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Chromophoric dissolved organic matter and microbial enzymatic activity. A biophysical approach to understand the marine carbon cycle

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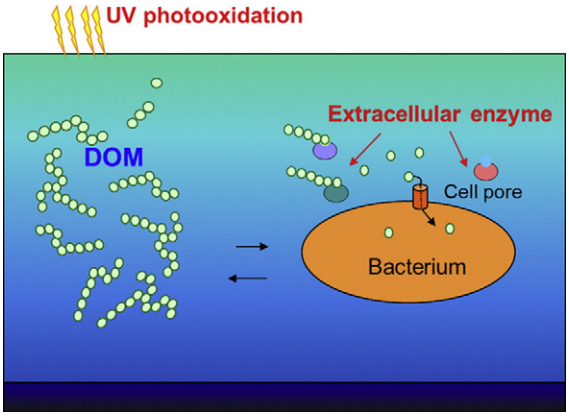
HIGHLIGHTS

- Distribution of dissolved organic carbon at Arno River mouth
- Characterization of chromophoric dissolved organic matter by absorption and fluorescence spectroscopy
- Extracellular enzyme activity in this coastal water

GRAPHICAL ABSTRACT



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ABSTRACT

This study reports the first information on extracellular enzymatic activity (EEA) combined with a study of DOM dynamics at the Arno River mouth. DOM dynamics was investigated from both a quantitative (dissolved organic carbon, DOC) and a qualitative (absorption and fluorescence of chromophoric DOM, CDOM) perspective. The data here reported highlight that the Arno River was an important source of both DOC and CDOM for this coastal area. CDOM optical properties suggested that terrestrial DOM did not undergo simple dilution at the river mouth but, other physical–chemical and biological processes were probably at work to change its molecular characteristics. This observation was further supported by the "potential" enzymatic activity of β-glucosidase (BG) and leucine aminopeptidase (LAP). Their Vmax values were markedly higher in the river water than in the seawater and their ratio suggested that most of the DOM used by microbes in the Arno River was polysaccharide-like, while in the seawater it was mainly protein-like.

1. Introduction

Dissolved organic matter (DOM) is the largest reservoir of organic carbon on the Earth and it plays a key role in the global carbon cycle. The concentration of dissolved organic carbon (DOC, ~93% DOM)

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can give quantitative information on DOM while optical properties (absorption and fluorescence) of its chromophoric fraction (CDOM) can give indirect qualitative information on the main groups of chromophores occurring in its pool [13]. Absorption and fluorescence measurements are relatively straightforward and inexpensive and they can be undertaken with small volumes of water; however the information coming from these analyses may help to understand the DOM cycle in coastal ecosystems.

Coastal areas are important sites for biological productivity and carbon cycle [1–4]. Here DOM is produced by several autochthonous processes (e.g. phytoplankton extracellular release, excretion, sloppy feeding, cell lysis) [5] and its dynamics is strongly influenced by terrestrial inputs. Because of the high mixing rate between terrestrial and coastal water it is difficult to discriminate between the different DOM sources. Two hypotheses have been formulated; the conservative one suggests that the main source of DOM in coastal area is the river input and the decrease in DOM concentration going toward the open sea is simply due to the dilution of river water. The linear inverse relationship between DOC and salinity, usually observed in coastal systems [6–9], supports this idea. The other hypothesis [10,11] suggests that in estuarine regions, terrestrial DOM is partially removed and substituted with the DOM produced "in situ". Studies on DOC isotopic composition in the York River Estuary showed the existence of a dynamic DOC cycle with a non-conservative behavior of DOC [11,12]. The authors, applying an isotopic mixing model, suggested that only 20–38% of DOC at the York River mouth was of terrestrial origin, while 38–56% was produced "in situ". The model also indicated a concomitant loss (27–45%) of riverine DOC within the estuary.

