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Short communication

Molecular fluorescence analysis of rainwater: Effects of sample preservation

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ABSTRACT

Very different filtration and preservation procedures may be found in the literature on the study of the rainwater dissolved organic fraction. Thus, the influence of sample filtration and preservation procedures on the fluorescence of rainwater dissolved organic matter (DOM) was studied in this work. Rainwater was filtered through different filters (quartz 0.22 μm or PVDF 0.45 μm) and excitation (λ_{em} = 415 nm) and synchronous ($\Delta\lambda$ = 70 nm) fluorescence spectra were obtained at the same day of collection, or after preservation by refrigeration (1–7 days) or by freezing (1–4 weeks). The excitation–emission matrix (EEM) spectra of rainwater showed six types of fluorescent bands: two corresponding to humic-like bands, and four resembling proteins. Then, the excitation and synchronous spectra were chosen in order to monitor changes in the humic-like and protein-like bands, respectively. The filtration procedures adopted in this work did not affect the fluorescence properties of the rainwater samples. However, these properties were differently preserved by refrigeration or freezing: after refrigeration, filtered rainwater maintained the original fluorescent properties for at least 4 days, while after freezing fluorescent properties were not always preserved since it occurred a decrease of protein-like fluorescence intensity.

1. Introduction

Molecular fluorescence spectroscopy is an excellent tool for tracing the origin and nature of chromophoric dissolved organic matter (CDOM) and may be used to reveal important information about its composition and biogeochemical cycling [1]. Compared with other spectroscopic techniques, fluorescence spectroscopy is a simple, sensitive, non-destructive, and rapid analytical technique that does not require separation, and needs only a small volume of aqueous sample [2].

Given its sensitiveness, fluorescence spectroscopy results may be affected by sample processing before analysis, namely, filtration and preservation. Dissolved organic matter (DOM) is operationally defined as the organic matter that passes through the pores of filters, usually of 0.2–0.7 μm pore size [3]. Although there is an almost universal consensus about 0.45 μm [4,5], the size limit that is used to differentiate DOM from particulate organic matter is somewhat arbitrary. On the other hand, DOM fluorescence is sensitive to changes in the environmental conditions and, ideally, water samples should be analysed immediately after sample collection. However, it is often desirable, or necessary, because of logistical constraints, to store water samples before processing.

Regarding rainwater, the work by Willey et al. [6] was key to highlighting the importance of the study of the dissolved organic fraction. Table 1 shows the subsequent works published on this matter together with the filtration and preservation procedures adopted. A few of these works have highlighted the valuable properties of molecular fluorescence for the characterization of rainwater DOM [14,18,21,22,25,27,28] and it has been demonstrated that fluorescence has a great potential for fingerprinting of rainwater DOM [22].

Although references in Table 1 are all quite recent, separation and preservation procedures used differ a lot. Filters of different materials and pore sizes, or, also, no filtration, have been used to separate DOC and different ways of sample preservation have been followed by different research groups. Still, different procedures have been followed in different works by the same research group. Even when focussing only on those works using such a sensitive technique as molecular fluorescence spectroscopy, this situation remains. Kieber et al. [14,18] and Miller et al. [25] filtered rainwater samples through 0.2 μm filters (polysulfone) immediately after collection and kept them at 4 °C in the dark until fluorescence analysis, but, while Kieber et al. [14] analysed the samples within 3 h after collection, Miller et al. [25] did not specify the sample preservation time. Miller et al. [21] and Muller et al. [22] did analyse samples without filtration and analysed them by fluorescence spectroscopy immediately or within a maximum of 24 h, respectively, but without specifying how samples were kept meanwhile. In order to use the same operational definition of DOM for its quantification and for its characterization by fluorescence spectroscopy, filtration

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Table 1
Works published on the rainwater dissolved organic fraction during the last decade.

