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Microbial extracellular enzyme activity in marine sediments: extreme pH to terminate reaction and sample storage

Christian Bélanger, Brigitte Desrosiers, Kenneth Lee*

Fisheries and Oceans Canada, Marine Environmental Sciences Division, Maurice Lamontagne Institute, PO Box 1000, Mont-Joli, Québec, Canada G5H 3Z4

ABSTRACT: The current analytical technique for measuring microbial extracellular enzyme activity within natural samples of water, soil or sediment involves incubation of samples with specific substrates conjugated with a fluorophore such as 7-amino-4-methylcoumarin (AMC) or methylumbelliferone (MUF). In this procedure, the extracellular enzymatic reaction is usually terminated by freezing and the reaction mixture is then stored frozen for later analysis. Unfortunately, the precision of this assay is limited by the reactivation of extracellular enzymes which can occur when samples are thawed and by the adsorption of liberated fluorophore onto the sediment which cannot be quantified without proper controls. To overcome these limitations, we describe a simple method using extreme pH (<5.5; >11) to terminate extracellular enzymatic reactions. Studies of aminopeptidase (AMPase) and β-glucosidase (βGase) activity in marine sediments have also shown that addition of HCl and NaOH, respectively, eliminated the reactivation of extracellular enzymes when frozen samples were thawed. Furthermore, acetone and NaOH additions also reduced the interference caused by the adsorption of fluorophores to the sediment. Linearity of standard curves for fluorophores in the presence of these added chemicals were maintained and additional use of acetone increased AMC fluorescence (~20%). Modifications described in this report significantly reduced the level of variability in the determination of extracellular enzyme activity in marine sediments and allowed the simultaneous treatment of numerous samples.

KEY WORDS: Bacteria · Exoenzyme · Extracellular enzyme · Marine sediment · Fluorescence · Adsorption · pH · Preservation

INTRODUCTION

In the marine environment, extracellular enzymes play a central role in the recycling of organic carbon and nitrogen compounds. High molecular weight organic compounds cannot be transported directly into bacteria to be catabolized. Thus, bacteria must hydrolyze these organic polymers to smaller molecules before they are incorporated into the cell for subsequent metabolism. This extracellular hydrolytic activity is performed by bacterial extracellular enzymes.

Fluorogenic substrates have been widely used since the early 1980s to assess extracellular enzyme activity in water and sediment (Chróst 1991). Extracellular enzyme activity can be measured by monitoring the hydrolysis of specific fluorogenic substrates which produce highly fluorescent end products, such as 7-amino-4-methylcoumarin (AMC) or methylumbelliferone (MUF) which can be easily quantified by fluorometry (Kanaoka et al. 1977, Hoppe 1983). Use of these fluorometric methods which are more sensitive than colorimetric assays will reduce the incubation times of extracellular enzyme activity assays from days to hours.

It is necessary to measure extracellular enzyme activity rates *in situ* to determine their ecological significance. This last restriction is due to the sensitivity of the enzyme systems to temperature changes (Chróst & Velimirov 1991) and the fact that activity generally decreases rapidly after sampling. When using fluorogenic substrates to quantify extracellular enzyme activity under field conditions, due to logistical reasons

*Addressee for correspondence. E-mail: leek@dfo-mpo.gc.ca

such as restrictions in space, time and fluorometer availability, it is often more convenient to stop the enzymatic reaction in the field by freezing and complete fluorescence analysis in laboratory. Chróst & Velimirov (1991) showed that MUF fluorescence is not affected by freezing. However, reactivation of extracellular activity after thawing prior to fluorescence analysis contributes to an increase in fluorescence which results in overestimating activity. Under most conditions, this experimental error may be minimized by maintaining the thawed tubes at low temperature (<4°C). However, the contribution of reactivated extracellular enzyme activity may be significant in situations where the assay is performed at low *in situ* temperature environments. Heat has been used in previous studies to inactivate extracellular enzymes at the end of incubation (King 1986, Marxsen & Fiebig 1993, Vetter & Deming 1994). However, heating will increase the background fluorescence and thus decrease the sensitivity of the assay. In terms of field work, heating is not a preferred protocol, when compared to chemical stop reagent, as it requires both space and time. Recently, poisoning of samples with mercuric chloride has been suggested as a method to stop extracellular enzyme activity in sea water (Christian & Karl 1995). This chemical did not significantly affect the fluorescence of fresh and/or frozen conserved samples containing MUF. For sediment analysis, Delmas & Garet (1995) used sodium dodecyl sulfate (SDS, 1% w/v final concentration) as a denaturing agent to stop the reaction. Unfortunately, the large volume added (4 times that of the assay volume) decreased the assay's sensitivity by diluting the concentration of liberated AMC. Sediment adsorption may also reduce the concentration of liberated fluorophore in liquid phase. This reduction can be corrected with proper controls but these additional steps in the analytical process may introduce other errors.

We described the use of extreme pH (HCl and NaOH additions) as a rapid method to block extracellular enzymatic aminopeptidase (AMPase) and β-glucosidase (βGase) activity of marine sediments respectively. While acetone and NaOH can be used to desorb AMC and MUF respectively from sediment samples, separation of the liquid phase from the sediment by centrifugation is a simple way to avoid the increasing loss of fluorescence attributed to the adsorption of the fluorophores onto sediment during storage time, even in the presence of added chemicals.

