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Measurement of enzyme kinetics in water samples: effect of freezing and soluble stabilizer

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ABSTRACT: The effect of freezing on the kinetics of enzymatic activity in water samples from both marine and limnic environments was tested in order to decide whether analysis of thawed samples could be used to estimate the magnitude of enzyme activity in samples. Samples stored at –20 °C over 1 to 65 d were assayed using the fluorogenic substrates 4-methylumbelliferyl-β-D-glucopyranoside (MUFGlp) and L-leucine-4-methyl-7-coumarinylamide (Leu-MCA). The establishment of enzyme-substrate saturation in thawed samples and the calculation of kinetic parameters for β-D-glucosidase and leucine aminopeptidase showed: (1) a fast decrease in V_{max} and (2) a fast increase of K_m for seawater and freshwater samples. The observed decrease of V_{max} and the loss in enzyme affinity led us to the conclusion that samples frozen at –20 °C could not be used for an estimation of enzyme activity in the water samples. Attempts to stabilize the enzyme with 0.1 % and 1 % (v:v) glycerol at –20 °C brought no significant improvement in obtaining higher enzyme activities. Also, freezing of the samples at –70 °C or a combination of 10 % glycerol and storage at –70 °C gave no satisfactory results in the determination of enzyme activities in thawed samples. In searching for an alternative, an attempt was made to recover fluorescence by adding the fluorogenic substrate, and freezing the sample at –20 °C after the incubation. The recovery of fluorescence (i.e. concentration of product of enzymatic reaction) after 1 and 10 d of storage was 100 % for both β-D-glucosidase and leucine aminopeptidase in seawater and freshwater samples. Fluorescence in water samples was not affected by repeated freezing and thawing processes. The method is recommended for storage of seawater and freshwater samples for enzyme activity measurement which cannot be immediately analyzed.

INTRODUCTION

Information on the functioning of microbial ectoenzyme systems is a prerequisite to understanding of the microbial loop in aquatic ecosystems (Chróst 1990). Artificial fluorogenic substrates, introduced for determination of enzyme activity and specificity in aquatic environments, have been considered a useful and sensitive tool for monitoring degradation processes within this compartment (Hoppe 1983, Somville & Billen 1983, Somville 1984, Chróst & Krambeck 1986, Chróst 1989, Münster et al. 1989). Although the acitivities registered when applying fluorogenic substrates are not necessarily identical to those measured when using natural substrates, the ease of the methodology gives rapid and valuable information on the degradation potential of a specific aquatic system at a given time (Jacobsen & Rai 1988, Chróst 1990). Since it was demonstrated that ectoenzyme activity is mainly associated with the particulate fraction within water samples for both marine and freshwater systems (Hollibaugh & Azam 1983, Hoppe 1983, Rego et al. 1985, Hoppe et al. 1988, Chróst 1989), and that only little activity is detected in the dissolved, extracellular, phase (Chróst & Overbeck 1987, Chróst 1989), the method found a wide application in the field of microbial ecology.

The choice of a method for ectoenzyme assay depends on the enzyme being studied and the information required. If information about the potential activity of the ectoenzyme in the aquatic habitat is required, there are reasons for using both low and high concentrations of the substrate in assay. The enzyme should be substrate-saturated. Possible competition of artificial substrate with natural substrates should be minimized, as should competitive inhibition of the substrate with inhibitors in samples (Chróst 1989, 1990). For such assays, the kinetic approach is recommended,

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which enables calculation of the maximum reaction velocity (V_{max}), and the Michaelis constant (K_m), which indicates the enzyme affinity to the substrate. The kinetic approach, however, requires rate measurements at low and high substrate concentrations to determine first-order (the reaction velocity increases proportionally to the increase in substrate concentrations) and zero-order (reaction velocity remains constant, not affected by the concentration of substrate) enzyme kinetics.

