



Research article

Formaldehyde preservation for deferred measurements of alkaline phosphatase activities in marine samples

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ARTICLE INFO

Keywords:

Environmental science
 Biochemistry
 Alkaline phosphatase activity
 Formaldehyde
 Inhibition
 Enzymes
 Aquatic studies
 Deferred measurements

ABSTRACT

Alkaline phosphatases are the main enzymes required by microorganisms to hydrolyse organic phosphorus into available phosphate in aquatic environments. The investigations of alkaline phosphatase activity (APA) usually generate numerous samples (size fractionation, Michaelis-Menten kinetics). Therefore, convenient and reliable preservation of incubated samples for a deferred analysis would be very useful when measurements cannot be performed right away. The APA of marine pond waters was measured using 4-Methylumbelliferyl phosphate (MUF-P) as the fluorogenic substrate modelling natural organic phosphorus compounds. Where typical inhibitors of other enzymatic activities, such as 1% sodium dodecyl sulfate, mercuric chloride, or buffered solutions of ammonium and glycine, failed to stop APA, the addition of formaldehyde efficiently inhibited APA. The effect of formaldehyde was the strongest with the highest concentration tested (4% final concentration) and in buffered (pH 8) solutions. Since a slow and gradual increase in APA may persist with time, the combination of the addition of 4% buffered formaldehyde with immediate freezing is the best method to entirely inhibit APA. The maximal rate of hydrolysis (V_{max}) and the Michaelis constant (K_m) of formaldehyde (4%)-inhibited samples did not significantly change during storage at $-20\text{ }^{\circ}\text{C}$ for 11 days. The method was successfully tested on samples with extremely high values of APA ($15000\text{--}40000\text{ nM h}^{-1}$) that were preserved for 1 month at $-20\text{ }^{\circ}\text{C}$ (98% inhibition). This method is a reliable and useful means of preserving incubated samples, and it provides convenient controls for background fluorescence of water and substrate, without provoking abiotic hydrolysis of the substrate.

1. Introduction

The ability of aquatic microorganisms to acquire phosphorus (P) from organic phosphorus compounds requires the action of alkaline phosphatases (APs), which hydrolyse phosphomonoesters into available orthophosphate and organic matter. Alkaline phosphatases are present in aquatic environments as ectoenzymes and extracellular enzymes. Ectoenzymes are enzymes secreted by microorganisms that cross the cytoplasmic membrane but remain associated with their producer and that hydrolyse substrates outside the cell (Chrost, 1990). Extracellular enzymes are released into the surrounding environment. Alkaline Phosphatase Activity (APA) has been detected in both phytoplankton and bacterioplankton (Chrost and Overbeck, 1987; Martinez and Azam, 1993). Similar to most of ectoenzymes synthesized by aquatic microorganisms, APs are mainly inducible, catabolic enzymes. The induction or repression of their synthesis is regulated by substrate or end-product availability or depletion (Chrost, 1990). AP synthesis is usually

induced under phosphate (PO_4)-limited conditions in order for microorganisms to access the organic P source. As such, APA has often been used as an indicator of plankton P limitation. However, more recently, the occurrence of high APA has been associated with PO_4 replete waters and bacteria-colonizing particles (Labry et al., 2016; Davis and Mahaffey, 2017).

Even though APA investigations have been conducted in lakes for several decades and, more recently, in marine waters, the protocols used still vary substantially between studies with different substrates, concentrations, pH values, temperatures, methods of measuring product absorbance or fluorescence (immediate or differed measurements; Chrost, 1990; Labry et al., 2016). Several biases in these protocols might affect the interpretation of the results and the comparison between studies. Urvoy et al. (2020) highlighted the issues concerning the substrate concentration used (trace or saturating concentration) and the occurrence of a potential artifact in fluorometric measurements, which has never been considered in marine studies. The present study focuses

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on the issue concerning immediate fluorescence measurements of the enzymatic reaction product or their deferred analysis, requiring a reliable activity inhibitor.

