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RESEARCH ARTICLE

Influence of pH, EDTA/Fe(II) ratio, and microbial culture on Fe(II)-mediated autotrophic denitrification

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Abstract Fe(II)-mediated autotrophic denitrification with four different microbial cultures under different pH and EDTA/Fe(II) conditions was investigated in batch bioassays. Initially, the highest nitrate removal (72%) was achieved with an activated sludge inoculum. The use of pure cultures of *Pseudogulbenkiania* strain 2002 and *Thiobacillus denitrificans* resulted in a 55 and 52% nitrate removal, respectively. No denitrification was observed for a mixed culture dominated by *Thiobacillus thioparus* and *T. denitrificans*. A longer enrichment on Fe(II) and the supplementation of thiosulfate as additional electron donor were needed to stimulate the denitrifying activity of the *Thiobacillus*-mixed culture. A second subculture on Fe(II) as sole electron donor resulted in higher denitrification efficiencies for all microbial cultures. In particular, nitrate removal reached up to 84% with a specific nitrate removal rate of 1.160 mM·(g VSS·day)$^{-1}$ in the bioassays seeded with the *Thiobacillus*-mixed culture. All cultures were favored by decreasing the EDTA/Fe(II) molar ratio from 2.0 to 0.5. The most significant denitrification enhancement was observed for the *Pseudogulbenkiania* species, indicating a lower tolerance to EDTA. The two pure cultures effectively maintained denitrification at pH 7.0 and were more sensitive to a pH decrease. Conversely, the optimal pH was 6.0 for the *Thiobacillus*-mixed and activated sludge cultures.

Keywords Ferrous iron · Nitrate · Autotrophic denitrification · EDTA · *Thiobacillus* · *Pseudogulbenkiania*

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Introduction

Nitrate is one of the most common pollutants in water resources worldwide (Park and Yoo 2009; Zhang et al. 2015). Nitrate contamination is mainly caused by the use of agricultural fertilizers (Viers et al. 2012; Qambrani et al. 2013) and the uncontrolled discharge of industrial and domestic wastewaters (Zhang et al. 2015; Bhandari et al. 2016). Moreover, nitrate is often associated with mining activity due to use of large amounts of explosives such as "ammonium nitrate fuel oil" (ANFO) (Zaitsev et al. 2008). In mining environments, nitrate co-occurs with several metal species, including iron (Papirio et al. 2014).

Nitrate is mainly removed from wastewaters by heterotrophic denitrification (Ashok and Hait 2015). Denitrification can also be used for the treatment of metal-containing wastewaters (Zou et al. 2014, 2015). Fe(II) has been demonstrated to stimulate heterotrophic denitrification (Papirio et al. 2014) and is also an effective electron donor for autotrophic denitrification (Straub et al. 1996). The first microorganisms capable of maintaining biological nitrate-dependent Fe(II) oxidation were discovered only 20 years ago (Straub et al. 1996). The use of denitrifying Fe(II) oxidizers results in the reduction of nitrate to nitrogen gas and the bioprecipitation/biorecovery of

Fe(III) (hydr)oxides, with the possible co-precipitation or adsorption of other metals (Hohmann et al. 2009; Ahoranta et al. 2016). The complete reduction of nitrate to dinitrogen gas with Fe(II) as electron donor is as suggested by Sorensen (1987):

$$10Fe^{2+}(aq) + 2NO_3^-(aq) + 24\,H_2O \rightarrow N_2(g) + 10Fe(OH)_3(s) + 18H^+(aq) \quad (1)$$

Fe(II)-mediated autotrophic denitrification is advantageous over classical heterotrophic denitrification for the treatment of low-organic wastewaters. The addition of simple organic compounds would increase the operational costs and induce secondary organic pollution (Zhang et al. 2015). Nevertheless, the use of waste activated sludge fermentation liquid as carbon source can enhance the denitrification efficiency, by keeping the treatment costs low as no organic substrates are supplemented (Ji and Chen 2010). Moreover, the anaerobic methane oxidation coupled to denitrification can be considered as an alternative for the treatment of organic-deficient wastewaters (Wang et al. 2017).

Nitrate-dependent Fe(II) oxidation has been reported in both mixed enrichments and pure cultures isolated from various habitats (Weber et al. 2006a; Kiskira et al. 2017). A circumneutral feed pH and a molar Fe(II)/NO_3^- ratio of 5 have mostly been used in the existing literature (Straub et al. 1996; Weber et al. 2006b; Blöthe and Roden 2009). None of the known Fe(II)-oxidizing denitrifiers has been reported to be acidophilic, and the knowledge of their possible acclimation to acidic environments still remains limited.

Thiobacillus denitrificans and *Pseudogulbenkiania* strain 2002 are microorganisms capable of performing Fe(II)-driven autotrophic denitrification. The two microbial species are both located in the subclass of the *Proteobacteria* and are reported to grow as anaerobic chemolithotrophs (Kelly and Wood 2000; Weber et al. 2006b). *T. denitrificans* can be found in soil, mud, freshwater, marine sediments, and also in domestic sewage and industrial wastewater treatment ponds. It has widely been reported to use reduced sulfur compounds (e.g., thiosulfate, elemental sulfur, and sulfide) as electron donors, but contradictory results have been obtained for the capability of *T. denitrificans* to use Fe(II) (Straub et al. 1996; Muehe et al. 2009). *Pseudogulbenkiania* strain 2002 has been observed in freshwater and paddy soils used for the cultivation of rice and soybean (Tago et al. 2011). Unlike *T. denitrificans*, *Pseudogulbenkiania* strain 2002 is a strictly nitrate-dependent Fe(II)-oxidizing microorganism (Weber et al. 2006b, 2009).

As Fe(II) is not stable at circumneutral pH, ethylenediaminetetraacetic acid (EDTA) is commonly employed as chelating agent in order to promote a higher Fe(II) solubilization. An attentive supplementation of EDTA is required, as EDTA is an organic pollutant which is persistent in the environment (Oviedo and Rodríguez 2003). Moreover, EDTA often leads to the inhibition of the activity of several denitrifying Fe(II)-oxidizing species (Kumaraswamy et al. 2006; Kanaparthi et al. 2013; Klueglein et al. 2015). Further research is therefore needed in order to evaluate the most suitable EDTA/Fe(II) ratio that does not result in microbial inhibition, while enhancing Fe(II) solubility. For instance, EDTA can be recycled and reused, mitigating the organic pollution and lowering the operational costs (Juang and Wang 2000).

