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Influence of manganese and ammonium oxidation on the removal of 17α-ethinylestradiol (EE2)

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ABSTRACT

Flow-through reactors with manganese oxides were examined for their capacity to remove 17α-ethinylestradiol (EE2) at μg L^{-1} and ng L^{-1} range from synthetic wastewater treatment plant (WWTP) effluent. The mineral MnO_2 reactors removed 93% at a volumetric loading rate (B_V) of 5 μg EE2 L^{-1} d^{-1} and from a B_V of 40 μg EE2 L^{-1} d^{-1} on, these reactors showed 75% EE2 removal. With the biologically produced manganese oxides, only 57% EE2 was removed at 40 μg EE2 L^{-1} d^{-1}. EE2 removal in the ng L^{-1} range was 84%. The ammonium present in the influent (10 mg N L^{-1}) was nitrified and ammonia-oxidizing bacteria (AOB) were found to be of prime importance for the degradation of EE2. Remarkably, EE2 removal by AOB continued for a period of 4 months after depleting NH_4^+ in the influent. EE2 removal by manganese-oxidizing bacteria was inhibited by NH_4^+. These results indicate that the metabolic properties of nitrifiers can be employed to polish water containing EE2 based estrogenic activity.

1. Introduction

Endocrine disrupting compounds (EDCs) have become a concern over the past decades. Several studies have shown that these substances interact with the endocrine system of many aquatic species and thereby affecting their growth, development and reproduction (Jones and de Voogt, 1999; Rodgers-Gray et al., 2000). Even at less than 1 ng L^{-1}, 17α-ethinylestradiol (EE2) can interfere with the hormonal systems of male rainbow trout (Purdom et al., 1994) and male fathead minnows (Lange et al., 2001). Municipal wastewater is considered as one of the main sources of EDCs in the environment and the most important estrogenic compounds are the natural estrogens 17β-estradiol (E2) and estrone (E1), the synthetic contraceptive pill hormone 17α-ethinylestradiol (EE2), the xenoestrogens nonylphenol (NP) and octylphenol (OP) and a number of pesticides and phytoestrogens (Desbrow et al., 1998; Snyder et al., 2001).

Several studies on the occurrence of sex hormones in the influents and effluents of WWTPs indicate their presence in the lower ng L^{-1} range, with values ranging from 1 to 10 ng L^{-1} (Cargouët et al., 2004; Vethaak et al., 2005; Johnson et al., 2007). However, up to 20 ng EE2 L^{-1} was detected in surface water (Barel-Cohen et al., 2006). The maximum measured levels of

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E2, E1 and EE2 in raw domestic wastewater were 150, 34 and 28 ng L^{-1} respectively; while in WWTP effluent, respectively, 64, 72 and 106 ng L^{-1} were detected (Ternes et al., 1999a; Vethaak et al., 2002; Clara et al., 2005). E1, previously identified as the main intermediate of E2 degradation in activated sludge, is more stable than E2 (Ternes et al., 1999b). The higher concentration for EE2 in the WWTP effluent is explained by both the cleavage of conjugated forms during sewage treatment and the poor EE2 biodegradability under normal wastewater treatment conditions (Panter et al., 1999).

Three different mechanisms are involved in the fate of EE2 in an activated sludge system, namely conjugate cleavage, sorption and biodegradation (Panter et al., 1999; Carballa et al., 2007). The biodegradation pathways for EE2 described in literature are (1) degradation as the sole C-source (Shi et al., 2002; Haiyan et al., 2007) (2) cometabolism with E2 or its degradation product E1 (Pauwels et al., 2008) and (3) metabolism during nitrification (Vader et al., 2000; Shi et al., 2004).

Manganese (III/IV) oxides are among the strongest oxidizing agents that may be encountered in the absence of molecular oxygen (Laha and Luthy, 1990) and have been extensively studied as oxidative agents in soils and sediments. MnO_2 catalyses many chemical reactions and removal by manganese oxides has been shown for different kind of compounds, such as substituted phenols, chlorophenol, aromatic amines, aniline, chloroanilines, humic substances, atrazine, triclosan, and antibacterial N-oxides (Stone, 1987; Ulrich and Stone, 1989; Laha and Luthy, 1990; Sunda and Kieber, 1994; Pizzigallo et al., 1998; Wang et al., 1999; Zhang and Huang, 2003; Zhang and Huang, 2005). A general surface reaction mechanism involving the oxidation of organics with manganese oxides has been extensively reported (Stone, 1987; Laha and Luthy, 1990). The organic compounds are first adsorbed to the oxide surface, forming a precursor complex. Electrons are transferred within this complex, finally followed by the release of organic oxidation products and Mn^{2+} (Zhang and Huang, 2005). De Rudder et al. (2004) explored the use of manganese oxides as an oxidative removal substrate for the removal of EE2 in flow-through reactors. The MnO_2 reactor removed significantly more EE2 than expected from its adsorption capacity, probably due to its catalytic properties. More recently, EE2 removal was shown by manganese-oxidizing strains *Leptothrix* sp. and *Pseudomonas putida* (Sabirova et al., 2008). Manganese-oxidizing bacteria are a well-studied microbial group and they are used for the removal of Mn, Fe and/or recalcitrants for potable water production (Gouzinis et al., 1997; Bruns et al., 2001; Bodour et al., 2003; Raynal and Pruden, 2008). Manganese oxidation is negatively affected by the presence of ammonium (Tekerlekopoulou and Vayenas, 2008). In this study, the influence of manganese oxides on EE2 removal was examined in continuous-flow reactors. Since the reaction with MnO_2 is surface dependent, it was postulated that biological produced manganese would be more reactive because of its higher available surface (Tebo et al., 2004; Zhang and Huang, 2005). Therefore, two types of reactors, one containing mineral MnO_2 (Aqua-mandix) and the other receiving Mn^{2+} to biologically produce manganese oxides, were evaluated. The following additional points were investigated: (1) regeneration of manganese to prevent loss of the catalyst, (2) the influence of E2 on EE2 removal, and (3) the influence and mutual interaction of Mn^{2+} and NH_4^+ on EE2 elimination.