In the process of experimental work and especially during periods of intensive field work, the accumulation of water samples awaiting processing has been experienced by most microbial ecologists. The problem of adequate sample storage and preservation for later enzymatic assay is a central one. Storage facilities in many field stations are restricted to cooling units with a limited temperature range of +4 to −20 °C; in some cases units achieving −70 °C are available. In this study, we tested whether water samples which were immediately frozen and stored at −20 or −70 °C could still be used for an evaluation of enzyme kinetic parameters (V_{max} and K_m) and activity using fluorogenic substrates. We tested whether changes in enzyme activity were predictable in manner, thus allowing the establishment of correction curves (Olsson 1983). We also determined the effect of soluble additive (glycerol) for the stabilization of enzymes (Torchilin & Martinek 1979) in water samples.

MATERIAL AND METHODS

Sampling. Seawater samples were taken during high tide from the intertidal region of the Baltic Sea near the fishing harbour at Lippe and at Scharbeutz (Schleswig-Holstein, Germany); freshwater samples were taken from the highly eutrophic brackish lake Große Binnensee, situated close to the sea, as well as the moderately eutrophic lake Plußsee. Sampling was done with sterilized 5 l PVC containers and achieved within 1 h for both localities. The containers were immediately taken to the laboratory and water samples were filtered through a 56 µm plankton net to avoid interference of large particles.

Subsamples of both seawater and lake water were taken for determination of bacterial abundance (AODC; Hobbie et al. 1977) and chlorophyll *a* (Wetzel & Likens 1979). Dissolved organic carbon (DOC) in 0.2 µm filtered (Nuclepore membrane filters) samples was measured with the use of a Beckman organic carbon computational system (Tocomaster model 915-B). Inorganic nutrients and other physicochemical parameters (pH, conductivity, temperature) were analyzed according to Strickland & Parsons (1972). Subsamples (4.5 ml) were used for measurements of enzyme initial activity (within 1.5 h after sampling). Portions of 500 ml of both waters (i.e. minimum volume necessary for further enzyme assays) were transferred into sterilized PVC containers and immediately frozen and stored at −20 or −70 °C. Frozen samples of both sea and freshwater were thawed after 1, 3, 10, 24 and 65 d and assayed for enzyme activity as described below.

Determination of enzyme activity. β-D-Glucosidase activity (βGlc) was measured from the increase in fluorescence as the nonfluorescent substrate 4-methylumbelliferyl-β-D-glucopyranoside (MUFGlp; Sigma) was hydrolyzed enzymatically yielding the fluorescent product 4-methylumbelliferone (MUF). The amount of substrate cleaved was equivalent to the amount of highly fluorescent MUF anion generated after hydrolysis. To measure leucine aminopeptidase (Leu-amp) activity we used the model substrate L-leucine-4-methyl-7-coumarinylamide hydrochloride (Leu-MCA; Fluka), yielding the fluorescent product 7-amino-4-methylcoumarin (AMC) after hydrolysis of the substrate.

Stock solutions of MUFGlp and Leu-MCA were prepared to a concentration of 2 mM each in ultrapure water (Millipore Q) and stored at −20 °C. The stock MUFGlp and Leu-MCA solutions were thawed at room temperature and diluted to 0.125, 0.250, 0.5 and 1.0 mM immediately before assay. For all enzyme assays, 0.5 ml of substrate solutions were added to triplicate 4.5 ml water samples yielding final MUFGlp and Leu-MCA concentrations of 12.5, 25, 50, 100 and 200 µM. Subsamples for determination of dissolved, free (extracellular) enzyme activity were filtered (0.2 µm Nuclepore membrane filters; < 0.2 atm) and assayed separately as described above. All samples were incubated at room temperature (20 °C) in the dark for 2 to 20 h depending on enzyme activity.

Background fluorescence of the sample water and fluorescence of the substrates were measured. Triplicate 4.5 ml water sample blanks were kept as the incubated samples, and 0.5 ml of each substrate concentration were added at the end of the incubation time. The fluorescence of blanks was measured immediately after substrate supplementation (time zero) and subtracted from the sample fluorescence.

MUF fluorescence was measured at 365 nm excitation and 460 nm emission wavelength; AMC fluorescence was measured at 380 nm excitation and 440 nm emission. The spectrofluorometer (Kontron SFM-25) was calibrated with a standard solution of MUF (Sigma) or AMC (Fluka) prepared in 0.2 µm filtered (Nuclepore) water sample.