The objectives of this work were (1) to investigate the efficiency of Fe(II)-mediated autotrophic denitrification in terms of Fe(II) oxidation and nitrate removal with different microbial cultures in batch bioassays; (2) to evaluate the effects of decreasing pH on the process; and (3) to determine the optimal EDTA/Fe(II) ratio.

Material and methods

Sources of microorganisms and cultivation mineral media

The chemolithotrophic denitrifying cultures used in this study were as follows: (1) a *Thiobacillus*-dominated mixed culture previously enriched on thiosulfate and nitrate (Di Capua et al. 2016; Zou et al. 2016); (2) an activated sludge inoculum collected from the municipal wastewater plant in Cassino (Italy); (3) a pure culture of *Pseudogulbenkiania* strain 2002 (DSM 18807); (4) a pure culture of *T. denitrificans* (DSM 12475). Both pure cultures were purchased from the "Leibniz-Institute DSMZ-German collection of microorganisms and cell cultures" in Braunschweig (Germany).

The two denitrifying mixed cultures were enriched under anaerobic conditions for 1 month in batch mode in 125 mL serum flasks. Fe(II) and NO_3^- concentrations were 10 and 2 mM, respectively. The basal medium was prepared with the following components ($g \cdot L^{-1}$): 2.00 $NaHCO_3$, 0.25 NH_4Cl, 0.30 KH_2PO_4, 0.40 $K_2H_2PO_4$, and 0.10 NaCl. The trace mineral solution was added from a sterile stock solution and prepared by dissolving the following in a 1.5 $g \cdot L^{-1}$ nitrilotriacetic acid disodium salt solution ($g \cdot L^{-1}$): 3.00 $MgSO_4 \cdot 7H_2O$, 0.50 $MnSO_4$, 1.00 NaCl, 0.10 $FeSO_4 \cdot 7H_2O$, 0.10 $CaCl_2 \cdot 2H_2O$, 0.10 $CoCl_2 \cdot 6H_2O$, 0.13 ZnCl, 0.01 $CuSO_4 \cdot 5H_2O$, 0.01 $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 H_3BO_3, 0.025 $Na_2MoO_4 \cdot 2H_2O$ (Weber et al. 2009).

The two pure cultures were preliminary cultivated by using two different mineral media. The mineral medium used for the *Pseudogulbenkiania* strain 2002 culture contained the following components ($g \cdot L^{-1}$): 5.0 peptone, 2.0 meat extract, and 15.0 agar. pH was adjusted to 7.0. The medium was sterilized by autoclaving at 121 °C for 15 min. The medium used for the activation of the *T. denitrificans* culture consisted of four different solutions, as reported by Zou et al. (2016). Fe(II) and

NO_3^- concentrations were the same of those used for the cultivation of *Thiobacillus*-mixed and activated sludge cultures.

All the incubations were maintained in absence of light at 22 ± 2 °C on a gyratory shaker (80 rpm). After the enrichment, all the microbial cultures were seeded in the serum bottles used for the batch experiments.

Preparation of the experiments

Fe(II)-mediated autotrophic denitrification was studied in batch bioassays by using 125 mL serum bottles. All the bottles were maintained at room temperature (22 ± 2 °C). Each bottle contained the basal medium and trace elements, as described in "Sources of microorganism and cultivation mineral media" section. Fe(II) and NO_3^- were added in concentration of 10 and 2 mM, respectively. Thiosulfate ($S_2O_3^{2-}$) in concentration of 0.5 mM was used as an additional electron donor in the experiments performed with the *Thiobacillus*-mixed and pure *T. denitrificans* cultures. Fe(II), NO_3^-, and $S_2O_3^{2-}$ were added in the form of iron(II) chloride ($FeCl_2{\cdot}4H_2O$), sodium nitrate ($NaNO_3$), and sodium thiosulfate (NaS_2O_3), respectively. EDTA in molar ratios of 2:1, 1:1, and 0.5:1 with Fe(II) was used as chelating agent. All the chemicals were of analytical grade (Sigma Aldrich, Germany).

The feed pH (7.0, 6.0, and 5.0) was adjusted by adding NaOH and HCl before flushing the bottles with He in order to maintain anoxic conditions. The dissolved oxygen (DO) was below 0.3 $mg{\cdot}L^{-1}$. Bicarbonate (2 $g{\cdot}L^{-1}$ as $NaHCO_3$) was added to each bottle as buffer and inorganic carbon source. The enrichment cultures were added to the bottles in the amount of 10% *v/v*. This resulted in an initial volatile suspended solids (VSS) concentration of 180, 300, 380, and 720 mg $VSS{\cdot}L^{-1}$ in the bottles inoculated with *Thiobacillus*-mixed, pure *T. denitrificans*, activated sludge, and *Pseudogulbenkiania* strain 2002 cultures, respectively. Finally, the bottles were sealed with butyl rubber stoppers and aluminum crimps and placed on a gyratory shaker at 250 rpm. Microcosms were prepared in duplicate. For each microbial culture, controls without electron donors were carried out to monitor the degradation of NO_3^-, which was not associated with chemolithotrophic denitrification. Abiotic controls were also performed for possible chemical reactions between Fe(II), NO_3^-, and/or $S_2O_3^{2-}$.

Batch bioassays

The batch experiments were conducted as described in Table 1. The four inocula were individually investigated in each experiment. In experiment 1, Fe(II) was used as sole electron donor for chemolithotrophic denitrification using all the four cultures at pH 7.0 and a EDTA/Fe(II) ratio of 2.0. In experiment 2, the potential of thiosulfate as additional electron donor was investigated in the bioassays seeded with the *Thiobacillus*-mixed and pure *T. denitrificans* cultures under the same operating conditions of experiment 1. Thiosulfate was added in concentration of 0.5 mM, lower than the theoretical amount indicated by the molar $NO_3^-/S_2O_3^{2-}$ ratio of 1.6:1.0 (Manconi et al. 2007).