The major consumers of DOM are heterotrophic prokaryotes [10,14], however they are not capable of using an important fraction of the molecules occurring in the DOM pool, because their size is too large for penetrating through the cell wall or being transported across the cytoplasmic membrane [15]. A critical step for DOM degradation is the synthesis of hydrolytic extracellular enzymes by heterotrophic prokaryotes (EEA) [15–17]. These enzymes are used by microbes to reduce the molecular weight of molecules at <600 Da, so that they can be transported across the cytoplasmic membrane and used for metabolic and/or catabolic processes. Any enzyme, freely dissolved in the water column or bound to the cell surface, is defined as an extracellular enzyme. Free EEA may be secreted into the water column or released during cell lysis [18]. The free enzymes can access distant substrates, but the hydrolysis products may not return to the enzyme producer, thus raising the energetic cost. Previous studies have shown that the amount of EEA free in water varies widely [19–22]. Understanding the substrate specificities of EEA, the rates at which they function and the factors that control their production, distribution, and active lifetimes, is of central importance in the study of the marine carbon cycle. The occurrence of different EEAs can also give information about the substrates that can be readily transformed and metabolized and those that are not used by prokaryotes.

In this paper, the first EEA data are reported for the Arno River, together with a study on DOC and CDOM spatial distribution at the river mouth.

2. Materials and methods

2.1. Study area and sampling stations

The study area is located in the northern Tyrrhenian Sea at the Arno River mouth. Three stations, differently affected by terrestrial input, were investigated (Arno, Mix and Sea) (Fig. 1). Arno is the largest river in Tuscany and the fifth in Italy, it has a highly variable discharge (6 m^3/s to 2000 m^3/s) and covers a total length of 241 km with a drainage basin of 8228 km^2. It flows through high populated (e.g. Firenze) and industrialized areas (e.g. chemical industry in Santa Croce), making it strongly polluted and the coastal area at its mouth strongly affected by anthropogenic pressure.

Sample collection was performed June 26th 2012 when the discharge was at its minimum (6 m^3/s) (data from ARPAT, Regional Agency for Environmental Protection of Tuscany).

Salinity and temperature were measured with a portable Hanna 9033 conductivity instrument.

2.2. Samples collection and filtration

Samples were collected in amber glass bottles, preconditioned with filtered, open-seawater and rinsed 3 times with the sample before its collection. Samples were filtered through sterile 0.2 μm nylon filters (Sartorius) under low pressure of high-purity air. Samples were filtered quickly in order to avoid alteration by microbial activity. The filters were washed with 250 ml of Milli-Q water and 50 ml of sample before its collection in order to avoid any DOC and/or CDOM contamination. Filtered samples were analyzed within 6 h.

2.3. Dissolved organic carbon measurements

DOC measurements were carried out by using a Shimadzu TOC-VCSN. Samples were acidified with 2 N HCl and sparged for 3 min with CO_2-free pure air, in order to remove inorganic carbon before the high temperature catalytic oxidation. From 3 to 5 replicate injections were performed until the analytical precision was within 1% (±1 μM). A calibration curve was done by the injection of standard solutions of potassium hydrogen phthalate in the same concentration range as the samples. The system blank was measured every day at the beginning and end of the analysis using the low-carbon water (2–3 μM) produced by a Milli-Q system. The reliability of measurements was controlled twice daily by comparison of data with a DOC reference seawater sample kindly provided by Prof D.A. Hansell of the University of Miami. This procedure also assures the goodness of the calibration curve [23]. A detailed description of the procedure is reported in [24].

2.4. CDOM optical properties

2.4.1. Absorbance measurements

CDOM absorbance was measured throughout the UV and visible spectral domains (230–800 nm) using a JASCO Spectrophotometer V-550 and a 10 cm quartz cell. Milli-Q water was used as reference [25]. The absorbance (A) was converted into absorption coefficients (a) by using the Eq. (1):

$$a_\lambda = 2.303 \cdot \frac{A_\lambda}{L} \quad (1)$$

where A_λ is the absorbance at wavelength λ and L is the path length expressed in meters. The dependence of a_λ on λ is typically described using Eq. (2):

$$a_\lambda = a_{\lambda_0} e^{-S(\lambda-\lambda_0)} \quad (2)$$

where S is the spectral slope coefficient in the λ–λ_0 nm spectral range. Spectral slope coefficients were estimated using a linear fit of the log-linearized a_λ spectrum over the 275–295 nm spectral range according to [26].

