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Extraction and Concentration of Freshwater – and Seawater – Derived Dissolved Organic Matter for Use in Aquatic Toxicology Studies

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ABSTRACT

Dissolved organic matter (DOM) is defined as the organic matter that passes through a 0.45 μm-mesh filter. Recent studies have shown the DOM importance in mitigating trace metals and organic pollutants toxicity. In general, studies with DOM are performed using commercial organic matter. However, it has been demonstrated that this organic matter has little structural similarity with the aquatic humic acids. Furthermore, a natural DOM is composed by different fractions, which can exhibit different complexing properties with metals. Thus, it is important to evaluate the effect of different sources of natural DOM on pollutants toxicity. To use natural DOM in aquatic toxicity tests, it is necessary to extract and provide suitable storage for samples in the laboratory. The ideal process for DOM isolation from natural waters should be capable of rapidly and effectively extracting large quantities of DOM from water without fractionation, chemical alteration and/or other losses. Therefore, in the present paper we describe the methodological approaches currently in use in our laboratory for extraction and concentration of both freshwater and seawater-derived DOM using reverse osmosis (freshwater) and solid phase extraction (seawater).

Key-words: aquatic toxicology, freshwater, organic matter, PPL resin, reverse osmosis, seawater, sewage, solid phase extraction.

RESUMO

Extração e concentração de matéria orgânica dissolvida de águas doce e marinha para uso em estudos de toxicologia aquática

A matéria orgânica dissolvida (MOD) é definida como a matéria orgânica que passa através de uma membrana de 0,45 μm. Estudos recentes têm mostrado a importância da MOD na proteção contra a toxicidade de metais e poluentes orgânicos. Em geral, os estudos com MOD são realizados com matéria orgânica comercial. No entanto, tem sido demonstrado que esta matéria orgânica tem pouca semelhança estrutural com os ácidos húmicos aquáticos. Além disso, a MOD natural é composta por diferentes frações, que podem exibir diferentes capacidades de complexação com os metais. Portanto, é importante avaliar o efeito de diferentes fontes de MOD natural na toxicidade dos poluentes. Porém, para usar a MOD natural em testes de toxicidade é necessária adequada extração e armazenagem das amostras em laboratório. O processo ideal para isolar MOD de águas naturais deve ser capaz de extrair rápida e efetivamente grandes quantidades de MOD da água sem fracionamento, alteração química e/ou outras perdas. Portanto, no presente artigo, nós descrevemos as estratégias metodológicas atualmente utilizadas em nosso laboratório para a extração e concentração de MOD de águas doce e marinha, através de osmose reversa (água doce) e extração em fase sólida (água do mar).

Palavras-chave: água doce, água do mar, efluente, extração em fase sólida, matéria orgânica, osmose reversa, resina PPL, toxicologia aquática.

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INTRODUCTION

Dissolved organic matter (DOM) is defined as the organic matter that passes through a 0.45 μm-mesh filter. It is expressed in mg L^{-1} as dissolved organic carbon (DOC), since carbon is its major component, and because its structure is complicated and not well defined. Furthermore, DOM composition can vary according to origin, season, and physicochemical characteristics of the surrounding environment. Carbon accounts for about 50% of the natural organic matter (NOM) weight. The term "total organic carbon" (TOC) refers to all organic carbon species found in organic structures, present in the water, from methane with a molecular weight of 16 Da to the large and complex humic substances (500-100,000 Da). DOM had generally been considered as being composed mainly of humic substances. Humic substances are generally described as heterogeneous polyfunctional polymers formed through the breakdown of plant and animal tissues by chemical and biological processes, and generally comprise one-third to one-half of the DOC present in natural waters (Thurman, 1985). Actually, they are reported as non-stable polymers. For example, at neutral or alkaline pHs, humic substances can be present as supra-molecular associations of relatively small heterogeneous molecules held together by weak dispersive forces such as van der Waals interactions (Conte *et al*., 2006). Furthermore, non-humic and humic solutes are not completely distinguishable from each other, because some natural non-humic solutes, such as soil carbohydrates, can be an integral part in the structural composition constructing humic solutes (Peuravuori *et al*., 2005).