In experiment 3, the *Thiobacillus*-mixed and pure *T. denitrificans* cultures cultivated on Fe(II) and thiosulfate and the activated sludge and *Pseudogulbenkiania* strain 2002 cultures cultivated on the sole Fe(II) were subcultured in a new medium prepared at pH 7.0 and with a EDTA/Fe(II) ratio of 2.0. In experiment 4, the effect of decreasing EDTA concentrations (20, 10, and 5 mM) and pH (7.0 and 6.0) was evaluated by using the enrichment cultures from experiment 3. In experiment 5, the use of Fe(II) as sole electron donor at pH 5.0 and EDTA/Fe(II) ratio of 0.5 was investigated by using the enriched *Thiobacillus*-mixed, pure *T. denitrificans*, and activated sludge cultures from experiment 3.

Sampling and analytical methods

Ferrous iron, nitrate, thiosulfate, and pH were analyzed at $t = 0$, after 6 h on day 1 and, subsequently, every 24 h after day 1 until day 10. Samples were taken with 5-mL disposable syringes. NO_3^- and $S_2O_3^{2-}$ concentration was analyzed by ion chromatography (IC) with chemically suppressed conductivity using a 883 Basic IC Plus system equipped with a Metrosep A Supp 5-150/4.0 column and a 863 Compact IC Autosampler (Metrohm, Switzerland). The liquid samples were filtered with 0.22 μm syringe cellulose membranes (EMD Millipore, USA) prior to IC analysis. Ferrous iron was quantified photometrically by using a Lambda 10 UV-Vis spectrometer (Perkin Elmer, USA), following the analytical method reported by Ahoranta et al. (2016). Fe(II) determination was performed immediately after the sampling, for avoiding Fe(II) chemical oxidation. DO and pH measurements were performed with a Multimeter 3410 (WTW, Germany) equipped with a FDO® 925 and a SenTix® 140-3 pH electrode, respectively. VSS were analyzed according to the standard methods (APHA 1992). Gas samples were not taken from the headspace of the bottles. The production of NO and N_2O was not evaluated.

Results and discussion

Fe(II)-mediated autotrophic denitrification with pH 7.0 and EDTA/Fe(II) 2.0

Supplementation of Fe(II) as sole electron donor with the four initial cultures

Fe(II)-mediated autotrophic denitrification was investigated in batch experiments under different operating conditions.

Table 1 Operating conditions used in the batch experiments. Feed nitrate was 2 mM in all the bioassays

Experiment	Microbial cultures	Electron donor	EDTA/ Fe(II)	pH
1	*Thiobacillus-dominated* mixed culture (TM)	Fe(II)—10 mM	2.0	7
	T. denitrificans pure culture (TDP)			
	Activated sludge (AS)			
	Pseudogulbenkiania strain 2002 (PG)			
2	TM (enriched on Fe(II) from experiment 1)	Fe(II)—10 mM	2.0	7
	TDP (acclimated to Fe(II) from experiment 1)	$S_2O_3^{2-}$— 0.5 mM		
3	TM (enriched on $S_2O_3^{2-}$ and Fe(II) from experiment 2)	Fe(II)—10 mM	2.0	7
	TDP (acclimated to $S_2O_3^{2-}$ and Fe(II) from experiment 2)			
	AS (enriched on Fe(II) from experiment 1)			
	PG (acclimated to Fe(II) from experiment 1)			
4	TM (from experiment 3)	Fe(II)—10 mM	2.0	7
	TDP (from experiment 3)		1.0	6
	AS (from experiment 3)		0.5	
	PG (from experiment 3)			
5	TM (from experiment 4)	Fe(II)—10 mM	0.5	5
	TDP (from experiment 4)			
	AS (from experiment 4)			

Table 2 reports the nitrate removal and Fe(II) oxidation achieved in all the batch bioassays after 10 days. In all experiments, no nitrite was detected as intermediate of denitrification and pH remained stable at 7.0.

Figure 1a shows the results obtained in experiment 1, using Fe(II) as sole electron donor in the bioassays seeded with the four initial inocula. The pure *T. denitrificans* culture was capable of maintaining Fe(II)-mediated autotrophic denitrification. The specific average nitrate removal rate was 0.362 mM·(g VSS·day)$^{-1}$. The molar Fe(II)/NO_3^- ratio constantly ranged between 3.5 and 5.0, indicating that denitrification proceeded in a good agreement with the stoichiometry (Eq. 1). After 10 days, Fe(II) oxidation reached 66%, whereas nitrate removal was 52%. Conversely, denitrification did not occur in the bioassays with the *Thiobacillus*-mixed culture. Fe(II) oxidation was 35%, but not associated with nitrate removal. Fe(II) was oxidized most likely due to the chemical reaction with residual DO, in agreement with what observed in abiotic controls. The profile of Fe(II) in the abiotic controls was as reported in Fig. 1a. Fe(II) concentration decreased by 35% in the first 4 days due to chemical oxidation. NO_3^- concentration did not significantly change over 10 days in both free electron donor and abiotic controls.

The feasibility of Fe(II)-driven denitrification with a pure culture of *T. denitrificans* is still contradictory. Straub et al. (1996) observed nitrate-dependent Fe(II) oxidation under strictly autotrophic conditions with a Fe(II)/NO_3^- molar ratio of 5:1. Fe(II) oxidation occurred in 8 weeks, but no information for nitrate removal was presented. In contrast, Muehe et al. (2009) did not observe Fe(II) oxidation coupled to autotrophic denitrification. The addition of organic compounds, i.e., humic substances, was needed to stimulate both Fe(II) and NO_3^- removal, likely due to the enhanced Fe(II) complexation and bioavailability (Kanaparthi and Conrad 2015).

In this study, the specific nitrate removal rate obtained with activated sludge inoculum was 0.321 mM·(g VSS·day)$^{-1}$, slightly lower than that achieved with *T. denitrificans* (Fig. 1a). Fe(II) oxidation and nitrate removal were 83 and 72%, respectively, at the end of the experiments. The molar ratio between oxidized Fe(II) and removed nitrate was in the range 2.2–4.8, in agreement with Nielsen and Nielsen (1998). However, Nielsen and Nielsen (1998) reported a much faster Fe(II)-based denitrification as 3 mM of Fe(II) was oxidized in 5 h coupled to the removal of 1 mM of nitrate.