The measurement of APA is usually carried out using a fluorogenic substrate modelling organic P compound. 4-Methylumbelliferyl phosphate (MUF-P) is the preferred fluorogenic substrate for environmental AP studies because of its high sensitivity. In addition, other MUF-derivative substrates are available to study different ectoenzymes, using the same final product, 4-Methylumbelliferone (MUF), for calibration curves and the same wavelength settings (Ammerman, 1993). The kinetic approach, which allows for determination of the maximal rate of hydrolysis (V_{\max}), the Michaelis constant (K_m) and the turnover time of dissolved organic P compounds, is the best way to study these activities. However, it requires numerous measurements of hydrolysis rates for each sample, using different substrate concentrations. In addition, since APA is linked to phytoplankton and bacterioplankton and is partly released into the surrounding environment, size fractionation is recommended to investigate APA regulation by microorganisms (Hoppe, 2003). This involves the simultaneous incubation of a large number of samples. It makes the subsequent fluorometric measurements of each sample difficult and prone to errors. Errors may come from the time lag between the first and the most recently analyzed samples, which involves a difference in the incubation time. Microtiter plate spectrofluorometers are increasingly used as they allow high-throughput assays. However, the small volume and long incubation time may generate containment issues. This might impact the meaningful interpretation of the results when the purpose is to obtain an APA that is as close as possible to the activity expressed by natural communities.

Thus, to correctly assess the APA of numerous samples, a convenient and reliable means of preservation for deferred fluorescence reading is needed. To our knowledge, no investigation has been conducted on the effect of potential inhibitors of APA in environmental studies. Chrost and Velimirov (1991) found that freezing ($-20\text{ }^{\circ}\text{C}$) incubated samples did not affect the kinetic parameters (V_{\max} and K_m) of β -glucosidase or leucine aminopeptidase activities over 10 days. However, activity may persist until the samples are completely frozen and may start again upon thawing (Belanger et al., 1997). This artifact can be important when activities are high. Christian and Karl (1995) found that mercuric chloride (HgCl_2) was a reliable preservative in oligotrophic waters for determining β -glucosidase activity and potentially other activities measured with MUF derivatives. However, they showed that the use of HgCl_2 requires the addition of NaCl or other salts in freshwaters and estuarine waters, and it should be avoided in waters with high particle-associated activities, for which activities are not totally stopped. Finally, HgCl_2 was found to reduce MUF fluorescence (Skórczewski et al., 1999). Delmas and Garet (1995) found that 1% sodium dodecyl sulfate (SDS), a strong protein-denaturing agent, stopped exoproteolytic activity, and storage of the SDS fixed samples at $-20\text{ }^{\circ}\text{C}$ did not alter the kinetic parameters, allowing deferred measurement after incubation. A solution of ammonium and glycine was also used as an inhibitor of β -glucosidase activity, which was measured with MUF derivatives (Chrost et al., 1989; Labry et al., 2020). Formaldehyde is known to be a fixative of proteins (Fox et al., 1985) and has been found to inhibit thymidine and leucine incorporation in bacteria (Tuominen et al., 1994). Besides its use on acid and alkaline phosphatases for histochemical studies of human and animal tissues (Abul-Fadl and King, 1948; Christie and Stoward, 1974; Porat-Ophir et al., 2013), to our knowledge, its effect on the inhibition of APA in aquatic samples has not been tested.

The objective of the present study was to find an inhibitor capable of stopping APA for storage and deferred analysis, without affecting MUF fluorescence or provoking abiotic hydrolysis of the substrate. The inhibitor should affect both individual dissolved enzymes and ectoenzymes. Several potential inhibitors of enzymatic activities such as SDS, HgCl_2 , ammonium and glycine were firstly tested before investigating different concentrations of formaldehyde in pure and buffered solutions. Finally, the combination of freezing with the addition of the best

formaldehyde concentration found to inhibit APA was tested on the preservation of APA kinetic parameters (V_{\max} , K_m) and on samples with exceptionally high APA levels.

2. Methods

2.1. Sampling

Marine water samples for the tests in Sections 2.3, 2.4 and 2.5, were collected in a man-made, experimental, marine coastal pond on the Atlantic coast of France, near La Rochelle. Incubations were carried out within 1 h of collection.

