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Marion Urvoy, Claire Labry, Daniel Delmas, Layla Creac'H, Stéphane L'Helguen. Microbial enzymatic assays in environmental water samples: Impact of inner filter effect and substrate concentrations. *Limnology and Oceanography: Methods*, 2020, 18, pp.725-738. 10.1002/lom3.10398 . hal-02989835

HAL Id: hal-02989835

<https://hal.science/hal-02989835>

Submitted on 19 Nov 2020

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1 ***Microbial enzymatic assays in environmental water samples: impact of Inner***

2 ***Filter Effect and substrate concentrations***

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7 ***Running head***

8 Enzymatic assays in environmental water samples

9 ***Key words***

10 Microbial enzymatic activities, Fluorimetry, Inner Filter Effect, Biogeochemical studies

11 *Abstract*

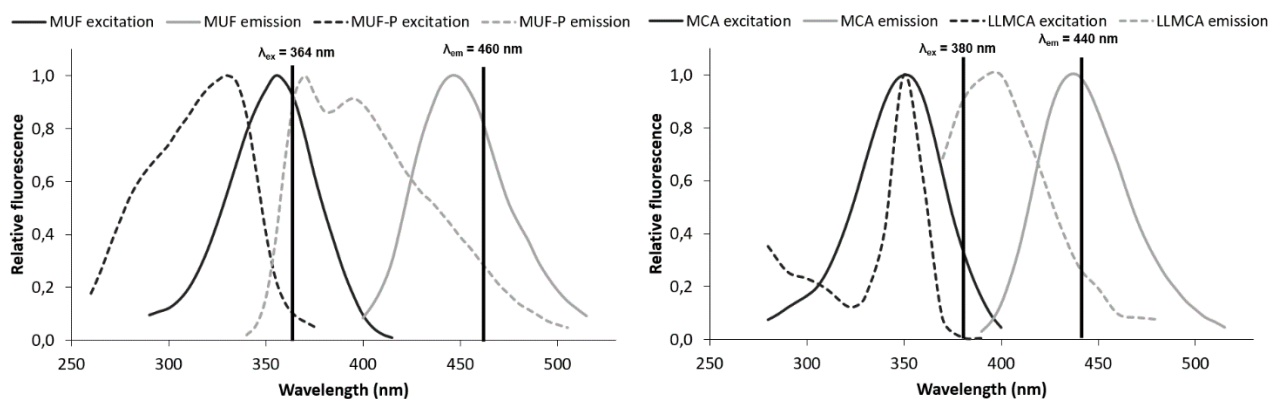
12 As microbial enzymatic activities initiate the mineralization of organic matter through the microbial
13 loop, it is important to correctly measure those activities and be able to perform inter-study comparisons.
14 Enzymatic activity assays are typically carried out using fluorogenic substrate analogs, such as 4-
15 Methylumbelliferone and 7-Amino-4-methylcoumarin linked to sugar monomers, phosphate group or
16 amino acids. However, methodological divergences can be found in aquatic science literature,
17 potentially leading to misestimated activities. To highlight some of those methodological key points, we
18 first addressed the potential occurrence of an Inner Filter Effect (IFE), a fluorometric artifact that affects
19 the relationship between fluorophore concentration and fluorescence intensity, due to absorption of
20 exciting or emitted light. It has never been considered in the context of environmental water studies
21 before, despite significantly affecting measured activities. IFE occurred with 2 out of 3 tested
22 spectrofluorometers when assaying proteases, although no IFE was detected for phosphatase assays. We
23 also evaluated how substrate concentration ranges might affect kinetic parameters estimation, revealing
24 that many existing studies might use insufficient maximum substrate concentration. Finally, for single
25 substrate concentration assays, we argued for the use of saturating substrate concentration, as naturally
26 occurring substrates might compete with the fluorogenic analog at trace level. The amendment of a
27 molecule mimicking natural substrates generated a significant inhibition of natural seawater
28 phosphatases and proteases assayed with trace concentrations of fluorogenic substrate, while almost no
29 inhibition occurred at higher concentrations. Those key points need to be addressed in order to assess
30 enzymatic rates and allow inter-study comparison.

31 *Introduction*

32 Extracellular enzymes are mostly produced by heterotrophic prokaryotes that hydrolyse polymeric
33 organic matter into units smaller than 600 Dalton, transportable across their cell membranes (Payne
34 1980). Although they are the only entity capable to significantly affect both dissolved and particulate
35 organic matter (Chróst 1990), other organisms can release extracellular enzymes as well. For instance,
36 phytoplankton, phototrophic prokaryotes, metazoa and macroalgae are able to contribute to the pool of
37 alkaline phosphatases (Hoppe 2003; Niell et al. 2003; Labry et al. 2005).

38 Microbial enzymes play a major role in the ocean: they initiate the mineralization of complex
39 organic matter through the microbial loop, transforming both dissolved and particulate organic matter
40 into living biomass, dissolved organic carbon or carbon dioxide. The activity and specificity of
41 extracellular enzymes therefore affect global carbon and nutrient cycling, carbon flow through aquatic
42 food web as well as carbon export to the deep ocean (Azam et al. 1983; Azam 1998; Bidle 2010). As
43 hydrolysis is considered the limiting step of organic matter utilization, any factors affecting enzyme
44 activity might affect the entire mineralization pathway. As such, enzymatic assays are widespread and
45 degradation capacity has been investigated in relation to diverse factors, such as for instance: substrate
46 composition and size, microbial community structure or environmental conditions (Azam et al. 1983;
47 Chróst 1990; Kirchman 2008).

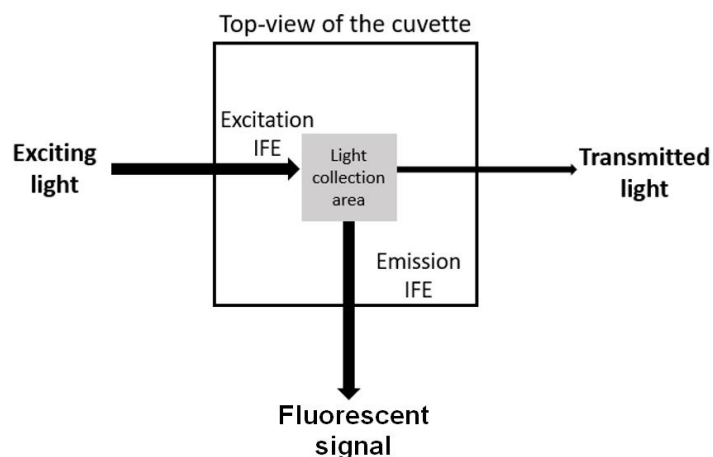
48 Measurements of enzyme activity are typically carried out using fluorogenic molecules consisting
49 of a substrate moiety covalently linked to a fluorophore (or fluorochrome). Non-hydrolysed substrate
50 has a low background fluorescence, while upon hydrolysis the fluorescence spectra of the released
51 fluorophore is considerably modified, allowing its selective detection (Fig. 1). The most commonly used
52 molecules include 4-Methylumbelliferone (MUF) linked to monosaccharides (glycosidase assays) or
53 phosphate groups (phosphatase assays) and 7-Amino-4-methylcoumarin (MCA) linked to amino acids
54 (protease assays) (Arnosti 2003; Hoppe 1983; Kirchman 2008). Enzymatic activity is determined by
55 following the increase in fluorescence over time and using standards of known fluorophore
56 concentration. Even though the use of simple substrate analogs suffers from limitations (Arnosti 2011;
57 Steen et al. 2015), they have been widely used thanks to their sensitivity and ease of use (Chróst 1990).



58

59 *Fig. 1. (A) Normalized fluorescence spectra of MUF-P (alkaline phosphatase substrate, dashed lines) and MUF (product, solid*
 60 *lines) over emission (gray lines) or excitation wavelength (black lines). Excitation spectra of MUF and MUF-P were obtained*
 61 *using a constant emission wavelength (460 nm) while excitation wavelength varied. Emission spectra were obtained using a*
 62 *constant excitation wavelength (364 nm) with variable emission wavelength. (B) Normalized fluorescence spectra of LLMCA*
 63 *(protease substrate, dashed lines) and MCA (product, solid lines) over emission (gray lines) or excitation wavelength (black*
 64 *lines). To obtain excitation spectra of MCA and LLMCA, emitted light was respectively collected at a wavelength of 440 and*
 65 *410 nm while excitation wavelength varied. To obtain emission spectra, emitted light was collected over a range of wavelength*
 66 *while excitation wavelength was set to 350 and 325 nm for MCA and LLMCA, respectively. em: emission, ex: excitation.*
 67 *Vertical lines represent excitation and emission wavelengths used during enzymatic assays.*

68 However, several biases might affect result interpretation and impede inter-study comparison. The first
 69 one is a measurement artifact intrinsic to fluorimetry known as the Inner Filter Effect (IFE), which has
 70 been completely overlooked in the literature assaying enzymatic activity in environmental water
 71 samples. With conventional spectrofluorometers, a fluorescent molecule is excited by a light source at
 72 a specific wavelength, selected by a monochromator. The emitted fluorescence is collected at right
 73 angle with respect to the incident beam and detected by a photomultiplier at the emission wavelength,
 74 also selected by a monochromator (Valeur 2001). The IFE reduces the fluorescence signal due to the
 75 absorption of exciting (excitation IFE) or emitted light (emission IFE), which affects the relationship
 76 between fluorophore concentration and fluorescence intensity (Kao et al. 1998; Valeur 2001; Eccleston
 77 et al. 2005, see Fig. 2 for a conceptual representation). IFE can result from the fluorophore itself or any
 78 other light-attenuating molecules naturally present in the analysed sample.



