



Effects of Fe(III) and quality of humic substances on As(V) distribution in freshwater: Use of ultrafiltration and Kohonen neural network



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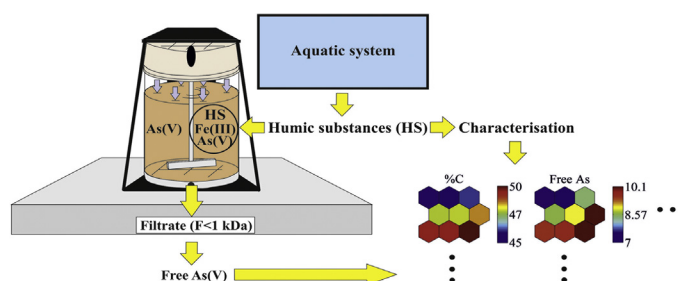
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HIGHLIGHTS

- Evidence of associations of As(V)-Fe(III)-HS by ultrafiltration studies.
- Most As, DOC and Fe appeared in the higher molecular size fraction of HS.
- Different behaviour of HS toward As complexation in relation to HS quality.
- Kohonen neural network enabled the detection of particular characteristics of HS.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 21 March 2017

Received in revised form

25 August 2017

Accepted 28 August 2017

Available online 29 August 2017

Handling Editor: Martine Leermakers

Keywords:

Ternary complex

Dissolved organic matter

Arsenic distribution

Ultrafiltration

Self-organising maps (SOM)

ABSTRACT

Humic substances (HS) are ubiquitous organic compounds able to affect mobility and availability of arsenic (As) in aquatic systems. Although it is known that associations between HS and As occur mainly via iron (Fe)-cationic bridges, the behaviour and distribution of this metalloid in HS- and Fe-rich environments is still not fully understood. In this paper, the quality of HS from different rivers in Brazil and Germany and its influence on the behaviour of As(V) under different Fe(III) concentrations were investigated. HS were extracted from four different rivers (Cascatinha, Holtemme, Selke and Warne Bode), characterised and fractionated into different molecular weight sizes (10, 5 and 1 kDa). Complexation tests were performed using an ultrafiltration system and 1 kDa membranes. All data was analysed using the Kohonen neural network (SOM – Self organising maps). All samples, except Selke, exhibited similar results of free As (<1 kDa). The results suggested that associations between HS, Fe and As were dependent on nitrogen (N)–aromatic carbon (C), amount of sulphur (S) and the molecular size of the HS. Although all HS appeared to be similar after looking at most variables analysed, the SOM could discriminate them into three different groups. Characterisation of the HS indicated that they had terrestrial material (from C₃ plants) as precursor material. Most of the As and Fe was distributed in the fractions of higher (>10 kDa) and lower (<1 kDa) size. HS quality is an important factor to take into account when studying the behaviour of As in HS-rich environments.

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1. Introduction

Arsenic (As) is a very toxic metalloid of global concern due to the contamination of aquatic systems and its effects on environment and health (Azizur Rahman and Hasegawa, 2012). It usually appears as an oxyanion, either as arsenite (As(III)) under reducing conditions or arsenate (As(V)) under oxidising conditions (Sharma et al., 2010). Poisonous levels of this element can be found in surface and groundwater in many areas around the world from Asia (Mladenov et al., 2015; Pfeiffer et al., 2015) to Latin America (Matschullat et al., 2000). These contamination problems are typically associated with local lithology and/or human activity, especially in relation to mining. A complete understanding of the behaviour of As in natural aquatic systems and the role of humic substances (HS) is not yet achieved and more effort is needed. As part of natural dissolved organic matter, HS are ubiquitous organic compounds with molecules of different sizes and weight. They are formed via degradation of organic debris and influence the mobility, toxicity and bioavailability of nutrients and contaminants in aquatic ecosystems, including As (Lu et al., 2000).

HS can originate from both autochthonous (in-stream processes) and allochthonous sources (McDonald et al., 2004). Their characteristics and the overall contribution from each of these sources are dependent on several factors, including catchment, climate and seasonality (Steinberg, 2003). Furthermore, their composition also reflects the transformation processes that take place once the organic material reaches aquatic systems (Wagner et al., 2015). Therefore, it is essential to study for example the functional groups within the HS structure, since these groups are known to change their lability and chemical reactivity in the environment (Mladenov et al., 2015). The variability of S content among HS from different locations for instance will determine the amount of As complexed on HS (Catrouillet et al., 2015). Consequently, it is essential to investigate the chemical quality of HS and how changes in time and space affect the behaviour and distribution of As in aquatic systems.

Knowledge of the various As species in natural waters is crucial since different element species exhibit different behaviours in terms of bioavailability, mobility and toxicity. These species can be distinguished through fractionation of their total fraction (TF) into different sizes or molecular weight ranges. In this approach, it is known that most HS molecules can be retained by ultrafiltration using 1 or 3 kDa membranes depending on the operational definition (Bauer and Blodau, 2009; Liu et al., 2011; Gontijo et al., 2016) since they occur mainly in the colloidal fraction (CF). The permeate in these systems corresponds to free (hydrated) ions or small complexes which can pass through 1 or 3 kDa filters. This fraction is generally defined as the free fraction (FF) and is the most mobile fraction in natural waters (Buffle, 1991; Monteiro et al., 2016).

Concerning the nature of HS and As interactions, the main known mechanisms are competitive sorption, electron shuttling and cation bridging binding (ternary complexes) (Mladenov et al., 2015). In these mechanisms, iron (Fe) has a key role in As mobilisation and distribution in aquatic systems. For instance, HS can compete with As for adsorption sites on Fe mineral surfaces releasing As to the solution and increasing its mobility (Sharma et al., 2010; Liu et al., 2011). Regarding the cation bridging mechanism, dialysis experiments showed evidence of the formation of either HS-Fe(III)-As complexes or HS-Suspended Fe(III) (oxy)hydroxides-As(V) associations (Ritter et al., 2006). Direct binding between As and HS is also possible but is not significant due to their charge at around neutral pH (neutral for As(III) and negatively charged for HS and As(V)). Buschmann et al. (2006) suggested that this direct interaction can be explained by a higher formal central charge for As(V), stabilising effects, and phenolate and carboxylic

functional groups of HS. Another explanation is the presence of thiol groups in HS, which may be a possible binding site to As(III) (Catrouillet et al., 2015).

