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The determination of total dissolved free primary amines in seawater: Critical factors, optimized procedure and artefact correction

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The paper reviews the methods described in the literature for the determination of total dissolved free primary amines (TDFPA) by fluorescence. A wide set of reaction conditions can be found, but they rely on few experiments for their validation. Among fluorogenic compounds, *o*-phthaldialdehyde (OPA) is more sensitive than fluorescamine and was thus examined here. However, the use of mercaptoethanol (ME) in the reaction (as an additional derivatization compound) is able to generate unreliable results, in particular when standardization relies on glycine. We suggest replacing ME with 3-mercaptopropionic acid (MPA) which induces more stable and comparable fluorescence among amine compounds. A systematic study was therefore undertaken to define reagent concentrations and pH effects on the reaction rates for a variety of primary amines with particular focus on amino acids. The reaction rate is increased by increasing OPA concentration and pH, but slowed by excess MPA. Ammonium interference is influenced by several factors, but spectral investigation showed that the choice of conditions can drastically reduce it. The magnitudes of natural and OPA-induced background fluorescence signals have been assessed in various mediums and it is shown that their contribution to the signal amounts to a large fraction, when not most, of the measured fluorescence. A segmented flow method is proposed with a protocol for adequate correction of biases.

Keywords: Total dissolved free primary amines; Seawater; Determination; Fluorescence

THE DETERMINATION OF TOTAL DISSOLVED FREE PRIMARY AMINES IN SEAWATER: CRITICAL FACTORS, OPTIMIZED PROCEDURE AND ARTEFACT CORRECTION

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ABSTRACT

The paper reviews the methods described in the literature for the determination of total dissolved free primary amines (TDFPA) by fluorescence. A wide set of reaction conditions can be found, but they rely on few experiments for their validation. Among fluorogenic compounds, *o*-phthaldialdehyde (OPA) is more sensitive than fluorescamine and was thus examined here. However, the use of mercaptoethanol (ME) in the reaction (as an additional derivatization compound) is able to generate unreliable results, in particular when standardization relies on glycine. We suggest replacing ME with 3-mercaptopropionic acid (MPA) which induces more stable and comparable fluorescence among amine compounds. A systematic study was therefore undertaken to define reagent concentrations and pH effects on the reaction rates for a variety of primary amines with particular focus on amino acids. The reaction rate is increased by increasing OPA concentration and pH, but slowed by excess MPA. Ammonium interference is influenced by several factors, but spectral investigation showed that the choice of conditions can drastically reduce it. The magnitude of natural and OPA-induced background fluorescence signals have been assessed in various mediums and it is shown that their contribution to the signal amounts to a large fraction, when not most, of the measured fluorescence. A segmented flow method is proposed with a protocol for adequate correction of biases.

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1 INTRODUCTION

Naturally occurring dissolved free primary amines include several classes of compounds such as aliphatic amines, hexosamines and amino acids, resulting from metabolic processes of degradation, hydrolysis and excretion at various levels of the food chain. Although no data are available on the specific composition of the whole group of compounds, dissolved free amino acids (DFAA) have been widely studied. Determination of total dissolved free primary amines (TDFPA) can be operationally defined as the response of an amine-sensitive analytical method standardized against glycine. The only comparison of TDFPA and chromatographic analysis of DFAA made on a wide set of samples (Delmas et al., 1990) led to a relatively good correlation between these two pools, indicating that variations of TDFPA can mostly be attributed to DFAA. Daumas (1976) stated that amino acids are involved in abiotic (metal complexation) and biotic (excretion, assimilation, enzymatic regulation) processes (Fuhrman, 1987; Jørgensen, 1987; Crottereau and Delmas, 1999). While their main source in seawater is primary production, they account for only a minor fraction of dissolved organic matter (DOM) because of their high turnover rate which makes them an important source of carbon and nitrogen for heterotrophic bacteria (Fuhrman and Bell, 1985), and to a lesser extent for phytoplankton.

In order to measure the concentrations of tens to hundreds of nanomoles of total DFAA in seawater generally reported (Fuhrman and Bell, 1985), initial methods required a pre-concentration step (e.g. Siegel and Degens, 1966) followed by conventional liquid chromatography with colorimetric detection. Progress in high performance liquid chromatography (HPLC) coupled with fluorometric detection has enabled seawater to be analyzed without the need for pre-concentration (Lindroth and

Mopper, 1979). While some studies require the determination of individual DFAA, the total concentration is often the pertinent parameter when considering the overall class of DFAA, for instance as a source of nitrogen and carbon for microplankton. Additionally, a fairly constant distribution of individual amino acids has been commonly observed in marine and estuarine waters (Laanbroek et al., 1985). Glycine, serine, alanine, threonine and ornithine generally account for one half to two thirds of total dissolved free amino acids (Macko and Green, 1982; Garrasi et al., 1979; Jørgensen, 1987). Hence, methods have been described for the determination of total DFAA by adding the fluorogenic reagents directly to the seawater sample and measuring the resulting fluorescence. Unfortunately, the reagents used to detect amino acids are not specific to the amino acid function (-(NH_2)COOH), but to the amine group alone (-NH_2). Therefore, in contrast to chromatographic methods, which separate amino acids from each other and from the matrix, the response of 'total' methods includes that of amines other than amino acids and should be called total dissolved free primary amines (TDFPA; North, 1975; Jørgensen, 1979; Delmas et al., 1990). Josefsson et al. (1977) mentioned that they should be regarded as procedures that give a quick picture of which samples should be further studied by chromatography and Dawson and Liebezeit (1981), stating that the values obtained by 'total' methods are somewhat ill-defined as to the nature of compound analyzed, called the result 'ORS, i.e. *o*-phthaldialdehyde reactive substances'. No attempt was made to check the comparability of methods, but all published values have invariably been expressed in glycine-equivalents, which gives the data apparent consistency.

Several fluorogenic compounds are available for amines and amino acids (e.g., Aminuddin and Miller, 1995). OPA, in the presence of various SH-groups, has been well documented and does not reveal any major problem in our experience. Since the work by Roth (1971) describing the reaction of OPA, 2-mercaptoethanol (ME) and amino acids, reaction conditions were all developed for HPLC analysis (see Mopper and Dawson, 1986, for example). In contrast,no precise optimization experiments could be found in the literature for TDFPA determination. Our experience in this field suggested that re-examining the procedure could improve the data's comparability. The aim of this paper is therefore to review the literature on OPA–amino acid derivatization in order to clarify the analytical background of TDFPA methods, with special attention given to biases and artefacts. Experiments were performed to identify critical factors for satisfactory reaction conditions and signal detection, with regard to sensitivity and interfering sources of fluorescence. Accordingly, a segmented flow analysis (SFA) of TDFPA is proposed, with the appropriate controls and corrections.

2 LITERATURE REVIEW

2.1 General features

The few papers which have dealt with TDFAA methodology in seawater have been closely derived from that of North (1975), a manual method relying on fluorescamine derivatization, and that of Josefsson et al. (1977), a continuous flow automated method using *o*-phthaldialdehyde (OPA).

Fluorescamine was used by Jørgensen (1979), Jørgensen et al. (1980) and Sellner and Nealley (1997). According to North (1975), the sample is buffered at pH = 9 with sodium borate then fluorescamine is added as an acetone solution. Immediate and thorough mixing is necessary because fluorescamine degrades rapidly in water into a non-fluorescent compound. The time period of fluorescence recording was not specified, but, according to Fourche et al. (1976), it is not very important since the fluorochromes formed are stable.

OPA, like fluorescamine, has been widely used in chromatographic analysis. Fourche et al. (1976) showed that OPA derivatives, while relatively unstable, produced maximal fluorescence intensities 5 to 10-fold higher than those produced by fluorescamine. This large increase in sensitivity reduces the signal/noise ratio as well as the relative background fluorescence of natural DOM. In addition, Fourche et al. (1976) showed that the relative intensities of most OPA-amino acid derivatives were in a narrower range than those obtained with fluorescamine. OPA methods also require thiols for the formation of the fluorochromes, the most widely used of which is 2-mercaptoethanol, which has a very unpleasant odor. Despite some disadvantages, OPA became popular as a reagent for total DFAA

determination and several versions of Josefsson et al.'s (1977) method have been described. These include a manual method by Parsons et al. (1984), and automated versions like segmented flow analysis (SFA) by Dawson and Liebezeit (1981, 1983) and Liebezeit and Behrends (1999) and flow injection analysis (FIA) by Petty et al. (1982) and Delmas et al. (1990). A FIA-like method including molecular weight column separation of OPA-positive compounds was described by Nagel and Liemann (1987).

2.2 Reaction conditions and relative response of amino acid with OPA

Reaction conditions of amino acid derivatization with OPA significantly differ according to authors (Table 1): pH = 9-10.5, [OPA] = 0.25-33 mmol/L, [ME] = 1.1-47 mmol/L and [ME]/[OPA] ~ 1-10. The reaction time is as low as 6 s in FIA and up to 10 min in manual analysis.

A pH of 9.5-10.5 is generally given as optimal for the reaction of amino acids with OPA, but fluorescence intensity is nearly constant in the range of 8-11 for most amino acids (Roth, 1971). Increased intensities obtained in seawater at pH = 11.5 by Liebezeit and Dawson (1981) were subsequently attributed to the enhancing effect of calcium at pH > 9 (Mopper and Dawson, 1986).

Roth (1971) mentioned that fluorescence intensity peaks in 5 min, followed by a slow decrease, allowing routine determination up to 25 min after mixing the sample and reagents. Fourche et al. (1976) indicated that the maximum was reached in 1-2 min, Lindroth and Mopper (1979) in 2-3 min and Liebezeit and Dawson (1981) in 10-20 min. These differences had not been discussed in the literature. The optimal reaction time is governed by the rates of the fluorochromes' formation and decomposition. The high instability of some amino acids, such as glycine, lysine and ornithine, is sometimes mentioned (Fourche et al., 1976; Lindroth and Mopper, 1979).

Using an excess of 200 times the reagent was suggested to overcome the reagent being consumed by high concentrations of non-amino acid amines in physiological fluids (Lindroth and Mopper, 1979), but an overly large OPA excess was reported to catalyze the instability of OPA-derivatized amino acids (Mopper and Dawson, 1986). According to Roth (1971), fluorescence intensity sharply increases with increasing OPA concentrations, then is nearly constant (slow decrease) in the range from 2.4 to 14 mmol/L OPA in the reaction medium ([OPA]/[AA] = 150 to 900; values calculated from Roth's data). For ME, Roth mentioned little effect in the range of 0.003-1.8 mmol/L ME ([ME]/[AA] = 11 to 5700), but fluorescence decreases when [ME] > 28 mmol/L (Dorresteijn et al., 1996). The high [ME]/[OPA] molar ratios used in various methods (up to 10; see table 1), despite equimolar reaction stoichiometry, seem unwarranted (unless it is to overcome ME decay). While the OPA-ME pair was widely used, better derivative stability can be obtained by replacing ME by other thiols (Mopper and Dawson, 1986) such as 3-mercapto-propionic acid (MPA) or N-acetyl-L-cysteine (Molnár-Perl and Bozor, 1998).