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80 *Fig. 2. Conceptual illustration of excitation IFE (also termed primary IFE) and emission IFE (also termed secondary IFE)*

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using a right angle geometry, one of the most common for cuvette system.

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The predominant effect, excitation or primary IFE, is caused by the absorption of the exciting light, which is attenuated as it progresses through the solution, inefficiently exciting the fluorophore. Absorption can be performed by the hydrolysis product, but also by the non-hydrolysed substrate. For instance, in the case of phosphatase assays, both MUF-Phosphate (MUF-P, alkaline phosphatase substrate) and MUF (hydrolysis product) absorb exciting light at the routinely used excitation wavelength ($\lambda_{ex} = 364$ nm, Fig. 1). Substrate absorption seems minimal and is often completely neglected by investigators, but it can be significant in enzymatic assays performed with a high substrate to product ratio. Excitation IFE is favored by the use of concentrated solutions (absorbance > 0.1), which are almost inevitable in enzymatic assays (Valeur 2001; Eccleston et al. 2005). IFE is likely to occur with the conventional right angle geometry as detection system collects light on an area restricted to the center of the cuvette: light might be absorbed before even reaching the collection area (Kao et al. 1998; Valeur 2001). Different cell configurations, such as front face illumination, were specifically developed in order to avoid this and reduce IFE. Long optic path systems (i.e. 1 cm cuvette) are especially affected by IFE, although shorter optic path systems such as microtiter plate spectrofluorometers can be impacted as well (Pinto et al. 2015, Marathe et al. 2013). The age and efficiency of the exciting lamp can also play a role in the occurrence of this effect (Valeur 2001).

A secondary IFE might also arise if the emitted light is re-absorbed by surrounding molecules. Absorption by the fluorophore itself depends on its Stokes shift, which is the difference between

100 emission and excitation maxima (Eccleston et al. 2005). A small Stokes shift implies that absorption
101 and emission spectra strongly overlap, so the emitted light might be absorbed by non-excited
102 fluorophore molecules, decreasing the measured signal. For molecules with large Stokes shift, such as
103 MUF and MCA, emission IFE is not likely to occur (Fonin et al. 2014).

104 The IFE has been described before in biochemical studies using substrate analogs (Liu et al. 1999;
105 Palmier and Van Doren 2007; Puchalski et al. 1991) but, to our knowledge, has never been considered
106 in the context of marine studies. Sebastián and Niell (2004) reported a reduction of the reaction velocity
107 by excess of substrate when assaying high substrate concentrations. . However, they did not mention a
108 possible IFE.

109 The second bias is linked to the wide differences in substrate concentrations used, for both Michaelis-
110 Menten kinetics and single point assays. Before performing a single point enzymatic assay, kinetic
111 parameters (maximum velocity - V_{max} - and Michaelis affinity constant - K_m) should ideally be
112 determined with a kinetic experiment using several substrate concentrations. However, there are wide
113 differences in maximum substrate concentration used in the literature. They vary between 0.1 and 300
114 μM for alkaline phosphatase activities (APA) assayed with MUF-P (see Table 1 for references) and
115 between 25 and 1000 μM for exoproteolytic activities (EPA) assayed with L-Leucine-MCA (LLMCA,
116 see Table 2 for references). Insufficient maximum substrate concentration can affect parameters
117 determination as the saturation of enzymatic active sites might not be reached.

118 As kinetics are laborious, time consuming, expensive and require large sample volume, they are
119 often set aside in favor of single point assays, using a single substrate concentration. A huge variability
120 in the concentrations used is also noticed: between 0.1 and 250 μM for APA assayed with MUF-P (see
121 Table 1 for references) and between 2.5 and 1000 μM for EPA assayed with LLMCA (see Table 2 for
122 references). Those differences may be related to the existence of different perspectives in the current
123 oceanographic community. Trace substrate concentration ($< 1 \mu\text{M}$) might be used in order to determine
124 enzymatic rates and substrate turnover in conditions as close as possible to those prevailing in situ. This
125 is opposed to a more conventional approach using a saturating substrate concentration, several times
126 higher than the enzymes K_m which allows the determination of maximal velocity rate (Chróst 1990;

127 Hoppe 2003). Single point assays using low substrate concentration results in several issues. (i) The
 128 substrate might not greatly exceed the enzyme concentration, which is a necessary condition to satisfy
 129 the steady-state assumption made by Michaelis-Menten. (ii) Slow product formation leads to
 130 fluorometric sensitivity issue (Chróst 1990). (iii) Those assays are subject to higher errors as velocities
 131 correspond to the first order part of the Michaelis-Menten equation, which is the most variable region.
 132 In consequence, small pipetting errors could result in large differences in estimated activity. (iv) At low
 133 concentration, naturally occurring substrates might compete with the fluorogenic substrate analog,
 134 leading to significantly underestimated activities (Chróst 1990). Consequently, measured activities
 135 might be dependent on natural substrate concentration, potentially altering results and preventing intra
 136 and inter-study comparison.

137 *Table 1. Literature examples showing phosphatase activity assay conditions used in different environments. All*
 138 *measurements were carried out using 4-Methylumbelliferyl phosphate (MUF-P) substrate.*

| | Study | Environment | Substrate range for kinetic (μM) | Substrate concentration for single point assay (μM) |
|---------------------------|------------------------------|--|---|--|
| OLIGOTROPHIC ENVIRONMENTS | Sohm and Capone 2006 | Tropical and sub tropical north Atlantic | - | 0.1 |
| | Sisma-Ventura and Rahav 2019 | Mediterranean Sea (microcosms) | - | 0.1 |
| | Duhamel et al. 2014 | North pacific subtropical gyre | 0.025 - 1 | 1 |
| | Sala et al. 2001 | Mediterranean Sea | - | 200 |
| | Van Wambeke et al. 2002 | Mediterranean sea | 0.025 - 1 | - |
| | Thingstad et al. 1998 | Mediterranean sea | 0.005 - 0.2 | - |
| | Yamaguchi et al. 2019 | Central north Pacific | 0.100 - 2 | - |
| | Bogé et al. 2012 | North west Mediterranean | 0.03 - 30 | - |
| EUTROPHIC ENVIRONMENTS | Rees et al. 2009 | English channel | - | 0.25 |
| | Strojsova et al. 2008 | Eutrophic reservoir | - | 100 |
| | Carlsson et al. 2012 | Coastal tropical Atlantic | - | 250 |
| | Koch et al. 2009 | Coastal waters Florida bay | 0.05 - 2 | - |
| | Davis et al. 2014 | Celtic sea | 0.8 - 2 | - |
| | Chrost and Overbeck 1987 | Eutrophic Lake | 10 - 200 | - |
| | Labry et al. 2005 | Coastal estuarine waters | 0.5 - 250 | 250 |
| | Nausch et al. 2004 | Baltic sea | 0.1 - 300 | - |