MATERIALS AND METHODS

Sampling and conditioning of marine sediment. The first 10 cm of marine sediment was collected with a Shipek grab at a depth of approximately 50 m from the St. Lawrence Estuary off Rimouski, Québec (Canada) in September 1995 (48° 33.96' N, 68° 33.08' W). Sediments from this region typically contain equal amounts of silt and clay and less than 5 to 10% sand. The sediments were sieved (inner diameter 5 mm) to remove large particles. Sediments were placed under sea water in a reservoir receiving fresh sea water pumped from the St. Lawrence Estuary to maintain microbial activity during storage. Seasonal temperature and salinity of the sea water varied from −1 to 10°C and from 23 to 31‰ respectively during the experimental period.

Extracellular enzyme activity determination. AMPase and βGase activities were determined using a modification of the techniques described by Mayer (1989) and King (1986) respectively. Substrates for AMPase and βGase activity determination were L-leucine-4-methyl-7-coumarinylamide (AMC-Leu) (Fluka Biochemika) and 4-methylumbelliferyl-β-D-glucoside (MUF-Glu) (Sigma Chemical) respectively. Stock solutions of 5.25 mM AMC-Leu and MUF-Glu were prepared in 10% methanol. Standard solutions of 50 µM MUF and 50 µM AMC (Sigma Chemical) were prepared in methanol 100% (v/v). Seven sediment slurry samples were prepared by combining 1.2 cm^3 of well-mixed sediment with 3 ml of filtered (0.22 µm pore size) natural sea water (FSW). The assay was performed in triplicate. Slurry samples were preincubated at 5°C for 30 min before starting the reaction by the addition of 40 µl of the analogous substrate (50 µM final concentration). After 1 h of incubation at 5°C, the reaction was stopped by freezing tubes at −85°C. To provide an estimate of the fluorescence produced by residual extracellular enzyme activity following the incubation period (from freezing to fluorescence reading) and by the slight background fluorescence of the substrate, a slurry sample was incubated in absence of substrate. At the end of the incubation period, the substrate was added to this sample prior to immediate freezing at −85°C. The fluorescence value from this sample was subtracted from each of the triplicate assay values to evaluate the fluorescence produced by extracellular enzyme activity during the 1 h incubation. Adsorption of the fluorophore to sediment was evaluated by adding 50 µl of the standard solution (AMC or MUF) in duplicate to the slurry sample and to 4.2 ml of FSW separately (588 nM final concentration). A blank of FSW and a blank of slurry sample were also included to evaluate their natural fluorescence. Fluorescence of the assay was corrected for adsorption. All controls and sediment slurry samples used in the assay were incubated and frozen under the same conditions. Tubes were thawed just prior to fluorescence readings. For MUF measurement (in the case of βGase activity determination), 100 µl of 2 N NaOH was added to each tube to increase the pH value above 10 for optimal

MUF fluorescence. Tubes were centrifuged at 2500 × *g* for 15 min at 2°C. Fluorescence of the supernatant was determined on a Perkin-Elmer LS50 fluorometer under excitation/emission wavelengths of 365/455 and 370/440 nm for MUF and AMC respectively. All tubes were maintained in an ice bath during the period between thawing and measurement to minimize residual activity. After fluorescence analysis, the sediment pellet within the tubes was dried at 70°C for 36 h. Activity was expressed as the quantity of MUF or AMC produced per gram of dry sediment per hour.

Extracellular enzyme activity stop reagent. To verify the various experimental methods used to stop extracellular enzyme activity, different treatments to terminate enzyme activity were performed on the slurries and all controls before the preincubation time. A regular extracellular enzyme activity determination was performed as before on both treated and untreated slurries. Extracellular enzyme activities of treated slurries were compared to the results obtained with non treated slurries. For AMPase activity, sediment slurries were pretreated with the detergent sodium dodecyl sulfate (SDS), the denaturing agents guanidine isothiocyanate, guanidine hydrochloride and mercuric chloride, organic solvents phenol and acetone, and with acids including formic acid, trichloroacetic acid (TCA) and HCl. For βGase activity, sediment slurries were pretreated with NaOH. The different test treatments are outlined in Tables 1, 2 & 3. When acids (TCA, HCl

Table 1. Relative AMPase activity of sediment slurries following different heat treatments

Treatment	Conditions/time[a]	Relative AMPase activity[b] (SD)
None (control)	–	100.0% (3.0)
Boiling	100°C/15 min	0.4% (0.1)
Heating	80°C/15 min	1.7% (0.3)
Microwave	Max. power[c]/20 s	7.6% (2.8)

[a]Temperature or power/time of treatment
[b]Activity compared to the control (standard deviation, n = 3)
[c]A total of 10 tubes (capacity of 15 ml) containing 4.2 ml of slurry were placed in an 800 W microwave (CEM Corporation, model MDS-81D) set at maximum power

Table 2. Relative AMPase activity and proportion of free AMC in sea water portion of sediment slurries following different chemical treatments. NV: not verified