2.2. Measurement of alkaline phosphatase activity (APA)

Alkaline phosphatase activity (APA) was measured using the fluorogenic substrate analogue, 4-Methylumbelliferyl phosphate (MUF-P, Hoppe, 1983). The water sample was incubated with the substrate and the fluorescent 4-Methylumbelliferone (MUF) released by the enzymatic hydrolysis of MUF-P was measured by flow injection analysis (FIA; Delmas et al., 1994) on a subsample (50 μL). The FIA system is a liquid chromatography injection system (without the chromatographic column), connected to a Kontron SFM25 spectrofluorometer with a 1 mm optic path and to an integrator for data processing. A buffered solution (0.1 M boric acid, pH 10.5) was pumped at a flow rate of 1 mL min^{-1} , and full loops of sample (50 μL) were manually filled and injected. Sample was diluted about 10 times by the carrier fluid and the run took less than a minute per sample. Thus, regardless of the pH of incubation, the final measurement of the MUF fluorescence was carried out at optimum pH > 10 (Hoppe, 1983; Chrost and Krambeck, 1986). Fluorescence was measured using excitation at 364 nm and emission at 460 nm. For the experiments in Sections 2.3 and 2.4, the MUF solutions (20 nM –2 μM) used to calibrate the system were prepared in deionized Milli-Q water. For the experiments in Sections 2.5 and 2.6, the MUF solutions for calibration were prepared in deionized Milli-Q water with a 4% final concentration of buffered formaldehyde. 50 μL of each MUF solution was injected through the FIA system and the corresponding fluorescence was used for calibration. All chemical products were purchased from Sigma-Aldrich, Darmstadt, Germany. APA was expressed as concentration of the enzymatic reaction product in the initial water sample volume per time.

2.3. Effect of different inhibitors on APA

The inhibition of APA was first studied by testing the following different potential preservatives: 1% (w/v) SDS, 100 μM HgCl_2 , and a buffered solution (pH 10.5) of NH_4Cl /glycine. Seawater collected in the marine pond was divided into subsamples to test each preservative. 10 mL of marine pond water was amended with either 1 mL of a 10% SDS solution, 1 mL of a 1 mM HgCl_2 solution, or 1 mL of a buffered solution (pH 10.5) of 0.2 M NH_4Cl /0.05 M glycine before the addition of 250 μL of 10 mM MUF-P (final concentration of 250 μM). An untreated sample and an autoclaved control (120 $^{\circ}\text{C}$ for 20 min) were prepared with the same MUF-P concentration as the samples treated with inhibitors. All samples were incubated in the dark, at in situ pH and temperature (20 $^{\circ}\text{C}$) and periodically analyzed (after 0.5, 1, 2, 3, 6 and 7 h of incubation) for MUF fluorescence with the FIA system.

2.4. Effect of different concentrations of pure or buffered formaldehyde on APA

Given that none of the preservatives (SDS, HgCl_2 , NH_4 /glycine) inhibit APA substantially, a second set of experiments testing the effects of several concentrations (2, 3 and 4%) of formaldehyde in pure or buffered solutions on the inhibition of APA was performed. Seawater collected in the marine pond was divided into subsamples to test each

concentration of pure or buffered formaldehyde. A stock solution of buffered formaldehyde (pH 8) was prepared with 50:50 v/v of a pure formaldehyde solution (37% w/w = 40% w/v) and a 0.1 M sodium tetraborate solution. Samples treated with pure formaldehyde were prepared as follows: 222, 333 or 444 μL of a pure formaldehyde solution (40% w/v) were added to 4 mL of marine pond water to reach a final formaldehyde concentration of 2, 3 and 4% w/v, respectively. For samples treated with buffered formaldehyde, 444, 666 and 888 μL of the buffered formaldehyde solution were added to 4 mL of marine pond water to reach a final formaldehyde concentration of 2, 3 and 4% w/v, respectively. In parallel, a sample without formaldehyde and untreated and treated autoclaved controls with 2, 3, and 4% pure or buffered formaldehyde were prepared. The time-course experiment began with the addition of 100 μL of 10 mM MUF-P (final concentration of 250 μM). All samples were incubated in the dark, at in situ pH and temperature (20 $^{\circ}\text{C}$) and were periodically analyzed (after 2, 4, 5 and 6 h of incubation) for MUF fluorescence. After 4 h of incubation, 2 mL was removed from the untreated sample, 444 μL of buffered formaldehyde (4% final concentration) was added, and the MUF fluorescence was measured 1 h and 2 h later. Finally, all remaining samples were frozen (-20 $^{\circ}\text{C}$) and analyzed 6 days later (at 144 h).