Although *Pseudogulbenkiania* strain 2002 is a more specialized microbial culture in maintaining Fe(II)-based autotrophic denitrification, the use of *Pseudogulbenkiania* strain 2002 resulted in the lowest specific NO_3^- removal rate, i.e., 0.130 mM·(g VSS·day)$^{-1}$ (Fig. 1a). Fe(II) oxidation reached 54%, whereas nitrate removal was 55%. The molar Fe(II)/NO_3^- ratio was in the range 4.6–5.5, in a good agreement with the stoichiometry (Eq. 1). The high EDTA concentration most probably repressed the microbial activity of *Pseudogulbenkiania*

Table 2 NO_3^- removal and Fe(II) oxidation rates and efficiency achieved in all the bioassays

Experiment	Microbial culture	EDTA/ Fe(II)	pH	Time [days]	NO_3^- removal [%]	Specific NO_3^- removal rate [mM·(g VSS·day)$^{-1}$]	Fe(II) oxidation [%]	Specific Fe(II) oxidation rate [mM·(g VSS·day)$^{-1}$]
1	TM	2.0	7	0–10	–	–	35	2.092
	TDP				52	0.362	66	1.867
	AS				72	0.321	83	1.421
	PG				55	0.130	54	0.688
2	TM	2.0	7	0–4	73	1.976	37	1.822
				5–10	9	0.159	44	1.446
				0–10	82	0.886	81	1.597
	TDP			0–5	49	0.627	43	2.462
				6–10	26	0.335	30	1.721
				0–10	75	0.481	73	2.091
3	TM	2.0	7	0–10	83	1.160	68	1.678
	TDP				82	0.597	95	2.640
	AS				65	0.347	45	0.757
	PG				54	0.168	64	0.695
4	TM	2.0	7	0–10	80	1.179	54	3.034
		1.0			76	1.389	74	4.405
		0.5			98	1.417	83	4.501
		2.0	6		85	1.392	65	3.515
		1.0			98	1.355	60	3.383
		0.5			98	1.414	56	3.960
	TDP	2.0	7		83	0.630	81	2.815
		1.0			78	0.817	70	2.314
		0.5			93	0.795	89	2.530
		2.0	6		47	0.323	78	2.314
		1.0			56	0.353	60	1.798
		0.5			66	0.437	65	1.903
	AS	2.0	7		91	0.574	64	1.565
		1.0			89	0.627	60	1.429
		0.5			100	0.644	89	1.798
		2.0	6		84	0.653	59	1.464
		1.0			100	0.690	82	2.165
		0.5			100	0.701	100	1.804
	PG	2.0	7		61	0.248	40	0.557
		1.0			96	0.342	57	0.754
		0.5			100	0.439	53	0.830
		2.0	6		64	0.226	43	0.581
		1.0			77	0.292	42	0.581
		0.5			89	0.333	29	0.299
5	TM	0.5	5	0–10	45	0.498	40	2.075
	TDP				30	0.200	32	1.048
	AS				39	0.200	49	1.171

strain 2002. A slow nitrate removal with *Pseudogulbenkiania* strain 2002 was also observed by Weber et al. (2006b), even in the absence of EDTA. In their study, *Pseudogulbenkiania* strain 2002 only oxidized 25% of the initial 10 mM Fe(II) and 22% of the fed 2.2 mM nitrate in 7 days.

Supplementation of $S_2O_3^{2-}$ as additional electron donor to the Thiobacillus-mixed and pure T. denitrificans cultures

The capability for Fe(II)-mediated autotrophic denitrification of the previously Fe(II)-enriched *Thiobacillus*-mixed culture