The fluorescence intensity resulting from derivatization of individual amino acids with OPA has been determined by several authors. Relative intensities have been normalized to glycine in order to obtain a fair comparison of these data (figure 1). This points out the low or insignificant response of some amino acids (½Cys, Cys, Lys, Pro, Orn) which actually account for a small part of total DFAA in seawater, with the exception of ornithine. We also noted a large dispersion of the relative responses recorded by each of the authors, along with a marked discrepancy for a given amino acid from one author to another.

2.3 Fluorescence due to compounds other than amino acids

Ammonium is acknowledged to be the compound which interferes most frequently. Using fluorescamine, North (1975) mentioned no detectable ammonium interference, but Jørgensen (1979; 1980) measured about 1 % of the ammonium concentration as glycine equivalents. Using OPA, the ammonium derivative is relatively more fluorescent and its interference (with respect to glycine) was reported as 3 % (Delmas et al., 1990), 5 % (Lindroth and Mopper, 1979; Dawson and Liebezeit, 1981; Liebezeit and Behrends, 1999), 7 % (Tada et al., 1998), 6-19 % (Petty et al., 1982) and 18 % (Josefsson et al., 1977; Parsons et al., 1984).

Table 1. Reaction conditions for OPA-amino acid derivatization in TDFPA determination methods (Ref. 1-9) and other applications (Ref. 10-12). Abbreviations: RM, reaction medium; ns, not specified.

Characteristic[b]	Reference[a]											
	Ref. 1-6	Ref. 2	Ref. 3	Ref. 4	Ref. 5	Ref. 6	Ref. 7	Ref. 8	Ref. 9	Ref. 10	Ref. 11	Ref. 12
Method type	manual	manual	SFA LR[c]	SFA HR[c]	SFA	SFA	FIA	FIA	(FIA) NSFA[d]	manual	HPLC	HPLC
Sample/reagent	1/1	1/1	1/0.4	1/2.7	1/0.5	1/0.5	-	-	-	1/30	1/5[e]	1/0.1
[Borate] in RM	400	190	105	285	140	140	< 420	< 395	315	47	300	ns
pH of buffer	10.5	9.5	9.5	9.5	10.5	10.5	10.4	10.0	10.6	9.0	9.5	ns
pH in fresh water[f]	10.35	9.4	9.35	9.45	10.3	10.3	-	-	10.6	9.0	9.45	ns
pH in seawater[f]	10.15	9.2	9.05	9.3	10.05	10.05	-	-	10.6	-	9.4	9.4[g]
[OPA] in RM	0.7	1.8	0.5	1.3	0.25	0.13	< 5.0	< 4.9	ns	1.2	33	6.7
[ME] in RM	7	17	1	2.6	2.4	4.8	< 29	< 28	30	1.1	47	6.4
[ME]/[OPA]	10	9.5	2	2	9.5	38	6	6	-	0.9	1.5	0.95
Reaction time, min	10	2	1.5	1.5	2 (1.1[h])	1.1[h]	0.2[h]	0.1[h]	6	1-2	2	10
Excitation, nm	340	342	320-400	320-400	340	340	360	340	350	340	330	360
Emission, nm	455	452	400-700	400-700	455	420	> 550	450	455	455	> 418	400-700

a: Ref.1: Dawson and Liebezeit, 1983; Ref. 2: Parsons et al., 1984; Ref. 3 and 4: Josefsson et al., 1977; Ref. 5: Dawson and Liebezeit, 1981, 1983; Ref. 6: Liebezeit and Behrends, 1999; Ref. 7: Petty et al., 1982; Ref. 8: Delmas et al., 1990; Ref. 9: Nagel and Liemann 1987; Ref. 10: Fourche et al., 1976; Ref. 11: Lindroth and Mopper, 1979; Ref. 12: Liebezeit and Dawson, 1981.

b: the concentration unit of compounds in square brackets is 'mmol/L'.

c: LR, low range (0.05-2.5 µmol/L); HR, high range (2-15 µmol/L).

d: after injection, samples pass through a separation column, thus removing the matrix; the next step corresponds to non segmented flow analysis (NSFA).

e: condition for standards in fresh water (valid for data in table 2 of Ref. 11); unclear conditions for seawater ("stronger reagent", sample/reagent = 10/1).

f: stated in Ref. 12 and experimentally determined in this work on the basis of reaction conditions provided for the others.

g: pH valid for data in table 2 of Ref. 12 (maximum fluorescence found at pH 11.5).

h: computed from the manifold characteristics provided in the corresponding papers.

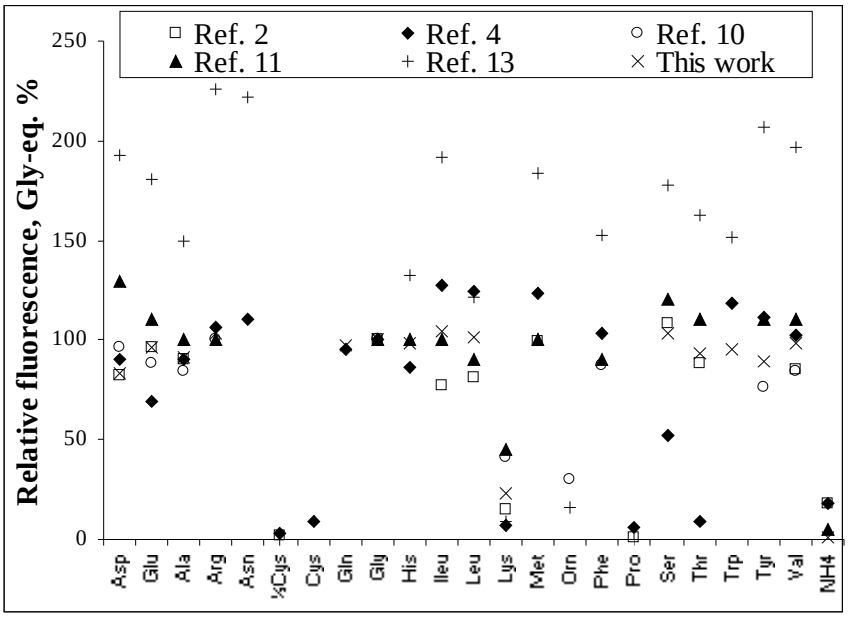

Figure 1. Comparison of glycine-normalized relative fluorescence intensities of amino acids and ammonium from literature data and this work. References and reaction conditions as in Table 1: Ref. 2: Parsons et al., 1984; Ref. 4: Josefsson et al., 1977; Ref. 10: Fourche et al., 1976; Ref. 11: Lindroth and Mopper, 1979. Ref. 13 is Roth (table 2, 1971), reaction conditions: pH = 9, [OPA] = 2.5 µmol/L and [ME] = 5.9 µmol/L. For amino acid abbreviations, see section 3.2 'Reagents'.

Data is scarce for OPA-induced fluorescence from non-amino acid amine compounds. Josefsson et al. (1977) checked 5 aminosugars and 8 dipeptides which respectively produced responses up to 65 % and 32 % that of glycine.

Interference of the natural background fluorescence (NBF) of the sample, essentially due to the humic-like dissolved organic matter, is measured by replacing the fluorogenic reagent with distilled water (Josefsson et al., 1977) or a reagent without OPA (North, 1975). Because of the higher fluorescence intensities obtained with OPA than with fluorescamine, NBF interference is minimized with the former, so that Dawson and Liebezeit (1983) and Liebezeit and Behrends (1999) indicated that this signal could be ignored (given value: ~ 0.01 µmol/L glycine-equivalent).

The last type of interference could be inferred from data provided by North (1975). They observed that half of the fluorescamine-positive material was not removed in uptake experiments, whereas glycine disappeared within a few dozen minutes. Similarly, comparisons of TDFPA to the sum of chromatographied amino acids (Jørgensen et al, 1980; Delmas et al, 1990) also indicated a high TDFPA intercept when extrapolating the sum of AA to zero. This remaining fluorescence can be attributed to the reaction of the reagent with amino groups of the dissolved organic matter (other than TDFPA), but none of the published procedures suggested that this artefact should be corrected for. It is referred to hereafter as OPA-induced residual fluorescence (OIRF).

2.4 Background for TDFPA determination

The accurate determination of TDFPA implies that i) the compounds of interest and the standard produce nearly equal signal intensities (so that mixtures with equal total amounts but different compositions do not produce different concentration values), ii) all types of interference are measured and corrected (ammonium, NBF, OIRF). In addition to undesired fluorescence responses, the TDFPA signal might be altered by variations in the matrix composition, i.e. mainly the salinity of the sample when analytical conditions are not optimized.

3 MATERIAL AND METHODS

3.1 Experimental conditions

For kinetic studies ('manual experiments'), 52 mL of the amino acid standard solutions (10 µmol/L) were put into an opaque vial, stirred with a magnetic stirrer and drawn (1 mL/min) through a capillary Teflon tube to the filter fluorometer cell using a peristaltic pump. Once 2 mL had been pumped to establish the baseline, the flow was stopped during a few seconds to allow 5 mL of the reagent to be added and mixed, then resumed. The signal was recorded for up to 30 min. Blank fluorescence from reagent and seawater was negligible compared to the DFAA signal intensity.

Bran+Luebbe (Technicon) Autoanalyzer 3 material was mostly used for the segmented flow manifold (Figure 2). The sampler IV was a modified version accommodating large volume vials to minimize contamination (Kérouel and Aminot, 1987).