Calculation of enzyme kinetic parameters. The kinetic parameters (V_{max} and K_m) were calculated from

Table 1 Physicochemical properties of the sampled habitats

Parameter	Lakes		Baltic Sea coast	
	Große Binnensee	Plußsee	Lippe	Scharbeutz
Temperature (°C)	6.5	16.5	5.0	13.2
pH	7.9	8.1	8.3	9.0
Conductivity (µS cm^{-1})	725	253	12 970	12 150
P-PO_4^{3-} (µg P l^{-1})	158	25	7.5	8.2
N-NO_3^- (µg N l^{-1})	35	15	5.8	5.2
N-NH_4^+ (µg N l^{-1})	25	145	1.5	3.5
SiO_2 (µg Si l^{-1})	255	35	155	165
DOC (mg C l^{-1})	24.5	12	2.7	5.5
Chlorophyll *a* (µg l^{-1})	68.5	15	1.8	8.5
Bacteria (cells ml^{-1})	4.45×10^7	8.01×10^6	1.01×10^6	2.89×10^6

the direct plot of reaction velocity (v) versus substrate (S) concentration using the computer program 'Enzfitter' (Elsevier Biosoft, United Kingdom, software for IBM PC) to determine the best fit of the rectangular hyperbola (Leatherbarrow 1987).

RESULTS AND DISCUSSION

Characterization of sampling sites

The characteristics of the sampled marine and freshwater environments are given in Table 1. It can be seen that the parameters of the water column sampled from the Baltic Sea at Lippe reflect a situation of low productivity, contrary to Große Binnensee which was sampled the same day during November 1989. Bacterial numbers in Große Binnensee were highest (4.45×10^7 cells ml^{-1}), and chlorophyll *a* and DOC concentrations amounted to 68.5 µg l^{-1} and 24.5 mg C l^{-1}, respectively, indicating an event of high primary production in November. In Plußsee (May 5, 1990) bacterial density reached 8.01×10^6 cells ml^{-1}. Chlorophyll *a* and DOC concentrations were 15 µg l^{-1} and 12 mg C l^{-1}, respectively. Again, seawater samples from the Baltic Sea (Scharbeutz) sampled on May 5, 1990 had a lower bacterial density and lower chlorophyll *a* and DOC concentrations than Plußsee water.

Effect of sample freezing at –20 °C on enzyme kinetic parameters

In unfrozen, fresh seawater samples V_{max} of β-glucosidase (βGlc) was low (5.08; SD ± 4.8 nmol l^{-1} h^{-1}) and decreased to 2.62 ± 0.2 nmol l^{-1} h^{-1} after freezing for 24 h and thawing, while K_m changed from 20.34 ± 1.9 to 21.7 ± 5.3 µM. The decrease in activity corresponds to a loss of 48.4 % of the initial V_{max} of βGlc. In the dissolved, extracellular fraction, an increase of βGlc activity from 12.1 to 18.2 % was noticed. In all following samples thawed after 3, 10, 24 and 65 d, up to 40 h incubation times were necessary to obtain a detectable activity. Moreover, the response of enzyme activity to increased substrate concentrations did not follow Michaelis-Menten enzyme kinetics.

β-Glucosidase activity in thawed freshwater samples from Große Binnensee was also characterized by a decrease of V_{max} and an increase of K_m (Fig. 1). Although the decrease of V_{max} was low (from 43.3 ± 1.7 to 38.7 ± 2.6 nmol l^{-1} h^{-1}) after 24 h storage at –20 °C, there was a drastic loss in enzyme affinity indicated by an increase (from 21.3 ± 3.2 to 104.6 ± 14 µM) of the Michaelis constant that reached 125 ± 15 µM after 65 d of storage. At this time, V_{max} was reduced to one third of its initial value of the unfrozen sample. The decrease of V_{max} and the loss of βGlc affinity to substrate was accompanied by an elevation of enzyme activity in the extracellular fraction of the samples (see Fig. 4). The major shift from bound to dissolved enzyme activity