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140 *Table 2. Literature examples showing exoproteolytic activity assay conditions used in different environments. All*
 141 *measurements were carried out using L-Leucine-7-amido-4-methylcoumarin (LLMCA) substrate.*

| | Study | Environment | Substrate range for kinetic (μM) | Substrate concentration for single point assay (μM) |
|----------------------------------|-------------------------|--|---|--|
| OLIGOTROPHIC ENVIRONMENTS | Van Wambeke et al. 2009 | Mediterranean Sea | 0.05 - 100 | 50 |
| | Talbot et al. 1997 | Strait of Magellan | - | 200 |
| | Fukuda et al. 2000 | Subartic Pacific | - | 200 |
| | Misic et al. 2002 | Antartica | 1 - 100 | - |
| | Caruso et al. 2019 | Mediterranean Sea | 20 - 160 | - |
| EUTROPHIC ENVIRONMENTS | Gonnelli et al. 2013 | Arno river mouth (Italy) | 0.05 - 8.5 | - |
| | Song et al. 2019 | Brackish water microcosms | 0.1 - 20 | - |
| | Karner et al. 1992 | Adriatic Sea | - | 2.5 |
| | Rath et al. 1993 | Caribbean Sea | 0.1 - 25 | 2.5 |
| | Chappell et al. 1995 | Ouse and Derwent Rivers (UK) | 0.5 - 100 | 50 |
| | Foreman et al. 1998 | Maumee River (USA) | - | 120 |
| | Bullock et al. 2017 | Neuse and Tar-Pamlico Rivers (USA) | - | 400 |
| | Cunha et al. 2001 | Estuarine ecosystem (Portugal) | - | 1000 |
| | Patel et al. 2000 | Semi-enclosed coastal ecosystem | 2.5 - 40 | - |
| | Sinsabaugh et al. 1997 | Ottawa, Maumee and Hudson Rivers (USA) | 5 - 120 | - |
| | Shi et al. 2019 | Coastal waters, Northern South China Sea | 1 - 350 | - |
| | Ory et al. 2011 | Charente River (France) | 2 - 1000 | - |

142

143 The accumulation of these experimental biases might lead to severely misestimated enzymatic activities.

144 The purpose of this study is to address these issues by quantifying their effect on fluorometric enzymatic

145 assays. First, we will show how to detect the IFE occurrence, its effect on enzymatic measurements and

146 how it can be corrected. Secondly, we will address the effect of the substrate concentration ranges upon

147 the determination of kinetic parameters of pure enzymes, phytoplankton cultures and natural bacterial

148 communities. We will finally argue in favor of using saturating substrate concentrations when

149 performing single point assays, which allows the determination of the enzymatic equipment of the cells,

150 rather than the in situ degradation rate.

151 ***Material and procedures***

152 **Reagents and solutions**

153 All chemical products were purchased from Sigma-Aldrich.

154 **Substrate solutions.** Stock solutions of 10 mM 4-Methylumbelliferyl-phosphate (MUF-P) and 40 mM
155 L-Leucine-7-amido-4-methylcoumarin (LLMCA) were prepared in half a volume of 2-methoxyethanol,
156 dissolved using sonication and volume was adjusted to final concentration with Milli-Q water (Millipore
157 purification system). Working solutions from 0.5 to 500 μ M for MUF-P and from 3.9 to 1000 μ M for
158 LLMCA were obtained by successive dilutions of stock solutions in Milli-Q water and stored at – 20
159 $^{\circ}$ C.

160 **Product solutions.** Stock solutions of 2 mM 4-Methylumbelliferone (MUF) and 4 mM 7-Amino-4-
161 methylcoumarin (MCA) were prepared as described previously and used to prepare standards, as
162 described in the following section.

163 **Competitors for inhibition tests.** Stock solutions of 4.2 mM Glucose-6-phosphate (G6P), 10 mM
164 Leucyl-glycine (Leu-gly), 10 mM L-Leucyl-glycyl-glycine (Leu-gly-gly) and 10 mM Hexaglycine
165 (Hexagly) were made in Milli-Q water, serially diluted to appropriate concentrations and stored at – 20
166 $^{\circ}$ C.

167 **IFE detection**

168 **Standards preparation.** As non-hydrolysed substrate may affect fluorescence measurement,
169 calibration curves of each product (MUF and MCA) were prepared in various concentration of substrate
170 (MUF-P and LLMCA, respectively). Substrate concentrations are the ones used when determining
171 enzymatic parameters. In practice, standards of MUF ranging from 8 nM to 2 μ M were prepared in
172 Milli-Q water or MUF-P concentration ranging from 0.5 to 500 μ M, resulting in 12 calibrations for
173 APA. Buffered formaldehyde (18%, pH 8) was added to each standard to respect assay dilution
174 conditions (3.3% final concentration), as formaldehyde was used here to stop APA (C. Labry unpubl.).
175 Standards of MCA ranging from 20 nM to 2 μ M were prepared in Milli-Q water and in LLMCA
176 concentration ranging from 3.9 to 500 μ M resulting in 9 calibrations for EPA. 10% sodium dodecyl

177 sulfate (SDS) was added to each standard to respect assay dilution conditions (1% final) as it is used to
178 stop EPA. This is particularly important for EPA as 1% SDS was shown to result in a 20% increase in
179 MCA fluorescence (Delmas and Garet 1995). All standards were stored at -20°C .

180 **Fluorometric measurements.** In this study, we compared classical cuvette readings with Flow Injection
181 Analysis (FIA) reading (Delmas et al. 1994). Briefly, FIA is a liquid chromatography injection system
182 (without the chromatographic column), connected to a Kontron SFM25 fluorescence spectrometer with
183 a 1 mm optic path. This system allows a quick, sensitive and reproducible sample processing (Delmas
184 et al. 1994). The use of a carrier fluid (here a 0.1 M buffered borate solution adjusted to pH 10.5 and
185 delivered at 1 mL min^{-1}) provides the possibility of setting the pH during fluorescence reading,
186 independently from the pH of incubation. This is important as MUF fluorescence yield greatly varies
187 with pH and is maximum at $\text{pH} > 10$ (Chróst and Krambeck 1986). For cuvette readings, pH was
188 adjusted to 10 by adding 0.98 and 0.20 mL of a 0.5 M pH 12 buffered borate solution per 4 mL sample
189 (containing preservative) for APA and EPA respectively. Measurements were carried out in a 1 cm
190 cuvette using two spectrofluorometers, SFM25 and Perkin Elmer LS50.

191 All measurements were performed with a 90° angle illumination. Excitation and emission
192 wavelength were respectively 364 nm and 460 nm for APA and 380 nm and 440 nm for EPA.

193 **Michaelis-Menten kinetics**

194 Michaelis-Menten kinetics were carried out on various samples to evaluate the impact of IFE and of
195 substrate concentration ranges on kinetic parameters determination.

196 **Sample preparation.** Tested samples were either purified enzymes, phytoplankton cultures or natural
197 microbial communities. Purified enzymes from *Escherichia coli* and shrimp (acquired from
198 Sigma/Aldrich) were diluted to a stock concentration of 10 mU mL^{-1} in Milli-Q water and stored at 4
199 $^{\circ}\text{C}$. For each activity assay, stock enzyme was diluted to $250\text{ }\mu\text{U mL}^{-1}$ in $0.2\text{ }\mu\text{m}$ filtered natural seawater
200 (Whatman Nucleopore filters).

201 *Alexandrium minutum* and *Thalassiosira weissflogii* were precultured in F/4 medium (Guillard and
202 Ryther 1962) and inoculated in phosphate-free F/4 medium for 3 days at 18°C , under a 12:12 light cycle

203 (120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Activities were measured on total fraction comprising algal-attached and
204 dissolved enzymes.

205 Natural seawater was collected from the Brest station of the Service d'Observation en Milieu
206 Littoral program (SOMLIT, French marine monitoring network, <http://somlit.epoc.u-bordeaux1.fr>).
207 and filtered through 0.8 μm Whatman Nucleopore filters to remove eukaryotes and larger cells.

208 **Incubations.** In order to determine kinetic parameters, 2 mL samples were incubated in the dark with
209 50 μL substrate solutions, with final concentrations ranging from 0.5 to 500 μM for MUF-P and from
210 1.95 to 1000 μM for LLMCA). Incubation time for each type of sample was previously determined so
211 that less than 25% of the substrate was hydrolysed, in order to measure initial linear velocity. Natural
212 bacterial communities were incubated at in situ temperature (between 12 and 16 $^{\circ}\text{C}$), purified enzymes
213 and phytoplankton cultures at 20 $^{\circ}\text{C}$. At the end of the incubation, reaction was stopped by adding 18%
214 buffered formaldehyde (pH 8, 3.3% final concentration) for APA or 10% SDS for EPA (1% final
215 concentration). Samples were then frozen at - 20 $^{\circ}\text{C}$ until fluorescence measurement, which was
216 performed as previously described. These preservatives stop enzymatic activities and allow storage at -
217 20 $^{\circ}\text{C}$ for deferred sample analysis, if necessary, without any changes on the kinetic parameters (APA:
218 C. Labry unpubl., EPA: Delmas and Garet 1995).

219 **Controls.** As natural seawater and substrates (MUF-P and LLMCA) do produce fluorescence, a blank
220 sample was prepared for each substrate concentration by directly mixing samples, substrate and reaction
221 inhibitor (formaldehyde for APA, SDS for EPA) and immediately freezing them at - 20 $^{\circ}\text{C}$. Blank
222 fluorescence was then subtracted from sample fluorescence and results were converted to degradation
223 rates using product standards diluted in Milli-Q water.