Sample filtration	Preservation procedure	Time until analysis	Fluorescence analysis	Origin	References
N.F.	Dark at 4 °C	N.S.	None	São Paulo State (Brasil)	Lara et al. [7]
N.F.	Freezing	N.S.	None	South Island (New Zealand)	Kieber et al. [8]
0.45 μm (Gelman GN-6)	4 °C	Within 24 h	None	Guandaushi Forest (Taiwan)	Liu and Sheu [9]
0.7 μm (Whatman GF/F)	Freezing	N.S.	None	New Brunswick, NJ (USA)	Seitzinger et al. [10]
0.45 μm (Gelman GN-6)	4 °C	Within 24 h	None	Hui-Sun Forest (Taiwan)	Wang et al. [11]
N.F.	N.S.	Within minutes	None	Wilmington, NC (USA)	Kieber et al. [12]
N.F.	N.S.	Within 12 h	None	Wilmington, NC (USA)	Avery et al. [13]
0.2 μm (Supor® polysulfonone)	Dark at 4 °C	Within hours (2–3)	EEM	Wilmington, NC (USA)	Kieber et al. [14]
N.F.	N.S.	Within minutes	None	Wilmington, NC (USA)	Willey et al. [15]
0.7 μm (Millipore GF/F)	4 °C	7 days[a]	None	Riberão Preto and Araraquara (SP, Brasil)	Campos et al. [16]
N.F.	N.S.	N.S.	None	N.S.	Ćosović et al. [17]
0.2 μm (Supor® polysulfonone)	Dark at 4 °C	Within hours (2–3)	EEM	Wilmington, NC (USA)	Kieber et al. [18]
0.6 μm (Whatman GF/F)	Freezing	N.S.	None	Twin Cays (Belize)	Wanek et al. [19]
0.45 μm (Millipore cellulose acetate)	Freezing	N.S.	None	Riberão Preto and Araraquara (SP, Brasil)	Coelho et al. [20]
N.F.	N.A.	Immediately	EEM	Wilmington, NC (USA)	Miller et al. [21]
N.F.	N.S.	Within 24 h	EEM	Birmingham (UK)	Muller et al. [22]
0.7 μm (Whatman GF/F)	Dark at 4 °C	N.S.	None	Šibenik (Croatia)	Plavšić et al. [23]
1 μm (Glass fiber filters)	N.S.	Within 48 h	None	Guangzhou (China)	Xu et al. [24]
0.2 μm (Supor® polysulfonone)	Dark at 4 °C	N.S.	EEM	Wilmington, NC (USA)	Miller et al. [25]
0.7 μm (Whatman GF/F)	Dark at 4 °C	N.S.	None	Zagreb and Šibenik (Croatia)	Orlović-Leko et al. [26]
0.45 μm (Millipore PVDF)	Freezing	N.S.	EEM	Aveiro (Portugal)	Santos et al. [27]
0.45 μm (Millipore PVDF)	N.S.	Within few hours	EEM	Aveiro (Portugal)	Santos et al. [28]
0.45 μm (membrane filters)	At 4 °C	Within 1 month	None	Tönnersjöheden (Sweden)	Rosenqvist et al. [29]
0.45 μm (Sartorius Minisart SRP)	Freezing	N.S.	None	Beijing, Baoding, Cangzhou, Luancheng, Tianjin, Tanggu, Tangshan, Xinglong, Yucheng and Yangfang (China)	Pan et al. [30]

N.F. = not filtered; N.S. = not specified; N.A. = not applicable; EEM = excitation–emission matrix.
[a] DOC integrity was proved.

through 0.45 μm has been used either for analysis within a few hours after collection [28] or for analysis after freezing preservation [27].

The production environmental data without the influence of operational variables such as filtration and storage of samples is of utmost importance, namely when applying molecular fluorescence spectroscopy to such a low concentration of DOM as in the case of rainwater. Thus, this work aims to study the effects of filtration and preservation procedures on the fluorescence properties of DOM from samples of rainwater in order to choose the most appropriate procedures.

2. Experimental

2.1. Rainwater sampling and preservation

Rainwater samples were collected in spring and autumn, during April (A08) and October 2008 (O08), respectively, at a sampling station (40°38′ N, 8°39′ W) in the western part of the town of Aveiro, Portugal. Samples were collected at 70 cm above the ground, through glass funnels (30 cm diameter) into glass bottles (5 L). Sampling containers were left out open in order to collect both wet and dry depositions on a 24 h basis. Prior to use, all glass materials were immersed for 30 min, in a solution of NaOH (0.1 M), then rinsed with distilled water, followed by another immersion for 24 h in a solution of HNO_3 (4 M), and finally rinsed with ultrapure (Milli-Q) water. Rainwater samples, after finishing the sampling period of 24 h, were transported within less than 1 h to the laboratory for further processing.