2.5. Effect of the addition of 4% buffered formaldehyde and freezing on APA kinetic parameters

Considering that the addition of 4% buffered formaldehyde was the most effective at inhibiting APA, a third set of experiments to study the effects of both 4% buffered formaldehyde addition and freezing on the kinetic parameters of APA was performed. 50 μL of 0.07, 0.15, 0.3, 0.6, 1.25, 2.5, 5 and 10 mM MUF-P were added to 2 mL of marine pond water (1 sample and 1 autoclaved control for each MUF-P concentration) to give a final concentration of 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, and 250 μM MUF-P, respectively. After 6 h of incubation in the dark at in situ pH and temperature (20 $^{\circ}\text{C}$), samples were treated with a 4% final concentration of buffered formaldehyde and, immediately analyzed and frozen (-20 $^{\circ}\text{C}$). The samples were periodically thawed (after 3, 5, and 11 days) for MUF fluorescence analysis and refrozen.

2.6. Effect of the addition of 4% buffered formaldehyde and freezing on samples with very high APA

Finally, we tested the use of 4% buffered formaldehyde on samples with particularly high bacterial enzymatic activities. Natural bacterial communities collected in the Bay of Brest were inoculated with dead cells of *Thalassiosira weissflogii*, which had been cultured under nutrient replete conditions, Si stress (f/2 medium without silicate), or N stress (f/2 medium without nitrate) conditions. *T. weissflogii* biomass equivalent to 3000 μM particulate C was frozen, thawed, and inoculated to bacterial communities. These degradation batches were incubated at 16 $^{\circ}\text{C}$ for 30 days. The protocol of these degradation experiments is detailed in work by Suroy et al. (2015). Michaelis-Menten kinetics of APA was determined 2 days after inoculation, corresponding to the bacterial biomass and APA maxima, which were regularly measured during the course of the experiment. The protocol for kinetic parameter determination is described in Section 2.5. Controls treated with 4% buffered formaldehyde were prepared for each MUF-P concentration and immediately frozen (-20 $^{\circ}\text{C}$) after substrate addition. Samples were incubated for 1 h in the dark at 16 $^{\circ}\text{C}$, treated with 4% buffered formaldehyde, frozen (-20 $^{\circ}\text{C}$), and analyzed 1 month later.

2.7. Statistical analysis

The non-parametric Friedman test and pairwise comparisons analysis using Nemenyi test in R software were used to evaluate for a statistical difference in APA due to effects of different inhibitors (Sections 2.3 and 2.4). The level of significance was set at $p < 0.05$. In Section 2.5, the

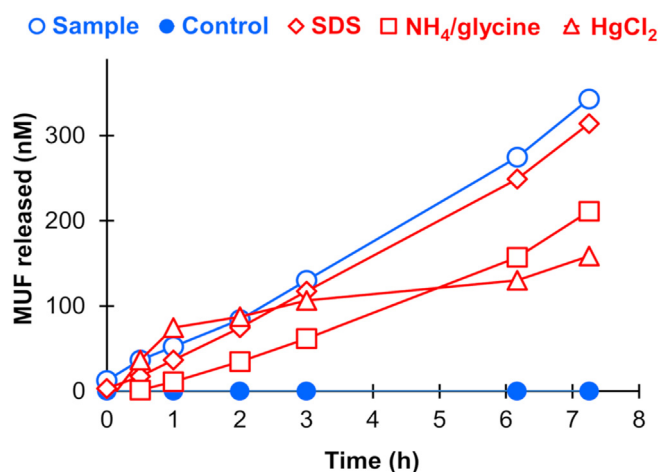


Figure 1. Effect of several potential inhibitors (SDS, HgCl_2 , $\text{NH}_4/\text{glycine}$) on the concentration of 4-Methylumbelliferone (MUF) released by the action of alkaline phosphatases of marine pond water. Comparison with an untreated water sample and an autoclaved water control. All Samples were incubated in the dark, at in situ pH and temperature (20 $^{\circ}\text{C}$) and periodically analyzed for MUF fluorescence with the FIA system.

kinetic parameters of APA, V_{max} and K_m , were calculated by non-linear least-squares regression of velocity versus substrate concentration plots using XLSTAT (Microsoft) software.

