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Partial Nitritation-Anammox Granules: Short-Term Inhibitory Effects of Seven Metals on Anammox Activity

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Abstract The inhibitory effect of seven different metals on the specific anammox activity of granular biomass, collected from a single stage partial nitritation/anammox reactor, was evaluated. The concentration of each metal that led to a 50% inhibition concentration (IC_{50}) was 19.3 mg Cu^{+2}/L, 26.9 mg Cr^{+2}/L, 45.6 mg Pb^{+2}/L, 59.1 mg Zn^{+2}/L, 69.2 mg Ni^{+2}/L, 174.6 mg Cd^{+2}/L, and 175.8 mg Mn^{+2}/L. In experiments performed with granules mechanically disintegrated (flocculent-like sludge), the IC_{50} for Cd^{+2} corresponded to a concentration of 93.1 mg Cd^{+2}/L. These results indicate that the granular structure might act as a physical barrier to protect anammox bacteria from toxics. Furthermore, the presence of an external layer of ammonia oxidizing bacteria seems to mitigate the inhibitory effect of the metals, as the values of IC_{50} obtained in this study for anammox activity were higher than those previously reported for anammox granules. Additionally, the results obtained confirmed that copper is one of the most inhibitory metals for anammox activity and revealed that chromium, scarcely studied yet, has a similar potential inhibitory effect.

Keywords Ammonia oxidizing bacteria · Anammox · Granules · IC_{50} · Nitrogen removal

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1 Introduction

The anammox-based processes are the object of intensive studies in the last years, being considered appropriate for the removal of nitrogen rich effluents, such as landfill leachate (Anfruns et al. 2013). This type of wastewater contains high ammonia concentrations that can vary between 0.2–13,000 mg N/L depending on the age of the landfill. It also may contain high recalcitrant organic matter and significant metal concentrations (Renou et al. 2008; Wang et al. 2014) that can negatively affect the activity of the anammox process (Jin et al. 2012; Yin et al. 2016).

It is known that excessive concentrations of metals are normally inhibitory for microorganisms, due to the fact that they are not biodegradable and can accumulate inside the organisms, causing toxicity (Yin et al. 2016). However, in certain cases, trace concentrations of particular metals can improve microbial activity because they are the components of many enzymes or coenzymes (Yin et al. 2016). Chen et al. (2014) observed that the addition of Fe^{+3}, Cu^{+2}, and Ni^{+2} at concentrations of 6.61, 1.18, and 1.11 mg/L, respectively, increased the nitrogen removal efficiency in an anammox continuous reactor by approximately 50%.

The effects of inhibitory compounds are expressed in general as values of 50% inhibition concentration (IC_{50}). The IC_{50} values found in literature for metals effects on anammox activity are highly variable. The reported IC_{50} corresponding to Cu^{+2} vary from 30 mg Cu^{+2}/L (Zhang et al. 2016) to only 4.2 mg Cu^{+2}/L (Daverey et al. 2014a) or 1.9 mg Cu^{+2}/L (Lotti et al. 2012). The different values of IC_{50} reported can be attributed to several factors: the anammox species present in the sludge are different, physical properties of seed sludge (flocculant or granular), biomass concentration, exposure time to the toxic, temperature, pH, etc. (Daverey et al. 2014a). These different conditions for the experiments underline the necessity to perform specific studies with each type of biomass and fixed conditions to more precisely evaluate the effect of different metals over anammox activity.

