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Inhibition of respiration and distribution of Cd, Pb, Hg, Ag and Cr species in a nitrifying sludge

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ABSTRACT

The study investigated the inhibitory effects of the heavy metals Cd, Pb, Hg, Ag and Cr (as Cr^{3+} and Cr^{6+}) on a nitrifying sludge. The aim was to assess the IC_{50} concentrations leading to 50% inhibition. The method is based on respiration of nitrifying sludge in the presence of these metals. Both O_2 consumption and CO_2 production were taken into account. The order of the inhibitory effect was Ag > Hg > Cd > Cr^{3+} = Cr^{6+}. Metal speciation was calculated in terms of free metal, inorganic metal complexes and bound metal. Pb largely precipitated and 50% inhibition was never reached. Ag was always in the form of the free ion or labile complexes. Hg had apparently a lower toxicity than Ag, since most of it was initially highly complexed with ammonia. Cd was present in the form of free ion and complexes which caused inhibition although a large part of them were precipitated. The inhibitory effects of trivalent chromium (Cr^{3+}) and hexavalent chromium (Cr^{6+}) were similar. The latter was present in the form of the anion CrO_4^{2-} and was not taken up by biomass. The study highlighted that IC_{50} values alone do not have an explanatory power of inhibition unless speciation is also considered.

1. Introduction

Heavy metals enter aquatic systems from sources such as industrial effluents, landfill leachate and municipal wastewaters. Depending on the type and concentration of heavy metal, the inhibitory effects may show great variations in natural, contaminated, or man-made systems [1]. In water quality studies it is often emphasized that the characteristics of water modify the speciation of a metal, its bioavailability and mobility [1,2]. This fact is also becoming more evident for regulatory authorities and a better understanding is required for the speciation of pollutant metals in wastewater effluents and surface runoff for the development of water quality regulations [3].

The same type of approach as in water quality studies has to be followed in wastewater treatment to predict the fates and control the effects of individual metals in municipal and industrial wastewaters. Industrial effluents containing various types of heavy metals are discharged into either receiving waters or publicly owned treatment works (POTWs). In both cases, in discharge standards total heavy metal is taken into consideration. However, in order to gain an insight into the effects of metals in treatment systems and receiving media, it is essential to understand their speciation in the presence of ligands. This approach may also help the development of regulations for wastewater discharge and sludge use.

Cadmium is a metal exhibiting a toxic effect at even low concentrations and is therefore considered to be a nonessential metal. The major sources of cadmium are electroplating, smelting, alloy manufacturing, pigments, plastic, battery, mining and refining processes.

Pb is a toxic metal and the major industrial sources of lead are battery manufacturing, printing and pigment, metal plating and finishing, ammunition, soldering material, ceramic and glass industries, iron and steel manufacturing.

Among heavy metals discharged into receiving waters and POTWs, the most stringent discharge standard is set for Hg due to bioaccumulation and toxicity properties.

Silver is primarily used in electronics, jewelry, solder, bearings, and for medical and dental applications. In one industrial wastewater derived from the photographic film industry, the Ag concentration was 0.077 mg/L, whereas other constituents such as COD, BOD, total nitrogen, As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn were also present [4]. Photoprocessing facilities produce wastewaters having concentrations of 0.4 and 1.1 mg/L depending on the presence or absence of recovery, respectively. The total silver concentration in POTWs is reported to range from 0.004 to 0.10 mg/L [5]. Data about silver toxicity is variable since Ag may be in the form of species such as Ag_2S and elemental silver. It is reported that operational problems were not encountered in an activated sludge system when the silver concentration in a photoprocessing wastewater was 1.85 mg/L [5]. However, Ag was present in a medium

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containing sulfate and thiosulfate leading to the formation of stable complexes and probably the free ion concentration was too low to cause toxicity.

In the case of Cr two different forms are of interest in wastewater treatment. Generally, hexavalent chromium (Cr^{6+}) toxicity is regarded to be much higher than the trivalent (Cr^{3+}) form. The usual practice in Cr^{6+} removal is the reduction to Cr^{3+} followed by precipitation. Soluble and particulate Cr^{3+} is a particularly important pollutant in tannery wastewaters. The total chromium concentration of the primary settling effluent of tannery wastewaters may be as high as 40–65 mg/L [6] whereas much higher values about 100–500 mg Cr^{3+}/L were also reported [7]. Aerobic biological treatment is usually carried out for simultaneous removal of organic carbon and nitrogen from tannery effluents which have strong wastewater characteristics. Trivalent chromium salts are also used in textile dying, in the ceramic and glass industry, and in photography. On the other hand, hexavalent chromium (Cr^{6+}) salts are used extensively in the metal finishing and plating industries, in the leather industry as a tanning agent, and in the manufacture of paints, dyes, explosives, and ceramics.

As known nitrification is the most crucial step in biological nutrient removal. Nitrifiers are sensitive to most heavy metals as well as to other substances. However, studies addressing metal speciation are rather rare. In particular, in nitrifying activated sludge and biofilm systems speciation of heavy metals is usually ignored. This fact may be one of the reasons of the wide IC_{50} range reported for heavy metals. Another reason is that toxicity of heavy metals has been rarely tested in a nitrification system alone [8–11], but in organic carbon removal systems having a high heterotrophic activity. Although this is the real case, in such a system the effect of heavy metals on nitrification may be influenced by the presence of organic matter which also complexes with metals.

In accordance with this discussion, the aim of the present study is to assess the individual inhibitory effect of the heavy metals Cd, Pb, Hg, Ag and Cr (as Cr^{3+} and Cr^{6+}) on a nitrifying sludge and to evaluate the IC_{50} concentrations in a comparative way by taking into consideration also the speciation properties. These metals are regarded as potentially toxic and differ from others in the way that they are not micronutrients for growth except for Cr^{3+} which is regarded as such [12]. Respiration is one of the most applicable methods in assessing the toxicity of several compounds to aerobic bacteria. Almost all studies use oxygen consumption as an indicator of respiration whereas CO_2 measurements are rarely employed. In the present study, the response of a nitrifying sludge to individual metals was evaluated using a respirometric procedure relying on both O_2 and CO_2 measurements. IC_{50} values were calculated for each metal based on O_2 and CO_2 measurements. Further, a novelty of this study was that in interpretation of metal toxicity, also theoretical speciation of metals was taken into consideration along with analytical measurements.