2. Experimental procedures

2.1. Reactors

Two types of semi-continuously fed lab-scale reactors were installed to study the catalytic removal of EE2 with manganese oxides, one containing mineral MnO_2 (Aqua-mandix, Aqua-Techniek, The Netherlands) and the other receiving

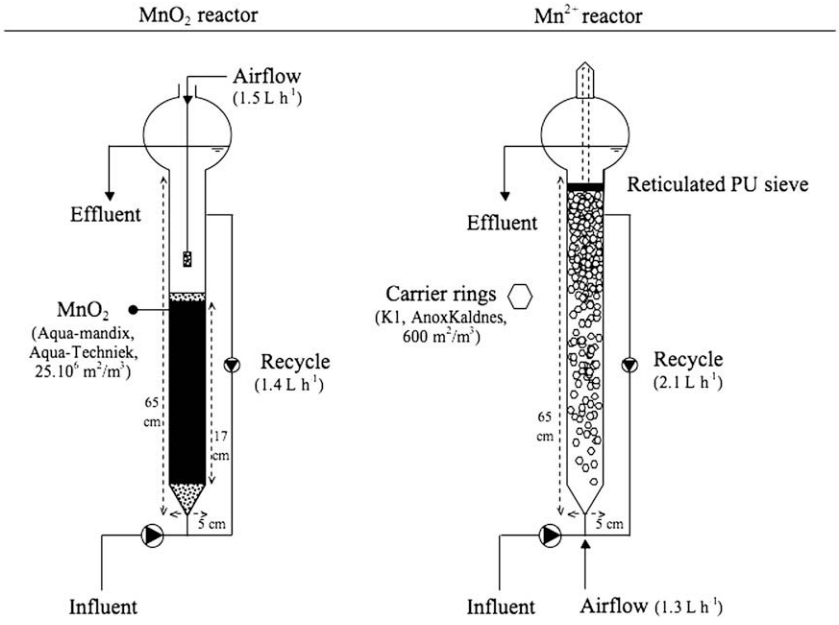

Fig. 1 – Scheme of the MnO_2 and Mn^{2+} reactors.

Mn^{2+} ($MnSO_4.H_2O$) to test the reactivity of biologically produced manganese oxides (Fig. 1). The void volumes of both reactors were 1.85 and 1.4 L, respectively. The combined removal of EE2 and E2 was tested at the same time in separate setups, one only with EE2 and the other setup with EE2 and E2, resulting in 2 MnO_2 and 2 Mn^{2+}-reactors. To retain the small MnO_2 granules, sand was used as a barrier above and below the manganese column and pumice stone (40 g) was mixed with the Aqua-mandix (300 g) to increase pore size and prevent clogging by bacterial growth. This resulted in a 17-cm long manganese column with a void volume of 250 mL. In the reactors receiving Mn^{2+}, 130 g of plastic polyethylene carrier rings (model K1, AnoxKaldnes, Sweden) were added to provide a surface of 0.6 m^2 for biofilm growth and manganese precipitation. A reticulated polyurethane (PU) sieve was included in the upper part of the reactor to keep the rings submerged. The reactors were operated in an upflow mode with a minimum upflow velocity of 1 m h^{-1} obtained by means of a recycle pump (Watson Marlow), which provided sufficient mixing and shear for biofilm growth. Periodical aeration (10 min h^{-1} at 1.5 L h^{-1}) in the MnO_2 reactors was installed at the top of the reactor and continuous aeration (1.3 L h^{-1}) was installed at the bottom of the Mn^{2+} reactors to obtain O_2 levels near saturation. Dissolved oxygen, pH and temperature were measured weekly (HACH portable multimeter HQ40d, Consort C532).

2.2. Influent

The influent was prepared in glass bottles using tap water and 5 mineral stock solutions containing macro- and micronutrients (Forrez et al., in press) in order to simulate the characteristics of a WWTP effluent. As a result, the concentrations in the influent in terms of nutrients (N and P) were 10.6 mg NH_4^+-N L^{-1} and 0.6 mg PO_4^{3-}-P L^{-1}. To investigate the influence of NH_4^+, the N-source was replaced by 10.6 mg NO_3^- L^{-1} and subsequently no N-source was added during the last period of reactor operation. Estrogens were spiked from two ethanol stocks (EE2 with and without E2) resulting in identical chemical oxygen demand (COD) concentrations in the feeding of all reactors, i.e. 0.2 g COD L^{-1}. An E2:EE2 concentration ratio of 5:1 was used, reported as optimum for the cometabolism of EE2 by estrogen degrading bacteria (Pauwels et al., 2008). From urine concentrations, the ratio natural vs. synthetic hormones in raw sewage is expected to be about 2–3, but measured ratios range from 1 up to 25 (de Mes et al., 2005). Influent was supplemented with 1% (v/v) of natural pond water as a source of bacteria for the first 100 days.