DOM has various functions and plays important roles in aquatic ecosystems. For instance, it interacts with trace metals and controls their dynamics. Furthermore, it fuels the microbial loop, generates gases and nutrients with biological and photochemical reactions, absorbs and extinguishes light, and affects satellite images (Ogawa & Tanoue, 2003). Several studies have also shown the DOM importance in mitigating the toxicity of trace metals and organic pollutants (*e.g.* Erickson *et al*., 1996; Fliedner, 1997). DOM forms complexes with metals, thus reducing their bioavailability and toxicity (Erickson *et al.,* 1996; Ma *et al.,* 2001; Kramer *et al*., 2004; De Shamphelaere *et al.,* 2004). In general, studies regarding the DOM influence on metals toxicity are performed using commercial organic matter, usually as humic acid (Aldrich) from soils (De Shamphelaere *et al*., 2005). However, it has been demonstrated that this humic acid has little structural similarity with the aquatic humic acids (Malcolm & MacCarthy, 1986). Furthermore, a natural DOM is composed by different fractions, as the humic and fulvic acids, which can exhibit different complexing properties with metals (Ma *et al.,* 2001).

Natural DOM concentrations depend primarily on the type of water, origin, vegetation and climate, among other factors (Kramer *et al.,* 2004). For example, the humic substances constitute 10-30% of the marine DOM and 70-90% of the freshwater marsh DOM (Thurman, 1985). DOMs from different sources can be composed by molecules with different characteristics, since they are formed from different precursors (Ryan *et al.,* 2004). Thus, their complexing characteristics and capacities can vary from one place to another (Kramer *et al*., 2004; Ryan *et al*., 2004). Consequently, the DOM source can influence the bioavailability and toxicity of metals to aquatic organisms (Kramer *et al.,* 2004).

To use natural DOM in toxicological tests is necessary to extract and provide suitable storage for samples in the laboratory. The ideal process for DOM isolation from natural waters should be capable of rapidly and effectively extracting large quantities of DOM from water without fractionation, chemical alteration and/or other losses (Sun *et al*., 1995). DOM has been isolated from water by various methods, including precipitation, vacuum evaporation, ultrafiltration, solvent extraction, freeze-drying, freeze-concentration, charcoal, strong anion-exchange resins, reverse osmosis, and by adsorption chromatography with macroporous resins, such as XAD resins (for review, see Malcolm, 1989). However, there are clear differences in the practicality, extraction efficiency, and characteristics and amount of DOM extracted using these different techniques. In this context, XAD resins are widely used by hydrology researchers since early 1970's, because they overcame some of the limitations of older used methods (Thurman & Malcolm, 1981; Malcolm, 1989). XAD-2, XAD-4, and XAD-8 (Amberlite®) are the most common adsorbers used for extraction and concentration of DOM from both freshwater and seawater (Peuravuori *et al*., 1997; Maurice *et al*., 2002; Engbrodt, 2001; Engbrodt & Kattner, 2005). However, XAD resin cleaning procedure previous to DOM extraction is quite laborious and time consuming (Standley & Kaplan, 1998). Therefore, in the last years reverse osmosis became one of the most used methods for extraction of large amounts of DOM from natural freshwater (Maurice *et al*., 2002; De Shamphelaere *et al*., 2005). In addition, some different adsorbers such as polypropylene (PPL) resins for extraction of large amounts of DOM from seawater were recently tested (Boris *et al*., 2007).

In light of the above, in the present work we describe the methodological approaches recently used in our laboratory for extraction and concentration of natural DOM for use in aquatic toxicology studies. Procedures employed are based on the reverse osmosis and solid phase extraction with PPL resin methodologies for freshwater- and seawater-derived DOM extraction, respectively. Results obtained are compared with those from the literature when XAD resins were used for freshwater- and seawater-derived DOM extraction.

