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Alkaline Phosphatase Activity in Seawater: Influence of Reaction Conditions on the Kinetic Parameters of ALP

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Alkaline phosphatase (ALP) is an extracellular enzyme enabling utilisation of phosphomonoesters as the source of inorganic phosphate (P_i) required for the maintenance of cellular metabolism. Its catalytic properties have been extensively studied in aquatic environments in an attempt to describe its influence on phosphorous cycling in natural waters. Despite its significance and frequent determination, no standard method has been developed so far. In order to examine the effect of various reaction conditions on the kinetic properties of ALP in seawater, the stability of ALP in chloroform-stabilised samples has been studied in addition to the influence of the buffer type, its concentration and pH, the metal-ion content, P_i and incubation time. It might be concluded that the kinetic properties of ALP are significantly influenced by the buffer pH, the concentration of Mg^{2+} and Zn^{2+} ions and the incubation period, requiring a precise definition of these reaction parameters in determining a standard method for the measurement of ALP activity in water media.

Key words: aquatic enzymes, alkaline phosphatase, kinetic properties, method optimisation.

INTRODUCTION

Alkaline phosphatase (ALP, EC 3.1.3.1) is present in almost all forms of life, from algae, bacteria, and protozoa to higher plants and animals, but

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also as a free enzyme in natural waters and sediments.[1] Its exact biological function is not yet known, however it has been suggested that ALP may be associated with the transport of inorganic phosphate (P_i) into the cell. It is an adaptive enzyme whose biosynthesis is controlled by the concentration of P_i in the medium[2,3] and its activity is often used as an indicator of the P_i requirement in the planktonic community.[4–8] Influence of various organic phosphates on the biocenosis depends on their availability to biological degradation, which is determined by the kinetic properties of the planktonic extracellular enzymes. Investigations are being carried out to determine the distribution of phosphatase activity between different planktonic fractions and free phosphatases. A variety of organic phosphomonoesters may be hydrolysed by ALP, however the natural substrates for ALP in water and the rate of their hydrolysis have been only partially described.[9–11] Despite the significance of establishing the kinetic properties of ALP and measuring its activity in water, no standard method for accurate determination of its kinetic parameters has been established to date.

Phosphatase activity can be monitored by measuring the hydrolysis rate of organic phosphorus compounds found in the natural environment. This method may be applied to measure the actual activity of free phosphatases and to determine the concentration of phosphates susceptible to enzymatic hydrolysis. However, it does not permit accurate activity measurements of enzymes bound to the planktonic organisms, since the required sample stabilisation with chloroform increases the concentration of natural substrates by removing them from the planktonic cell wall. On the other hand, the cells in the sample may assimilate free orthophosphate, and hence obstruct its measurement. Alternatively, ALP activity can be determined by measuring the hydrolysis rate of an artificial substrate under controlled reaction conditions.[12] Such results are suitable for a quantitative estimation of the effects that ecological parameters may have on phosphatase activity. During longer field investigations, the reaction must be stopped after an optimal incubation period, and the released product measured when possible. Occasionally, the reaction is terminated by addition of NaOH, causing precipitation of organic matter in the seawater sample and acceleration of spontaneous *p*-nitrophenyl phosphate (*p*NPP) hydrolysis, which greatly hampers activity measurements. Although a vast amount of data is available, kinetic results obtained from measurements performed under different reaction conditions are not comparable. In an attempt to approach the standard conditions for the measurement of ALP activity in seawater samples, the influence of various reaction parameters on the kinetic properties of ALP has been investigated.

EXPERIMENTAL

Materials

Trizma base and *p*-nitrophenyl phosphate (*p*NPP) were purchased from Sigma (St. Louis, USA). $MgSO_4 \times 7H_2O$, $ZnSO_4 \times 7H_2O$, and KH_2PO_4 were obtained from Kemika, Zagreb.

Inorganic Phosphate Determination

The concentration of inorganic phosphate in seawater samples was determined according to Murphy and Riley.[13] The P_i concentration range was estimated at 0.07 to 0.14 μmol dm^{-3}, which is in agreement with the data presented by Kušpilić *et al*.[14]

Sample Preparation

Seawater samples were collected from the surface layer at a localised site of the Kaštela Bay (Central Adriatic Sea) in the summer of 1998 (June to September). The sampling season was characterised by an *in-situ* temperature range of 20–27 °C. The chlorophyll *a* (chl *a*) concentration range was 0.31 to 1.60 mg m^{-3} (I. Marasović).[15] Unfiltered samples were stabilised immediately after sampling by adding 15 ml chloroform per 1 litre of sample. Filtered samples were prepared by vacuum filtration, utilising a polycarbonate filter unit (Nalgene) with nitrocellulose filter membranes, pore size 0.2 and 3 μm (Advantec MFS Inc.), followed by stabilisation with chloroform.