2.3. Operational conditions

Feeding was supplied to the reactors with a peristaltic pump (Watson Marlow) every hour during 3 min at different flow rates depending on the operational parameters hydraulic retention time (HRT) and loading rates for COD, N, estrogens and Mn^{2+}. Tables 1 and 2 show these parameters during the time-course of the experiments in the MnO_2 and Mn^{2+} reactors, respectively. In the saturation (SA) period, adsorption of EE2 to the PVC-tubing and Aqua-Mandix was allowed. This period was longer in the MnO_2 reactors because of the EE2 adsorption capacity of Aqua-Mandix as determined by de Rudder et al. (2004) while the K1 carrier rings did not contribute to EE2 removal (Forrez et al., in press). During an adaptation period (AD), bacterial enrichment was obtained by operating at a long HRT of 7–11 days. Subsequently, in a first phase of dynamic biodegradation testing (periods D_1–D_4), the reactors were tested for their maximum EE2 removal capacity by increasing loading rate and decreasing HRT. In a second phase, after a recovery (period *R*), focus was put on the influence of biological manganese oxidation (period *L*) and nitrification (periods N_1–N_2), respectively, by reducing the Mn^{2+} concentration in the influent and shifting from ammonium to nitrate as a N-source or no N-source at all. During this second experimental phase only the reactors running on EE2 ($MnO_{2(EE2)}$ and $Mn^{2+}_{(EE2)}$) were used to focus on the manganese and nitrogen processes. In a reactor running on EE2 and E2 ($MnO_{2(EE2+E2)}$), the EE2 removal at ng L^{-1} range and without E2 was examined during this second phase. The reactor was operated at 100 ng EE2 L^{-1} and an HRT of 1 day for 3 months prior to sampling. During the following 3 weeks, 24-h

Table 1 – Operational parameters and influent concentrations for the MnO_2 reactors ($MnO_{2(EE2)}$ and $MnO_{2(EE2+E2)}$). SA: saturation period, AD: adaptation period, D: degradation period, R: recovery period, N: period with focus on N-processes. Results are expressed as averages ± standard deviation; *n* is the number of data points used. NA: not applicable.

Code	Days	*n*	HRT (d)	$MnO_{2(EE2)}$	$MnO_{2(EE2+E2)}$	
				EE2 (μg L^{-1})	EE2 (μg L^{-1})	E2:EE2 ratio
SA_1	D 0–35	22	1	105 ± 5	119 ± 11	4.0 ± 0.5
SA_2	D 35–63	17	1	48 ± 4	61 ± 4	3.7 ± 1.2
AD	D 63–128	26	11	45 ± 6	56 ± 6	2.0 ± 1.6
D_1	D 128–155	10	7	37 ± 3	48 ± 1	1.9 ± 1.5
D_2	D 155–195	16	4	51 ± 10	59 ± 6	3.3 ± 1.4
D_3	D 195–202	5	2	43 ± 2	51 ± 3	3.2 ± 0.5
D_4	D 202–250	26	1	40 ± 4	50 ± 5	2.7 ± 1.5
R	D 0–43	18	1	37 ± 5	NA	NA
N_1	D 43–147	35	1	40 ± 7	NA	NA
N_2	D 147–175	10	1	42 ± 6	NA	NA

Table 2 – Operational parameters and influent concentrations for the Mn^{2+} reactors ($Mn^{2+}_{(EE2)}$ and $Mn^{2+}_{(EE2+E2)}$). SA: saturation period, AD: adaptation period, D: degradation period, R: recovery period, L: period with lower Mn^{2+} levels, N: period with focus on N-processes. Results are expressed as averages ± standard deviation; n is the number of data points used. If the latter is not applicable, the number is reported between brackets.

Code	Days	n	HRT (d)	$Mn^{2+}_{(EE2)}$		$Mn^{2+}_{(EE2+E2)}$		
				EE2 (μg L^{-1})	Mn^{2+} (mg L^{-1})	EE2 (μg L^{-1})	E2:EE2 ratio	Mn^{2+} (mg L^{-1})
SA	D 0–18	6	1	107 ± 11	57 ± 6 (3)	113 ± 9	3.0 ± 1.3	60 ± 1 (3)
AD	D 18–83	30	7	97 ± 15	55 ± 6	117 ± 16	3.4 ± 0.9	55 ± 7
D_1	D 83–110	16	4	81 ± 3	59 ± 3 (10)	103 ± 3	3.0 ± 0.8	58 ± 4 (10)
D_2	D 110–150	16	4	46 ± 6	73 ± 9	61 ± 7	3.8 ± 0.7	75 ± 7
D_3	D 150–157	5	2	42 ± 1	74 ± 5	54 ± 1	4.3 ± 0.2	74 ± 5
D_4	D 157–217	26	1	45 ± 4	73 ± 8 (16)	55 ± 4	3.8 ± 1.5	74 ± 7 (18)
R	D 217–265	13	2	39 ± 2	58 ± 14	57 ± 2	4.4 ± 0.5	55 ± 5
L	D 265–312	9	2	42 ± 4	5.4 ± 0.5 (6)	52 ± 15	5.2 ± 2.3	5.4 ± 0.5 (6)
N_1	D 312–371	13	2	45 ± 5	5.0 ± 0.3	44 ± 4	4.8 ± 1.3	5.0 ± 0.3
N_2	D 371–395	6	2	41 ± 7	5.1 ± 0.3	45 ± 2	4.8 ± 0.3	5.1 ± 0.2

composite samples of 500 mL were taken weekly and analyzed using Gas Chromatography coupled to multiple Mass Spectrometry (GC–MS–MS).

