Chemosphere 333 (2023) 138853

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

To analyze or to throw away? On the stability of excitation-emission matrices for different water systems

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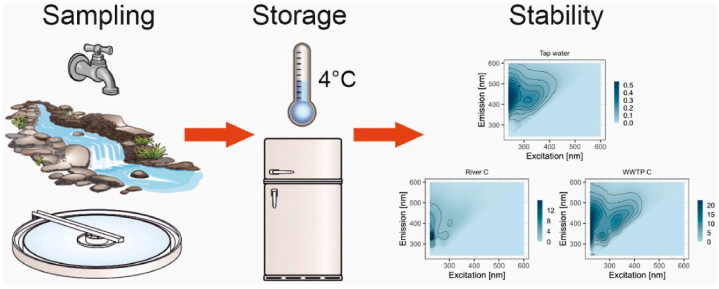
HIGHLIGHTS

- Systematic investigation of the stability of EEM of various water systems.
- Stronger organically and microbially polluted samples require faster processing.
- HPLC data suggest an alteration rather than a decrease in DOM during storage.
- Highest difference between days zero and one, causing a bias between lab and on site.
- Relative fluorescence metrics are more robust than absolute fluorescence intensity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Y Yeomin Yoon

Keywords:
3D fluorescence spectroscopy
Standard protocol
Dissolved organic matter
Storage conditions
Fluorescence indices
High-performance liquid chromatography

ABSTRACT

Fluorescence spectroscopy has numerous applications to characterize natural and human-influenced water bodies regarding dissolved organic matter (DOM) and contamination. Analyzing samples in a timely manner is crucial to gaining valid and reproducible excitation-emission matrices (EEM) but often difficult, specifically in transnational projects with long transport distances. In this study, eight samples of different water sources (tap water, differently polluted rivers, and wastewater treatment plant (WWTP) effluents) were stored under standardized conditions for 59 days and analyzed regularly. With this data set, the sample and fluorescence spectra stability was evaluated. Established analysis methods such as peak picking and fluorescence metrics were compared over time and benchmarked against dissolved organic carbon (DOC) and a maximal change of 10% in terms of their variability. Additional high-performance liquid chromatography (HPLC) data to identify single organic compounds provides insights into these DOM alterations and allows for conclusions about the underlying biological processes. Our results corroborate in a systematic way that the higher the organic or microbial load, the faster the sample must be processed. For all water sources, considerable changes were found between days zero and one, indicating a potential systematic bias between in-situ and laboratory measurements. The absolute signals of individual peaks vary substantially after only a few days. In contrast, relative metrics are robust for a much longer time. For specific metrics, when filtered and stored under cool and dark conditions, tap water may be stored for up to 59 days, non-polluted river water for up to 31–59 days, and WWTP effluents for up to 14–59 days. The storability thus depends both on the specific water source and the analytical plan. By systematizing our

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https://doi.org/10.1016/j.chemosphere.2023.138853
Received 24 February 2023; Received in revised form 2 May 2023; Accepted 3 May 2023
Available online 8 May 2023

understanding of how the specific water source and DOM concentration determine the stability of samples during storage, these conclusions facilitate efforts to establish a standardized protocol.

1. Introduction

As an essential tool for assessing and monitoring water quality fast and relatively inexpensively in terms of dissolved organic matter (DOM), fluorescence spectroscopy is becoming increasingly popular. Applications range from real-time monitoring of karst springs (Frank et al., 2017) and drinking water treatment (Li et al., 2020) to the tracking of pollution sources in groundwater or surface waters (Henderson et al., 2009; Wasswa et al., 2019) and the characterization of DOM during high-flow events (Peer et al., 2022), all the way to evaluating the effectiveness of wastewater treatment (Cohen et al., 2014; Carstea et al., 2016; Mesquita et al., 2017). Comparative results are a key requirement for innovations in fluorescence spectroscopy, as demonstrated, in particular, by the OpenFluor platform (Murphy et al., 2014). Major transnational research collaborations that bundle joint laboratory resources for the economical use of available resources are highly promising. However, it can be challenging to promptly deliver the samples to the laboratory and process them.

For this reason, it is necessary to establish common standards not only for the analysis and interpretation but also for handling samples on-site and in the laboratory. In the case of DOC, it has been known for many years that samples can be reliably analyzed after one week (Heinz and Zak, 2018) or up to five months (Tupas et al., 1994) if adequately prepared and cooled at 4 °C or frozen (−20 °C). In addition, it is also necessary to filter the samples before storage (Otero et al., 2007).

Regarding fluorescence spectroscopy, there are literature and protocols on how to prepare samples, i.e., filtering with pre-washed or non-bleeding filters (Sgroi et al., 2020) and how to transport samples, i.e., cooled at 4 °C and protected from light. Numerous studies have explored factors influencing the resulting excitation-emission matrix (EEM), such as temperature, turbidity (Goffin et al., 2020), or pH (Patel-Sorrentino et al., 2002; Spencer et al., 2007; Timko et al., 2015). However, limited literature examines the storability, or stability, of samples to be analyzed by fluorescence spectroscopy. There is widespread consensus on a rule of thumb regarding processing times: processing should be as quick as possible (Carstea et al., 2016). However, there is yet to be a consensus on how to quantify the upper threshold for storage time and how to proceed when this storage time is exceeded, e.g., due to a considerable transport distance or a large number of samples (Spencer and Coble, 2014). Despite technical advances in recent years, in-situ fluorescence measurements currently do not offer the full potential of 3D fluorescence measurements in the laboratory (Carstea et al., 2020). Even if emerging research points to the correlation between in-situ measurements and standard water quality parameters (Carstea et al., 2020), these limitations may not allow a shift to in-situ fluorescence spectra, depending upon the research question. A further reason for delayed sample processing is retained samples, which are only analyzed as required when findings emerge later, e.g., as is the case for monitoring drinking water and wastewater treatment.

For instance, Sgroi et al. (2020) demonstrated that carefully considering storage conditions and timely sample processing is crucial to obtaining high-quality 3D fluorescence spectra from WWTP effluents. As organic load varies in different water systems, one can wonder if these results can be generalized to all kinds of water systems. There may be samples for which it is still possible to gain reliable measurements even after they have been stored for more than two days. Although there are at least a handful of related research results regarding storing samples of different water sources concerning EEM, they vary in terms of their conclusions and the metrics being considered.

Park and Snyder (2018) concluded that changes in total fluorescence values (the sum of intensities established by fluorescence regional integration) do not exceed 7.5% of the original signal when samples of a secondary wastewater effluent are filtered and stored at 4 °C for 21 days. Other works suggest only seven-day stability for surface waters (Spencer and Coble, 2014), lakes, and leachate samples (Heinz and Zak, 2018). In particular, substantial variability in some peaks and fluorescence metrics was observed, but only for a few of them was this effect significant (Heinz and Zak, 2018). Similar results were obtained by Spencer and Coble (2014), who looked at tryptophan-like and fulvic-like fluorescence in freshwater samples. There were no alterations during the first seven days of storage. Still, there was a microbial-induced decrease of the fluorescence intensity by 10–35% of the initial value after 21 days (Spencer and Coble, 2014). By contrast, Murphy et al. (2010) reported no significant storage effects in solutions mimicking various aquatic environments after 43 days. Furthermore, there is even evidence that it is feasible to obtain EEM from frozen samples (Otero et al., 2007; Spencer and Coble, 2014), although this is generally only recommended for some source waters (Spencer et al., 2007; Spencer and Coble, 2014). Especially wastewater effluent should not be frozen (Sgroi et al., 2020), and also, for freshwater samples, immediate analysis without freezing is recommended (Heinz and Zak, 2018). Again, however, a consensus about sample freezing has yet to be agreed upon.