Treatment	Final conc./added vol.[a]	Final pH	Relative AMPase activity[b] (SD)	Free AMC in sea water[c] (SD)
None (control)	–	7.72	100.0% (3.0)	40.0% (5.5)
Sodium dodecyl sulfate	0.91% (w/v)/200 µl	7.79	37.7% (0.9)	NV
Guanidine thiocyanate	7.45% (w/v)/500 µl	7.71	10.9% (0.5)	60.8% (5.9)
Guanidine hydrochloride	7.45% (w/v)/500 µl	7.37	24.5% (0.3)	NV
Phenol	0.64% (w/v)/500 µl	7.67	85.7% (3.1)	NV
Acetone 1×	10.6% (v/v)/500 µl	7.81	26.6% (0.4)	80.6% (1.1)
Acetone 2×	19.2% (v/v)/1000 µl	7.87	19.6% (1.1)	NV
Formic acid 1×	0.021% (v/v)/10 µl	6.18	9.5% (0.7)	NV
Formic acid 5×	0.104% (v/v)/50 µl	4.36	0.1% (0.2)	23.3% (1.4)
TCA 1×	2.35 mM/50 µl	6.88	36.7% (1.8)	NV
TCA 5×	11.6 mM/50 µl	5.57	0.5% (1.2)	30.1% (2.1)
HCl 1×	2.35 mM/10 µl	6.89	45.7% (3.8)	NV
HCl 5×	11.2 mM/50 µl	5.51	0.01% (0.3)	29.7% (3.4)
Mercuric chloride	4.0 mM/100 µl	7.70	41.2% (3.2)	NV

[a]Final concentration/quantity of stock solution added to 4.2 ml of slurry
[b]Activity compared to the control (standard deviation, n = 3)
[c]Proportion of free AMC in sea water portion of the slurry (standard deviation, n = 3)

Table 3. Relative βGase activity and proportion of free MUF in sea water portion of sediment slurries following NaOH treatments

Treatment	Conditions[a]	Final pH	Relative βGase activity[b] (SD)	Free MUF in sea water[c] (SD)
None (control)	–	7.72	100.0% (9.0)	43.5% (2.7)
NaOH 1×	45.0 mM/100 µl	10.2	6.3% (0.2)	93.3% (0.4)
NaOH 2×	90.0 mM/100 µl	11.0	2.7% (0.4)	100.0% (0.2)

[a]Final concentration/quantity of NaOH stock solution added to 4.2 ml of slurry
[b]Activity compared to the control (standard deviation, n = 3)
[c]Proportion of free MUF in sea water portion of the slurry (standard deviation, n = 3)

or formic acid) were used, 200 µl of buffered solution containing between 0.25 and 0.7 M NaOH (depending on quantity of acid added) and 0.25 M phosphate buffer pH 8.0 (0.5 M KH_2PO_4 adjusted to pH 8.0 with NaOH) was added before centrifugation to obtain a final pH between 7 and 8.

Among all treatments tested, the selected stop reagent for AMPase activity was HCl at a final concentration of 11.2 mM. When this specific treatment was applied, a pH value between 7 and 8 was obtained by adding 200 µl of neutralizing buffer (0.25 M NaOH and 0.25 M of phosphate buffer pH 8.0) before fluorescence reading.

Evaluation of residual post incubation AMPase activity. To evaluate residual activity following thawing and centrifugation, 3 sets of tubes normally used for a single AMPase assay were required. The first set was used to perform a standard AMPase activity determination on fresh sediment slurry as previously described and its value was designated 'original activity' of fresh slurry. The second and third sets of tubes, containing sediment slurries or sea water only (without substrate or MUF/AMC), were frozen at −85°C for 18 h and then thawed. A standard assay was performed on thawed slurries of the second set of tubes at 5°C or in an ice bath to evaluate residual activity after thawing but prior to centrifugation. The third set of tubes was centrifuged at 2500 × *g* for 15 min at 2°C. A standard assay was then performed directly on the supernatant within the centrifuged tubes, containing the sediment pellet, at 5°C or in an ice bath to evaluate residual activity following centrifugation prior to fluorescence reading. Activity of the second and third sets were compared to the 'original activity' of fresh slurry.

To evaluate the effect of chemical stop agents on residual activity, HCl (11.2 mM final concentration) was added to the slurries of the second and third sets of tubes before freezing at −85°C for 18 h. After thawing, acetone (10.6% v/v final concentration) was added to the second and third sets of tubes. A standard assay was performed on thawed slurries of the second set of tubes at 5 and 20°C. Tubes of the third set were centrifuged at 2500 × *g* for 15 min at 2°C and 200 µl of neutralizing buffer (0.25 M NaOH and 0.25 M of phosphate buffer pH 8.0) was added and then centrifuged a second time. A standard assay was then performed on supernatant within the centrifuged tubes, containing the sediment pellet, at 5 and 20°C. Activity of the second and third sets were compared to the 'original activity' of fresh slurry.

Relative fluorescence of AMC as a function of pH. Solutions of FSW were adjusted to different pH levels using concentrated HCl or NaOH (<1.7% v/v of acidic or alkaline solution was added to minimize variations in salinity). AMC was added to these adjusted FSW solutions to yield a final concentration of 658 nM and fluorescence was monitored as previously described.

Standard curves. Stock solutions 250 µM AMC or MUF in methanol were diluted in methanol to produce 125, 50, 37.5, 25, 12.5 and 5 µM working solutions. For AMC, 50 µl aliquots of these working solutions were placed in triplicate in 4.2 ml of FSW with and without the addition of 50 µl of 1 M HCl and/or 200 µl of neutralizing buffer (0.25 M NaOH and 0.25 M of phosphate buffer pH 8.0) and 500 µl of acetone. Similarly, for MUF, 50 µl of the working solutions was placed in triplicate in 4.2 ml of FSW and supplemented with 100 µl of NaOH (2 or 4 M). Fluorescence readings of the 2 fluorophores were carried out as previously described.