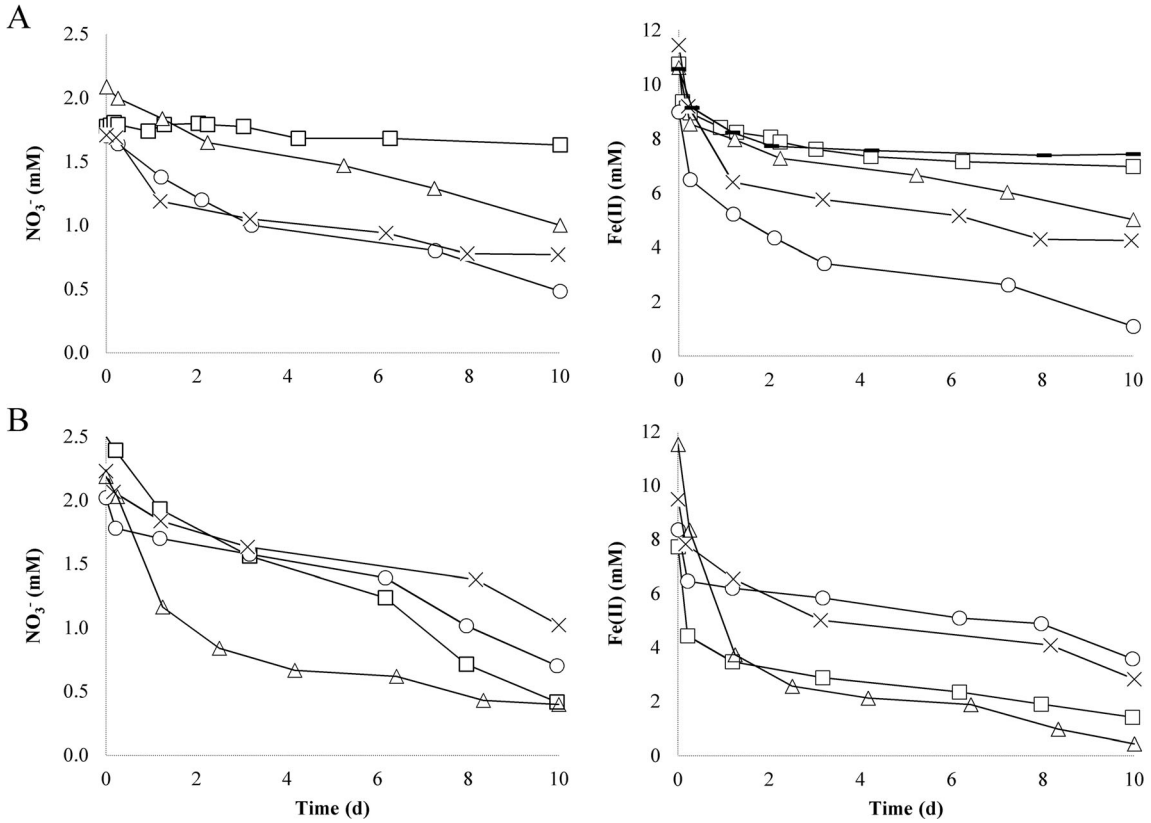

Fig. 1 NO_3^- and Fe(II) profiles in experiment 1 (**a**) and experiment 3 (**b**) in abiotic controls (*solid line*) and bioassays with the *Thiobacillus*-mixed culture (TM) (*empty square*), pure *T. denitrificans* culture (TDP) (*empty triangle*), activated sludge inoculum (AS) (*empty circle*), and *Pseudogulbenkiania* strain 2002 culture (PG) (*multiplication sign*). Standard deviations are in the range 0.02–0.65 mM

was investigated by supplementing thiosulfate in experiment 2 (Fig. 2). Thiosulfate was completely oxidized in 4 days. At this stage, nitrate removal reached up to 73% with a specific nitrate removal rate of 1.962 mM·(g VSS·day)$^{-1}$. About 46% of the initial nitrate was removed by thiosulfate-driven denitrification, according to the stoichiometry reported by Manconi et al. (2007). The remaining 27% of nitrate was removed by Fe(II)-oxidizing metabolism. Aside the initial Fe(II) drop to 4 mM due to chemical oxidation, Fe(II) oxidation was 37% after 4 days, indicating that Fe(II) oxidation was almost completely associated with autotrophic denitrification. From day 5 on, nitrate removal was only 9% with Fe(II) as sole electron donor. As also observed in experiment 1, the *Thiobacillus*-mixed culture was not able to maintain Fe(II)-mediated autotrophic denitrification in absence of thiosulfate. The specific nitrate removal rate dropped to 0.157 mM·(g VSS·day)$^{-1}$, whereas the iron oxidation rate remained constant.

The effect of thiosulfate as supplementary electron donor was also tested on the pure *T. denitrificans* culture (Fig. 2). As long as thiosulfate was present, the specific nitrate removal rate was 0.627 mM·(g VSS·day)$^{-1}$. In the absence of thiosulfate, denitrification proceeded with a nitrate removal rate of 0.335 mM·(g VSS·day)$^{-1}$. The overall nitrate removal rate was 0.481 mM·(g VSS·day)$^{-1}$, i.e., 34% higher than that achieved in experiment 1.

Both the *Thiobacillus*-mixed and pure *T. denitrificans* cultures were stimulated by supplementing thiosulfate as additional electron donor. However, the increase of nitrate removal rate was more significant for *Thiobacillus*-mixed culture, as the prolonged enrichment of the *Thiobacillus*-mixed culture on $S_2O_3^{2-}$ in a previous study (Di Capua et al. 2016) resulted in a microbial community specialized in using thiosulfate as sole electron donor. Conversely, the pure *T. denitrificans* culture demonstrated to use Fe(II) more efficiently than the *Thiobacillus*-mixed culture.

Supplementation of Fe(II) as sole electron donor with all the acclimated and enriched cultures

After enriching and acclimating the *Thiobacillus*-mixed and pure *T. denitrificans* cultures on thiosulfate, Fe(II)-based denitrification was again investigated with Fe(II) as sole electron donor. The use of a more enriched *Thiobacillus*-mixed culture resulted in a higher biological Fe(II) oxidation coupled to denitrification (Fig. 1b). The specific nitrate removal rate significantly increased and reached 1.160 mM·(g VSS·day)$^{-1}$. Fe(II) oxidation and NO_3^- reduction were 68 and 83%,

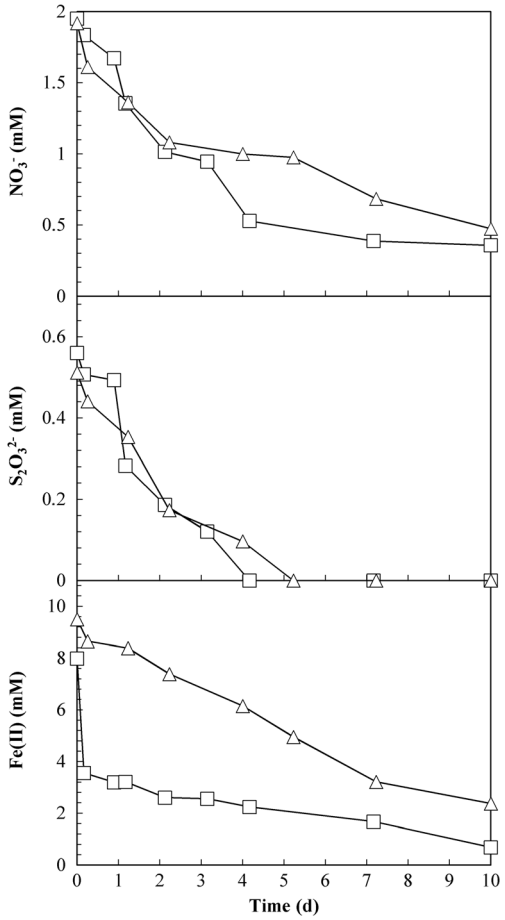

Fig. 2 The evolution of NO_3^-, $S_2O_3^{2-}$, and Fe(II) during experiment 2 for TM (*empty square*) and TDP (*empty triangle*). Standard deviations are in the range 0.02–0.65

respectively. *Thiobacillus thioparus* predominated over *T. denitrificans* in the microbial community (Di Capua et al. 2016). *T. thioparus* has previously been reported as an obligate thiosulfate-oxidizing chemolithotrophic denitrifier, not capable of using Fe(II) (Robertson and Kuenen 2006). However, after a prolonged enrichment on Fe(II) and $S_2O_3^{2-}$, the combined Fe(II)-oxidizing activity of *T. thioparus* and *T. denitrificans* was considerably enhanced, also in the presence of Fe(II) as sole electron donor.