2.4. Batch tests

A batch test with the effluent of reactor $MnO_{2(EE2)}$ was performed to investigate the presence of ammonia-oxidizing bacteria (AOB) and their capability to remove EE2. 500 mL of effluent from period D_4 were placed in 1-L glass Erlenmeyers and spiked with 100 μg EE2 L^{-1} from an ethanol stock. Ammonium was added in all treatments at 10.6 mg N L^{-1}. To inhibit nitrification, 5 mg L^{-1} allylthiourea (ATU) was dosed (Ginestet et al., 1998) and 1-min boiled effluent served as a control.

Similarly, the effluent was examined for the removal of EE2 with and without nitrification and in the presence of Mn^{2+}. 500 mL of effluent from period N_1 (no ammonium in the feed) was put in 1-L glass Erlenmeyers and spiked with 80 μg EE2 L^{-1}. Different assays, with and without 5 mg Mn^{2+} L^{-1} and with and without 10.6 mg NH_4^+-N L^{-1} were performed and 1-min boiled effluent served as controls. The test was repeated with effluent from period N_2 (no N-source in the feed).

To get a view on the interference of nitrification and manganese oxidation, removal of EE2 with a monoculture of *Ps. putida* MnB6 (LMG 2322), which is a manganese oxidizer, and with a mixed culture of *Ps. putida* and a nitrifying enrichment culture (ABIL, Avecom nv, Belgium) was set up. The *Ps. putida* was cultivated on a medium described by Boogerd and de Vrind (1987) and 2 mL of an overnight preculture with 5 mg Mn^{2+} L^{-1} was used as inoculum. ABIL is an aqueous suspension containing a highly active, nitrifying microbial consortium, commercially available for the start-up of biofilters in aquaria systems (Grommen et al., 2002). An inoculum of 0.1 mL ABIL was applied to mimic the presence of nitrifiers in the effluent of the reactors. *Ps. putida*, with and without ABIL, was added to 500 mL of tap water spiked with 100 μg EE2 L^{-1} and 5 mg Mn^{2+} L^{-1} and interference of ammonium on manganese oxidation was tested by adding 10.6 mg N L^{-1}. 5-min boiled treatments served as controls.

All batch tests were incubated at 28 °C on a shaker (Platform shaker, 120 rpm, Innova 2300, New Brunswick Scientific, Belgium) so passive aeration occurs by agitation. Samples were taken every 2 or 3 days for nitrate/nitrite, Mn^{2+} and EE2 determination.

2.5. Analytical methods

Samples preparation and HPLC analysis for E1, E2 and EE2 determination were performed as previously described by Forrez et al. (in press). Determination of EE2 at ng L^{-1} concentrations with GC–MS–MS was performed according to Noppe et al. (2005). Influent samples were diluted 5 times and the clean-up step was omitted.

Ammonium was determined colorimetrically with Nessler reagent according to standard methods (Greenberg et al., 1992). Both nitrite and nitrate were determined using a Metrohm 761 Compact Ion Chromatograph equipped with a conductivity detector. The operational parameters were as follows: column metrosep A supp 5; eluent 1.06 g Na_2CO_3 L^{-1}; flow 0.7 mL min^{-1}; sample loop 20 μL.

Soluble manganese was determined colorimetrically with the formaldoxime method (Bartley et al., 1957). Briefly, samples of 3 mL were rapidly mixed with 1 mL of freshly made alkalic formaldoxime hydrochloride solution (Sigma–Aldrich, 0.5 g L^{-1} in 10 mL of 10 N NaOH). After 10 min reaction time, the absorbance was measured at 450 nm against a similarly treated blank and the Mn^{2+} concentration was calculated from a standard calibration curve between 0.5 and 5 mg Mn^{2+} L^{-1}. A solution of 2 mg Mn^{2+} L^{-1} was used as a reference. In addition, manganese in the effluent of the reactors was routinely measured with the Aquaquant analysis system (Merck, Germany).

3. Results

The pH was stable in all reactors during the whole experimental period with average values of 7.2 ± 0.3 and 6.9 ± 0.3 for

the MnO_2 reactors, fed with EE2 and EE2 + E2, respectively, and 7.8 ± 0.3 and 7.9 ± 0.3 for the reactors receiving Mn^{2+}. Dissolved oxygen in the reactors containing mineral MnO_2 was around 6.1 ± 1.0 and 5.5 ± 1.0 mg O_2 L^{-1} for $MnO_{2(EE2)}$ and $MnO_{2(EE2+E2)}$, respectively. For the reactors receiving Mn^{2+}, oxygen levels were near saturation except during an aeration failure in period D_2 for the $Mn^{2+}_{(EE2)}$-reactor. The temperature for the reactors containing MnO_2 was around 32 ± 2 °C and 26 ± 2 °C for the reactors receiving Mn^{2+}, which is in the optimal range for bacterial processes. During period D_2, a short unexpected rise in room temperature up to 39 °C was observed for the MnO_2 reactors, which negatively affected both the EE2 removal and manganese oxidation.

Sorption of EE2 occurred in all reactors during the first weeks, which was reflected in an EE2 removal efficiency of around 30%. Using the adsorption isotherms obtained by de Rudder et al. (2004), 2.35 mg EE2 was calculated to adsorb on 300 g mineral MnO_2 (Aqua-mandix) with 43 μg EE2 L^{-1} as the equilibrium concentration. Considering the total amount adsorbed in the first saturation period SA_1 (days 0–35), which was 2.13 mg EE2, saturation of the MnO_2 was expected. This was reflected in the EE2 removal decrease to almost zero in the subsequent period SA_2 (data not shown).