Fig. 1 shows a diagram of the experimental procedure adopted in this work. Once in the laboratory, rainwater was divided into two aliquots. One of the aliquots was filtered through 0.22 μm quartz filters, GSWP Millipore, in a glass filtration apparatus and the other one was filtered through 0.45 μm hydrophilic PVDF Millipore membrane filters in a stainless steel filtration apparatus. For both filtration systems, blanks (Milli-Q water) were obtained and analysed by fluorescence spectroscopy in the same way as samples. The filtrate from each aliquot was then divided in nine sub-aliquots, one to be analysed at the day, four to be analysed 1–7 days under refrigeration (4 °C), and four to be analysed 1–4 weeks freezing (−18 °C). In the case of samples from April, they were only filtered through 0.45 μm and then subjected to the subsequent preservation procedures here considered (Fig. 1). In all cases, rainwater was stored in glass vials. Optical measurements were conducted within 2–3 h of rainwater collection and/or after reaching room temperature (20 °C), in the cases of refrigerated and/or frozen sub-aliquots.

2.2. Laboratory analytical procedures

The molecular fluorescence spectra were obtained by a Fluoromax 3 (JobinYvon-Spex Instruments S.A., Inc., now HORIBA Jobin Yvon Inc., Edison, NJ, USA) with a xenon lamp source. Fluorescence analyses were carried out under thermostated 20 °C conditions and spectra were recorded using 1 cm cells and 5 nm bandpasses on both the excitation and emission monochromators. Excitation–emission matrix (EEM) fluorescence spectra were obtained by concatenating emission spectra measured every 5 nm from 290 to 510 nm using excitation wavelengths (λ_{ex}) from 240 to 400 nm (5 nm intervals). Synchronous spectra ($\Delta\lambda$ = 70 nm) and excitation spectra (λ_{em} = 415 nm) were also acquired using λ_{ex} from 240 to 400 nm (5 nm intervals). Scans were corrected for instrument configuration using factory supplied correction factors [31]. Data were normalized to a daily-determined water Raman intensity ($275_{ex}/303_{em}$, 5 nm bandpasses) and converted to Raman normalized quinine sulfate (QS) equivalents in ppb [32]. For each way of filtration, the corresponding averaged blank (Milli-Q water) spectrum was subtracted from rainwater spectra. Replicate scans within 5% agreement in terms of intensity and within bandpass resolu-

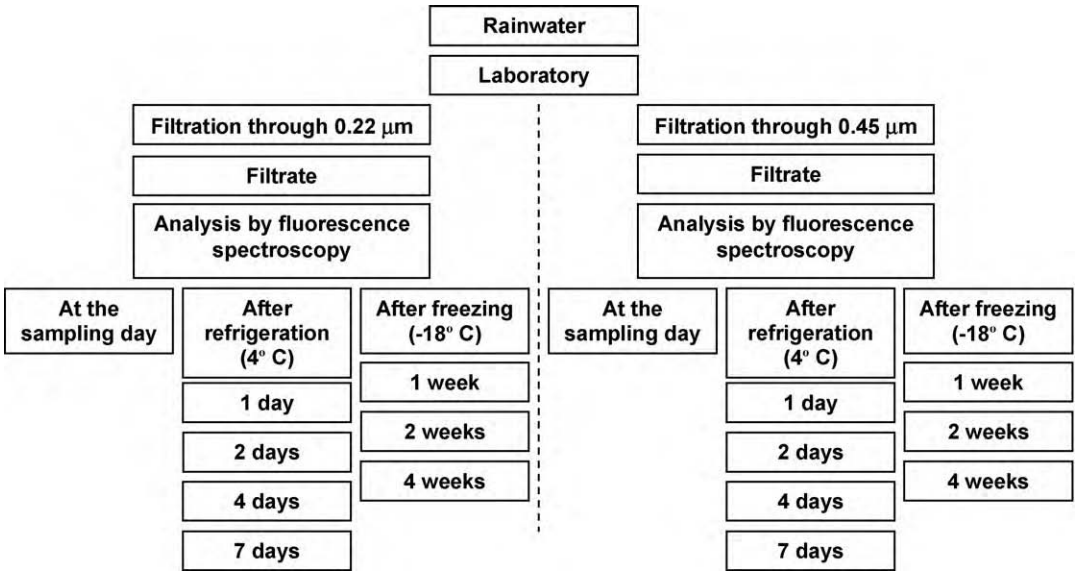

Fig. 1. Schematic diagram of the experimental procedure adopted.

tion in terms of band location were obtained. However, at low excitation wavelengths ($\lambda_{ex} \leq 250$ nm), instrumental variability on fluorescence intensity was always higher than at $\lambda_{ex} > 250$ nm.