3. Results and discussion

3.1. Effect of different inhibitors on APA

The effects of several preservatives known to stop some enzymatic activities (i.e., exoproteolytic and β -glucosidase activities) on APA of marine water were first tested, by comparing samples with inhibitors to an untreated sample and an autoclaved control. The untreated sample showed a linear increase in MUF concentration during 7 h of incubation, corresponding to an APA of 45 nM h^{-1} . None of the tested preservatives succeeded in stopping the APA, whereas no APA was detected in the

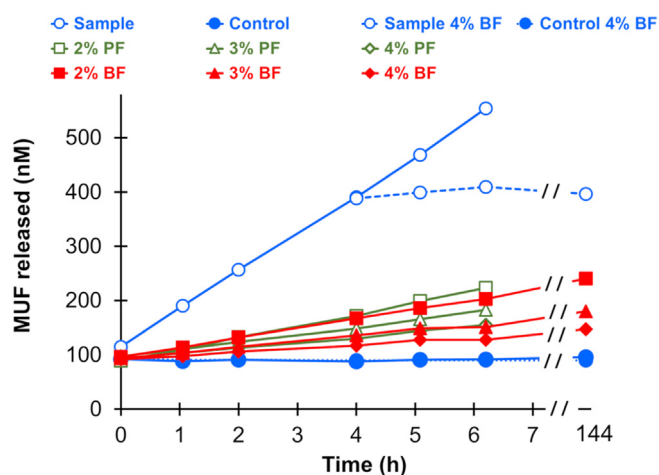


Figure 2. Effect of preservation with 2, 3 and 4% pure formaldehyde (PF, empty square, triangle and diamond symbols) or buffered formaldehyde (BF, full square, triangle and diamond symbols) on the concentration of 4-Methylumbelliferone (MUF) released by the action of alkaline phosphatases present in marine pond water. Comparison with untreated sample (empty circle, full line), sample inhibited after 4 h of incubation (empty circle, dashed line), autoclaved sample (control, full circle and line), and autoclaved sample treated with 4% buffered formaldehyde (full circle, dashed line).

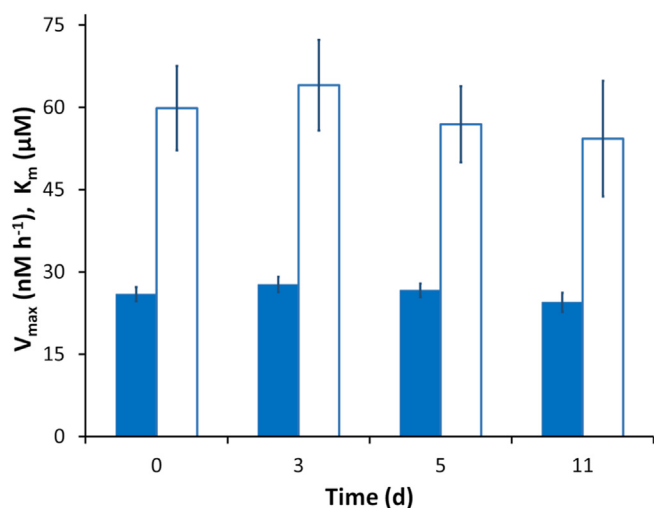


Figure 3. Effect of the storage duration ($-20\text{ }^{\circ}\text{C}$) of marine pond water samples, fixed by the addition of 4% buffered formaldehyde after incubation, on the kinetic parameters (V_{max} , filled histogram; K_m , empty histogram) of alkaline phosphatase activity. Bars are the standard errors of the estimated parameters.

autoclaved control (Figure 1). According to the Friedman and pairwise comparison tests, the buffered solution of $\text{NH}_4/\text{glycine}$ was the only treatment that showed a significant difference with the untreated sample ($p = 0.005$). The addition of 1% SDS had almost no effect on APA inhibition ($p > 0.05$), while it was shown to entirely inhibit exoprotoleolytic activity (Delmas and Garet, 1995). It seems that 1% SDS does not change the conformation of APs in the vicinity of their active site. Alkaline phosphatases might belong to the part of enzymes that do not bind to SDS molecules, which explains why they can retain activities (Nelson, 1971; Otzen, 2011). The addition of HgCl_2 had no effect during the first hour of incubation, then reduced activity was observed in the following hours. However, the difference with sample was not statistically significant ($p > 0.05$). Finally, the buffered (pH 10.5) solution of $\text{NH}_4/\text{glycine}$ only partially reduced the APA, while it totally stopped β -glucosidase activity (Chrost et al., 1989; Labry et al., 2020), since these enzymes are active at acidic pH (Robinson, 1956; Belanger et al., 1997). This emphasizes the fact that preservatives are actually enzyme specific and should be tested before their use as inhibitors.