During the saturation period (SA_1 and SA_2), considerable amounts of reduced manganese (40 mg Mn^{2+} L^{-1} and 20 mg Mn^{2+} L^{-1}, respectively) were released in the effluent of the MnO_2 reactors. In the Mn^{2+} reactors, manganese oxidation was not observed during the start-up period. In addition, the Mn^{2+} in the effluent was sensitive to changes especially when stress (temperature, aeration or increased loading rate) was exerted. Average values for Mn^{2+} concentrations in the effluent during the subsequent experimental periods are given in Table 3.

During the adaptation period AD (days 63–128) in the mineral MnO_2 reactor, E2 supplementation resulted in a faster response with respect to the EE2 removal than its counterpart, which received only EE2 (Fig. 2). The stimulatory effect of E2 was corroborated by the observations during days 170–200 (period D_2) when EE2 removal decreased from 93 to 53% due to temperature stress. The recovery of the MnO_2 reactor supplemented with E2 was about 2 times faster than its counterpart (slopes of EE2 removal efficiency were 0.46 (R^2: 0.40) for reactor $MnO_{2(EE2)}$ and 1.00 (R^2: 0.63) for reactor $MnO_{2(EE2+E2)}$ from days 177–195). The natural hormone E2 itself was removed below detection limit (3 μg L^{-1}) in both reactors ($MnO_{2(EE2+E2)}$ and $Mn^{2+}_{(EE2+E2)}$) after 20 days of operation. Estrone (E1), a degradation product of E2, was only detected occasionally in the influent during storage at 4 °C.

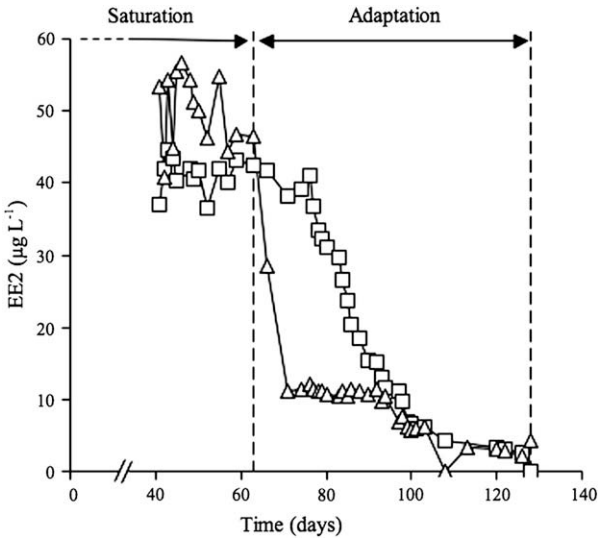

Fig. 2 – EE2 concentrations in effluent of reactor $MnO_{2(EE2)}$ (□) and $MnO_{2(EE2+E2)}$ (△) during the start-up period.

When comparing the reactor receiving Mn^{2+} with the mineral MnO_2 reactor in the first phase of dynamic biodegradation testing, the reactor with biogenic manganese oxide performed better than the mineral MnO_2 reactor at 21 μg EE2 L^{-1} d^{-1} (Table 3, period D_3). But it became evident that the removal of EE2 was consistently lower in the one receiving Mn^{2+} from period D_4 on, i.e. at the highest loading rate. Increase of the EE2 loading rate to 40 μg L^{-1} d^{-1} negatively affected the reactor receiving Mn^{2+} while the reactor containing the mineral MnO_2 was improving up to 75% removal. Recovery of the EE2 removal in the Mn^{2+} reactors could not be achieved by lowering the loading rate

Table 3 – EE2 removal efficiency and Mn^{2+} leakage in the effluent during the different operational periods for the 2 types of manganese reactors (MnO_2 and Mn^{2+}) fed with EE2. NA: not applicable. Arrows + value indicate a trend and the result at the end of the period.

Code		HRT (d)		$B_{V,EE2}$ (μg L^{-1} d^{-1})		EE2 removal (%)		Mn^{2+} (mg L^{-1})	
MnO_2	Mn^{2+}	MnO_2	Mn^{2+}	MnO_2	Mn^{2+}	MnO_2	Mn^{2+}	MnO_2	Mn^{2+}
D_1	AD	7	7	5 ± 1	14 ± 2	93 ± 4	95 ± 3	1.5 ± 0.5	↓6
D_2	D_2	4	4	13 ± 3	12 ± 1	53±5[a]	80±3[b]	↑5[a]	50 ± 6[b]
D_3	D_3	2	2	21 ± 1	21 ± 1	60 ± 6	83 ± 2	8 ± 1	27 ± 4
D_4	D_4	1	1	40 ± 4	45 ± 4	75 ± 2	57 ± 5	3 ± 1	43 ± 5
R	R	1	2	37 ± 5	20 ± 1	68 ± 6	61 ± 3	1.1 ± 0.7	26 ± 4
L	L	1	2	NA	21 ± 2	NA	67 ± 6	NA	4 ± 1
N_1	N_1	1	2	40 ± 7	23 ± 2	85 ± 7	57 ± 9	0.05 ± 0.06	0.9 ± 0.5
N_2	N_2	1	2	42 ± 6	20 ± 3	74 ± 6	68 ± 8	0.03 ± 0.01	1.9 ± 0.6

a T-stress.
b Aeration failure.

in the subsequent periods. When the influent concentration of Mn^{2+} was lowered with a factor 10 (period *L*, Table 2), no effect was seen on the EE2 removal in these reactors (Table 3). EE2 removal efficiency at ng L^{-1} concentrations was similar, i.e. 84 ± 21% ($n = 3$), with influent and effluent concentrations of 115 ± 48 and 12 ± 11 ng EE2 L^{-1} ($n = 3$), respectively. Mn^{2+} concentrations were below LOD, i.e. 0.03 mg L^{-1}.