2. Materials and methods

2.1. Nitrifying sludge and feed

The sludge was taken from the recycle line of the municipal wastewater treatment plant Paşaköy in Istanbul. Paşaköy WWTP has a design flow rate of 100 000 m^3/day and is operated for carbon and nutrient removal according to the A_2/O (anerobic–anoxic–oxic) process. The total sludge age is around 15 days and the whole system is low-loaded. Sludge is wasted from the aerobic tank which based on extended aeration operation.

The sludge taken from this plant was then fed in a laboratory-size batch activated sludge reactor of 14 L on a fill-and-draw mode. The initial concentrations were 7.14 mM $(NH_4)_2SO_4$ (equivalent to 100 mg/L NH_4^+–N), 14.14 mM $NaHCO_3$, 0.1 mM $MgSO_4{\cdot}7H_2O$, 0.013 mM $CaCO_3$, 0.018 mM $FeSO_4{\cdot}7H_2O$, 0.015 mM $MnSO_4{\cdot}H_2O$, 0.022 mM K_2HPO_4 after daily feeding. The feed did not contain any organic matter in order to avoid heterotrophic activity. Regularly, the ammonium removal capacity of sludge was monitored by the measurement of ammonium (NH_4^+–N) concentrations, pH, DO and MLVSS concentrations. As a result of this feeding, the sludge became enriched in nitrifiers. The bacteria in this main sludge were occasionally monitored during the experimental period using the fluorescence *in situ* hybridization (FISH) technique. The ammonium oxidizing species *Nitrosomonas* were initially present in the sludge and became dominant with respect to time. As time progressed, the nitrite oxidizing *Nitrobacter* species became more dominant in the sludge than the *Nitrospira* that were initially present. All sludge samples used in inhibition studies in Section 2.2 were taken from this main reactor which was not acclimated to metal.

2.2. Inhibition measurements on the nitrifying sludge using respirometry

2.2.1. Respirometer operation and raw results

The effects of Cd, Pb, Hg, Ag, and two different forms of Cr, namely Cr^{3+} and Cr^{6+}, were tested individually using the automated Columbus Oxymax-ER 10 Gas Respirometer equipped with O_2 and CO_2 sensors [13]. The principle of operation relies on the circulation of air through chambers whereas the liquid in chambers remains static (static liquid-flowing gas respirometer). The aerated respirometer chambers function as bioreactors and O_2 and CO_2 levels in the gas phase are continuously measured with respect to time.

The medium in respirometric chambers is explained in Section 2.2.2. Nitrifiers in the sludge consumed O_2 in response to ammonium and nitrite oxidation. During this reaction also bicarbonate in the liquid phase was consumed, alkalinity was reduced and CO_2 was produced. Neglecting the cell synthesis this reaction may be written as shown in Eq. (1).

$$NH_4^+ + 2O_2 + 2HCO_3^- \rightarrow NO_3^- + 2CO_2 + 3H_2O \quad (1)$$

As a result of nitrification, in the liquid phase of respirometric chambers pH decreased slightly. The net effect was that CO_2 was released into the gas phase which was then recorded as CO_2 production. Thus, O_2 consumption from the gas phase was paralleled by production or release of CO_2 into the gas phase which was directly related to the nitrification rate. In each run, data were obtained in the respirometer with respect to time in terms of O_2 consumption rate (in mgO_2/h), cumulative O_2 consumption (in mgO_2), CO_2 production rate (in $mgCO_2$/h), and cumulative CO_2 production (in $mgCO_2$). The total duration of each run was adjusted to 16 intervals in the instrument which approximately corresponded to 21 h. Elongation of time to 24 h did not change the general pattern of O_2 and CO_2 exertion. Addition of metal inhibited both O_2 consumption and CO_2 production. Fig. 1 illustrates the course of cumulative O_2 consumption and CO_2 production in a typical run.

2.2.2. Composition of feed in respirometric chambers

In total, 30 different runs were conducted with the metals Cd, Pb, Hg, Ag, Cr^{3+} and Cr^{6+} to find the inhibitory range and to ensure reproducibility of results. With each metal, at least three sets of runs were conducted. The widest concentration range tested depended on the individual effect of each metal and was as follows—Cd: 0–50 mg/L; Pb:0–50 mg/L; Hg: 0–15 mg/L; Ag: 0–5 mg/L; Cr^{3+}: 0–100 mg/L; Cr^{6+}: 0–200 mg/L.

Ten respirometer chambers were operated in parallel where two of them served as control reactors. The initial ammonium concentration was adjusted to 3.57 mM NH_4^+–N (equivalent to

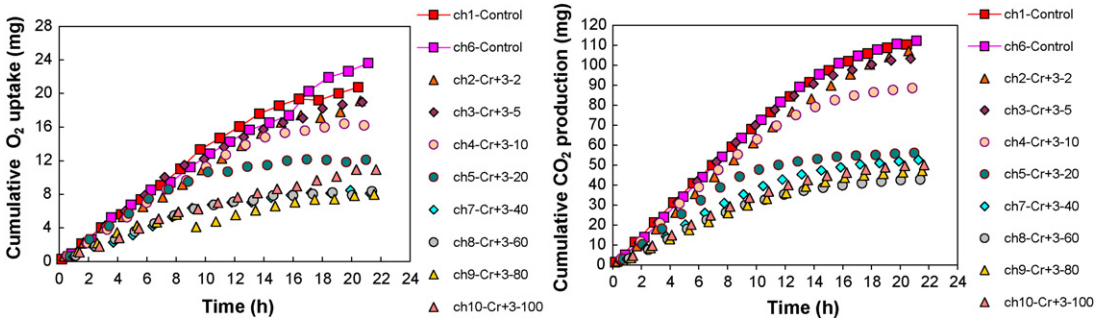

Fig. 1. Typical cumulative O_2 consumption (left) and cumulative CO_2 production (right) during the course of a metal inhibition test with Cr^{3+}.