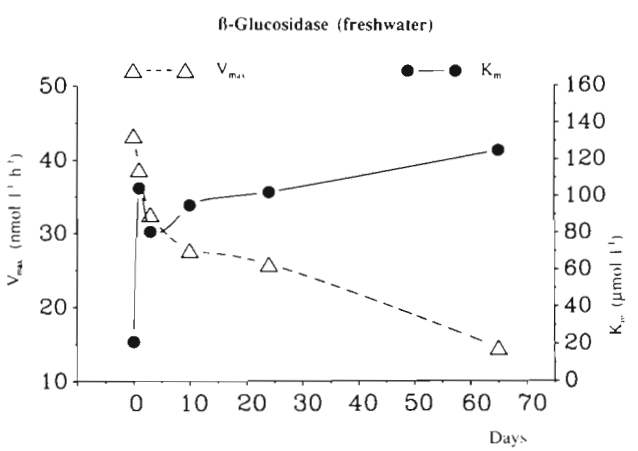

Fig. 1. Changes of V_{max} and K_m of β-D-glucosidase (βGlc) in thawed freshwater samples from lake Große Binnensee (samples were kept frozen at –20 °C from 1 to 65 d)

took place when thawing the first sample after 24 h. An increase of extracellular enzyme activity from 11.2 to 32.7 % was observed. Highest dissolved, extracellular enzyme activity (41.2 %) was noted after 24 d storage of the frozen samples.

Leucine aminopeptidase (Leu-amp) activity in seawater samples (Fig. 2) was 5 times lower than in freshwater samples (Fig. 3), but V_{max} values showed the same pattern of decrease in frozen samples as those from Große Binnensee. Increase of K_m values indicated a loss in enzyme substrate affinity and increased from 206 ± 12 in unfrozen samples to 482 ± 139 µM in thawed samples. The changes in extracellular enzyme activity in seawater samples was similar to that of freshwater samples and increased from 4.51 % to a maximum of 32 % after 24 d storage of frozen samples (Fig. 4). V_{max} of Leu-amp in lakewater was the same in 24 h frozen samples as in unfrozen samples, but decreased after 3 d storage of frozen samples (Fig. 3). In samples thawed after 65 d, enzyme V_{max} had dropped to 556 ± 53 nmol l^{-1} h^{-1}. This was 11.5 % of the initial value. Michaelis constant changed markedly after 24 h freezing, showing lowest affinity (391 ± 79 µM) in 3 d old samples. Although K_m decreased again its value remained above 150 µM for samples kept frozen for 10, 24 and 65 d. As for βGlc, an important increase of Leu-amp extracellular activity (4.62 to 26 % increasing to a maximum of 37 % after 24 d) was noted after 24 h of freezing (Fig 4).

Our observations agree well with studies by Daniels & Glew (1984) and demonstrated that these unpredictable patterns of the non-linear decrease of enzyme V_{max} and the increase of K_m do not allow extrapolation to estimate the magnitude of the original enzyme kinetic parameters of fresh samples. Contrary to findings of other authors, stating that freezing did not significantly influence enzymatic activity (Meyer-Reil 1991) we can neither recommend storage of water samples at −20 °C for later enzymatic assay, nor encourage the establishment of a correction curve for the frozen samples (Olsson 1983).

Determination of enzyme V_{max} and K_m parameters is recommended when comparing enzyme activities measured at different sites and sampling time (Hoppe 1983, Chróst 1989, Chróst & Overbeck 1987, 1990, Chróst et al. 1989). Natural water samples consist of a variety of organic compounds that fluctuate significantly (Münster & Chróst 1990) and can compete with artificial substrate for enzyme active center. Therefore, the saturation of enzyme measured by means of artificial substrate is necessary to avoid the possible competitive and/or non-competitive inhibition of its activity by natural substrates (Hoppe 1983, Somville 1984, King 1986, Chróst 1990). When the active center of enzyme is saturated with an artificial substrate, V_{max} is an independent parameter (i.e. reaction velocity does not rely on changing substrate concentrations) and thus can be compared among different enzyme assays of varying water samples. The magnitude of K_m value reflects both the presence of variable pools of natural enzyme substrates (which compete with an artificial substrate) in water samples and the affinity of assayed enzyme to its substrate.