224 **Statistical analysis.** Affinity constant (K_m) and maximum velocity (V_{max}) and their standard deviations
225 were calculated using nonlinear least squares regression of the data fitted to the Michaelis-Menten
226 equation, using R (“nls” function in the “stats” package). Non-linear regression seems to be the best
227 method to estimate kinetic parameters (Chróst 1990).

228 **Inhibition tests**

229 To assess the effect of natural competitors on APA and EPA of natural bacterial samples, we monitored
230 the fluorescence emitted by 3 mL seawater sample amended with 75 μL substrate (0.125 to 2 μM of
231 MUF-P, 0.125 and 2 μM of LLMCA) in a 1 cm optic path cuvette (SFM25 spectrofluorometer) during
232 short periods of time (2 to 10 min). A molecule competing with the substrate analog was then added and
233 fluorescence was monitored using the same procedure. The competitor used for APA was G6P (75 μL ,
234 final concentration from 50 nM to 1.5 μM), a natural compound involved in bacterial metabolism which
235 is susceptible to be present in both natural environments and cultures. For EPA, three competitors were
236 tested: Leu-gly, Leu-gly-gly and Hexagly (30 μL , final concentration of 8 and 16 μM). Fluorescence
237 increased linearly over time and the presence of competitors visibly affected the slopes, which
238 correspond to reaction velocities. pH was not adjusted during this measurement as slopes were not
239 converted to actual velocities. Slopes were corrected for the dilution due to competitor's addition.

240 *Assessment and discussion*

241 **Influence of IFE on activity assays**

242 IFE occurs when exciting or emitted light is absorbed, decreasing the fluorescence signal and resulting
243 in underestimated enzymatic activities. It includes all light-attenuating processes, caused by the
244 fluorogenic substrate analog itself or by any other chromophores naturally present in samples. This study
245 is limited to IFE caused by fluorogenic substrate as it is important during enzymatic assays using large
246 concentration of substrate, but IFE resulting from natural organic matter may also affect fluorometric
247 assays (Kothawala et al. 2013). IFE resulting from natural compounds should theoretically be corrected
248 using calibration curves prepared in the same matrix as the samples (for instance 0.2 μm filtered
249 seawater).

250 *Detection of IFE caused by fluorogenic substrate*

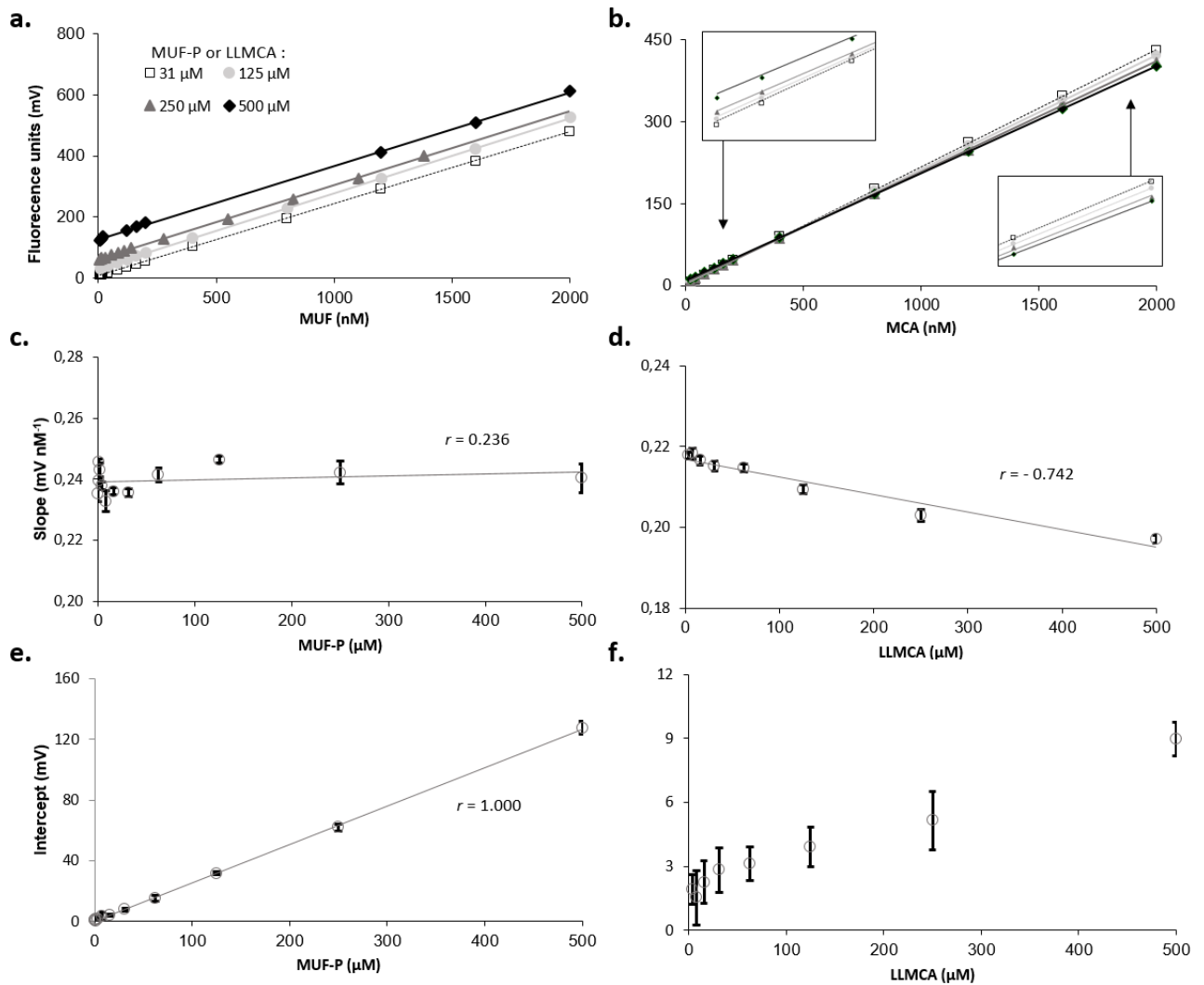
251 MUF-P/MUF and LLMCA/MCA have similar excitation and emission spectra, characterized by a large
252 Stokes shift, meaning that emission IFE should not occur. However, both non-hydrolysed substrates and
253 hydrolysis products can absorb exciting light and produce an excitation IFE.

254 The occurrence of an excitation IFE from fluorescent products was tested by measuring calibration
255 standards of products (MUF and MCA) diluted in Milli-Q water. If this effect occurs, the released
256 product will absorb exciting light, with a greater impact at high product concentration. Consequently,
257 the emitted signal will not be linear over the product range of concentration. As the curves obtained
258 were linear (determination coefficient for linear fitting: $R^2 > 0.999$, data not shown) for the three
259 instruments (FIA-SFM25, SFM25 and LS50), it appears that there is no IFE from the product between
260 0 and 2 μM , for both MUF and MCA.

261 Excitation IFE resulting from non-hydrolysed substrates can be detected by measuring fluorescence
262 of the reaction product in varying substrate concentrations. If an IFE occurs, we expect that a given
263 substrate concentration will affect the fluorescence of all product concentrations in a similar way. The
264 linearity of the relationship between fluorescence and product concentration would be unaffected.
265 However, the IFE would increase with increasing substrate concentration which would result in a
266 reduction of the calibration slope.

267 Figure 3 shows the results obtained for APA (no IFE detected, Fig. 3a, c, e) and EPA (IFE detected,
268 Fig. 3b, d, f) with LS50 spectrofluorometer. Without IFE, the slope of those calibration curves is
269 independent from MUF-P concentration (slope = -6.77×10^{-6} , correlation coefficient $r = 0.236$, $df = 10$,
270 not significant, Fig. 3c) as MUF-P substrate does not affect fluorescence reading. The intercept is
271 linearly proportional to this concentration (slope = 0.25 , $r = 1.000$, $df = 10$, significant at 0.1%, Fig. 3e),
272 reflecting the natural fluorescence of the substrate. These results also show the necessity of using a blank
273 for each substrate concentration assayed. When an IFE occurred, the slope of the calibration curves
274 decreases with increasing LLMCA concentration (slope = -4.37×10^{-5} , $r = 0.742$, $df = 8$, significant at
275 5%, Fig. 3d): as exciting light is absorbed, it becomes limiting and the fluorochrome is not fully excited.
276 As expected, this effect is more pronounced at higher concentration of LLMCA, affecting calibration
277 curve slopes in a greater manner. For similar reasons, the intercept does not vary linearly at high LLMCA
278 concentrations (Fig. 3f).