2.4.2. Fluorescence measurements

A FluoroMax4 Horiba Spectrofluorometer (model FP770), with 10 × 10 mm^2 quartz cuvettes, was used to measure fluorescence emission spectra; the slit-widths were set at 5 nm for both the excitation and the emission wavelength. Two spectral regions were investigated [3,4]: (1) the protein-like fluorescence was excited at 280 nm

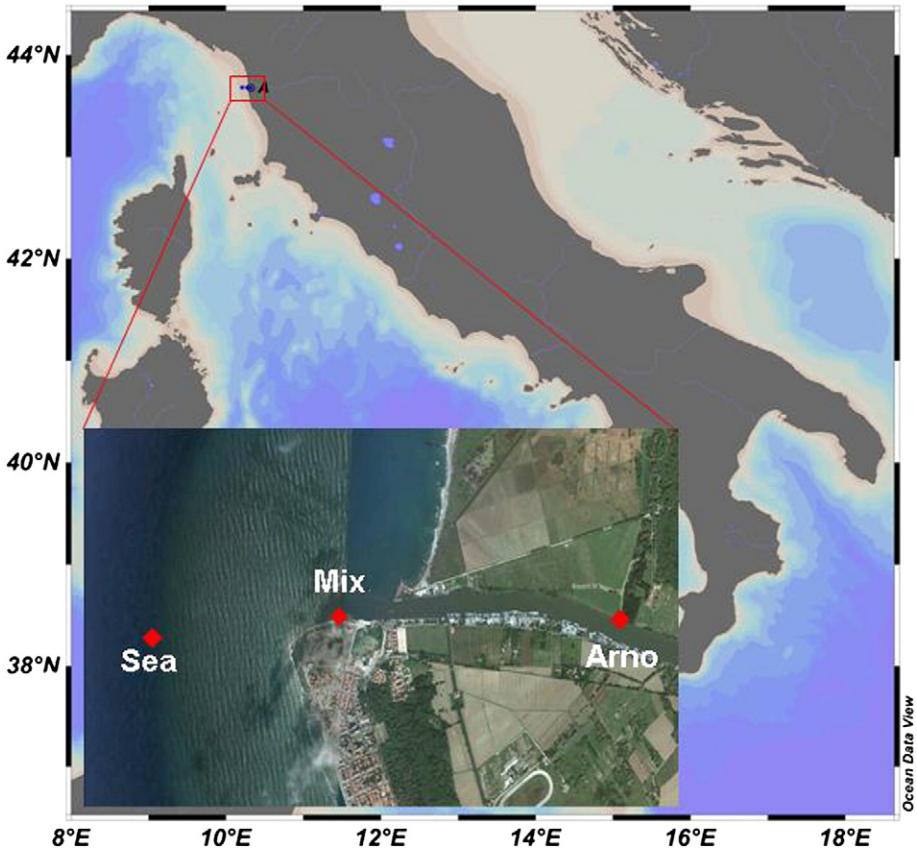

Fig. 1. Study area and sampling stations at the Arno River mouth (red diamonds). The inset map clearly shows the mixing between the river water and the seawater.

and the emission was recorded between 290 and 500 nm (F_{280}); (2) the humic-like fluorescence was excited at 355 nm and the emission was recorded between 365 and 600 nm (F_{355}). Milli-Q water was used as a blank and its spectrum was subtracted throughout. Each fluorescence spectrum was corrected for any inner-filtering effect [27]. The Raman scatter peak was removed by using the monotone cubic interpolation (shape-preserving) [28,29], since water subtraction did not completely removed its signal. Finally, the fluorescence intensity value was normalized by the integrated Raman band of Milli-Q water ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 371$–428 nm), measured at the same day of the analysis [30]. The fluorescence intensity is reported as equivalent water Raman units (R.U.) that can be compared among laboratories, being corrected for instrumental response. No significant variation was observed in the integral of the water Raman band from repeated measurements (<2%).

