Assessing the microbial bioavailability and rate constants of dissolved organic matter by fluorescence spectroscopy in the coastal upwelling system of the Ría de Vigo

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Abstract

The time course of colored dissolved organic matter (CDOM) absorption and fluorescence were monitored during 50 to 70 days of laboratory incubations with water collected in the coastal upwelling system of the Ría de Vigo (NW Iberian Peninsula) under contrasting hydrographic conditions. CDOM fluorescence at peak-T (Ex/Em, 280/350 nm), characteristic of protein-like materials, decayed at a 1st order rate constant (k_T) of 0.28 ± 0.13 day^{-1} (average ± SD). k_T covaried (R^2 = 0.86, p<0.0002) with the rate constant of the bulk DOC (k_{DOC}), but the protein-like materials degraded 72 ± 23% faster than DOC. Therefore, this study confirms that the CDOM fluorescence at peak-T can be used as a proxy to a DOM fraction significantly more labile than the bulk bioavailable DOC. In parallel with the decay of DOC and protein-like fluorescence, an increase in CDOM fluorescence at peak-M (Ex/Em, 320/410 nm) during the course of the incubations verified the production of marine humic-like substances as a by product of the microbial metabolism. CDOM fluorescence at peak-M built up at a production rate (k_M) of 0.06 ± 0.01 day^{-1} (average ± SD) in the Ría de Vigo. Furthermore, the slope of the linear regression between k_{DOC} and k_M (R^2 = 0.64, p< 0.001) revealed that the formation of marine humic-like substances occurred at about one fifth of the rate of net DOC consumption.

Keywords: DOC, bioavailable, refractory, rate constant, fluorescence spectroscopy

1. Introduction

Dissolved organic carbon (DOC) is the major form of organic carbon in aquatic environments playing a key role in global biogeochemical cycles (Carlson 2002). DOC is a heterogeneous pool of molecules resulting from multiple biotic processes of both autochthonous and allochthonous origin (Scully et al. 2004). Most of the DOC pool is refractory to microbial degradation over time-scales of years but a variable fraction can be used by micro-organisms (e.g. Lønborg and Søndergaard 2009), with autochthonous DOC considered more bioavailable than allochthonous DOC (Cauwet 2002).

Fluorescence spectroscopy has been applied to study dissolved organic matter (DOM) dynamics, as the fluorescence intensity depend on the concentration but also on the chemical composition of DOM (Coble et al. 1990). Earlier studies have given information about the main DOM fluorophores: protein- and humic-like compounds (Coble et al. 1990; Stedmon and Markager 2005). Protein-like fluorescence, associated with the aromatic amino acids (tyrosine, tryptophan and phenylalanine), has been suggested as indicators of the dynamics of total hydrolyzable amino acids, THAA (Yamashita and Tanoue 2003). The THAA pool is considered bioavailable and can contribute substantially to the bacterial carbon and nitrogen demand in marine systems (Coffin 1989). In addition, the fluorescence of humic-like compounds has been suggested as a suitable proxy for refractory DOM, but has also been identified as a by-product of in situ microbial degradation processes (Nieto-Cid et al. 2006; Yamashita and Tanoue 2008). These studies suggest that the protein- and humic-like fluorescence could be used to study labile and refractory DOM in the marine environment. However, quantitative relationships between these variables are still lacking.

The coastal upwelling area of the Ría de Vigo (NW Iberian Peninsula) produces and processes large amounts of DOC (Álvarez-Salgado et al. 2001), and is therefore an

appropriate area to establish if a quantitative relationship between fluorescence spectroscopy measurements and the bioavailability and rate constant of DOC exists. Complementing the study by Lønborg et al. (2009b) on the kinetics and C: N: P molar ratios of DOM degradation in the Ría de Vigo, we show here new insights on the dynamics of the consumption of labile and the production of refractory DOM based on fluorescence spectroscopy measurements during the course of the same experiments.

2. Material and methods

2.1 .Study area and sampling program

The Ría de Vigo is a large (3.32 Km^3) coastal embayment on the NW Iberian Peninsula influenced by wind-driven upwelling and downwelling periods. Upwelling favourable northerly winds dominate from April to October bringing cold and nutrient-rich water into the ría. Southerly winds dominate the rest of the year, resulting in downwelling forcing warm and nutrient-poor shelf surface water into the embayment (Álvarez-Salgado et al. 2003). Fig. 1 shows the study site, which was near the main channel in the middle section of the embayment. Samples from this location are influenced by both continental and oceanic contributions and have proved to be representative of the processes occurring in the embayment (Nogueira et al. 1997).

As described in Lønborg et al. (2009b), water for the laboratory incubation experiments was collected in autumn (20 and 27 September, and 4 October 2007), winter (31 January, 7 and 14 February 2008), spring (17 and 24 April 2008), and summer (26 June, 3 and 7 July 2008) with a 25 L Niskin bottle at 5 meters depth, and combined into a 50 L acid washed container. Salinity and temperature profiles were recorded prior to water

81 collection with an SBE 9/11 CTD probe. Aliquots of the 50L container were taken for
82 chlorophyll *a* (Chl *a*) and inorganic nutrients determination. For Chl *a* between 100 and
83 200 mL of the water samples were filtered through a GF/F filter, which were frozen (-
84 20°C) until analysis with a Turner Designs 10000R fluorometer after 90% acetone
85 extraction (Yentsch and Menzel, 1963). Water samples for dissolved inorganic nitrogen
86 (DIN; NH_4, NO_2^-, and NO_3^-) and phosphate (DIP; HPO_4^{-2}) were collected in 50 mL acid
87 washed (HCl) polyethylene bottles and kept frozen (-20ºC) until determination with an
88 Alpkem segmented flow autoanalyser.

89 Daily offshore Ekman transport values ($-Q_X$, $m^2\ s^{-1}$) were calculated according to
90 Wooster et al. (1976) from average daily geostrophic winds estimated from atmospheric
91 surface pressure charts provided at 6 h intervals by the Spanish "Instituto Nacional de
92 Meteorología". Positive values of $-Q_X$ indicate upwelling and negative values downwelling.
93 Lønborg et al. (2009b) estimated the renewal time of the embayment from $-Q_X$ as:

94 $$t = \frac{n}{\sum_{i}^{n} |Qx_i|} \cdot \frac{V}{L} \qquad (1)$$

95 Where $|Qx_i|$ is the absolute value of the daily offshore Ekman transport, a rough estimate of
96 the volume of water upwelled/downwelled per kilometre of coast, V is the volume of the
97 embayment from the inner reaches to the sampling site (0.53×10^9 m^3) and L (2.50×10^3
98 m) is the length of the open end of the embayment at the sampling site, a 7 days running-
99 mean of $|Qx_i|$ centred on the sampling date was used (n = 7) (see Fig. 1). The 7 days
100 running-mean was chosen as previous studies have shown that the average flushing time of
101 the Ría de Vigo is about 1 week (e.g. Alvarez-Salgado et al. 2001).
102