RESULTS AND DISCUSSION

AMPase activity termination

Enzyme activity can be terminated by denaturation of the catalytic protein. Disruption of the quaternary structure of an enzyme modifies the conformation of its active site, thus inhibiting its ability to attach to the substrate as required to mediate its transformation into products. Denaturation can also be achieved by physical treatment such as heating or by chemicals. Different compounds were investigated to identify chemicals capable of effectively terminating extracellular enzyme reactions in marine sediment.

Boiling sediments completely inactivated AMPase activity (Hoppe 1993; Table 1). However, heating samples to 80°C for 15 min was sufficient to terminate the enzymatic reaction (Table 1). The absence of observed activity showed that heat treatments were sufficient to denature AMPase in an irreversible manner. However, heating elevated background fluorescence within the sediment by up to 5 times and thus reduced the detection limit of the assay. Detergent, denaturing agents and organic solvents inhibited AMPase activity to varying degrees, but never completely. On the other hand, AMPase activity was completely blocked when sufficient acid was added to reduce the pH of the sediment to <5.57 (Table 2). This result is consistent with a previous study by Mayer (1989) that showed very low AMPase activity in marine sediments when the assay was performed at pH 6.0 (optimum activity observed at pH 9.0). Based on our results, HCl (11.2 mM final concentration) was selected as the optimal stop reagent for AMPase activity in marine sediment.

It has recently been shown that 4 mM of mercuric chloride can be used as a preservative for extracellular enzymes in sea water environments (Christian & Karl 1995). However, in our study with sediment, $HgCl_2$

failed to completely block extracellular enzyme AMPase activity (Table 2). This phenomenon was also reported by Christian & Karl (1995) in water containing particulate material. The mercuric ion may be scavenged by reactive groups associated with the sediment and thus not be available to inhibit extracellular enzyme activity. In contrast to Delmas & Garet (1995) who reported the hydrolytic reaction in marine sediment was stopped by adding SDS (1% final concentration), in our study, SDS (0.91% w/v final concentration) failed to completely stop exoprotease activity. This difference may be attributed to the precipitation of SDS observed in FSW at 5°C (Delmas & Garet 1995 worked at 20°C). In addition, Delmas & Garet (1995) added 8 ml of 1.25% SDS in 2 ml of the assay resulting in a 5× dilution of enzyme and substrate. Such dilution alone may also be sufficient to decrease enzyme activity. In our study, we minimized the volume of added SDS and other chemicals (Table 2) to avoid dilution of the liberated fluorophore. Such dilution decreased the fluorophore detection limit.

AMC fluorescence versus pH

Fluorescence of MUF decreases dramatically at pH levels below 10 (Chróst & Krambeck 1986), but to our knowledge, the effect of pH on AMC fluorescence has not been studied. To verify if AMC fluorescence is dependent of the pH, FSW containing a known amount of AMC was adjusted to different pH levels using HCl or NaOH, and fluorescence was measured. As for MUF (but in a lower range), fluorescence of AMC was pH dependent (Fig. 1). AMC fluorescence begins to decrease at a pH below 3.5 and reaches 1.8% of its maximum fluorescence at pH 1. At this low pH, the NH_2 radical of AMC is converted to NH_3^+. It is known that the NH_3^+ form of different amino substituted cyclic compounds, such as aniline, is non fluorescent in contrast to the substituted NH_2 form (Undenfriend 1962). In most field studies with water or sediment from fresh water or marine environments, AMPase activity is measured at ambient pH levels which rarely fall below pH 3.5. Adding HCl to stop AMPase activity lowers the pH of the sediment slurries up to 5.5 (Table 2) and between 1.9 and 2.5 for AMC in FSW only. Depending on buffer capacity of different sediments, HCl addition may decrease the pH lower than 3.5. Because low pH affects AMC fluorescence, a neutralizing buffer was added to the test samples (sediment slurries and FSW of controls) to readjust the pH to 8 prior to fluorescence reading.

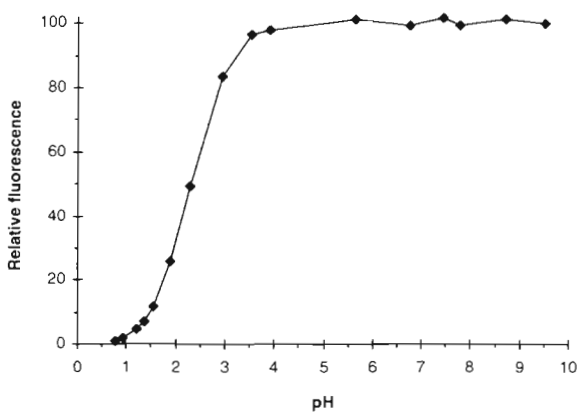

Fig. 1. Relative fluorescence intensity (pH 7.8 = 100) of 658 nM AMC in filtered sea water (FSW) adjusted to different pH

AMC adsorption

Adsorption of the fluorophore to sediment is another cause of error in extracellular enzyme activity determinations. Depending on the physicochemical nature of the molecule and the sediment, adsorption can occur on solid matrix of sediment and on organic matter associated with sediment. Approximately 40% of the AMC remains free in the sea water portion of sediment slurry within our sediment (Table 2). Depending on the type of sediment, as little as 25% of free AMC in the sea water portion has been observed in our laboratory studies. Acidification (including HCl, TCA and formic acid) tends to increase the adsorption of the fluorophore onto sediment while acetone addition (10.6% v/v final concentration) contributed to the liberation of AMC from sediment (Table 2). The presence of organic cosolvents in water increased the affinity of organic compounds (such AMC) for the water-acetone phase and reduced the proportion adsorbed on organic matter (Schwarzenbach et al. 1993).