50 mg/L NH_4^+–N) using 1.78 mM $(NH_4)_2SO_4$ to simulate a municipal wastewater. Alkalinity was added in the form of $NaHCO_3$ in stoichiometric proportions (7.1 mM). The concentrations of other constituents in the final medium were 0.05 mM $MgSO_4{\cdot}7H_2O$, 0.0064 mM $CaCO_3$, 0.009 mM $FeSO_4{\cdot}7H_2O$, 0.0074 mM $MnSO_4{\cdot}H_2O$, 0.011 mM K_2HPO_4.

Following the adjustment of pH to about 7, the metal was added to each chamber at variable concentrations. ICP standards (Merck) having a concentration of 1000 mg/L metal in nitric acid were used for this purpose. In the case of Cr^{6+}, standard solutions were prepared using K_2CrO_4. The pH of the medium was checked before addition of biomass such that it was not exposed to extreme pH conditions. A sludge sample taken from the nitrification reactor was settled and washed thoroughly with deionized water. Then, 20 mL from this sludge was added into each respirometer chamber. The liquid volume in all chambers was finally adjusted to 100 mL with deionized water. The initial pH was adjusted nearly to 7.5 with NaOH. In chambers the average MLVSS and MLSS concentrations were about 520 and 690 mg/L, respectively.

2.3. Analytical determinations

At the end of each run, the ammonium nitrogen concentrations, pH, MLSS and MLVSS in each respirometer chamber were measured. Ammonium nitrogen was determined according to the Nessler Method shown in the HACH Method 8038 [14]. pH was measured with the pH meter WTW Inolab. MLSS and MLVSS analyses were carried out in accordance with Standard Methods [15]. After the run, the samples were filtered through 0.45 μm Whatman GF 6 filters and the residual soluble metal was measured by Atomic Absorption Spectrometry (AAS) using Perkin Elmer AAnalyst 300. In some Cd sets, soluble Cd was also measured by the voltammetry apparatus VA 797 Computrace, Metrohm Inc. using the differential pulse polarography (DPP) in the dropping mercury electrode (DME) mode. The results obtained by voltammetry were compared with AAS analyses. Hg and Pb were analyzed using the ICP Instrument Perkin Elmer OES Optima 2100 DV since their residual concentrations were very low. Hg analyses were made by the ICP boron hydride generation technique present in Standard Methods [15]. Final soluble Cr^{6+} was determined by the 3500-Cr Colorimetric method indicated in Standard Methods [15].

2.4. Evaluation of raw respirometric data and calculation of IC_{50} values

The accuracy of respirometric readings was checked with control tests in the absence of metal. Statistical analyses of 10 parallel controls showed that cumulative O_2 and CO_2 measurements yielded less scatter than the momentary rates in mg O_2 or CO_2/h. Therefore, in all evaluations the cumulative values were taken into consideration rather than the rates. As time progressed or the number of intervals increased, the 95% confidence intervals about the mean O_2 consumption became narrower. In any case, raw data obtained in terms of CO_2 production exhibited less fluc-

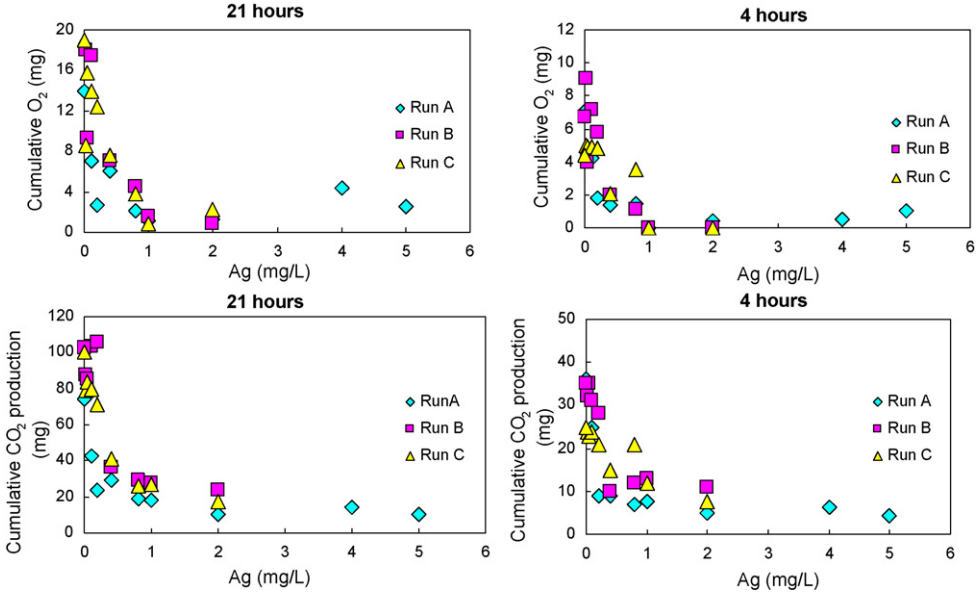

Fig. 2. Cumulative O_2 consumptions (upper figures) and CO_2 productions (lower figures) exerted up to 4 and 21 h in the presence of Ag.

tuations than O_2. For example, in the last interval of control tests the variation in CO_2 production was 2.46% about the mean, much lower than in the case of O_2 consumption which was about 9.75%. Obviously, the exchange of O_2 between the liquid and gas phases is more subject to disturbances while a much steadier equilibrium is reached between the bicarbonate in the medium and the CO_2 in the gas phase.

Further, all results indicated that short-term inhibition tests below 4 h should be avoided since they may lead to misinterpretations. Therefore, only data from 4 h onwards were used in the calculation of IC_{50} values.

In our case complete inhibition was not reached for each metal. At the maximum dose the inhibition amounted to about 64% for Cr^{3+}, 96% for Ag, 80% for Hg, 70% for Cr^{6+}, nearly 100% for Cd. The extent of inhibition was even lower when calculations were made on CO_2 basis. Therefore, the metal concentrations corresponding to 50% inhibition reflect the IC_{50} values and not the EC_{50} values which are considered when a substance leads to 100% inhibition [16].