2.4.3. Extracellular enzymatic activity (EEA)

The "potential" activity of two enzymes, the β-glucosidase (BG) and the leucine aminopeptidase (LAP), was measured by using the fluorogenic proxies substrates 4-methylumbelliferyl β-D-glucopyranoside (MUF-Glu) and 7-amino-4-methylcoumarin (MCA-Leu), respectively [16]. Stock solutions (5 mM) of both substrates were prepared in methyl-cellosolve and stored at −20 °C until use.

The enzymatic assays were started by adding 3 ml of filtered seawater into 10 × 10 mm^2 disposable methacrylate cuvettes containing 5 increasing concentrations of MUF-Glu or MCA-Leu fluorogenic substrates. The range of substrate concentrations, estimated in previous experiments as non-saturating concentrations, was 0.05 to 8.5 μM for MCA-Leu and 2 to 35 μM for MUF-Glu. The assays, in duplicate, were carried out at room temperature and incubated in the dark. Autoclaved seawater was used as reference. Fluorescence spectra were recorded by Aqualog fluorometer (Horiba) by using an excitation wavelength of 365 nm. The fluorescence intensity was measured at 450 nm for MUF-Glu and at 440 nm for MCA-Leu, immediately after the addition of fluorogenic substrate (t_0) and this was repeated every 2 h for the first 10 h and every 6 h for the following two days. The fluorescence intensities were then converted into the rate of substrate hydrolysis (velocity = V) by using a standard curve made with the two free fluorophores (MUF and MCA). The values of V ($nM \cdot h^{-1}$) were plotted against the substrate concentrations (S, μM) and fitted by using a non-linear regression according to the Michaelis–Menten expression (3)

$$V = \frac{Vmax \cdot S}{Km + S} \tag{3}$$

where Vmax is the maximal rate of the reaction and Km is the Michaelis–Menten constant, a measure of the affinity between the enzyme and substrate (in other words its binding affinity).

The double-reciprocal Michaelis–Menten Eq. (4):

$$\frac{1}{V} = \frac{Km}{Vmax} \cdot \frac{1}{S} + \frac{1}{Vmax} \tag{4}$$

was used to make a Lineweaver–Burk plot (1/V versus 1/S) and to calculate the values of the kinetic parameters Vmax and Km (Fig. 2).

3. Results and discussion

3.1. DOC distribution

DOC showed the highest concentration in the Arno River (351.8 ± 2.9 μM) and a marked decrease toward the open sea where a value 4-fold lower (77.6 ± 0.5 μM) was observed (Table 1).

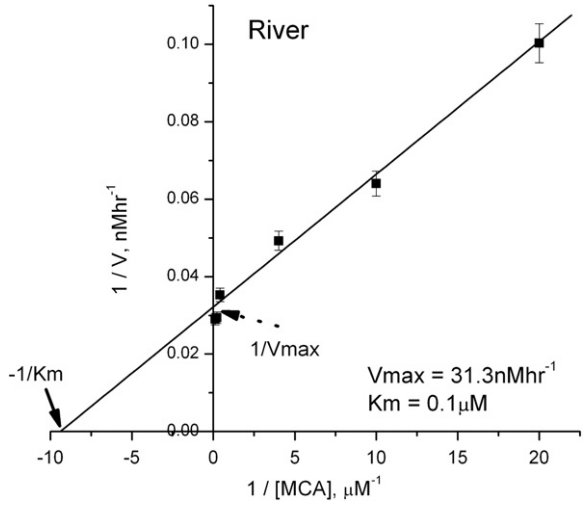

Fig. 2. "Lineweaver–Burk" plot for leucine aminopeptidase (LAP) in the Arno River. The graphical way to compute Vmax and Km, as well as their values, is also reported.

The mixing between river water and seawater was characterized by a DOC concentration of 137.0 ± 0.3 μM, suggesting that the Arno River represented an important source of organic matter for the area. These values were similar to those observed in the same area in September 1997 and in January 1999 [3,4].