3.2. Effect of different concentrations of pure or buffered formaldehyde on APA

3.2.1. Effect on the time-course of APA

The inhibition of APA by formaldehyde was tested, since formaldehyde is known to be a fixative of proteins (Fox et al., 1985), and has been found to inhibit thymidine and leucine incorporation in bacteria (Tuominen et al., 1994). In addition, previous studies have investigated its effect on acid and alkaline phosphatases for histochemical study of

human and animal tissues (Abul-Fadl and King, 1948; Christie and Stoward, 1974; Porat-Ophir et al., 2013). Several concentrations of formaldehyde were tested in pure or buffered solutions. The untreated sample showed a linear increase in MUF concentration during 6 h of incubation, corresponding to an APA of 80 nM h^{-1} . In contrast, the APA was reduced in all formaldehyde-treated samples, even if they showed a small and gradual increase in MUF concentration (Figure 2). Statistical tests showed that the untreated sample was significantly different ($p < 0.01$) from the autoclaved control, the buffered formaldehyde (4%) control and sample. However, even if the differences between the 2, 3 and 4% treated samples were not statistically significant ($p > 0.05$), the inhibition effect tended to increase with the concentration of formaldehyde used. In addition, for each concentration of formaldehyde, the inhibition tended to be always higher with buffered formaldehyde compared to non-buffered formaldehyde (Figure 2). This can be explained by the chemical forms of formaldehyde presents in pure or buffered solutions. Chemical studies have indicated that formaldehyde (HCHO) in aqueous solutions combines chemically with water to form methylene hydrates ($\text{HO}-\text{CH}_2\text{OH}$), which have the same chemical reactivity as the aldehyde form (Kiernan, 2000). Methylene hydrate molecules react with one another to form polymers. The fixative power of formaldehyde comes from these monomeric formaldehyde or methylene hydrate molecules. Dilution with a buffered solution at physiological pH instantaneously breaks up the small polymers into monomers, whereas this process takes a couple of days in plain water (Kiernan, 2000). The hydrolysis of polymers is catalysed by the hydroxide ions present in slightly alkaline solutions. Moreover, buffered alkaline conditions, as used in the present study, must have reduced the spontaneous formation of formic acid, as often occurs in dilute formaldehyde solutions (Fox et al., 1985).

Thus, the addition of 4% buffered formaldehyde was the most effective at inhibiting the APA ($p < 0.01$). The APA was instantaneously stopped despite a slight increase in MUF concentration up to 5 h of incubation. The addition of 4% buffered formaldehyde to a subsample after 4 h of incubation entirely inhibited the APA (Figure 2). Finally, after 6 days of freezing ($-20\text{ }^{\circ}\text{C}$) the remaining samples, no increase in MUF concentration was observed (Figure 2). The combination of freezing and formaldehyde preservation enabled the complete inhibition of APA in our samples. According to chemical studies, formaldehyde, as a reactive electrophilic species, reacts with various functional groups of biological macromolecules, such as proteins, glycoproteins, phospholipids, nucleic acids, and polysaccharides (Fox et al., 1985). However, it seems that the fixative action of formaldehyde is mostly due to its reactions with proteins (Kiernan, 2000). The aldehyde group combines strongly with primary amines (at the end of the peptide chain) and thiols (i.e., cysteine) and, by covalent bindings, creates cross-links (in the form of CH_2 called a methylene bridge) with the nitrogen atom of the peptide linkages inside the protein (French and Edsall, 1945; Kiernan, 2000). Such intramolecular cross-links were actually observed in insulin (Metz et al., 2006) and bovine serum albumin (Liu et al., 2011), changing their conformations. Other biological molecules, such as polysaccharides, lipids and nucleic acids could be trapped in a matrix of cross-linked protein