MATERIALS AND METHODS

Water collection for DOM extraction

Approximately 200 L of water from freshwater or seawater were collected in the field and brought to the laboratory in polyethylene containers previously cleaned with 1% HNO_3. Freshwater was then filtered using a sequence of polypropylene filters (nominal pore sizes = 10, 5, and 0.5-μm mesh filters;

Cuno, Polyclean®, Brazil). For seawater, only the 0.5 µm-mesh filter was used.

When it was not possible to extract and concentrate DOM immediately, collected water was kept at 4°C in the dark to avoid photochemical transformation (Bertilsson & Tranvik, 2000; Ma *et al.*, 2001).

Freshwater-derived DOM extraction and concentration using reverse osmosis

Freshwater-derived DOM was extracted from water collected at the Vieira Stream (Rio Grande, RS, Southern Brazil) (Fig. 1) in March 2006. The first source of DOM was the water collected before the "Navegantes" Public Sewage Treatment Plant (BSTP). The second one was the water collected about 2 m after the effluent discharge from the Treatment Plant (ASTP). The effluent from the Treatment Plant goes directly into the Vieira Stream, which flows into the Patos Lagoon Estuary (Rio Grande, RS, Southern Brazil).

Approximately 200 L of freshwater were collected in the field and brought to the laboratory in polyethylene containers previously cleaned with 1% HNO_3. Water was then filtered using a sequence of polypropylene filters (nominal pore sizes = 10, 5, and 0.5-µm mesh filters; Cuno, Polyclean®, Brazil). The filterable fraction obtained was considered as the DOM source. DOM was then isolated and concentrated by reverse osmosis (RO). The experimental procedures were based on the methodology described by De Schamphelaere *et al.* (2005).

After water collection, procedures for DOM extraction and concentration were done as quickly as possible. These procedures are summarized in Figure 2. Briefly, the permeate solution from the RO system, *i.e.* the purified water, was discarded, while the concentrated solution was recycled into the sample reservoir and mixed with additional water from the sampling site. The concentrated solution, which would be discarded from the RO system, was considered as the concentrated DOM. RO extraction was performed until DOM was concentrated as desired.

Total organic carbon (TOC) concentration in DOM stock solutions was measured using a TOC analyzer (TOC 5000, Shimadzu). DOM stock solutions were filtered again prior to TOC measurements using 0.45-µm mesh acetate-cellulose filters (Sartorius).

In this case, we considered that the DOM was completely dissolved, being TOC considered as DOC. DOC concentration in the concentrated solution was determined using a TOC analyzer.

Seawater-derived DOM extraction and concentration using solid phase extraction with PPL cartridges

Seawater-derived DOM was extracted from the water collected approximately 20 miles away from the Cassino Beach (Rio Grande, RS, Southern Brazil), representing a more autochthonous source of DOM (Fig. 1). Water was collected in December 2006.