In such a context of fragmented and partially contrasting findings, this study aims to comprehensively and systematically investigate the stability of EEM of samples of various water sources. The changes during the first five days after sampling are specifically addressed, reflecting typical periods during which samples are transported to the laboratory and analyzed. The samples were stored and analyzed by fluorescence spectroscopy for 59 days to collect data on extreme cases. Several standard evaluation methods, fluorescence metrics, and high-performance liquid chromatography (HPLC) analyses were combined in an integrated assessment to provide valuable insights into the alteration of DOM in samples and the underlying biological processes beyond solely evaluating stability.

2. Materials and methods

2.1. Samples and storage conditions

Samples were collected from eight different water sources. Tap water, as supplied in the laboratory, represents drinking water. Four samples were collected from three differently polluted rivers: river A, unaffected by wastewater treatment plant (WWTP) effluent and partially affected by agriculture; river B (before), unaffected by municipal WWTP effluent; the same river B (after) being affected by effluent from one municipal WWTP (see WWTP B below); river C strongly affected by effluent from several municipal and industrial WWTPs as well as agriculture. In addition, effluents from three WWTPs of different design capacities were stored: a large municipal WWTP with 180,000 population equivalents (PE) based on COD_{120} (WWTP A), a small municipal WWTP with 6,500 PE (WWTP B) which discharges into river B, and a lab-scale WWTP with 1.7 PE (WWTP C). All river and effluent samples were collected under dry weather conditions. Deionized water served as a control sample to reflect variability common in measurement.

Samples were immediately filtered using a 0.7 μm glass microfibres filter (698, VWR, Belgium) as the experimental design in this study required a fairly high volume of 1 L per sample. Considering WWTP effluents in particular, it may not have been feasible to prepare this sample volume promptly using a 0.45 μm or 0.2 μm filter. To maintain identical conditions for all water sources examined, the same kind of filter was also used for tap and river water samples. These conditions

enable the maximum possible DOM alteration to happen in order to evaluate these effects visibly. Afterward, they were stored in glass bottles of 1 L at 4 °C in the dark for a period of up to 140 days. Prior to spectroscopic measurement, subsamples of 20 mL were placed in glass test tubes and warmed to room temperature (~20 °C). The samples were analyzed on days zero, one, three, ten, 24, 31, and 59 of the storage period. Additionally, HPLC data were also measured on day 132.

2.2. Analytical methods

Standard water quality analysis of the samples included pH, dissolved organic carbon (DOC) (DIN EN 1484), ammonia nitrogen (NH_4–N) (DIN EN ISO 11732), nitrate nitrogen (NO_3–N) (DIN EN ISO 13395), nitrite nitrogen (NO_2–N) (DIN EN ISO 13395), and orthophosphate phosphorus (PO_4–P) (DIN EN ISO 6878).

2.2.1. Fluorescence spectroscopy

EEM measurements were conducted using a HORIBA Scientific Aqualog® spectrofluorometer with a Xenon lamp and a quartz cell with an optical path length of 1 cm. The measurements cover excitation wavelengths of 200–600 nm in 3 nm steps. Detected emission wavelengths ranged from 246 to 824 nm, with a slit width of 5 nm, and integration times of 5 s for deionized and tap water samples, 1 s for river samples and 0.5 s for WWTP effluent samples. The dilution of the river and WWTP effluent samples using Milli-Q was chosen to ensure that the maximum absorbance was < 1.5 to limit the inner filter effect. Integration times and dilutions were computationally normalized so that the measured values correspond to undiluted samples at an integration time of 1 s to establish comparability of the different samples. In each case, the signal from Milli-Q, measured daily, was used as the blank value. Correction for the dark signal, spectral correction, normalization to the reference detector, and subtraction of the blank was processed in Aqualog software. EEMs were then exported and analyzed using the statistical software R (R Core Team, 2022). Modified functions of the eemR (Massicotte, 2019) package were used to import the data into R. Rayleigh and Raman masking, as well as correction for inner filter effects, were performed as proposed by Lakowicz (2006) using functions of the package staRdom (Pucher et al., 2019). For this purpose, absorbance was measured with the same equipment and settings. Finally, the fluorescence intensities of the corrected EEM were transformed into Raman units (R.U.) (Lawaetz and Stedmon, 2009). This was done by daily determination of the area of the Raman peak of Milli-Q at an excitation wavelength of 350 nm and emission wavelengths between 383 and 410 nm with the same spectrofluorometer which is then used for normalization.

2.2.2. Chromatographic separation

For each water sample, 100 µL were directly injected without further preparation. Chromatographic separation was performed on a Phenomenex Luna C18 (150 mm × 3 mm, 5 µm particle size) column with a temperature of 40 °C and Phenomenex C18-Security guard cartridges (40 mm × 3 mm). The eluents were (A) 0.1% acetic acid solution in Milli-Q and (B) 0.1% acetic acid in acetonitrile solution, and all solvents were of gradient grade for liquid chromatography (Sigma Aldrich). The flow rate was set at 0.8 mL/min and the gradient program was as follows: 0 min, 90% A, 10% B; 8 min, 90% A, 10% B; 8.5 min, 60% A, 40% B; 17 min, 60% A, 40% B; 19 min, 5% A, 95% B; 25 min, 60% A, 40% B; 30 min, 90% A, 10% B.

2.2.3. HPLC with MS/MS detection

The high-performance liquid chromatography (HPLC) analyses were performed using an AB SCIEX 3200 QTRAP® system consisting of binary pumps, a degasser to degas the eluents, and a CTC PAL autosampler with Peltier cooled trays (4 °C). A linear hybrid triple quadrupole ion trap tandem mass spectrometer (MS/MS) with electrospray ionization (ESI) in full scan MRM (multiple reaction monitoring) EMS (enhanced mass spectra) mode was used to screen the unknown analytes in negative and positive polarity using a scan from m/z 50 to 600 and a scan speed of 1000 Da s^{-1}.

2.3. Coble peaks and fluorescence metrics

As a first step, changes in the absolute fluorescence signal are considered. For this, the Coble peaks T and A are used as indicators, which can be assigned to certain compounds (Coble, 1996). The tryptophan-like fluorescence (TLF, Coble peak T) is located at $\lambda_{275/340}$ in the EEM and suggests the presence of protein-associated dissolved organic matter and is also associated with microbial material (Coble, 1996). Humic-like fluorescence (HLF, Coble peak A), the highest intensity at emission wavelengths between 380 nm and 460 nm at an excitation of 260 nm, is predominantly associated with humified material, often derived from terrestrial input (Coble, 1996).