103 *2.2. Incubation experiments*

104 Filtration of the sample water started within 10 min of collection; one part was
105 filtered through a dual-stage (0.8 μm and 0.2 μm) filter cartridge (Pall-Acropak supor
106 Membrane) which had been pre-washed with 10 L of Milli-Q water; the second part was
107 filtered through pre-combusted (450°C for 4 h) GF/C filters to establish a microbial culture.
108 After filtration, the water was kept in the dark until arrival in the base laboratory, within 2 h
109 of collection. The water was transferred into a 20 L carboy and the microbial inoculum was
110 added to the 0.2 μm filtrate corresponding to 10% of the total volume. Water was then
111 siphoned from the carboys into calibrated 110 mL biological oxygen demand (BOD) glass
112 bottles, which were filled and allowed to overflow and then capped with ground-glass
113 stoppers. Four replicate bottles were fixed with Winkler reagents immediately after filling
114 for initial O_2 concentrations, while 4 other bottles were incubated at 15°C and fixed after 53
115 or 70 days (summer experiments only). The remaining water was transferred into 24 glass
116 500 mL glass bottles (headspace ~100 ml), four replicate bottles being analyzed for each
117 sub-sampling at day 0, 4, 12 and 53 or 70. Additional sub-samples for DOM fluorescence
118 measurements were taken at days 1 and 2. Incubators were kept in the dark, at 15°C. All
119 glassware used in the experiments was acid washed and then rinsed with Milli-Q water
120 prior to use.

121 After fixation, four replicated dissolved oxygen (O_2) samples were kept in the dark
122 until analyzed 24 h later by Winkler potentiometric end-point titration using a Titrino 720
123 analyzer (Metrohm). The total BOD was calculated as the difference between the initial and
124 final O_2 concentrations (in μmol L^{-1}). Since the initial and final concentrations of NH_4^+,
125 NO_2^- and NO_3^- were different because of nitrification during the course of the incubations

126 (data not shown), oxygen concentrations were referred to the oxidation state of nitrate: O_2c
127 $= O_2 - 0.5 \cdot NO_2^- - 2 \cdot NH_4^+$.

128 Bacterial production (BP) was determined at day 0, 4, 12 and 53 or 70 by [^{3}H]
129 thymidine (Tdr) incorporation (Fuhrman and Azam 1980). 100 µl of an aqueous stock
130 solution of [^{3}H - methyl] thymidine (46 Ci mmol) was added to 9.9 mL of sample and the
131 contents were mixed. Four bottles were left and two received 10 mL trichloroacetic acid
132 (TCA) to serve as a killed control. All samples were incubated in the dark at 15°C for 2 h;
133 after which 10 mL of ice-cold TCA was added to extract the soluble thymidine pools from
134 the cells. Samples were filtered onto 0.2 µm polycarbonate filters (pre-soaked in thymidine)
135 and washed with 95% ethanol and autoclaved Milli-Q water. Filters were then placed in
136 scintillation vials, dried at room temperature for 24 h and mixed with 10 mL of scintillation
137 fluid (Sigma-Flour). Radioactivity was measured using a spectral liquid scintillation
138 counter, with the efficiency of counting determined by the external standard method. The
139 conversion factors 2×10^{18} cells mol^{-1} thymidine (Smits and Riemann 1988) and 30 fg C
140 cell^{-1} (Fukuda et al. 1998) were used to convert thymidine incorporation rates into bacterial
141 carbon production.

142 Samples for analyses of the dissolved phase were collected form each of the 4
143 replicate incubation bottles by filtration through 0.2 µm filters (Pall, Supor membrane Disc
144 Filter) to follow dissolved organic carbon (DOC) and the optical properties of colored
145 dissolved organic matter (CDOM): absorption and fluorescence. Sub-samples (10 mL) for
146 DOC analysis were collected in pre-combusted (450ºC, 12 hours) glass ampoules at day 0,
147 4, 12 and 53 or 70 of the incubations and preserved by adding 50 µL 25 % H_3PO_4. DOC
148 sub-samples were analyzed in four replicates using a Shimadzu TOC-CSV organic carbon
149 analyzer. Three to five injections of 150 µL were performed per replicate. Concentrations

were determined by subtracting a Milli-Q blank and dividing by the slope of a daily standard curve made from potassium hydrogen phthalate. To avoid the small error associated with day-to-day instrument variability, all samples from a given experiment were analyzed on a single day. All samples were checked against deep Sargasso Sea reference water (2,600 m). The deep sea reference gave an average (± SD) concentration of 46.0 ± 2.0 μmol L^{-1} with the nominal value for DOC provided (D.A. Hansell's laboratory) being 44.0 ± 1.5 μmol L^{-1}.

The CDOM absorption was measured in four replicates on a Beckman Coulter DU 800 spectrophotometer using Milli-Q water as a blank. Before analysis samples were warmed to room temperature. The absorption was measured at a wavelength of 350 nm using a 10 cm quartz cuvette. The absorption coefficient (a_λ) was calculated as:

$$a_\lambda = \frac{2.303 \cdot A_\lambda}{L} \qquad (2)$$

Where A_λ is the optical density measured at 350 nm (m^{-1}) corrected for background absorption measured at 700 nm, the factor 2.303 converts from base 10 to base e logarithms and the denominator L is the cell path-length in meters (Stedmon and Markager 2001).

The CDOM fluorescence was measured in four replicates on a Perkin Elmer LS 55 luminescence spectrophotometer equipped with a xenon discharge lamp, equivalent to 20 kW for 8 μs duration. The detector was a red-sensitive R928 photomultiplier, and the photodiode works as a reference detector. Measurements were performed at a constant temperature of 20°C in a 1 cm quartz fluorescence cell. Milli-Q water was used as a reference, and the intensity of the Raman peak was checked daily. Excitation/emission (Ex/Em) measurements were performed at peak-T (aromatic amino acids, average Ex/Em, 280/350 nm; termed FDOMt), peak-A (general humic compounds, average Ex/Em 250/435

nm; termed FDOMa), peak-C (terrestrial humic substances, average Ex/Em = 340/440 nm; termed FDOMc) and peak-M (marine humic substances, average Ex/Em 320/410 nm; termed FDOMm), obtained from Coble et al. (1990). Fluorescence measurements were expressed in quinine sulphate units (QSU), i.e. in μg eq QS L^{-1}, by calibrating the LS 55 Perkin Elmer at Ex/Em: 350 nm/450 nm against a quinine sulphate dihydrate (QS) standard dissolved in 0.05 mol L^{-1} sulphuric acid.

Linear regression analyses were performed using the best-fit between the two variables X and Y obtained by model II regression as described in Sokal and Rohlf (1995). Prior to the regressions, normality was checked and the confidence level was set at 95%, with all statistical analysis conducted in Statistica 6.0.

