



## Using fluorescence-based microplate assay to assess DOM-metal binding in reactive materials for treatment of acid mine drainage

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### Abstract

One potential drawback of compost-based passive bioreactors, which is a promising biotechnology for acid mine drainage (AMD) treatment, is the transport of dissolved organic matter (DOM)-metal complexes in surface waters. To address this problem, the objective of this study was to assess the maximum capacity of organic substrates to release soluble DOM-metal complexes in treated water. The reactivities of DOM in maple wood chips and sawdust, composted poultry manure, and leaf compost were quantified toward  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Cu}^{2+}$  using fluorescence quenching. The DOM showed the highest reactivity toward Fe, but a limited number of available sites for sorption, whereas DOM-Cd complexes exhibited the lowest fluorescence quenching. Overall, the DOM from a mixture of wastes formed higher concentrations of DOM-metal complexes relative to sole substrates. Among DOM-metal complexes, the concentrations of DOM-Ni complexes were the highest. After reaching steady-state, low concentrations of DOM-metal complexes were released in treated water, which is in agreement with theoretical predictions based on geochemical modeling. Therefore, in addition to physicochemical characterization, fluorescence quenching technique is recommended for the substrate selection of bioreactors.

**Key words:** passive bioreactors; acid mine drainage; DOM reactivity; fluorescence quenching; metal complexation

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### Introduction

Passive bioreactor is an environmentally sustainable technology to address the acid mine drainage (AMD) problem (Neculita et al., 2007). The AMD (acidic, sulfate, and metal polluted water) treatment is achieved through complex biogeochemical processes using (reactive) mixtures of organic and inorganic materials. Metal removal mechanisms involve sorption and precipitation. The use of inexpensive and available organic wastes (ex. manure, compost, wood waste, cereal straws) in the filling-mixture composition and the precipitation of metals in their most stable form (sulfides) are two of the main advantages of this technology (Johnson and Hallberg, 2005). Passive bioreactor relies on sulfate-reducing bacteria (SRB), which, in order to thrive and help the AMD treatment by pH increasing and the precipitation of biosulfides, require a source of available organic carbon. The challenge for having an effective passive bioreactor is thus to select suitable substrates. Dissolved organic matter (DOM) is a major energy source for bacteria as available organic

carbon. However, DOM also contains compounds with large surface area and several functional groups with strong capacity to bind and co-transport metals out of the AMD treatment system (Camobreco et al., 1996; Dudal et al., 2005; Knoth de Zarruk et al., 2007), thereby decreasing its efficiency. In addition, in anoxic environments the dissolved organic carbon (DOC) and metals in low oxidation states are active redox-constituents (Hakala et al., 2009) or can easily adsorb dissolved phosphorus and limit its availability to phytoplankton (Johnson et al., 2010). Hence, the quantification of DOM-metal complexes is of great interest, especially upon the start-up of bioreactors when soluble organic compounds are prevalent. Because there is no direct measurement method for these complexes, the treatment monitoring generally focuses on DOC concentration. However, only a portion of DOC is responsible for metal binding, and thus important in estimating metal mobility (Guo and Chorover, 2003; Dudal et al., 2005). Among available techniques to quantify the capacity to form DOM-metal complexes, fluorescence spectroscopy is increasingly used (Dudal et al., 2005). The DOM fluorescence quenching during metal addition can be used

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to quantify the affinity between DOM and metal cations and to calculate a complexation value (Senesi, 1990). A relatively new fluorescence microplate-based procedure (Dudal et al., 2006) allows for an easy comparison of DOM samples from different origins and is highly relevant for a rapid screening of different metal affinities for binding.

This work is a continuation of a previous study, in which a mixture of wastes (maple wood chips and sawdust, leaf compost, and poultry manure) efficiently treated highly contaminated AMD in column bioreactors for more than one year (Neculita et al., 2008a, 2008b). The evaluation of metal speciation by chemical analysis and thermodynamic modeling suggested that limited DOM-metal complexes were released into the treated water (Neculita et al., 2008c). This new research focused on the quantification of initial DOM reactivity in wastes, alone or in mixtures, toward four metals generally found in AMD ( $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Cu}^{2+}$ ). The main objective was to assess the ability of the fluorescence quenching technique to evaluate maximum capacities of each carbon source to release mobile metal-complexes in treated water especially upon the start-up of passive bioreactors.