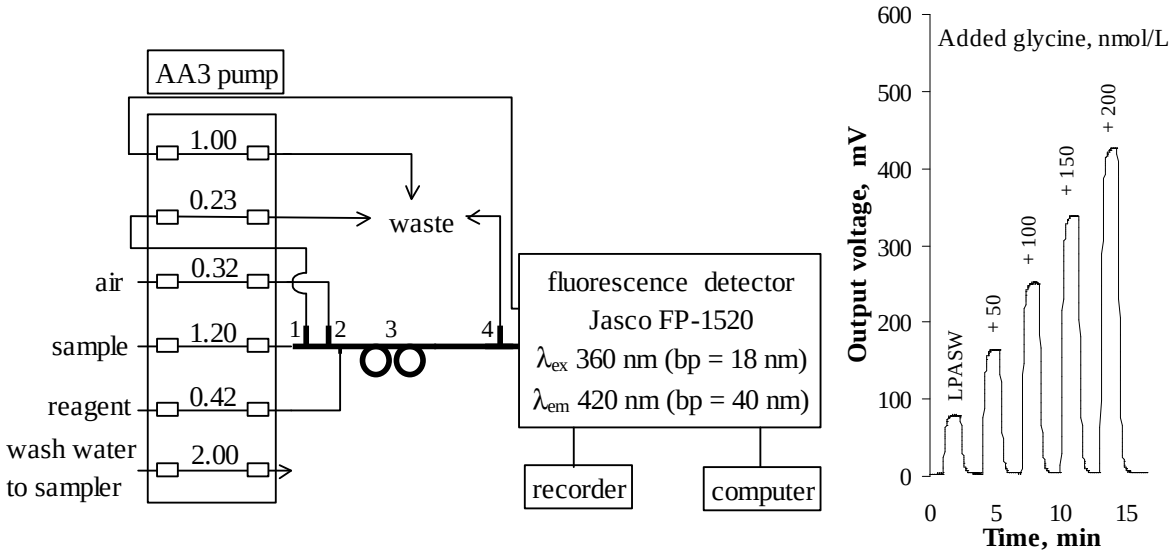

Figure 2. Automated manifold for the determination of TDFPA in seawater. Part # 1: low dead-volume debubbler (e.g. Alpkem A 303-0103-00); part # 2: 3-way injector (B+L 116-0489-01); part # 3: 20 turn delay coil; part # 4: A2 debubbler (B+L 116-0200-02). Right: Recordings of a typical calibration in low primary amine seawater (LPASW). Detector gain/attenuation, 100/32; output voltage resolution, 1 mV. Regression: Net voltage (mV) = 1.724 × C(nmol/L) + 75.8 (R2 = 0.99999).

Until recently, a filter fluorometer LDC Fluoromonitor III from Laboratory Data Control was used for field studies, according to Josefsson et al. (1977). It was equipped with a 360 nm Black Fluorescence lamp, a 370 nm excitation filter, a long pass (418-700 nm) emission filter. The excitation filter band-pass of about 20 nm led to a bell shape wavelength range of 340-400 nm. Similarly, emission started at about 400 nm. The flow cell (30 µl) was adapted for use with SFA (low-pressure) by replacing metallic capillary tubes connected to the cell against 0.8 mm i.d. Teflon tubes. The time constant for output signal was set at 1 second. Subsequently, a Model FP-1520 Jasco spectrofluorometer replaced the filter detector. The flow cell (16 µl) was fitted with low pressure connectors and fed through a 0.8 mm i.d. Teflon tube. The excitation and emission band-pass were 18 nm (fixed) and 40 nm (selected). Wavelength precision was about ± 1 nm. A recorder was connected to the 10 mV output of the fluorometer and a personal computer with in-house software helped measure peak height and compute concentrations.

Gloves were always worn when handling reagents, samples and materials and during on-board sampling. All glassware was combusted at 480 °C and protected from ambient air and dust (wrapped

in aluminum foil and kept in boxes) until use. Samples were collected, from the sampling bottle, into 1 liter glass vials with on-line screening through a 10 µm polypropylene membrane held in a Millipore Swinnex device. These samples were cooled, out of light, until subsequent (within one hour) vacuum-filtration ($\Delta P < 0.15$ bar) through Whatman GF/F glass-fiber filters held in a glass filter-holder. Filtered water was collected in a glass ampoule fitted directly under the holder. The 50 ml glass sample vials were filled from the ampoule via its Teflon tap and immediately placed into a freezer (-25 °C) until analysis.

Salinity was measured using a Guildline Portasal 8410 salinometer, DOC according to Aminot and Kérouel (2004) and ammonium according to Kérouel and Aminot (1997).

3.2 Reagents

Conditions for the manual experiments were based on those described by Josefsson et al. (1977) for the low concentration range (see Table 1), but with a less concentrated buffer. Two thiols were tested, the widely used ME and MPA, and the same volume of either thiol was added to the reagent. The 'number of drops' of ME given by Josefsson et al. (1977) was converted into volume after the delivery of a Pasteur pipette was measured as being ~ 0.013 ml per drop. Aqueous solutions were prepared with high quality demineralized water (Milli-Q system). Boric acid, NaOH and absolute ethanol were of analytical grade from various producers. OPA was obtained from Sigma (ref. P1378), 2-mercaptoethanol from Fluka (ref. 63690) and 3-mercapto-propionic acid from Sigma (ref. M6750). All chemicals were used as purchased, but we avoid using aged OPA crystals (insoluble particles). The OPA-ethanol solution and the thiols were delivered using positive displacement pipettes.

Reagent for manual experiments: 2 mL of the OPA stock solution (40 g/L in absolute ethanol) and 80 µL of ME or MPA were added in 100 mL of borate buffer (0.4 mol/L H_3BO_3 + NaOH, pH = 9.5). The reagent was left standing a few hours before use. Then 5 ml of reagent were added to 50 ml of sample. Concentrations in the reaction medium were (mmol/L): [borate] = 37, [OPA] = 0.51, [MPA] = 0.8, [ME] = 1.0, pH ~ 8.9.

For the automated segmented flow analysis the buffer is prepared separately, as follows, in order to be used as reagent for the determination of the natural background fluorescence. To prepare 1 liter of buffer, 7 g of H_3BO_3 and 3.2 g (or 40 ml of 2 mol/L) NaOH are dissolved in demineralized water (resulting pH ≅ 9.65). The mixed fluorogenic reagent is prepared by adding 600 µL of OPA solution (40 g/L in ethanol), 20 µL MPA and 50 µl Brij 35 per 100 mL of this buffer. The fluorogenic reagent is prepared daily and used without delay. Concentrations in the reaction medium are (mmol/L): [borate] = 34, [OPA] = 0.54, [MPA] = 0.69.

The selection of the amino acids and other amine compounds tested in the experiments relied mainly on their relative abundance in seawater as stated in the literature. The conventional amino acid abbreviations will be used and amino acids listed in alphabetic order: alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cystine (Cys), cysteine (½Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ileu), leucine (Leu), lysine (Lys), methionine (Met), ornithine (Orn), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophane (Trp), tyrosine (Tyr), valine (Val). The following other amine compounds were checked: mono-, di- and tri-methylamine (MMA, DMA, TMA), mono-, di- and tri-ethylamine (MEA, DEA, TEA), glucosamine, galactosamine, glycyl-alanine, alanyl-glycine, glycyl-leucine, bovine serum albumin, ovalbumin, bovine pancreas trypsine. Amino acids were obtained from various producers, amines from Fluka and peptides and proteins from Sigma.

Aged seawater used for experiments was collected at the end of summer near the Ifremer pier, at high tide with immediate screening through a 50 µm plankton net. After storage in light in a polyethylene carboy for several months, it was virtually nutrient- and primary amine-depleted when used.

4 RESULTS

4.1 Kinetics studies

The rate of OPA-amine derivatization in seawater was checked using a selection of 10 amino acids, monomethylamine (MMA) and ammonium, which are among the most abundant amino compounds in seawater. Subsequent investigations indicated that the pH ~ 8.9 in our experiments was slightly lower than the optimal one but it allowed better discrimination among amino acids.

The results are shown in figure 3. Three groups of compounds differ from each other, whatever the thiol, with roughly comparable derivatization rates. They are in decreasing rate order: Gly > (Ala, His, Leu, Orn, Ser, Try, MMA) > (Asp, Thr, Val, NH_4). There is no apparent relationship between derivatization rates and the nature of radicals or end-groups. Maximum intensity, independent of the derivatization rate, is reached in less than 1 min for Gly, ~ 2 min for the second group and ~ 5 min for the third group. Fluorescence intensity then slowly decreases with ME, while it is more stable or continues to increase slowly with MPA (e.g. Gly, His, MMA). MPA remarkably enhances the stability and the fluorescence intensity of the Gly and MMA derivatives. Unfortunately, ornithine produces low intensity with both thiols.

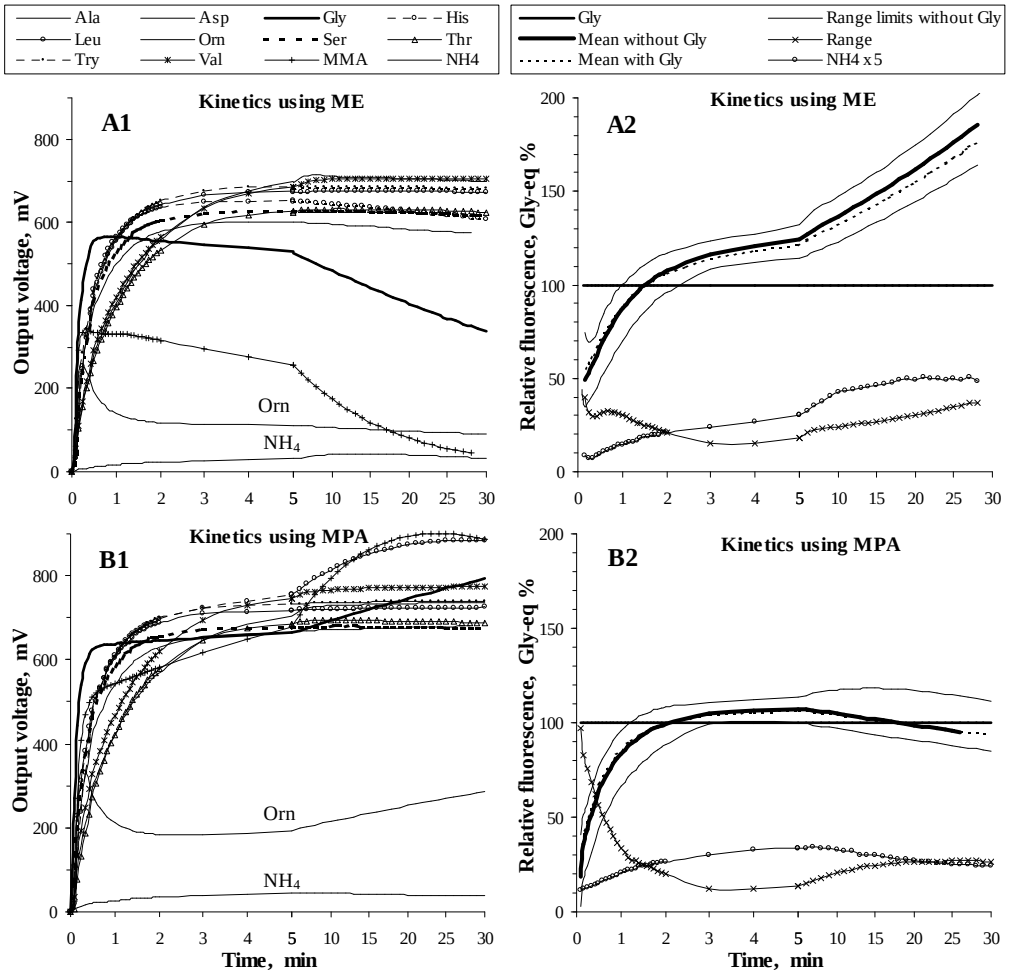

Figure 3. Kinetics of OPA-derivative formation with amino acids, MMA and ammonium, using two thiols ME (A1 and A2 graphs) and MPA (B1 and B2 graphs). A1 and B1: behavior of individual compounds with ME and MPA, respectively. A2 and B2: summary of the response of amino acids and ammonium (Orn and MMA excluded) relative to glycine with ME and MPA, respectively. Note the time scale change after 5 min.