Further, the stability of four relative fluorescence metrics is assessed. A fluorescence metric is a dimensionless ratio derived from a high-dimensional EEM for easier summary characterization. Different metrics have been proposed in the literature, each using data points from different EEM regions. The fluorescence index (FIX) is the ratio of the fluorescence intensity of the wavelength combinations $\lambda_{370/450}$ and $\lambda_{370/500}$. As an indicator of the dissolved organic compounds, it suggests whether they are more likely to be of microbial origin (FIX ~ 1.9) or dissolved from terrestrial sources (FIX ~ 1.4) (McKnight et al., 2001). It is important to note here, however, that the FIX does not employ the TLF as a range for identifying material of microbial origin but instead uses a range at much higher excitation and emission wavelengths. The humification index (HIX) is the ratio of the sum of fluorescence intensity at emission wavelengths between 435 nm and 480 nm, and between 300 nm and 345 nm, each at an excitation of 254 nm (Zsolnay et al., 1999; Huguet et al., 2009). Comparing these two broad aromatic-dominated areas provides evidence for the input of humified material derived primarily from soils, with higher values representing a higher degree of humification in the input material (Zsolnay et al., 1999; Ohno, 2002). The biological fluorescence index (BIX), the ratio of the fluorescence intensity of $\lambda_{310/380}$ and $\lambda_{310/430}$ (Huguet et al., 2009), indicates the proportion of recently produced DOM compared to older, more decomposed DOM. The ratio between microbial and humic material is called T/C ratio (Baker, 2001). Specifically, peak C is the highest intensity at emission wavelengths between 420 nm and 480 nm at an excitation of 350 nm and is thus an additional indicator of humic-like fluorescence. Values greater than one imply a higher proportion of microbial material, possibly indicating input from point sources like WWTP effluents into rivers. Values below one suggest a higher proportion of humic-like material from soil erosion.

All calculations were carried out using R (R Core Team, 2022) and applied the corresponding functions from the package eemR (Massicotte, 2019) to calculate the BIX, FIX, and HIX.

3. Results and discussion

3.1. Stability of samples over time

Fig. 1 illustrates the fluorescence spectroscopic signal of all stored samples at baseline, i.e., at the sampling date, also called day zero. The high-dimensional structure is represented by a contour plot which plots the range of excitation and emission wavelengths on the X and Y axes, respectively, and indicates the fluorescence intensity for each excitation-emission combination in Raman units (R.U.) on a color scale. Expectedly, the control sample of deionized water (DW) shows no signal besides slight scattering due to optical effects. Only very little DOM evenly distributed in all areas of the EEM can be detected in the tap water. River A shows a signal for a non-polluted river, with the highest signal at about $\lambda_{220/450}$ and a secondary peak at $\lambda_{370/450}$. This typical signal is echoed in the EEM of river B, too, especially before the discharge of the WWTP

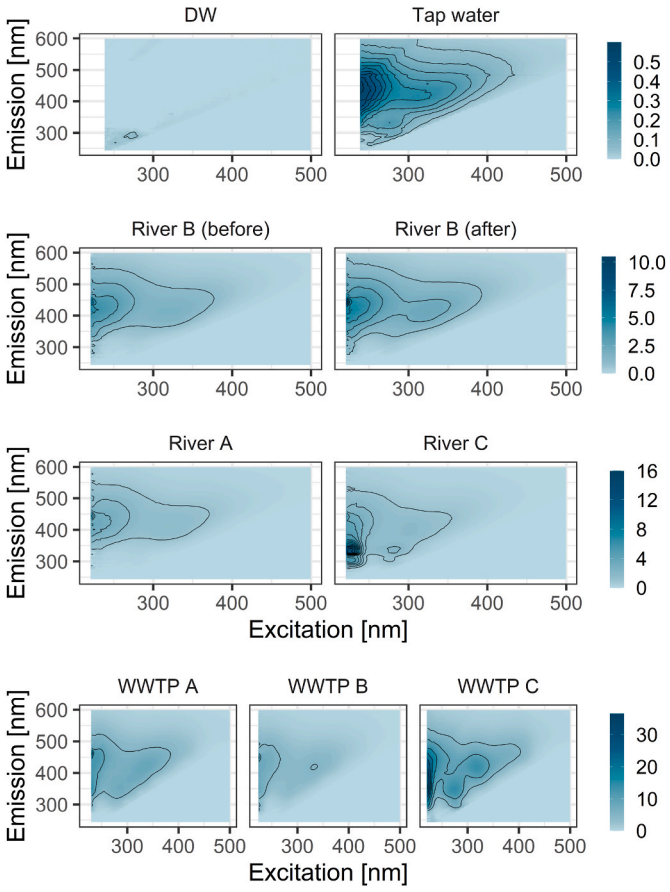

Fig. 1. Contour plots of the samples on day zero of the storage period.

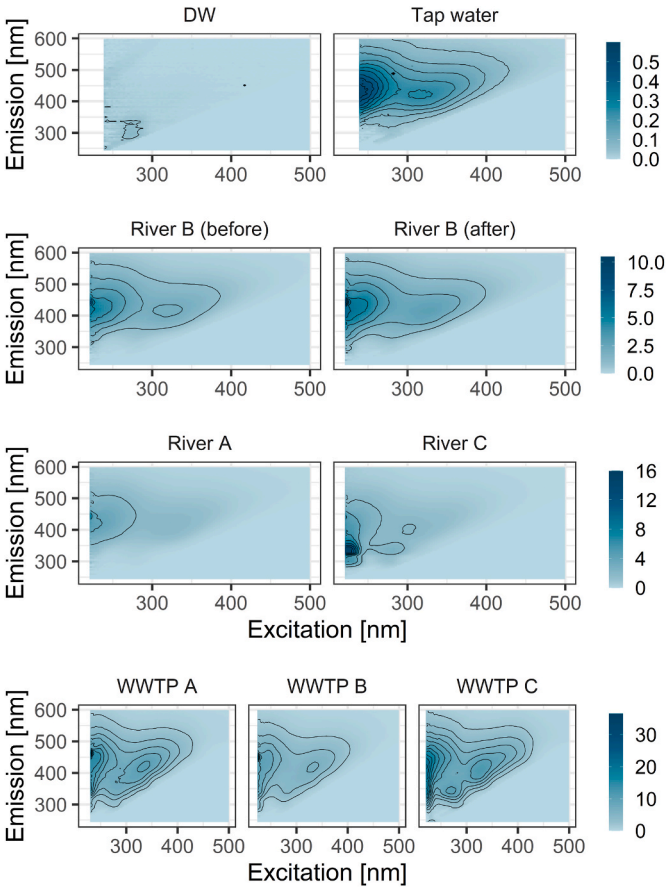

Fig. 2. Contour plots of the samples on day 59 of the storage period.