Two important parameters to consider are the aggregation state of the biomass and the presence of other microbial populations, apart from those responsible for the anammox process. The biomass that grows as granular aggregates is known to resist the effects of toxic compounds better than the flocculent one (Adav et al. 2008). The granular structure exerts a mass transfer resistance for the diffusion of toxics from the bulk liquid to the core of the granule. Therefore, the microorganisms located in the inner layers are more protected against the effects of the metals at higher toxic concentrations. It is known that the anammox biomass tends to form biofilm or granules under high nitrogen substrate environments (Ni et al. 2015). Furthermore, in most of the full-scale applications, the anammox process occurs simultaneously to the partial nitritation process in a single stage configuration (Lackner et al. 2014). Therefore, the study of the metal inhibition over granular biomass, where these both processes take place simultaneously is of interest, as the ammonia oxidizing bacteria (AOB) mainly grow in the outer layer of the granular structure where dissolved oxygen is present (Vázquez-Padín et al. 2010). Recently, the capacity of the granular sludge to resist the toxicity of certain compounds, like metals or chlorophenol, was related to the presence of extracellular polymeric substances (EPS) on the surface of the aggregates (Wei et al. 2015; Li and Yu 2014). In this sense, anammox bacteria have been found to secrete more EPS than anaerobic/aerobic, which play an important role in the granulation process and the stability of the granular structure (Ni et al. 2015).

There is quite an amount of research works that reported on the inhibitory effect of different metals on anammox bacteria activity (Li et al. 2015; Lotti et al. 2012; Bi et al. 2014; Daverey et al. 2014b; Zhang et al. 2016). Most of them used granular sludge; however, little information is available on this effect on granular biomass performing the partial nitritation-anammox processes.

The use of granular sludge performing the partial nitritation and anammox processes in a single unit to remove nitrogen from landfill leachate is a good alternative to explore. However, the resistance of this granular biomass to metal toxicity is hard to predict. In the present study, specific anammox activity (SAA) tests were performed to evaluate the effects of different metal concentrations on the anammox activity of granular biomass where the partial nitritation-anammox processes take place. The experimental results will help to determine the suitability of a specific inoculum to treat effluents containing metal concentrations.

2 Materials and Methods

2.1 Granular Sludge from Partial Nitritation-Anammox Reactor

The granular sludge was collected from a pilot plant of 200 L containing *Brocadia Fulgida* as the main anammox population and with an average diameter of 3.2 mm (Morales et al. 2015). The granular sludge performed the partial nitritation-anammox processes in a single unit, in the so called ELAN® process developed by the company FCC Aqualia in collaboration with the Universidade de Santiago de Compostela. The pilot plant treated the reject water from an anaerobic sludge digester located in an urban WWTP (Guillarei-Galicia, Spain) containing 540–1045 mg NH_4^+-N/L and operated at 30 °C. The concentration of metals present in this granular sludge is presented in Table 1.

2.2 Batch Activity Tests

The SAA tests were carried out in closed vials of 25 mL, according to the methodology described by Dapena-Mora et al. (2007). The biomass samples were washed with phosphate buffer, and the corresponding concentration of metal was added. The pH of the medium was set at 7.8 by adding HCl (1 M). Then the headspace of hermetically closed vials was flushed with helium gas to avoid the presence of oxygen. The vials were incubated at 30 °C and 150 rpm for an acclimation time of 12 h. Then the substrates, 70 mg N/L of ammonium and

Table 1 Concentration of heavy metals in the partial nitritation/anammox granular sludge

Metal	mg/g VSS
Fe	7.604 ± 0.010
Al	5.411 ± 0.006
Zn	0.592 ± 0.011
Mn	0.232 ± 0.010
Ti	0.097 ± 0.006
Cu	0.087 ± 0.010
Pb	0.026 ± 0.001
Se	0.016 ± 0.001
Cr	0.012 ± 0.009
Cd	< 0.001

nitrite, respectively, were added. Afterwards, the overpressure evolution of the gas produced in the vial headspace throughout time was measured and recorded using a differential pressure transducer 0–5 psi, linearity 0.5% of full scale (Centerpoint Electronics). The maximum SAA (expressed as mg N/(g VSS·d)) was determined from the slope of the curve described by the cumulative N_2 production with the time and related to the biomass concentration in the vials measured as volatile suspended solids (VSS). The biomass concentration in the tests was of 4.5 ± 0.2 g VSS/L.