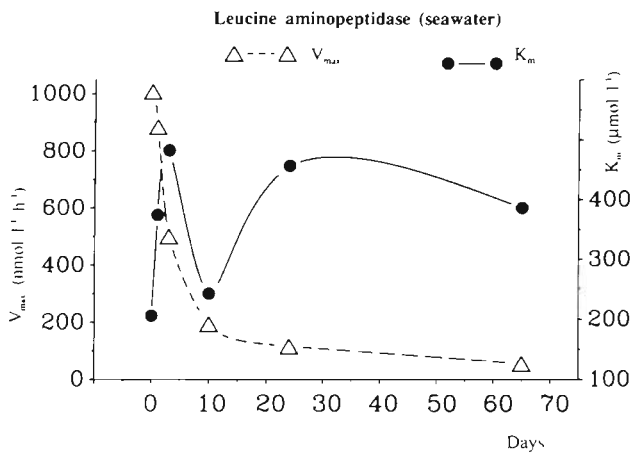

Fig. 2. Changes of V_{max} and K_m of leucine aminopeptidase (Leu-amp) in thawed seawater samples from the Baltic Sea (samples were kept frozen at −20 °C from 1 to 65 d)

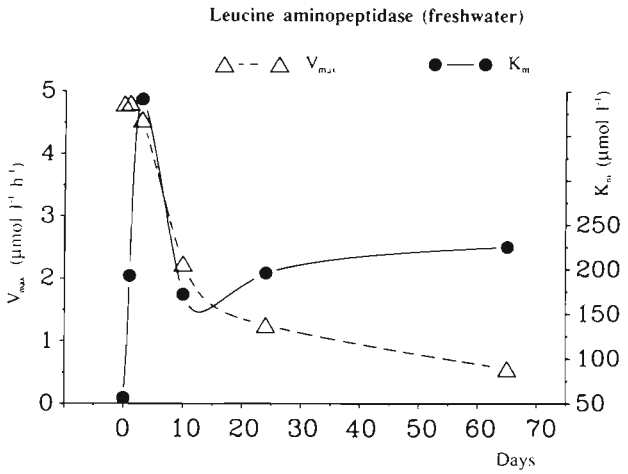

Fig. 3. Changes of V_{max} and K_m of leucine aminopeptidase (Leu-amp) in thawed freshwater samples from lake Große Binnensee (samples were kept frozen at −20 °C from 1 to 65 d)

Several interacting processes taking place during freezing and thawing of water samples may cause the measured decrease in enzyme activity and affinity. The major effect of freezing is that water availability for microbes is substantially reduced, thus lowering microbial activity. In addition, the rate of chemical reactions is slowed down. However, at a temperature as low as −40 °C, up to 5 % of the water can remain unfrozen (Charm & Matteo 1971). This unfrozen water is mostly

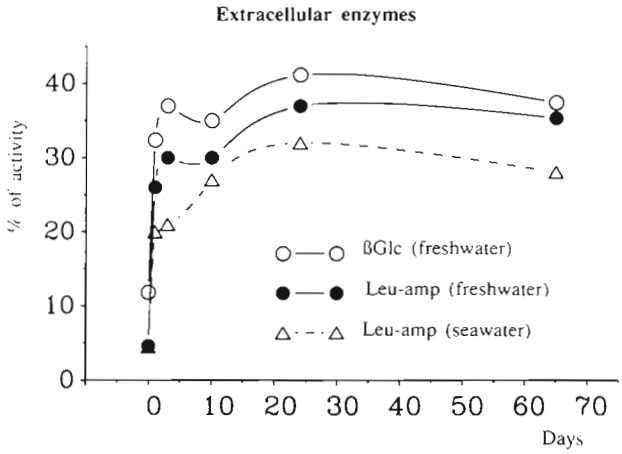

Fig. 4. Dissolved, extracellular enzyme (Leu-amp, βGlc) activity (0.2 µm filtrate) in thawed seawater and freshwater samples which were kept frozen at −20 °C from 1 to 65 d (expressed as percent of the total initial V_{max} of unfiltered sample)

in close association with particles and thereby behaves differently from 'free' water in this respect. In these sites, microbial activity may continue, even though at very low rate. Extracellular proteases, present in these sites, may hydrolyze enzymes and this process may also occur during the thawing of the samples. A further process to consider is the exposure of enzymes to shearing during freezing and thawing. Damage or modification of the protein's tertiary structure may occur, resulting in a change of the binding site structure, thus leading to a decrease or even complete loss in substrate binding capacity. Alternatively, a modification of the binding site could result in binding of other substrates, being structurally similar to artificial substrates (MUFGlp, Leu-MCA), thereby blocking the enzyme and leading to enzymatic inhibition.