effluent. Considering the EEM of the sample after the discharge of the WWTP effluent, one can observe an increase in the overall fluorescence signal. More specifically, the region at $\lambda_{225/275}$ is more prominent. This particular feature can also be observed in the EEM of WWTP B, which discharges its effluent in river B. WWTP B is operated as low loaded activated sludge plant with nitrification/denitrification and simultaneous aerobic sludge stabilization (ASS) with high sludge age. Three dominating peaks ($\lambda_{225/450}$, $\lambda_{320/430}$, $\lambda_{225/275}$) can be seen at the same positions, resulting from complete nitrification and semi-complete denitrification with low concentrations of nitrogen in the effluent (NH_4–N = 0.024 mg L^{-1}, NO_3–N = 9.7 mg L^{-1}). River C is most representative of rivers impacted by municipal and industrial effluents. In the domain of higher emission wavelengths, the EEM of river C is similar to that of the other rivers. However, the highest signal here is in wavelength ranges below 350 nm, with the primary peak at $\lambda_{225/342}$ and the secondary peak at $\lambda_{275/310}$. WWTP A is a low-loaded activated sludge plant with nitrification/denitrification and anaerobic sludge digestion (AD), resulting in higher ammonium and nitrate values (NH_4–N = 1 mg L^{-1}, NO_3–N = 22.25 mg L^{-1}) than WWTP B, yielding slight differences in EEM, i.e., hardly any signal at $\lambda_{275/310}$. WWTP C has been operated at the time of sampling as a high-load activated sludge plant with a short sludge age and without nitrification (NH_4–N = 47.01 mg L^{-1}, NO_3–N = 0.4 mg L^{-1}). For this reason, the fluorescence spectroscopic signal differs from the other two WWTPs. It is generally higher, and besides the expected peaks at $\lambda_{220/450}$ and $\lambda_{370/450}$, an entire peak area can be found at an excitation of 220 nm, which spans an emission of 300–500 nm. Moreover, peaks at an excitation of 295 nm and an emission between 300 nm and 400 nm are very pronounced.

For a direct visual comparison with the fluorescence spectroscopic signal after storage over 59 days, see Fig. 2. The EEM of deionized and tap water largely resemble the baseline situation. In qualitative terms, the fluorescence spectroscopic signal of river A barely changed overall. In contrast, river C has an additional tertiary peak compared to the baseline that emerged at $\lambda_{310/400}$. River B (before) reveals a slightly more pronounced secondary peak at $\lambda_{320/420}$ after 59 days than right after sampling. This difference is also evident, though to a lesser extent, for river B (after). As expected, the EEMs of the WWTP effluents show the most significant changes in the overall visual assessment, and the signals are even more similar in composition. For example, WWTP A has a secondary peak at $\lambda_{350/415}$, while at $\lambda_{275/310}$ even though there is no other peak, the fluorescent signal is still apparent, in contrast to the baseline. Concerning WWTP B, the three peaks already detected after the sampling are generally more intense. WWTP C remains the quantitatively highest signal of all WWTP effluents. Interestingly, the primary peak at $\lambda_{220/450}$ and the peak range at an excitation of 220 nm and an emission of 300–500 nm have hardly changed. In contrast, the secondary peak has shifted to somewhat lower wavelengths at $\lambda_{320/420}$. Also, unlike the baseline, a specific partition separates the secondary peak from the tertiary peak, which now has its highest signal at $\lambda_{275/310}$.

3.1.1. *Absolute fluorescence intensity*

The stability or variation of quantitative fluorescence indicators and metrics is more revealing than the qualitative alteration. Fig. 3 depicts the temporal development of the absolute intensities of HLF and TLF, compared against DOC as a reference parameter. To efficiently compare the different water sources, they are grouped as deionized and tap water, rivers, and WWTP effluents, respectively. All results are reported in detail as relative changes with respect to day zero in Table S1. Further reference is provided by standard water quality parameters that vary by biological process throughout the storage period in Table S2. Although deionized and tap water contain only low concentrations of DOC, a reduction is evident throughout the first 10–17 days. While each river

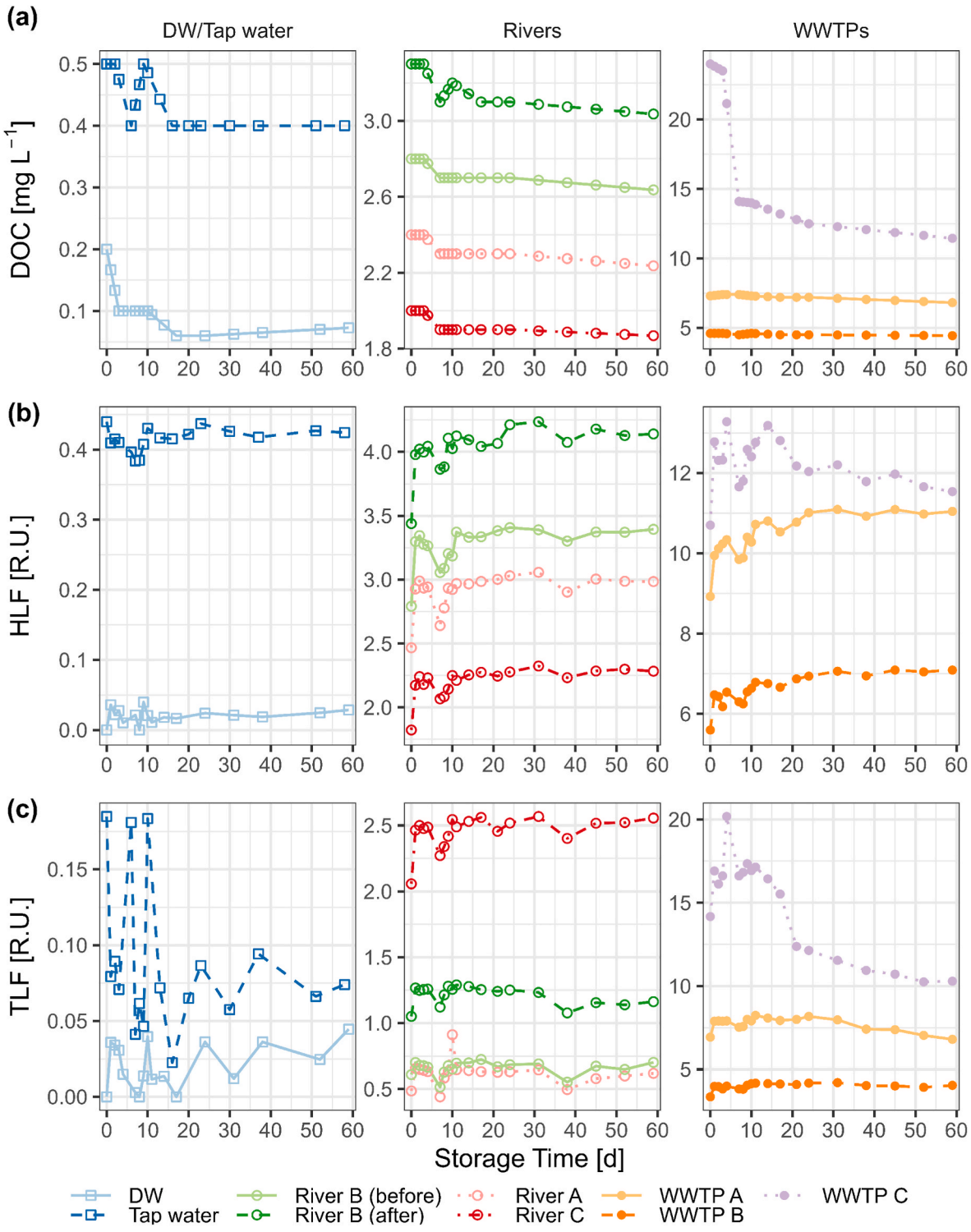

Fig. 3. DOC (a), HLF (b), and TLF (c) during 59 days of storage.