Spectrophotometric Assay

The catalytic activity was followed by monitoring the absorbance increase at 405 nm from *p*-nitrophenol (*p*NP) generation in an enzyme catalysed hydrolysis of *p*-nitrophenyl phosphate (*p*NPP), using the Perkin-Elmer Lambda Bio 40 spectrophotometer. Under the requirement that no more than 5% of the substrate should be hydrolysed during the kinetic experiment, the sensitivity of the method restricts the lower *p*NPP concentration limit to 0.01 mmol dm^{-3}. Activity was monitored, following a 24-hour incubation, in a standard assay containing 0.1 mol dm^{-3} Tris, pH = 8.2, 0.01 to 0.1 mmol dm^{-3} *p*NPP and 3 ml of filtered (0.2 μm) seawater sample in a total volume of 5 ml, unless otherwise indicated. Measurements were run against a blank containing an equivalent amount of the relevant seawater sample previously autoclaved at 121 °C for 30 min. The amount of hydrolysed substrate, calculated from the equimolar amount of released *p*NP using the molar absorption coefficient ε of 17042 $(\text{mol dm}^{-3})^{-1}\ \text{cm}^{-1}$, was expressed as μmol *p*NPP per dm^3 of sample and hour (μmol *p*NPP $dm^{-3}\ h^{-1}$). Catalytic constants were determined by fitting the experimental data to the Michaelis-Menten equation using non-linear regression analysis. Inhibition constants were calculated from apparent K_m and V_m values in the presence of inhibitor.

The stock solution of *p*NPP in distilled water was sterilised by filtration over a sterile 0.2 μm syringe filter (Minisart RC 25, Sartorius AG, Götingen, Germany). Lower substrate concentrations were prepared by dilution with autoclave-sterilised

distilled water. Stock solutions of $MgSO_4$, $ZnSO_4$, $CaCl_2$ or KH_2PO_4 were prepared by dissolving their respective effector substance in 0.5 mol dm^{-3} Tris, pH = 8.2.

Sample Dilution

Activity was monitored in reaction mixtures containing different amounts of filtered (0.2 μm) seawater sample. To ensure a uniform sample volume, seawater, preheated at 121 °C for 30 min, was added to the assay solution as required.

TCA Inactivation

The reaction was initiated immediately after sampling, allowed to proceed for 24 hours, and terminated by adding 4 mol dm^{-3} of trichloracetic acid (TCA) to the assay solution (v/v 1:20). Measurements were conducted at intervals, following adjustment of the pH to 8.2 with 4 mol dm^{-3} NaOH.

RESULTS

Enzyme Stability

ALP activity was monitored during a period of 8 days in samples stabilised with chloroform, using unfiltered seawater samples or samples filtered over a nitrocellulose membrane, pore size 0.2 and 3 μm (Table I). The 0.2 μm size fraction contains soluble ALP, whereas the 3 μm size fraction consists of both free ALP and ALP bound to the bacterial cell wall. Measurements were performed using 0.2 mmol dm^{-3} *p*NPP. The reaction was initiated either directly after sampling or at intervals, and was allowed to proceed for 24 hours at room temperature. The phosphatase activity of the

TABLE I

ALP activity expressed as μmol *p*NPP hydrolysed in 1 dm^3 of seawater sample per hour[a]

Preincubation time / d	Phosphatase activity / μmol *p*NPP dm^{-3} h^{-1}		
	0.2 μm size fraction	3 μm size fraction	unfiltered sample
1	3.47	11.81	148.61
2	1.80	5.69	211.81
4	1.15	4.44	130.69
8	–	3.06	51.80

[a] The sample was collected on 05.06.1998 in the Kaštela Bay.

unfiltered sample increases initially, followed by reduction to approximately 30% of the original activity. The activity of both the 0.2 and the 0.3 μm size fractions decreases to 50% of the original value within 2 days. The enzyme inactivation reaction of free ALP is a first order rate reaction with an inactivation rate constant of $k = 0.42\ d^{-1}$ and half-life time $t_{1/2} = 1.64$ d.

Incubation Time

The reaction was initiated immediately after sampling, and activity was monitored following variable incubation periods, as specified in Table II. The V_m values were extrapolated to an incubation time of 24 hours. The *p*NP concentration, recorded after a 6-hour incubation, was too low for accurate measurements. Using the lowest substrate concentration of 0.01 mmol dm^{-3} *p*NPP, the absorbance obtained after 35 hours of incubation indicates that approximately 7% of the substrate has been hydrolysed. The maximum velocity decreases with incubation time, suggesting enzyme inactivation.