## 1 Material and methods

### 1.1 Dissolved organic matter samples

The DOM samples originated from maple wood chips (MWC) (P. W. I. Industries, Canada), composted poultry manure (CPM) (Fertilo, Fafard, Canada), leaf compost (LC) (city of Montreal, Canada), as well as a mixture (MIX). The MIX contained 60% organic materials (20% LC, 20% maple sawdust, 10% CPM, and 10% MWC) and 40% inorganic materials (20% sand, 15% creek sediment, 3% urea, and 2% calcium carbonate). The extraction was carried out following literature (Dudal et al., 2006). Briefly, 30 g of dried organic material were mixed with 60 mL of ultra pure water (18.2 M $\Omega$ -cm) in a 100-mL amber glass bottle, shaken for 15 min on an end-over-end shaker, and centrifuged for 10 min at 11,800  $\times g$  at 4°C. The supernatant was then filtered through a 0.45- $\mu\text{m}$  membrane filter (Durapore, Millipore) and the extracts were stored in amber glass bottles for a maximum of 12 hr at 4°C. The carbon content of the filtered sample was measured with a total carbon analyzer (TOC 5050A, Shimadzu, Japan). The DOC concentrations of the samples were as follows (mg/L): 48.6 (LC), 104 (CPM), 145 (MWC), and 692 (MIX).

### 1.2 Column bioreactors operation

The MIX tested in this study was previously proved efficient for the long-term treatment (11–15 months) of highly contaminated AMD, under continuous flow in column bioreactors (Neculita et al., 2008a, 2008b). To this end, the MIX was used for filling six 3.5-L plexiglas column bioreactors, which were operated in triplicates and downward flow at two hydraulic retention times (HRTs) (7.3 and 10 days) (Fig. 1).

After packing with MIX, the bioreactors were saturated

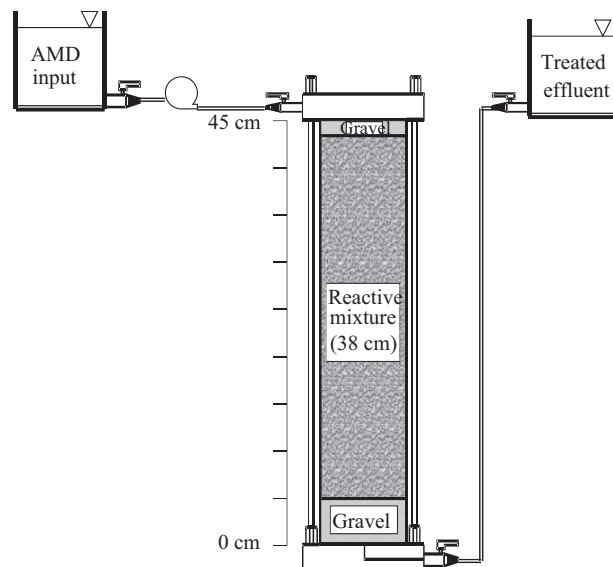


Fig. 1 Design of down-flow column bioreactors.

with Postgate B medium (Postgate, 1984) and incubated for 5 weeks to allow the SRB acclimation, before the continuous flow started. The fed consisted of synthetic AMD (Table 1), which quality was similar to a real AMD from a former mine site in northern Quebec (Canada).

The artificial AMD was prepared using distilled water and ACS chemicals, as per Zagury et al. (2006). During bioreactors operation, weekly sampling and analysis were performed. All parameters were analyzed according to standard methods (APHA, 1998). More details about this part of the study are provided by Neculita et al. (2008a). After seven months of bioreactors operation, metal speciation in the effluent was evaluated by chemical analysis and geochemical modeling (VMINTEQ, version 2.52 and WHAM VI) (Neculita et al., 2008c).