Denitrification coupled to Fe(II) oxidation was also stimulated by subculturing the pure *T. denitrificans* culture in a fresh medium. The specific nitrate removal rate was 0.597 mM·(g VSS·day)$^{-1}$, compared to 0.360 mM·(g VSS·day)$^{-1}$ obtained in experiment 1. Nitrate removal was 82% and Fe(II) oxidation was 95%.

In contrast, nitrate removal in the bioassays with activated sludge and *Pseudogulbenkiania* strain 2002 cultures was less significantly enhanced after the subculture of the microbial cells (Fig. 1b). Compared to experiment 1, the specific nitrate removal rate only increased by 8% for activated sludge inoculum, whereas it rose from 0.130 to 0.168 mM·(g VSS·day)$^{-1}$ for *Pseudogulbenkiania* strain 2002. Nitrate removal and Fe(II) oxidation were 66 and 45% for activated sludge inoculum and 54 and 64% for *Pseudogulbenkiania* strain 2002, respectively. A slower Fe(II) oxidation was observed for the activated sludge inoculum than that obtained in experiment 1, most probably due to an optimization of denitrification that required a lower Fe(II) amount to achieve the same nitrate removal.

The rates of Fe(II)-mediated autotrophic denitrification obtained in this work were lower than those reported in similar batch experiments aimed at investigating classical heterotrophic denitrification or sulfur-driven autotrophic denitrification. For instance, Papirio et al. (2014) observed a nitrate removal rate up to approximately 400 mg·L^{-1}·day^{-1} by using denitrifying cultures enriched on ethanol. A N-NO_3^- removal rate of 52.2 mg·L^{-1}·day^{-1}, i.e., 3-fold higher than the highest achieved in this study, was obtained by Di Capua et al. (2016) under chemolithotrophic conditions with $S_2O_3^{2-}$ as electron donor. However, the implementation of Fe(II)-mediated autotrophic denitrification in continuous-flow bioreactors is expected to result in higher nitrate removal rates. Under these operating conditions, a higher biomass concentration can be used leading to an enhanced denitrification efficiency (Zhang et al. 2015).

In comparison with heterotrophic and sulfur-based autotrophic denitrification, Fe(II)-mediated autotrophic denitrification does not result in nitrite and this represents a major advantage as nitrite is reported to be inhibitory for many denitrifiers and nitrogen can be entirely removed from the liquid phase (Straub et al. 1996; Zhang et al. 2015).

Effect of the EDTA/Fe(II) ratio

EDTA is widely used in many environmental applications such as metal recovery from wastewaters and soils (Di Palma et al. 2003). The use of EDTA results in increasing treatment costs, but EDTA can be effectively recycled and suitable for reuse without losing its chelating properties (Juang and Wang 2000; Di Palma et al. 2003). In Fe(II)-mediated autotrophic denitrification, EDTA is used to improve Fe(II) solubility and bioavailability. An EDTA-recycling step after denitrification can be an interesting option to enhance the economic feasibility of the overall process (Zeng et al. 2005).

The dosing of EDTA is of major importance in Fe(II)-based denitrification. Microbial activity can be influenced by the molar EDTA/Fe(II) ratio as microbial cultures differently tolerate the inhibitory effects of free EDTA and Fe-EDTA species (Klueglein et al. 2015). In this study, the effect of decreasing EDTA on Fe(II)-mediated autotrophic denitrification was investigated in experiment 4. The NO_3^- removal efficiency achieved at EDTA/Fe(II) ratios of 2.0, 1.0, and 0.5 was as shown in Fig. 3.

When using the *Thiobacillus*-mixed culture, the specific nitrate removal rate was 1.179, 1.389, and 1.417 mM·(g VSS·day)$^{-1}$ at pH 7.0 and 1.392, 1.355, and 1.414 mM·(g VSS·day)$^{-1}$·at pH 6.0 with an EDTA/Fe(II) ratio of 2.0, 1.0, and 0.5, respectively. The specific nitrate removal rate increased from 0.629 to 0.817 mM·(g VSS·day)$^{-1}$ by decreasing the EDTA/Fe(II) ratio from 2.0 to 1.0 with the pure *T. denitrificans* culture at pH 7.0. A similar trend was observed at pH 6.0, with the specific nitrate removal rate increasing from 0.325 to 0.353 and 0.437 mM·(g VSS·day)$^{-1}$ with an EDTA/Fe(II) ratio of 2.0, 1.0, and 0.5, respectively.

The specific nitrate removal rate was 0.574 and 0.534 mM·(g VSS·day)$^{-1}$·for activated sludge inoculum at pH 7.0 and 6.0, respectively, with an EDTA/Fe(II) ratio of 2.0. An increase of the nitrate removal rate by 9 and 29% was observed by decreasing the EDTA/Fe(II) ratio to 1.0 at pH 7.0 and 6.0, respectively. The highest nitrate removal rate was obtained with an EDTA/Fe(II) ratio of 0.5, i.e., 0.644 and 0.701 mM·(g VSS·day)$^{-1}$ at pH 7.0 and 6.0, respectively.

The most significant effect of the EDTA concentration was observed with the *Pseudogulbenkiania* strain 2002 culture, indicating the lower tolerance of this species to EDTA. The lowest molar EDTA/Fe(II) ratio resulted in an almost double nitrate removal rate compared to that achieved with an EDTA/Fe(II) ratio of 2.0. The specific nitrate removal rate increased from 0.248 to 0.348 and 0.439 mM·(g VSS·day)$^{-1}$, with a decreasing EDTA/Fe(II) ratio. A less significant increase was observed at pH 6.0, with nitrate removal rates of 0.226, 0.292, and 0.335 mM·(g VSS·day)$^{-1}$·with an EDTA/Fe(II) ratio of 2.0, 1.0, and 0.5, respectively.