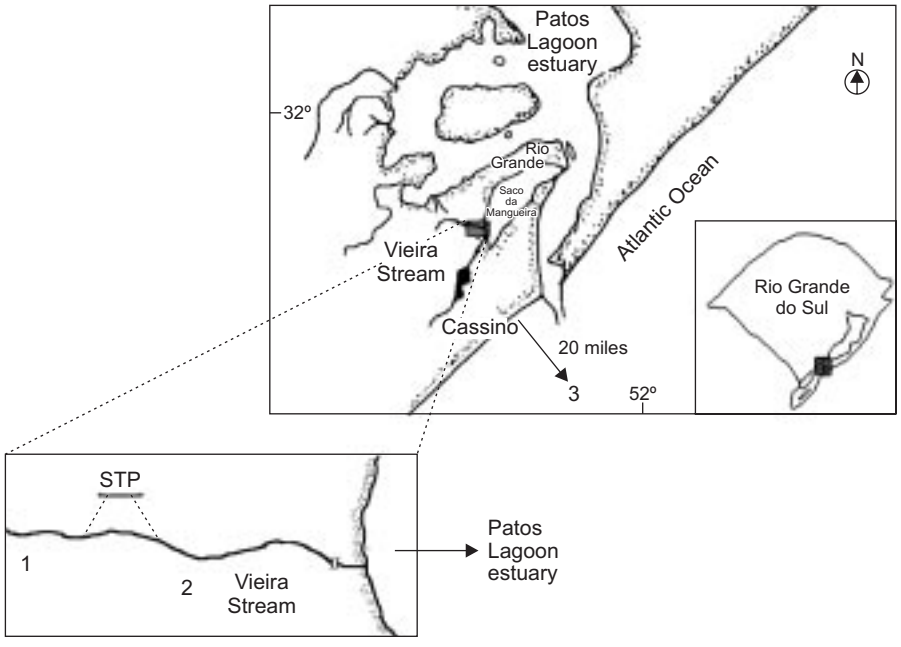

Figure 1 – Sampling sites where water was collected to extract natural organic matter from both the Vieira Stream and Cassino Beach (Rio Grande, RS, Southern Brazil). 1 = before the "Navegantes" Public Sewage Treatment Plant (BSTP); 2 = about 2 m after the "Navegantes" Public Sewage Treatment Plant (ASTP); 3 = about 20 miles away from the Cassino Beach. *Source*: Gilberto Fillmann (Fundação Universidade Federal do Rio Grande, RS, Brazil).

Approximately 200 L of seawater were collected as previously described for freshwater DOM, and filtered using a 0.5 µm-mesh filter (Cuno, Polyclean®, Brazil). The filterable fraction obtained was considered as source of DOM. Marine-derived DOM was then concentrated using PPL cartridges (Mega Bond Elut PPL, 5 GM 60 mL, 16/PK, Varian), as recommended by Koch *et al.* (2007). Bond Elut PPL is a functionalized styrene-divinylbenzene polymer, which has been optimized for the extraction of highly polar species from large-volume water samples. Featuring a proprietary high-purity spherical polymer with an extremely non-polar surface, Bond Elut PPL retains even the most polar compounds, such as phenols, and achieves high recoveries and fast extraction speeds (Varian Inc., Palo Alto, CA, USA). PPL cartridges were activated using methanol (Merck) and Milli-Q water before its use for DOM extraction. Two successively fillings of each solution were performed to activate the PPL cartridges. Filtered seawater was then acidified (pH 2) with HCl, and placed in 10-L glass flasks previously cleaned with 1% HNO_3. Each flask was connected to one PPL cartridge through a plastic tube and sealed with a plastic cap. Flow rate should not exceed 20 mL min^{-1}, and up to 60 L of seawater can be allowed to pass through each cartridge. Any pumping device was used to accelerate the methanol and Milli-Q water flows during cartridge activation or the seawater flow during the marine-DOM concentration and elution.

After all seawater passed through cartridges, the next step was to remove salts from the resin, before DOM elution. Thus, cartridges were rinsed with 100 mL of Milli-Q water acidified (pH 2) with HCl. Then, DOM was eluted with 120 mL of methanol at a maximum flow rate of 10 mL min^{-1}. Methanol eluate was dried at room temperature (~20ºC). Afterwards, a DOM stock-solution was prepared diluting the DOM powder extracted using Milli-Q water. DOC concentration in the DOM stock solution was determined using a TOC analyzer, as previously described. This solution was then stored at 4ºC in the dark until its use in toxicological tests. All procedures for seawater-derived DOM extraction and concentration are summarized in Figure 2.