IC_{50} values were calculated for different exposure times to find out whether this had a pronounced effect. For this purpose, the cumulative O_2 consumption and CO_2 production corresponding to 4, 8, 12, and 21 h were taken into consideration. As an example, Fig. 2 demonstrates the cumulative O_2 and CO_2 values in relation to metal dose at an early period (4 h) and at the end (21 h) of a typical test. A software was used for the calculation of IC_{50} from O_2 and CO_2 data by nonlinear regression [17]. The inhibition in ammonium removal was also compared with O_2 and CO_2 data. At least three runs were conducted with each metal and the results of at least two runs were selected in the estimation of IC_{50} for each metal.

2.5. Theoretical heavy metal speciation under test conditions

As shown in Tables 2–7, metal speciation was theoretically calculated in terms of free metal, inorganic metal complexes and metal bound to biomass using a chemical equilibrium program [18]. Throughout the speciation tables, the biomass-bound metal was expressed as CdB, PbB, HgB, AgB and CrB, respectively. Initial speciations shown in these tables were calculated based on metal addition, the concentrations of anions and cations in the medium and the pH value. The error in metal addition was approximately 5–10%. The uptake of metal onto biomass was disregarded. In all initial calculations the assumption was made that precipitation was negligible. Speciation of metals exhibited a dynamic pattern during respirometric measurements since both pH and composition changed as a result of nitrification. Therefore, metal speciation was recalculated under final conditions, using the metal dose, final pH, final ammonium concentrations, etc. In this case, the sorption onto biomass or formation of biomass-bound metal, was also included in theoretical calculations. In these calculations the metal-biomass partition coefficients (K_p) for each metal were estimated from the data pairs on biosorbed metal concentration (mg metal/g SS) and soluble metal concentration (mg/L) by using the linear regression method. The partition coefficients were 3.77, 48.36, 43.23 and 14.83 L/g SS for Cd, Hg, Ag, and Cr^{3+}, respectively. A sorption reaction was included for each metal in the MINTEQA2 program [18] to calculate the concentration of biosorbed metal.

3. Results and discussion

3.1. IC_{50} values for each metal

Table 1 shows the IC_{50} values along with their 95% confidence intervals expressed on the basis of O_2 consumption and CO_2 production. Usually a better fit was obtained when CO_2 data were used. As mentioned in Section 2.4, raw CO_2 data exhibited less variation

Table 1
Calculation of IC_{50} values based on O_2 and CO_2 measurements until 4, 8, 12 and 21 h.

Metals	IC_{50} basis	21 h-IC_{50} (mg/L)	Confidence range	R^2	12 h-IC_{50} (mg/L)	Confidence range	R^2	8 h-IC_{50} (mg/L)	Confidence range	R^2	4 h-IC_{50} (mg/L)	Confidence range	R^2
Cd	O_2	10.65	8.20–13.11	0.91	9.94	7.67–12.22	0.92	8.84	6.38–11.30	0.87	12.01	9.87–14.15	0.91
	CO_2	19.90	15.86–23.93	0.91	17.42	14.60–20.25	0.96	16.53	13.77–19.29	0.94	20.02	16.94–23.11	0.95
Hg	O_2	10.82	8.28–14.14	0.81	10.72	5.27–16.17	0.66	9.19	3.80–14.58	0.62	8.62	2.19–15.06	0.53
	CO_2	14.94	10.79–20.69	0.87	12.29	8.02–16.55	0.86	12.91	7.95–17.87	0.84	18.39	2.43–34.35	0.57
Ag	O_2	0.33	0.26–0.42	0.91	0.75	0.37–1.50	0.68	0.40	0.24–0.67	0.73	0.44	0.27–0.73	0.76
	CO_2	0.42	0.28–0.56	0.85	0.38	0.29–0.50	0.90	0.39	0.30–0.51	0.92	0.80	0.48–1.34	0.74
Cr^{3+}	O_2	37.38	28.41–49.17	0.90	57.21	36.95–77.46	0.85	76.39	26.09–126.70	0.71	75.43	30.27–120.60	0.64
	CO_2	44.49	31.62–57.36	0.89	55.07	40.56–69.58	0.93	65.75	46.59–84.91	0.91	59.60	40.91–78.29	0.89
Cr^{6+}	O_2	38.97	26.71–51.23	0.88	61.15	42.93–79.37	0.81	81.07	61.90–100.20	0.87	83.61	56.44–110.80	0.77
	CO_2	204.30	139.60–268.90	0.89	220.40	163.60–277.20	0.93	343.40	186.20–500.60	0.86	746.70	370.40–1123	0.91

Table 2
Theoretical speciation of Cd under initial and final test conditions.

Cd in species (mg/L)									
Total Cd (mg/L)	0	2	5	10	15	20	30	40	50
Initial speciation									
Cd^{2+}	0.00	1.51	3.79	7.55	11.24	15.13	22.91	30.19	37.92
$CdOH^{+}$	0.00	0.00	0.01	0.02	0.02	0.03	0.04	0.06	0.08
$CdSO_4$ (aq)	0.00	0.25	0.63	1.24	1.84	2.47	3.70	4.83	6.00
$Cd(SO_4)_2^{2-}$	0.00	0.01	0.01	0.03	0.04	0.06	0.09	0.11	0.14
$CdCO_3$ (aq)	0.00	0.22	0.52	1.08	1.68	2.15	3.02	4.48	5.47
$CdHCO_3^{+}$	0.00	0.01	0.04	0.07	0.11	0.15	0.22	0.29	0.36
$Cd(CO_3)_2^{2-}$	0.00	0.00	0.01	0.01	0.02	0.02	0.03	0.05	0.06
Final speciation									
Cd^{2+}	0.00	0.31	0.84	1.92	2.88	4.78	7.51	12.10	16.83
$CdOH^{+}$	0.00	0.00	0.00	0.00	0.02	0.01	0.05	0.07	0.10
$CdSO_4$ (aq)	0.00	0.02	0.06	0.20	0.41	0.52	1.18	1.93	2.73
$Cd(SO_4)_2^{2-}$	0.00	0.00	0.00	0.00	0.01	0.01	0.03	0.04	0.06
$CdCO_3$ (aq)	0.00	0.01	0.02	0.11	1.40	0.60	3.75	4.87	7.05
$CdHCO_3^{+}$	0.00	0.00	0.01	0.02	0.03	0.05	0.07	0.12	0.17
$Cd(CO_3)_2^{2-}$	0.00	0.00	0.00	0.00	0.05	0.01	0.15	0.16	0.24
CdB	0.00	1.65	4.07	7.75	10.16	14.03	17.27	20.73	22.84
Analytical measurement									
Final soluble Cd (mg/L)	0.00	0.02	0.20	0.35	0.25	0.61	0.53	0.56	0.79

