Inhibitory effect of heavy metals on nitrogen production by anaerobic ammonium oxidation bacteria

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Abstract
Heavy metals (HMs) are common components of landfill leachates and piggery wastewater, which correspond with possible applications of the anaerobic ammonium oxidation (anammox). For this reason, the study of the fate and effect of HMs on the anammox process is of utmost importance. In this work, the effect of several HMs on the specific anammox activity (SAA) was studied. For this purpose, the normalized SAA was compared with soluble concentrations of several HMs, and the following inhibition constants (K_i) were calculated (μg L^{-1}): 6,400 (Zn^{2+}), 4,070 (Cu^{2+}), 9,760 (Cd^{2+}), 37,210 (Ni^{2+}) and 6,510 (Pb^{2+}).

Keywords
Anaerobic ammonium oxidation, heavy metals, inhibition, toxicity.

INTRODUCTION

Anaerobic ammonium oxidation (anammox) is a promising alternative to treat waste off-streams containing high concentrations of ammonium and relatively low organic matter composition (Ahn 2006). This chemolithoautotrophic process involves the oxidation of ammonium with nitrite as the electron acceptor (Jetten *et al.* 2001).

Wastewater streams that are good candidates for anammox treatment include landfill leachates, sludge concentrates, and several industrial wastewater with high ammonium content, such as slaughterhouse and piggery wastewater (Ahn 2006; Kartal *et al.* 2010). Among them, landfill leachates and piggery wastewater share a common feature: elevated content of heavy metals (HMs) (L'Herroux *et al.* 1997; Baun & Christensen 2004). Although concentrations of HMs in landfill leachates have generally been reported to be in several hundreds of μg L^{-1} (Baun & Christensen 2004), there are some cases in which much higher concentrations have been found, including landfills in some arid areas and under-developed waste-importing countries (Oshode *et al.* 2008; Al-Muzaini 2009). Piggery wastewater can also bear elevated concentrations of Cu^{2+} and Zn^{2+} derived from their supplementation as essential micronutrients for pig metabolism (L'Herroux *et al.* 1997).

HMs are common inhibitors of anaerobic metabolism (Chen *et al.* 2008), and there is some evidence that they can cause inhibition of the anammox process (Jin *et al.* 2012; Lotti *et al.* 2012). However, the lack of specific studies concerning the effect of HMs on the anammox process limits its scalability to field applications. For these reasons, understanding of the fate and inhibitory impact of HMs on the anammox process is of utmost importance for the correct control of full-scale anammox reactors. The aim of this work was to evaluate the effect of a wide range of concentrations of several HMs on the activity of granular anammox biomass.

MATERIALS AND METHODS

Anammox biomass

Granular anammox biomass was retrieved from a laboratory-scale expanded granular sludge bed reactor. This sludge had a specific anammox activity of 0.424 ± 0.014 mmol N_2 g^{-1} VSS h^{-1}.

Batch toxicity bioassays

Batch experiments were conducted by using 100-mL serum bottles hermetically closed and inoculated with 1.88 g VSS L^{-1} of the EGSB sludge. Nitrite and ammonium were added at 3.57 and 2.70 mM, respectively. A standard anammox medium was used according to a previous work (Sun *et al.* 2011). $NaHCO_3$ (0.6 g L^{-1}) was added as C source and for pH control. The media and headspace were flushed with $He:CO_2$ (80:20, v/v) to ensure anaerobic conditions and adjust the pH at 7.0. Five HMs were spiked as follows (in μg L^{-1}): Zn^{2+} (500-12,500), Cu^{2+} (1,000-10,000), Cd^{2+} (100-75,000), Ni^{2+} (250-100,000), and Pb^{2+} (250-75,000). All the HMs where supplemented as hydrated chlorides with the exception of Pb^{2+} ($Pb(NO_3)_2 \cdot 2H_2O$). The biomass was exposed to the HM for 2 h before adding the N substrates. Control experiments lacking HM addition were carried out in parallel under the above described conditions. All the assays were conducted in duplicate.