In the presence of the selected stop reagent (HCl, 11.2 mM final concentration), adsorption of AMC on sediment increased to a point at which only 30% of the AMC was free in the sea water portion of the sediment slurry. This negative effect of HCl could be minimized by the addition of 10.6% (v/v) of acetone which increased the free portion of AMC to about 60% (data not shown). Because higher concentrations provided no additional net benefit, acetone additions at a final concentration of 10.6% (v/v) were selected as the optimal concentration to reduce adsorption of AMC to sediment.

AMC fluorescence versus added chemicals

Fluorescence of AMC increased by 20% when it was added to FSW supplemented with 10.6% (v/v) of acetone (data not shown). No difference was observed

between AMC fluorescence in FSW and in FSW containing HCl and neutralizing buffer. To verify if light emission or excitation of AMC was affected by acetone, fluorescence excitation/emission scans were performed on AMC in FSW containing HCl and neutralizing buffer and with or without acetone. The peak of the emission scan under 370 nm of excitation of AMC in the presence of acetone was at the same wavelength, but fluorescence intensities were higher than in FSW alone (Fig. 2A). The excitation pattern was not the same for AMC in FSW in the presence and absence of acetone (Fig. 2B). While fluorescence intensities were almost identical between 315 and 335 nm in the presence of acetone, fluorescence was elevated at wavelengths above 335 nm and lower at wavelengths below 315 nm. No difference was observed between excitation and emission scans of AMC in FSW only compared to AMC in FSW supplemented with HCl and neutralizing buffer. We concluded that the increase of AMC fluorescence in the presence of acetone was due to changes in the excitability of this molecule. In the absence of changes in sediment background fluorescence, acetone increases the detection limit for AMC in sediments, thus increasing the sensitivity of the AMPase assay.

Linearity ($r^2 > 0.999$) of the AMC standard curve in FSW containing HCl and neutralizing buffer in the presence or absence of acetone was observed at between 50 and 3000 nM AMC. At all concentrations tested, acetone increased AMC fluorescence by about 20% (Fig. 3). No difference was observed between the standard curve of AMC in FSW with or without HCl and neutralizing buffer supplements. A slight precipitate observed following neutralizing buffer additions did not interfere with AMC fluorescence.

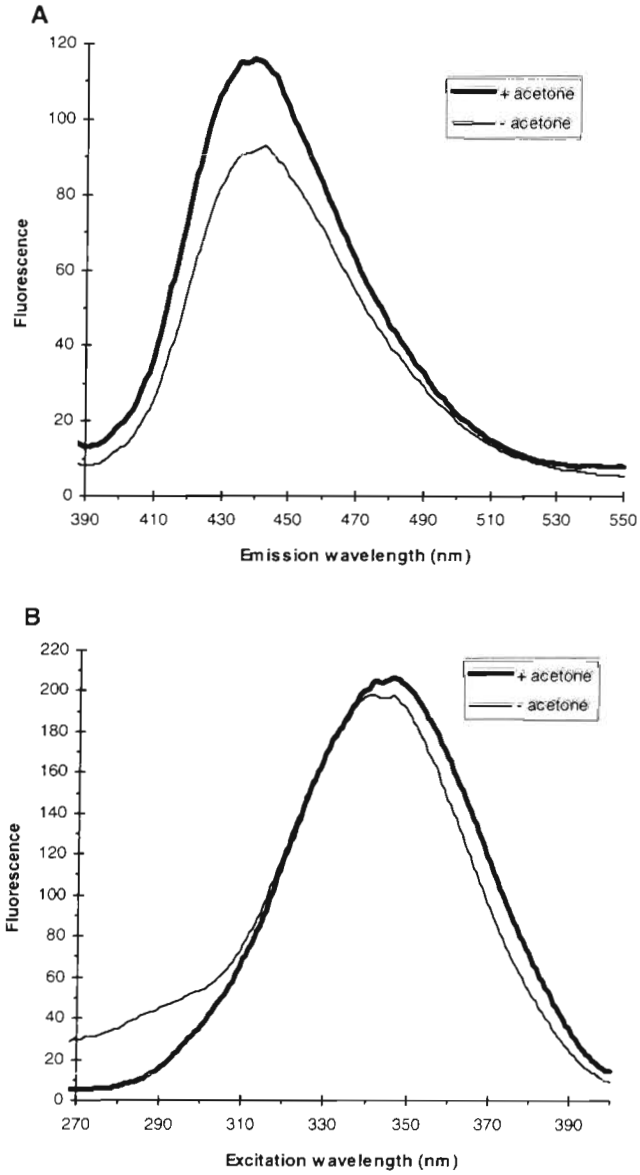

Fig. 2. Relative fluorescence spectra of AMC in FSW supplemented with HCl (11.2 mM final concentration) and neutralizing buffer in the presence or absence of 10.6% acetone (v/v). No difference in spectra was noted between AMC in FSW supplemented with or without HCl (11.2 mM final concentration) and neutralizing buffer (not shown). (A) Emission spectrum of AMC under excitation at 370 nm. (B) Excitation spectrum of AMC under emission at 440 nm