sample had a different DOC concentration, all samples showed the same evolution: scarcely any change in DOC in the first seven days; after that, a rapid but minor decrease followed by another relatively constant modest reduction until the end of the storage period. A stable and minimal decrease of the DOC concentration occurs in the effluents of WWTPs A and B. Differently, in the case of the highly loaded WWTP C, an abrupt drop of about 8 mg L^{-1} appears after seven days, after which the DOC concentration continues to decrease to a smaller extent. All river samples show a similar pattern of the HLF, namely a sharp increase of approximately 0.5 R.U. in HLF between day zero and day one, followed by a modest drop and by a stabilization around the level of day one with only slight fluctuations as storage continues. Similarly, for all WWTP effluent samples, an increase in HLF of 2 R.U. also arises between day zero and day one. Onward trends are similar for WWTPs A and B as HLF increases fairly constantly for a total of 1 R.U. until day 59. Once again, WWTP C differs significantly. Between days one and 14, the HLF fluctuates considerably to descend afterward again towards the baseline level. Yet, the changes after day one in the WWTP effluent samples are

below 15% in relative terms. Seemingly large fluctuations in the TLF can be detected in deionized and tap water. The TLF of the tap water sample changed by over 50% from day zero to day one, and after another major fluctuation on day 10, it stabilized around the value of day one. However, these should not be misinterpreted, as neither deionized nor tap water exhibits any relevant signal in this range, so the tiniest changes can seem spuriously important. The difference between day zero and day one in TLF is less pronounced than HLF in the river samples, although a subtle ascent of more than 10% has been observed. During the rest of the storage time, all river samples tend to have a slight variation of less than 10%. Similar to the results of Spencer and Coble (2014), the storage experiments for river samples reveal little change in TLF during days one and seven. Yet, in contrast to these earlier results, a slight decrease was observed as early as ten days after sampling, but this decrease was much smaller than the postulated 10–35% at 21 days (Spencer and Coble, 2014). WWTPs A and B present a remarkable increase of more than 10% in TLF between day zero and day one. After this, the signal remains stable for a substantial time until, for WWTP A, the signal reverses towards baseline intensity from day 30 onwards by more than 10%. WWTP C, however, shows an entirely different trajectory and less stability in the TLF. During the first five days of storage, the signal increases by 5 R.U., then decreases until it reaches the baseline level again after 17 days, and then decreases further by 5 R.U. According to Park and Snyder (2018), total fluorescence can gradually increase during the first nine days of storage, but then it drops and maintains this level throughout further storage. The same can be generalized to HLF and TLF in the present work, but not for all different water sources and only limited to specific samples. Even if the described increase can be observed, especially for WWTP C, the process does not stabilize. Instead, it reverses after about 14 days, demonstrating that for some samples, the signal for individual peaks can drop significantly below the one at the beginning. However, if the DOC standardized total fluorescence is considered for WWTP C, it precisely follows the pattern reported in Park and Snyder (2018). The other water sources reveal a moderate increase of the DOC standardized total fluorescence during the first five to ten days of storage, which then stabilizes.

3.1.2. *Fluorescence metrics*

Fig. 4 shows the variation of standard fluorescence metrics, i.e., BIX, HIX, FIX, and T/C ratio, during storage. Deionized and tap water are not depicted since they have a very low fluorescence signal overall. Hence, changes in these ratios have an overly strong effect and do not withstand

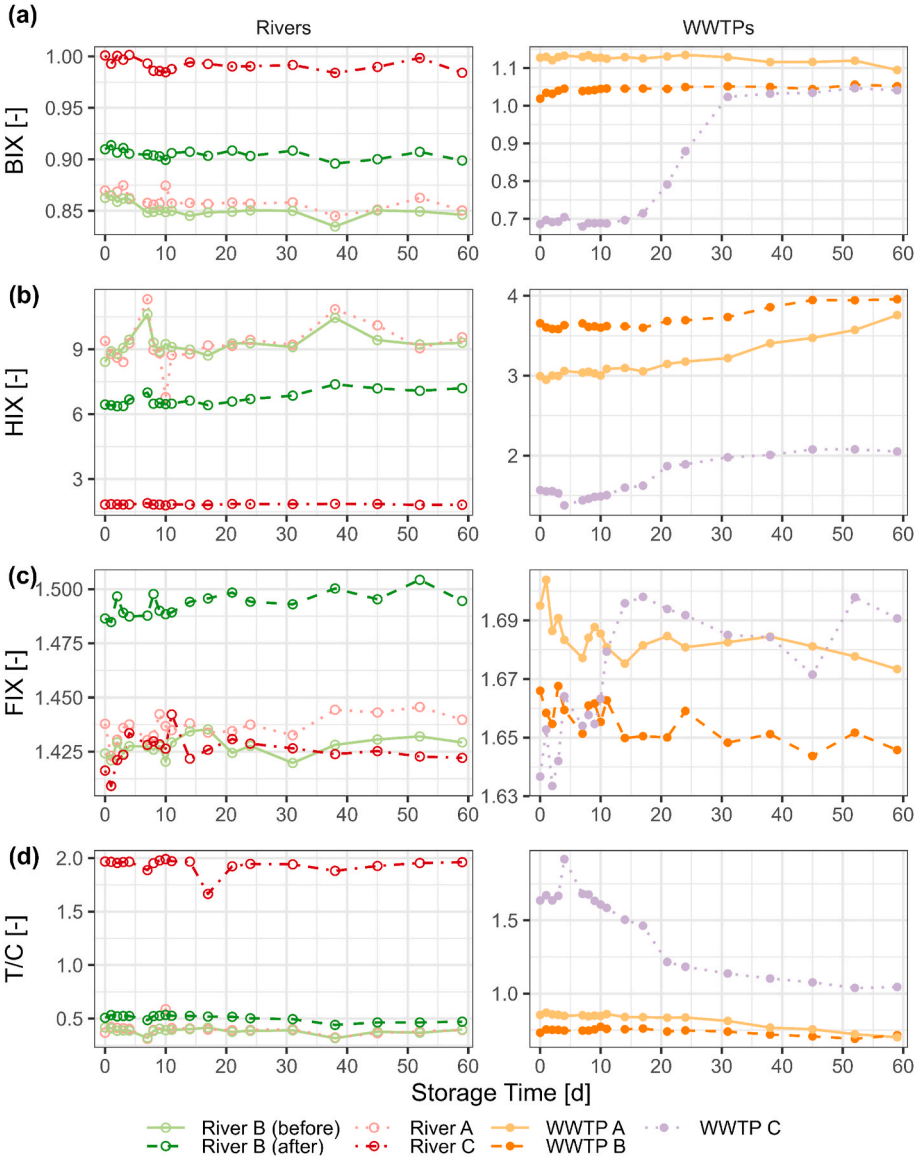

Fig. 4. BIX (a), HIX (b), FIX (c), and T/C ratio (d) during 59 days of storage.