Kohonen neural network (also known as self-organising maps, SOM) is a multivariate method which recognizes patterns in a group of data. It is an artificial intelligence technique that has the ability to project complex high dimensional data in a two dimensional array, which is an important feature in environmental studies with a large number of variables (Kowalski et al., 2013; Gontijo et al., 2014; Deljanin et al., 2016). This ability is one of the main advantages of the technique when compared to other multivariate statistical approaches, together with an easy visualisation of the results and standardisation of the samples (da Silva et al., 2008; Deljanin et al., 2015). SOM technique has been used in several environmental studies, including analysis of the behaviour and relationship among surface water quality parameters, distribution and bioavailability of As, performance of a wastewater treatment plant and studies of applicability of leaves of different species as biomonitor of trace elements (Çinar, 2005; Çinar and Merdun, 2009; Deljanin et al., 2015; Gontijo et al., 2016).

This study aimed to examine the quality of HS in rivers from Brazil and Germany and understand how it affects the behaviour of As(V) in surface waters under different Fe(III) concentrations. Ultrafiltration was used to distinguish free (<1 kDa) from bound/complexed species in the colloidal fraction (>1 kDa and <0.45 µm). This technique was chosen because it is a faster and easier way to evaluate HS interactions compared to the time-consuming dialysis methods. Furthermore, all HS were fractionated into several molecular sizes to investigate their chemical characteristics and the distribution of As and Fe. The SOM technique was used as a tool to investigate HS-As interactions and possible relationships among samples and variables. The topic of this paper is relevant because a deeper understanding of the processes governing As dynamics in environments rich in Fe and HS is still needed. Moreover, our findings are of value for further studies of As bioavailability, mobility and toxicity.

2. Materials and methods

2.1. Sampling and previous analyses

Water samples were taken from 4 different points located in Southeast Brazil (Cascatina, in dry and rainy seasons) and in Central Germany (rivers Holtemme, Selke and Warme Bode) (Fig. 1). These specific locations were chosen due to the broad amount of HS and/or presence of As in the water. Different sources of HS were selected because they could reflect different structural characteristics, which may influence the complexation and fate of metals and metalloids in rivers and lakes. Cascatina represents a pristine area on the southern hemisphere with low concentrations of total Fe (<250 µg L⁻¹) but elevated concentrations (ca. 5 mg L⁻¹) of dissolved organic carbon (DOC) (Gontijo et al., 2016). The Holtemme river, which flows to Bode River in central Germany, drains a rewetted fen in its catchment which results in high DOC concentrations (Kamjunke et al., 2013; Wollschläger et al., 2016; Herzsprung et al., 2017). Warme Bode is a stream which is also part of the Bode catchment (Kamjunke et al., 2015). All these three rivers/streams show only very low concentrations of As (Kamjunke et al., 2013; Gontijo et al., 2016). In contrast, the Selke river (tributary of Bode river), which receives effluents from former medieval mining activities, showed high As concentrations (ca. 20 µg L⁻¹) in past studies (Kamjunke et al., 2013).

All water samples were analysed for their hydrochemical composition before the extractions of HS. Conductivity, dissolved oxygen (DO), pH and temperature were measured *in situ* (WTW

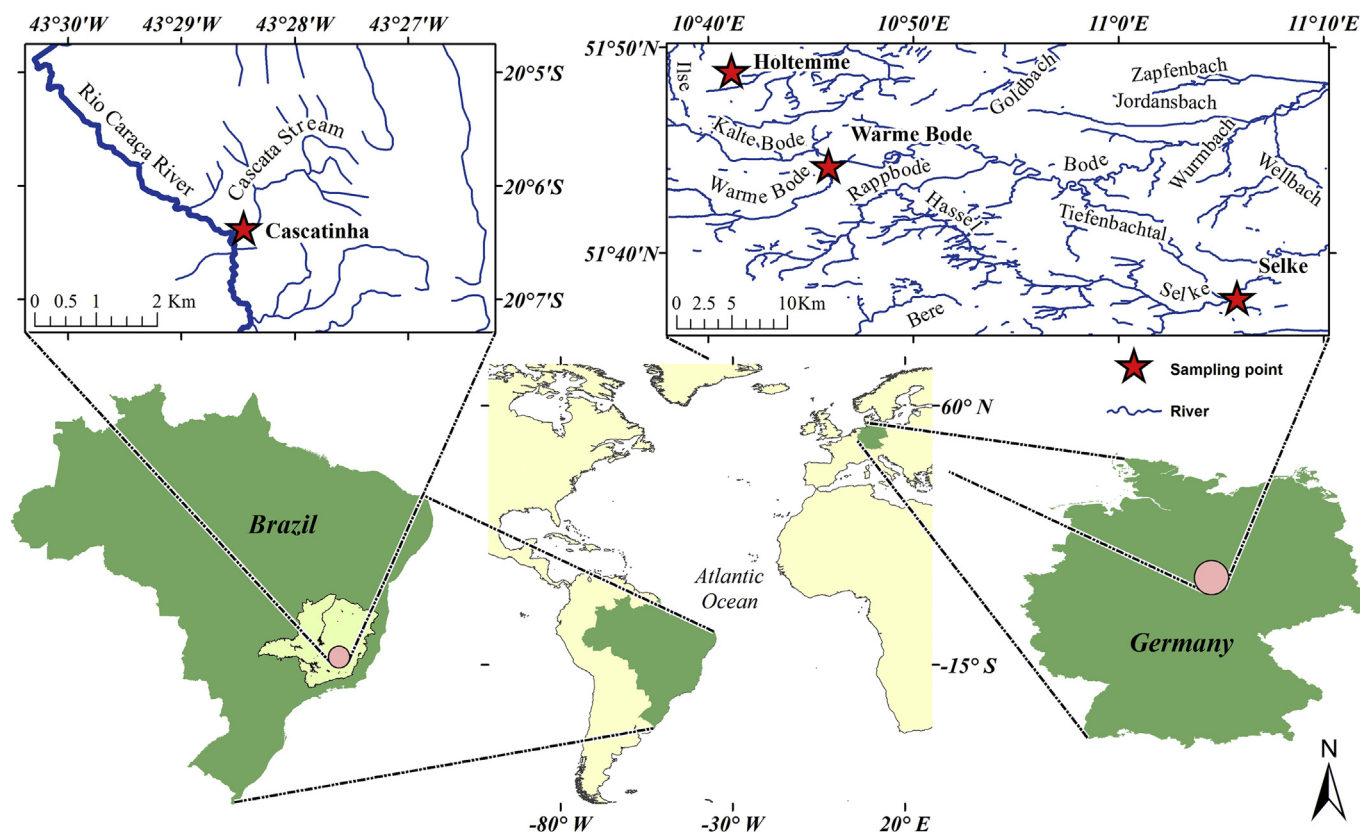


Fig. 1. Localisation of the four sampling points (stars) in Brazil and Germany. Cascatinha was sampled two times (in dry and rainy seasons).