and correlated well with nitrification rate. IC_{50} values corresponding to 50% inhibition in CO_2 production were always slightly higher than on O_2 basis indicating a lesser inhibition. The reason may be attributed to the relative CO_2 production rates and cumulative CO_2 production in uninhibited and metal inhibited chambers. The bicarbonate consumption is initially high in uninhibited (control) chambers where the nitrification proceeds rapidly. As known, the bicarbonate ion is converted into carbonic acid. This results finally in a high release of CO_2 into gas phase. After some period, as nitrification continues and pH drops slightly, the release rate of CO_2 from the liquid phase, or the CO_2 production rate in the gas phase decreases. On the other hand, in metal inhibited chambers, alkalinity consumption is less since nitrification is inhibited. This leads generally to a lower CO_2 production in the gas phase compared to the control. In such cases the pH in the entire period is at a higher level than in uninhibited controls. Finally, when the cumulative CO_2 in uninhibited (control) and metal inhibited chambers is compared, the CO_2 recordings in inhibited cases are slightly overestimated with respect to control. This results that CO_2-based IC_{50} values are higher than those determined on O_2 basis.

3.2. Inhibitory effects of metals and distribution of metal species in sludge

3.2.1. Cd

Throughout the study the stability of metal complexes was evaluated by considering the complex stability constants reported in the literature [19]. In Table 2 the typical Cd speciation is shown under initial and final conditions of a test. All species demonstrated in this table are regarded as soluble except the biomass-bound metal (CdB) at the end of the test. According to these calculations, the major part of Cd should initially be in the form of the free ion Cd^{2+}. At the end of the test, a significant part should remain in soluble phase and some part should be present as CdB (Table 2). However, measurements (last row in Table 2) showed that the final soluble Cd was much lower than theoretically calculated. Thus, in this nitrification medium a significant fraction of the initial Cd precipitated in the form of bicarbonate or carbonate species which have a relatively high complex stability. Precipitation of Cd was particularly evident above 4 mg/L. Therefore, initial and final free and labile Cd levels were actually lower than those shown in Table 2.

As shown in Table 1, when total Cd was considered, the 21 h-IC_{50} value was 10.65 mg/L (0.09 mM) as concluded from O_2 consumption while the CO_2-based IC_{50} value was higher as explained before.

Inhibition was only then obvious when this metal was added in a high range of 0–50 mg/L. In spite of precipitation of Cd, elevation of dose still increased the inhibitory effect. For example, at a dose of 40 mg/L total Cd, although the major part of Cd had precipitated, the inhibition was about 80%. 90% inhibition was observed at values exceeding 40 mg/L. On the other hand, in some runs in the low total Cd range of 0–5 mg/L, 50% inhibition could not be assessed clearly. The inhibitory effect of Cd became particularly evident at doses exceeding 10 mg/L. This leads to the assumption that also Cd precipitates are effective besides the labile forms. For Cd, the concentration leading to 10% inhibition (IC_{10}) was about 3.8 mg/L.

In the low Cd range of 0–5 mg/L, in some runs the final soluble Cd was measured by both AAS and voltammetry. At 5 mg/L total Cd, approximately less than 10% stayed finally in the dissolved phase. AAS measures all forms of Cd whereas the latter reflects labile Cd consisting of free Cd and Cd in weak complexes. The comparison showed that the soluble Cd levels measured by both methods had the same order of magnitude. This reflected that most of the final soluble Cd consisted of labile species. The stable part of Cd was eliminated from the soluble phase either by sorption or precipitation.

A characteristic of Cd was that the inhibitory effect was seen at early hours. The same type of behavior was also seen in the case of Ag which was however much more toxic than Cd.

3.2.2. Pb

As in the case of Cd, theoretical calculations showed that Pb formed initially complexes with hydroxide, sulfate and carbonate, but not with ammonia as seen in Table 3. The Pb–hydroxide complexes are rather weak whereas carbonate and bicarbonate complexes are rather strong as concluded from basic data [19]. But, unlike the case of Cd, initially the free ion levels (Pb^{2+}) were very low. In the range of 0–6 mg/L, the free ion amounted theoretically to about 10% of the total. The major part of Pb existed in the form of the carbonate complex $PbCO_3$ (aq) (about 45%) which is stable and has a precipitation potential as $PbCO_3$(s) ($\mathrm{Log}\,K = 13.13$). About 35% of Pb existed as $PbHCO_3^{+}$. Since Pb precipitated, the biosorption could not be calculated and no speciation could be predicted for final test conditions.

In several Pb runs an obvious inhibition was not recorded even in the range of 0–50 mg/L. This may be attributed to the very low free

Table 3
Theoretical speciation of Pb under initial test condition.

Pb in species (mg/L)									
Total Pb (mg/L)	0	0.1	0.4	0.6	0.8	1	2	4	6
Initial speciation									
Pb^{2+}	0.00	0.01	0.05	0.07	0.09	0.08	0.23	0.49	0.78
$PbOH^{+}$	0.00	0.00	0.01	0.01	0.02	0.02	0.04	0.08	0.11
$Pb(OH)_2$ (aq)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$Pb(OH)_3^{-}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pb_2OH^{3+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$Pb_3(OH)_4{}^{2+}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$Pb(OH)_4{}^{2-}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$Pb_4(OH)_4{}^{4+}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$PbSO_4$ (aq)	0.00	0.00	0.02	0.02	0.03	0.03	0.08	0.17	0.28
$Pb(SO_4)_2{}^{2-}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$Pb(CO_3)_2{}^{2-}$	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.02
$PbCO_3$ (aq)	0.00	0.05	0.20	0.31	0.41	0.61	1.00	1.91	2.74
$PbHCO_3^{+}$	0.00	0.03	0.13	0.18	0.25	0.24	0.64	1.33	2.08

ion concentrations at the beginning. Control tests in the absence of biomass showed that in the total Pb range of 2–40 mg/L the remaining soluble Pb was initially only about 1.5 mg/L. In the presence of biomass the final soluble Pb levels were even reduced below 0.05 mg/L. Since the inhibitory effect of Pb was generally less than 50%, IC_{50} values could not be calculated for this metal.