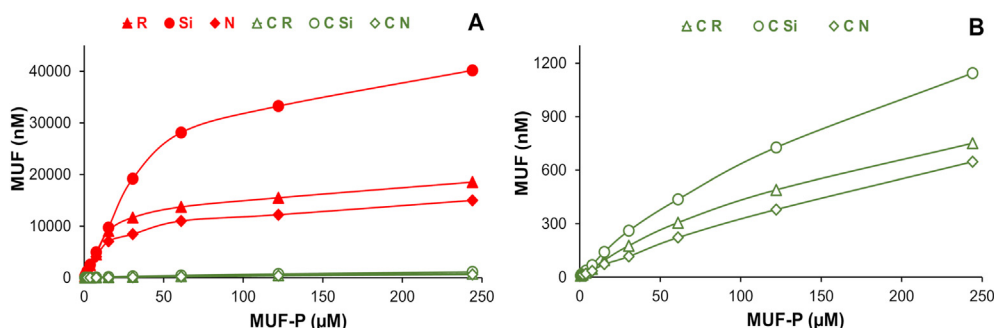


Figure 4. A. Concentration of the released 4-Methylumbelliferone (MUF, nM) versus the concentration of 4-Methylumbelliferyl phosphate (MUF-P, μM) for samples containing bacterial communities inoculated with dead cells of *Thalassiosira weissflogii* cultured under nutrient replete (R, triangle), Si stress (Si, circle) or N stress (N, diamond) conditions. The fluorescence of the associated formaldehyde (4%) treated controls (C) converted to the MUF equivalent (nM) is illustrated in A. for comparison and is magnified in B.

molecules. Thus, formaldehyde could have denatured both dissolved and membrane linked APs, in addition to its fixative action inside microorganisms. Whereas formaldehyde penetrates tissues or cells quickly, its reactions with proteins, especially cross-linking, can occur more slowly (Helander, 1994; Kiernan, 2000). This could explain the gradual increase in the MUF concentration of formaldehyde-treated samples incubated at 20 °C and supports the idea that the use of formaldehyde as a preservative must be accompanied with freezing (-20 °C).

3.2.2. Effect on the controls

Since 4% buffered formaldehyde instantaneously inhibits APA (Figure 2), controls can be easily prepared by adding 4% formaldehyde before substrate addition and freezing, instead of autoclaving the samples. In field studies especially, adding a chemical reagent to inhibit activities is easier than heating seawater, which requires equipment, space, and time. Moreover, boiling seawater could modify the nature of the dissolved organic substances present in the sample water and, therefore, modify the background fluorescence of these organic substances. This was not the case in the present study since the MUF equivalent concentration of formaldehyde-treated autoclaved controls and formaldehyde-treated samples was very similar after substrate addition, with values of 94.0 ± 2 and 93.8 ± 3 nM ($n = 6$) on average, respectively. Boiling seawater did not change the fluorescence of the seawater. This is probably due to the low concentration of organic matter present in the marine pond water used for the tests. By contrast, boiling seawater may affect the fluorescence of controls in water with high concentrations of dissolved organic matter, such as in estuarine waters, sediment samples, or phytoplankton cultures.

Another notable result is that the fluorescence of the controls and samples, expressed as the MUF equivalent concentration, was substantial just after substrate addition. This highlights, as underlined by Urvoy et al. (2020), that non-hydrolysed MUF-P has a low but substantial natural fluorescence, which must be subtracted from the sample fluorescence. Unfortunately, numerous authors have not explained how they consider this substrate fluorescence.

Finally, the similarity between the autoclaved control and the autoclaved control treated with 4% buffered formaldehyde during the time-course of the experiment highlights that formaldehyde does not induce abiotic hydrolysis of MUF-P (Figure 2). Thus, the use of a reliable preservative, such as formaldehyde, provides convenient controls for determining the natural fluorescence of waters due to dissolved organic matter and the background fluorescence of the substrate, without abiotic hydrolysis of the substrate.