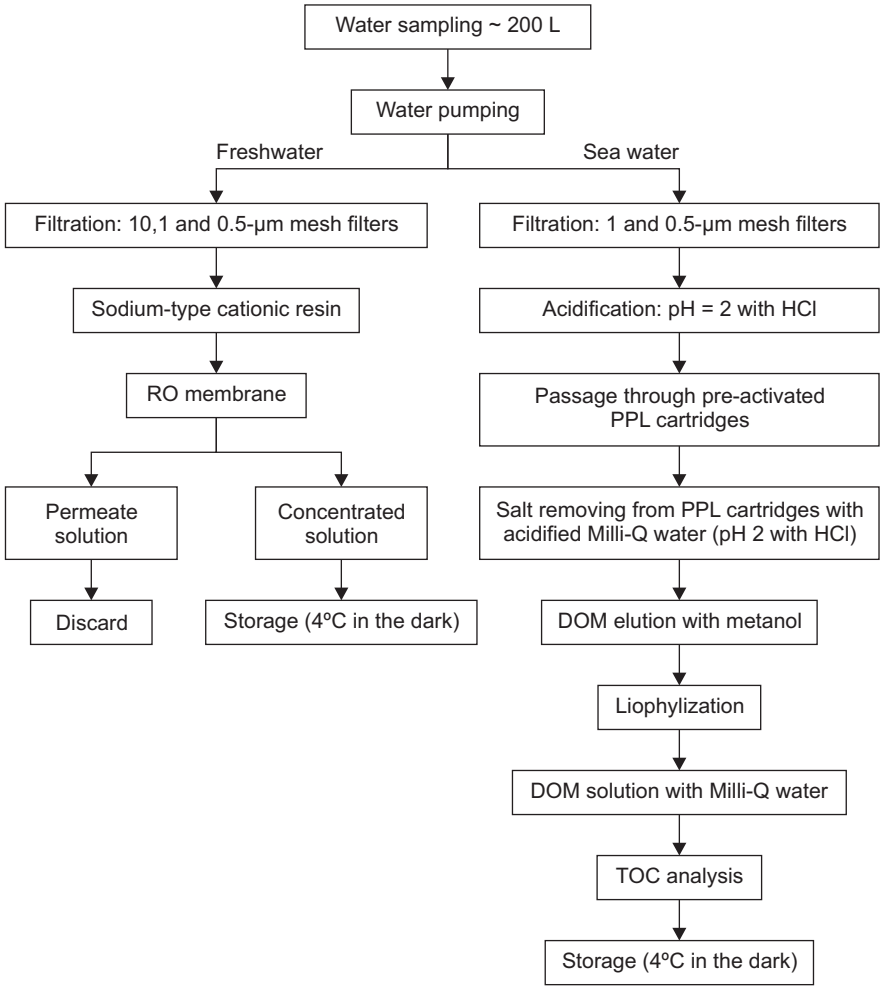

Figure 2 – Procedures for collection, concentration and storage of freshwater – and seawater – derived DOM using reverse osmosis (RO) and PPL cartridges techniques, respectively.

RESULTS AND DISCUSSION

Water samples collected for freshwater-derived DOM extraction was concentrated by ~15-fold. The DOC concentration in the water from the Vieira stream was 18 mg C L^{-1} at both sampling sites, and increased to 121.8 and 126.3 mg C L^{-1} in the DOM stock solutions prepared with water collected before (BSTP) and after (ASTP) the public sewage treatment plant, respectively. Therefore, DOC concentration increased ~7-fold for both DOM stock solutions, and the recovery yielded was ~50% (Table 1). This result is similar to that obtained by Maurice *et al.* (2002) using XAD-8 resins to concentrate DOM from a freshwater fen in a pine region. However, other studies using RO observed higher recoveries (between 80 and 100%) (Serkiz & Perdue, 1990; Sun *et al*., 1995; Maurice *et al.*, 2002; De Shamphelaere *et al.*, 2005). Although RO isolation gives, in general, higher recoveries of carbon than XAD-8, it was reported that it gives also higher ash content, especially Si and S, and it can promotes condensation and coagulation (Maurice *et al.*, 2002). On the other hand, XAD-8 resins is designed to isolate only the humic fraction of DOM, and it apparently isolates more hydrophobic compounds and causes ester hydrolysis, thus leading to DOM chemical alterations (Maurice *et al.*, 2002).

Although high recovery yield is an important reason for the successful use of RO for DOM extraction, there are other factors to be considered. Among the main advantages of this sampling technique, when compared to another one like XAD-8 resin, we can mention the low probability of chemical alteration and the relatively quickness of concentration of large quantities of DOM from natural surface waters (Serkiz & Perdue, 1990; Sun *et al*., 1995). In turn, one of the main disadvantages of using XAD-8 resin is the need for a careful cleaning of the resin prior its use. Cleaning procedures have been reported to be laborious and time consuming (Standley & Kaplan, 1998).