The decrease of feed EDTA from 20 to 5 mM resulted in an increase of Fe(II) oxidation by 48, 14, and 49% at pH 7.0 in the experiments with *Thiobacillus*-mixed, activated sludge, and *Pseudogulbenkiania* strain 2002 cultures, respectively. Fe(II) oxidation increased by 12, 17, and 23% in the experiments with *Thiobacillus*-mixed, pure *T. denitrificans*, and activated sludge cultures, respectively, at pH 6.0. A decrease of 10% was observed at pH 7.0 for the pure *T. denitrificans* culture, whereas Fe(II) oxidation was 48% lower at pH 6.0 for *Pseudogulbenkiania* strain 2002. The Fe(II) oxidation rate was not significantly affected by the decreasing EDTA concentrations at pH 6.0 in the other microbial cultures. Denitrification was also maintained at the lowest EDTA/Fe(II) ratio, indicating that the low EDTA efficiently chelated Fe(II) and promoted its bioavailability.

This study demonstrated that all the investigated cultures were alleviated by the decrease of EDTA. Free EDTA is generally the most toxic EDTA form to bacteria as it disrupts the cell membranes (Oviedo and Rodríguez 2003). However, Chakraborty and Picardal (2013) reported a negligible amount of free EDTA at an EDTA/Fe(II) ratio of 2.0, simulated by the Visual MINTEQ software. At a lower extent, EDTA toxicity

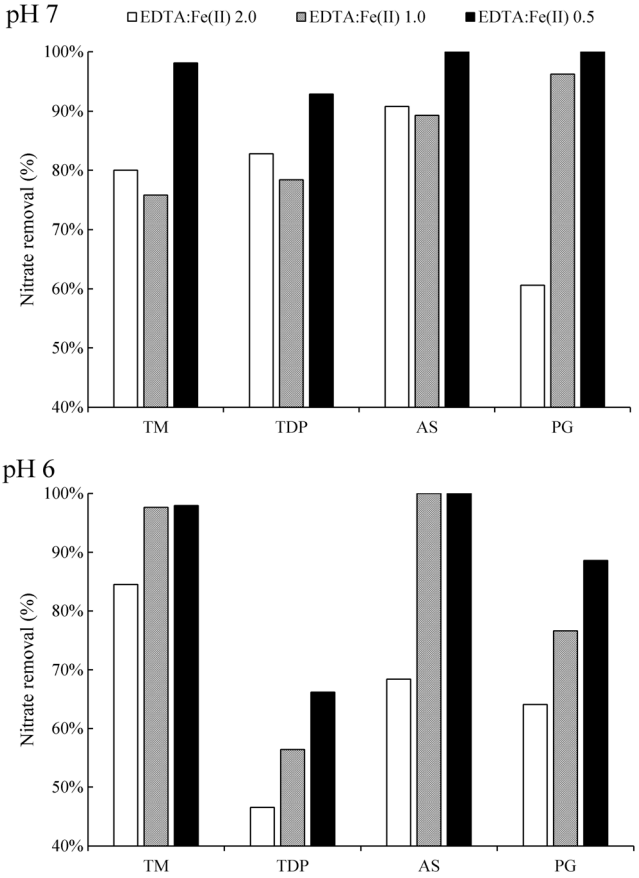

Fig. 3 Nitrate removal obtained with TM, TDP, AS, and PG in experiment 4 at pH 7.0 and 6.0 with EDTA/Fe(II) ratios of 2.0, 1.0, and 0.5

can also be associated with chelated Fe(II)-EDTA and Fe(III)-EDTA species (Klueglein et al. 2015). In this study, the mechanism of inhibition by EDTA at the higher EDTA/Fe(II) ratios remains unclear. In spite of this, a faster metabolic activity was observed during the enrichment of all cultures.

Influence of the initial pH

The effect of decreasing pH from 7.0 to 6.0 on Fe(II)-mediated autotrophic denitrification was investigated in experiment 4. A further pH decrease to 5.0 was assessed with the *Thiobacillus*-mixed, pure *T. denitrificans*, and activated sludge cultures in experiment 5.

It is known that pH affects microbial activity and iron speciation (Hedrich et al. 2011). At pH < 4.0, Fe(II) is more stable but the inhibition of most denitrifiers occurs. Conversely, a neutral pH is favorable for biological activity but Fe(II) is quickly oxidized with oxygen (Johnson et al. 2012). The known Fe(II)-oxidizing denitrifiers are neutrophilic (Hedrich et al. 2011), and therefore up to now the majority of studies was performed at pH between 6.0 and 8.0 (Kiskira et al. 2017).

Studies investigating the optimal pH on the process with pure and mixed cultures reported that pH should not be below 6.0 (Straub et al. 2004; Oshiki et al. 2013; Zhang et al. 2015). Only some uncultured *Actinobacteria* were found to be capable of performing Fe(II)-driven denitrification at pH 4.5 (Kanaparthi et al. 2013).

In this study, the activity of the *Thiobacillus*-mixed and activated sludge enrichments was enhanced by decreasing pH from 7.0 to 6.0 (Fig. 3). With an EDTA/Fe(II) ratio of 2.0, the specific nitrate removal rate increased from 1.179 to 1.392 mM·(g VSS·day)$^{-1}$ for the *Thiobacillus*-mixed culture. A slight increase of denitrification efficiency was also observed for the activated sludge inoculum, with the nitrate removal rate increasing from 0.644 mM·(g VSS·day)$^{-1}$ at pH 7.0 to 0.701 mM·(g VSS·day)$^{-1}$ at pH 6.0, with an EDTA/Fe(II) ratio of 0.5. Nielsen and Nielsen (1998) reported a more significant pH dependence of an activated sludge inoculum, with an optimal pH of 8.0. At pH 8.0, the Fe(II) oxidation rate was 0.132 mM Fe(II)·(g VSS·h)$^{-1}$, which was two times higher than that at pH 7.0, and almost four times higher than that at pH 6.0 and 5.0. Nielsen and Nielsen (1998) did not perform any previous enrichment on Fe(II), most likely inducing a higher pH dependence of Fe(II)-mediated denitrification.