3.3. Effect of the addition of 4% buffered formaldehyde and freezing on APA kinetic parameters

The effect of both 4% buffered formaldehyde addition and freezing during storage on the preservation of the kinetic parameters of APA was tested. Neither 3 days, 5 days, nor 11 days of storage (-20 °C) of formaldehyde (4%)-inhibited samples altered the kinetic parameters of APA (Figure 3). V_{\max} varied between 26.0 ± 1.3 nM h⁻¹ (estimation \pm standard error) for the first immediate analysis and 24.5 ± 1.8 nM h⁻¹, after 11 days of storage at -20 °C without any trend of evolution (Figure 3). V_{\max} values are reproducible, as shown by the low relative standard deviation, e.g., 5% (mean = 26.2 nM h⁻¹; standard deviation [SD] = 1.4; $n = 4$). Similarly, no trend of evolution could be discerned according to K_m . Values ranged between 59.9 and 54.3 μ M (mean = 58.8 μ M; SD = 4.2; $n = 4$), with a low relative SD of 13%. Both formaldehyde addition and freezing completely stopped APA and preserved their kinetic parameters. Moreover, the addition of formaldehyde to MUF solutions for calibration curves did not reduce the MUF fluorescence (data not shown), contrary to effect of HgCl₂ (Skórczewski et al., 1999). As a consequence, the sensitivity of the APA assay was not reduced with the addition of formaldehyde.

3.4. Effect of the addition of 4% buffered formaldehyde and freezing on samples with very high APA

Finally, we tested the use of 4% buffered formaldehyde to inhibit samples showing particularly high levels of bacterial enzymatic activities. Bacterial communities were inoculated with high concentration of organic matter (here dead *T. weissflogii* cells). The V_{\max} of the APA of these communities reached extremely high values of between 15000 and 40000 nM h⁻¹ (Figure 4A), which are the largest values we have ever measured. They are 100 times higher than values found in coastal water showing severe P limiting conditions (100–500 nM h⁻¹; Labry et al., 2005; Ivancic et al., 2016). Figure 4A shows a drastic inhibition of APA in all controls, with a percent inhibition of $98.1 \pm 0.9\%$ ($n = 30$). Thus, even for samples with very high APA, the addition of 4% buffered formaldehyde combined with freezing has a strong power of inhibition. The remaining fluorescence of the controls, expressed in MUF equivalent concentration, corresponds to the background fluorescence of MUF-P and to the MUF fluorescence derived from residual APA (Figure 4B). Actually, with 100% inhibition, the MUF equivalent concentrations of the controls would have been proportional to the MUF-P concentrations (background fluorescence of MUF-P). As seen in Figure 4B, they did not vary linearly with the MUF-P concentrations. They tended to saturate at high MUF-P concentrations, which indicates residual APA. However, the controls remained very low compared with the MUF concentrations of the samples (1.9%; Figure 4A). Thus, even for exceptionally high APA values, the controls remained insignificant compared to the samples.

4. Conclusion

Classical inhibitors of exoproteolytic and β -glucosidase activities (SDS, HgCl₂, NH₄/glycine) did not succeed at inhibiting the APA of marine waters. On the contrary, the addition of 4% buffered formaldehyde instantaneously stopped the APA and the slight and gradual increase in MUF fluorescence with time was counterbalanced by immediate freezing of the samples. Storage of formaldehyde (4%)-treated samples at -20 °C did not change the APA kinetic parameters (V_{\max} , K_m) for up to 11 days. In addition, the use of formaldehyde provides convenient controls for determining the background fluorescence of water and substrate, without provoking abiotic hydrolysis of the substrate. This method provides a reliable and useful means of preserving incubated samples. During field studies, this method is easy to perform, requires minimal materials, and avoids loss of samples since spectrofluorometer breakdown might occur. Instead of measuring sample fluorescence on board, this treatment enables better use of time for determining other parameters (for instance, kinetic parameters of other ectoenzymatic activities). Despite being tested with marine samples, this method could be applied to freshwater and estuarine water as well and in phytoplankton and bacterioplankton cultures. This method of preservation enables extensive studies on APA in aquatic environment, both in PO₄ deplete and replete waters, to better understand the relationship between microbial dynamics and the phosphorus cycle.

Declarations

Author contribution statement

Claire Labry: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Marion Urvoy: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by the French National Program for Coastal Environment (EC2CO).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We thank Tania Hernandez-Farinas for her assistance on statistics, and Daniel Delmas for all his perspicacious comments on the manuscript.

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