The tested metals were Cu^{+2}, Cr^{+2}, Pb^{+2}, Zn^{+2}, Ni^{+2}, Cd^{+2}, and Mn^{+2}. For each metal, ten different concentrations were applied in triplicate from 0 (control) to 240 mg/L.

In the case of Cd^{+2} tests the influence of the biomass structure on the estimated SAA was also verified. For this purpose SAA tests were performed with granular sludge completely disintegrated and homogenized with a blender.

2.3 Calculations

The determination of the inhibition percentage of anammox activity was calculated according to the following equation:

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{SAA}}{\text{SAA}_0}\right) \cdot 100$$

Where SAA and SAA_0 are the specific anammox activities measured in the batch tests with different metal concentrations and without metal (control test), respectively. The IC_{50} was determined as the concentration of the metal which led to a 50% inhibition of the SAA compared to that of the control test without metal (SAA_0) and was determined by using regression analysis of the curves shown in Fig. 1.

2.4 Analytical Methods

At the end of each activity test, the biomass concentration (g VSS/L) was determined according to Standard Methods (APHA-AWWA-WPCF 2005). The average diameter of the granules was determined using a stereomicroscope (Stemi 2000-C, Zeiss) for image acquisition and the software Image ProPlus® for image analysis. The concentration of metals in the granular sludge was determined by triplicate through ICP-MS spectroscopy (Agilent 7700x). The sludge was freeze and dried and then it was digested with NO_3H and H_2O_2 in a microwave (20 min, 190 °C) previous to the metal analysis.

3 Results and Discussion

3.1 Metals with High Inhibitory Effect: Copper and Chromium

The Cu^{+2} and Cr^{+2} ions caused the highest inhibitory effects on the anammox activity among the tested metals (Fig. 1), with IC_{50} values of 19.3 and 26.9 mg/L, respectively. From previous studies (Table 2), it can be observed that the inhibition of anammox activity by copper has been widely studied, because it is an important component of the nitrite reductase enzyme in the anammox bacteria (Daverey et al. 2014a). However, previous research works about the effect of chromium are scarce, despite of the fact that this can be one of the most problematic metals regarding anammox activity inhibition.

The curve describing the anammox activity inhibition at different copper concentrations (Fig. 1a) shows that the inhibition persisted at values of approximately 90–95% for concentrations higher than 75 mg Cu^{+2}/L. Daverey et al. (2014a) found that anammox bacteria activity was strongly affected by the presence of copper for biomass concentrations lower than 2 g VSS/L with an IC_{50} value of 4.2 mg Cu^{+2}/L. The type of biomass used by these authors corresponded to sludge performing simultaneous partial nitritation, anammox, and denitrifying processes, although no information was

