Both Mn(III) and Mn(IV)oxides precipitate on the cell surface and surrounding environment (Tebo et al., 2005). In the presence of an organic micro-pollutant, Mn(III)oxides react as electron acceptor and are reduced to Mn^{2+} while the micro-pollutant is oxidised (de Rudder et al., 2004). Bacteria produce a mixture of Mn(III)- and Mn(IV)oxides as nanoscale particles with excellent sorption- and oxidation properties due to their high specific surface area and high number of Mn(IV) vacancies (Villalobos et al., 2003).

Consequently, BioMnOx is gaining attention as an attractive solution for degradation of compounds such as 17α-ethinylestradiol (Sabirova et al., 2008), diclofenac (Meerburg et al., 2012; Forrez et al., 2010, 2011) and several biocides and iodinated x-ray contrast media (Forrez et al., 2011). Practical application of MnOx, however, requires the continuous addition or regeneration of MnOx. The chemical oxidation of dissolved Mn^{2+} to Mn(III)- or Mn(IV)oxide particles is slow (rate constant: 10^{-2} to 10^{-5} h^{-1}) when compared to bacterial oxidation (rate constant 10 to 10^{-1} h^{-1}) Morgan (2000, 2005). A continuous regeneration in WWTPs can thus realistically only occur if catalysed by bacteria. If microbial Mn^{2+} oxidation by bacteria can be controlled, BioMnOx can theoretically be used as a continuously self-regenerating oxidant for the removal of micro-pollutants.

There are strong indications of BioMnOx's ability to remove organic micro-pollutants from wastewater effluents, but many unknowns still exist. Most studies have so far investigated the removal of micro-pollutants at high concentrations, exceeding levels typical for municipal wastewater (Sabirova et al., 2008; Forrez et al., 2010) – only one paper, i.e., Forrez et al. (2011) used realistic concentrations of micro-pollutants in a hollow fibre membrane reactor into which the pre-isolated BioMnOx was introduced. There is a need for a deeper understanding how effective BioMnOx is for removing micro-pollutants at more realistic (i.e. μg L^{-1}) concentrations. Until now, there are no studies on the removal of micro-pollutants with BioMnOx produced *in situ*, and only Forrez et al. (2010) compared removal of diclofenac by BioMnOx produced *in situ* and by pre-oxidised BioMnOx.

In this study, we investigated the potential of BioMnOx's to remove selected organic micro-pollutants *in situ* and at low concentrations (10 μg L^{-1}). For this reason, we did not separate the generated BioMnOx from the cell suspension before addition of organic micro-pollutants but used the suspension as is.

2. Materials and methods

2.1. Growth of Pseudomonas putida and formation of biogenic manganese oxides (BioMnOx)

P. putida MnB6 (BCCM/LMG 2322) specimen were kindly provided by Nico Boon, Laboratory of Microbial Ecology and Technology (Lab MET), Ghent University. A pre-culture was grown overnight in half strength Luria Broth at 30 °C, 120 rpm. One milliliter of this pre-culture was used to inoculate the main incubation performed in 500 mL Erlenmeyer flask, containing 200 mL growth medium without manganese (Villalobos et al., 2003) at 28 °C. During this phase, the pH of the growth medium was adjusted to 6.8 using sodium hydroxide solution. The flasks were kept under the same temperature on a rotary shaker set at 120 rpm. Oxygen was supplied by a passive aeration by shaking. Manganese (II) chloride ($MnCl_2$) was added to a final concentration of 2 mg Mn^{2+} L^{-1} when the cells entered the stationary phase after approximately 7 h (OD_{600} = 0.7, data not shown).

2.2. Transmission electron microscopy (TEM) images of BioMnOx

Cell suspension were prepared for TEM by fixation in 4% glutaraldehyde solution prepared in HEPES buffer (pH 6.8) for 1 h at room temperature followed by three washing steps and resuspension in MilliQ water. The cells were stored at 4 °C until analysis by TEM. Formvar-coated copper grids (mesh 300) with carbon film were loaded with approximately10 μL of the suspension and air-dried before imaging. The grids were glow discharged. The cells were visualised using a Phillips CM 12 transmission electron microscope equipped with a LaB6 filament operating at 80 kV and a Mega View III FW camera (FEI Company, Eindhoven, The Netherlands). TEM energy dispersive spectroscopy (TEM-EDS) was performed with the same instrument at 200 kV accelerating voltage with Si(Li) detector (EDAX Inc., Mahwah, NJ, USA), with 0.55 eV energy resolution per channel and Super Ultra Thin Window (SUTW).

2.3. Measurements of manganese concentrations

Samples for the determination of dissolved and particulate manganese were taken every 4 h during the first day of incubations and every 12 h on the following days. After 4 d sampling was reduced to once a day. Total manganese was measured in the beginning and in the end of each test. All measurements were performed with an ANALYST 800 flame atomic absorption spectrophotometer (AAS), Perkin–Elmer, Waltham (MA), USA, at a wavelength of 279.5; with the flame being maintained by air-acetylene. LOD was 0.05 mg Mn L^{-1}. Under the experimental conditions, the Mn^{2+} was soluble and the Mn(III) and (IV) oxides were particulate.

The determination of dissolved manganese was performed by centrifuging 10 mL samples of bacteria suspension (10 min at 500 g/1500 rpm) to remove the biomass and particulate manganese. The supernatants were collected, acidified with 1% HNO_3 (7 M) and stored at 4 °C prior to measurement with AAS.

For the determination of particulate manganese, pellets from the centrifugations were collected and washed three times in MilliQ water. Successively they were resuspended in 10 mL 20 mM hydroxylamine hydrochloride solution. Hydroxylamine hydrochloride reduces particulate manganese oxides to Mn^{2+} (Chao, 1972). These suspensions were reacted at 28 °C on a rotary shaker (120 rpm) for approximately 12 h. The resulting suspensions were centrifuged (see above) and the supernatants were collected, acidified with 1%, vol/vol HNO_3 and stored at 4 °C until measurement with AAS.

The total manganese concentrations were measured from the bacteria suspensions without centrifuging and the reduction procedure was the same as for the particulate manganese (10 mL 20 mM hydroxylamine hydrochloride, 1% vol/vol HNO_3).

2.4. Sample preparation and analyses of micro-pollutants

1 mL samples from the batch experiments described in the section below were centrifuged for 10 min at 500 g. Supernatants