a comparison with the other water sources. Furthermore, the metrics under consideration do not apply to deionized and tap water as they were developed for use only within particular ecosystems (Gabor et al., 2014). As opposed to the absolute fluorescence intensity, the relative fluorescence metrics indicate no sudden alterations between day zero and day one, irrespective of the water source. Nevertheless, some differences are found. The pattern of the BIX appears very similar for all river samples and for WWTPs A and B, whose samples indicate either unsystematic fluctuations or slightly increasing or decreasing trends, but in all cases, limited to minimal changes below 10%. Matters differ for the high-load WWTP C. At baseline, it starts with a considerably lower BIX than the other two effluent samples. However, after 14 days of minimal fluctuations, it converges and stabilizes around a value of 1, i.e., approximately the BIX value of WWTP A and B. The HIX of river B (after) increases relatively steadily to a small extent. At a somewhat higher level, rivers A and B (before) follow a similar trend, additionally characterized by two sudden spikes of almost 10% around day seven and day 38. In contrast, river C remains constant during storage with a HIX near zero because the organic matter in river C derives mostly from WWTP discharges and, to a very low extent, from the input of terrestrial origin. All three WWTP effluents show an increase in HIX during storage, with the slope differing between the samples. The FIX remains stable throughout storage for both river and WWTP effluent samples. Therefore, the fluctuations range from 0.01 to a maximum of 0.06 and are negligible. Previous work has found similar, significant changes, especially in HIX, for example, in lake and leaf litter leachate samples, while observed changes in FIX were also not substantial (Heinz and Zak, 2018). Two river samples also show stable T/C ratios during the 59 days. The only exception is river C, showing a significantly higher T/C ratio at the beginning, which decreases slightly just before day 20, but then quickly returns to the initial level. In relative terms, the T/C ratio for river A increases by 60% from the beginning of storage to the 10th day but then approaches the initial value again after about 31 days. WWTPs A and B show a moderate, continuous decrease in the T/C ratio during storage. Comparing the course of the T/C ratio of WWTP C with its TLF, it becomes clear that the peak T intensity primarily dominates the T/C ratio. As it decreases during storage, the T/C ratio decreases correspondingly until peaks T and C have reached a balanced state. Heinz and Zak (2018) suggest from a low HIX and a higher T/C ratio like in the present work, especially for WWTP C, that the corresponding changes in DOC concentration are related to microbial DOM. The characterization of WWTP C as a high-load plant and the increasing BIX further backs this assumption. However, this relationship is reversed from the 20th day of storage onwards, indicating that microbial activity stagnates. It might seem counterintuitive that the HIX slightly increases simultaneously, but it is still found in a range dominated by biological activity (Huguet et al., 2009).

These results are not surprising considering the differences between the WWTPs. AD plants, such as WWTP A, have higher effluent nitrogen and carbon concentrations than plants with ASS, such as WWTP B, since the latter typically have a higher sludge age (Svardal and Kroiss, 2011). Furthermore, WWTP A contains a higher refractory fraction in the chemical oxygen demand (COD) in the effluent due to industrial influences than WWTP B, handling solely municipal wastewater (DWA, 2016). Once more, the condition is different for WWTP C, a high-load plant with short sludge age, resulting in partial treatment. Although the stability of the WWTP effluent samples is not as robust as that of the other samples, the data still show valid measurements after 14–59 days, i.e., well beyond the 2-day storage period suggested earlier (Sgroi et al., 2020), for both absolute and relative fluorescence indices. Depending on the context, changes in fluorescence metrics generally indicate biological or microbial activity (Gabor et al., 2014), both of which are still present here despite filtration and dark, cooled storage. Cooling can slow or delay microbial activity (Park and Snyder, 2018), but not prevent it entirely. Furthermore, using biocides for sample preservation is not recommended, as those are known to affect the optical properties of samples (Park and Snyder, 2018). River samples, in particular, are more stable for extended periods than WWTP effluents, which suggests that initial DOM concentration is a critical factor in this respect.

These findings support the assumption that the higher the DOM concentration and the more complex the DOM composition, the shorter the recommended storage time for samples (Heinz and Zak, 2018). For this reason, tap water is storable the longest and WWTP effluent the shortest, while the storability of river samples is somewhere in between. This result applies across all fluorescence peaks and metrics considered. As already described by Sgroi et al. (2020), a graded stability depending on the DOM concentration is also apparent within the WWTP samples, with samples from WWTPs that perform only partial treatment having shorter storage times. Distinguished by water sources, these observations clearly emphasize the recommendation of Spencer and Coble (2014) that the storage length of a sample, until measured, requires consideration of the water source and the corresponding DOM composition. Moreover, it becomes clear that the metrics considered also need to be incorporated into this decision, especially if the absolute fluorescence intensity of individual peaks is to be applied in particular referencing WWTP effluent samples (Sgroi et al., 2020). Nevertheless, it is evident from the results presented here that the guideline of two days storage until processing of the samples given by Sgroi et al. (2020) concerning WWTP effluents is not generalizable to other water sources. In fact, it is possible to process water sources containing less DOM beyond this period and to perform valid measurements. Under certain circumstances, obtaining valid measurement results for effluents from WWTPs with full biological treatment after up to 14 days of storage is possible, whereby the admissibility should be checked for each situation.

3.2. *Characterisation of DOM alterations during storage using HPLC MS/MS*

Should an alteration in the EEM occur, tracking the corresponding processes during this time can be helpful, as this provides valuable information about the composition of the DOM and interactions within the DOM. Especially when the position of the highest peak in the EEM fingerprint changes, one might hypothesize that this is due to a change in the DOM composition (Coble, 1996). HPLC MS/MS analysis provides more in-depth insights into such alterations. After full-scan EMS data acquisition in negative and positive polarity mode, the entire chromatogram spectra were used to determine the qualitative difference between samples from various water sources. Representative EMS spectra of the whole chromatogram of a sample of each water source, i.e., tap water, river C, and WWTP C, are displayed in Fig. S1 in the supplementary material. The example indicates well-visible differences between the profiles of the different water sources.