3.2. CDOM distribution

3.2.1. Absorption

CDOM absorption spectra were unstructured and showed the classical near-exponential decrease at increasing wavelength from the ultraviolet to the visible region (Fig. 3A). A marked decrease in the absorption coefficients was observed moving from the Arno River (a_{280} = 21.4 m^{-1}; a_{355} = 5.8 m^{-1}) to the seawater (a_{280} = 1.3 m^{-1}; a_{355} = 0.3 m^{-1}). This behavior can be due to a decrease in CDOM concentration and/or to the occurrence of different kinds of fluorophores in river water. Although the shape of the three spectra looked similar, when they were normalized, interesting differences were highlighted (Fig. 3B). The absorption spectra of river and mixing water showed a noticeable shoulder in the 250–350 nm region; this feature was not visible in the seawater. The absorbance in this UV region is usually attributed to π–π* electronic transitions in phenolic arenes, aniline derivatives, polyenes and polycyclic aromatic hydrocarbons with two or more rings [31,32]. This finding may indicate the occurrence of a higher concentration of these compounds, that can be considered as tracers of anthropogenic pollution, in the river and mixing water. The absence of this signal in the seawater suggests that these molecules were quickly removed or transformed during their transfer to the sea.

The spectral slope in the range 275–295 nm ($S_{275–295}$) has been recently reported to be a useful biogeochemical indicator. An increase in $S_{275–295}$ was attributed to a decrease in molecular weight and aromaticity degree of CDOM [33]. In addition, a strong correlation was observed between $S_{275–295}$ and the concentration of lignin in ocean margins affected by river input [26]. These authors used $S_{275–295}$ as a tracer of the percentage of terrigenous DOC (%tDOC), in particular an increase in $S_{275–295}$ was indicative of a decrease in the %tDOC in surface water. In our study, $S_{275–295}$ showed values of 0.0160 nm^{-1} in the Arno River and 0.0275 nm^{-1} in the seawater (Table 1). The Arno values are in agreement with those observed by Fichot and Benner in Mississippi and Atchafalaya rivers (0.0135–0.0169 nm^{-1}) while the seawater values are similar to those reported for fresh DOM produced during a diatom bloom (0.0259 nm^{-1}) [26,34]. The increase in $S_{275–295}$ moving from the river to the sea supports the idea that the lignin content decreased going toward the sea and the chromophores in riverine and marine CDOM were different. In the seawater, CDOM was probably constituted by chromophores with a molecular weight and aromaticity degree lower than in the river water. A simple dilution of chromophores would in fact result in a decrease in absorption coefficient but would leave the value of $S_{275–295}$ unchanged, as a simple dilution of river water in Milli-Q water can easily demonstrate [26].

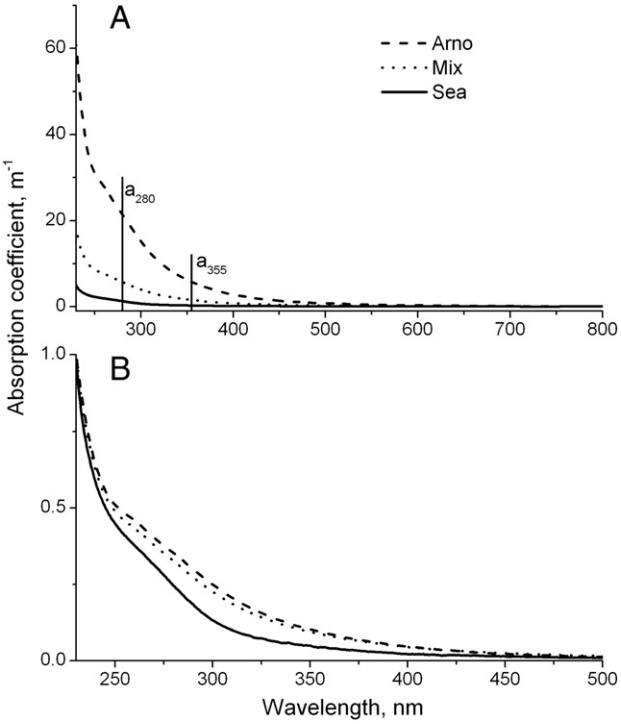

Fig. 3. CDOM absorption spectra at the three stations; each spectrum is the average of three replicates (A). Normalized CDOM absorption spectra (B). The different shape is clearly visible, in particular between 250 and 350 nm.