Research with natural DOM is greatly facilitated if significant quantities of DOM can be extracted and concentrated relatively fast, providing samples to prepare, for months after collection, large volumes of experimental media with various nominal DOC concentrations, if necessary. Thus, the use of RO for freshwater-derived DOM extraction is highly recommended for this purpose in spite of the fact that some problems can occur during DOM extraction. Some of these problems are discussed below.

A possible problem associated with the use of the RO extraction is the increasing intermolecular interactions at higher DOM concentrations (Zsolnay, 2003). In addition, pressure can also influence DOM solubility during concentration procedures in the RO system. Pressure changes can result in cavitation with the formation of small gas bubbles. Surface-active DOM can then adsorb on the bubble surfaces, and once bubbles collapse they adsorb DOM as particulate organic matter (Zsolnay, 2003). To ensure that DOM concentrated by RO was really dissolved, in the present work DOM solutions were filtered again using 0.45-µm mesh filters. As filters can release organic materials, a blank test was performed filtering Milli-Q water and measuring DOC, as previously described. In our study with DOM derived from freshwater collected at the Vieira Stream, DOC in Milli-Q water was only 3.27 mg C L^{-1}, being this attributable to a DOC releasing from the acetate-cellulose filters used. In this case, the contribution of the acetate-cellulose membrane to the total DOC in the stock solutions was only of 2.7 and 2.6% for the BSTP and ASTP DOMs. Despite these adversities, De Shamphelaere *et al*. (2005) showed that DOM extraction and concentration using the RO technique does not affect the physicochemical characteristics of the experimental media prepared with the DOM extracted and concentrated, as well as the protective effects of the DOM against metal toxicity in freshwater organisms.

To ensure the quality of the DOM extracted, some precautions should be take into account when using the RO technique. One of them is related to how often the RO membrane should be changed. In our laboratory, we used one RO membrane for each extraction. However, the fact that only ~50% recovery was obtained suggests that the RO membrane should be changed more frequently along the DOM extraction, to avoid membrane saturation. As previously mentioned, cavitation can lead to particulate organic matter formation, thus increasing probability for membrane saturation, although water was passed through a sodium type cation exchange resin before to reach the RO membrane.

Another precaution is related to the complete cleaning of the RO system before and after each new extraction. For example, Sun *et al.* (1995) described a thorough cleaning procedure conducted in their laboratory. The process consisted in recirculating a 0.5 g L^{-1} solution of a detergent (Alconox) at low operating pressure, followed by a thorough rinse with pure water. In our case, after the extraction of BSTP-derived DOM, we let tap water followed by ASTP water flows through the RO equipment before ASTP extraction. No problems associated with the cleaning procedure adopted would be expected, since BSTP water should contain less suspended solids and other particulate materials than the ASTP water, and also because both sources of DOM were collected in the same stream (Vieira Stream).

Despite the clear advantages showed by the RO system for extraction of freshwater-derived DOM, this equipment cannot be used for DOM extraction from seawater, since salts start to precipitate right away (*e.g.*, calcium is nearly its solubility limit in seawater). In addition, seawater represents, in terms of DOC recovery yielded after extraction and concentration, one of the most challenger sources of DOM, since inorganic matter can be approximately 30,000 times more concentrated than organic matter, and DOC was estimated to be only between 0.3 and 2.0 mg C L^{-1} in seawater (Thurman, 1985). Despite these difficulties, some different adsorbers for extraction of large amounts of DOM from seawater were recently tested (adsorbers with varying hydrocarbon chains bonded to a silica structure and styrene

divinil benzene polymer type adsorbers). Solid phase extraction through PPL cartridges was the most efficient adsorber among those tested (Boris *et al.*, 2007).