The comparison between the EMS spectra obtained promptly after sampling and those obtained after storage for more than 130 days affirm that each water source undergoes some alteration during storage. However, the extent varies depending on the DOM present, as shown above. The EMS spectra indicate that the varying degree of expression per water source is due to differences in the number of organics still present in the samples. Thus, little change can be seen in the tap water spectrum between the two time points. This result is in complete agreement with the results of the fluorescence spectroscopic analysis, where no permanent changes were observed in the absolute peaks or the relative ratios. In the spectrum of river C, the shift from higher to lower mass is already evident, with particularly those organics with an m/z above 230 decreasing in negative ionization. The most considerable change in the EEM of river C could be observed between day zero and day one, with a second slightly smaller change occurring around day 7 of storage. Based on the fluorescence spectroscopy results, this alteration may have already happened at these relatively early times and subsequently changed little, even with more extended storage. As expected, the shift towards higher mass is even more explicitly pronounced in the

spectrum of WWTP C. Organics with an m/z of 300–400 in the negative ionization decrease during storage. In contrast, in positive ionization, there is an increase in all masses. From this, it could be hypothesized that organics may be converted from negative to positive ionization. In this sense, these results reflect the observations in the corresponding EEM of WWTP C, where one major peak region splits into three separate, smaller peaks during storage. At the same time, a slight increase in HLF and a substantial rise in BIX contrast with a noticeable decrease in TLF and T/C ratio. Thus, the mentioned alteration can also be detected in the fluorescence spectroscopic analyses. However, to verify the hypotheses formulated here, an in-depth study with a more extensive sampling and several coordinated measurement times is required, enabling the alterations to be traced in detail and concrete conclusions to be drawn about different DOM compositions.

4. Conclusions

This study systematically investigates the stability of excitation-emission matrices of various water systems stored under standardized conditions. Its results contribute to creating a standardized protocol by systematizing our understanding of how the specific water source and the DOM concentration determine the stability of samples during storage. An important implication of these findings is the improved design of fluorescence spectroscopic studies, especially in transnational collaborations. In particular, the parallel HPLC analysis contributes significantly to understanding DOM alteration in different water sources under standardized conditions without external influences. The higher the organic and microbial content of the sample, the more quickly it must be analyzed, even though samples are filtered and stored in the dark in locked bottles at 4 °C. The fluorescence signal changes by less than 10% for tap water for 59 days, rivers for 31–59 days, and WWTP effluent samples for 14–59 days, depending on the metric considered. Relatively stable DOC suggests an alteration in the samples rather than degradation. Irrespective of the water source and the indicator or metric considered, the most considerable change occurs between the day of sampling and the first storage day because biological processes are still active until filtration and cooling to storage temperature. As samples are usually processed on day one, a constant bias between on-site and laboratory measurements is anticipated. This may be neglected if samples are measured only on-site or in the laboratory. However, this discrepancy could be relevant for research questions requiring looking at fluorescence spectroscopic data in situ and in the laboratory. Samples should be filtered on-site and cooled quickly to minimize this effect. The stability also depends on the indicator or metric applied. Indices representing ratios between peaks are stable for longer periods, while absolute signals from single peaks tend to fluctuate more within a few days. Consequently, it is preferable to use ratio indices when timely sample processing presents difficulties. In this context, using the appropriate metrics according to the literature for the respective water matrix is essential. In this respect, it is worth noting that ratio indices were developed as limit values rather than quantitative indicators. Thus, extended storability is associated with restricted interpretability.

However, in this study, no comparison between different sample preparation regimes was performed. Beyond the indicators (HLF and TLF) and metrics (BIX, HIX, FIX, and T/C ratio) presented here, the effect of storage on multivariate analytical methods such as parallel factor analysis (PARAFAC) or self-optimizing models (SOM) might be essential to investigate. Still, those require a considerably higher number of samples per water source to establish stable global models. Potentially, the stability of fluorescence spectroscopy needs to be assessed differently in this regard. The same holds true for metrics incorporating a higher number of wavelength pairs, for example, fluorescence regional integration (FRI) or total fluorescence intensity (TF). As they are the sum of multiple wavelength pairs, fluctuations in the intensity of single wavelength pairs tend to be less influential. Similarly, evaluating stability and DOM alteration using additional standard water quality parameters, e.g., nitrate or ammonium, and a corresponding description of the effects on fluorescence spectroscopic measurements would be interesting. Further research is needed to characterize the changes in DOM, specifically over a longer observation period. The systematic combination of EEM and non-target analyses ideally suits this purpose.

Credit author statement

Sandra Peer: Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing – original draft, **Anastassia Vybornova**: Data curation, Investigation, Software, Writing – review & editing, **Joseph Tauber**: Conceptualization, Investigation, Writing – review & editing, **Ernis Saracevic**: Conceptualization, Investigation, Writing – review & editing, **Jörg Krampe**: Resources, Writing – review & editing, **Matthias Zessner**: Funding acquisition, Supervision, Writing – review & editing, **Ottavia Zoboli**: Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank the cooperating wastewater treatment plants for providing effluent samples and Zdravka Saracevic for her additional work in the lab. This research was funded by the TU Wien via the competitive funding program for innovative projects. The authors acknowledge TU Wien Bibliothek for financial support through its Open Access Funding Programme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138853.

References

Carstea, E., Popa, C., Baker, A., Bridgeman, J., 2020. In situ fluorescence measurements of dissolved organic matter: a review. Sci. Total Environ. 699 https://doi.org/10.1016/j.scitotenv.2019.134361.

Baker, A., 2001. Fluorescence excitation-emission matrix characterization of some sewage-impacted rivers. Environ. Sci. Technol. 35, 948–953. https://doi.org/10.5531021/es000177t.

Carstea, E.M., Bridgeman, J., Baker, A., Reynolds, D.M., 2016. Fluorescence spectroscopy for wastewater monitoring: a review. Water Res. 95, 205–219. https://doi.org/10.1016/j.watres.2016.03.021.

Coble, P.G., 1996. Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. Mar. Chem. 51, 325–346. https://doi.org/10.1016/0304-4203(95)00062-3.

Cohen, E., Levy, G.J., Borisover, M., 2014. Fluorescent components of organic matter in wastewater: efficacy and selectivity of the water treatment. Water Res. 55, 323–334. https://doi.org/10.1016/j.watres.2014.02.040.

DWA, 2016. DWA-A 131E – Dimensioning of Single-Stage Activated Sludge Plants. Standard. Hennef, Germany.

Frank, S., Goeppert, N., Goldscheider, N., 2017. Fluorescence-based multi-parameter approach to characterize dynamics of organic carbon, faecal bacteria and particles at alpine karst springs. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2017.09.095.

Gabor, R.S., Baker, A., McKnight, D.M., Miller, M.P., 2014. Fluorescence indices and their interpretation. In: Coble, P.G., Lead, J., Baker, A., Reynolds, D.M., Spencer, R. G.M. (Eds.), *Aquatic Organic Matter Fluorescence* Cambridge Environmental Chemistry Series. Cambridge University Press, pp. 303–338. https://doi.org/10.1017/CBO9781139045452.015.