3. Results

3.1. Seasonal and short–time scale hydrographic variability during the survey periods

Lønborg et al. (2009b) have shown that the values of $-Q_X$ in Table 1 indicate a transition from strong upwelling-favorable to moderate downwelling-favorable winds during the autumn surveys. In winter, the embayment evolved from wind relaxation to strong downwelling-favorable winds. The spring surveys occurred under moderate downwelling-favorable winds, whereas initial strong upwelling-favorable winds were followed by moderate downwelling during the summer surveys. Therefore, apart form the seasonal variability, the range of hydrographic conditions that occur in the Ría de Vigo in association with the highly variable wind regime has been sampled. In order to estimate the efficiency of the ría as a DOM digestor, flushing times were calculated from $-Q_X$ (see

195 section 2.1). An average value of 7 days was obtained, ranging from 3 days when coastal
196 winds were strong to 18 days for surveys that coincided with calm winds (Table 1).
197 As indicated above, the combination of the seasonal cycle and the short-time scale
198 variability imposed by coastal winds create contrasting hydrographic conditions at the
199 sampling site. During the autumn surveys surface (5 m) temperature decreased from > 16ºC
200 on 20-Sep-2007 to < 14ºC during the following two weeks. Concomitantly, DIN increased
201 from 3 to 13 μmol L^{-1} and Chl *a* was constant at around 3 mg m^{-3}. During the winter
202 surveys, surface temperatures were the lowest, between 13.0 and 13.5ºC, Chl *a* levels were
203 < 1.5 mg m^{-3} and DIN concentration was maintained above 8 μmol L^{-1}. During the spring
204 surveys, salinities were relatively low because of intense precipitation during April (153.7
205 mm from 01-Apr-2008 to 24-Apr-2008 recorded in the terrace of the host laboratory).
206 Salinity on the 24-Apr-2008 was as low as 25, coinciding with the highest chlorophyll
207 levels, > 8 mg m^{-3}, and relatively low phosphate, < 0.1 μmol L^{-1}. Conversely, DIN levels
208 were > 5 μmol L^{-1}, because of the high N: P molar ratio in the nutrient salts transported by
209 the continental waters. Finally, during the summer surveys, the highest temperatures, >
210 17ºC, and low DIN levels, < 3 μmol L^{-1}, were recorded with Chl *a* concentrations ranging
211 from 1.1 to 4.5 mg m^{-3}.
212
213 *3.2. CDOM dynamics during the course of the incubations*
214 Earlier studies have found that the microbial degradation of DOC can be limited by
215 inorganic nutrients (Del Giorgio and Davies 2003). The low DIN:DIP ratio typical of the
216 Ría de Vigo (Nogueira et al. 1997) suggests that nitrogen rather than phosphorus was likely
217 to limit DOC uptake. To test for nutrient limitation additional incubations were conducted
218 each sampling date enriched with carbon (glucose) and nitrate (data not shown). These

219 experiments showed no effect on DOC degradation, suggesting that the microbial
220 community in the incubations did not experienced nitrogen limitation.

221 The concentration of DOC decayed exponentially during the course of all the
222 incubations performed in this study (Fig. 2). Lønborg et al (2009b) adjusted the kinetics of
223 DOC utilization to a first-order exponential decay function using the Marquardt-Levenberg
224 algorithm taking the refractory pool into account:

225 $$DOC(t) = BDOC \cdot \exp(-k_{DOC} \cdot t) + RDOC \quad (3)$$

226 Where DOC(t) is the concentration of DOC at time t (0, 4, 10 and 50 or 70 days),
227 BDOC the bioavailable pool (μmol L^{-1}), k_{DOC} the rate constant (day^{-1}), t the time (days)
228 and RDOC the residual pool at the end of the incubations (μmol L^{-1}). BDOC is defined as
229 BDOC = DOC(0) - RDOC, where DOC(0) is the initial DOC concentration. Therefore,
230 k_{DOC} was the only parameter to be adjusted.

231 DOC(0), BDOC, RDOC and k_{DOC} values are reported in Table 2a. Initial DOC varied
232 between 73 and 94 μmol L^{-1}, 17 ± 6% (average ± SD) of which was bioavailable with an
233 average half-life time, $\ln 2/k_{DOC}$, of 3.3 ± 0.9 days (average ± SD). As reported by Lønborg
234 et al. (2009b) (i) the initial concentrations are within the values previously reported for the
235 surface layer of the Ría de Vigo; (ii) the bioavailable fraction is comparable with the values
236 found in other coastal waters, 22 ± 13%, and within the range previously suggested for this
237 ecosystem, 10-30%; (iii) the k_{DOC} values are higher than in other coastal waters but
238 resembled the rates found on George Bank; and (iv) the refractory pool, 67 ± 4 μmol L^{-1}
239 (average ± SD), is not significantly different from the DOC concentration reported for the
240 Eastern North Atlantic Central water found in the bottom layer of the ría during upwelling
241 events.

As for the case of DOC, the protein-like fluorescence decayed exponentially (Fig. 2) and could be was modelled by:

$$FDOMt(t) = BFDOMt \cdot \exp(-k_T \cdot t) + RFDOMt \quad (4)$$

Where FDOMt(t) is the protein-like fluorescence (QSU) at incubation time t (0, 1, 2, 4, 10 and 50 or 70 days), BFDOM(t) is the bioavailable FDOMt (QSU), k_T the rate constant (day^{-1}), t the time (days) and RFDOMt the remaining pool at the end of the incubations. We assumed that RFDOMt was constant throughout the incubations.

The parameters of the equations that describe the 1st order decay of protein-like substances are summarized in Table 2b. A decrease of 0.55 ± 0.22 QSU (average ± SD) was observed during the course of the incubations, which indicates that 28 ± 7% of the initial protein-like fluorescence is bioavailable. This bioavailable fraction decayed at a rate of 0.28 ± 0.13 day^{-1} (average ± SD) that represents a half-life time of 3.0 ± 1.3 days. It is worth noting that, despite the labilility of the protein-like substances, a residual signal of 1.35 ± 0.13 QSU (average ± SD) remains at the end of the incubation time.

In parallel with the decay of DOC and FDOMt, an increase in marine humic-like fluorescence of 0.56 ± 0.18 QSU (average ± SD) was observed during the course of the incubations (Fig. 2), which can be modelled by:

$$FDOMm(t) = PFDOMm \cdot [1 - \exp(-k_M \cdot t)] + FDOMm(0) \quad (5)$$

Where FDOMm(t) is the marine humic-like fluorescence (QSU) at incubation time t (0, 1, 2, 4, 10 and 50 or 70 days), PFDOMm is the amount of FDOMm produced (QSU), k_M the formation rate (day^{-1}), t the time (days) and FDOMm(0) the initial FDOMm pool. Using this formula it is assumed that the initial FDOMm was refractory.

264 Equation parameters summarized in Table 2c show that initial FDOMm values ranged
265 from 1.2 to 2.8 QSU and that the exponential model used to describe the FDOMm built up,
266 gave production rates (k_M) between 0.04 and 0.08 day^{-1}.

267 BFDOMt and PFDOMm are both significantly correlated with BDOC (eq 1 & 2 of
268 Table 3) and their corresponding rates, k_T and k_M are significantly correlated with k_{DOC}
269 (Fig. 3). These results indicate that (i) fluorescence spectroscopy, specifically the protein-
270 like fluorescence, can be used to follow the kinetics of degradation of bioavailable DOC;
271 (ii) fluorescent humic-like substances are a by-product of the degradation of bioavailable
272 DOC; (iii) the significant origin intercept of the relationship between k_T and k_{DOC} indicates
273 that there is a fraction of bioavailable DOC that does not cycle with bioavailable FDOMt
274 and the regression slope lower than 1 suggests that bioavailable DOC cycles slower than
275 bioavailable FDOMt; and (iv) for the case of the relationship between k_{DOC} and k_M (Fig.
276 3b), the significant origin intercept indicates the bioavailable DOC is not the only source of
277 humic-like fluorescence and the regression slope suggests that PFDOMm built-up is much
278 slower than BFDOMt decay.