The oxidation of the ammonium present in the influent, initially not taken into account, was addressed in the last stage of operation of the reactors (periods N_1 and N_2). The influent of all reactors contained 10.6 mg NH_4^+-N L^{-1} and it was observed that this ammonium was converted into NO_3^-. However, in the reactor receiving both Mn^{2+} and NH_4^+, the nitrification was partly inhibited, giving rise to residual NH_4^+ levels of 1–3 mg N L^{-1} in the effluent (data not shown). When the effluent of the reactor containing MnO_2 was tested in a separate batch test, it was found that NH_4^+ was converted to NO_3^- and EE2 was removed simultaneously. Control batch tests with inhibition of AOB by ATU or by boiling did not show nitrification or removal of EE2 (data not shown). To examine if NH_4^+ was essential as a substrate, the reactors were run without NH_4^+ for a period of 4 months and it was observed that the AOB survived. The latter was evidenced by a series of additional batch tests. One batch test with and without ammonium was performed after 1 month (Fig. 3) and these results were confirmed after 4 months. In both tests, nitrification and simultaneous removal of EE2 occurred. Remarkably, EE2 removal in the MnO_2 reactors even improved up to 85% in the absence of NH_4^+ in the influent. From Fig. 3 it appears that removal of EE2 without addition of ammonium is enhanced compared to the test with ammonium. After 5 days, 75% and 50% of the EE2 was removed in the absence and presence of nitrification, respectively. Addition of Mn^{2+} did not improve the removal rate of EE2.

Additional tests with tap water and a mixture of a known manganese oxidizer and a known nitrifying consortium were performed (Fig. 4). These tests clearly showed that Mn^{2+} oxidation by the *Ps. putida* promote EE2 removal. However, the additional supplementation of nitrifiers increased the EE2 removal considerably, even without ammonium addition.

4. Discussion

Aqua-mandix and manganese dioxide in general are able to adsorb EE2 (Bernard et al., 1997) and based on the observations of de Rudder et al. (2004), it could be calculated that EE2 was removed beyond the sorption capacity of the MnO_2 (Aqua-mandix). Sorption onto the manganese oxide makes EE2 available for chemical oxidation, since the surface complex formation is considered to be the rate-limiting step (Zhang and Huang, 2005). In contrast, EE2 in the solid phase is less available for biological removal.

In the saturation period SA_2, EE2 and COD concentrations in the influent were decreased to half, which is reflected in the reductive dissolution of manganese dioxide. The high Mn^{2+} concentrations could be explained by the lack of a mature biofilm with manganese-oxidizing bacteria in the reactors. From the adaptation period (AD) on, manganese oxidation occurred in all reactors and it was possible to obtain manganese effluent concentrations below drinking water guidelines (<0.4 mg L^{-1}) (WHO, 2006). However, ammonium interfered with the manganese oxidation, both in the batch tests as in the reactors, especially those receiving Mn^{2+}. The inhibitory effect of NH_4^+ on manganese oxidation has been reported by Vandenabeele et al. (1995) with a 50% inhibition when NH_4^+ exceeded 0.7 mg N L^{-1}.

Initially, the results seemed to be in accordance with the hypothesis that the biologically produced manganese oxides performed better in terms of EE2 removal than the mineral MnO_2 because of its higher available surface for the complex formation between EE2 and the surface-bound $Mn^{(IV)}$ (Tebo et al., 2004; Zhang and Huang, 2005). At loading rates (B_V) up to 20 μg EE2 L^{-1} d^{-1}, the reactors with biologically produced manganese oxides showed a better removal than those with mineral MnO_2 (83 and 60%, respectively). Further increase in the B_V up to 40 μg EE2 L^{-1} d^{-1} resulted in an increase of Mn^{2+} in the effluent of the reactor with biologically produced manganese and a significant decrease of the EE2 removal rate. In the

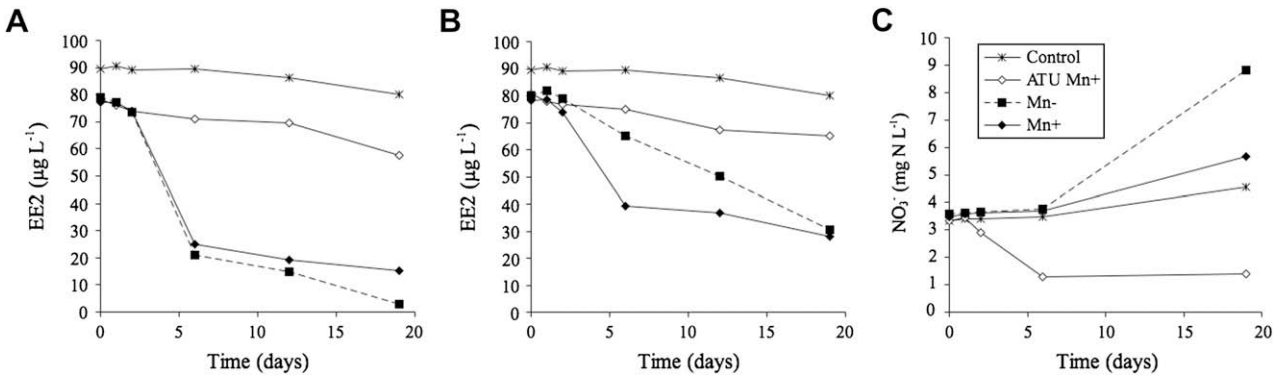

Fig. 3 – Removal of EE2 and nitrification in the batch test with the effluent from the reactor containing MnO_2 (period N_1). Boiled effluent (1 min) served as a control. ATU (allylthiourea) was added to inhibit the nitrification enzymes. Mn−: 0.03 mg Mn^{2+} L^{-1} and Mn+: 5 mg Mn^{2+} L^{-1}. (A): without ammonium addition; (B): with 10.6 mg NH_4^+-N L^{-1}; (C): nitrate concentrations during the test with 10.6 mg NH_4^+-N L^{-1} addition. In the latter test, the residual levels of NH_4^+ for the control, ATU Mn+, Mn− and Mn + were 3.7, 3.0, 0.3 and 0.7, respectively.