Multi 3430 and Ultrameter – Myron L Company). Samples (about 100 mL) were taken in plastic bottles and acidified (concentrated HNO_3) for subsequent laboratory analysis of Al, Fe and As. About 15 mL of water was collected in brown bottles for total organic carbon (TOC) analysis. The same amount of water was filtered using 0.45 μm membranes for analysis of DOC. Both DOC and TOC samples were analysed in a total carbon analyser (DIMATOC[®] 2000, Dimatec Analysentechnik GmbH, Germany). About 160 L of water was collected in plastic bottles to perform extraction of HS, ultrafiltration and optical spectroscopic measurements.

2.2. Fractionation using ultrafiltration

In the laboratory, water samples (10 L) were filtered sequentially in 0.45 μm and 1 kDa regenerated cellulose membranes using a Millipore Pellicon[®] System (Germany) and a Teflon home-made ultrafiltration system (Brazil). An aliquot (approx. 10 mL) of the dissolved fraction (DF, <0.45 μm) and FF (<1 kDa) was stored after acidification (HNO_3) for determination of metals and As. Acid digestion (method 3005A, USEPA (1992)) was performed for all unfiltered samples (TF) and DF of Cascatinha. Furthermore, the TF and DF samples from Cascatinha were pre-concentrated in a factor of six, while the FF samples were pre-concentrated in a factor of four. All analyses were done in an Agilent 8800 ICP-MS Triple Quad (As) and Optima 7300DV PerkinElmer ICP-OES (metals), except for the original and filtered waters of Cascatinha, where it was used an Agilent 700 Series ICP-OES (metals) and a Varian AA240Z GFAAS (As).

2.3. Extraction of aquatic humic substances

HS were extracted following the method of Thurman and

Malcolm (1981) using chromatographic columns filled with Supelite[®] DAX-8 resin. Before extraction, the resin was cleaned using methanol, 0.1 M HCl and 0.1 M NaOH. The original water samples (ca. 150 L) were previously acidified with 6 M HCl to pH < 2 and these samples were passed through the columns to retain HS. The columns were eluted using 0.1 M NaOH and the extracts were acidified thereafter to the original pH of the water samples. All extracts were stored in a refrigerator at 4 °C until use. Part of these HS was dried in a drying oven (40 °C) for characterisation analyses. The volume of original water passed through the columns and the volume of HS extracted for each sample are shown in the supplementary section (Table A.1).

2.4. Elemental analysis

The elemental composition (C, N, H, S) of the dried material (HS extracts) was analysed using the elemental analysers CHN628 (LECO), CS230 (LECO) and Vario EL Cube (Elementar Analysensysteme GmbH). Oxygen was calculated via the equation % O = 100 – %C – %H – %N – %S (Lu et al., 2000). The ash content corresponded to the remaining mass after heating the sample in a crucible to 750 °C and was determined using a thermogravimetric analyser (TGA701, LECO). The elemental composition of all HS extracted was corrected by ash content. The elemental analysis provides useful information about the origin and structure of the organic matter through the ratios H/C, O/C and C/N. The H/C and O/C ratios provide an estimation of aliphaticity and humification of organic matter. The C/N ratio provides information about the origin of the HS, where low values (4–10) indicate protein-rich algae origin and higher values (>20) indicate cellulose and lignin-rich terrestrial sources (Wolfe et al., 2002).

2.5. Nuclear magnetic resonance

^{13}C NMR solid state spectra of the HS extracts were recorded using a Bruker AV 300 WB operating at 75.47 MHz. Magic angle spinning (MAS) was performed at 15 kHz with zirconium dioxide rotors (diameter 4 mm). Cross polarisation (CP) contact time was 2000 μs and the number of accumulated scans was 10,000. The ^{13}C NMR spectra were divided into different chemical shift regions according to chemical type of carbon (Rodríguez-Murillo et al., 2011): 0–45 ppm (alkyl carbon), 46–58 ppm (methoxyl carbon), 59–65 (C6 carbon in glucopyranoside structures), 66–94 ppm (O-alkyl carbon), 95–110 (C1 carbon in glucopyranoside structures), 111–146 (H-aromatic carbon), 147–167 (N-aromatic carbon), 168–188 (carboxyl carbon), 189–200 (carbonyl carbon). The aliphaticity and aromaticity of the samples corresponded to the relative area (%) from 0 to 100 and 111–167, respectively.

2.6. Isotope analysis

Isotope analyses ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of all HS extracts were performed with the EA Eurovector/Delta V Advantage. The analysis of the stable isotopes in the delta-notation $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ is a proxy for the precursor material of organic matter related to C and N cycles (including availability of water and nutrients) and processes that affect HS. The isotopic composition may indicate whether HS are of terrestrial or plankton origin or whether the precursor material is derived from C_3 or C_4 plants. The distinction between C_3 and C_4 is related to the different photosynthetic cycles in which plants fix atmospheric CO_2 , where more depleted $\delta^{13}\text{C}$ values (around -27.0‰) are related to C_3 plants and higher values (around -12.5‰) are related to C_4 plants (Amon and Meon, 2004; Zhao et al., 2010). Concerning $\delta^{15}\text{N}$, Amon and Meon (2004) indicate that these values may be used as a proxy to differentiate between plankton and terrestrial sources. In addition, Kendall (1998) reported that depleted $\delta^{15}\text{N}$ values (-3 to $+1\text{‰}$) are evidence of N_2 fixation.

2.7. Fractionation of organic matter into different molecular weight ranges

The extracts of HS were fractionated sequentially into different molecular sizes by 1-, 5- and 10 kDa membranes. A Stirred Ultrafiltration Cells (SUC) System (Model 8200, Millipore™) and regenerated cellulose membranes were used to perform these experiments. All fractionations were carried out in inert atmosphere using nitrogen as a gas. For each extract, 200 mL of HS solution was prepared. The retentate of each filtration step was adjusted to 25 mL whereas the permeate was filtrated in the next smaller membrane. The permeate of the last membrane (1 kDa) was concentrated using a rotary evaporator and the volume was adjusted to 25 mL. An illustration summarising all steps is shown in the supplementary section (Fig. A.1). From all samples achieved by the described fractionation As, Fe, organic carbon content (OCC) and the optical properties were determined by the methods described in chapter 2.1, 2.2, and 2.9.

2.8. Complexation experiments using ultrafiltration

To investigate the influence of DOC and Fe in the complexation of As, a lab experiment was designed using the same SUC system (Model 8200, Millipore™) and regenerated cellulose membranes as for the HS extracts fractionation (see chapter 2.7 and Fig. A.3 in supplementary section). All experiments were carried out for the free fraction (<1 kDa) in an inert atmosphere using nitrogen as gas. A possible loss of As by the ultrafiltration system was evaluated

before the experiment. For this purpose, 50 mL As solution ($100 \mu\text{g L}^{-1}$) was prepared and tested at different pH (4, 6 and 8) and conductivities ($500, 1000$ and $2000 \mu\text{S cm}^{-1}$) in the absence of HS. Fe(III) was not added because it would precipitate due to its low solubility above pH 3.5. However, the addition of Fe in the experiments in the presence of HS would not be a problem since organic matter would keep the Fe added in solution as either HS-Fe(III) cation-As(V) or HS-suspended Fe(III) (oxy)hydroxides-As(V) associations (Ritter et al., 2006). The results of these tests showed a loss of As by the ultrafiltration system of about 11% and are presented in the supplementary section (Fig. A.2).