Table 1
Physical characteristics, DOC concentrations (average of three replicates ± standard deviation) and optical properties (absorption coefficients and spectral slope) observed at the three stations. The values of the absorption coefficients (a_{280}, a_{355}) and the spectral slope ($S_{275–295}$) are the average of three replicates; the standard error was ~1%.

Station	Salinity	Temperature °C	DOC μM	a_{280} m^{-1}	a_{355} m^{-1}	$S_{275–295}$ nm^{-1}
Arno	6.88	29.4	351.8 ± 2.9	21.44	5.80	0.0160
Mix	25.8	25.2	137.0 ± 0.3	5.77	1.56	0.0176
Sea	36.1	25.5	77.6 ± 0.5	1.29	0.29	0.0275

3.2.2. Fluorescence

F_{280} and F_{355} strongly decreased from the river to the sea, according to DOC concentration and to the absorption coefficient pattern (Fig. 4). The spectral shape of F_{280} was similar at the Arno and Mix stations, with two resolved peaks at 340 and 414 nm; in contrast, the F_{280} in the seawater showed only one resolved peak at 340 nm. The ratio between the two peaks was ~2 in both the river and mixing water (Table 2). F_{355} showed only one peak at 450 nm in all the samples; even if in the seawater, this peak was broader. All these observations suggest a higher heterogeneity in the chemical composition of CDOM in the seawater probably due to the occurrence of both simple and complex fluorophores.

The groups of fluorophores found in this study were roughly identified by a comparison with the information available in the literature