An overall trend was a shift of enzyme activity towards the dissolved fraction. This could be explained by the action of shear forces, separating cell- or particle-bound enzymes from their original sites during thawing, or by mechanical rupture of cells, releasing both ectoenzymes and intracellular enzymes into the DOM pool. The decrease of total enzyme activity was therefore often accompanied by a corresponding increase of the activity in the dissolved, extracellular fraction. However, this observation cannot be generalized since in many samples the decrease of enzyme activity was not proportional to an increase of the activity in the dissolved fraction. In some cases, the activity in the dissolved fraction was higher than the relative decrease of total activity from the initial activity.

Modification of enzyme activity and affinity during freezing and thawing water samples may result from the molecular structure and physical properties of water (Stillinger 1980). Water expands when freezing so that the ice formed requires more volume than the liquid phase. This disrupts microbial cells which start to leak and release intracellular enzymes and surface-bound ectoenzymes during thawing of the sample. Moreover, a variety of organic constituents present in water samples additionally act as surfactants and reduce surface tension thus leading to liberation (Chróst 1991) and reconfiguration of enzyme molecules and loss of enzyme activity and affinity to substrate.

Effect of glycerol and low temperature freezing on enzyme activity

At present, it is not clearly understood why some enzymes are stabilized by freezing while others are not. Soluble additives such as polyalcohols (e.g. glycerol) have been used to reduce enzyme inactivation due to freezing (Daniels & Glew 1984). In the following experiments we tested the effect of glycerol as a stabilizing additive during the freezing of both seawater and freshwater samples. We added 0.1 % and 1 % (v:v) glycerol to the water samples and froze them at −20 °C for 48 h. After thawing and temperature stabilization (20 °C) we incubated the triplicate samples for 2 to 4 h at final MUFGlp and Leu-MCA concentrations of 100 and 200 µM at 20 °C.

The enzymatic activity of β-glucosidase (Table 2) in both seawater and lakewater samples was far below the initial enzyme activity determined after sample collection. Highest enzymatic activity was 1.25 nmol l^{-1} h^{-1} in seawater samples with 0.1 % glycerol, amounting to 32 % of the initial activity. The enzyme activity of glycerol frozen samples was only 6.4 % greater than controls. Freshwater samples, treated with 1 % glycerol, had the highest enzyme activity (1.97 nmol l^{-1} h^{-1}). However, this was 31 % of the initial activity. Glycerol treatment increased enzyme activity only 11 % above frozen control samples.

Initial enzyme activity of Leu-amp was higher in both seawater and lakewater samples as compared to β-glucosidase, but enzyme activity in glycerol treated samples was still low after thawing (Table 3). Highest activity for seawater samples was observed for 0.1 % glycerol treated samples and was 34.6 % of the initial activity. The addition of 1 % glycerol to lakewater samples increased to 51 % of the initial activity. Nonetheless, the increase in activity was only 10 % as compared to the control sample.

The effect of sample freezing and storage at −70 °C on enzymatic activity is presented in Table 4. Highest activity was obtained for βGlc and corresponded to 80 % of the initial activity. In the remaining samples β-glucosidase activity ranged from 26 to 68 %.

Table 2. Change of β-glucosidase activity in seawater (Baltic coast; Scharbeutz) and freshwater (lake Plußsee) samples after storage at –20 °C for 24 h and treatment of samples with 0.1 % and 1.0 % glycerol before freezing. Values in parentheses are ± standard deviations of means; control: water sample without glycerol

Substrate concentration (μM)	Initial activity (nmol l^{-1} h^{-1})	Activity after freezing and thawing (nmol l^{-1} h^{-1})			% of initial activity		
		Control	0.1 % glycerol	1.0 % glycerol	Control	0.1 % glycerol	1.0 % glycerol
Seawater samples							
100	3.86 (± 0.18)	1.00 (± 0.17)	1.25 (± 0.05)	0.70 (± 0.13)	25.9	21.3	18.1
200	5.74 (± 0.35)	1.31 (± 0.12)	1.51 (± 0.27)	1.08 (± 0.13)	22.8	26.3	18.8
Freshwater samples							
100	6.29 (± 0.63)	1.25 (± 0.0)	1.32 (± 0.04)	1.97 (± 0.24)	19.8	20.9	31.3
200	10.53 (± 0.03)	2.07 (± 0.23)	1.70 (± 0.17)	2.87 (± 0.22)	19.6	16.1	27.2