The influence of DOC concentration for As complexation was studied using HS from river Holtemme. Solutions of 50 mL with $100 \mu\text{g L}^{-1}$ As and 5.5 mg L^{-1} Fe were prepared and pH and conductivity were adjusted to 6 and $1000 \mu\text{S cm}^{-1}$ respectively (for details see SI). Aliquots of Holtemme HS extracts were added to achieve final DOC concentrations of 10, 20 and 40 mg L^{-1} , respectively. Experiments using the same conditions were performed without addition of Fe to evaluate its role in As distribution between CF and FF (note, all HS extracts had their original inherent Fe concentration).

The influence of different extracts in the complexation of As was also evaluated using the same solution as described above. These experiments were performed using HS from Cascatinha (dry and rainy seasons), Holtemme, Selke and Warme Bode. Aliquots of the HS extracts were added to a final DOC concentration of 20 mg L^{-1} . Analyses for As, Fe, and OCC were done as described in 2.1 and 2.2.

2.9. Optical spectroscopic measurements

Absorbance spectra (240–600 nm) and excitation-emission matrices (EEM) fluorescence spectra were obtained using an Aqualog® Spectrofluorometer (Horiba) in a 1 cm quartz cuvette. The results were used as a proxy to evaluate the quality of HS extracted and their 4 fractions from the fractionation experiments (<1 kDa, 1–5 kDa, 5–10 kDa, >10 kDa). Optical spectroscopic analyses are an easy, quick and inexpensive way to evaluate the source, composition and reactivity of HS (Wagner et al., 2015). After the measurements, several indexes were calculated. The fluorescence index (FI) (Hansen et al., 2016) was used as an indicator of the source of the material analysed (relative contribution of microbial- or terrestrial-derived components). The freshness index (β/α) indicates the contribution of recently produced organic matter (higher values indicate higher proportion of fresh dissolved organic matter) (Hansen et al., 2016). The humification index (HIX) is related to the content of HS or degree of humification and generally varies between 0.6 and 0.9 (the higher its value, the higher the degree of humification) (Ohno, 2002). The ratio of absorbance at 254 and 365 nm (E2:E3 ratio) was used as a proxy for the molecular weight of the HS. The higher the molecular weight of HS, the lower the E2:E3 ratio as a result of stronger light absorption of higher size humic molecules at longer wavelengths (Helms et al., 2008).

2.10. Fluorescence quenching

Dilutions of HS were prepared to evaluate the quenching effect of HS in the presence of As and Fe. Fluorescence quenching occurs when there is a decrease in the fluorescence emission of a sample due to several molecular interactions. The formation of complexes (static mechanism) can produce this effect due to the formation of a ground state complex between a fluorophore and a quencher (Manciualea et al., 2009). The dynamic quenching involves collisions between the quencher and fluorophore and is dependent on the concentration (Senesi, 1990). Five dilutions of the HS extract of Holtemme (10 mg L^{-1} OCC) were prepared: inherent As and Fe

concentrations (A), 200 $\mu\text{g L}^{-1}$ As and inherent Fe concentration (B), 160 $\mu\text{g L}^{-1}$ As and 2.2 mg L^{-1} Fe (C), 100 $\mu\text{g L}^{-1}$ As and 5.5 mg L^{-1} Fe (D), and inherent As concentration and 11 mg L^{-1} Fe (E). Fluorescence analyses were carried out using an Aqualog Spectrofluorometer (Horiba), where EEM were acquired. The EEM were divided into 5 regions based on Chen et al. (2003).

2.11. Multivariate analysis

The whole data set was analysed using SOM to evaluate possible associations among all variables and among all samples. The software MatLab 2015b (MathWorks, Natick, MA) and the SOM toolbox (freeware, available on www.cis.hut.fi/somtoolbox/) were used to perform all analyses (Alhoniemi et al., 2000). The dataset was autoscaled before analysis so that all variables would have the same level of importance. The structure of each neuron was hexagonal and the shape was planar.

The discrimination properties of the SOM are related to the total number of neurons (hexagonal units) in the maps, since samples in the same or neighbouring neurons are considered similar. These related samples are recognized to be in a same group according to data provided (da Silva et al., 2008; Kowalski et al., 2013), what is directly related to the investigated variables. In this way, all (or the majority of) samples would be clustered together (same group) if the maps have a small number of neurons and no discriminant information would be extracted. On the other hand, big maps (higher number of neurons) would have samples too far apart, with no possibility to extract information of similarity (Kowalski et al., 2013; Gontijo et al., 2016). Therefore, architectures from 2×2 to 4×4 were tested and the most informative one (which allowed samples to be distributed in the map in a way without getting most of the samples in the same neuron or most in separate neurons) was chosen in order to detect the variability information of the samples.

3. Results and discussion

All samples taken in Cascatinha had very low electric conductivities (6 and 8 $\mu\text{S cm}^{-1}$ in the dry and rainy seasons, respectively) compared to samples from Germany where water from the Selke river showed the highest conductivity (414 $\mu\text{S cm}^{-1}$) and pH (7.6). The lowest pH (4.9) was measured in Cascatinha (rainy season). Other results of the physicochemical measurements and the sampling date are available in the supplementary section (Table A.2).

The results for water analyses of Al, As, Fe, and organic carbon in the TF, DF and FF are also presented in the supplementary section (Table A.3). Selke exhibited the highest amount of total As (28.6 \pm 1.1 $\mu\text{g L}^{-1}$) but the lowest amount of total Fe (115 \pm 6 $\mu\text{g L}^{-1}$). Holtemme showed the highest amount of total Fe (605 \pm 10 $\mu\text{g L}^{-1}$). Total As was below 1 $\mu\text{g L}^{-1}$ at Cascatinha, Holtemme and Warme Bode. Concerning the distribution, the relative amount of dissolved Fe in the FF for all samples was very low (less bioavailable). It

indicates that Fe was predominantly in the CF (DF – FF) either as Fe-minerals or complexed Fe. HS probably helped to keep this Fe suspended in solution (Ritter et al., 2006). A higher amount of total Fe in the Selke River (83%) occurred in the particulate fraction. On the other hand, total As in the Selke River was mainly in the FF (27.5 \pm 1 $\mu\text{g L}^{-1}$) and therefore more mobile and bioavailable at this sampling point. DOC concentrations of CF ranged from 2.0 mg L^{-1} (Selke river) to 7.1 mg L^{-1} (Holtemme river) whereas the variation was much smaller in FF (1.2–1.7 mg L^{-1} ; see Table A.3 in the SI).