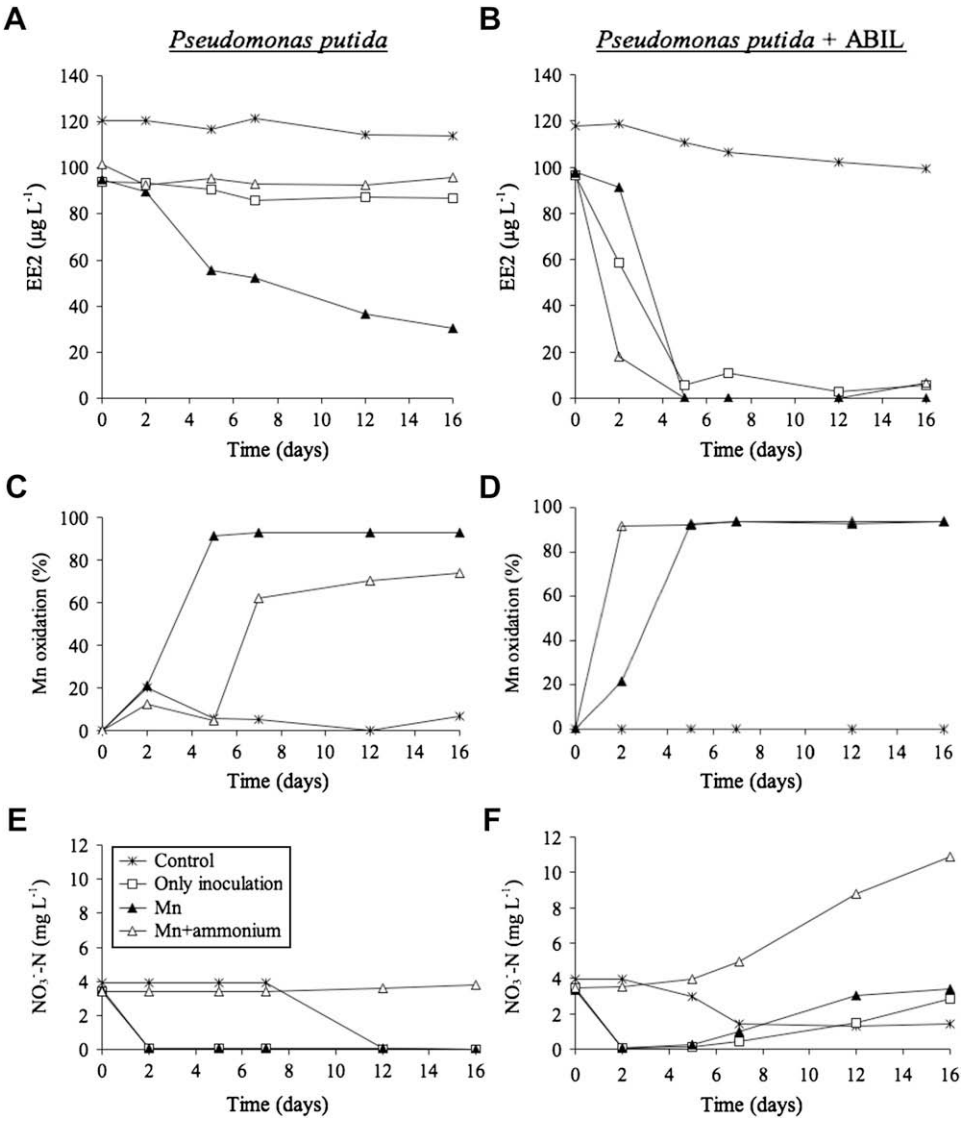

Fig. 4 – Removal of EE2 upon inoculation with a monoculture of *Pseudomonas putida* with (B, D, F) and without (A, C, E) the nitrifying consortium ABIL. Controls were boiled for 5 min. Mn^{2+} and NH_4^+-N were dosed at 5 mg L^{-1} and 10.6 mg L^{-1}, respectively.

subsequent period at 20 μg EE2 L^{-1} d^{-1}, Mn^{2+} oxidation was restored, but recovery of the reactor in terms of EE2 removal was not obtained, indicating that the maximum capacity was reached. However, the mineral MnO_2 reactor showed more stability and EE2 removal remained over a period of several months at a B_V of 40 μg EE2 L^{-1} d^{-1}.