Goffin, A., Vasquez-Vergara, L.A., Guérin-Rechdaoui, S., Rocher, V., Varrault, G., 2020. Temperature, turbidity, and the inner filter effect correction methodology for

analyzing fluorescent dissolved organic matter in urban sewage. Environ. Sci. Pollut. Res. 27, 35712–35723. https://doi.org/10.1007/s11356-020-09889-5.
Heinz, M., Zak, D., 2018. Storage effects on quantity and composition of dissolved organic carbon and nitrogen of lake water, leaf leachate and peat soil water. Water Res. 130, 98–104. https://doi.org/10.1016/j.watres.2017.11.053.
Henderson, R.K., Baker, A., Murphy, K.R., Hambly, A., Stuetz, R.M., Khan, S.J., 2009. Fluorescence as a potential monitoring tool for recycled water systems: a review. Water Res. 43, 863–881. https://doi.org/10.1016/j.watres.2008.11.027.
Huguet, A., Vacher, L., Relexans, S., Saubusse, S., Froidefond, J.M., Parlanti, E., 2009. Properties of fluorescent dissolved organic matter in the Gironde Estuary. Org. Geochem. 40, 706–719. https://doi.org/10.1016/j.orggeochem.2009.03.002.
Lakowicz, J.R., 2006. Principles of Fluorescence Spectroscopy, third ed. Springer US. https://doi.org/10.1007/978-0-387-46312-4.
Lawaetz, A.J., Stedmon, C.A., 2009. Fluorescence intensity calibration using the Raman scatter peak of water. Appl. Spectrosc. 63, 936–940. https://doi.org/10.1366/000370209788964548.
Li, L., Wang, Y., Zhang, W., Yu, S., Wang, X., Gao, N., 2020. New advances in fluorescence excitation-emission matrix spectroscopy for the characterization of dissolved organic matter in drinking water treatment: a review. Chem. Eng. J. 381, 122676 https://doi.org/10.1016/j.cej.2019.122676.
Massicotte, P., 2019. eemR: tools for pre-processing emission-excitation-matrix (EEM) fluorescence data. r package version 1.0.1. https://CRAN.R-project.org/package=eemR.
McKnight, D.M., Boyer, E.W., Westerhoff, P.K., Doran, P.T., Kulbe, T., Andersen, D.T., 2001. Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. Limnol. Oceanogr. 46, 38–48. https://doi.org/10.4319/lo.2001.46.1.0038.
Mesquita, D.P., Quintelas, C., Amaral, A.L., Ferreira, E.C., 2017. Monitoring biological wastewater treatment processes: recent advances in spectroscopy applications. Rev. Environ. Sci. Biotechnol. 16, 395–424. https://doi.org/10.1007/s11157-017-9439-9.
Murphy, K.R., Butler, K.D., Spencer, R.G.M., Stedmon, C.A., Boehme, J.R., Aiken, G.R., 2010. Measurement of dissolved organic matter fluorescence in aquatic environments: an interlaboratory comparison. Environ. Sci. Technol. 44, 9405–9412. https://doi.org/10.1021/es102362t.
Murphy, K.R., Stedmon, C.A., Wenig, P., Bro, R., 2014. OpenFluor– an online spectral library of auto-fluorescence by organic compounds in the environment. Anal. Methods 6, 658–661. https://doi.org/10.1039/C3AY41935E.
Ohno, T., 2002. Fluorescence inner-filtering correction for determining the humification index of dissolved organic matter. Environ. Sci. Technol. 36, 742–746. https://doi.org/10.1021/es0155276.
Otero, M., Mendonça, A., Válega, M., Santos, E.B.H., Pereira, E., Esteves, V.I., Duarte, A., 2007. Fluorescence and DOC contents of estuarine pore waters from colonized and non-colonized sediments: effects of sampling preservation. Chemosphere 67, 211–220. https://doi.org/10.1016/j.chemosphere.2006.10.044.
Park, M., Snyder, S.A., 2018. Sample handling and data processing for fluorescent excitation-emission matrix (EEM) of dissolved organic matter (DOM). Chemosphere 193, 530–537. https://doi.org/10.1016/j.chemosphere.2017.11.069.
Patel-Sorrentino, N., Mounier, S., Benaim, J.Y., 2002. Excitation–emission fluorescence matrix to study pH influence on organic matter fluorescence in the Amazon basin rivers. Water Res. 36, 2571–2581. https://doi.org/10.1016/S0043-1354(01)00469-9.
Peer, S., Vybornova, A., Saracevic, Z., Krampe, J., Zessner, M., Zoboli, O., 2022. Enhanced statistical evaluation of fluorescence properties to identify dissolved organic matter dynamics during river high-flow events. Sci. Total Environ. 851, 158016 https://doi.org/10.1016/j.scitotenv.2022.158016.
Pucher, M., Wünsch, U., Weigelhofer, G., Murphy, K., Hein, T., Graeber, D., 2019. staRdom: versatile software for analyzing spectroscopic data of dissolved organic matter in R. Water 11, 2366. https://doi.org/10.3390/w11112366.
R Core Team, 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing Vienna, Austria. https://www.R-project.org/.
Sgroi, M., Gagliano, E., Vagliasindi, F.G.A., Roccaro, P., 2020. Absorbance and EEM fluorescence of wastewater: effects of filters, storage conditions, and chlorination. Chemosphere 243, 125292. https://doi.org/10.1016/j.chemosphere.2019.125292.
Spencer, R.G.M., Bolton, L., Baker, A., 2007. Freeze/thaw and pH effects on freshwater dissolved organic matter fluorescence and absorbance properties from a number of UK locations. Water Res. 41, 2941–2950. https://doi.org/10.1016/j.watres.2007.04.012.
Spencer, R.G.M., Coble, P.G., 2014. Sampling design for organic matter fluorescence analysis. In: Baker, A., Reynolds, D.M., Lead, J., Coble, P.G., Spencer, R.G.M. (Eds.), *Aquatic Organic Matter Fluorescence* Cambridge Environmental Chemistry Series. Cambridge University Press, Cambridge, pp. 125–146. https://doi.org/10.1017/CBO9781139045452.008.
Svardal, K., Kroiss, H., 2011. Energy requirements for waste water treatment. Water Sci. Technol. 64, 1355–1361. https://doi.org/10.2166/wst.2011.221.
Timko, S.A., Gonsior, M., Cooper, W.J., 2015. Influence of pH on fluorescent dissolved organic matter photo-degradation. Water Res. 85, 266–274. https://doi.org/10.1016/j.watres.2015.08.047.
Tupas, L.M., Popp, B.N., Karl, D.M., 1994. Dissolved organic carbon in oligotrophic waters: experiments on sample preservation, storage and analysis. Mar. Chem. 45, 207–216. https://doi.org/10.1016/0304-4203(94)90004-3.
Wasswa, J., Mladenov, N., Pearce, W., 2019. Assessing the potential of fluorescence spectroscopy to monitor contaminants in source waters and water reuse systems. Environ. Sci.: Water Res. Technol. 5, 370–382. https://doi.org/10.1039/C8EW00472B.
Zsolnay, A., Baigar, E., Jimenez, M., Steinweg, B., Saccomandi, F., 1999. Differentiating with fluorescence spectroscopy the sources of dissolved organic matter in soils subjected to drying. Chemosphere 38, 45–50. https://doi.org/10.1016/S0045-6535(98)00166-0.