The particular behavior of glycine must be examined: its derivatization rate is the highest, but, when ME is used, its maximum fluorescence intensity is lower than for most other amino acids and its derivative rapidly decomposes. Figure 3 (graphs A2 and B2) illustrates the relative responses in glycine equivalents (Gly-eq), i.e. when glycine is selected as the standard for calibration of the method. This highlights the fact that reaction times below ~ 2 min (in the present conditions) will underestimate the total concentration (either with ME or MPA), while longer reaction times will overestimate the total concentration if ME is used, but not if MPA is used.

So this study indicates that some published reaction times (Table 1) may be too short and therefore lead to a large range of relative fluorescence intensities among amino acids. Since MPA produces more comparable fluorescence intensities over a broader range of reaction times, it was the thiol selected for subsequent work (it is also worth mentioning that MPA is much less malodorous than ME).

4.2 Effect of pH and reagent concentrations

Glycine, histidine and serine (10 µmol/L) were selected as test compounds for their typical behavior shown by the previous tests. Ammonium was also checked. The conditions were similar to those described in the previous section for the OPA/MPA reaction, except when otherwise specified.

Kinetics were monitored at pH values (in the reaction medium) of 9.0, 9.5 and 10.0, in demineralized water, demineralized water spiked with Ca^{2+} at seawater concentration ($CaCl_2$, 10.5 mmol/L) and natural seawater (S = 35) aged in the laboratory. High pH values (> 10) were not tested since the risk of precipitation of calcium and magnesium salts (Pai et al., 2001) makes them inappropriate for marine waters. To prevent potential contamination from pH electrodes, preliminary tests allowed the pH of the buffer to be adjusted to make the reaction medium pH correspond to the expected value within ± 0.03 pH unit.

The results (figure 4) show that the glycine derivatization (rate and fluorescence intensity) was almost unaffected by pH in any medium. Histidine derivatization was hardly affected within the first minutes, but the subsequent intensity drift is minimized by an increase in pH. Serine derivatization is slightly slowed at pH = 9.0 with respect to higher pH values (9.5 and 10) at which the results are comparable. The overall effects of pH on the derivatization of amino acids were markedly lower around a pH = 9.5 than a pH of about 9.0, and in seawater compared to fresh water. The rate of ammonium derivatization was comparable at pH = 9.0 and pH = 9.5 (pH = 10 not checked), but the intensity increased by 12-14 % from 9.0 to 9.5. Unlike the amino acids tested, the fluorescence intensity of the ammonium derivative was lower in seawater with respect to fresh water.

The results when Ca^{2+} was added to demineralized water are not plotted in figure 4 since no difference (± 2 %) was found with pure demineralized water. The enhancing effect of Ca^{2+} on fluorescence intensities at pH > 9 (Mopper and Dawson, 1986) was not confirmed here.

The pH experiments indicate that a pH value of ~ 9.5 in the reaction medium is favorable for obtaining a restricted range of responses for DFAAs over a broad range of reaction times. Higher values would require excessive pH or borate concentrations in the reagent, with the undesirable risk of precipitation when mixed with the sample. A pH value around 9.2-9.3 is a compromise that provides satisfactory reaction rate and low ammonium interference.

Borate is the common buffering substance used for OPA-amino group derivatization (Fiorino et al., 1989; Dorresteijn et al., 1996; Molnar-Perl and Bozor, 1998). Replacing it was not considered here since it has proven to be satisfactory for reactions requiring slightly alkaline conditions in seawater, however its buffering strength was checked. Buffers were prepared by adding NaOH to 0.1 to 0.4 mol/L boric acid to obtain a pH = 9.5. When the buffers were mixed with seawater (salinity S = 35), the pH of the mixtures decreased nearly linearly as a function of the proportion of seawater in the mixture down to 9.0 for about 80 % of seawater. It should be mentioned that the initial borate concentration had only a minor effect on the final pH of the reaction medium (± 0.03 pH unit). However, tests of the responses of amino acids and ammonium at various borate concentrations (with the same pH) indicated a significant enhancing effect of borate on the response of ammonium with

respect to glycine. The ammonium response increased linearly from 1.4 to 2.7 % between 36 and 175 mmol/L borate, while the amino acid responses were nearly constant. When various reaction times were investigated, the borate effect was attributed to a magnification of the ammonium fluorescence intensity rather than an acceleration in the reaction rate. We also suspect that high borate concentrations amplify the baseline drift in the automated method.

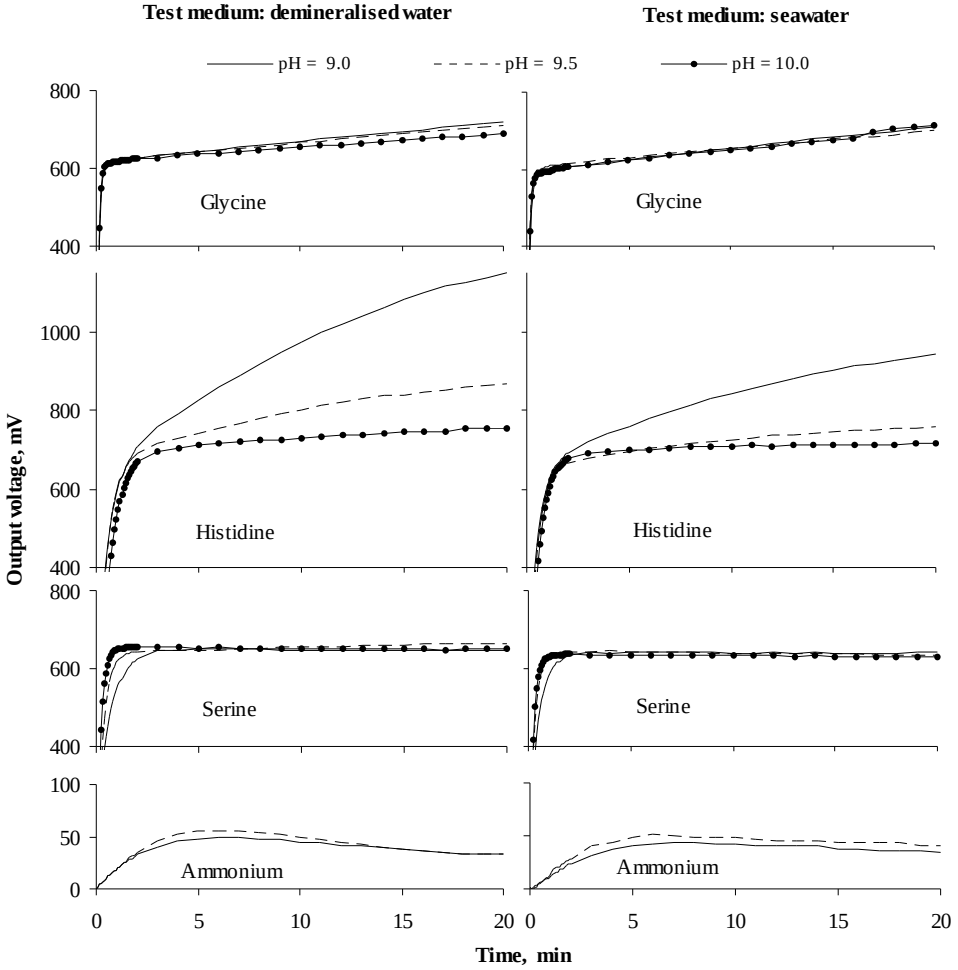

Figure 4. Kinetics of derivatization of glycine, histidine, serine and ammonium with the OPA-MPA combination at pH 9.0, 9.5 and 10.0, in demineralized and sea water.

The concentrations of OPA and AMP were separately tested. While keeping the initial concentration for one compound, the other compound was tested at half and twice its initial value, which resulted in the following combinations: i) [OPA] = 0.5 mmol/L with [MPA] = 0.4 and 1.6 mmol/L, ii) [MPA] = 0.8 mmol/L with [OPA] = 0.25 and 1 mmol/L. The pH of 8.9 provided easy comparison with previous experiments and magnified differences by slowing reaction rates. In the tested range, the reaction rate is a direct function of the OPA concentration (Figure 5), but maximum intensities are similar, except for glycine after the first 2-3 min. This may point out a secondary reaction concurrent to the main derivatization process. The combined effect of the OPA concentrations and pH was checked on the derivatization of serine in seawater. As shown in figure 5, the reaction rate is almost doubled at pH = 9.5, compared to pH = 8.9.

Within the tested concentration range, MPA has a minor effect on the reaction rate (results not shown), but high concentrations tend to slow the reaction rate. This was verified using the automated manifold (reaction time of 2.2 min) with serine (the most sensitive to MPA concentration). Only 60 % of the initial signal was obtained with 7 mmol/L MPA in the reaction medium and no detectable fluorescence with 42 mmol/L MPA.