### 1.3 Microplate preparation and reading

The maximum capacity of DOM for metal-binding was assessed by fluorescence quenching during the addition of increasing metal concentrations, as described by Dudal et al. (2006). Five opaque 96-well fluorescence-compatible microplates (OptiPlate, PerkinElmer, USA) were prepared for CPM, MWC, LC, and MIX, as well as for the control (10 mg/L phenol). Samples and control were tested (in triplicates) for four metals (Cu, Ni, Fe, and Cd). Seven in-well concentrations (0.008–1.67 mmol/L) were designed

Table 1 Selected physicochemical characteristics of AMD and treated water from column bioreactors

Parameter (mg/L)	AMD	7.3-day HRT effluent	10-day HRT effluent
pH	4.5 ± 0.8	6.2 ± 0.2	6.0 ± 0.2
Fe (mg/L)	504 ± 83	91 ± 47	201 ± 59
Ni (mg/L)	13.7 ± 1.0	0.08 ± 0.02	0.09 ± 0.02
Cd (mg/L)	9.8 ± 1.8	< 0.03	< 0.03
SO <sub>4</sub> <sup>2-</sup> (mg/L)	3756 ± 484	2942 ± 384	3227 ± 406
DOC (mg/L)	< 0.5	6.5 ± 1.6	5.3 ± 1.6

Values are presented as mean ± standard deviation of physicochemical parameters determined weekly during a 30-week period. AMD: acid mine drainage; HRT: hydraulic retention time.

to monitor the fluorescence quenching to assess both the affinity and saturation. The MIX contained calcium carbonate to increase the pH to an appropriate range (5 to 8) for SRB (Cocos et al., 2002; Zagury et al., 2006). Because the fluorescence and metal-binding capacity are strongly pH-related, all DOM samples were buffered (4:1 volume ratio) with 0.1 mol/L N-methylpiperazine (Aldrich) in 0.1 mol/L KNO<sub>3</sub>. In each well, 10 µL of metal solution and 250 µL of pH-buffered DOM sample were added. One well that contained ultrapure water instead of the metal solution was also added for each sequence. For complete complexation, the microplate was then mixed in a Thermomixer (Eppendorf) for 5 min, at 300 r/min and 20°C. Fluorescence readings were performed with a luminescence spectrometer (LS55, PerkinElmer, USA) that was equipped with a PerkinElmer microplate reader extension. The quenching measurements used appropriate excitation and emission wavelengths (330 and 430 nm, respectively). These values were determined based on excitation-emission matrices (obtained by scanning the whole fluorescence domain), which showed two common peaks:  $\lambda_{\text{ex}}230/\lambda_{\text{em}}430$  and  $\lambda_{\text{ex}}330/\lambda_{\text{em}}430$ . The latter was chosen for it enables the fluorescence readings to be acquired without any interference from the microplate polypropylene material.

#### 1.4 Metal complexation

A Langmuir-type model was used with the experimental data for the calculation of the binding constant (Dudal et al., 2005):

$$\frac{F_0 - F}{F_0} = \frac{Y_{\text{max}} \times C_{\text{Me}^{z+}}}{K_L + C_{\text{Me}^{z+}}} \quad (1)$$

where,  $F_0$  and  $F$  are the fluorescence of the DOM and water- and metal-containing well, respectively,  $Y_{\text{max}}$  is the maximum fluorescence quenching that corresponds to the saturation of all-complexing sites available,  $K_L$  is the affinity towards the metal cation (half-saturation concentration of metal, in µmol/L of cation  $\text{Me}^{z+}$ ), and  $C_{\text{Me}^{z+}}$  represents the molar concentration of free metal cations.

The model fitting and parameter estimation used least-square optimization and non-linear regression software (WinReg 3.2) (Debord, 2004). Metal-binding capacity constants ( $K_{\text{mb}}$  in L/mol for a 1:1 complex formation) were derived from  $K_L$  using the following Eq. (2):

$$K_{\text{mb}} = \frac{10^6}{K_L} \quad (2)$$

The  $K_{\text{mb}}$  values were then used to calculate the DOM-Me (DOM-Me) complex concentrations based on the following Eq. (3) (Dudal et al., 2005):

$$K_{\text{mb}} = \frac{C_{\text{DOM-Me}}}{C_{\text{DOM}} \times C_{\text{Me}^{z+}}} \quad (3)$$

where,  $C_{\text{DOM-Me}}$  and  $C_{\text{DOM}}$  represent the molar concentrations of DOM-metal complexes and free DOM, respectively.