The results of other studies also point to the importance of speciation in toxicity studies. In a study it is reported that Pb concentrations of 40 mg/L have no significant effect on nitrification [20]. In that study, the pH value was in the range of 7.2 ± 0.1 and the low toxicity was probably due to precipitation of Pb in the presence of bicarbonate alkalinity. Our study points out that in nitrification systems supplied with a stoichiometric amount of alkalinity, it is unlikely that Pb leads to a serious inhibition.

3.2.3. Hg

As seen in Table 1, the 21 h-IC_{50} value for Hg in terms of O_2 consumption was 10.82 mg/L (0.05 mM). Thus, on molar basis Hg proved to be the second most toxic metal after Ag. Nonlinear regression results showed that the Hg concentration leading to 10% inhibition (IC_{10}) was below 2 mg/L. In the studied range the inhibitory effect did not reach 90%, but nonlinear regression results suggest that 90% inhibition will be observed at values exceeding 30 mg/L.

As shown in Table 4, initially the free ion (Hg^{2+}) concentration was extremely low, at about 1.02×10^{-9} mg/L at 15 mg/L total Hg. Hg was mostly present in the form of the ammonium complex $Hg(NH_3)_2{}^{2+}$. This ammonium complex has a relatively high complex stability [15], it is therefore unlikely that it rapidly dissociates into free ion. In prediction of Hg toxicity on nitrifying biomass, the complexation of this metal with ammonia should be always considered which probably was a factor leading to a relative alleviation of inhibition. In terms of ammonium availability there were no restrictions. Calculations showed that only a very small fraction of the ammonium initially present (about 3.4%) was bound as the Hg complex, the remaining part was still available for nitrification.

The complex $Hg(OH)_2$ is initially also important and has a relatively high stability [19]. It is reported to have a high affinity for many adsorbents [21], therefore it may be removed by sorption onto biomass. Other Hg complexes in the form of carbonates and bicarbonates had negligible concentrations. According to analytical measurements, the final soluble Hg levels were always low. At the maximum dose of 15 mg/L Hg, the final soluble Hg was about 0.35 mg/L, lower than predicted from theoretical calculations. This also showed the possibility of precipitation or sorption of soluble Hg complexes onto biomass.

Compared to the metals Ag or Cd, the inhibitory effect of Hg was less immediate, probably because most of Hg was initially held in the form of relatively stable complexes. As seen in Table 1, the regression coefficients were low and confidence intervals around the IC_{50} values were rather large at early hours.

The concentration of mercury in the influent of POTWs is reported to be as low as 50–500 ng/L [22]. Since in our study the 21 h-IC_{50} for Hg is 10.82 mg/L, at levels found in POTWs mercury will not have an effect on nitrification and organic carbon removal in POTWs. However, mercury concentrations may be crucial in industrial wastewater treatment. Ni, Cd, and Hg are found in the wastewater from flue gas desulfurisation unit at concentrations of hundreds of μg/L [23]. Industrial effluents such as chloralkali contain total mercury concentrations between 1.6 and 7.6 mg/L. At such high concentrations mercury is usually removed by physicochemical processes. However, as reported in a study, there are also

Table 4
Theoretical speciation of Hg under initial and final test conditions.

Hg in species (mg/L)									
Total Hg (mg/L)	0	0.5	1	2	4	6	8	10	15
Initial speciation									
Hg^{2+}	0	1.15×10^{-10}	8.40×10^{-11}	1.21×10^{-10}	3.04×10^{-10}	9.84×10^{-10}	1.32×10^{-9}	6.97×10^{-10}	1.02×10^{-9}
$Hg(OH)^2$	0.00	0.09	0.17	0.34	0.69	1.05	1.40	1.77	2.70
$Hg(NH_3)_2{}^{2+}$	0.00	0.41	0.83	1.65	3.30	4.94	6.57	8.20	12.25
$HgCO_3$ (aq)	0.00	0.00	0.00	0.01	0.01	0.02	0.02	0.03	0.04
Final speciation									
$Hg(OH)^2$	0.00	0.01	0.02	0.05	0.10	0.14	0.19	0.19	0.35
$Hg(NH_3)_2{}^{2+}$	0.00	0.01	0.03	0.08	0.17	0.44	0.47	0.43	1.22
$HgCO_3$ (aq)	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01
HgB	0.00	0.48	0.94	1.87	3.72	5.41	7.34	7.38	13.43
Analytical measurement									
Final soluble Hg(mg/L)	0.00	0.00	0.02	0.13	0.15	0.14	0.21	0.27	0.35

Table 5
Theoretical speciation of Ag under initial and final test conditions.

Ag in species (mg/L)									
Total Ag (mg/L)	0	0.025	0.05	0.1	0.2	0.4	0.8	1	2
Initial speciation									
Ag^{1+}	0.00	0.02	0.04	0.08	0.16	0.33	0.66	0.83	1.60
$AgSO_4^-$	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.04
$AgNH_3^+$	0.00	0.00	0.01	0.01	0.02	0.04	0.08	0.10	0.23
$Ag(NH_3)_2^+$	0.00	0.00	0.00	0.00	0.01	0.02	0.04	0.05	0.13
Final speciation									
Ag^{1+}	0.00	0.00	0.00	0.00	0.01	0.02	0.03	0.04	0.07
$AgNH_3^+$	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.03	0.05
$Ag(NH_3)_2^+$	0.00	0.00	0.00	0.00	0.00	0.01	0.07	0.10	0.15
AgB	0.00	0.02	0.05	0.10	0.19	0.36	0.68	0.84	1.72
Analytical measurement									
Final soluble Ag (mg/L)	0.000	0.000	0.004	0.000	0.007	0.028	0.056	0.073	0.137

attempts to apply biological processes. A mercury-resistant bacterial strain which is able to reduce ionic mercury to metallic mercury was used to remediate mercury-containing wastewater [24]. A recent study has shown that *Nitrosomonas europaea* cells exposed to Hg^{2+} had the ability to recover quickly from toxic effects, apparently associated with upregulation of the mercury resistance genes and amoA, but no such recovery was evident in Cd^{2+}– exposed cells [25].