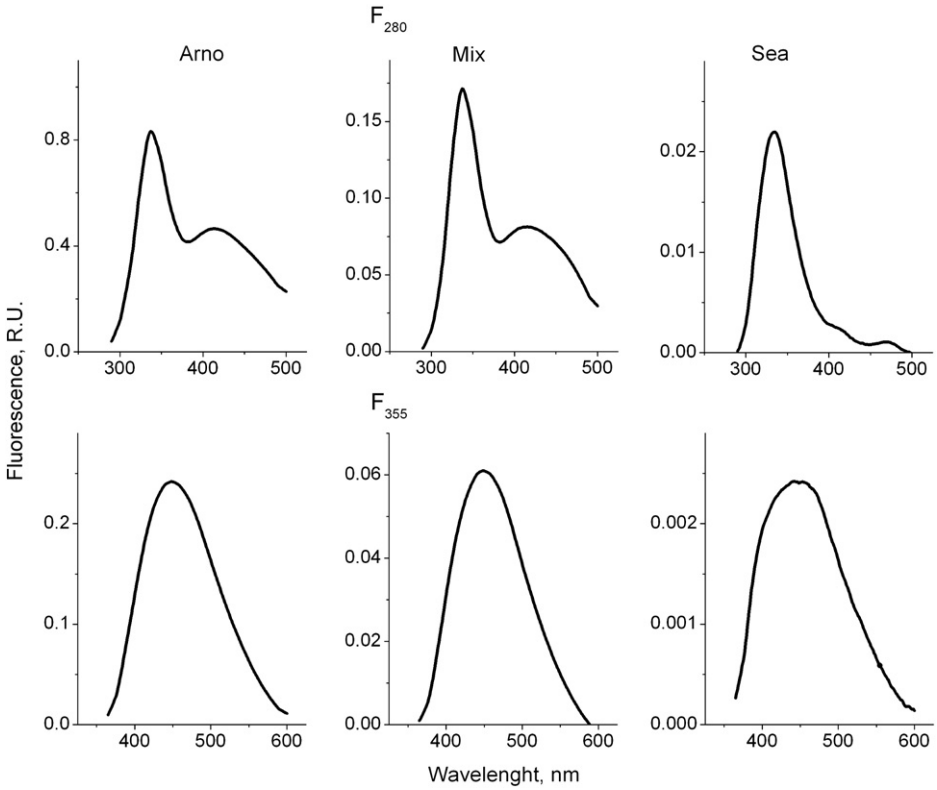

Fig. 4. Fluorescence emission spectra excited at 280 nm (F_{280}, upper panels) and at 355 nm (F_{355}, lower panels) at the three stations. Each spectrum is the average of three replicates. Please note that the scale of the fluorescence intensity is not the same in order to highlight the shape of the emission spectra between the different samples.

[13,35–37] (Table 2). Taking into account F_{280}, the peak at 340 nm was mainly due to the occurrence of a protein-like material. This is traditionally reported to be an autochthonous material, even if a terrestrial origin cannot be excluded. The peak at 414 nm was mostly related to human activity (wastewater and agriculture). Regarding F_{355}, the peak at 450 nm observed in all the samples has been traditionally defined as "peak C" and it has been related to the occurrence of humic-like material mostly of terrestrial origin [13].

3.3. EEA

LAP and BG "potential" activities were measured for the first time in this area. Enzymatic assays were carried out on filtered water in order to estimate the activity of free enzymes that represent an important fraction of the total enzymes [21,38]. All the methods, used to estimate EEA in the aquatic environment, have some limitations; (1) the artificial substrates, added in the samples, may compete for enzyme active sites with natural substrates (of unknown concentration); (2) most common artificial substrates are dimers and they do not properly represent the polysaccharide structure; in addition they lack the tertiary structure of a polysaccharide in solution [15,39,40]. As a consequence, the use of fluorogenic substrates provides just an estimate of enzymatic activities. Nevertheless, this information is of great ecological significance, since it contributes to understand the microbial potentialities of using the molecules available in the organic matter pool and therefore to define their role in the functioning of aquatic ecosystems.

The kinetic parameters (Vmax, Km) of "potential" enzymatic activity were calculated from the Lineweaver–Burk plot (Figs. 2 and 5). Vmax represents the maximum rate that the enzyme can reach, while Km is the substrate concentration at which the reaction rate is at half-maximum; in other words it is an inverse measure of the substrate's affinity of the enzyme. A small Km indicates high affinity; consequently the rate will approach Vmax very quickly.

The Vmax values for both LAP and BG were markedly higher in the river water than in the seawater (Table 3), suggesting that a higher concentration of DOM and/or a higher complexity (molecular weight and degree of aromaticity) of CDOM was related to an increase in EEA. The Vmax values, measured in this study for the enzymes freely dissolve in seawater (Table 3), are in agreement with those observed for the total enzymatic activity in different areas of the Mediterranean

Table 2
CDOM fluorescence emission properties at the three stations, by using an excitation wavelength of 280 and 355 nm. An identification of the main fluorescence peaks based on the literature [13] is also reported.

Station	Emission peaks (λ_{ex} = 280)	F_{340}/F_{414}	Component [13]	Source	Emission peaks (λ_{ex} = 355)	Component [13]	Source
Arno	340	1.9	Peak T; protein-like	Autochthonous	450	Peak C; humic-like	Fulvic acid, terrestrial
	414		Peak M; humic-like	Wastewater and agriculture			
Mix	340	2.1	Peak T; protein-like	Autochthonous	450	Peak C; humic-like	Fulvic acid, terrestrial
	414		Peak M; humic-like	Wastewater and agriculture			
Sea	340	–	Peak T; protein-like	Autochthonous	450	Peak C; humic-like	Fulvic acid, terrestrial