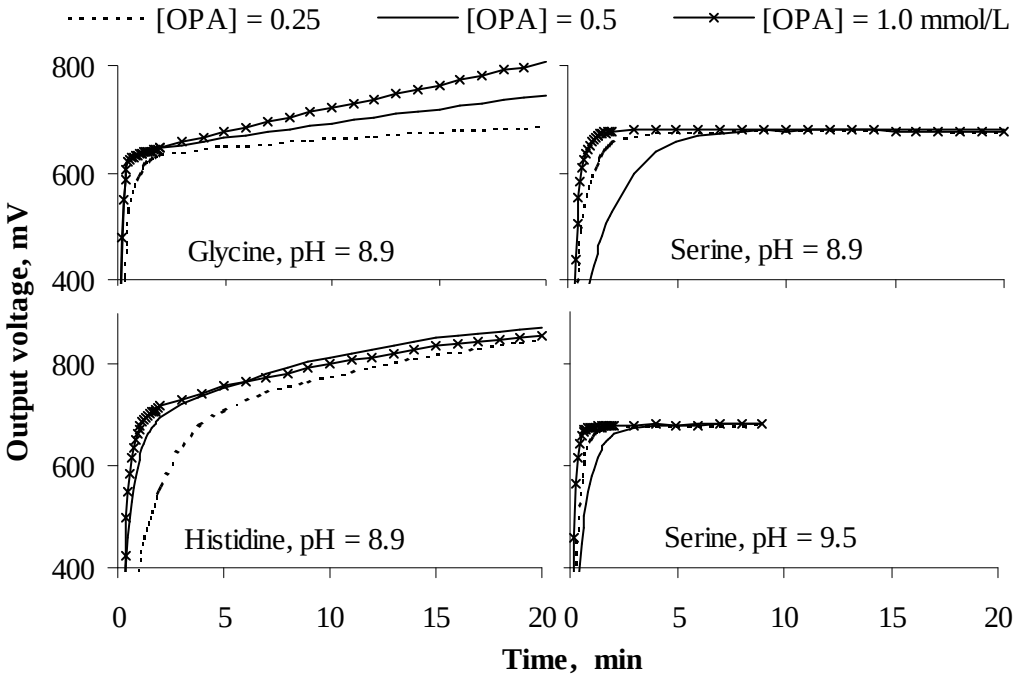

Figure 5. Effect of variable OPA concentrations on the rate of OPA-amino acid derivative formation ([MPA] = 0.8 mmol/L).

In these experiments with 10 µmol/L amino acid in natural seawater, the OPA molar excess was 25, 50 and 100 times the DFAA concentration. However, no loss of recovery was observed, even with the lowest OPA/DFAA molar ratio, contrary to the results of Lindroth and Mopper (1979). However, since high ammonium concentrations may be encountered in some waters, the potential reagent consumption by this compound was checked in the range of 0-100 µmol/L in aged seawater, with and without addition of 1 µmol/L glycine. Using the same reagent concentrations as in the initial kinetics studies, an OPA/NH_4 ratio as low as 8 was obtained in the reaction medium. Both the linear response for ammonium (no loss of recovery) and the 100 ± 0.5 % recovery for glycine up to 100 µmol/L NH_4 indicated that reaction conditions are not altered by ammonium in that range.

It was concluded that the starting OPA concentration of ~ 0.5 mmol/L OPA in the reaction medium did not need to be increased provided the pH was not lower than 9.1-9.2. Increasing the OPA concentration would also generate an undesirable drift in the maximum intensity of glycine if reaction times exceeding 2-3 min are required (in manual determination, for example). For MPA, the starting concentration (0.8 mmol/L) was reduced to ~ 0.69 mmol/L in the optimized procedure for operational convenience (pipette delivery).

4.3 Spectral investigation

Excitation and emission spectra of OPA/MPA amino compounds derivatives were investigated. The Jasco spectrofluorometer was coupled with the automated manifold in order to record all spectra at the same fixed reaction time (2.2 min). Since no significant pH effect was found in the range of 8.9-9.5 on Gly, Ser and ammonium spectra, reaction conditions were, for convenience's sake, kept identical to those used for the kinetics studies. Preliminary tests with a few compounds (Cys, Gly, His, Orn, Ser, Try, NH_4) confirmed the statement of Fourche et al. (1976) that, unlike the fluorescence induced by other compounds, OPA-induced fluorescence is poorly influenced by the molecular structure of the amino acids. Under the given conditions, excitation and emission maxima were found at 331-333 nm and 453-458 nm respectively for the six tested amino acids, values close to the expected 340/455 nm pair (Roth, 1971; Fourche et al., 1976). We noted that i) the range of fluorescence intensities was slightly dependent on the excitation and emission wavelengths, ii) for ammonium the emission maximum shifted to 461 nm. Extended experiments were therefore

undertaken with 10 amino acids (Ala, Asp, Glu, Gly, His, Leu, Ser, Thr, Try, Val) and ammonium, using 360 nm and 420 nm as excitation and emission wavelengths (figure 6). Despite being 20-35 nm away from optimal wavelengths, the corresponding excitation and emission maxima are not shifted by more than about 5 nm. Using this pair of wavelengths, the sensitivity for amino acids decreased, as expected, to only 20-25 % of that obtained under optimal conditions. However, thanks to the method's high sensitivity, this minor disadvantage is balanced out by the considerable reduction in ammonium interference. Indeed, the relative response of ammonium regularly decreases as a function of the emission wavelength from 570 nm to 410 nm, with a compromise value of ~ 1.5 % at 420 nm.

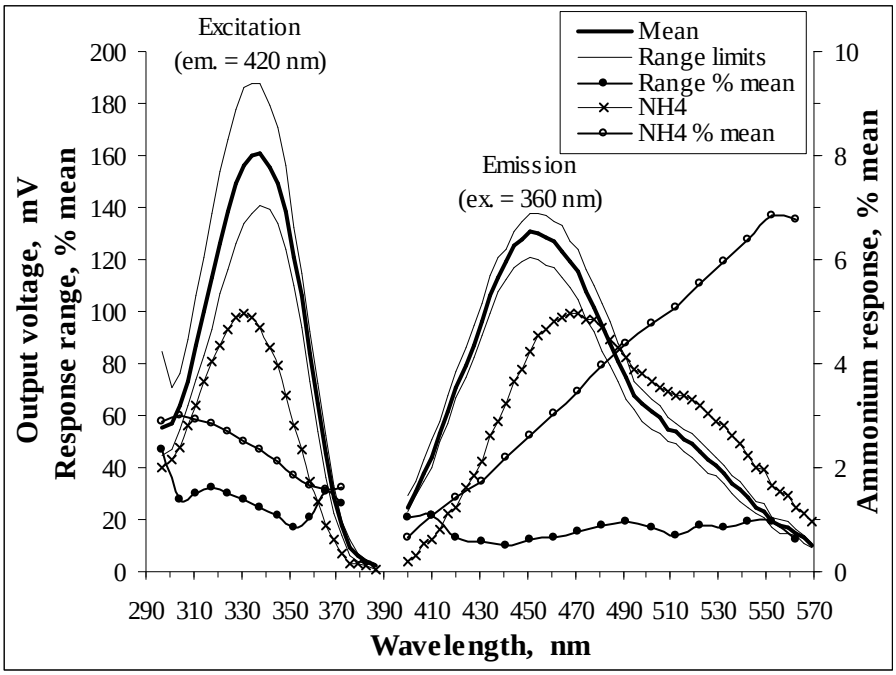

Figure 6. Excitation (λ_{em} = 420 nm) and emission (λ_{ex} = 360 nm) spectra (both band-pass: 18 nm) of OPA/MPA derivatives for a selection of amino acids (Ala, Asp, Glu, Gly, His, Leu, Ser, Thr, Try, Val) and for ammonium (at a concentration 25 times that of amino acids). Intensities were corrected for the seawater total blank signal.

The excitation and emission spectra of amines, aminosugars and peptides (monomethylamine, monoethylamine, glucosamine, galactosamine, glycyl-alanine, alanyl-glycine, glycyl-leucine) were found to be very close to those of the average amino acid spectrum within the spectrofluorometer's accuracy of ± 1-2 nm. Unlike for ammonium, a significant reduction in the relative response of these compounds could not be expected from a particular selection of the detection wavelengths.

In the literature, working wavelengths were essentially defined by the characteristics of the available filter for a type of fluorometer. Because of the shifted spectrum of ammonium towards higher wavelengths, interference of that compound will be increased if the measuring emission wavelength is increased or the band-pass enlarged. For instance, the use of a 550 nm cut-off emission filter (Petty et al., 1982) is far from optimal for the analysis of samples containing ammonium.

4.4 Magnitude of the natural background fluorescence

The natural background fluorescence (NBF) is produced by the medium components, apart from the fluorogenic reaction. After total fluorescence has been recorded with the complete reagent, the samples are re-analyzed using the borate buffer instead of the mixed OPA reagent. The output signal is converted in TDFPA-equivalent by comparison with the TDFPA standard for the same sample series previously analyzed. Various data have been compiled in order to assess the magnitude of NBF. The

automated method described in section 3 ‘Material and methods’ was used for these measurements with the LDC filter fluorometer. All the corrections described in this work were applied to compute net TDFPA concentrations. The data come from the Bay of Seine, a eutrophic area off the French coast of the English Channel, and from the continental shelf of the western French coast (Bay of Biscay, North-east Atlantic), including the Loire river estuary.

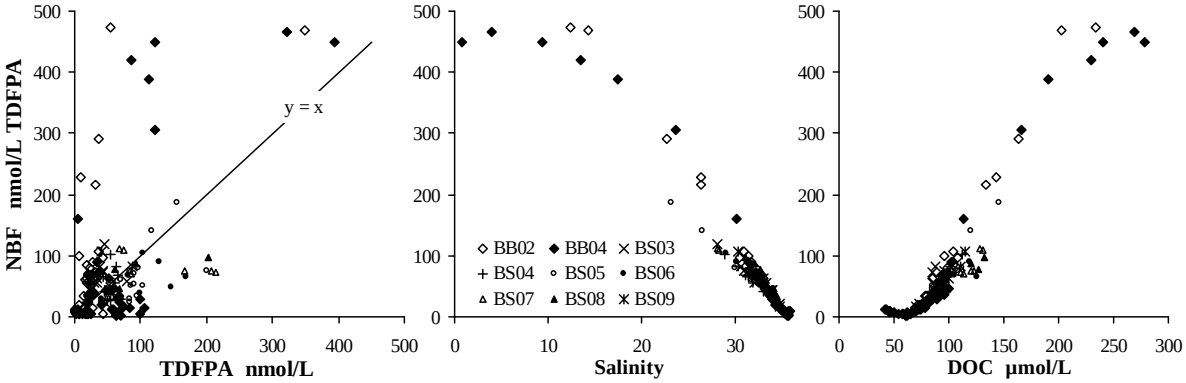

Figure 7. Left graph: compared magnitudes of the natural background fluorescence (NBF) measured by the automated method (LDC detector) and expressed in TDFPA concentration. Center and right graphs: relationships between NBF and salinity or dissolved organic carbon (DOC). Key: BB = Bay of Biscay; BS = Bay of Seine; 02, …, 09 = month of sampling.

The NBF signal magnitude is generally comparable to that of TDFPA, and even exceeds it in many cases (figure 7, left graph). The close examination of NBF vs salinity (figure 7, center graph) indicates that the value of 10 nmol/L TDFPA-eq (Dawson and Liebezeit, 1983), is only encountered in offshore waters with salinity > 35.3. In coastal waters (30 < S < 35) the NBF signal is usually between 10 and 100 nmol/L TDFPA equivalents, while in estuaries it can reach ~ 500 nmol/L TDFPA -eq. Neglecting the NBF, as suggested by Dawson and Liebezeit (1983), can therefore lead to highly biased TDFPA values. These variations are clearly related to the DOM (figure 7, right graph). However, an inverse relationship was found for DOC < 60 µmol/L throughout a vertical profile in oceanic water (0-4000 m; station A2 in Aminot and Kérouel, 2004), i.e. NBF increased with depth. Recent coastal and estuarine data (Bay of Biscay, spring 2004) using the Jasco spectrofluorometer detector (settings as in figure 2) led to NBF values lower by 20-30 %, compared with the LDC values.