3.2.4. Ag

Throughout our study, Ag proved to be the most toxic metal among all. As seen in Table 1, it had an 21 h-IC_{50} value of 0.33 mg/L (0.0031 mM) when O_2 consumption was considered. The IC_{50} value on CO_2 basis was higher and was calculated as 0.42 mg/L (0.0039 mM). On molar basis, the toxic effect of this metal was one to two orders of magnitude higher than other metals reported in this study. In the case of Ag, at already very low concentrations of this metal a high inhibitory effect was observed. The Ag concentration leading to 10% inhibition (IC_{10}) was about 0.07 mg/L while 90% inhibition was observed at 1.01 mg/L (IC_{90}).

Table 5 illustrates the typical theoretical speciation of Ag under initial and final test conditions. Initially, a significant part of Ag is present in the form of the free ion, Ag^+ in the nitrification medium. Also, the Ag–ammonia complexes shown in Table 5 are labile as concluded from stability constants in the literature [19] and can therefore easily dissociate into the free ion. This obviously led to the high toxicity of this metal. Ag did not form complexes with carbonates and sulfates which are considered stable.

According to the theoretical calculations shown in Table 5, after contact with biomass, most of the Ag is bound to biomass (AgB). The total soluble Ag concentrations finally measured were in agreement with the sum of theoretically soluble species shown in Table 5. Most probably it is the free form of Ag that is directly taken onto/into biomass causing toxicity, the key species emerge therefore as the free and biomass-bound Ag. Unlike the case of Hg, the IC_{50} values calculated based on total metal are meaningful indicators of real toxicity. Moreover, in all runs Ag exerted its toxicity immediately upon contact with biomass. Fig. 2 shows that at doses about 1 mg/L nearly complete inhibition of O_2 consumption was reached at 4 h.

According to these results, silver can be quite toxic on nitrifiers at levels usually found in POTWs if these receive silver input in a free form. Further, our speciation results demonstrate that a significant part of silver is bound to biological sludge which may be an important factor for sludge digestion. In the effluent of such a system, Ag is likely to remain in the form of the free ion and will further lead to toxicity in receiving waters.

3.2.5. Cr

3.2.5.1. Cr^{3+}. In terms of total Cr^{3+} added, the 21 h-IC_{50} was as 37.38 mg/L (0.72 mM), relatively higher than other metals indicating a lower toxicity on molar basis. As seen in Table 6, according to theoretical calculations, in the concentration range 0–100 mg/L

Table 6
Theoretical speciation of Cr^{3+} under initial and final test conditions.

Cr^{3+} in species (mg/L)									
Total Cr^{3+} (mg/L)	0	2	5	10	20	40	60	80	100
Initial speciation									
Cr^{3+}	0.00	1.38×10^{-5}	2.72×10^{-5}	6.28×10^{-5}	1.26×10^{-4}	2.31×10^{-4}	4.00×10^{-4}	4.90×10^{-4}	8.18×10^{-4}
$Cr(OH)_2^+$	0.00	1.7292	4.2819	8.5939	17.2347	34.2978	51.5741	68.8348	86.6673
$Cr(OH)^{2+}$	0.00	0.0585	0.1292	0.2781	0.5584	1.0632	1.7167	2.1924	3.1761
$Cr(OH)_3$ (aq)	0.00	0.1973	0.5480	1.0263	2.0572	4.2840	6.0048	8.3860	9.1918
$Cr(OH)_4^-$	0.00	0.0030	0.0094	0.0164	0.0329	0.0717	0.0939	0.1375	0.1313
CrO_2^-	0.00	0.0036	0.0111	0.0194	0.0390	0.0851	0.1114	0.1630	0.1557
$CrOHSO_4$ (aq)	0.00	0.0098	0.0217	0.0466	0.0932	0.1764	0.2829	0.3590	0.5164
Final speciation									
Cr^{3+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01
$Cr(OH)_2^+$	0.00	0.17	0.42	0.84	1.69	3.36	5.03	6.70	8.39
$Cr(OH)^{2+}$	0.00	0.03	0.11	0.29	0.53	1.17	1.90	2.58	3.16
$Cr(OH)_3$ (aq)	0.00	0.00	0.01	0.01	0.02	0.04	0.05	0.07	0.08
$CrSO_4^+$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$CrOHSO_4$ (aq)	0.00	0.00	0.01	0.03	0.07	0.15	0.26	0.34	0.42
CrB	0.00	1.79	4.45	8.83	17.70	35.28	52.77	70.29	87.92
Analytical measurement									
Final soluble Cr^3 (mg/L)	0.00	0.06	0.18	0.22	0.13	0.10	0.10	0.05	0.10

Table 7
Theoretical speciation of Cr^{6+} under the initial conditions of a test.

Cr^{6+} in species (mg/L)									
Total Cr (mg/L)	0	10	20	40	60	80	120	160	200
Initial speciation									
CrO_4^{2-}	0	9.21	18.47	36.77	55.11	73.72	110.58	146.87	183.52
$NaCrO_4^-$	0	0.20	0.40	0.79	1.16	1.53	2.24	2.89	3.53
$KCrO_4^-$	0	0.01	0.03	0.13	0.29	0.50	1.10	1.89	2.88
$HCrO_4^-$	0	0.55	1.11	2.28	3.22	4.26	6.12	8.36	10.03
$Cr_2O_7^{2-}$	0	0.00	0.00	0.01	0.02	0.03	0.07	0.12	0.18
Final analytical measurement									
Final soluble Cr^{6+} (mg/L)	0	7.74	16.59	32.86	43.68	68.27	117.26	156.35	207.73

almost none of soluble Cr^{3+} remained initially in the form of free ion Cr^{3+}, but about 86% was present as the hydroxide complex $Cr(OH)_2^+$. Also other hydroxide complexes such as $Cr(OH)_3$ (aq) and $Cr(OH)^{2+}$ were formed. $Cr(OH)_3$ (aq) constituted about 9% of the initial Cr and was relatively labile according to stability constants [19]. The other species $Cr(OH)^{2+}$ was stable but constituted only about 3% of initial Cr.