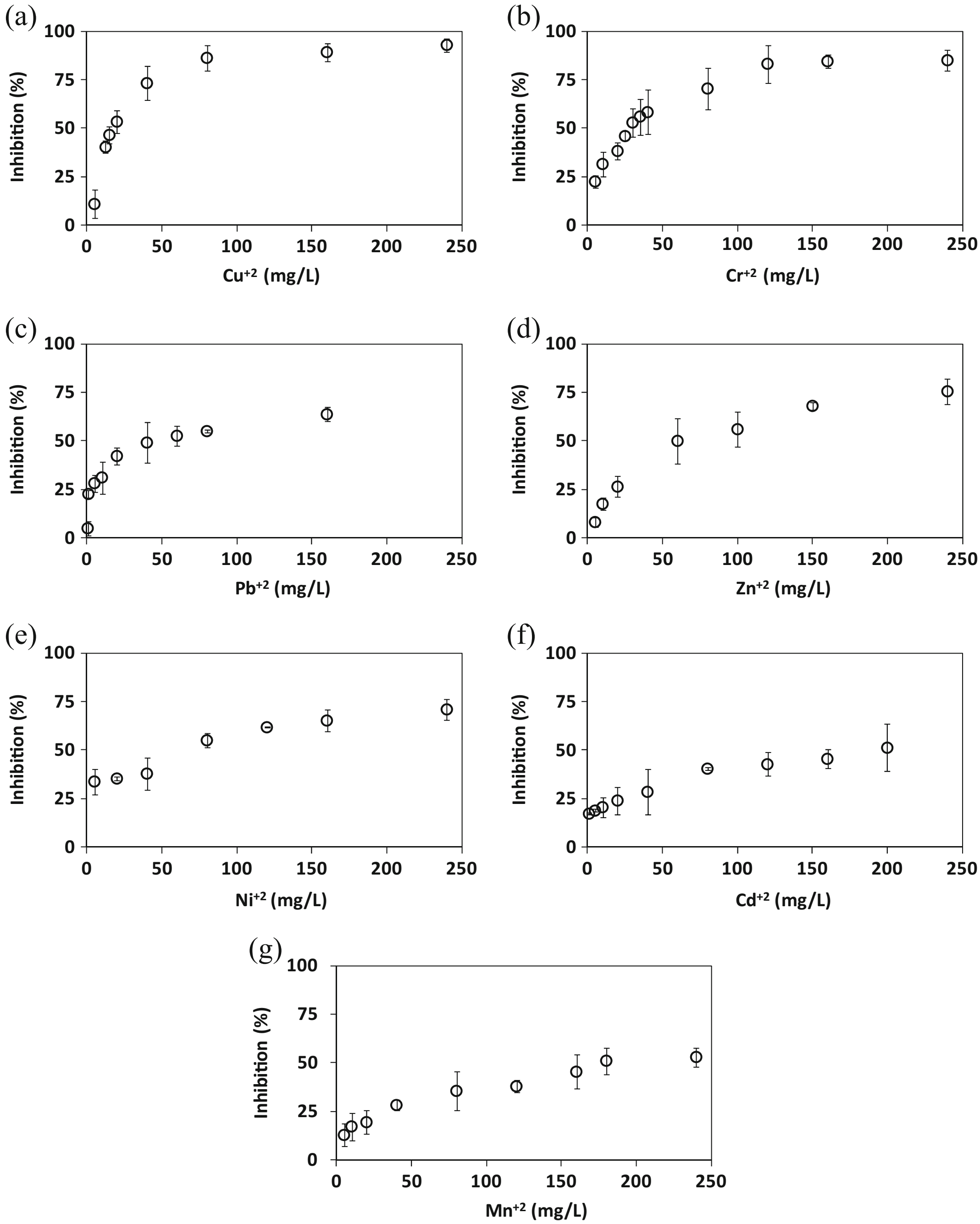

Fig. 1 Percentage of anammox activity inhibition at the different metal concentrations for: (**a**) copper, (**b**) chromium, (**c**) lead, (**d**) zinc, (**e**) nickel, (**f**) cadmium and (**g**) manganese

provided about its aggregation state (suspended, granular, biofilm, etc.). The results obtained by these authors are in the range of the IC_{50} values observed for copper by Li et al. (2015) (5.8 mg/L) and Lotti et al. (2012) (from 1.9 to 5 mg/L). Both studies were performed with anammox granules of moderate size (between 1 and 2 mm) containing mainly *Brocadia* sp. and with biomass concentrations lower than 1 g VSS/L (Table 2). Recently Zhang et al. (2016) also studied the effect of copper on anammox activity with anammox granules relatively large (5.1 mm) at a biomass concentration of 2 g VSS/L. These authors obtained a IC_{50} value of

Table 2 Overview of IC_{50} values for different heavy metals corresponding to experiments performed with anammox biomass in batch tests