279 Overall, no IFE was detected when assaying APA with all three tested instruments. An IFE was
280 detected for proteases assays performed using cuvettes (SFM25 and LS50) but not using FIA-SMF25.
281 The absence of IFE using FIA-SFM25, compared to the cuvette systems, might be due to both the sample
282 dilution by hydraulic system (as sample is diluted about 10 times by carrier fluid) and by the 10 times
283 shorter optic path, resulting in 100 times less substrate interfering with light flux. A greater overlap of
284 fluorescence spectra, as well as different samples dilution (see following discussion), might favor the
285 occurrence of IFE with the EPA assays compared to the APA assays.. Those results illustrate the fact
286 that this effect greatly depends on the type of substrate, concentrations and equipment used and should
287 be regularly tested for each assay protocol.



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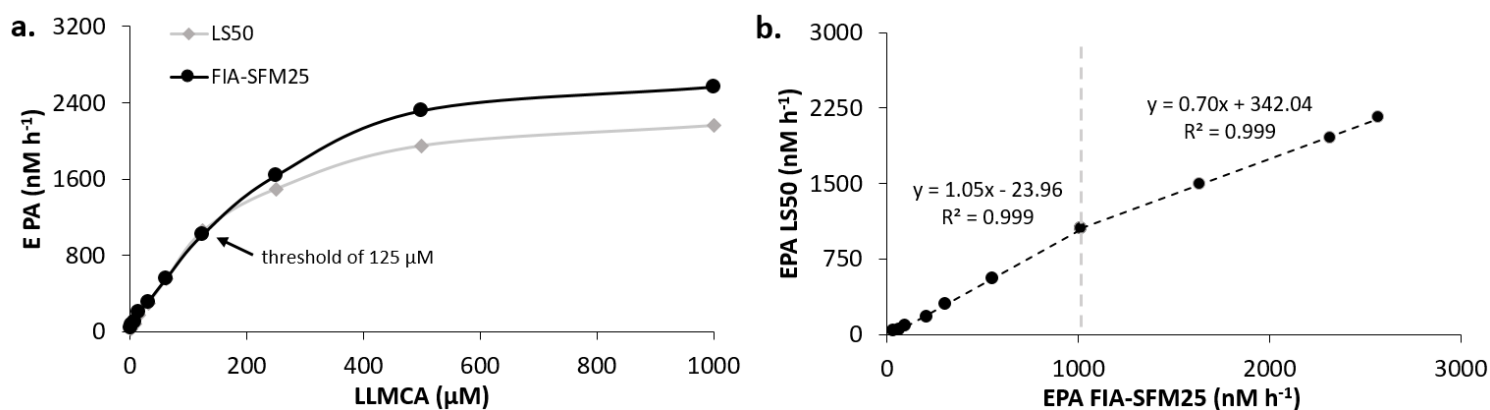
289 *Fig. 3. Detection of IFE using calibration curves of MUF (a, c, e) and MCA (b, d, f), with cuvette operated LS50*
 290 *spectrofluorometer. Calibration curves (a, b) were determined from solutions prepared in varying concentration of non-*
 291 *hydrolysed substrate (MUF-P, LLMCA). On panel a and b, only four calibration curves are shown to facilitate readability*
 292 *although 12 and 9 curves were respectively measured for APA and EPA. All calibrations are displayed on other panels.*
 293 *Calibration of MUF shows no IFE as the slope of each calibration is not dependent on MUF-P concentration (c) and intercept*
 294 *varies linearly with MUF-P concentration (e). Calibration of MCA exhibits an IFE as the slope of each calibration varies*
 295 *significantly with LLMCA concentration (d) and intercept does not vary linearly above 60 μM of LLMCA (f). r: correlation*
 296 *coefficient. Error bars (c, d, e, f) represent 95% confidence interval of the fitted parameters.*

297 *Consequences and correction of IFE*

298 To assess the consequences of the detected IFE on natural samples, we carried out a kinetic measurement
 299 of natural bacterial communities EPA. The samples were measured using both FIA-SFM25 and cuvette
 300 operated LS50 with pH correction.

301 With FIA-SFM25, unweighted hyperbolic regression gave a V_{\max} of $3386 \pm 198 \text{ nM h}^{-1}$ and a K_m
 302 of $275 \pm 40 \mu\text{M}$. When carried out with LS50 for which IFE occurs, V_{\max} only reached $2668 \pm 154 \text{ nM}$
 303 h^{-1} and K_m equalled $205 \pm 32 \mu\text{M}$, hence a respective difference of 21 and 26% on each parameter (Fig.
 304 4a). The correlation between the two measurements shows that, for the tested spectrofluorometers, the
 305 IFE appears at a LLMCA concentration of $125 \mu\text{M}$ (Fig. 4a), which roughly corresponds to a rate of
 306 1000 nM h^{-1} . Below this threshold, the two spectrofluorometers results correlate well (slope of 1.05, Fig.
 307 4b) whereas above, cuvette measurements yield lower activities (slope of 0.70, Fig. 4b).

308



309 Fig. 4. (a) Kinetics of natural bacterial communities EPA measured using FIA-SFM25 (black line) or with LS50 (using cuvette,
 310 grey line). (b) Correlation between EPA measured with LS50 (using cuvette) and using FIA-SFM25. Dashed line highlights
 311 the substrate concentration threshold.

312 This threshold value is close to the $150 \mu\text{M}$ self-quenching threshold observed by Saifuku et al.
 313 (1978) in the original method of LLMCA assay.

314 IFE can be avoided by using diluted solutions, shorter optic path, horizontal over vertical slits, a change
 315 in lamp geometry or in excitation/emission wavelengths (Valeur 2001; Eccleston et al. 2005; Fonin et
 316 al. 2014). However, it is not always possible to modify those parameters and this effect cannot always
 317 be avoided. In such cases, it should be corrected either experimentally or mathematically. If mathematic
 318 corrections are available, they can be quite complicated to implement (see Fonin et al. 2014 or Puchalski
 319 et al. 1991 for references). To experimentally correct the IFE in a simple way, each fluorescence value
 320 obtained with a given substrate concentration was converted into a concentration using the calibration
 321 curve of product prepared in the same given substrate concentration, instead of the usual calibration

322 curve prepared in Milli-Q water. For instance, the values measured when assaying EPA with 1000 μM
323 of LLMCA were converted using the MCA standards prepared in 1000 μM of LLMCA. The corrected
324 values with LS50 are: $V_{\text{max}} = 3267 \pm 183 \text{ nM h}^{-1}$ and $K_m = 288 \pm 40 \mu\text{M}$, hence a respective difference
325 of 4 and 5% on each parameter, which is within standard error inherent to fluorometric measurement
326 and model fitting.

327 *Importance of protocol and equipment used*

328 We would like to draw specific attention to the fact that IFE greatly depends on protocol as well as
329 equipment used.

330 The first methodological key point would be the preparation of calibration curves, which are
331 necessary to correctly assess and correct IFE. As this effect is dependent on dilution, calibration
332 solutions should be prepared in the exact same manner as the samples. In our protocol, all samples were
333 diluted 1.2 times by the preservative (formaldehyde or SDS), which was consequently also added to the
334 standards. Preservative is, to our knowledge, almost never amended in calibration solutions.

335 The second methodological key point concerns the pH adjustment when fluorescence is measured
336 using cuvette. Our samples were further diluted by buffered borate amendment (1.24 times for APA,
337 1.05 times for EPA) to adjust pH to 10, which maximizes MUF and MCA fluorescence and allows the
338 comparison between FIA and cuvette measurements. As such, IFE is minimized in our protocol. This is
339 especially true for APA, for which larger volumes of buffer were necessary to reach $\text{pH} = 10$. However,
340 in the literature, it is often unclear if pH adjustment is performed when using cuvettes or microplates. If
341 not, IFE could be much more severe.

342 Another methodological key point would be the choice of excitation wavelength. For instance,
343 Christie et al. (1978) noted that an excitation wavelength of 350 nm was necessary to overcome an IFE
344 that was observed at 320 nm (maximum excitation wavelength) with MUF- α -D-mannopyranoside and
345 MUF- α -D-glucopyranoside with a spectrofluorometer using cuvette. This is especially important for
346 MCA as an excitation wavelength of 360 nm (maximum of excitation) will favor IFE due to a bigger
347 absorbance by LLMCA (Fig. 1).

348 The occurrence of IFE strongly depends on the equipment used. For instance, Briciu-burghina et al.
349 (2015) encountered a pronounced IFE in 1 cm optic path cuvette using MUF- β -D-glucuronide substrate,

350 whose fluorescence spectra resemble MUF-P spectra, for which we found no IFE. This could be due,
351 for example, to differences in cell geometry, slits orientation, fluorescence observation angle, lamp
352 power and age (Valeur 2001; Eccleston et al. 2005; Fonin et al. 2014).