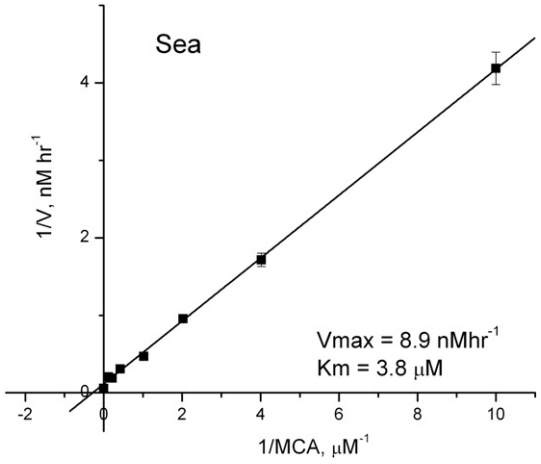

Fig. 5. "Lineweaver–Burk" plot for leucine aminopeptidase (LAP) in the seawater. The values of Vmax and Km are also reported on the graph.

Sea (BG = 35 nM · h^{-1} [40]; BG = 0.006–9.51 nM · h^{-1}; LAP = 5.24–5558 nM · h^{-1} [41,42]).

There are interesting differences between the two enzymatic activities in the river water and seawater. In the Arno River the rate of carbohydrate hydrolysis (Vmax of BG) was ~7 times higher than that of protein (Vmax of LAP), while in the seawater it was lower (Vmax BG/Vmax LAP = 0.6). In addition, the Vmax of BG was very high in the river (235 nM · h^{-1}) and it dropped ~41 times going to the sea, suggesting that the substrates of polysaccharidic origin [40] were markedly more concentrated in the river water than in the seawater. Interestingly, the rate of protein hydrolysis was just 3.5 times higher in the river water than in the seawater, suggesting the occurrence of labile, freshly produced DOM in both the river water and seawater. In fact high levels of LAP activity have been usually observed in the surface layers, where the supply of labile and freshly produced DOM is probably at its maximum [42].

The ratio of the potential activity of protein vs. carbohydrate hydrolysis (Vmax LAP/Vmax BG) provides information on the kind of DOM mainly used by microbes. Low ratios suggest a preferential flow of organic matter through polysaccharides while high ratios indicate a preferential flow through proteins [42]. The values observed in this study (Table 3) suggest that most of the DOM used by microbes in the Arno River was polysaccharide-like (0.13), while in the seawater it was mainly protein-like (1.56).

The values of Km for BG activity were almost the same in the river water and seawater (Table 3), while those for LAP activity showed a 38-fold increase moving from the river to the sea. These data suggest a possible shift in the dominant prokaryote species or in the level of the enzyme expression by the same species in the two areas and this could be related to the different molecular characteristics of DOM in the two environments.

Table 3
Kinetic parameters of the two extracellular enzymatic activities (EEAs) in the Arno River and in the seawater. The values are the average of two replicates. The standard error was ~5%. LAP: leucine aminopeptidase; BG: β-glucosidase.

Station	EEA	Vmax nM · h^{-1}	Km μM	Vmax LAP/ Vmax BG
Arno	LAP	31.3	0.1	0.13
	BG	235.0	32.2	
Sea	LAP	8.9	3.8	1.56
	BG	5.7	35.7	

4. Conclusion

The data reported in this study indicate that the Arno River represents an important source of both DOC and CDOM for this coastal area. Absorption and fluorescence spectra showed not only a marked decrease moving from the river water to the seawater, but also an interesting change in their shape as well as an increase in $S_{275-295}$. These findings suggest that terrestrial DOM did not undergo to a simple dilution at the river mouth but other physical–chemical and biological processes were probably at work to change its molecular characteristics. Chromophoric compounds can vary because they originate from different sources (terrigenous vs. marine) or as result of photochemical and/or biological transformations in the water column. Our data are not enough to assess what is the most important process. Potential enzymatic activities showed interesting differences between the river water and the seawater and suggested that most of the DOM used by microbes in the Arno River was polysaccharide-like, while in the seawater it was mainly protein-like. This study highlights the importance of merging information coming from DOC and CDOM with those from EEA. Seasonal studies, coupled with mineralization and photodegradation experiments, as well as some biological information are crucial in order to better understand the influence of the Arno input on the coastal biogeochemical processes.

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