In this study, the two pure cultures of *T. denitrificans* and *Pseudogulbenkiania* strain 2002 demonstrated to be less tolerant to decreasing pH. The decrease of pH from 7.0 to 6.0 resulted in a lower denitrification efficiency in the experiments with *Pseudogulbenkiania* strain 2002, with all the EDTA/Fe(II) ratios tested. In agreement, Weber et al. (2009) reported a faster growth of *Pseudogulbenkiania* strain 2002 at a pH ranging between 6.8 and 8.0. A significant decrease of nitrate removal rate was also observed for the *T. denitrificans* culture at pH 6.0. The nitrate removal rate dropped from 0.629, 0.817, and 0.795 to 0.325, 0.353, and 0.437 mM·(g VSS·day)$^{-1}$ by decreasing pH from 7.0 to 6.0 with an EDTA/Fe(II) ratio of 2.0, 1.0, and 0.5, respectively. The optimal pH for maintaining sulfur-driven denitrification with *T. denitrificans* is approximately 6.9 (Kelly and Wood 2000). A better activity of *T. denitrificans* at neutral pH was confirmed in this study, by using Fe(II) as electron donor. However, to the best of the authors' knowledge, no information concerning the optimal pH in Fe(II)-mediated denitrification by *T. denitrificans* had previously been reported.

The effect of pH 5.0 with an EDTA/Fe(II) molar ratio of 0.5 was also investigated for the *Thiobacillus*-mixed, pure *T. denitrificans*, and activated sludge cultures in experiment 5 (Fig. 4). *Pseudogulbenkiania* strain 2002 was not tested at pH 5.0, as nitrate removal rate was significantly low at pH 6.0. The efficiency of denitrification considerably decreased at pH 5.0 for all the microbial enrichments. The specific nitrate removal rate dropped from 1.470, 0.437, and 0.439 mM·(g VSS·day)$^{-1}$·to 0.498, 0.200, and 0.335 mM·(g VSS·day)$^{-1}$, by decreasing the pH from 6.0 to 5.0 with the *Thiobacillus*-mixed, pure *T. denitrificans*, and activated sludge cultures, respectively.

Both chemical and biological Fe(II) oxidation coupled to denitrification were repressed at decreasing pH, resulting in lower Fe(II) oxidation rates in almost all the experiments.

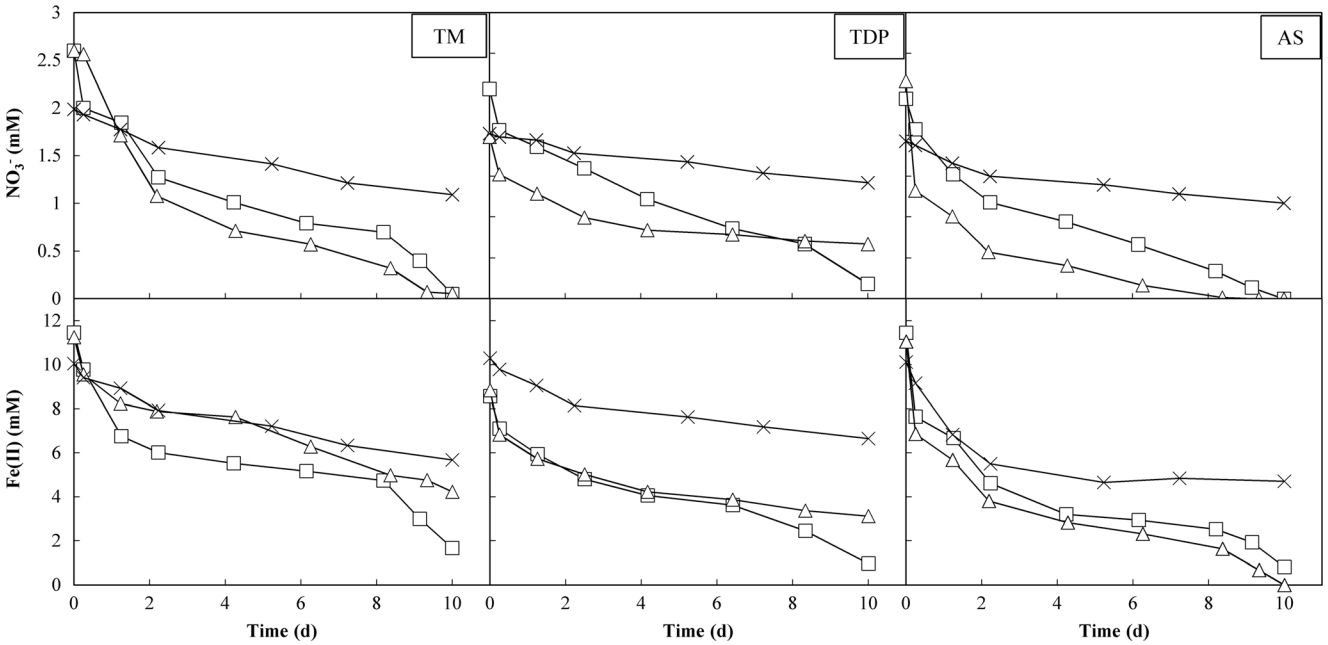

Fig. 4 Fe(II) and NO_3^- profiles for TM, TDP, and AS at pH 7.0 (*empty square*), 6.0 (*empty triangle*), and 5.0 (*multiplication sign*) in experiment 5. Standard deviations are in the range 0.02–0.60 mM

Conclusions

Fe(II)-mediated autotrophic denitrification was effectively maintained with two pure and two enriched mixed cultures. Nitrate removal was above 60% in all the bioassays operated at pH 7.0 and an EDTA/Fe(II) ratio of 2.0. After a longer acclimation to Fe(II) and stimulation with $S_2O_3^{2-}$, the *Thiobacillus*-mixed culture resulted in the highest specific nitrate removal rate, equal to 1.179 mM·(g VSS·day)$^{-1}$. Decreasing EDTA/Fe(II) ratios resulted in higher nitrate removal efficiency and rates. With EDTA/Fe(II) ratios of 1.0 and 0.5, denitrification was particularly enhanced for *Pseudogulbenkiania* strain 2002, which less tolerated EDTA. At pH 6.0, the activity of *T. denitrificans* and *Pseudogulbenkiania* strain 2002 was repressed, whereas a faster denitrification was observed for the *Thiobacillus*-mixed and AS cultures. The use of pH 5.0 resulted in a 65, 75, and 69% slower nitrate removal than at pH 7.0 for the *Thiobacillus*-mixed, pure *T. denitrificans*, and activated sludge cultures, respectively.

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