Both, elemental analysis (Table 1) and ^{13}C NMR (Fig. 2 and spectra showed in Fig. A.4, supplementary section) showed that HS from the Selke River had the highest aliphaticity among all samples analysed (higher H/C ratio and relative area between 0 and 110 ppm in ^{13}C NMR). Furthermore, it had also the highest percentage of O and S. The finding that it had the highest O/C ratio may indicate a higher amount of carbohydrates. HS from Cascatinha (dry season) was the most aromatic sample as indicated by the highest relative area between 111 and 167 ppm in ^{13}C NMR. Looking only at the results of ^{13}C NMR from all samples analysed, the difference between maximum and minimum values for each integration region (Δxy) was small, indicating some similarity among all HS even though the samples were taken from different regions (Selke river was the sample location with the highest difference of (Δxy)). All HS samples showed C/N ratios higher than 20, indicating cellulose and lignin terrestrial material as precursors.

Results of FI ranging from 0.87 to 1.28 indicated that all HS were predominantly terrestrially derived (originated from degraded plants and/or soil organic matter), which agrees with the C/N ratios. Cascatinha (rainy season) showed the lowest FI (0.87), suggesting a higher contribution of these terrestrially-derived materials as precursor. In contrast, HS from Selke River exhibited the highest FI (1.28), which indicates a greater contribution from microbial sources. The HIX values of the samples were similar and high (around 0.90), suggesting a high degree of humification for all extracted HS. From the β/α -ratio it is concluded, that the HS from Cascatinha (0.36 - lowest value) was more decomposed ("less fresh") than HS from the Selke River which showed the highest β/α ratio (0.58), indicating that the material was produced more recently.

All HS extracted had depleted $\delta^{13}\text{C}$ values of about -27‰ (see supplementary section, Table A.4) indicating a higher terrestrial contribution of C_3 plants as precursor material. HS from Cascatinha (dry season) showed less negative values of $\delta^{13}\text{C}$ (-25.97‰) whereas HS from Warme Bode exhibited the most depleted $\delta^{13}\text{C}$ values (-27.93‰). All HS samples presented low values of $\delta^{15}\text{N}$ (around or below 3‰) indicating terrestrial origin and N_2 fixation.

The distribution of As, Fe, OCC and optical indexes of the 4 fractions of extracted HS are shown in Fig. 3. Most of the OCC in Cascatinha, Holtemme and Warme Bode HS appeared in fraction F1 (>10 kDa). HS from the Selke River showed overall lower molecular weight since most of its OCC occurred in fraction F2 ($5 < \text{F2} < 10$ kDa). Almost all Fe in the extracts was detected in

Table 1
Results from elemental analysis (free of ash) and optical spectroscopic properties of all HS extracted.

Sample	OCC (mg L^{-1}) ^a	Elemental content								Optical spectroscopic properties			
		C (%)	H (%)	N (%)	O (%)	S (%)	H/C	C/N	O/C	E2/E3 ^a	FI ^a	HIX ^a	β/α ^a
Cascatinha (dry)	479 \pm 13	47.2	7.2	1.3	43.7	0.7	1.8	42.9	0.7	3.4	0.96	0.91	0.36
Cascatinha (rainy)	812 \pm 59	52.1	6.8	1.3	39.3	0.6	1.6	48.0	0.6	3.2	0.87	0.95	0.36
Holtemme	2028 \pm 163	52.5	7.5	1.5	37.7	0.7	1.7	39.8	0.5	3.7	1.01	0.90	0.38
Selke	1130 \pm 5	42.8	9.4	1.5	45.3	1.1	2.6	33.5	0.8	5.4	1.28	0.89	0.58
Warme Bode	887 \pm 68	47.5	6.6	2.3	42.8	0.8	1.7	24.1	0.7	4.5	1.02	0.94	0.44

^a OCC = organic carbon in the humic substances extracted; E2 = absorbance in 254 nm; E3 = absorbance in 365 nm; FI = fluorescence index; HIX = humification index; β/α = freshness index.

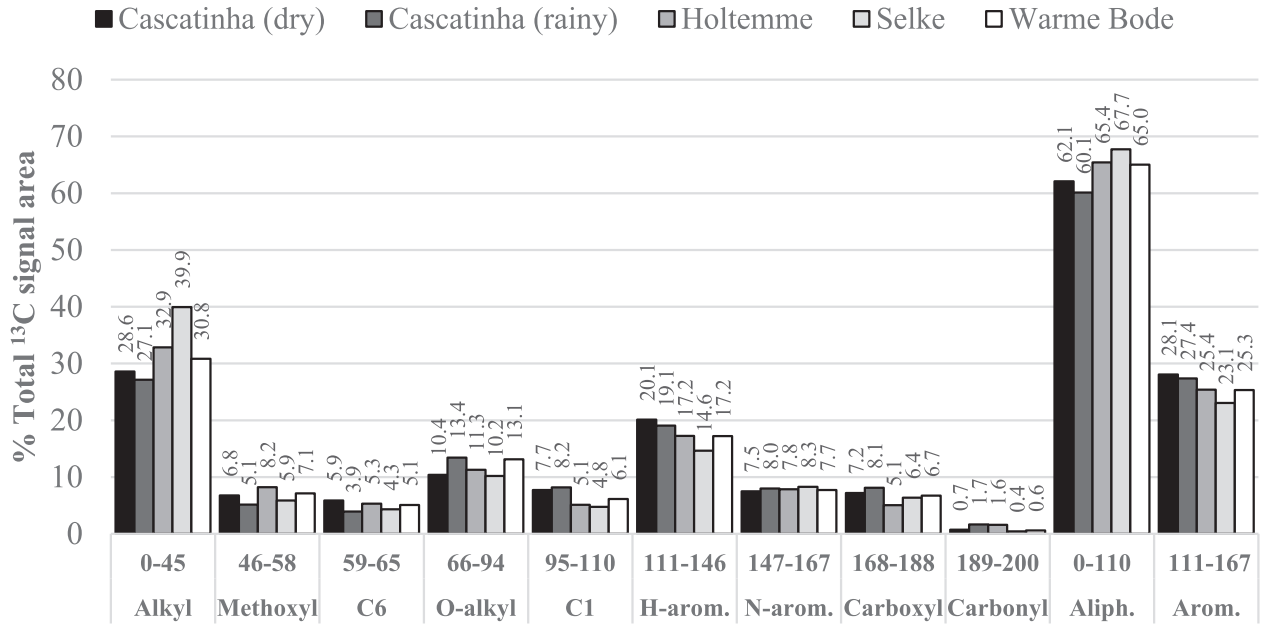


Fig. 2. – Integration values of ¹³C NMR areas of HS.