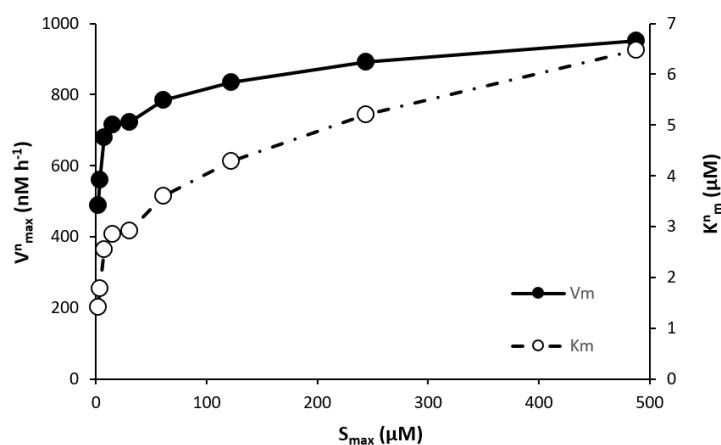
353 Microplate spectrofluorometer have been increasingly used as they allow high throughput assays
354 and limit the substrate volume, although the small incubation volume and long incubation time may lead
355 to bottle wall-effects (higher enzyme and substrate adsorption). IFE seems less likely to occur with those
356 set-ups thanks to front-face optics and small optic path. However, studies using 96-wells microplates
357 have shown its occurrence with MUF derivative substrates. For instance, Pinto et al. (2015) reported an
358 IFE using MUF-galactoside, without specifying the importance of the effect. Marathe et al. (2013) found
359 an IFE using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid, which was an important
360 interference in their assay. Even though microplates are less prone to IFE, its occurrence should be
361 checked in every spectrofluorometer.

362 **Influence of substrate concentration range on kinetic parameters determination**

363 To illustrate the effect of substrate concentration range on kinetic parameters determination, we
364 conducted APA measurements on purified enzymes (*E. coli* and shrimp), phytoplankton cultures (*A.*
365 *minutum* and *T. weissflogii*) and natural bacterial populations. Several fittings were consecutively made
366 on each kinetics to determine V_{\max}^n and K_m^n each time eliminating the highest concentration of substrate
367 (S_{\max}). This process aims to illustrate the impact of substrate concentration range by decreasing the
368 maximum concentration used from $S_{\max} = 500 \mu\text{M}$ ($n = 11$ points) to $S_{\max} = 2 \mu\text{M}$ ($n = 3$). The resulting
369 variation of parameters is due to fitting artifact. It should be noted that only an apparent K_m can be
370 determined, as natural substrates might be present in the medium, competing with the substrate analog.

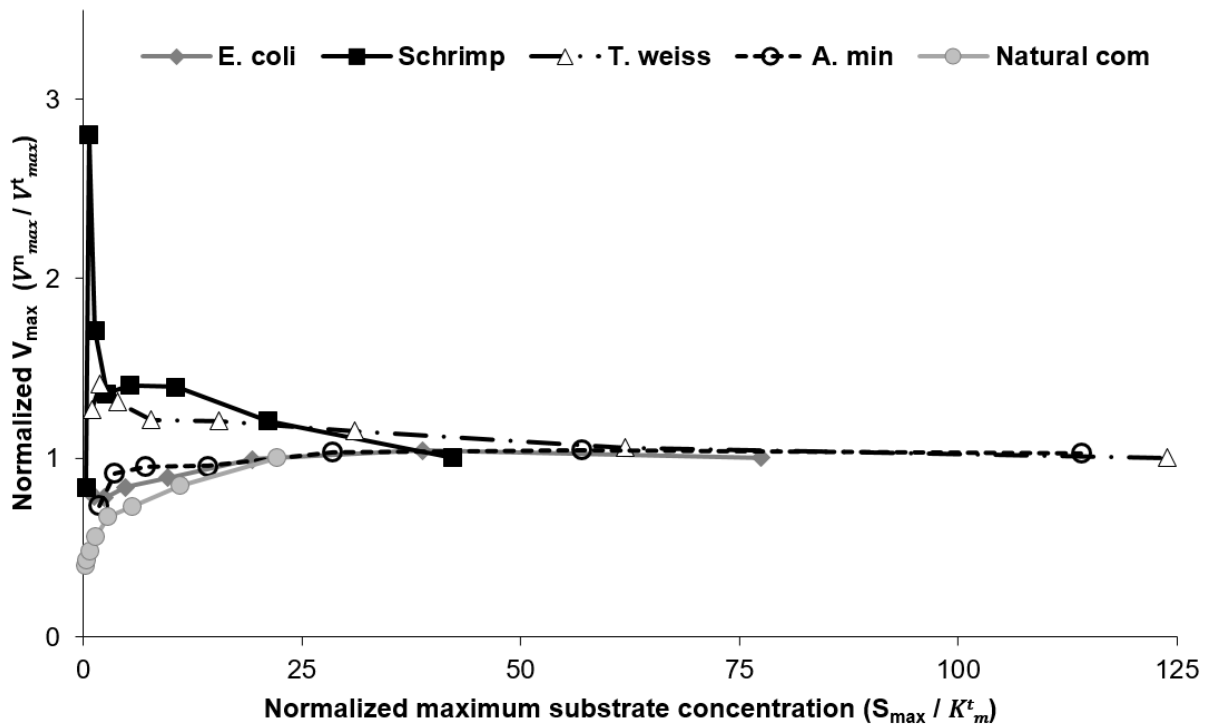
371 Figure 5 shows the results obtained for the purified *E. coli* APA. Both V_{\max}^n and K_m increase
372 (respectively + 61% and + 157%) when S_{\max} increases from $2 \mu\text{M}$ to $60 \mu\text{M}$. Both parameters then tend
373 to plateau at higher S_{\max} , reaching what can be considered as their “true” value when $S_{\max} = 500 \mu\text{M}$
374 ($V_{\max}^t = 952 \text{ nM h}^{-1}$ and $K_m^t = 6.5 \mu\text{M}$), even though K_m^n still seems to vary consequently. The threshold
375 concentration of $60 \mu\text{M}$ roughly corresponds to $10 K_m^t$. This value is generally recommended as a

376 saturating concentration even though it deviates from true enzyme saturation since “only” 91% of active
377 sites are occupied, as dealing with concentrated solutions can be complicated (Bisswanger 2014) .



378
379 *Fig. 5. Evolution of kinetic parameters (V_{max}^n , K_m^n) of purified E. coli alkaline phosphatase over the maximum substrate*
380 *concentration (S_{max}) used. Several fittings were done on the same assay data, iteratively removing the highest substrate*
381 *concentration. Correlation coefficient indicated significant results for $n > 4$ (at 0.1%).*

382 In order to compare the results of consecutive fittings performed on different enzymes, kinetic
383 parameters (V_{max}^n and K_m^n) were normalized by the “true” kinetic parameters (V_{max}^t and K_m^t) determined
384 using the largest substrate concentration range ($S_{max} = 500 \mu\text{M}$). Figure 6 presents the normalized
385 velocities (V_{max}^n / V_{max}^t) plotted against the normalized maximum substrate concentration (S_{max} / K_m^t),
386 which allows to observe the impact of S_{max} independently from the enzyme’s affinity. It clearly shows
387 the same pattern as before: V_{max}^n greatly varies with low S_{max} , then tends to stabilize around the correct
388 parameter estimation at higher S_{max} ($> 25 K_m^t$). Normalized K_m follows the exact same pattern (data not
389 shown).



390

391 Fig. 6. Normalized V_{max} (V^n_{max} / V^t_{max}) over normalized maximum substrate concentration (S_{max} / K^t_m) of APA assays conducted
 392 on various samples (purified enzymes from shrimp and *E. coli*, cultures of *T. weissflogii* and *A. minutum* and natural bacterial
 393 communities). Each dot represent a non-linear fitting done on the same kinetic experiment data, but iteratively reducing S_{max} ,
 394 which is represented normalized on the X-axis. Velocities are normalized by the “true” V^n_{max} obtained at $S_{max} = 500 \mu M$ and
 395 substrate concentrations are normalized by the “true” K^t_m obtained at $S_{max} = 500 \mu M$.

