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Research article

Effect of Cu, Ni and Zn on Fe(II)-driven autotrophic denitrification

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ABSTRACT

Fe(II)-mediated autotrophic denitrification in the presence of copper (Cu), nickel (Ni) and zinc (Zn) with four different microbial cultures was investigated in batch bioassays. In the absence of metals, complete nitrate removal and Fe(II) oxidation were achieved with a *Thiobacillus*-dominated mixed culture and *Pseudogulbenkiania* sp. 2002 after 7 d. A nitrate removal of 96 and 91% was observed with a pure culture of *T. denitrificans* and an activated sludge enrichment, respectively, after 10 d of incubation. Cu, Ni and Zn were then supplemented at an initial concentration of 5, 10, 20 and 40 mg Me/L. A decrease of approximately 50% of the soluble metal concentrations occurred in the first 4 d of denitrification, due to metal precipitation, co-precipitation, sorption onto iron (hydr)oxides, and probably sorption onto biomass. A higher sensitivity to metal toxicity was observed for the microbial pure cultures. *Pseudogulbenkiania* sp. 2002 was the least tolerant among the biomasses tested, resulting in only 6, 8 and 6% nitrate removal for the highest Cu, Ni and Zn concentrations, respectively. In contrast, the highest nitrate removal efficiency and specific rates were achieved with the *Thiobacillus*-dominated mixed culture, which better tolerated the presence of metals. Averagely, Cu resulted in the highest inhibition of nitrate removal, followed by Zn and Ni.

1. Introduction

Nitrate is a common pollutant in municipal and industrial wastewaters. The extensive use of nitrogen-based fertilizers and chemicals is among the main reasons of nitrate contamination of water bodies (Viers et al., 2012). Moreover, nitrate often co-occurs with metals and heavy metals (HMs), such as iron, copper, cobalt and nickel in mining water streams (Papirio et al., 2014; Zou et al., 2014). The presence of HMs in the water resources is a major concern (Ochoa-Herrera et al., 2011; Santos and Judd, 2010), with several industrial activities being mostly responsible for their discharge in the hydrosphere (Karvelas et al., 2003).

The microbial activity of microorganisms capable for denitrification can be either stimulated or inhibited by the presence of HMs. The supplementation of trace metals can enhance metabolic degradation, whereas the excess of metals usually represses the activity of most denitrifiers (Gikas, 2007). Microbes can tolerate increasing metal concentrations by developing particular mechanisms such as the efflux of metal ions outside the cell, the accumulation and complexation of the metal ions inside the cell, and the reduction of the HM ions to a less toxic state (Spain, 2003). However, at higher concentrations, metals can change the microbial enzyme conformation and block essential functional groups (Giller et al., 2009). Besides the amount and type of HM, the toxicity of HMs can be influenced by many factors such as the temperature, the presence of specific chemical substances, the metal speciation and bioavailability, and the concomitant presence of other HMs or major elements such as calcium (Gikas, 2008).

Copper (Cu) and zinc (Zn) are among the most common polluting metal ions in industrial effluents and are associated with toxicity issues. In experiments with anoxic-membrane bioreactors, the presence of Cu and Zn at a concentration of approximately 2.6 and 32.3 mg/L repressed the denitrification rate by 20 and 34%, respectively (Feng et al., 2013). In contrast, nickel (Ni) has been found to enhance the growth of microorganisms at concentrations

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below 5 mg Ni/L (Gikas, 2008), and denitrifiers have been observed to tolerate Ni concentrations up to 500 mg Ni/L (Zou et al., 2015). A longer exposure of the bacteria to metals can increase their metal tolerance, thus promoting denitrification (Sakadevan et al., 1999; Zou et al., 2015).

The effect of HMs has been mostly investigated on nitrifying (Hu et al., 2004; Lee et al., 2009) and heterotrophic denitrifying microbial cultures (Lawrence et al., 2004; Ochoa-Herrera et al., 2011; Sakadevan et al., 1999; Wang et al., 2013). However, the knowledge about the metal influence on Fe(II)-oxidizing autotrophic denitrifiers remains limited (Kiskira et al., 2017a). Hohmann et al. (2009) studied the effect of arsenic(III) as inhibiting element and reported that the enriched cultures of *Acidovorax* strain BoFeN1, *Rhodobacter ferrooxidans* strain SW2 and the enrichment culture KS tolerated up to 20–50 mM of As(III), resulting in a complete Fe(II) oxidation. To the authors' knowledge, the impact of Cu, Ni and Zn as inhibitors of Fe(II)-mediated autotrophic denitrification and the possibility of precipitating them with the biogenic Fe(III) hydro(-oxides) have not yet been investigated.