Residual activity

HCl treatment completely inhibited AMPase activity. However, 31% of the activity was recovered when neutralizing buffer was added (Fig. 4A). Acid denaturation can be either reversible or irreversible, depending on the enzyme. In partially reversible denaturation,

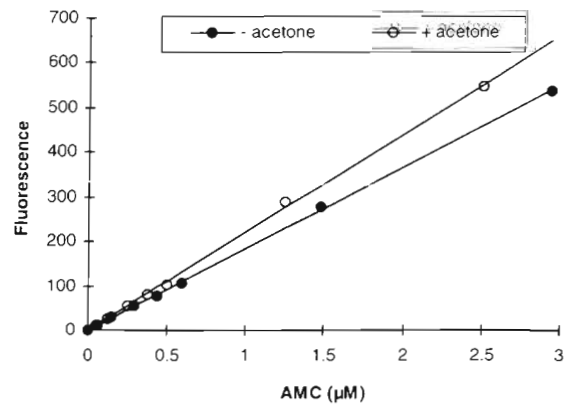

Fig. 3. Fluorescence of AMC vs its concentration in FSW containing HCl (11.2 mM final concentration) and neutralizing buffer with or without 10.6% acetone (v/v)

as we observed here, the enzyme recovers its activity when the pH is returned to its original value. Thus, when HCl is used to terminate the AMPase reaction, the neutralizing buffer should not be added until just prior to fluorescence determination in order to maintain the blocking effect of the acid. In the presence of 10.6% (v/v) acetone, only 7.4% of the enzyme is reactivated relative to the control (Fig. 4A).

Residual post incubation AMPase activity present after thawing and centrifugation operations performed during fluorescence analysis was quantified. Residual post incubation activity following thawing prior to centrifugation was 124% of its original activity (activity of the fresh slurry before freezing) when incubated at 5°C and 55% when incubated in an ice bath. Higher activity observed at 5°C can be explained by the liberation of intracellular AMPase by the freezing process. Even in an ice bath, the residual activity remained elevated before centrifugation (Fig. 4B). However, residual activity dropped to 31 and 15% of its original activity at 5°C and in an ice bath respectively following centrifugation. Centrifugation considerably reduced AMPase activity because an important portion of the extracellular enzyme activity is associated with particles (Rego et al. 1985, Mayer 1989).

Addition of HCl and acetone completely eliminated residual post incubation activity within the sediment slurry at 5°C. However, at 20°C, residual post incubation activity was 5.6 and 1.3% of the 'original activity' before and after centrifugation respectively (Fig. 4B).

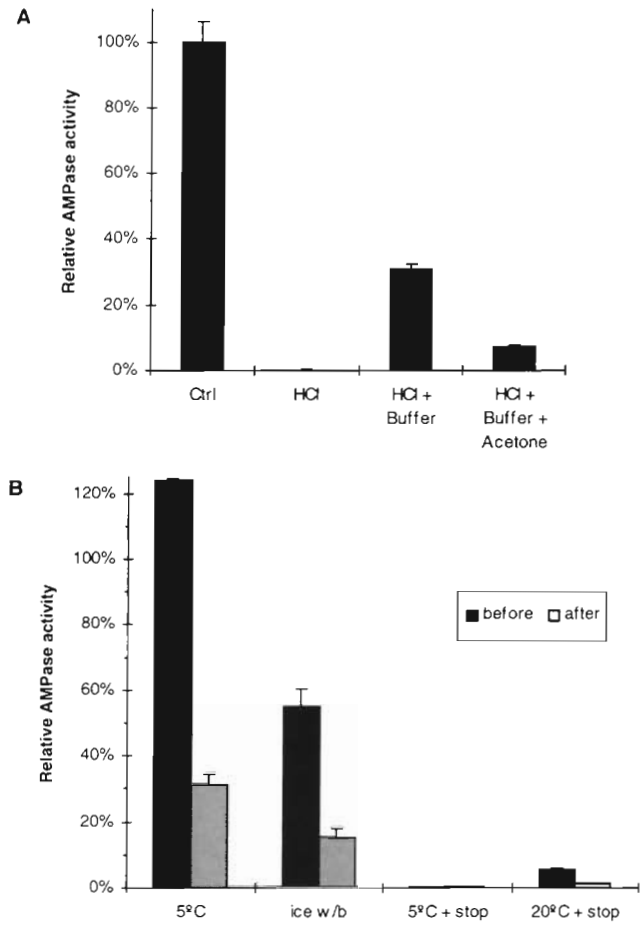

Fig. 4. (A) Effect of neutralizing buffer on AMPase activity in sediment slurry pretreated with HCl. The slurry was pretreated with HCl (11.2 mM final concentration) and then treated with or without neutralizing buffer and acetone. AMPase activity was determined for treated slurries and compared to a non treated control (Ctrl). Error bars represent standard deviation for triplicate samples used in the assay. (B) Evaluation of residual AMPase activity of sediment slurries at different temperature following freezing and thawing in the presence and absence of chemical stop agents (see 'Materials and methods'). This residual activity was measured from the slurry before centrifugation and from supernatant within the tube after centrifugation. Stop: 11.2 mM HCl was added before freezing, 10.6% acetone (v/v) was added after thawing and neutralizing buffer was added after centrifugation. Activity was compared to the AMPase activity of a fresh slurry measured at 5°C before the freezing and thawing process. Error bars represent standard deviation for triplicate samples used in the assay