279 Contrary to the changes observed in FDOMt and FDOMm, the CDOM absorption
280 coefficient at 350nm (a350), did not change significantly during the course of the
281 incubations (data not shown).

282 Apart from DOC and the CDOM absorption and fluorescence,, bacterial production
283 and dissolved oxygen changes were also monitored during the course of the incubations.
284 Initial bacterial production rates (BP) ranged from 0.3 to 2.0 μg C L^{-1} day^{-1}. Maximum
285 values were recorded in autumn and summer. BP decreased exponentially in parallel to the
286 decay of DOC and FDOMt, reaching values not significantly different from zero at the end
287 of the incubations (Fig. 2; Table 2d). The oxygen demand during the course of the 50 to 70

288 days of incubation (BOD) was converted to organic carbon consumption units using the
289 theoretical R_C = -O_2:Corg stoichiometric molar ratios obtained by Lønborg et al. (2009b)
290 for the same experiments (Table 2e). Within the errors of estimation of BDOC, BOD and
291 R_C (± 0.15 mol O_2 mol C^{-1}), it can be stated that the bacterial degradation of BDOC is
292 behind the observed dissolved oxygen consumption.
293
294 **4. Discussion**
295 Differences in the initial CDOM absorption and fluorescence point to temporal
296 changes in the DOM chemical composition of the water we collected to perform the
297 incubation experiments (Nieto-Cid et al. 2005; 2006). On the one hand, protein-like
298 fluorescence has been suggested as a useful indicator of the presence of THAA, which
299 potentially could be used to trace the dynamics of labile DOM (Yamashita and Tanoue
300 2003; Stedmon and Markager 2005, Nieto-Cid et al. 2006). In this sense, significant linear
301 relationships have been found in this study between the initial protein-like fluorescence,
302 FDOMt(0), the bioavailable DOC and k_{DOC} (eq 3-4 of Table 3), suggesting that the key
303 parameters that define the bioavailability and rate constants of DOC can be derived directly
304 from the initial protein-like fluorescence of the Ría de Vigo. It is also remarkable the
305 significant positive linear relationship between the initial FDOMt and its decomposition
306 rate, k_T (eq 5 of Table 3), demonstrating that higher FDOMt concentrations would lead to
307 faster mineralization rates as observed for the case of DOC in the Ría de Vigo (Lønborg et
308 al. 2009b) and in other coastal systems (Hopkinson et al. 1997, Lønborg et al. 2009a).
309 On the other hand, the humic-like fluorescence has been used to trace the presence of
310 both allocthonous and autochthonous refractory DOM (Yamashita and Tanoue 2004; Nieto-
311 Cid et al. 2005; 2006; Yamashita and Tanoue 2008). In agreement with this, the initial

312 fluorescence of the humic-like fluorophores correlated significantly with the refractory
313 DOC (eq 6-8 of Table 3). The origin intercepts of these relationships indicated that a large
314 fraction of RDOC (52-56 μmol L^{-1}) was non-colored material. FDOMa and FDOMc were
315 inversely correlated with salinity (eq 9-10 of Table 3), and remained constant throughout
316 the incubation time (data not shown), suggesting a predominantly allochthonous refractory
317 nature for these fluorophores (Yamashita et al. 2008). Furthermore, the initial absorption
318 coefficient at 350 nm correlated significantly with the initial fluorescence of the humic-like
319 fluorophores and with the refractory DOC (eq 11-14 of Table 3), suggesting that CDOM
320 absorption could be used to trace the refractory humic substances in the water collected to
321 perform the incubation experiments (Stedmon and Markager 2001). Conversely, during the
322 course of the experiments, the observed increase in FDOMm is not accompanied by a
323 significant increase in a350. This result suggests that the humic substances produced by the
324 incubated microbial cultures differ from those present in the initial waters. Note that apart
325 from possible allochthonous sources, the whole community of organisms could have
326 contributed to the production of humic substances in the initial waters.

327 The significant correlation of the initial bacterial production with the bioavailable
328 DOC (eq 15 of Table 3), together with the parallel evolution of BP and DOC during the
329 course of the incubations (Fig. 2), suggest that the inoculated natural bacterial assemblages
330 were growing only when bioavailable DOC was present in the incubated water. Therefore,
331 it confirms that the observed DOC decay was due to bacterial utilization. Furthermore, the
332 balance between the concentration of bioavailable DOC and the oxygen consumption after
333 50 to 70 days of incubation when the stoichiometric molar ratio R_C is considered (Table 2e)
334 suggest that BDOC is essentially respired by bacteria.

335 The slope of the correlation between k_T and k_{DOC} (Fig. 3a) indicated that the
336 bioavailable fraction of DOC is consumed at a rate corresponding to only 58 ± 8% of the
337 bioavailable FDOMt, i.e. that BFDOMt cycled 72 ± 23% faster than BDOC, agreeing with
338 a preferential mineralization of nitrogen over carbon-rich compounds as found in other
339 studies (Garber 1984; Hopkinson et al. 1997; 2002). The origin intercept of this correlation
340 (0.05 ± 0.02 day^{-1}) represents the average rate constant of BDOC not coupled to the
341 consumption of protein-like substances. Therefore, k_{DOC} can be viewed as the combination
342 of a basal rate constant of 0.05 ± 0.02 day^{-1} plus a rate constant corresponding to k_T linked
343 to the consumption of the labile protein-like compounds. It should be noted that the
344 observed relationship between k_{DOC} and k_T in Fig. 3a is specific for the coastal upwelling
345 area of the Ría de Vigo. This experimental approach therefore has to be repeated in any
346 other area of interest to establish the relationship between the two rate constants and,
347 therefore, to differentiate between the very labile and labile fraction in that area.
348 It has been suggested that coastal upwelling systems export labile DOC to the
349 adjacent ocean (Hansell and Carlson 1998; Álvarez-Salgado et al. 2001). Using the rate
350 constants of Table 2b and the average flushing times of water of Table 1, it results that 80 ±
351 13% (average ± SD) of the BFDOMt was consumed within the embayment. Therefore, 20 ±
352 13% of the BFDOMt produced in the Ría de Vigo was exported to the adjacent shelf, i.e.
353 this embayment exports horizontally a significant fraction of labile organic matter.
354 The RFDOMt pool, which represents as much as 72 ± 7% of the initial FDOMt,
355 could have two likely causes: i) a large fraction of the protein-like fluorophores are of a
356 refractory nature and, therefore, FDOMt fluorescence does not only trace labile material;
357 and ii) the high FDOMt levels at the end of the incubations are caused by interference of
358 the tails of the humic-like fluorophores on the FDOMt peak. The last suggestion is

359 supported by the significant linear relationships of RFDOMm, FDOMa(0) and FDOMc(0)
360 with RFDOMt (eq 16-18 of Table 3).