Even at very high Cr additions (up to 100 mg/L), the final soluble Cr levels were measured very low (<0.5 mg/L). Cr visibly precipitated above the dose of 20–30 mg/L, most probably in the form of hydroxides, which was then a factor for reduced inhibition. In addition, some of initial Cr complexes such as $Cr(OH)_2^+$ may be sorbed onto biomass. However, as in the case of Cd, in spite of precipitation, the inhibitory effect of Cr^{3+} increased with increasing dose showing that also particulate Cr had an effect. But, total inhibition was not observed in the range 0–100 mg/L. The maximum inhibition recorded was about 60% and was reached at 40 mg/L. IC_{50} values calculated at periods below 21 h had usually a large confidence interval indicating that 50% inhibition was not very pronounced (Table 1). After 21 h, 10% inhibition seemed to occur at a Cr^{3+} concentration below 10 mg/L while 90% inhibition could never be observed and safely predicted in the studied range.

3.2.5.2. Cr^{6+}. The inhibitory effect of Cr^{6+} was studied using K_2CrO_4. In contrast to expectations, the inhibitory effect of Cr^{6+} proved to be similar to Cr^{3+} and the 21 h-IC_{50} for Cr^{6+} was found as 38.97 mg/L (0.75 mM). For Cr^{6+} both 10% and 90% inhibition were not seen in the studied range. However, nonlinear regression results predict that IC_{10} should be below 1 mg/L whereas IC_{90} exceeded 400 mg/L.

Table 7 shows the theoretical speciation of hexavalent chromium after addition of chromium in the form of CrO_4^{2-}. As seen in this table, most of the chromium remains in the form of CrO_4^{2-}. Since this species and other species shown in Table 7 are negatively charged, they might not be sorbed to biomass surface which usually has also a negative charge. As seen in Table 7, final analytical measurements confirmed this low biosorption. Due to low biosorption speciation calculations could not be carried out under final conditions. The fact that the major part of Cr^{6+} remained in soluble phase and was not sorbed onto biomass was probably the major factor in the relatively low inhibition. In some studies it is reported that under certain conditions Cr^{6+} is reduced to Cr^{3+} in the presence of an organic electron donor [26–28]. In the current nitrification study there was no organic carbon in the medium. Further, our results indicated that the majority of Cr^{6+} remained in the bulk phase. Thus, even if intracellular Cr^{6+} reduction takes place, this amount should be very small. In terms of CO_2 inhibition, the effect of Cr^{6+} was lower (Table 1). The large difference between O_2- and CO_2-based IC_{50} results could not be explained.

Several contradictory findings exist in the literature on the behavior of Cr^{6+} in heterotrophic and/or autotrophic systems. In an activated sludge system in the range of 1–50 mg/L Cr^{6+} had no inhibitory effect on organic carbon removal and nitrification [29]. In another study Cr^{3+} increased the growth rate of activated sludge up to a concentration of 25 mg/L, but was toxic in the range of 80–160 mg/L [30]. On the other hand, Cr^{6+} increased the growth rate up to a concentration of 15 mg/L, but was toxic in the range of 160–320 mg/L. Other researchers found out that in a heterotrophic system Cr^{6+} increased the yield positively up to a concentration of 25 mg/L [31]. Others report that nitrifiers are much more sensitive to Cr^{6+} compared to heterotrophs and that even Cr^{6+} at a dose of 0.5 mg/L affects ammonium removal [32]. It should be pointed out that in all these studies calculation of different metal species was not intended.

4. Conclusions

The IC_{50} values calculated based on O_2 consumption were as Ag-IC_{50}: 0.0031 mM; Hg-IC_{50}: 0.05 mM; Cd-IC_{50}: 0.09 mM; Cr^{3+}-IC_{50}: 0.72 mM; Cr^{6+}-IC_{50}: 0.75 mM. The order of inhibitory effect was thus as follows in this nitrification system: Ag > Hg > Cd > Cr^{3+} = Cr^{6+}. The same order was also valid when IC_{50} values were expressed on CO_2 basis. Our results indicated that CO_2-based measurements were less subject to fluctuations and correlated well with nitrification rates in the presence of metals. Pb did not lead to 50% inhibition, therefore the IC_{50} value could not be determined.

The general conclusion was that in a nitrifying system total heavy metal concentrations do not have an explanatory power from the point of inhibition. The primary factor for inhibition is not the total metal concentration but the distribution of metal among species. Based on the IC_{50} value, Hg appears to have a lower toxicity than Ag, since most of Hg is initially complexed in the nitrification medium. On the other hand, Ag is always present in the form of free ion or labile complexes dissociating into free ion. Pb is apparently the least toxic metal, since this metal initially forms complexes which have a high precipitation potential. For this metal 50% inhibition was never reached under experimental conditions since soluble levels were very low. On the other hand, Cd formed the same type of complexes as Pb, but the labile fraction of Cd was initially higher. Thus, not only the types of metal species formed are relevant, but also their relative concentrations and stabilities. Among the metals for which the IC_{50} value could be calculated, the least toxic proved to be the chromium. Contrary to expectations, both in the form of trivalent chromium (Cr^{3+}) and hexavalent chromium (Cr^{6+}), the toxic effects of chromium were similar although each metal formed completely different species. Most of the hexavalent chromium was present in the form of the anion CrO_4^{2-} and remained in bulk water rather then being taken up by biomass.

The study emphasized the irrelevance of toxicity expressions based on IC_{50} alone and showed that IC_{50} values should not be compared directly without paying attention to speciation properties of each metal. The results of this study may help in understanding the underlying factors in inhibition studies and in plant operation.

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