Conditions of the batch experiments						Values for IC_{50} (mg/L)							
Type of sludge	Main bacterial population	g VSS/L	Acclimation time	SAA_0 mg N / g VSS·d	Type of test[b]	Cu^{+2}	Cr^{+2}	Pb^{+2}	Zn^{+2}	Ni^{+2}	Cd^{+2}	Mn^{+2}	Reference
PN/anammox granules (3.2 mm)	*Brocadia Fulgida*	4.5	12 h	284.6	Gas (30 °C)	19.3	26.9	45.6	59.1	69.2	174.6 93.1[d]	175.8	This study
Anammox	KSU-1 strain	0.854	24 h	222.7	Liquid	–	–	40 (7.2%)[c]	–	–	11.16 ± 0.42	–	[1]
SNAD[a]	–	1.3–1.9	3–24 h	–	Gas (35 °C)	4.2	–	–	–	–	–	–	[2]
SNAD[a]	–	< 2	–	–	Gas (35 °C)	–	–	–	6.9–11.5	–	–	–	[3]
Anammox granules (1–2 mm)	*Brocadia* sp.	0.74	1–24 h	680.0	Gas (30 °C)	1.9–5	–	–	3.9–4.5	–	–	–	[4]
Anammox granules (2.4 ± 0.6 mm)	*Brocadia*	0.68	2 h	280.8	Liquid and gas (30 °C)	5.8	–	75 (32%)[c]	16.6	73.6	26.8	–	[5]
Anammox granules (5.1 mm)	*Kuenenia Stutt*	2	–	444.4	Liquid (35 °C)	30	–	–	25	–	–	–	[6]
Anammox in gel carriers	*K. stuttgartiensis* KSU-1, *Brocadia anammoxidans*	–	–	–	Continuous feeding (30 °C)	6.5	–	–	12.5	7.9	–	–	[7]
Anammox granules (2.5–5.0 mm)	*Brocadia* sp.	–	8	450	Liquid (37 °C)	–	9.8[e]	10.4	–	–	7	–	[8]
Anammox granules (2.62 ± 0.63 mm)	–	–	0	≈ 500	Liquid (35 °C)	–	–	–	–	–	–	7.33	[9]

[1] Bi et al. 2014; [2] Daverey et al. 2014a; [3] Daverey et al. 2014b; [4] Lotti et al. 2012; [5] Li et al. 2015; [6] Zhang et al. 2016; [7] Kimura and Isaka 2014; [8] Yu et al. 2016 ; [9] Xu et al. 2017

[a] SNAD simultaneous partial nitritation, anammox, and denitrification

[b] Batch tests performed following the composition of the liquid or the gas phase

[c] Percentage of anammox activity decrease at this metal concentration

[d] Value of IC_{50} for the mechanically disintegrated granules

[e] Value of IC_{50} for Cr(VI)

30 mg Cu^{+2}/L; that is much higher than the values obtained by the other authors and in the range of the value obtained in this study (19.3 mg Cu^{+2}/L). The differences in the IC_{50} values found can be attributed to different variables according to the review of Table 2: the type/size of sludge, the predominant anammox bacteria specie, the concentration of biomass in the tests, the acclimation period, and the maximum specific anammox activity of the sludge of reference (SAA_0).

The inhibition curve obtained for chromium was very similar to that for Cu^{+2} (Fig. 1b), as well as the IC_{50} with a value of 26.9 mg Cr^{+2}/L. For concentrations higher than 125 mg Cr^{+2}/L, the inhibition was maintained leveled at around 85%. Although the chromium is a metal commonly present in landfill leachate and its concentration vary between 0.01 and 0.17 mg/L (Mavakala et al. 2016), there are not many specific research works that reported on the effect of this metal on anammox activity. Yu et al. (2016) obtained an IC_{50} value of 9.8 mg Cr^{+6}/L. These authors studied the Cr^{+6} because it is more water soluble and toxic than Cr^{+3} and Cr^{+2}. However, the results obtained in the present research work show that the inhibition caused by Cr^{+2} is as relevant as the one expected for Cu^{+2}.