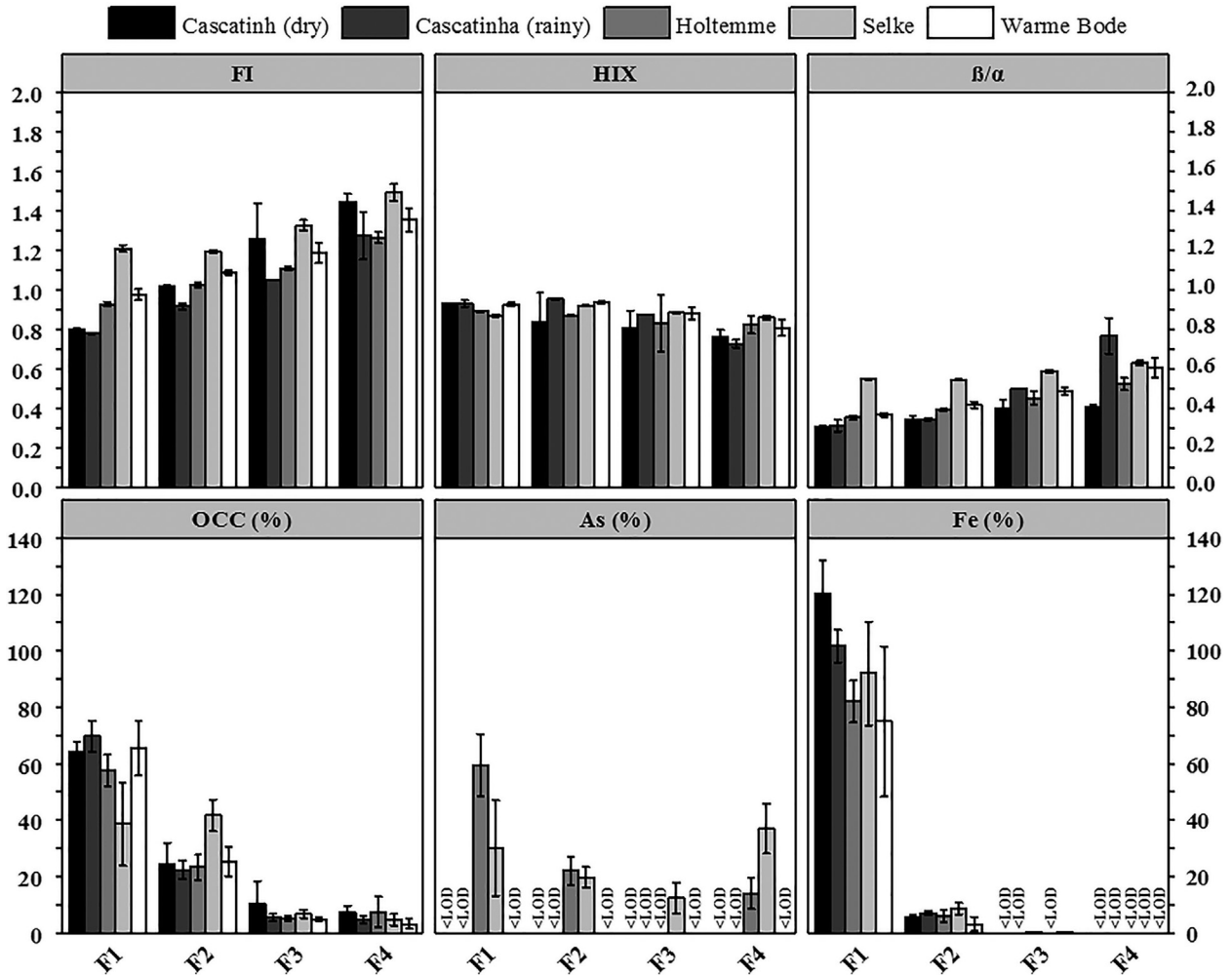


Fig. 3. – Distribution of As, Fe, OCC, and optical indices for all aquatic humic substances in four fractions: F1>10 kDa, 5 kDa < F2<10 kDa, 1 kDa < F3<5 kDa, F4<1 kDa (LOD = limit of detection).

fraction F1, even for Selke, indicating that Fe has more affinity to bind higher molecular weight fractions of HS. Probably this Fe was complexed or bound in aggregates to HS. It supports Nagao et al. (2009) who, in their studies with actinides, concluded that higher molecular weight sizes have a higher complexation ability. When detected, As was distributed especially between the highest and lowest molecular weight fractions (F1 and F4). The As distribution is probably linked to Fe, since the direct As binding is expected to be weak and/or not significant (Ritter et al., 2006; Sharma et al., 2010). Therefore, As in F1 was probably bound to colloidal Fe or as As-Fe-HS complexes. Fractions of higher molecular size showed lower FI and β/α -ratios reflecting respectively a greater terrestrial contribution and less fresh material than lower molecular size fractions, which had a higher autochthonous microbial contribution and were more recently produced. The higher molecular size fractions exhibited also higher HIX than lower molecular size fractions, indicating a higher degree of humification.

The results of the complexation experiments are shown in Fig. 4. From Fig. 4 (a) it can be seen that both, with and without addition of Fe, there was no influence of OCC on the complexation of As. The presence (spiked value) or absence (low inherent, natural concentrations) of iron was the determining factor for the amount of free As. A small decrease in the amount of free As could be observed when the concentration of OCC increased in both experiments (with and without Fe addition). The inherent Fe in the extract and availability of binding sites after increasing HS concentration may explain this trend. Fig. 4 (b) shows the influence of the HS extracts on the complexation of As with addition of Fe. Most of the extracts showed similar behaviour concerning the free As measured in the filtrate, except for the Selke river extract, which had the lowest amount of free As.

At first glance, most results indicated that all samples had similar characteristics and it would be difficult to explain why Selke samples had a lower amount of free As. However, the SOM analysis (Fig. 5) showed that the difference among these results was sufficient to discriminate the extracted HS into three groups (I, II and III in the map of samples, Fig. 5). Moreover, it helped to explain the different behaviour of Selke samples regarding As complexation. HS extracted from the Selke river belongs to group I, which had the highest values of alkyl C, N-aromatic C, aliphaticity, E2/E3, FI, β/α , % H, %O, %S, H/C, $\delta^{15}\text{N}$ and total Fe (see darker colours in the correspondent neuron of Selke in the map of each variable, Fig. 5). In

addition, it had lower intensities for C1, C6, free As, methoxyl, SUVA₂₅₄ and %C (see lighter colours in the correspondent neuron of Selke in the map of each variable, Fig. 5). HS from Holtemme and Warne Bode were grouped together (group II) and therefore considered similar. Group II had the highest intensities of the variables methoxyl, O-alkyl, HIX, %C, %N, total Fe and free As. All samples taken in Cascatinha (dry and rainy seasons) were grouped together (group III) in the same neuron reflecting no effect of seasonality, although small differences between the samples to some variables were recorded as discussed previously to $\delta^{13}\text{C}$. Group III had higher values of C1, C6, H-aromatic, carboxyl, carbonyl, aromaticity, HIX, SUVA₂₅₄, $\delta^{13}\text{C}$, %C and free As.