Table 3. Change of leucine aminopeptidase activity in seawater (Baltic coast; Scharbeutz) and freshwater (lake Plußsee) samples after storage at –20 °C for 24 h and treatment of samples with 0.1 % and 1.0 % glycerol before freezing. Values in parentheses are ± standard deviations of means; control: water sample without glycerol

Substrate concentration (μM)	Initial activity (nmol l^{-1} h^{-1})	Activity after freezing and thawing (nmol l^{-1} h^{-1})			% of initial activity		
		Control	0.1 % glycerol	1.0 % glycerol	Control	0.1 % glycerol	1.0 % glycerol
Seawater samples							
100	707 (± 25.7)	184 (± 13.5)	213 (± 1.1)	157 (± 20.2)	25.9	30.1	22.2
200	1075 (± 41.9)	331 (± 30.8)	372 (± 8.3)	290 (± 27.1)	21.9	34.6	26.9
Freshwater samples							
100	1189 (± 90.9)	229 (± 13.6)	229 (± 13.0)	397 (± 13.9)	19.2	19.3	33.4
200	1929 (± 84.1)	776 (± 72.2)	718 (± 61.5)	984 (± 48.0)	40.2	37.2	51.1

Aminopeptidase activity was low for all samples of both seawater and lakewater and remained below 3.8 % of the initial activity after 48 h storage at −70 °C.

The data in Tables 2 to 4 indicate that the addition of glycerol and freezing of the samples at −70 °C did not significantly increase the recovery of βGlc and Leu-amp activity. In a last experiment we combined the effect of freezing at −70 °C with the addition of 10 % glycerol to the water samples (Table 5). For both enzymes the loss of activity ranged from 30 to 40 % of the initial activity within 24 h and decreased to less than one-third after 7 d of freezing.

Effect of sample freezing on MUF and AMC fluorescence produced after incubation of samples with MUFGlp and Leu-MCA

Coastal seawater (Baltic Sea) and freshwater samples (Plußsee) were incubated at 5 concentrations of MUF-Glp and Leu-MCA (see 'Materials and methods') for 3 to 5 h. After incubation the fluorescence of enzymatically released MUF and AMC was measured to determine V_{max} and K_m of β-glucosidase and leucine aminopeptidase at time zero. Immediately after the fluorescence measurements the samples were frozen and kept at −20 °C for 1 and 10 d. Fig. 5 & 6 show that recovery of MUF and AMC fluorescence after 1 and 10 d was 100 % for β-glucosidase and leucine aminopeptidase, respectively, in both seawater and freshwater samples. The standard deviations of V_{max} values were low and the coefficients of variance varied between 2 and 6 %. MUF and AMC fluorescence in water samples was not affected by repeated freezing and thawing processes. No significant loss of fluorescence was detected over 10 d. Calculated βGlc and Leu-amp V_{max} remained almost constant in comparison to values determined at time zero. The same was true for estimated K_m values which were nearly constant for all measurements in −20 °C frozen samples stored for 1 and 10 d.

Conclusions – recommended assay

The fluorescence recovery data allow us to recommend the following method for the treatment of water

Table 4. Change of β-glucosidase and leucine aminopeptidase activity in seawater (Baltic coast; Scharbeutz) and freshwater (lake Plußsee) samples after storage at −70 °C for 48 h. Values in parentheses are ± standard deviations of means

Substrate concentration (μM)	Activity Seawater (nmol l^{-1} h^{-1})	% of initial	Freshwater (nmol l^{-1} h^{-1})	% of initial
	β-Glucosidase			
100	1.04 (± 0.12)	26.9	4.33 (± 0.64)	68.8
200	2.57 (± 0.34)	44.7	8.47 (± 0.12)	80.4
	Leucine aminopeptidase			
100	11.10 (± 1.66)	1.5	49.97 (± 0.98)	4.2
200	19.31 (± 2.73)	1.8	74.97 (± 1.35)	3.8