3.2 Metals with Moderate Inhibitory Effect: Lead, Zinc and Nickel

The IC_{50} values obtained for Pb^{+2}, Zn^{+2}, and Ni^{+2} were 45.6, 59.1, and 69.2 mg/L, respectively, which compared with the other tested metals in this research study can be considered as moderate inhibitory metals.

The value of IC_{50} obtained for lead was much higher than the one observed by Yu et al. (2016) (10.4 mg Pb^{+2}/L). Other research studies that reported on the effect of lead on the anammox activity do not provide the IC_{50} value because they did not test concentrations high enough to achieve the 50% inhibition. Bi et al. (2014) obtained an inhibition of 7.2% at 40 mg Pb^{+2}/L, and Li et al. (2015) an inhibition of 32% at 75 mg Pb^{+2}/L, which means even lower inhibitory effect than the observed in this study. However, Bi et al. (2014) explained that the medium used in their tests (a bicarbonate buffer) favored lead precipitation, resulting in lower concentration of soluble lead ion and, consequently, lower apparent anammox inhibition. Li et al. (2015) also observed that a considerable amount of cationic metals added in the inhibitory tests was not present in the soluble phase at the end of the assay, indicating possible precipitation processes. In the case of the lead these authors observed, among all metals tested, the higher disappearance of this cation from the soluble phase (only 8% of initial 75 mg Pb^{+2}/L was present at the end of the assay). Unfortunately, in the present research work, the possible precipitation of metals or their removal from the liquid phase (sludge adsorption, for example) from the beginning to the end of the experiments was not measured.

In the case of zinc, the value of IC_{50} obtained was of 59.1 mg Zn^{+2}/L (Table 2), and it is a low inhibitory effect compared with other research works. The higher inhibition for zinc was observed by Lotti et al. (2012), who determined an IC_{50} value between 3.9 and 4.5 mg Zn^{+2}/L. Daverey et al. (2014b) and Li et al. (2015) obtained similar IC_{50} values for concentrations of 11.5 and 16.6 mg Zn^{+2}/L, respectively. The lower inhibition value was observed by Zhang et al. (2016) with an IC_{50} of 25 mg Zn^{+2}/L. All these different values pointed out that it is not possible to have a consensus about the exact IC_{50}, and it will depend on different factors, as reflected in Table 2. It is clear that zinc is one of the reported metals that can be challenging for anammox process application.

The value for IC_{50} obtained for nickel was 70 mg Ni^{+2}/L, which was very close to the value observed by Li et al. (2015). However, in a continuous feeding system with anammox bacteria immobilized in gel carriers, Kimura and Isaka (2014) observed a strong inhibition with an IC_{50} value of only 7.9 mg Ni^{+2}/L. These authors pointed out that the characteristics of the experiments performed in the different studies provoked the wide difference in the results obtained for anammox bacteria activity.

3.3 Metals with Lower Inhibitory Effect: Cadmium and Manganese

The value of IC_{50} obtained for cadmium was 175 mg Cd^{+2}/L. This value is high in comparison with other research works that reported values of 7.0 mg Cd^{+2}/L (Yu et al. 2016), 11.16 mg Cd^{+2}/L (Bi et al. 2014), and 26.8 mg Cd^{+2}/L (Li et al. 2015). Experiments were also performed with Cd^{+2} ion, using granular sludge mechanically disaggregated to study the effect of the granular structure as a protection of anammox bacteria from the toxic compounds. After the granules disintegration, the average value of measured maximum anammox activity (SSA_0) was similar as before of 284 ± 10 mg N/g VSS·d. This result indicates that the mechanical disintegration did not affect the anammox bacteria activity. However, in the presence of cadmium ion, the obtained IC_{50} value (93.1 mg Cd^{+2}/L) was lower than that obtained for granular sludge. The comparison of the IC_{50} values of entire and disintegrated granules shows that the granular structure plays an important role in the protection of anammox bacteria from the toxic effect of metals.