Looking at the maps of variables it was possible to explain why HS from the Selke river had less free As (or more bound As) than the other samples analysed. The greater amount of complexed As can be attributed to a higher amount of N-aromatic C and a higher content of S (higher intensities of these variables at correspondent neuron of Selke, Fig. 5), since it is known that N- and S- containing functional groups can enhance the reactivity of HS (Mladenov et al., 2015). These interactions probably include the formation of HS-Fe-As and/or, to a small extent, direct HS-As associations. Thiol groups (-S containing) for instance can bind to As through Fe associations or, to a small extent, directly (Catrouillet et al., 2015, 2016). Another reason for the lower amount of free As in the Selke HS was the lower molecular size, indicated by the high E2/E3 ratio and confirmed by the fractionation experiments. The molecular size of HS is also an important factor for the ability to control HS reactivity (Bauer and Blodau, 2009), as opposed to Fe, where higher sized molecules have higher complexation ability (Nagao et al., 2009). However, at the same time it is known that low molecular weight molecules have higher density of carboxyl groups (and maybe other groups) (Bauer and Blodau, 2009) which are able to bind Fe and As. The alkyl content and aliphaticity probably did not contribute to higher binding, although these variables were higher in Selke river HS than in other samples. Total Fe was not the main factor contributing to the lowest concentration of free As in Selke river HS, since a higher total Fe concentration was measured in Warne Bode HS. HS from Warne Bode river had higher amount of free As, like the other HS samples (all theoretical and measured values of As and Fe in the solution before ultrafiltration are available in the SI (Table A.5)). Higher FI and β/α -ratio are also related to a higher amount of free As(V), probably indicating that recently produced

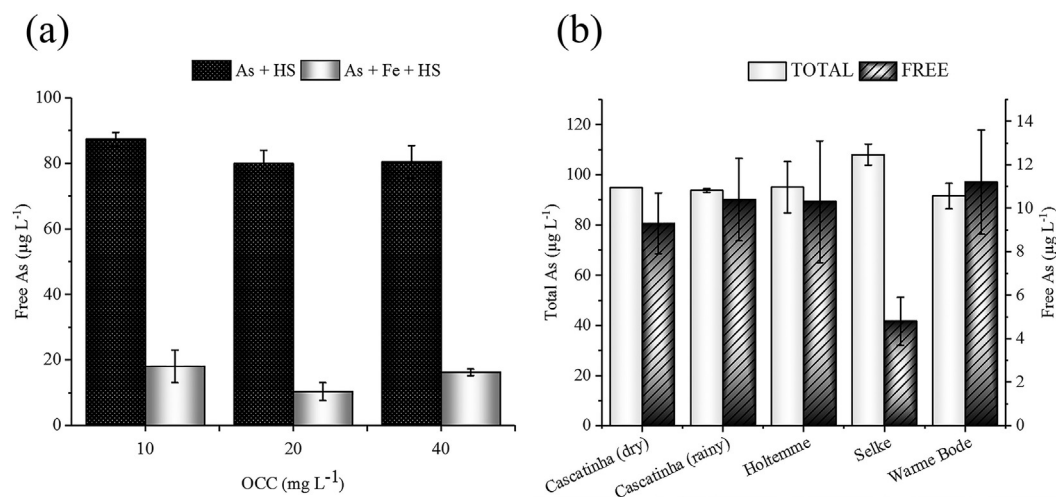


Fig. 4. – Results of the complexation tests. The left graphic (a) shows the influence of OCC (Holtemme HS extract) with (As + Fe + HS) and without (As + HS) addition of Fe. The right graphic (b) shows the influence of different HS extracts on the As distribution. All results for free As were corrected due to the loss of 11.3% in the system during ultrafiltration (see Fig. A.2 in the SI). The mean of total As for Holtemme HS was calculated from total concentration of all points.