A molecular weight of 12,000 Da was used for DOM transformation from mg/L to mol/L. This value was suggested by a previous study that analyzed CPM and LC for their molecular weight distribution in relation to the fraction with the highest fluorescence intensity (Knoth de Zarruk et al., 2007).

## 2 Results and discussion

### 2.1 Screening metal-binding ability of DOM

The decreasing intensity of natural fluorescence signals emitted by DOM (the quenching), is due to the increasing concentrations of DOM-Me complexes (Ryan and Weber, 1982a, 1982b). As results show, in the present study, the fluorescence quenching of Cu, Ni, Fe, and Cd exhibited classic affinity-saturation behavior at the tested concentrations (8–1670 µmol/L) (Fig. 2).

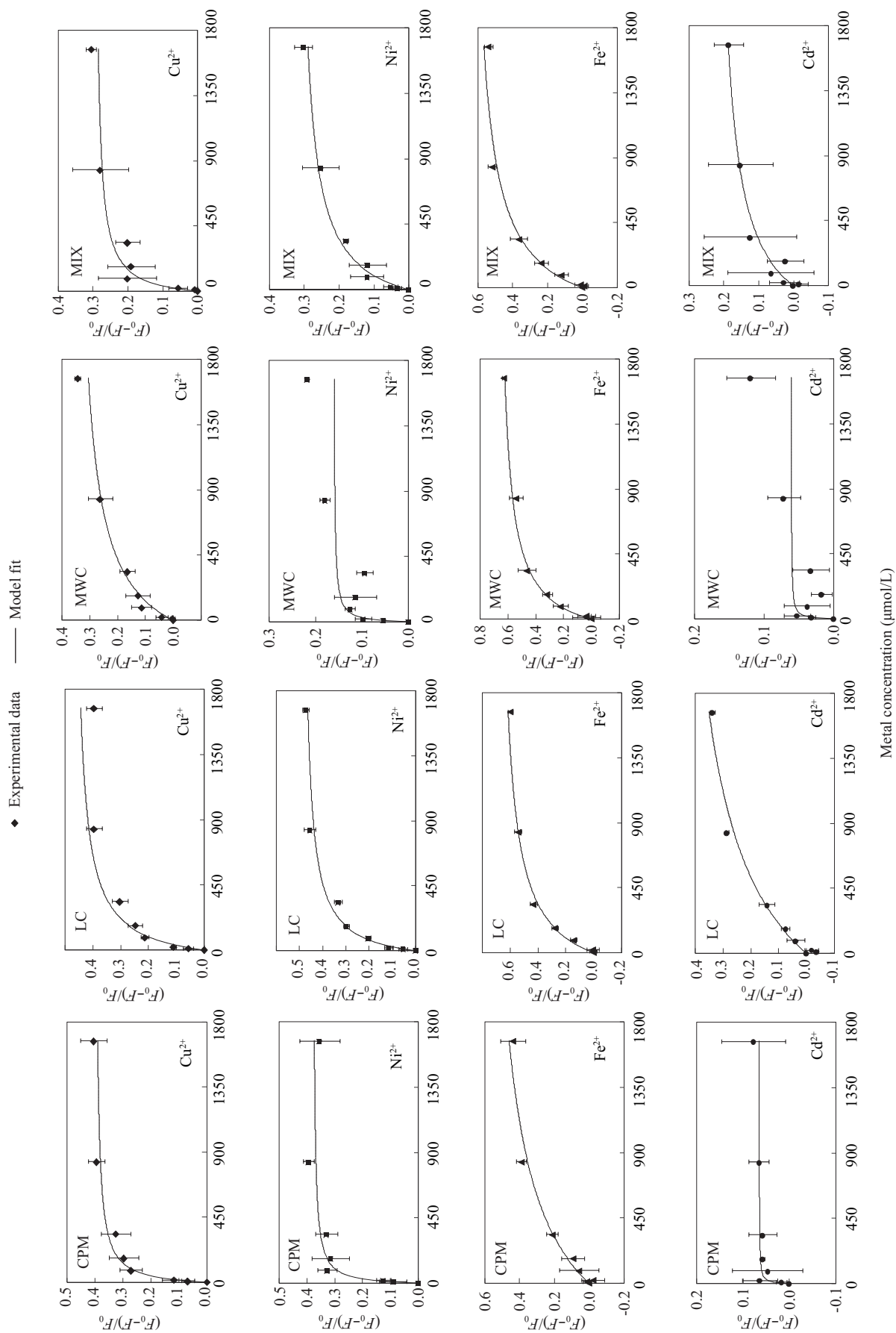
The analysis of QD20 values, i.e., metal concentration responsible for 20% of the quenching response, indicated that the DOM extracted from organic wastes (CPM and LC) behaved very similarly in their affinity toward metals (Cu–Ni > Fe). In contrast, the cellulosic materials (MWC) and the MIX exhibited higher affinities for Fe than for Cu and Ni. In other words, mine-impacted water dominated with Cu and Ni could generate higher concentrations of DOM-metal complexes with an organic waste substrate (ex. manure, leaf compost), whereas a higher content cellulosic substrate could produce more DOM-Fe complexes. The DOM from all substrates showed the highest reactivity toward Fe, but limited number of available sites for sorption, as suggested by the maximum fluorescence quenching values corresponding to the saturation of all of the complexing sites available (Fig. 2). The very low fluorescence quenching (< QD20) exhibited by the DOM-Cd complexes constituted the basis of this metal exclusion from further analysis. Sorption onto solid material and precipitation are important mechanisms of Cd removal that explain its very low concentrations (< 30 µg/L) in treated water during column bioreactor tests (Neculita et al., 2008a).