3. Results and discussion

Fig. 2 shows the EEM fluorescence spectra of A08 and O08 rainwater samples filtered through 0.45 μm. The corresponding spectra were obtained at the day of sample collection. Both samples have the same fluorescent bands: two humic-like bands, A ($\lambda_{ex}/\lambda_{em} \approx 240/415$ nm) and M ($\lambda_{ex}/\lambda_{em} \approx 300/415$ nm); and four protein-like bands, B_1 ($\lambda_{ex}/\lambda_{em} \approx 240/305$ nm), B_2 ($\lambda_{ex}/\lambda_{em} \approx 270/305$ nm), T_1 ($\lambda_{ex}/\lambda_{em} \approx 240/340$ nm) and T_2 ($\lambda_{ex}/\lambda_{em} \approx 275/330$ nm). Bands in the same range as A have already been identified in the emission–excitation matrix (EEM) fluorescence spectra of rainwater dissolved organic matter and have being assigned to humic-like compounds [14,22,27]. A band at similar $\lambda_{ex}/\lambda_{em}$ of M has already been identified in the EEM fluorescence spectra of rainwater, being assigned also to marine humic-like compounds [14,27]. B and T bands are attributed to protein-like compounds, such as tyrosine and tryptophan, respectively [1,33].

The $\Delta\lambda = 70$ nm for the synchronous spectra was chosen in order to highlight the protein-like fluorescence, and the excitation spectra with $\lambda_{em} = 415$ nm was chosen to study the behaviour of the humic-like bands [27]. These spectra are represented in Fig. 2 by the lines E and S, corresponding to excitation and synchronous spectra, respectively.

The comparison between spectra was done and the determination of the percentage differences calculated using the fluorescence intensities, as described below:

$$\text{Percentage difference } (\%) = \frac{x_i - \mu}{\mu} \times 100$$

where x_i is the fluorescence intensity of the rainwater spectrum to compare with μ that is the fluorescence intensity of the spectrum of reference, at the same wavelength. The percentage difference was calculated for all excitation wavelengths in the range 240–400 nm.

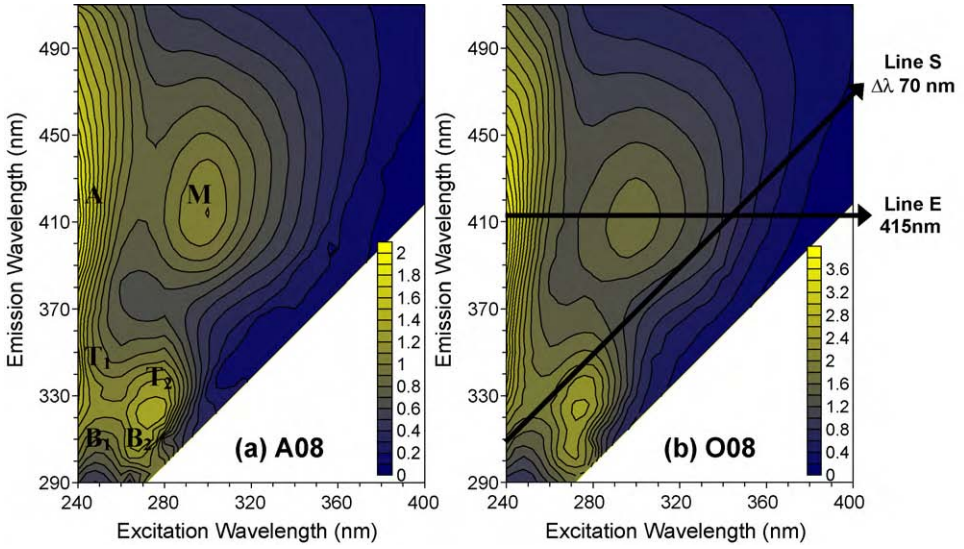

Fig. 2. EEM fluorescence contour profiles of (a) A08 and (b) O08 rainwater samples. Fluorescence intensities are presented in ppb QS. The line S indicates the spectral range covered by the synchronous mode using $\Delta\lambda = 70$ nm. The line E corresponds to the excitation spectrum at $\lambda_{em} = 415$ nm.