Therefore, the objectives of this work were 1) to investigate the efficiency and specific rates of nitrate removal during Fe(II)-mediated autotrophic denitrification in the presence of Cu, Ni and Zn in batch bioassays, and 2) to evaluate how four different microbial cultures, i.e. a *Thiobacillus*-dominated mixed culture, an activated sludge enrichment, and two pure cultures of *Pseudogulbenkiania* sp. 2002 and *T. denitrificans*, tolerated the presence of Cu, Ni and Zn.

2. Materials and methods

2.1. Sources of microorganisms and mineral growth medium

Four chemolithotrophic denitrifying cultures were used in this study. A *Thiobacillus*-dominated mixed culture, developed in experiments with thiosulfate as electron donor (Di Capua et al., 2016; Zou et al., 2016), and an activated sludge enrichment, originally collected from the municipal wastewater plant in Cassino (Italy), were used as mixed bacterial consortia. Then, two pure cultures of *Pseudogulbenkiania* sp. 2002 (DSM, 18807) and *T. denitrificans* (DSM 12475) were obtained from the 'Leibniz-Institute DSMZ - German collection of microorganisms and cell cultures' in Braunschweig (Germany). Prior to this study, all the cultures had been used in Fe(II)-based denitrification bioassays (Kiskira et al., 2017b).

The basal medium was prepared with the following components (g/L): 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 as detailed in Weber et al. (2009). The feed Fe(II) and NO_3^- concentrations were 600 and 120 mg/L, respectively.

2.2. Preparation of the experiments

Serum bottles of 125 mL were used for the batch experiments and maintained at room temperature (22 ± 2 °C). Fe(II) and NO_3^- were added in concentration of 600 and 120 mg/L, in the form of iron(II) chloride ($FeCl_2 \cdot 4H_2O$) and sodium nitrate ($NaNO_3$), respectively. EDTA in a molar ratio of 0.5:1.0 with Fe(II) was used as chelating agent. Cu, Ni and Zn were added in concentration of 5, 10, 20 and 40 mg/L in the form of copper chloride ($CuCl_2$), nickel chloride ($NiCl_2 \cdot 6H_2O$) and zinc chloride ($ZnCl_2$). All the chemicals were of analytical grade (Sigma Aldrich, Germany).

The feed pH was adjusted to 7.0 by adding NaOH and HCl before flushing the bottles with He in order to maintain anoxic conditions. Bicarbonate (2 g/L as $NaHCO_3$) was added to each bottle as pH buffer and inorganic carbon source. Both mixed and pure cultures were taken from the liquor of the bioassays of a previous experiment (experiment 4 in Kiskira et al., 2017b), which was properly stored at 4 °C for 1 month prior to performing the experiments of the present study. All the cultures were seeded in the serum bottles in a 10% (v/v) amount. The initial volatile suspended solids (VSS) concentration was 200, 320, 410 and 750 mg VSS/L in the bottles inoculated with the *Thiobacillus*-mixed culture, *T. denitrificans* pure culture, activated sludge enrichment and *Pseudogulbenkiania* sp. 2002, respectively. Then, the bottles were sealed with butyl rubber stoppers and aluminum crimps and placed on a gyratory shaker at 220 rpm. Microcosms were prepared in duplicate. For each microbial culture, controls without electron donors and metals were carried out to monitor the removal 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 Cu, Ni and Zn.

2.3. Thermodynamic modeling of metal speciation in the batch experiments

Metal speciation was predicted using Visual MINTEQ ver.3.1 (KTH, SEED, Sweden), a thermodynamic equilibrium modeling software (http://vminteq.lwr.kth.se/). Visual MINTEQ allows for simulating chemical processes, while it is not capable for taking into account biological processes (e.g. biodegradation and biosorption). However, the modeling by Visual MINTEQ can give important information on the metal speciation based on the experimental observations associated with a biological process. In this study, the dissolved and precipitated metal concentrations were simulated for the experiments performed with the *Thiobacillus* mixed culture as well as the abiotic control by using the concentrations measured after 4 days of incubation as input data. The simulations were performed accounting for Fe(II) and Fe(III), EDTA, nutrients, trace element concentrations as reported in section 2.1, and 40 mg/L of added Cu, Ni and Zn. The temperature was set to 22 °C, the pH was fixed at 6.3 and the oversaturated solids were allowed to precipitate.

2.4. Calculations

Nitrate removal and Fe(II) oxidation efficiencies were calculated based on the following equations:

$$Nitrate\ removal\ efficiency\ [\%] = \frac{Initial\ NO_3^-[mg/L] - Final\ NO_3^-[mg/L]}{Initial\ NO_3^-[mg/L]} \times 100$$

$$Fe(II) oxidation\ efficiency\ [\%] = \frac{Initial\ Fe(II)[mg/L] - Final\ Fe(II)\ [mg/L]}{Initial\ Fe(II)[mg/L]} \times 100$$