361 The production of marine humic-like substances during the course of the incubations
362 further confirms that they are a by product of microbial degradation processes (Yamashita
363 and Tanoue 2004; Nieto-Cid et al. 2006). The slope of the significant ($p < 0.01$) linear
364 correlation between k_{DOC} and k_M (Fig. 3b), showed that humic like materials were produced
365 at a rate corresponding to 17 ± 4% of k_{DOC}. The significant origin intercept of this linear
366 regression (0.02 ± 0.01 day^{-1}) further demonstrated that DOC is not the only source of
367 FDOMm during degradation. Although this study has been based on dark incubation
368 experiments, it should be noted that the in situ produced marine humic-like materials are
369 very sensitive to the natural UV radiation that causes a rapid photo bleaching of the
370 FDOMm fluorescence (Nieto-Cid et al. 2006). Consequently, our results regarding the
371 dynamics of marine humic-like fluorophores are not directly applicable to field conditions.
372

373 **5. Conclusions**

374 This study is, to our knowledge, the first to show quantitative relationships between
375 fluorescence of colored DOM and bioavailability of DOC. We demonstrate a positive linear
376 relationship between protein-like fluorescence and the bioavailable fraction of DOC as well
377 as its rate constants, suggesting that these key parameters can be derived directly from the
378 initial protein-like fluorescence of the Ría de Vigo. Furthermore, it has also been shown
379 that a large fraction of the protein-like fluorescence can be of a non-labile nature. The study
380 also demonstrates that the humic-like fluorescence was linearly related with the refractory
381 DOC pool and that the increase of these fluorophores during the course of dark incubations
382 as a by-product of bacterial respiration could be used as a proxy to DOM mineralization

383 processes. It is proposed that future studies should test the reliability of this approach in
384 other coastal areas, to verify the usability of fluorescence spectroscopy in characterizing
385 and assessing bioavailability of DOC in marine systems.

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480 **Figure legends**

481 Fig. 1. Map showing the sampling station (filled circle) in the middle Ría de Vigo (NW
482 Iberian Peninsula).

483 Fig. 2. Time course of dissolved organic carbon (DOC), protein-like (FDOMt) and marine
484 humic-like fluorescence (FDOMm) and bacterial production during the incubations
485 conducted in (a), (b), (c),(d) autumn, (e), (f), (g), (h) winter, (i), (j), (k), (l) spring and (m),
486 (n), (o), (p) summer. Incubation start dates are shown in legends. Error bars represent
487 standard errors.

488

489 Fig 3. Plots of the linear relationship between (a) the rate constants (day $^{-1}$) of FDOMt (k_T)
490 and DOC (k_{DOC}); and (b) k_{DOC} and k_M. Solid lines represent the corresponding regression,
491 the dashed line in panel a is the 1:1 line and the error bars are the standard errors. R^2 =
492 coefficient of determination, p = level of significance.

Table 1. Conditions in the Ría de Vigo at the sampling site (5 m depth) on the water collection dates. Salinity, temperature, chlorophyll *a*, dissolved inorganic phosphate (DIP) and inorganic nitrogen (DIN), and CDOM absorption coefficients (a350). Seven days running means of the offshore Ekman transport ($-Q_X$) and derived flushing times are also shown.

Date	Salinity	Temperature (°C)	Chlorophyll *a* (mg m^{-3})	DIP (µmol L^{-1})	DIN (µmol L^{-1})	a350 (m^{-1})	$-Q_X$ ($m^3\ s^{-1}\ km^{-1}$)	Flushing time (days)
20-Sep-2007	35.5	16.2	3.26	0.19 ± 0.02	3.17 ± 0.53	0.38 ± 0.04	551	4.4
27-Sep-2007	35.6	14.1	2.81	0.68 ± 0.01	10.34 ± 0.02	0.26 ± 0.03	-82	3.7
4-Oct-2007	35.4	13.8	2.80	0.54 ± 0.03	12.66 ± 0.38	0.25 ± 0.01	-133	11.0
31-Jan-2008	35.0	13.0	1.52	0.46 ± 0.01	9.60 ± 0.21	0.23 ± 0.02	6	5.3
7-Feb-2008	34.5	13.1	0.81	0.56 ± 0.02	11.34 ± 0.24	0.31 ± 0.01	-753	3.2
14-Feb-2008	35.2	13.4	1.13	0.42 ± 0.01	8.16 ± 0.30	0.26 ± 0.01	-127	18.0
17-Apr-2008	34.8	14.3	3.04	0.09 ± 0.02	0.40 ± 0.10	0.29 ± 0.01	-147	5.5
24-Apr-2008	25.0	15.5	8.42	0.09 ± 0.01	5.25 ± 0.20	0.60 ± 0.01	-87	8.6
26-Jun-2008	35.1	17.4	4.32	0.27 ± 0.01	0.72 ± 0.25	0.37 ± 0.09	782	3.0
3-Jul-2008	35.6	17.1	1.16	0.02 ± 0.01	1.20 ± 0.16	0.32 ± 0.01	-224	5.1
10-Jul-2008	35.4	18.4	4.52	0.38 ± 0.01	3.07 ± 0.20	0.39 ± 0.01	-36	9.0

Table 2. Initial (DOC(0), FDOMt(0)) used (BDOC, BFDOMt), end (RDOC, RFDOMt) concentrations and rate constants (k_{DOC}, k_T) of a) dissolved organic carbon (DOC) and b) protein-like fluorescence (FDOMt). c) Shows the initial (FDOMm(0)), produced (PFDOMm) and total (RFDOMm) pools of FDOMm fluorescence and build-up rates of FDOMm (k_M). Table 2d) Bacterial production measured at day 0 (BP(0)) , 4 (BP(4)), 12 BP(0) and 53/70 (BP(53/70) of the incubations. e) The initial ($O_{2C}(0)$) and final ($O_{2C}(53/70)$) oxygen concentrations and biological oxygen demand (BOD). The difference between the measured BOD and the expected BOD from the complete oxidation of BDOC (= BDOC · R_C) is also shown in Table 2.e). R_C is the theoretical stoichiometric molar ratio of O_2 consumption to CO_2 production obtained in Lønborg et al. (2009b) from the C:N:P composition of BDOM for the same experiments. Values are averages of 4 replicates ± standard error. R^2 = coefficient of determination.

a)

Date	DOC(0) (µmol L^{-1})	BDOC (µmol L^{-1})	RDOC (µmol L^{-1})	k_{DOC} (day^{-1})	R^2
20-Sep-2007	94 ± 1	29 ± 3	65 ± 1	0.35 ± 0.04	0.99
27-Sep-2007	79 ± 1	15 ± 1	62 ± 1	0.23 ± 0.05	0.99
4-Oct-2007	75 ± 2	12 ± 2	63 ± 1	0.18 ± 0.03	0.98
31-Jan-2008	75 ± 1	9 ± 1	67 ± 1	0.11 ± 0.03	0.97
7-Feb-2008	77 ± 1	7 ± 1	70 ± 1	0.11 ± 0.01	1.00
14-Feb-2008	73 ± 1	10 ± 2	63 ± 1	0.20 ± 0.01	0.95
17-Apr-2008	81 ± 1	13 ± 1	68 ± 1	0.20 ± 0.02	0.99
24-Apr-2008	85 ± 1	11 ± 1	73 ± 1	0.20 ± 0.02	0.99
26-Jun-2008	88 ± 1	17 ± 2	71 ± 2	0.30 ± 0.08	0.94
3-Jul-2008	82 ± 1	14 ± 1	68 ± 1	0.19 ± 0.05	0.93
10-Jul-2008	89 ± 2	18 ± 1	71 ± 1	0.30 ± 0.05	0.98

b)