Analytical

Analysis of N_2, O_2, NH_4^+, NO_2^- and NO_3^- were carried out as previously described (Sun *et al.* 2011). In order to measure soluble heavy metals concentrations, samples were taken at the initial and final times and at 1 h after adding every heavy metal, centrifuged at 13,000 rpm for 10 min and resulting supernatants were supplemented with HNO_3 to a final concentration of 2% (v/v). The acidified solutions were analyzed by inductively coupled plasma-optimal emission spectroscopy (ICP-OES Optima 2100 DV, Perkin–Elmer TM, Shelton, CT). All other analyses (VSS, pH, etc) were performed according to Standard Methods protocols (APHA 2005).

Specific anammox activity (SAA) and inhibition kinetics

SAA values were calculated by using the initial N_2 production rate in the linear range, thus considering them as maximum SAA (SAA_{max}, mmol N_2 g^{-1} VSS h^{-1}). Data were normalized based on the SAA of the uninhibited control experiments. The resulting SAA values were fitted to the Eq. [1] in order to calculate the inhibition parameters:

$$SAA = SAA_{\max} \cdot \frac{1}{1 + \left(\frac{I}{K_i} \right)^n} \qquad [1]$$

Where I and K_i represent the inhibitor concentration and the inhibition constant (μg L^{-1}) and n is the inhibition order (dimensionless). Data fitting and statistics calculations were carried out according to a previous work (Puyol *et al.* 2012).

RESULTS AND DISCUSSION

Solubility of HMs on anammox media

Fig. 1 shows the relationship between the added and soluble concentrations of every HM at 1 h and at the end of the experiment. As can be seen, the difference between concentrations at these two times is very low, suggesting that the contribution of sorption, precipitation or biological processes to HM fate under the conditions of our experiments can be considered negligible. Therefore, the effect of HMs on the SAA can be mainly attributed to the soluble HM concentration. In order to calculate the maximum experimental soluble concentration of every HM, these data were fitted to an empirical asymptotic-exponential equation with the form $y = a \cdot [1-e^{(-b \cdot x)}]$, where *a* represents the maximum soluble HM concentration. The resulting fitting curves are shown in Fig. 1.

Table 1 shows the maximum concentration of soluble HMs in the experimental conditions described. Some chemical processes, as precipitation of metal carbonates, could occur during the first two hours, just before the addition of the N substrates, which is suggested to cause the asymptotic relationship between the soluble and total HM concentration. Also, Pb^{2+} showed to have very low solubility in the anammox media.

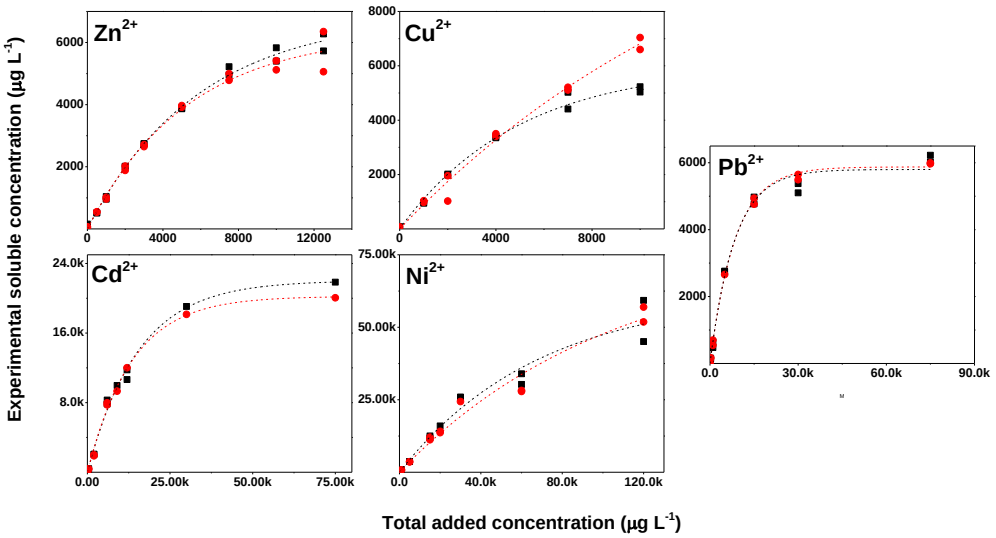

Figure 1. Comparison between added and experimentally measured soluble concentrations of the HMs tested. Dash-dot lines are fittings to an empirical equation.