It was hypothesized that EE2 is susceptible to manganese oxide-facilitated oxidation, generating Mn^{2+} and oxidized EE2 intermediates (de Rudder et al., 2004). These more readily biodegradable intermediates formed during the chemical oxidation with MnO_2 are postulated to be immediately removed by the manganese-oxidizing bacteria or other microorganisms present in the reactor, since no additional peaks in the HPLC-chromatograms could be detected. Sabirova et al. (2008) observed degradation of EE2 with pure cultures of manganese oxidizers and hypothesized it to be mainly a chemical process by the biologically formed manganese oxides. Hwang et al. (2008) elucidated a reaction product of the chemical oxidation of EE2 with Mn^{3+}. The tests performed with *Ps. putida* confirmed the involvement of manganese-oxidizing bacteria and pointed out the interference of ammonium on the manganese oxidation (Fig. 4A). It is well known that the presence of NH_4^+ hinders the biological oxidation of Mn^{2+} (Vandenabeele et al., 1995; Gouzinis et al., 1998; Tekerlekopoulou and Vayenas, 2008). EE2 removal was even completely inhibited when ammonium was added, despite the formation of manganese oxides. Positively charged ions like Mn^{2+} and NH_4^+ can adsorb onto the negatively charged MnO_2 surface when pH is higher than the zero-point of charge (around 4 for MnO_2), blocking the active sites for oxidation. As seen from Fig. 4C and E, only 78% of the Mn^{2+} was oxidized resulting in a residual concentration of 1.1 mg L^{-1} and no NH_4^+ was nitrified. Overall, it can be concluded that chemical oxidation of EE2 by means of

manganese oxides is not likely to happen in the biogenic reactor receiving Mn^{2+}, because of the inhibition caused by the residual concentrations of Mn^{2+} and NH_4^+. In the mineral MnO_2 reactor, nitrification and manganese oxidation were higher resulting in a lower inhibition.

Bacteria degrading the natural hormone E2 are able to cometabolize EE2. During the removal of estrone (E1), formed from the degradation of E2, simultaneous EE2 removal was observed (Pauwels et al., 2008). Although a stimulatory effect was noticed in the MnO_2 reactors, no improved removal was obtained on a long-term basis. It remains to be further investigated if this stimulatory effect is due to the induction of specific enzymes or because the presence of E2 supports the growth of estrogen degrading bacteria.

The activity of nitrifying bacteria has been reported to contribute to EE2 removal. Yi and Harper. (2007) showed that ammonia monooxygenase (AMO), which is the enzyme that catalyses the first step in nitrification, plays a role in the degradation of EE2. Forrez et al. (in press) reported 84% removal in an upflow aerated nitrifying reactor at 19 μg EE2 L^{-1} d^{-1} with NH_4^+ and 74% with NO_3^- as the nitrogen source. The MnO_2 reactor continued to remove EE2 with 85% when no NH_4^+ was added and 74% in the subsequent period without any N-source. Furthermore, after a 4-month operational period at 100–150 ng EE2 L^{-1} and 10.6 mg NH_4^+ L^{-1}, removal efficiencies remained at 84%, indicating the applicability of the process at environmental relevant concentrations in WWTP effluent.

Nitrifiers are known to be able to survive in the absence of their substrate. The normal decay rate for ammonium oxidizers in activated sludge is around 0.2 d^{-1}, resulting in a drop in the nitrification activity of 90% in 14 days (Salem et al., 2006). However, lower decay rates (0.02 d^{-1}) were observed for enriched cultures of nitrifiers. When grown in substrate-poor conditions, *Nitrosomonas europaea* required five times lower maintenance energy (Tappe et al., 1999). In this research, both conditions are fulfilled. During the long-term adaptation at oligotrophic conditions during period AD, nitrifiers originating from surface water were enriched at low NH_4^+ concentrations, which could result in an AOB community with low maintenance requirements and low decay rates. This could explain why AOB were maintained in the biofilm and were still active even after 4 months without supply of NH_4^+. In this context, Wilhelm et al. (1998) concluded that the activities of the energy-generating enzymes in *N. europaea* were not affected during long-term ammonia starvation.

The interesting outcome of our work was that even in the absence of their dedicated substrate, nitrifiers continued to remove EE2. In addition, when NH_4^+ was no longer dosed to the reactor receiving Mn^{2+}, it was noticed that the oxidation of manganese improved. However, despite the higher manganese oxidation, the EE2 removal did not increase indicating that the nitrifiers and not the manganese oxidizers were of prime importance in the EE2 removal. The latter is supported by the batch tests performed with *Ps. putida* and a nitrifying consortium (Fig. 4B). By comparing Fig. 4B with Fig. 4A (only inoculation, no addition of Mn^{2+} or NH_4^+), it can be concluded that nitrifiers are responsible for the fast EE2 removal. Although there were no significant effects, a slight difference can be seen after 2 days between "only inoculation" and "Mn" or "Mn + NH_4^+". When Mn^{2+} was added, EE2 removal was slightly inhibited and when Mn^{2+} and NH_4^+ were added, EE2 removal was enhanced. The latter suggests that the stimulating effect of ammonium on the nitrifiers is stronger than the inhibitory effect of Mn^{2+}. Yet, this inhibitory effect is less important in this treatment, because rapid manganese oxidation by *Ps. putida* occurs. These batch tests also demonstrated that the manganese-oxidizing bacteria can be used for the removal of EE2, if ammonium and nitrifiers are not present. It remains to be examined to what extent this process can bring about a different type of technology for EE2 removal.

5. Conclusions

- Ammonia-oxidizing bacteria (AOB) are of prime importance in the degradation of EE2 because selective inhibition of the latter resulted in the total abolishment of EE2 removal. Remarkably, once established in the reactor, AOB continued to exist without receiving NH_4^+ for a period of more than 4 months and continued to remove EE2.
- The capacity of manganese-oxidizing bacteria to contribute to EE2 removal was clearly demonstrated in the absence of nitrifiers.
- In effluents containing both Mn^{2+} and EE2, the removal of the latter by nitrifiers can be compromised because Mn^{2+} interferes with nitrification.
- Removal of EE2 continued at relevant WWTP effluent concentrations (100 ng L^{-1}) with removal efficiencies similar to those obtained in the μg L^{-1} range (75–85%).
- For practical purposes, a post-treatment nitrifying reactor supplemented with a few mg L^{-1} of ammonium offers the best window for biological removal of EE2.

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