The Langmuir-based model fitted the experimental values for Cu, Ni and Zn to an acceptable level of accuracy (Fig. 2). The goodness-of-fit model for the data, described as the degree of linear correlation of variables and computed as the coefficient of determination ( $R^2$ ), varied from 0.77 (MWC-Ni) to 0.96 (CPM-Ni). Based on the  $K_{\text{mb}}$  values (Table 2), the MIX respected the relative scale of classically reported stability of metal organic matter

**Table 2** Metal-binding capacity constants ( $\log K_{\text{mb}}$ ) and concentrations of possible DOM-Me complexes

Organic material	$\log K_{\text{mb}}$			DOM-Me complexes (µmol/L)		
	Cu	Ni	Fe	Cu	Ni	Fe
CPM	4.34	4.58	3.14	5.8	6.7	1.0
LC	3.89	3.96	3.58	1.8	1.9	1.1
MWC	3.45	4.74	3.74	2.6	10.0	4.1
MIX	4.14	3.66	3.52	28.7	15.9	12.9

DOM-Me complexes ( $C_{\text{Me}^{z+}} = 10^{-4}$  mol/L) are formed by substrates used in passive bioreactors.



**Fig. 2** Fluorescence quenching during metal (Cu, Ni, Fe and Cd) complexation by DOM extracted from different materials. CPM: composted poultry manure; LC: leaf compost; MWC: maple wood chips; MIX: their mixture. The solid lines represent the fits obtained from the affinity-saturation model.

interactions ( $\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Fe}^{2+}$ ) (Irving and Williams, 1948).

However, the ranking of  $K_{\text{mb}}$  values for each substrate was not in complete agreement with the data presented above (Table 2). The use of non-homogenous organic materials and the different natures of organic binding sites could explain these results (Wieder, 1990). Previous related work on the fractionation of CPM and LC also confirmed high differences in the chemical nature of the substrate binding sites (Knoth de Zarruk et al., 2007). Moreover, substrates characterization indicate higher content and complexity, as well as total organic carbon in cellulosic waste (MWC and sawdust) (44.0% and 45.3%, respectively) relative to CPM or LC (25.7% and 1.3%, respectively) (Neculita and Zagury, 2008). The hemicellulose (up to 37.2%) and cellulose (up to 64.9%) content of cellulosic waste were also higher compared to LC (10.3% and 4.8% for hemicellulose and cellulose, respectively) or CPM (21.5% and 25.3%, respectively).