Table 1. Maximum soluble concentrations of every HM in the anammox experiments, and goodness of fitting to an empirical asymptotic-exponential equation.

t	Zn^{2+}		Cu^{2+}		Cd^{2+}		Ni^{2+}		Pb^{2+}	
	Value	R^2	Value	R^2	Value	R^2	Value	R^2	Value	R^2
2 h	6,874 ± 184	0.99	6,022 ± 281	0.99	22,031 ± 369	0.99	61k ± 7k	0.96	5,799 ± 130	0.99
End	6,312 ± 257	0.99	14,103 ± 3272	0.99	20,228 ± 405	0.99	78k ± 12k	0.97	5,875 ± 51	1.00

Effect of HMs on SAA

The effect of the soluble concentration of selected HMs on the SAA was studied. As an example, the N_2 production at different added concentrations of Cd^{2+} is illustrated on Fig. 2. The inhibitory effect of every HM was analyzed by considering measured soluble concentrations at 2 h after the HM addition.

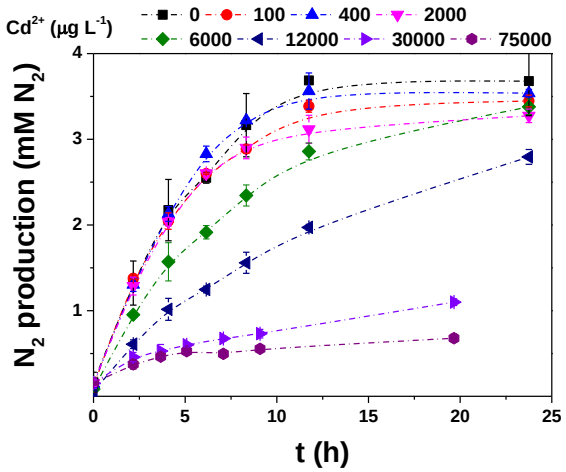

Figure 2. Effect of Cd^{2+} on nitrogen production by anammox granules. Error lines represents standard deviations from duplicates.

In all cases, HMs seemed to affect the SAA at concentrations above 2,000 μg L^{-1}. However, the trend for each particular HM is different. While Cu^{2+} exerted a critical effect at relatively low concentrations, with a decrease in the SAA of around 80% at 5,000 μg L^{-1}, this effect was much slighter for Cd^{2+} and, overall, for Ni^{2+}, reaching an inhibition of only 40% at a soluble Ni^{2+} concentration of around 25,000 μg L^{-1}.

In order to quantify the inhibitory effect of HMs on anammox activity, SAA data were fitted to Eq. [1]. The resulting parameters and goodness of fitting are presented in Table 2. As can be seen, these HMs can be ordered by toxicity as follows: $Cu^{2+} > Zn^{2+} \geq Pb^{2+} > Cd^{2+} > Ni^{2+}$. Relatively similar values have been recently reported for the inhibition constants of Cu^{2+} (1,900-5,000 μg L^{-1}) and Zn^{2+} (3,900-4,500 μg L^{-1}) (Lotti *et al.* 2012). To the best of our knowledge, this is the first study evaluating the effect of Cd^{2+}, Ni^{2+} and Pb^{2+} on anammox activity.

Table 2. Values of inhibition parameters and goodness of fitting of data from Figure 1 to the Eq. [1].

	Cu^{2+}	**Zn^{2+}**	**Cd^{2+}**	**Ni^{2+}**	**Pb^{2+}**
K_i (μg L^{-1})	4,069 ± 207	6,404 ± 354	9,762 ± 168	37,209 ± 5,135	6,511 ± 159
n	4.5 ± 1.1	4.1 ± 1.3	4.5 ± 0.4	0.86 ± 0.16	8.8 ± 2.1
SAA_{max} (%)	94.4 ± 4.2	96.4 ± 2.5	101.5 ± 1.5	98.8 ± 3.3	99.3 ± 2.1
R^2	0.92	0.81	0.99	0.92	0.90

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