Table 5. Effect of 10 % glycerol on leucine aminopeptidase and β-glucosidase activity in seawater (Baltic coast; Scharbeutz) and freshwater (lake Plußsee) samples stored at −70 °C. Enzyme activity was assayed at 200 μM of substrate. Values in parentheses are ± standard deviations of means

Days of storage	Activity (nmol l^{-1} h^{-1}) Seawater	Freshwater	% of initial activity Seawater	Freshwater
		Leucine aminopeptidase		
0	387 (± 7)	954 (± 5)	97.2	97.6
1	226 (± 18)	457 (± 25)	56.8	46.8
7	161 (± 23)	345 (± 22)	40.4	35.3
		β-Glucosidase		
0	178 (± 7)	645 (± 9)	95.5	94.3
1	113 (± 11)	271 (± 13)	60.6	39.6
7	67 (± 16)	156 (± 17)	35.9	22.8

samples to be stored for measurements of enzyme kinetics. The following procedure is proposed: (1) incubate triplicate 4.5 ml samples with 0.5 ml fluorogenic substrate solution (at least 5 concentrations; see 'Material and methods') in plastic vials (8 to 10 ml vials for radioactivity determination by liquid scintillation counting method are very convenient); (2) after incubation, freeze the samples at −20 °C; (3) store frozen samples until analysis; and (4) thaw the samples in a water bath (15 to 20 °C) and read the fluorescence immediately after thawing.

The advantage of the method is that all processes taking place during sample freezing and thawing which lead to a decrease of enzyme activity do not affect the intensity of fluorescence of final products of substrate hydrolysis. Product fluorescence results only from hydrolysis of the substrate at the temperature during the incubation time. The disadvantage of the

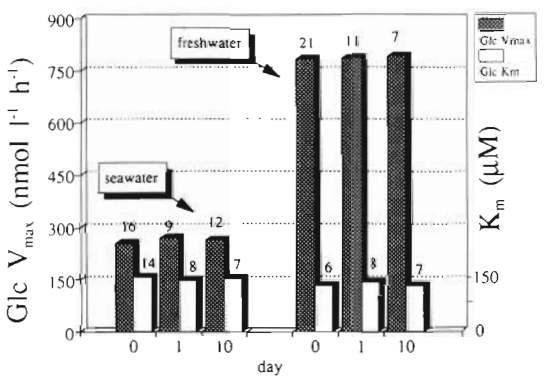

Fig. 5. Kinetics (V_{max} and K_m) of β-glucosidase (Glc) in thawed seawater and freshwater samples. After substrate addition the samples were incubated, immediately frozen and kept at −20 °C, thawed after 1 and 10 d, and the fluorescence of product was measured. Numbers above the bars are ± standard deviations

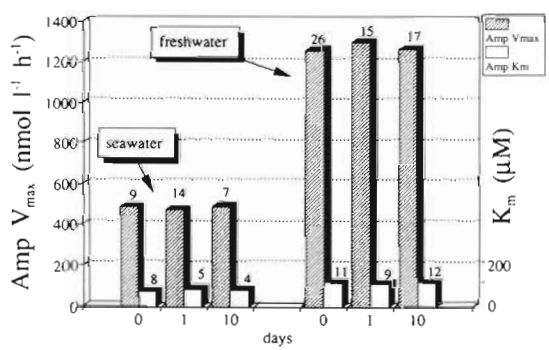

Fig. 6. Kinetics (V_{max} and K_m) of leucine aminopeptidase (Amp) in thawed seawater and freshwater samples. Details as in Fig. 5

method is primarily a theoretical one. After thawing of the water samples, enzyme activity may hydrolyse some of the remaining substrate, thus leading to an overestimation of V_{max}. However, we did not observe increase in fluorescence during thawing of samples and measurement procedure within 30 to 45 min, since the V_{max} was the same as for time zero (Figs 5 & 6). The high recovery of fluorescence of product of substrate hydrolysis indicates that it may remain stable over time periods longer than 10 d. Longer storage duration experiments are still being evaluated, but preliminary results indicate that the product fluorescence remains stable over at least 6 wk.

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