Based on the concentrations of DOM-Me complexes (Table 2), two important observations can be made. First, Ni gave the highest concentrations of complexes out of all single substrates (CPM, LC, and MWC). This might be a problem when high concentrations of Ni are found in the treated water. However, previously reported results, including first part of the present study, indicate that dissolved Ni remains at low levels in treated water because the solid organic material plays a major role in its retention by passive bioreactors (Wieder, 1990; Neculita et al., 2008b). Second, even though the DOM extracted from CPM was more selective for Cu and MWC for Fe, for all metals, the DOM extracted from MIX was the most selective. As a result, despite the selectivity of sole substrates, the mixtures of substrates can lead to DOC-metal complexes in the treated water. The high amounts of DOM-Fe complexes may seem odd, with respect to the reported minor retention of Fe in passive treatment systems by complexation (Wieder, 1990). Nevertheless, previous results on metal removal mechanisms in the MIX confirmed that Fe was mainly bound to the organic substrate, which includes other chemical forms besides sulfides (ex. sorption) (Neculita et al., 2008b). These results are well correlated with QD20, which indicate a high reactivity of organic materials toward Fe, but a limited number of available binding-sites (Fig. 2).

## 2.2 DOC and metal concentrations in treated water

Upon the start-up of passive bioreactors, high concentrations of DOC are usually leached out, giving the treated water a brownish-dark color. It is noticeable that a 10-fold higher DOC value (around 6.5 g/L) was measured in the water in the beginning of the column bioreactor tests performed in a previous step of this study using the same MIX (Neculita et al., 2008a), compared to the MIX extract used in the present research (692 mg/L). The bioreactors' saturation with Postgate medium (that contained lactate, an easily biodegradable organic compound) and a 5-week acclimation period before AMD feeding (which allowed for organic matter decomposition) accounted for

this difference. However, the DOC values decreased to  $< 10$  mg/L during the first three months of column bioreactors operation, before reaching the steady-state (Table 1). Likewise, Fe and Ni concentrations also decreased relative to the AMD values. Nevertheless, within the first month, the effluent water had high concentrations of both DOC and Fe, and, based on the present results, DOM-metal complexes. This is a very important finding, as DOC provides a good carrier for the export of iron to surface waters, readily adsorbing the dissolved phosphorus, a limiting nutrient to phytoplankton (Johnson et al., 2010). Moreover, geochemical modeling (VMINTEQ) results suggested that low concentrations of DOM-Ni and DOM-Cd complexes were formed (up to 20% of dissolved fraction) after seven months of bioreactors operation (Neculita et al., 2008c). For Fe, which had the highest concentrations of all metals in treated water, geochemical modeling (WHAM VI) indicated that up to 50% of  $\text{Fe}^{2+}$  would be complexed by dissolved ligands. These theoretical predictions were confirmed by the experimental results obtained in the present work (Table 2), which indicate the formation of DOM-Fe complexes in high concentrations.

## 3 Conclusions

The fluorescence quenching experimental approach provided useful quantitative data regarding the reactivity of DOM, which originated from the organic wastes used in passive bioreactors, towards metals. The DOM showed the highest reactivity toward Fe, but a limited number of available sites for sorption, whereas DOM-Cd complexes exhibited the lowest fluorescence quenching. Overall, the DOM from a mixture of wastes formed higher concentrations of DOM-metal complexes relative to single substrates. Among complexes, the concentrations of DOM-Ni complexes were the highest. After reaching steady-state, low concentrations of DOM-metal complexes were released into treated water in agreement with theoretical predictions based on geochemical modeling. Therefore, in addition to physicochemical characterization, the use of this technique prior to the set-up of organic carbon-based passive bioreactors is recommended, as it can provide valuable information on the concentration of DOM-metal complexes that can be potentially released upon the start-up. This information is important for selecting appropriate organic carbon sources.

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