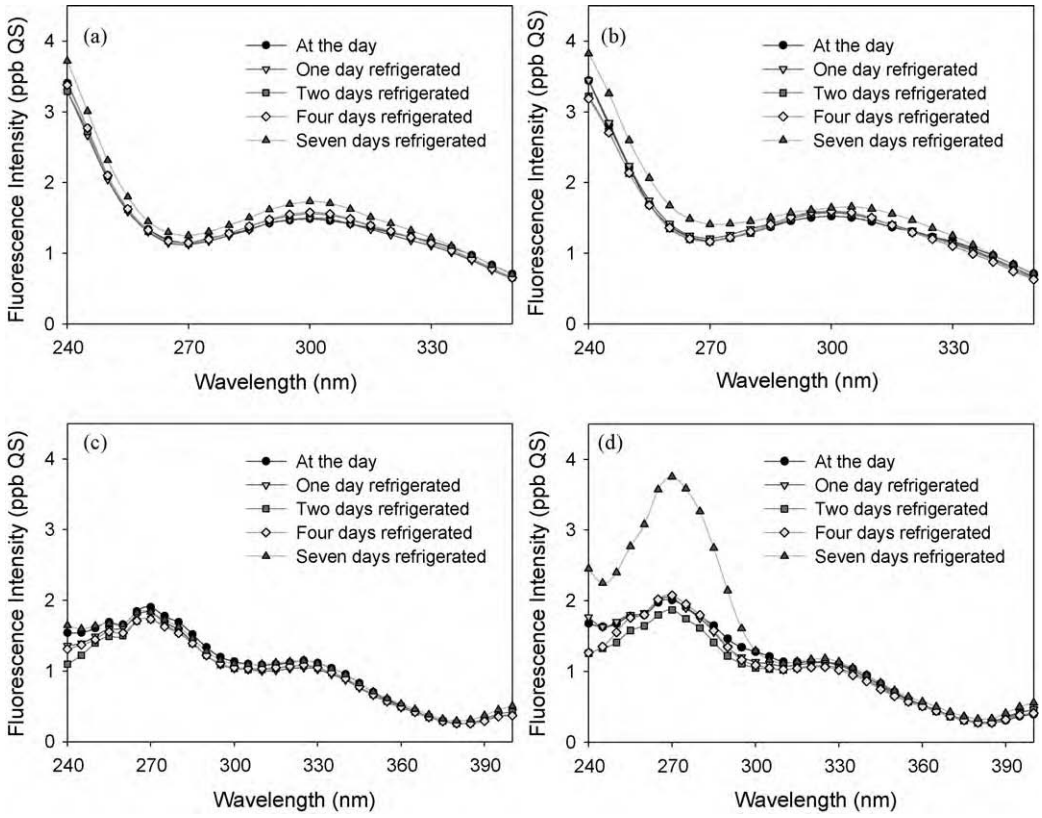

Fig. 3. Excitation, (a) and (b), and synchronous, (c) and (d), fluorescence spectra corresponding to O08 rainwater analysed after refrigeration (1–7 days), for the two filtrates (0.22 and 0.45 μm).

Fig. 3 shows the fluorescence spectra corresponding to the O08 rainwater sample obtained for the two filtrates (0.22 and 0.45 μm) after refrigeration (4 °C) during 1–7 days. Excitation and synchronous spectra at the sampling day were compared for rainwater filtered through quartz 0.22 μm and through PVDF 0.45 μm, obtaining a difference in fluorescence intensity lower than 7%. This value approximates the replication error, so the effect of rainwater filtration through different filters (quartz 0.22 μm and PVDF 0.45 μm) may be considered no significant.