In the case of the manganese the IC_{50} value obtained was 175.8 mg Mn^{+2}/L. As chromium, the toxic effect of manganese over anammox activity was scarcely studied. Recently, Xu et al. (2017) published a IC_{50} value of only 7.33 mg Mn^{+2}/L in batch tests, although they operated an anammox reactor treating an increasing concentration up to 200 mg Mn^{+2}/L with good nitrogen removal percentages (approximately 90%). The results of these authors regarding batch tests and continuous operation exposed to the metal (manganese) differ a lot. However, the successful operation of the anammox reactor under concentrations of manganese as high as 200 mg Mn^{+2}/L indicates that this metal has a low inhibitory effect over anammox activity, confirming the high IC_{50} value obtained in the present research study.

3.4 Analysis of Batch Tests Conditions to Determine the IC_{50} Value

The comparison of the IC_{50} values obtained in the present study with other published values (Table 2) underlines that the conditions to perform the tests strongly influence the obtained results. Therefore, although the batch tests can be useful to know the response of certain type of biomass to the presence of metals, the results obtained cannot be extrapolated to all type of anammox sludge.

For example the acclimation time is important in the case of granular sludge due to diffusion phenomena. If enough time is not provided before the beginning of the test, a gradient of metal concentration occurs between the surface and the center of the granule, which affects the results of SAA as demonstrated by Lotti et al. (2012). These authors studied the inhibition caused by copper and zinc over the anammox activity after 1, 8, and 24 h of exposition. They observed a high difference in the inhibition curves obtained in the case of copper after 1 and 24 h of exposition, although for zinc, this difference was not so high. In the present research work the exposure time of the partial nitritation-anammox granular biomass to the metal concentrations was of 12 h, which is enough time to discard diffusion effects according to the results obtained by other authors (Daverey et al. 2014a; Lotti et al. 2012).

Another factor that needs to be considered is the adsorption of the metal into the biomass. Therefore, the concentration of biomass plays an important role, because high concentrations involve large adsorption capacity. In the case of granular sludge the adsorption occurs mainly in the external layers, which facilitates the protection of the microorganisms located in the inner zones of the aggregates. The IC_{50} values for Cu^{+2}, Zn^{+2}, Cd^{+2}, and Ni^{+2} obtained in the present research work, performed with granules containing ammonia oxidizing and anammox bacteria, were higher than those previously obtained with anammox granules by Li et al. (2015). This fact might indicate that not only the granular structure but also the presence of an external layer of AOB could help to protect anammox bacteria from the toxic compounds, probably because the AOB layer acts as an adsorbent of the metals. Although, more research work is needed to confirm this initial observation.

Another important parameter to consider is the origin of the biomass used to perform the tests. The most part of the published studies used anammox biomass collected from laboratory reactors fed with synthetic media and without previous exposition to metals, apart from those normally present in the trace elements solutions added with the feeding. In the present research work, the granular partial nitritation-anammox biomass was obtained from a pilot plant treating the supernatant of a sludge anaerobic digester. Although the concentration of metals in this effluent was not known to consider the previous exposition of the biomass, the characterization of the granular sludge in terms of metal concentration (Table 1) indicates a high content of metals, which can also explain the high IC_{50} values obtained.

4 Conclusions

The values of IC_{50} for anammox bacteria activity, in the presence of different metals, found in the literature differ significantly from those obtained in the present study. The review performed highlights the importance of the experimental conditions (biomass concentration, type of sludge, acclimation period, etc.) in the final results. The experimental results from this research showed that the granular structure and the presence of ammonium oxidizing bacteria in the external layers of the granules could act as a barrier to protect anammox bacteria from the toxic effect of metals. Furthermore, the IC_{50} values obtained confirmed that copper exerts a high inhibitory effect on anammox activity and showed that chromium, not well studied up to date, can have a similar inhibition potential. These results provide an insight on the importance of the evaluation of the loss SAA due to the presence of metals in the effluent to be treated by certain sludge previous to its use as inoculum for the start-up of a reactor.

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