In the present study, we employed the solid phase extraction technique using PPL cartridges filled with Mega Bond Elut resin (Varian). The DOC concentration measured in the marine DOM stock solution obtained was 555.28 mg C L^{-1}. In turn, DOC in Milli-Q water used as a blank test was only 5.88 mg C L^{-1}. This concentration is attributable to a DOC releasing during filtration (acetate-cellulose membrane) of the DOM stock solution prior its use in toxicological experiments. In this case, the contribution of the acetate-cellulose membrane to the total DOC in the stock solutions was only of 1.1%, and the net concentration of DOC extracted from seawater was 549.40 mg C L^{-1}.

Unfortunately, the DOC concentration in the seawater collected at the sampling site was not measured. However, considering that: (1) the extraction efficiency of PPL cartridges with the resin employed is generally around 50% (Boris *et al.*, 2007); (2) the DOC concentration in our DOM stock solution was 549.40 mg C L^{-1}; (3) DOC obtained was extracted from 60 L of seawater; (4) the volume of DOM stock solution obtained was 100 mL; the concentration at our sampling site would be estimated as ~1.83 mg C L^{-1}. It must be pointed out that this value is very close to the mean DOC concentration found in seawater in the euphotic zone or near coastal regions (1.5 mg C L^{-1}; Malcolm, 1989). Furthermore, it falls into the range of DOC concentration generally reported for seawater (0.3-2.0 mg C L^{-1}; Thurman, 1985) (Table 1).

Studies reported in the literature show that 99% of the initial DOC content in seawater was recovered in the hydrophilic and hydrophobic fractions of the fractionation process using XAD-2 and XAD-4 resins, and hence no irreversible adsorption at the resin was observed (Engbrodt, 2001). Despite the higher recovery yielded with XAD resins than with the PPL resin used in the present study, solid phase extraction with XAD resins shows some disadvantages. For example, careful XAD resin cleaning is required in order to guarantee that possible incorporation of organic matter from the adsorbent does not occur (Thurman & Malcolm, 1981; Daignault *et al*., 1988; Malcolm, 1989; Standley & Kaplan, 1998). The equipment required for the cleaning procedure is expensive (high volume Soxhlet units and organic solvents) and the method is time consuming (the original method takes 5 consecutive days to be completed), demanding much effort (Daignault *et al*., 1988; Malcolm, 1989). Solvents used for cleaning are in general hazardous (methanol, dichloromethane, acetonitrile, diethyl ether, and acetone), demanding an efficient exhaustion system in the laboratory. Besides all of these factors, alterations of the natural DOM during extraction may occur associated with the extreme pH variations during the isolation process, irreversible interactions with resins, contamination from resin bleed, and size-exclusion effects (De Shamphelaere *et al*., 2005). Therefore, it is clear that, although solid phase extraction with PPL resin as employed here, has lower recovery yielded than the method described by Engbrodt (2001) using XAD-2 and XAD-4 resins, PPL resin show more advantages than disadvantages. DOM extraction is faster and cartridges are easier to manipulate than XAD resins. For example, there is no need for the thorough resin cleaning procedures described for XAD resins, and solid phase extraction with PPL resin demands the use of only a couple of reagents (methanol and HCl) during the extraction procedures.

Based on the data reported here and the experience developed in our laboratory, we believe that RO extraction and solid phase extraction with PPL resins are suitable techniques for extraction of freshwater- and seawater-derived DOM to be employed in toxicological studies, when compared to other available techniques using XAD resins. They clearly showed more advantages than disadvantages.

Table 1 – Data obtained during extraction and concentration of freshwater – and seawater – derived DOM using reverse osmosis and solid phase extraction techniques, respectively. Freshwater BSTP: freshwater collected before the sewage treatment plant discharge. Freshwater ASTP: freshwater collected after the sewage treatment plant discharge. *DOC concentration estimated based on data available (see text for explanation). #According to Boris *et al.* (2007).

	Freshwater BSTP	Freshwater ASTP	Seawater
Water collected	180	180	60
DOM solution obtained (L)	12	12	0.1
DOC in water (mg C L^{-1})	18	18	1.8*
DOC in the DOM solution (mg C L^{-1})	121.8	126.3	549.4
Recovery (%)	~50	~50	50#

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