4.5 Residual fluorescence of non-labile dissolved organic matter

Series of filtered samples were incubated in the dark at room temperature to determine the degradation rate of TDFPA and the OPA-induced residual fluorescence (OIRF) after some time. Examples are shown in figure 8 for offshore and coastal waters from the Bay of Biscay (N-E Atlantic, French continental shelf). They show that degradation occurs within a few days, then the signal is nearly constant (very slow decrease) over months. These results agree with published DFAA turnover times of tens of minutes to 1-2 days (Ferguson and Sunda, 1984; Fuhrman, 1987). According to this, the remaining fluorescence after about one or two weeks is not attributed to TDFPA.

The magnitude of the OIRF signal is usually in the same order of magnitude or larger than the TDFPA signal (figure 9, left graph), especially in offshore waters. In particular, the absolute minimum OIRF is about 30 nmol/L TDFPA-equivalents in the deepest oceanic waters. As for NBF, OIRF increases as a function of salinity and DOC (figure 9, center and right graph, but with maximum values around 100 µmol/L. If no correction is applied for this fluorescence, it will bias TDFPA significantly. The OIRF values were comparable whatever detector is used (LDC or Jasco).

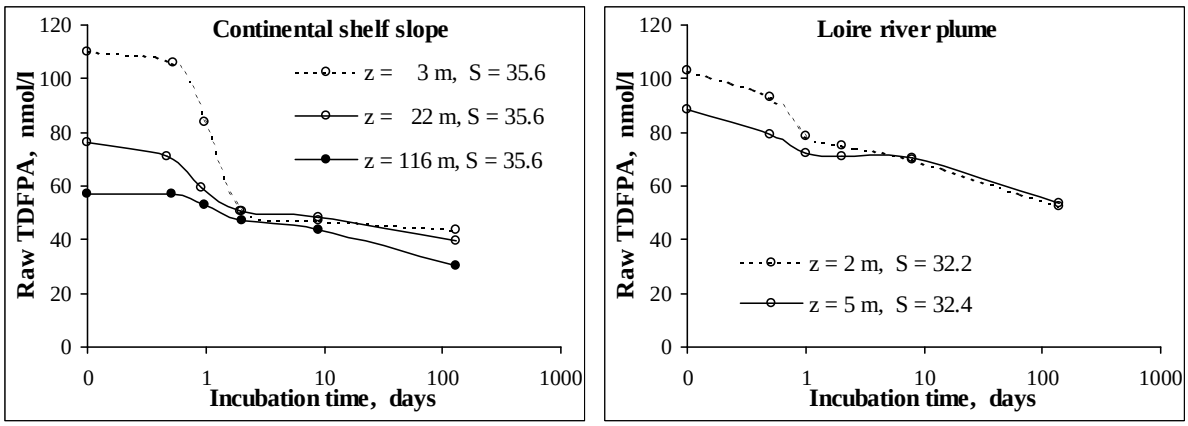

Figure 8. Change of 'raw' TDFAA concentrations of filtrated samples incubated at room temperature as a function of time. 'Raw' means that values were corrected for all but OIRF biases.

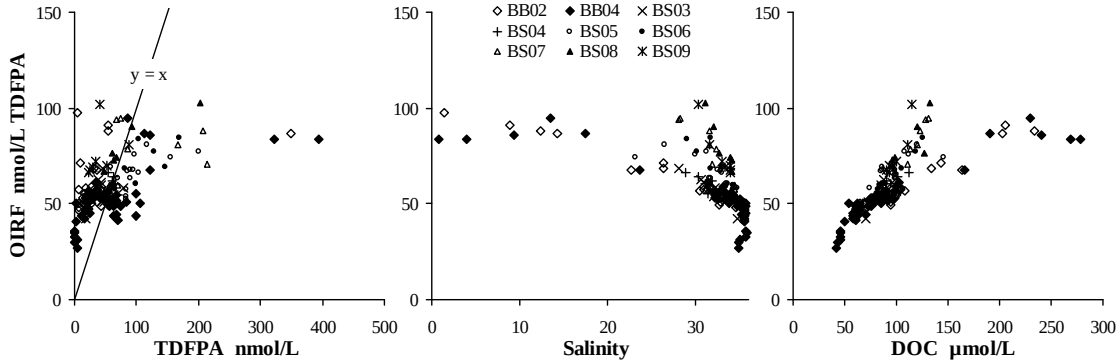

Figure 9. Left graph: compared magnitudes of the OPA-induced residual fluorescence (OIRF) measured by the automated method (LDC detector) and expressed in TDFPA concentration. Center and right graphs: relationships between OIRF and salinity or dissolved organic carbon (DOC). Key: BB = Bay of Biscay; BS = Bay of Seine; 02, …, 09 = month of sampling.

4.6 Selected analytical procedure

The automated method was designed to be applied to all types of marine waters, specially estuarine and coastal waters with variable salinity and pH as well as relatively high ammonium concentrations. The conditions given in section 3 'Material and methods' were selected to minimize the range of amino acid responses and reduce ammonium interference. Since reaction times are shorter in continuous-flow systems than in the corresponding manual method (Hansen and Grasshoff, 1983) medium reagent concentrations were not increased with regard to initial experiments, but the pH was raised to 9.2 (against 8.9) and the borate concentration maintained lower than in published methods. A 20 turn coil (reaction time: 2.2 min) was selected for work at 20-22 °C. A temperature test showed that the serine signal was stable (± 1 %) over the range 19-35 °C, but dropped by ~ 10 % at 10 °C. The manifold shown in figure 2, designed for the lowest range, includes a large sample delivery equipped with a debubbler to avoid subsequent flow perturbations. For high ranges requiring a small flow rate of sample, the debubbler can be removed, but the reagent and manifold should be adapted to maintain reaction concentrations and times. High quality demineralized water should be used for the baseline, otherwise, the TDFPA content of the baseline must be determined (see Josefsson et al., 1977). The baseline shift occurring when replacing pure water by the reagent does not exceed about 10 nmol/L TDFPA-equivalent.

A typical peak record is shown in figure 2 (20 samples per hour; sample/wash = 4/5). In the range 50-500 nmol/L, the intrinsic repeatability for successive peaks is ~ 1 mV output voltage (1 mV ≅ 0.6 nmol/L). The detection limit for the method itself need not be considered here, since any natural water will generate a background signal of at least 35 nmol/L TDFPA, even if no free amine is present. Linearity was confirmed up to 5 µmol/L.

The mean and standard deviation of the molar response of 14 amino acids (Ala, Arg, Asp, Gln, Glu, Gly, His, Ile, Leu, Ser, Thr, Trp, Tyr, Val) was 96 ± 6 % with respect to glycine. The robustness of the method was assessed by measuring the relative response of a reduced set of 9 amino acids (Ala, Asp, Gly, His, Leu, Ser, Thr, Trp, Val) under 12 different conditions, e.g. change of detector, excitation wavelength (350, 360 nm), emission band-pass (18, 40 nm), length of delay coil (5-45 turns, i.e. reaction time 33 s to 4 min 10 s), borate/pH concentration (7.6 mmol/L/9.1, 175 mmol/L/9.25). In the 9 tests using coils with more than 15 turns, the standard deviation of the 9 amino acids was found in the range of 4-7 % in glycine equivalent. As expected from the kinetics study, the standard deviation increased inversely to reaction time (10 % for 10 turns, 15 % for 5 turns) while the average recovery dropped with respect to glycine (4 % lower for 10 turns, 25 % for 5 turns). With the LDC detector, the average response was 10 % higher (106 ± 5 %), but ammonium interference was 3 times greater, than with the Jasco detector. Selecting the excitation wavelength at 350 instead of 360 nm decreased the average relative response to 90 ± 5 %. The two borate/pH conditions provided very close results (within ± 2 %).

The molar response rates of aliphatic amines, hexosamines and di-peptides were: MMA (82 %), MEA (75 %), glucosamine (66 %), galactosamine (82 %), glycyl-alanine (38 %), alanyl-glycine (14 %), glycyl-leucine (27 %). For secondary and tertiary aliphatic amines the responses were below 0.4 %. The interference rate of ammonium was 1.4 %. For proteins, the molar response was insignificant, but their interferences were computed per mole of combined amino acids, the usual way of expressing these compounds in seawater: 1.7 % for trypsin, 2.8 % for ovalbumin and 2.5 % for bovine serum albumin. This narrow range for protein responses contrasts with the values found by Nagel and Liemann (1987), i.e. ovalbumin and bovine serum albumin responses being 2 and 3 times that of trypsin.

The salt effect was determined by measuring the response of amino acids in depleted seawater (S = 35) and dilutions of this seawater with demineralized water to S = 17 and S = 1. Glycine, serine, ammonium and an equimolar mixture of the 9 amino acids used for the robustness test were analyzed at a total concentration of 400 nmol/L TDFPA. The response of amino acids remained within 0.5 % (i.e. no salt effect) whatever the salinity (pH of reaction medium varied from 9.2 for S = 35 to 9.6 for S = 1). For ammonium, the response relative to glycine decreased nearly linearly as salinity increased from 1.9 % (S = 1) to 1.4 % (S = 35). For accurate corrections in estuarine waters, ammonium interference should therefore be checked as a function of salinity.

4.7 Overall protocol, calculation of TDFPA concentration and precision

The overall protocol needs to split the filtered sample into two aliquots. One aliquot is immediately stored in a freezer (-25 °C) and the second placed in a clean box for a ~ 10 days incubation at room temperature. After incubation, the two aliquots are analyzed (preferably simultaneously) to determine uncorrected TDFPA and ammonium concentrations in parallel, then NBF. The TDFPA analysis is calibrated with standards of glycine and serine, to check recoveries, and ammonium to measure the ammonium interference rate (I_{NH4}). Six quantities have to be measured, i.e. i) in the non-incubated aliquot: (1) [$uTDFPA^{NI}$] (uncorrected TDFPA), (2) [NBF^{NI}], (3) [NH_4^{NI}], ii) in the incubated aliquot: (4) [uOIRF] (uncorrected OIRF), (5) [NBF^{I}], (6) [NH_4^{I}].