AMC fluorescence and adsorption vs storage

Fluorescence of AMC frozen in FSW at −20°C in the presence of HCl (90 mM final concentration) or HCl + acetone (10.6% v/v final concentration) did not apparently change over a storage period of 43 d (Fig. 5A). However, in sediment slurries, AMC adsorption to sediment increased during storage at −20°C in the presence and in the absence of HCl (Fig. 5B). This increase may be the result of AMC diffusion into sediment aggregates to saturate binding sites (Schwarzenbach et al. 1993). In the presence of HCl and acetone, centrifugation prior to the freezing reduced AMC diffusion within the sediment pellet for the first 5 d of storage, but this effect gradually disappeared with longer storage time. In contrast, when the supernatant was transferred to a new tube, the portion of free AMC in liquid phase remained constant over the 43 d of the storage (Fig. 5B). The only way to eliminate this increase in adsorption during the long term storage is to separate the liquid phase from the sediment before storage.

Suggested method for AMPase activity determination

Based on our results, we propose the following procedure to terminate the AMPase reaction at the end of AMPase activity determinations using AMC-Leu in marine sediment slurry samples composed of 1.2 cm^3

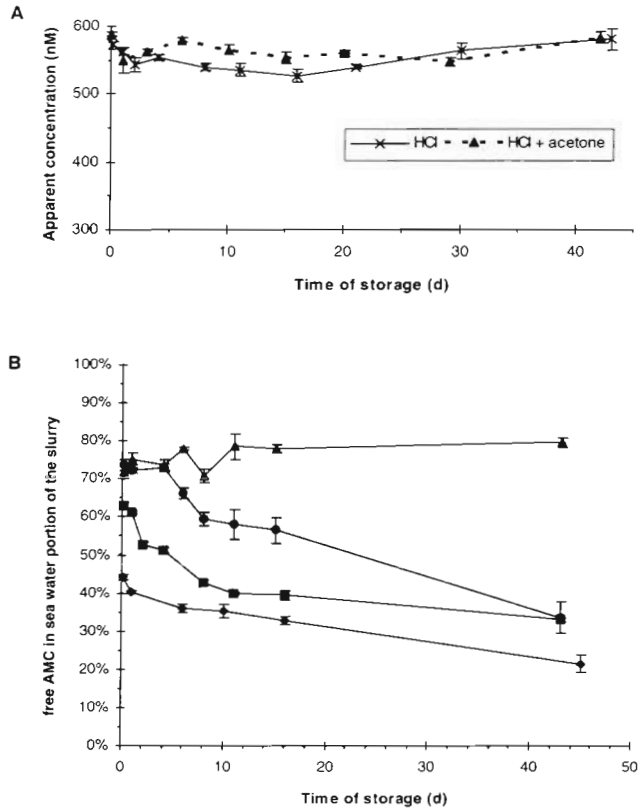

Fig. 5. (A) Stability of AMC in FSW during storage at –20°C in the presence of HCl (11.2 mM final concentration) or HCl + acetone (10.6% v/v final concentration). Apparent concentration is the fluorescence of a frozen standard compared to fluorescence of freshly prepared standard in same solution. Error bars represent standard deviation for triplicate samples. (B) AMC adsorption during storage at –20°C. The following treatments were done before storage: (◆) no treatment (Ctrl); (■) HCl (11.2 mM final concentration) was added; (●) HCl and acetone (10.6% v/v final concentration) was added and tubes were centrifuged; (▲) same as precedent but supernatants were transferred by decantation to new tubes following centrifugation. Immediately before fluorescence analysis, acetone was added to tubes from HCl treatment and neutralizing buffer was added to all treatments but the Ctrl and all tubes were centrifuged. Fluorescence of the supernatant was compared to fluorescence of AMC in FSW only containing the tested chemical. Error bars represent standard deviation for triplicate samples

of sediment and 3 ml of FSW. Following incubation, AMPase activity is blocked by the addition of HCl. For a short term storage (<5 d), the sample can then be frozen at –20°C. Immediately prior to fluorescence analysis, the tubes are thawed in an ice bath and acetone is added to extract sediment-bound AMC. Tubes are centrifuged and neutralizing buffer is mixed to the supernatant. Tubes are recentrifuged and a sample of supernatant is taken for fluorescence determination at room temperature. For long term storage (>5 d), after HCl addition, bound AMC is extracted from the sediment with acetone and tubes are centrifuged. The supernatant is then decanted to new tubes and stored at –20°C. Immediately prior to fluorescence determination, the tubes are thawed in an ice bath and neutralizing buffer is mixed into the supernatant. Tubes are then centrifuged and an aliquot of supernatant is taken for fluorescence determination at room temperature.

This new method for AMPase determination in sediment slurries significantly ($p < 0.01$) reduced the variability within the triplicate used in an extracellular enzyme activity determination. While the conventional method (without using HCl as stop agent and acetone as desorbant) had a coefficient of variation of 12.8% between triplicates (n = 12 activity determinations), this coefficient was reduced to 2.1% (n = 12 activity determinations) using the method described in this study.