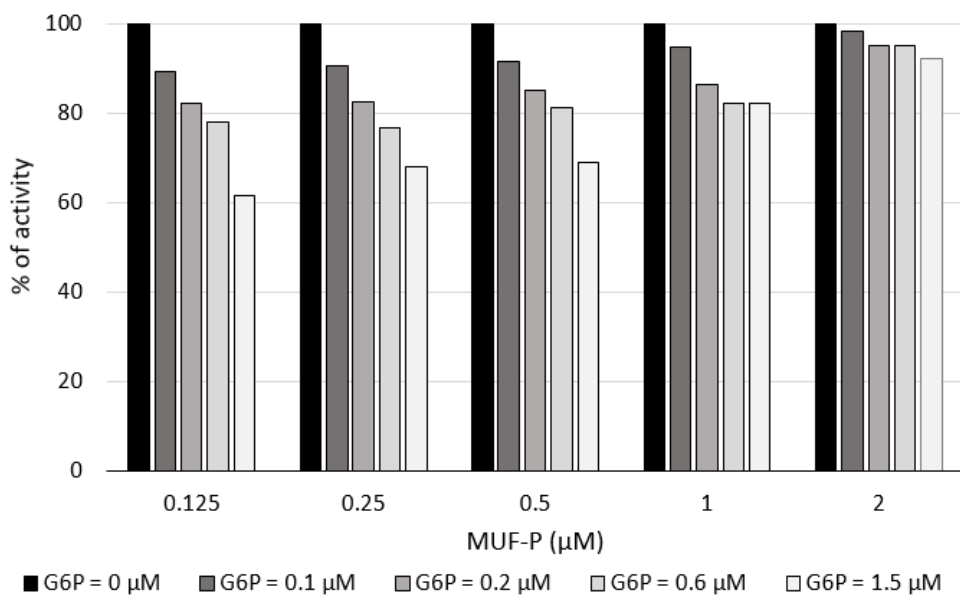
396 This experiment illustrates the consequences of the use of insufficient S_{max} to determine kinetic
 397 parameters. Both low and high substrate concentration are necessary to correctly fit all parts of the
 398 Michaelis-Menten equation (0 and 1st order, Chróst 1990). The width of the range depends on the
 399 enzyme affinity, since enzymes with low K_m are easily saturated and the curve’s plateau will be reached
 400 even at low S_{max} . High affinity enzymes (low K_m) are usually thought to occur in oligotrophic
 401 environments since they are supposed to process substrates at very low concentrations (Chróst 1991;
 402 Rath et al. 1993). However, even in those environments, S_{max} should be carefully chosen. For instance,
 403 all five studies determining enzymatic parameters in oligotrophic environments listed in Table 1
 404 contained at least one experiment where K_m was equal or greater than S_{max} . In contrast, enzymes are
 405 considered to have lower substrate affinity (high K_m) in eutrophic environments (Chróst 1991; Rath et
 406 al. 1993), so substrate ranges should be even wider. Similar results were obtained for EPA (not shown).

407 As the variation of kinetic parameters results from a fitting artifact, it should logically occur for all
408 enzymatic kinetics.

409 **Effect of natural substrates competition at trace concentration of analog substrate**

410 Substrate concentration used for single point assays varied from 0.1 μM to 250 μM for APA assayed
411 with MUF-P (see Table 1 for references) and from 2.5 to 1000 μM for EPA assayed with LLMCA (see
412 Table 2 for references). Low substrate concentrations are usually used in oligotrophic conditions, in
413 order to mimic mean environmental conditions. However, in such assays, naturally occurring substrates
414 might compete with the analog substrate. To illustrate this effect, we performed APA assays on natural
415 seawater microbial communities using trace substrate concentrations of MUF-P (0.125 – 2 μM , similar
416 to those in studied literature) amended with different concentrations of G6P (0.1 – 1.5 μM). G6P was
417 arbitrarily chosen to mimic dissolved organic phosphorus (DOP), the natural substrate of phosphatases.
418 Concentrations were chosen to be representative of DOP concentration in natural environments: it
419 usually ranges from 0 to 0.2 μM in open ocean surface waters (Ridal and Moore 1992; Karl and
420 Björkman 2015) while coastal and estuarine waters often contain more than 0.25 μM DOP (Karl and
421 Björkman 2015; Labry et al. 2016), with values sometimes exceeding 2 μM (Rinker and Powell 2006).

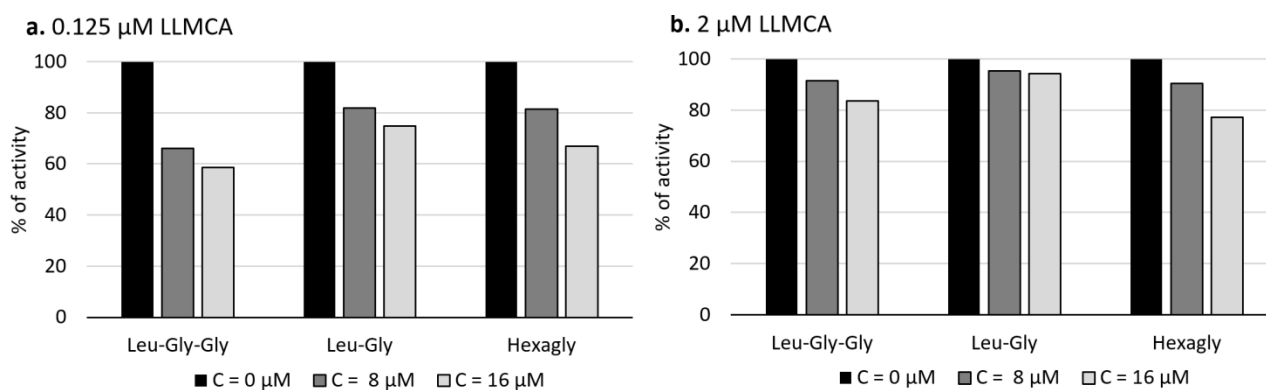
422 Activities were measured as slopes of fluorescence over time (in mV min^{-1}) and expressed as
423 percentage of maximum activity, measured without inhibitors, for each substrate concentration (Fig. 7).
424 With a MUF-P concentration of 0.125 μM , G6P concentrations of 0.1, 0.2, 0.6 and 1.5 μM decrease
425 MUF-P hydrolysis by 11, 18, 22 and 38%, respectively. Inhibition patterns are quite similar with 0.25,
426 and 0.5 μM MUF-P, while those effects are lower at 1 μM and tend to disappear at 2 μM MUF-P (1-8%
427 reduction). As measurement are carried out in seawater, naturally occurring DOP is also competing for
428 the enzymes' active sites, so the actual effect of G6P might be underestimated. However, results clearly
429 show that competition depends on the ratio of competitor to MUF-P and is not susceptible to happen at
430 saturating concentration of MUF-P.



431
 432 Fig. 7. APA measured using trace MUF-P concentrations with various concentrations of G6P, a natural non-fluorescent
 433 competitor. Results are given in $mV\ min^{-1}$ and normalized by the maximum activity obtained for each MUF-P concentration
 434 (without competitor).

435 Although G6P was used as a single competitor, samples often contain a mixture of compounds with
 436 their own affinity and inhibition constant, affecting enzymatic assays in different ways. The competitor's
 437 affinity for the enzyme (hence its effect on measurement) might especially vary for polymeric substances
 438 given their huge diversity (peptides or carbohydrates for example). We conducted similar tests on EPA,
 439 amending 8 and 16 μM Leu-Gly, Leu-Gly-Gly or Hexagly to 0.125 and 2 μM LLMCA. Although high,
 440 those competitor concentrations are representative of natural environments as combined dissolved
 441 amino acids may vary from 0.2 to 8 μM in oligotrophic to coastal waters (Keil and Kirchman 1991). As
 442 a result, 8 μM of Leu-Gly, Hexagly and Leu-Gly-Gly generated an inhibition of 18, 19 and 34%, while
 443 16 μM generated respectively 25, 33 and 41% of inhibition (Fig. 8a). Those results might be explained
 444 by the fact that measurements using peptidase substrates actually represent the concerted action of many
 445 distinct enzymes, as suggested by Steen et al. (2015), who performed competition experiments between
 446 12 amino acid-p-nitroanilide compounds and 3 fluorogenic substrates (LLMCA, L-arginine-MCA, L-
 447 proline-MCA). As expected, the use of higher concentration of LLMCA (2 μM) reduced the inhibition:
 448 8 μM of Leu-Gly, Hexagly and Leu-Gly-Gly generated 5, 10 and 8% inhibition respectively while 16

449 μM generated 6, 23 and 16% of inhibition respectively (Fig. 8b).