Date	FDOMt(0) (QSU)	BFDOMt (QSU)	RFDOMt (QSU)	k_T (day^{-1})	R^2
20-Sep-2007	2.63 ± 0.04	1.14 ± 0.06	1.49 ± 0.02	0.60 ± 0.10	0.94
27-Sep-2007	1.79 ± 0.05	0.52 ± 0.15	1.27 ± 0.10	0.30 ± 0.07	0.93
4-Oct-2007	1.68 ± 0.02	0.37 ± 0.05	1.32 ± 0.04	0.22 ± 0.03	0.96
31-Jan-2008	1.74 ± 0.03	0.43 ± 0.04	1.31 ± 0.01	0.15 ± 0.04	0.88
7-Feb-2008	1.74 ± 0.05	0.43 ± 0.08	1.31 ± 0.03	0.13 ± 0.01	0.98
14-Feb-2008	1.74 ± 0.04	0.58 ± 0.10	1.16 ± 0.06	0.22 ± 0.05	0.92
17-Apr-2008	1.94 ± 0.01	0.63 ± 0.06	1.31 ± 0.05	0.30 ± 0.03	0.98
24-Apr-2008	1.84 ± 0.01	0.27 ± 0.02	1.57 ± 0.02	0.18 ± 0.04	0.98
26-Jun-2008	2.00 ± 0.04	0.51 ± 0.06	1.48 ± 0.03	0.36 ± 0.10	0.76
3-Jul-2008	1.84 ± 0.02	0.64 ± 0.07	1.20 ± 0.05	0.22 ± 0.06	0.78
10-Jul-2008	1.99 ± 0.16	0.57 ± 0.28	1.42 ± 0.12	0.36 ± 0.04	0.98

c)

Date	FDOMm(0) (QSU)	PFDOMm (QSU)	RFDOMm (QSU)	k_M (day^{-1})	R^2
20-Sep-2007	1.85 ± 0.02	0.79 ± 0.08	2.64 ± 0.05	0.08 ± 0.02	0.86
27-Sep-2007	1.55 ± 0.06	0.67 ± 0.12	2.22 ± 0.06	0.06 ± 0.02	0.88
4-Oct-2007	1.21 ± 0.08	0.48 ± 0.10	1.69 ± 0.01	0.06 ± 0.01	0.94
31-Jan-2008	1.67 ± 0.05	0.47 ± 0.13	2.14 ± 0.09	0.06 ± 0.01	0.96
7-Feb-2008	2.08 ± 0.02	0.36 ± 0.07	2.43 ± 0.05	0.04 ± 0.01	0.95
14-Feb-2008	1.59 ± 0.02	0.27 ± 0.05	1.86 ± 0.02	0.04 ± 0.01	0.97
17-Apr-2008	1.55 ± 0.02	0.45 ± 0.06	2.00 ± 0.04	0.05 ± 0.01	0.94
24-Apr-2008	2.79 ± 0.06	0.77 ± 0.11	3.55 ± 0.05	0.06 ± 0.01	0.96
26-Jun-2008	1.84 ± 0.06	0.75 ± 0.09	2.59 ± 0.04	0.08 ± 0.02	0.90
3-Jul-2008	1.24 ± 0.03	0.48 ± 0.07	1.72 ± 0.04	0.05 ± 0.01	0.97
10-Jul-2008	1.91 ± 0.02	0.69 ± 0.07	2.60 ± 0.04	0.07 ± 0.01	0.98

Date	BP(0) (µg C L^{-1} day^{-1})	BP(4) (µg C L^{-1} day^{-1})	BP(12) (µg C L^{-1} day^{-1})	BP(53/70) (µg C L^{-1} day^{-1})
20-Sep-2007	2.0 ± 0.2	0.6 ± 0.1	0.4 ± 0.1	0.1 ± 0.1
27-Sep-2007	0.8 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
4-Oct-2007	0.7 ± 0.2	0.6 ± 0.1	0.4 ± 0.1	0.1 ± 0.1
31-Jan-2008	0.9 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
7-Feb-2008	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
14-Feb-2008	0.7 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.1
17-Apr-2008	0.7 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.2
24-Apr-2008	0.8 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.0 ± 0.1
26-Jun-2008	1.0 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.0 ± 0.1
3-Jul-2008	1.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.0 ± 0.1
10-Jul-2008	1.3 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	0.0 ± 0.0

d)

Date	O_{2C}(0) (µmol L^{-1})	O_{2C}(53/70) (µmol L^{-1})	BOD (µmol L^{-1})	BOD-BDOC·R_C (µmol L^{-1})
20-Sep-2007	232.7 ± 0.3	202.4 ± 2.0	30 ± 3	-7 ± 11
27-Sep-2007	236.4 ± 0.1	214.4 ± 0.7	22 ± 1	-1 ± 3
4-Oct-2007	237.8 ± 0.5	221.0 ± 1.0	17 ± 2	0 ± 8
31-Jan-2008	255.7 ± 0.4	234.0 ± 1.0	22 ± 2	8 ± 8
7-Feb-2008	255.2 ± 0.3	232.6 ± 0.8	23 ± 1	11 ± 7
14-Feb-2008	249.4 ± 0.3	225.5 ± 2.0	24 ± 2	9 ± 8
17-Apr-2008	251.5 ± 0.1	222.6 ± 2.4	29 ± 3	10 ± 9
24-Apr-2008	255.9 ±0.4	230.9 ± 0.6	25 ± 1	8 ± 7
26-Jun-2008	233.2 ± 0.4	199.8 ± 2.4	33 ± 3	8 ± 10
3-Jul-2008	238.9 ± 0.4	204.3 ± 0.2	35 ± 1	14 ± 7
10-Jul-2008	228.6 ± 0.3	194.4 ± 1.5	34 ± 2	8 ± 9

e)

Table 3. The obtained significant linear regressions between initial (DOC(0)), bioavailable (BDOC) and refractory (RDOC) DOC, absorption coefficient of CDOM (a350), initial (FDOMt(0), FDOMm(0) , FDOMa(0) and FDOMc(0)) and refractory (RFDOMt, RFDOMm) protein-like and humic-like fluorescence, rate constants of DOC (k_{DOC}) and FDOMt (k_T). Slope, intercept, and standard error (SE) are values found by Model II regression. R^2 = coefficient of determination, p = level of significance, n.s. - not significant.