The net concentration of TDFPA ([TDFPA]) is the net concentration in the non-incubated sample ([$TDFPA^{NI}$]) minus that in the incubated sample ([OIRF]):

$$[TDFPA] = [TDFPA^{NI}] - [OIRF],$$

where (all the signals being expressed in the TDFPA concentration unit):

$[TDFPA^{NI}] = [uTDFPA^{NI}] - [NBF^{NI}] - ([NH_4{}^{NI}] \times I_{NH4})$,

$[OIRF] = [uOIRF] - [NBF^{I}] - ([NH_4{}^{I}] \times I_{NH4})$.

A comparison of the magnitude of these components of the raw TDFPA signal for a variety of samples is shown in figure 10. With the LDC detector used at that time, ammonium interference was 4 %. The net TDFPA signal amount was 0-57 % (two third less than 30 %) of the raw TDFPA signal.

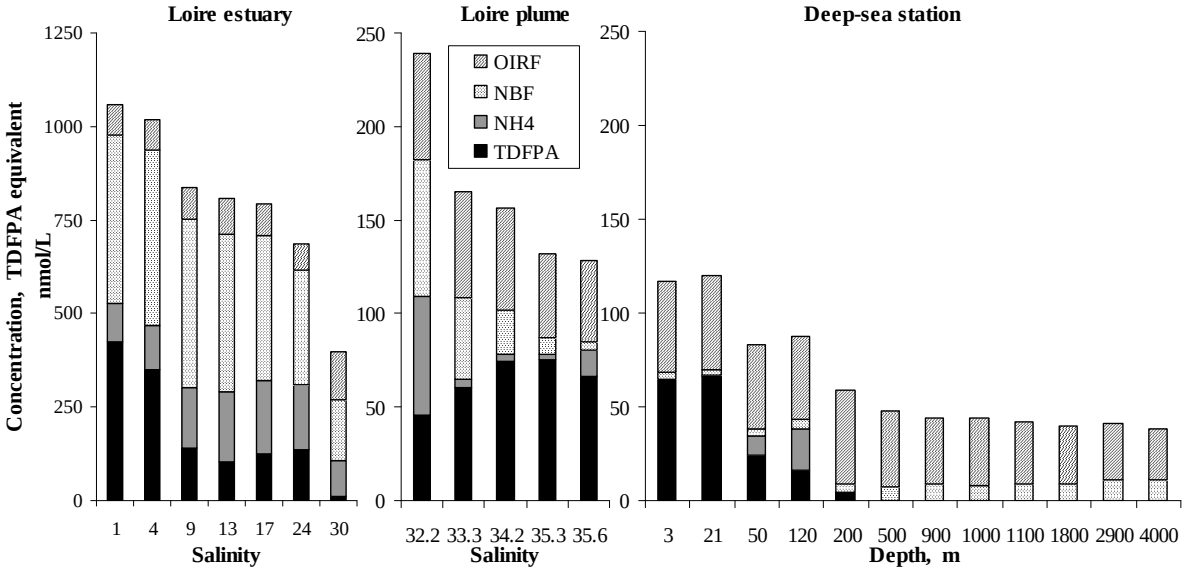

Figure 10. Composition of the raw TDFPA signal for various types of samples from the Bay of Biscay (North-east Atlantic), April 2002 (leg from upstream in the Loire river estuary, across the French continental shelf, to deep sea, 40 nautical miles off the shelf's edge).

The precision of TDFPA concentrations results from the six determinations above. Based on the principle of adding the variances of individual components (Taylor, 1990), the theoretical variance of a result (S^2) is (subscripts refer to parameters as above):

$S^2 = s_1{}^2 + s_2{}^2 + (s_3 \times I_{NH4})^2 + s_4{}^2 + s_5{}^2 + (s_6 \times I_{NH4})^2$.

Assuming that the various signal components measured with either method (TDFPA and ammonium) have a nearly equal absolute precision (specific to each method), it follows that:

$S^2 \cong 4s_{TDFPA}{}^2 + 2(s_{NH4} \times I_{NH4})^2$

This equation shows that an intrinsic standard deviation (s.d.) of *x* nmol/L in the TDFPA method leads to an s.d. of 2*x* nmol/L for a TDFPA result if the ammonium contribution is negligible. The ammonium contribution, for an interference rate of 1.4 %, begins to significantly alter the precision of TDFPA if its own s.d. exceeds 0.1 µmol/L (assuming unbiased ammonium values). If ammonium interference is 5 %, alteration will occur for the s.d. at as low as 0.02 µmol/L. Applying our precision data for the TDFPA components (about 1-2 nmol/L) and for the ammonium concentrations (≤ 0.02 µmol/L) encountered in coastal and oceanic waters led to a total standard deviation of 2-4 nmol/L for corrected TDFPA values. Verification from 6 deep oceanic samples (900-4000 m), assumed to be depleted in TDFPA, consistently provided a mean of 0 ± 2 nmol/L (range: -2 to +3 nmol/L).

5 DISCUSSION

This study showed that short reaction times (< 2 min) in the OPA-amine derivatization can produce very different relative fluorescence intensities among the compounds and that the differences

depend on pH and reagent concentrations. Mixtures of equal total concentration of amino acids, but with differing compositions, are thus liable to produce different apparent concentrations. Attention is given to the particular behavior of glycine in the OPA/ME reaction since it has invariably been used as a standard in all the published 'total' methods. The result is that overly short reaction times will underestimate the total concentration and generate high variability from the variable composition of amino acid mixtures, while overly high reaction times will overestimate the total concentration. Gly is therefore not the appropriate standard for calibrating total amino acid methods when ME is the thiol used. In contrast, when using MPA, standardization with Gly is acceptable, provided that the reaction time is not too short. In any case, very short reaction times (< 1 min) do not guarantee reliability for the analysis of natural samples, which are themselves essentially variable in composition.

The 200-fold excess of reagent suggested by Lindroth and Mopper (1979) is not justified in unpolluted marine waters, where the concentration of naturally occurring dissolved organic nitrogen rarely reaches 10 µmol/L (Bronk, 2002). In our experiments with 10 µmol/L DFAA, the recovery was not altered with only a 25-fold excess OPA. A large OPA excess is not recommended, since it will catalyse the instability of amino acid-OPA derivatives (Mopper and Dawson, 1986).

Although they have little effect on the maximum fluorescence intensity, modifications in reaction pH or OPA concentration within the range of values found in the literature (Table 1) may change reaction rates and, for short reaction times, produce highly different intensities for most amino acids. This may explain the inconsistency in relative fluorescence intensities found by different authors. Any change of the sample/reagent (S/R) ratio (to modify the analytical range, as suggested by Josefsson et al., 1977), without keeping medium reagent concentrations constant, can produce different total concentrations of TDFPA from the same sample, even though the same standard is used for each of the S/R ratio.

The reaction pH of 9.5, generally stated as optimal for OPA-amino acid derivatization, was found satisfactory for the determination of TDFPA in sea and estuarine waters and the borate concentration was not found to be critical. However, to minimize ammonium interference it is advisable to keep the pH ≤ 9.5 and to select a borate concentration lower than those proposed in the literature. Hence, a pH in the range of 9.1-9.3 appears to be suitable with regard to the derivatization rate and a ~2 min reaction time in automated analysis is satisfactory. In addition, the pH of the reagent itself can be below 10, which will avoid the risk of precipitation upon mixing with seawater samples.

While segmented flow analysis provides fixed sample-to-reagent proportions, fast mixing and adequate reaction time, FIA is based on short reaction times and generates changes in the sample-to-reagent ratio in the course of the reaction. Because too-short reaction times are liable to generate poor reproducibility, application of FIA requires a rather stable TDFPA composition in the samples (which is roughly valid for seawater) and checking of the response of the most abundant amino acids with respect to that of glycine.

Standardization of TDFPA methods should preferably include two of the most naturally abundant amino acids which exhibit different behaviors, such as Gly and Ser. Comparison of their responses is a quality assurance test which can alert us to any failure in the procedure. An ammonium standard should also be systematically run to determine its interference rate and allow subsequent correction when necessary. In coastal waters even a 1 % interference may, in some periods, produce a signal equivalent to tens of nanomoles per liter of amino acids.

Separation of OPA-reactive substances according to their molecular weight (MW) by Nagel and Liemann (1987) showed two main peaks, one of low (amino acid-like) MW and the other of high (protein-type) MW. It may thus be assumed that peptide-like compounds did not account for a significant amount of the TDFPA values in the samples studied by Nagel and Liemann (1987). Monoamines and hexosamines, if present, will contribute to the TDFAA signal with a recovery close to that of amino acids. Reported concentrations of monomethylamine start at a few nanomoles per liter in offshore waters (Gibb et al., 1995) and are mostly < 50 nmol/L in unpolluted coastal waters (Gibb et al., 1995; Gibb and Hatton, 2004). Monoethylamine was found < 2 nmol/L (Gibb and Hatton, 2004). We found no data on hexosamine concentrations in seawater, except an estimation of < 5 nmol/L for N-acetyl-glucosamine (Riemann and Azam, 2002). The measurement of TDFPA cannot be an exact

determination of free amines because of differences among amine compound responses and the impossibility of correcting for combined amino acids. The latter compounds, however, do not interfere much and, seeing their usual concentration in seawater, should not account for more than 5-10 nmol/L TDFPA-equivalents. Corrections for ammonium and NBF are well defined. However, the OIRF correction introduced in the present protocol requires further discussion. The correction refers to the DOM pools based on degradation rates (labile = hours/days, semi-labile = months/season, refractory = hundreds/thousands of years; Kirchman et al., 1993; Carlson and Ducklow, 1995). Our protocol implies that the OIRF signal is produced by the semi-labile and refractory DOM pools, while TDFPA belongs to the labile pool. Without OIRF correction, TDFPA in deep-sea water would amount 30 µmol/L although the DOM is thousands of year old and highly refractory. This uncharacterized DOM is not expected to contain TDFPA which is composed of biologically active substances with low molecular weight. OIRF is attributed to amine groups attached to the humic-like skeleton of the bulk DOM. The low level or absence of primary amines (with hydrocarbon chain) in oceanic sub-surface layers (Gibb and Hatton, 2004) allows us to assume that they belong to the labile pool, like amino acids and sugars. The stable OIRF signal obtained over an incubation period of 2-10 days indicates that the labile compounds are actually mineralized. The following slow decrease results from the degradation process of semi-labile compounds which progressively lose reactive amine groups. Although a minor contribution from potentially semi-labile TDFPA can not be excluded, correction according to the proposed protocol is reproducible and justified by the magnitude of the OIRF signal compared with net TDFPA.