βGase study

For βGase activity, we observed that simply increasing the slurry pH to 10, the optimal pH for MUF fluorescence, reduced βGase activity (Table 3). NaOH at final concentration of 90 mM in the slurry (final pH 11) was sufficient to reduce βGase activity to 2.7% of the control. This result is consistent with a previous study that showed low βGase activity in marine sediments at a pH greater than 10.5 (King 1986). A slight precipitate which appeared following NaOH addition to sea water did not interfere with MUF fluorescence. Furthermore, addition of NaOH, at a final concentration of 90 mM, completely eliminated the adsorption of MUF to the sediments (Table 3). The MUF standard curve in FSW with 45 or 90 mM NaOH was linear ($r^2 > 0.999$) over concentrations ranging between 50 and 3000 nM. No differences were observed in either emission or excitation spectra of MUF in the presence of 45 and 90 mM NaOH (data not shown).

Over an experimental period of 60 d, the freezing process for MUF at –20°C in the presence of NaOH (90 mM final concentration) affected its apparent concentration (Fig. 6A). While during the first 10 d the apparent concentration remained constant, a 34% increase was observed between Day 10 and the end of the experimentation (Day 60). While the addition of 90 mM NaOH was sufficient to eliminate the adsorption of MUF by the sediments, the storage process at –20°C produced a return and an increase in the adsorption (Fig. 6B) that may be caused by MUF diffusion in sediment aggregate as we observed for AMC. After a rapid increase between Days 4 and 8, the adsorption remained constant until the end of the

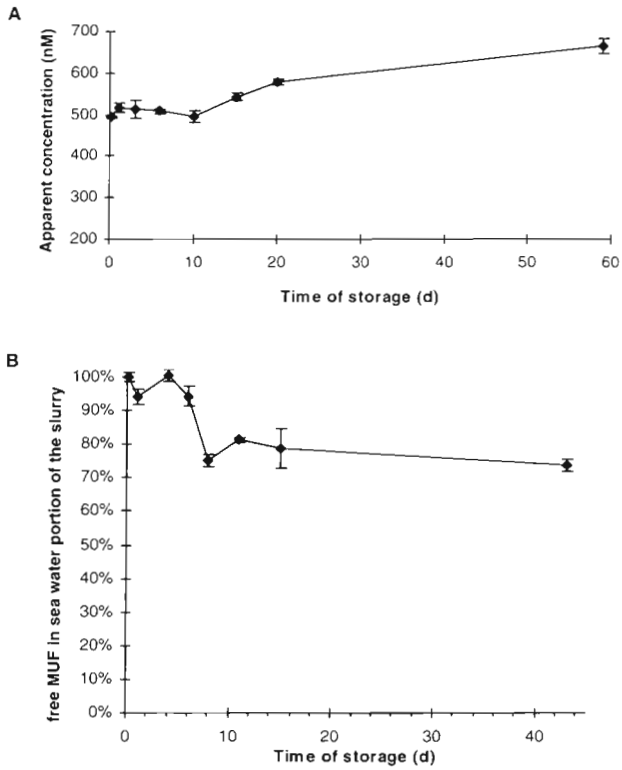

Fig. 6. (A) Stability during storage at −20°C of MUF in FSW in the presence of NaOH (90 mM final concentration). The apparent concentration is the fluorescence of frozen compared to fluorescence of freshly prepared standard in same solution. Error bars represent standard deviation for triplicate samples. (B) Stability of MUF adsorption during storage at −20°C in the presence of NaOH (90 mM final concentration). Fluorescence of the supernatant was compared to fluorescence of MUF in FSW containing NaOH. Error bars represent standard devia tion for triplicate samples

experiment (Day 43). Despite this observation, a large portion of MUF (72%) was present in the liquid phase after 43 d of storage.

Suggested method for βGase activity determination

For βGase, we propose the following procedure to terminate the enzymatic reaction using MUF-Glu in marine sediment slurry samples composed of 1.2 cm^3 of sediment and 3 ml of FSW. Following incubation, βGase activity is blocked by NaOH addition. For a short term storage (<6 d) slurry is immediately frozen at −20°C. Immediately prior to fluorescence analysis, tubes are thawed and centrifuged. Fluorescence is measured in the supernatant. For long term storage (>6 d), following NaOH addition, tubes are centrifuged, supernatants are transferred to new tubes and stored at −20°C. Immediately prior to fluorescence analysis, tubes are thawed and fluorescence is measured in the supernatant. Because of the remaining low βGase activity under alkaline conditions (Table 3), we suggest maintaining tubes in an ice bath upon analytical procedure.

CONCLUSION

When using AMC-Leu as artificial substrate for AMPase in sediment slurries, decreasing the pH below 5.5 using HCl is sufficent to terminate the reaction and acetone addition liberates AMC adsorbed onto sediment. When using MUF-Glu as artificial substrate for βGase in sediment slurries, increasing the pH to about 11 using NaOH is sufficient to terminate βGase activity and eliminate adsorption of MUF to the sediment. These chemical additives considerably reduce the variability among replicates and contribute to increased sensitivity of the AMPase and βGase activity determination.

Although the data presented are produced with 1 type of sediment (see 'Materials and methods'), we successfully used HCl and acetone to block the AMPase reaction and desorb AMC from different types of sediment (e.g. sandy sediment) from various locations.

The major advantage of chemical stop agents is that of simplicity. This treatment is easy and rapid to perform under field conditions, requires minimal materials, and allows the simultaneous treatment of numerous samples. With the absence of residual activity, multiple samples can be thawed and centrifuged at the same time in preparation for fluorescence analysis at room temperature. In conclusion, treatment of samples as described increased the reproducibility, precision and accuracy of the final extracellular enzyme assay.

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