Eq nº	X	Y	Slope (±SE)	Intercept (±SE)	R^2	p
1	BDOC	BFDOMt	0.037 ± 0.009	n.s.	0.61	<0.01
2	BDOC	PFDOMm	0.03 ± 0.01	n.s.	0.50	<0.02
3	FDOMt(0)	BDOC	23 ± 4	-29 ± 6	0.83	<0.0001
4	FDOMt(0)	k_{DOC}	0.29 ± 0.07	-0.3 ± 0.1	0.63	<0.004
5	FDOMt(0)	k_T	0.49 ± 0.07	-0.7 ± 0.1	0.84	<0.0002
6	FDOMa(0)	RDOC	3.7 ± 0.6	54 ± 2	0.80	<0.001
7	FDOMc(0)	RDOC	6 ± 2	56 ± 3	0.43	<0.03
8	FDOMm(0)	RDOC	9 ± 3	52 ± 4	0.46	<0.03
9	Salinity	FDOMa(0)	-0.3 ± 0.1	15 ± 3	0.54	<0.01
10	Salinity	FDOMc(0)	-0.20 ± 0.08	9 ± 2	0.39	<0.05
11	a350	RDOC	36 ± 11	56 ± 3	0.53	<0.02
12	a350	FDOMm(0)	4.2 ± 0.9	n.s.	0.70	<0.002
13	a350	FDOMa(0)	10 ± 2	n.s.	0.67	<0.003
14	a350	FDOMa(0)	6 ± 2	n.s.	0.40	<0.04
15	BP(0)	BDOC	15 ± 2	n.s.	0.82	<0.0002
16	RFDOMt	RFDOMm	4.3 ± 0.8	-3.4 ± 0.9	0.75	<0.0006
17	RFDOMt	FDOMa(0)	8 ± 3	-7 ± 3	0.38	<0.05
18	RFDOMt	FDOMc(0)	4 ± 1	-3 ± 1	0.64	<0.006*

*Data from the 07/02/08 have been omitted to reach significant levels

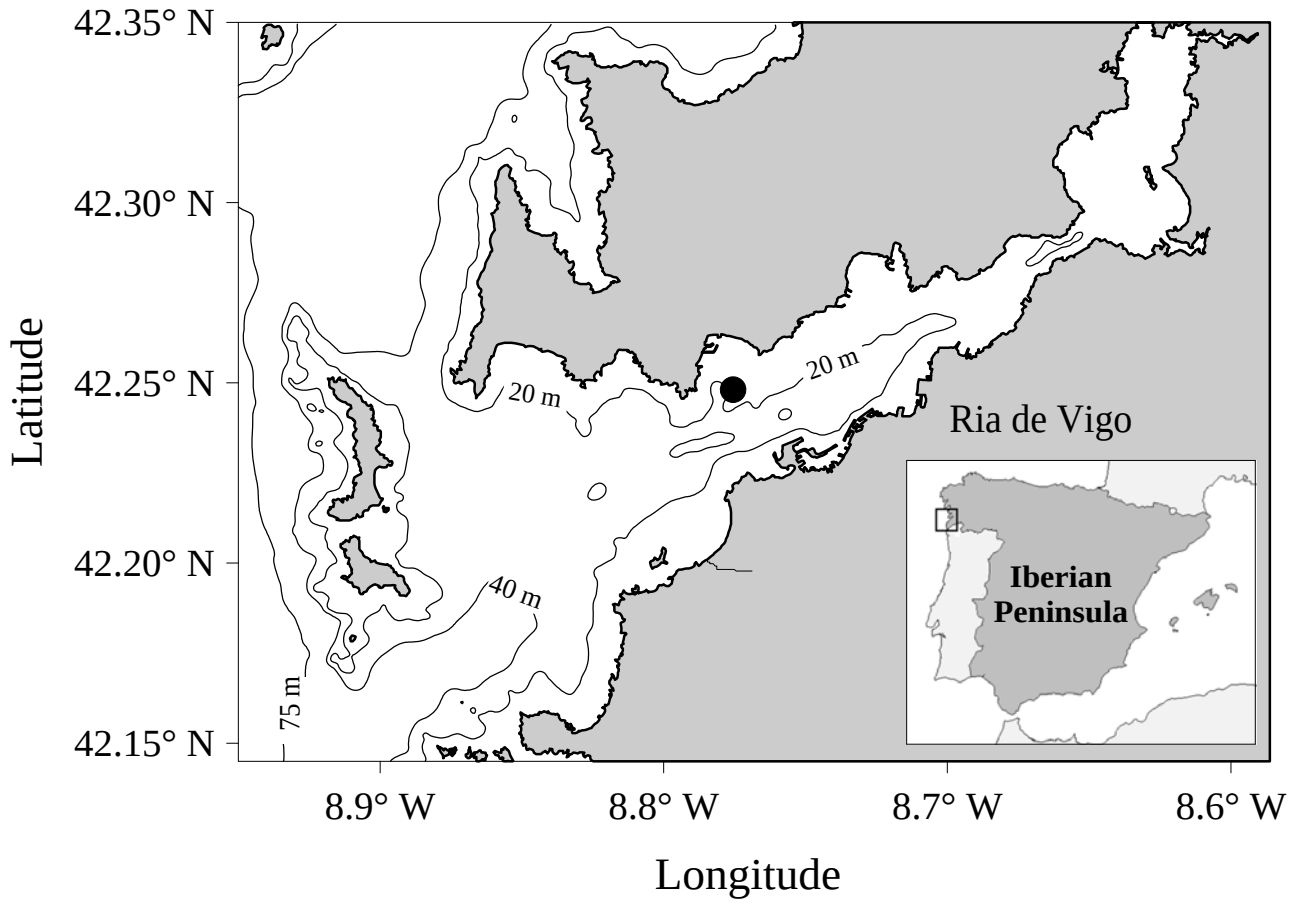
42.35° N
42.30° N
42.25° N
42.20° N
42.15° N
Latitude
8.9° W
8.8° W
8.7° W
8.6° W
Longitude
20 m
20 m
40 m
75 m
Ria de Vigo
Iberian
Peninsula

Fig. 1.