Excitation spectra in Fig. 3(a) and (b) show that, for both types of filters used, spectra keep the same until 4 days under refrigeration (differences in fluorescence intensity lower than 5%). However, when rainwater is preserved under refrigeration during 7 days, an intensity increase (up to 17%) of the excitation spectra was observed in the case of rainwater filtered through quartz 0.22 μm. For rainwater filtered through PVDF 0.45 μm, both an intensity increase of fluorescence (up to 21%) at low λ_{ex} and a shift to longer λ_{ex} were observed after 7 days under refrigeration. The A08 rainwater sample also kept the original spectra until 4 days under refrigeration, showing an intensity increase (up to 15%) at low λ_{ex} and a shift to longer λ_{ex} after 7 days under refrigeration.

Synchronous spectra in Fig. 3(c) and (d), show a marked increase of intensity of the protein-like fluorescence (87% at λ_{ex} = 270 nm) in the 0.45 μm sub-aliquot after 7 days under refrigeration while the spectra of the 0.22 μm sub-aliquots generally keep the original spectra obtained for rainwater at the day of sampling. The same behaviour was observed for the A08 rainwater sample filtered through 0.45 μm, which showed a slightly lower intensity increase than O08 (70% at λ_{ex} = 270 nm) after 7 days under refrigeration, but also kept the original spectrum for 4 days under refrigeration. Differences between filtrates may be related to differences on biological activity due to the different pore size of filters. Some bacteria, which may be trapped by the 0.22 μm filter, can pass through the 0.45 μm filter [34] and refrigeration may hold-up their activity but not completely stopped it [35,36]. Then, the fluorescence intensity increase in the 0.45 μm sub-aliquots may be related to microbiological activity, which may transform DOM to give new fluorophores, as it was pointed by Moran et al. [37].

Fig. 4 shows the fluorescence spectra corresponding to the O08 rainwater sample obtained for the two filtrates (0.22 and 0.45 μm) after freezing (−18 °C) during 1–4 weeks. In Fig. 4(a) and (b), for both types of filters used, excitation spectra maintain unaltered (differences in fluorescence intensity lower than 5%) for rainwater preserved under freezing during 4 weeks. However, synchronous spectra in Fig. 4(c) and (d) show that, although there are not important changes in humic-like fluorescence related to freezing, that is not the case for the protein-like fluorescence. Rainwater freezing caused a gradual decrease in intensity of the protein-like bands with time. The decrease after 4 weeks frozen was 37 and 18% at λ_{ex} = 270 nm for the rainwater filtered through 0.22 and 0.45 μm, respectively. These results confirm that the protein-like fraction of fluorescent DOM may be less stable in response to the freezing process in comparison to the humic-like fractions, which has been already proved by Spencer et al. [38]. The intensity decrease may be related to the cold denaturation of protein-like fluorophores, when water is not in liquid state [39,40]. The protein-like fluorophores may lose their 3D structure and then their spectral properties are altered [40,41] therefore affecting fluorescence intensity. The intensity decrease of the protein-like fluorescence after freezing and thawing was more remarkable for the O08 rainwater filtered through 0.22 μm than for that filtered through 0.45 μm. On the other hand, the decrease of the protein-like fluorescence intensity was not verified for the A08 rainwater sample, filtered through 0.45 μm, which fluorescence excitation and synchronous spectra, after freezing (up to 4 weeks) and thawing, kept unaltered (differences in fluorescence intensity lower than 5%). In a study on