The proposed protocol, which is based on adequate reaction conditions and corrections for ammonium, natural background fluorescence and OPA-induced residual fluorescence, leads to a measure of low molecular degradable amine compounds, most of which are probably DFAA. Provided the method is adequately standardized and the results corrected for major biases, TDFPA data should be reproducible throughout laboratories and go beyond a fluorescence signal obtained in the presence of OPA. The significance of TDFPA on the whole is therefore valid as an estimation of amine nitrogen which can be rapidly consumed by microplankton.

6 SUMMARY AND CONCLUSION

The following points were highlighted for the determination of TDFAA using OPA derivatization.

- Mercaptoethanol should be replaced by MPA, which produces more stable and more comparable responses.
- Standardization should not rely on glycine alone, since the behavior of this compound is not a good proxy for amino acids overall. The use of two standards, glycine and serine, which are among the most abundant amino acids in seawater, is recommended.
- Concentrations in the reaction medium should not be modified without reconsidering the response of various, naturally abundant amine compounds. Values of pH close to 9.2-9.3 seem to be a satisfactory compromise for all types of waters, considering the ammonium interference, the reagent preparation, and the risk of precipitation in seawater.
- Correction of undesired signals, i.e. the OPA-ammonium signal, the natural background fluorescence and the OPA-induced fluorescence of non-degradable material, is essential.
- Use of a spectrofluorometer detector (instead of a filter fluorometer) is advisable since it enables optimization of wavelength and band-pass settings to reduce interference from ammonium and natural background fluorescence.

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8 REFERENCES

Aminuddin, M., Miller, J.N., 1995. New fluorigenic reagents and their fluorescent reactions for amino acid measurements. Talanta 42(6), 775-778.

Aminot, A., Kérouel, R., 2004. Dissolved organic carbon, nitrogen and phosphorus in the N-E Atlantic and the N-W Mediterranean with particular reference to non-refractory fractions and degradation. Deep-Sea Research I 51, 1975-1999.

Bronk, D.A., 2002. Dynamics of DON. In: Hansell D.A., Carlson C.A. (Eds), Biogeochemistry of marine dissolved organic matter. Academic Press, Amsterdam, Boston, pp. 153-247.

Carlson, C.A., Ducklow, H.W., 1995. Dissolved organic carbon in the upper ocean of the central equatorial Pacific Ocean, 1992; Daily and fine-scale vertical variations. Deep-Sea Research Part II 42, 639-656.

Crottereau, C., Delmas, D., 1999. Régulation de l'activité exoprotéolytique bactérienne aux concentrations naturelles en matière organique dissoute dans un marais maritime. Comptes-Rendus de l'Académie des Sciences de Paris, Sciences de la vie 322, 551-556.

Daumas, R., 1976. Variation of particulate proteins and dissolved amino acids in coastal seawater. Marine Chemistry 4, 225-242.

Dawson, R., Liebezeit, G., 1981. The analytical approach for the characterisation of organics in seawater, 2.1 Methods for estimation of total amino nitrogen. In: Duursma and Dawson R. (Eds), Marine organic chemistry. Elsevier Oceanographic series 31, Elsevier Sci. Publ. Co., Amsterdam, pp. 448-450.

Dawson, R., Liebezeit, G., 1983. Determination of amino acids. In: Grasshoff K., Ehrhardt M. Kremling K. (Eds), Methods of seawater analysis, 2nd ed., Verlag Chemie, Weinheim, pp. 319-330.

Delmas, D., Frikha, M.G., Linley, E.A.S., 1990. Dissolved primary amine measurement by flow injection analysis with *o*-phthaldialdehyde: comparison with high-performance liquid chromatography. Marine Chemistry 29, 145-154.

Dorresteijn, R.C., Berwald, L.G., Zomar, G., de Gooier, C.D., Wieten, G., Beuvery, E.C., 1996. Determination of amino acids using *o*-phthalaldehyde-2-mercaptoethanol derivatization; Effect of reaction conditions. Journal of Chromatography A 724, 159-167.

Ferguson, R.L., Sunda, W.G., 1984. Utilization of amino acids by planktonic marine bacteria: importance of clean technique and low substrate additions. Limnology and Oceanography 29(2), 258-274.

Fiorino, A., Frigo, G., Cuchetti, E., 1989. Liquid chromatographic analysis of amino and imino acids in protein hydrolysates by post-column derivatization with *o*-phtalaldehyde and 3-mercapto-propionic acid. Journal of Chromatography 476, 83-92.

Fourche, J., Jensen, H., Neuzil, E., 1976. Fluorescence reactions of aminophosphonic acids. Analytical Chemistry 48(1), 155-159.

Fuhrman, J., 1987. Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach. Marine Ecology Progress Series 37, 45-52.

Fuhrman, J.F., Bell, T.M., 1985. Biological considerations in the measurement of dissolved free amino acids in seawater and implications for chemical and microbiological studies. Marine Ecology Progress Series 25, 13-21.

Garrasi, C., Degens, E.T., Mopper, K., 1979. The free amino acid composition of seawater obtained without desalting and preconcentration. Marine Chemistry 8, 71-85.

Gibb, S.W., Hatton, A.D., 2004. The occurrence and distribution of trimethylamine-N-oxide in Antartic coastal waters. Marine Chemistry 91, 65-75.

Gibb, S.W., Mantoura, R.F.C., Liss, P.S., 1995. Analysis of ammonia and methylamines in natural waters by flow injection gas diffusion coupled to ion chromatography. Analytica Chimica Acta 316, 291-304.

Hansen, H.P., Grasshoff, K., 1983. Automated chemical analysis. In: Grasshoff, K., Ehrhardt, M., Kremling, F. (Eds), Methods of seawater analysis (2nd ed). Verlag Chemie, Weinheim, pp. 347-361.

Jørgensen, N.O.G., 1979. Annual variation of dissolved free primary amines in estuarine water and sediment. Oecologia (Berl.) 40, 207-217.

Jørgensen, N.O.G., 1980. Uptake of glycine and release of primary amines by the polychaete *Nereis virens* (Sars) and the mud snail *Hydrobia neglecta* Muus. Journal of experimental marine Biology and Ecology 47, 281-297.

Jørgensen, N.O.G., 1987. Free amino acids in lakes: Concentration and assimilation rates in relation to phytoplankton and bacterial production. Limnology and Oceanography 32(1), 97-111.

Jørgensen, N.O.G., Mopper, K., Lindroth, P., 1980. Occurrence origin, and assimilation of free amino acids in an estuarine environment. Ophelia, suppl. 1, 179-192.

Josefsson, B., Lindroth, P., Östling, G., 1977. An automated fluorescence method for the determination of total amino acids in natural waters. Analytica Chimica Acta 89, 21-28.

Kérouel, R., Aminot, A., 1987. Procédure optimisée hors contaminations pour l'analyse des éléments nutritifs dissous dans l'eau de mer. Marine Environmental Research 22, 19-32.

Kérouel, R., Aminot, A, 1997. Fluorimetric determination of ammonia in sea and estuarine waters by direct segmented flow analysis. Marine Chemistry 57, 265-275.

Kirchman, D.L., Lancelot, C., Fasham, M, Legendre, L., Radach, G., Scott, M., 1993. Dissolved organic material in biogeochemical models of the ocean. In Evans, G.T., Fasham, M.J.R. (Eds), Towards a model of Ocean Biogeochemical Processes, Serie I : Global Environmental Change, vol. 10, 209-225. Berlin.

Laanbroek, H.J., Verplanke, J.C., de Visscher, P.R.M., de Vuyst, R., 1985. Distribution of phyto- and bacterioplankton growth and biomass parameters, dissolved inorganic nutrients and free amino acids during a spring bloom in the Oosterschelde basin, The Netherlands. Marine Ecology Progress Series 25, 1-11.

Liebezeit, G., Dawson, R., 1981. Isoindole derivatives of amino acids for HPLC separations – Effect of reaction pH and time on fluorescence yield. Journal of high Resolution Chromatography & Chromatography Communications 4, 354-356.

Liebezeit, G., Behrends, B., 1999. Determination of amino acids. In: Grasshoff, K., Kremling, K., Ehrhardt, M. (Eds), Methods of seawater analysis, 3rd ed., Wiley-VCH, Weinheim, pp. 541-546.

Lindroth, P., Mopper, K., 1979. High performance liquid chromatography determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. Analytical Chemistry 51(11), 1667-1674.

Macko, S.A., Green, E.J., 1982. An investigation of the dissolved free amino acids and their relation to phytoplankton cell density in the Damariscotta River estuary, Maine. Estuaries 5(1), 68-73.

Molnár-Perl, I., Bozor, I., 1998. Comparison of the stability and UV and fluorescence characteristics of the *o*-ptaldialdehyde/3-mercaptopropionic acid and *o*-ptaldialdehyde/N-acetyl-L-cysteine reagents and those of their amino acids derivatives. Journal of Chromatography A 798, 37-46.

Mopper, K., Dawson, R., 1986. Determination of amino acids in sea water – Recent chromatographic developments and future directions. The Science of the Total Environment 49, 115-131.

Nagel, K., Liemann, F., 1987. Automated assay for the quantification of dissolved proteins in natural seawater samples. Oceanologica Acta 10(2), 181-185.

North, B.B., 1975. Primary amines in California coastal waters: utilisation by phytoplankton. Limnology and Oceanography 20(1), 20-27.

Pai, S.-C., Tsau, Y.-J., Yang, T.-I., 2001. pH and buffering capacity problems involved in the determination of ammonia in saline water using the indophenol blue spectrophotometric method. Analytica Chimica Acta 434, 209-216.

Parsons, T.R., Maita, Y., Lalli, C.M., 1984. Determination of dissolved free amino acids by fluorimetic analysis. In: A manual of chemical and biological methods for seawater analysis, Pergamon Press, Oxford, pp. 40-44.

Petty, R.L., Michel, W.C., Snow, J.P., Johnson, K.S., 1982. Determination of total primary amines in seawater and plant nectar with flow injection sample processing and fluorescence detection. Analytica Chimica Acta 142, 299-304.

Riemann, L., Azam, F., 2002. Widespread N-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. Applied and Environmental Microbiology 68(11), 5554-5562.

Roth, M., 1971. Fluorescence reaction for amino acids. Analytical Chemistry 43, 880-882.

Sellner, K.G., Nealley, E.W.,1997. Diel fluctuations in dissolved free amino acids and monosaccharides in Chesapeake Bay dinoflagellate blooms. Marine Chemistry 56, 193-200.

Siegel, A., Degens, E.T., 1966. Concentration of dissolved amino acids from saline waters by ligand exchange chromatography. Science 151, 1098-1101.

Tada, K., Tada, M., Maita, Y., 1998. Dissolved free amino acids in coastal seawater using a modified fluorometric method. Journal of Oceanography 54, 313-321.

Taylor, J.K., 1990. Quality assurance of chemical measurements. Lewis Publishers, Inc., USA, 328 pp.