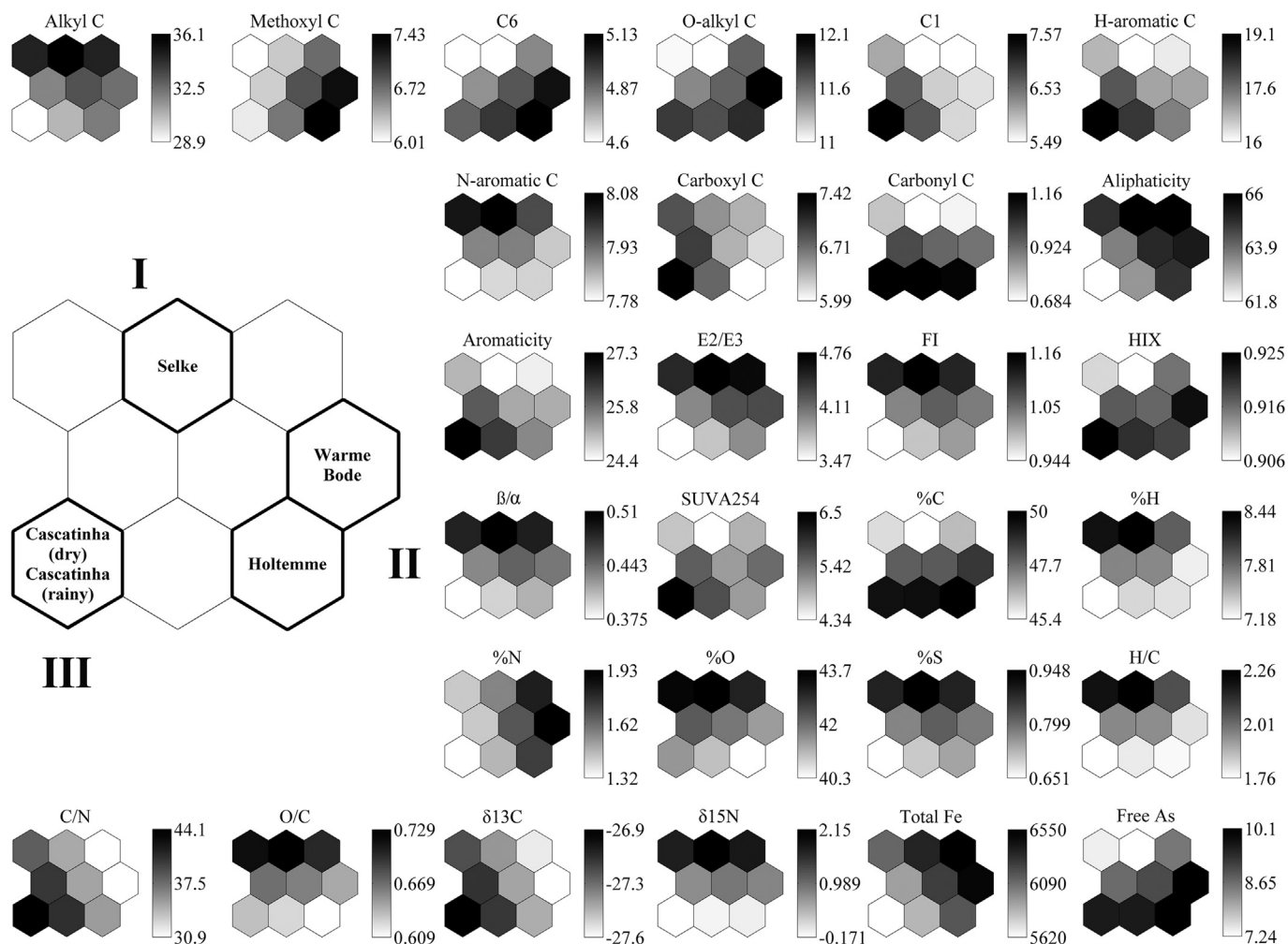


Fig. 5. – Kohonen neural network: the centre-left panel is the map of samples. Samples in the same or neighbouring neurons (hexagonal units) are considered similar and therefore were highlighted and numbered (3 groups). The other small maps are maps of variables. The bars beside the maps indicate the intensity of each variable. The darker the colour the higher the intensity to each variable. Total Fe corresponds to the Fe measured before ultrafiltration by 1 kDa. Free As corresponds to the As measured after ultrafiltration by 1 kDa.

HS and HS with a higher contribution from microbial sources have higher complexation ability.

The mean of fluorescence intensities in the region of humic acid-like organic matter (excitation/emission = 321–345/409–439 nm) of all diluted extracts (fluorescence quenching experiments) with and without addition of As and/or Fe is shown in Fig. 6. The mean of the fluorescence intensities decreased just a little when only As(V) ($200 \mu\text{g L}^{-1}$) was added (Fig. 6B) to the original sample with no additions (Fig. 6A). When Fe was added (Fig. 6C–E) the intensity decreased substantially due to static and dynamic quenching effects. This can also be seen in the EMM of all experiments presented in the SI (Fig. A.5).

When comparing field data and experimental data, it was possible to infer why the Selke river water (*in natura* sample) showed a higher concentration of free As (see Table A.3, in the SI), although its HS could complex more As than the HS extracted from the other rivers under consideration (Fig. 4b). Most of the Fe at this sampling point occurred in the particulate fraction and the concentration of DOC (and consequently HS) was very low. Therefore, the amount of HS and colloidal Fe were probably the most important factor limiting As binding in water samples from the Selke river. The waters of the other sampling points did not have significant amounts of As, although they had a higher concentration of dissolved Fe. The limiting factors, low Fe and/or low DOC may also

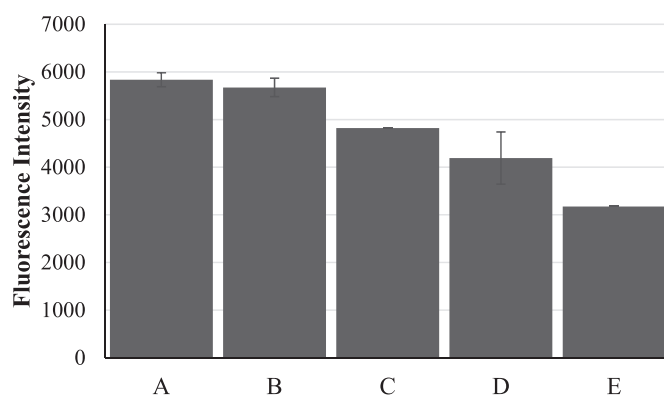


Fig. 6. – Mean of fluorescence intensity in the humic-like area (excitation/emission = 321–345/409–439 nm). All samples had the same OCC concentration of 10 mg L^{-1} (A): Inherent As and Fe concentrations; (B): $200 \mu\text{g L}^{-1}$ As and inherent Fe concentration; (C): $160 \mu\text{g L}^{-1}$ As and 2.2 mg L^{-1} Fe; (D): $100 \mu\text{g L}^{-1}$ As and 5.5 mg L^{-1} Fe; (E): inherent As concentration and 11 mg L^{-1} Fe.

explain the higher amount of free As found at some points in our previous work on rivers in Brazil (Gontijo et al., 2016), where a higher As concentration was detected close to mine operations.

4. Conclusions

Associations among HS, Fe and As were mainly related to the chemical characteristics of HS, like the amount of N-aromatic C, presence or absence of -S functional groups or even their molecular size. Although all extracts of HS analysed had apparently similar characteristics, the Kohonen neural network analysis proved to be a useful tool for discriminating all samples into 3 groups and point out all their chemical differences. It also helped to explain the lower amount of free As in the complexation experiments with HS from the Selke river. The chemical characterisation (^{13}C NMR, $\delta^{13}\text{C}$ isotope analyses) suggested that all samples extracted had predominantly cellulose and lignin terrestrial material from C_3 plants as precursor material. The fractionation into different sizes showed that most of the Fe and As occurred in the fractions of higher molecular weight size (>10 kDa). In addition, fluorescence analyses suggested that higher size fractions had a greater terrestrial contribution, a higher degree of humification and less fresh material than lower size fractions.

In our experiments, colloidal Fe was probably a limiting factor for As complexation in natural waters. The amount of free As did not change in the range of the DOC investigated in the complexation tests. However, field results from our previous works indicated that DOC can be a limiting factor when present only in very low concentrations (Gontijo et al., 2016). HS play an important role in the dynamics, behaviour and distribution of As in aquatic systems. At higher concentrations, HS can keep Fe in the colloidal phase and increase As mobility. Kohonen neural networks can be an important and cheap tool for such investigations and could support studies of bioavailability and toxicity of As, especially for poor countries rich in Fe and HS, and contaminated with As.

Acknowledgements

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant number 2012/17727-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Deutscher Akademischer Austauschdienst (DAAD, CAPES/DAAD grant number BEX10452/14-1) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant number 400572/2013-3). We thank the Otto Von Guericke Universität Magdeburg for the analyses of NMR and elemental analyses and especially Ms. Liane Hilfert for her assistance. We acknowledge Mr. Michael Herzog for his assistance in the laboratory at UFZ and Dr. Peter Herzsprung, for his help with the fluorescence analyses.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2017.08.143>.

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