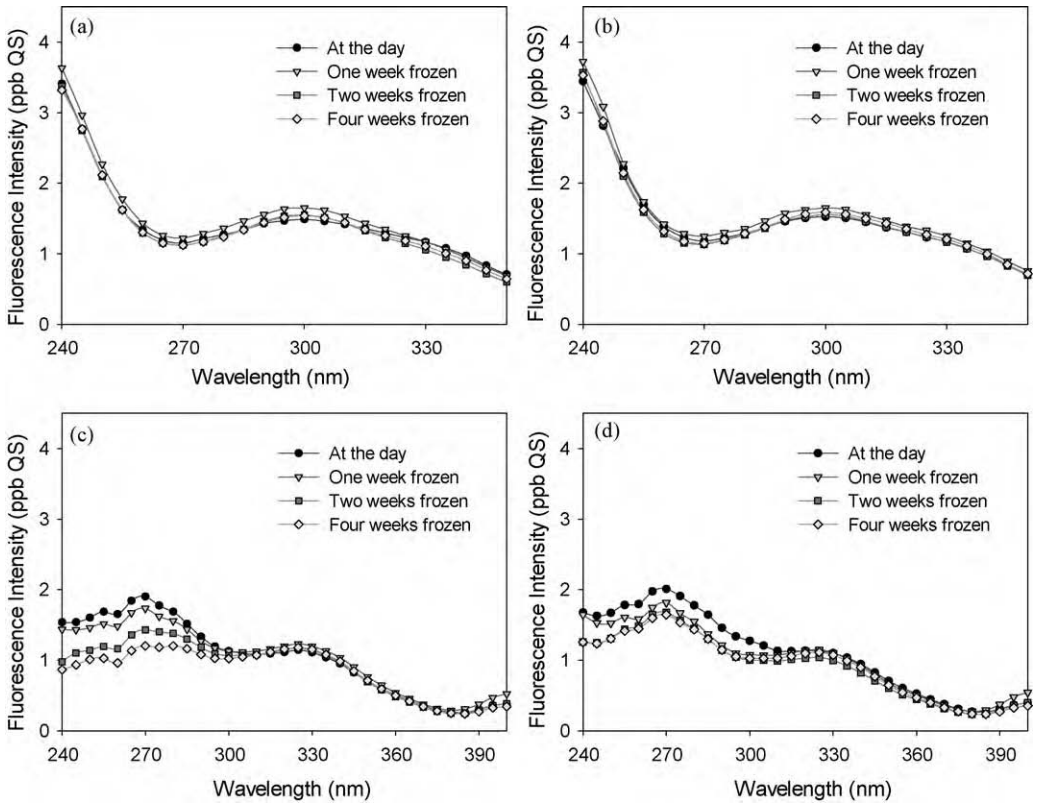

Fig. 4. Excitation, (a) and (b), and synchronous, (c) and (d), fluorescence spectra corresponding to O08 rainwater analysed after freezing (1–4 weeks), for the two filtrates (0.22 and 0.45 μm).

estuarine pore waters, no changes on fluorescence DOM (separated by filtration through 0.45 μm Durapore membranes) were found upon freezing [42]. Also, Spencer et al. [38] when studying the freeze/thaw effects on freshwater DOM (separated by filtration through 1.2 μm Whatman GF/C filters) from 35 different UK locations found large and variable responses of spectrophotometric measurements. Spencer et al. [38] highlighted that knowledge of the original properties could not be used to determine the amount of DOM fluorescence change that would occur with freezing and subsequent thawing.

4. Conclusions

Different filtration and preservation procedures have been adopted in different published works for the study of the dissolved organic fraction of rainwater. However, in the present work, on rainwater collected in Aveiro (Portugal), it has been found that these procedures may affect rainwater fluorescent properties:

(1) No significant differences were observed in original fluorescence properties between rainwater filtered through quartz 0.22 μm or PVDF 0.45 μm.
(2) No matter the filter used for DOM separation (quartz 0.22 μm or PVDF 0.45 μm), fluorescence properties keep unaltered for rainwater preserved under refrigeration during 4 days.
(3) Freezing may preserve rainwater fluorescent properties but may also cause an intensity decrease of the original protein-like fluorescence, depending on the rainwater sample. Differences must be related to the nature of the rainwater protein-like fluorophores, which may be less or more sensitive to denaturation.

The above results confirm that rainwater DOM fluorescent properties, mainly those related to the presence of protein-like compounds, are very sensitive and may be altered depending on the way of preservation and the time elapsed until analysis.

On the whole, authors would recommend filtration through 0.45 μm to separate the soluble from the particulate organic matter since this is the pore size which gets more consensus for the operational definition of dissolved organic matter and it has been shown that there are no alterations in samples filtered through 0.45 μm during the time recommended for preservation (4 days maximum). With respect to preservation, rainwater samples should be kept at dark 4 °C after collection and until molecular fluorescence analysis, which should be carried out as soon as possible but no longer than 4 days after collection. In any case, results on rainwater DOM fluorescence should always refer, apart from the filter type used for DOM separation, the preservation procedure, proving that it did not affect the original properties.

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