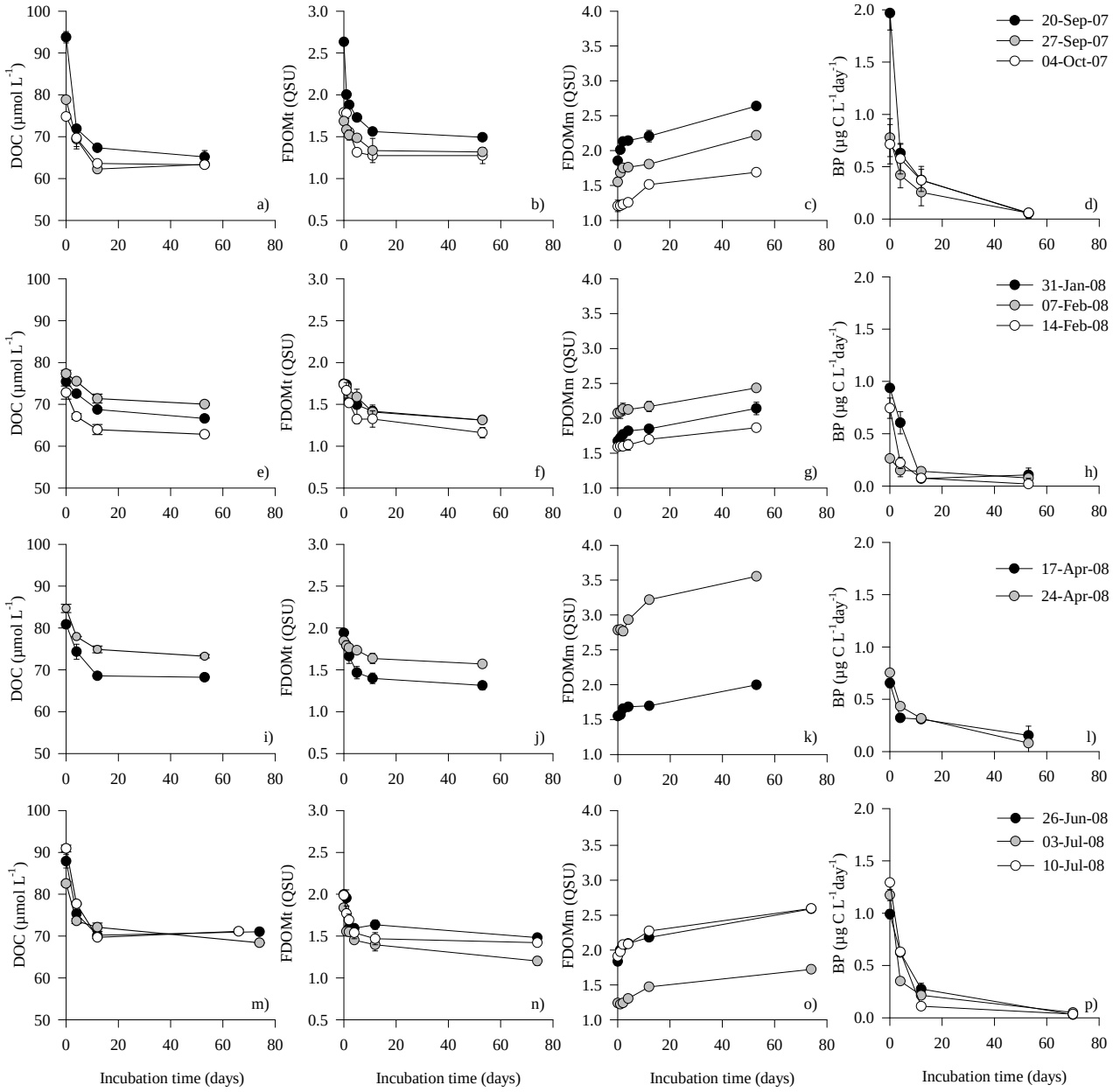
DOC (µmol L-1)
FDOMt (QSU)
FDOMm (QSU)
BP (µg C L-1day-1)
Incubation time (days)
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
m)
n)
o)
p)
20-Sep-07
27-Sep-07
04-Oct-07
31-Jan-08
07-Feb-08
14-Feb-08
17-Apr-08
24-Apr-08
26-Jun-08
03-Jul-08
10-Jul-08

Fig. 2.

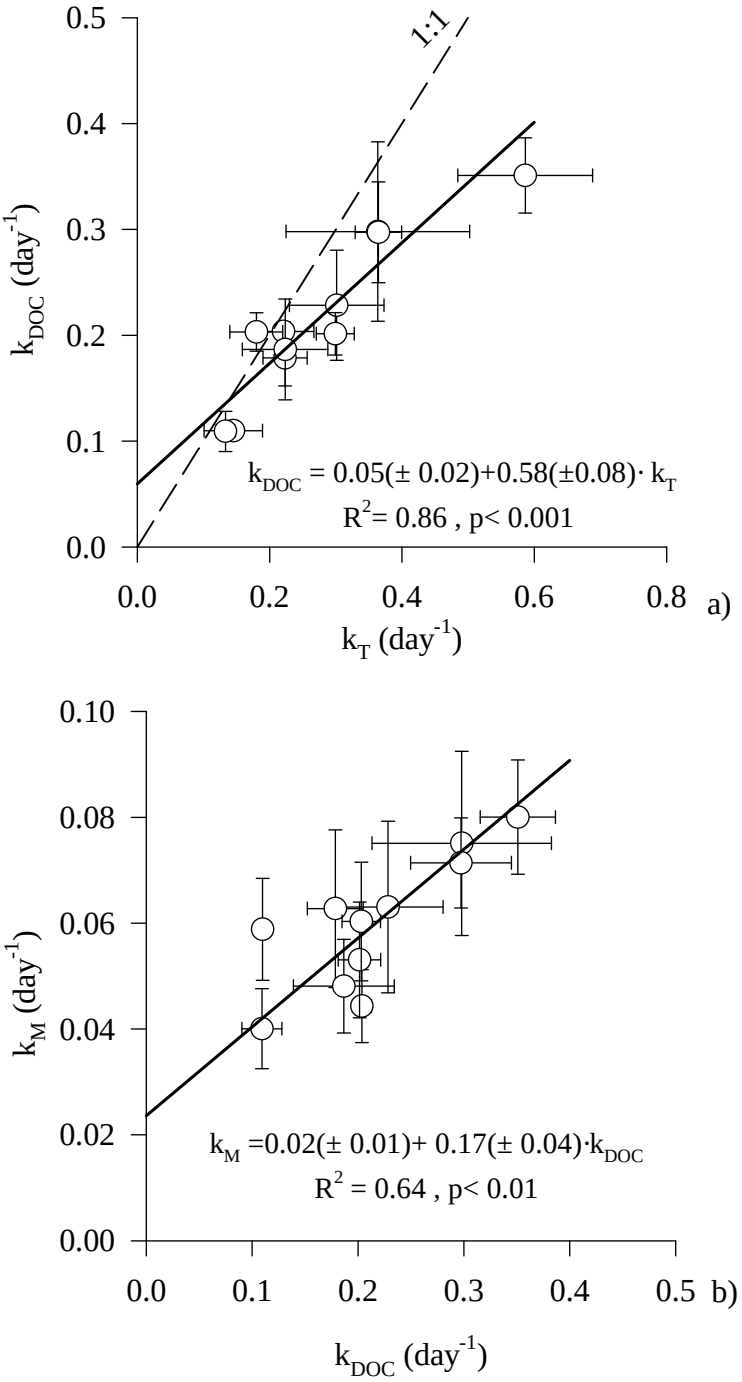
0.5
0.4
0.3
0.2
0.1
0.0
1:1
k_{DOC} (day^{-1})
k_{DOC} = 0.05(± 0.02)+0.58(±0.08)· k_T
R^2= 0.86 , p< 0.001
0.0
0.2
0.4
0.6
0.8
a)
k_T (day^{-1})
0.10
0.08
0.06
0.04
0.02
0.00
k_M (day^{-1})
k_M =0.02(± 0.01)+ 0.17(± 0.04)·k_{DOC}
R^2 = 0.64 , p< 0.01
0.0
0.1
0.2
0.3
0.4
0.5
b)
k_{DOC} (day^{-1})

Fig. 3.