450

451 *Fig. 8 : EPA measured using 0.125 μM LLMCA (a) or 2 μM LLMCA (b), with various concentrations of Leu-Gly-Gly, Leu-*
452 *Gly or Hexagly, acting as competitors (C). Results are measured in mV min^{-1} and normalized by the maximum activity*
453 *obtained without competitor.*

454 Several other studies have shown the competition between natural and fluorogenic substrates when
455 assaying APA (see for instance Chróst 1990; Fernley and Walker 1967; Hoppe 1983), EPA (Hoppe
456 1983; Somville and Billen 1983; Christian and Karl 1998) or glycosidases (Hoppe 1983; Somville
457 1984), which confirms our results showing that natural substrates may significantly alter the measured
458 enzymatic activities at low analog substrate concentrations.

459 This issue arises from the legitimate need to measure in situ degradation rates. However, as natural
460 substrates compete with the fluorogenic analog, those measurements are unreliable and depend on
461 sample composition, which completely impedes inter-study comparison. It raises questions regarding
462 what we really are interested in quantifying when measuring enzyme activity rates. Single point assays
463 using a saturating substrate concentration ($> 10 K_m$) allow the determination of a potential degradation
464 rate (V_{max}). This can be considered as a determination of enzyme concentration (Billen 1991). When it
465 is normalized by the biomass or number of cells, it reflects the enzymatic equipment of the cells. Those
466 measurements are comparable across studies and allow, for instance, to study the regulation of enzyme
467 synthesis in response to environmental trophic conditions. In any case, fluorogenic substrate assays
468 should be interpreted cautiously in terms of real substrate utilization, keeping in mind that activities are
469 measured with respect to naturally occurring substrates (Billen 1991).

470 *Conclusion and recommendations*

471 The starting point of this study was the comparison of existing literature on enzymatic activity
472 measurement in aquatic environments. We noticed substantial divergences in existing methodologies,
473 the most notable one being the difference in substrate concentrations used for assays. Furthermore, the
474 occurrence of IFE, a fluorometric artifact, has never been considered despite being likely to occur,
475 especially at saturating concentrations of substrate. Those factors might alter results and therefore,
476 investigation of past articles and inter-study comparison should be carried out cautiously. In this study,
477 we tried to highlight several methodological key points of enzymatic assays, illustrating them with APA
478 and EPA, although results should apply to all fluorometric enzymatic assays.

479 (1) We would like to emphasize the possible occurrence of IFE, a fluorometric artifact that has been
480 completely ignored in environmental water studies until now, despite having the potential to
481 significantly affect measurements. In this study, it was not detected for APA assays but occurred
482 for EPA assays with the two cuvette based spectrofluorometers tested, at a LLMCA threshold
483 concentration of 125 μM . This effect can also occur with microplate readers, to a lesser extent.
484 IFE depends on sample dilution and the equipment used and can be corrected by using
485 appropriate calibration curves.

486 (2) Ideally, before any single substrate assay, a kinetic analysis should be performed to determine
487 the apparent K_m and to choose a saturating concentration. In practice, this step is laborious.
488 However, when such experiments are carried out, a wide substrate concentration range (up to
489 several hundred micromolar) should be used in order to reach the enzyme's active sites
490 saturation and correctly fit both first and zero-order part of enzyme reaction. The maximum
491 substrate concentration should be at least 10 K_m to ensure that most enzymes are sufficiently
492 saturated.

493 (3) Finally, when performing a single substrate assay, saturating substrate concentrations ($> 10 K_m$)
494 should be preferred, rather than trace concentrations mimicking natural conditions. Indeed, this
495 latter approach might not fulfill Michaelis-Menten conditions (i.e. excess substrate compared to

496 enzyme concentration) and may lead to higher uncertainties. Furthermore, it is highly dependent
497 on both natural substrate concentrations and composition, as natural substrate might compete
498 with the substrate analog for the enzyme active sites. Up to 34% and 38% inhibition was
499 observed in the present study inhibition tests with natural substrates for EPA and APA,
500 respectively.

501 All these methodological issues must be addressed in order to correctly measure enzymatic rates and
502 allow inter-study comparison.

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- 699

700 **Figure captions**

701 **Fig. 1.** Fig. 1. (A) Normalized fluorescence spectra of MUF-P (alkaline phosphatase substrate, dashed
702 lines) and MUF (product, solid lines) over emission (gray lines) or excitation wavelength (black lines).
703 Excitation spectra of MUF and MUF-P were obtained using a constant emission wavelength (460 nm)
704 while excitation wavelength varied. Emission spectra were obtained using a constant excitation
705 wavelength (364 nm) with variable emission wavelength. (B) Normalized fluorescence spectra of
706 LLMCA (protease substrate, dashed lines) and MCA (product, solid lines) over emission (gray lines) or
707 excitation wavelength (black lines). To obtain excitation spectra of MCA and LLMCA, emitted light
708 was respectively collected at a wavelength of 440 and 410 nm while excitation wavelength varied. To
709 obtain emission spectra, emitted light was collected over a range of wavelength while excitation
710 wavelength was set to 350 and 325 nm for MCA and LLMCA, respectively. em: emission, ex: excitation.
711 Vertical lines represent excitation and emission wavelengths used during enzymatic assays.

712 **Fig. 2.** Conceptual illustration of excitation IFE (also termed primary IFE) and emission IFE (also
713 termed secondary IFE) using a right angle geometry, one of the most common for cuvette system.

714 **Table 1.** Literature examples showing phosphatase activity assay conditions used in different
715 environments. All measurements were carried out using 4-Methylumbelliferyl phosphate (MUF-P)
716 substrate.

717 **Table 2.** Literature examples showing exoproteolytic activity assay conditions used in different
718 environments. All measurements were carried out using L-Leucine-7-amido-4-methylcoumarin
719 (LLMCA) substrate.

720 **Fig. 3.** Detection of IFE using calibration curves of MUF (a, c, e) and MCA (b, d, f), with cuvette
721 operated LS50 spectrofluorometer. Calibration curves (a, b) were determined from solutions prepared
722 in varying concentration of non-hydrolysed substrate (MUF-P, LLMCA). On panel a and b, only four
723 calibration curves are shown to facilitate readability although 12 and 9 curves were respectively
724 measured for APA and EPA. All calibrations are displayed on other panels. Calibration of MUF shows
725 no IFE as the slope of each calibration is not dependent on MUF-P concentration (c) and intercept varies

726 linearly with MUF-P concentration (e). Calibration of MCA exhibits an IFE as the slope of each
727 calibration varies significantly with LLMCA concentration (d) and intercept does not vary linearly
728 above 60 μM of LLMCA (f). r : correlation coefficient.

729 **Fig. 4.** (a) Kinetics of natural bacterial communities EPA measured using FIA-SFM25 (black line) or
730 with LS50 (using cuvette, grey line). (b) Correlation between EPA measured with LS50 (using cuvette)
731 and using FIA-SFM25. Dashed line highlights the substrate concentration threshold.

732 **Fig. 5.** Evolution of kinetic parameters (V_{max}^n , K_m^n) of purified *E. coli* alkaline phosphatase over the
733 maximum substrate concentration (S_{max}) used. Several fittings were done on the same assay data,
734 iteratively removing the highest substrate concentration. Correlation coefficient indicated significant
735 results for $n > 4$ (at 0.1%).

736 **Fig. 6.** Normalized V_{max} ($V_{\text{max}}^n / V_{\text{max}}^t$) over normalized maximum substrate concentration (S_{max} / K_m^t)
737 of APA assays conducted on various samples (purified enzymes from shrimp and *E. coli*, cultures of *T.*
738 *weissflogii* and *A. minutum* and natural bacterial communities). Each dot represent a non-linear fitting
739 done on the same kinetic experiment data, but iteratively reducing S_{max} , which is represented normalized
740 on the X-axis. Velocities are normalized by the “true” V_{max}^t obtained at $S_{\text{max}} = 500 \mu\text{M}$ and substrate
741 concentrations are normalized by the “true” K_m^t obtained at $S_{\text{max}} = 500 \mu\text{M}$.

742 **Fig. 7.** APA measured using trace MUF-P concentrations with various concentrations of G6P, a natural
743 non-fluorescent competitor. Results are given in mV min^{-1} and normalized by the maximum activity
744 obtained for each MUF-P concentration (without competitor).

745 **Fig. 8.** EPA measured using 0.125 μM LLMCA (a) or 2 μM LLMCA (b), with various concentrations
746 of Leu-Gly-Gly, Leu-Gly or Hexagly, acting as competitors (C). Results are measured in mV min^{-1} and
747 normalized by the maximum activity obtained without competitor.

748

749 ***Acknowledgments***

750 This work was supported by the French National Program for Coastal Environment (EC2CO) and by
751 the ISblue project, Interdisciplinary graduate school for the blue planet (ANR-17-EURE-0015), co-
752 funded by a grant from the French government under the program "Investissements d'Avenir". We wish
753 to thank M. Latimier for the preparation of phytoplankton cultures.