TABLE II

Dependence of the kinetic constants of free ALP in seawater samples on the time of incubation[a]

Incubation time / h	K_m / mmol dm^{-3}	V_m / μmol *p*NPP dm^{-3} h^{-1}
6	–	–
15	0.0199 ± 0.0013	21.25 ± 0.64
21	0.0196 ± 0.0028	17.91 ± 0.49
35	0.0218 ± 0.0015	17.22 ± 0.55

[a] The sample was collected on 15.06.1998 in the Kaštela Bay.

Sample Dilution

Activity was measured following a 24-hour incubation in reaction solutions containing different amounts of the 0.2 μm size fraction in a total volume of 5.4 ml (Table III). The most prominent reduction of the initial substrate concentration was observed in the reaction solution containing the highest sample ratio, and consequently the highest K_m value.

Reaction Termination with TCA

When the reaction was terminated by adding TCA, the kinetic properties of free ALP did not change significantly over a period of 120 hours,

TABLE III

Dependence of the kinetic constants of free ALP in seawater samples on sample dilution[a]

Sample volume / ml	K_m / mmol dm^{-3}	V_m / μmol *p*NPP dm^{-3} h^{-1}
3	0.0081 ± 0.0009	20.13 ± 0.24
4	0.0082 ± 0.0012	22.60 ± 0.17
5	0.0107 ± 0.0007	22.41 ± 0.11

[a] The sample was collected on 03.07.1998 in the Kaštela Bay.

whereas the kinetic parameters of ALP in the unfiltered sample were substantially altered already within 24 hours (data not shown).

Buffer Type, Concentration and pH

The influence of buffer type (Table IV) and concentration (Table V) on the kinetic properties of soluble ALP was determined using either a Tris buffer, supporting the transphosphorylation reaction, or a borate or diethylbarbiturate buffer, which are not phosphate acceptors. For comparison of the kinetic parameters with respect to buffer type, measurements were conducted in 0.01 mol dm^{-3} buffer, pH = 8.2. Free ALP exhibits the lowest affinity and the lowest activity in borate buffer. The highest affinity for the substrate was measured in diethylbarbiturate buffer, whereas the highest maximum velocity was recorded in Tris buffer. An increase in Tris concentration from 0.01 to 1 mol dm^{-3}, measured at pH = 8.2, had a minor effect on the kinetic properties of soluble ALP. Increasing borate concentrations reduce the V_m and increase the affinity for the substrate. Opposite effects were observed in diethylbarbiturate buffer. The pH dependence of the K_m

TABLE IV

Influence of buffer type on the kinetic parameters of free ALP in seawater[a]

Buffer (0.01 mol dm^{-3}, pH = 8.2)	K_m / mmol dm^{-3}	V_m / μmol *p*NPP dm^{-3}h^{-1}
Tris	0.0188 ± 0.0023	0.87 ± 0.01
Borate	0.0285 ± 0.0027	0.60 ± 0.03
Diethylbarbiturate	0.0108 ± 0.0015	0.82 ± 0.03

[a] The sample was collected on 02.06.1998 in the Kaštela Bay.

TABLE V

Dependence of the kinetic constants of free ALP in seawater on buffer concentration, pH = 8.2 [a]

	[Buffer] / mol dm^{-3}	K_m / mmol dm^{-3}	V_m / μmol *p*NPP dm^{-3} h^{-1}
Tris	0.010	0.0113 ± 0.0021	7.36 ± 0.31
	0.100	0.0135 ± 0.0015	7.63 ± 0.43
	0.500	0.0157 ± 0.0031	7.36 ± 0.33
	1.000	0.0139 ± 0.0027	8.06 ± 0.17
Borate	0.010	0.0636 ± 0.0071	133.19 ± 6.26
	0.025	0.0563 ± 0.0022	121.11 ± 3.18
	0.050	0.0522 ± 0.0040	106.67 ± 5.11
Diethyl-barbiturate	0.010	0.1510 ± 0.0134	22.91 ± 0.72
	0.025	0.1635 ± 0.0232	25.00 ± 1.17
	0.050	0.1960 ± 0.0181	53.19 ± 3.65

[a] Samples used in measurements with Tris, borate, and barbiturate buffer were collected in the Kaštela Bay on 23.06.1998, 13.07.1998, and 23.08.1998, respectively.

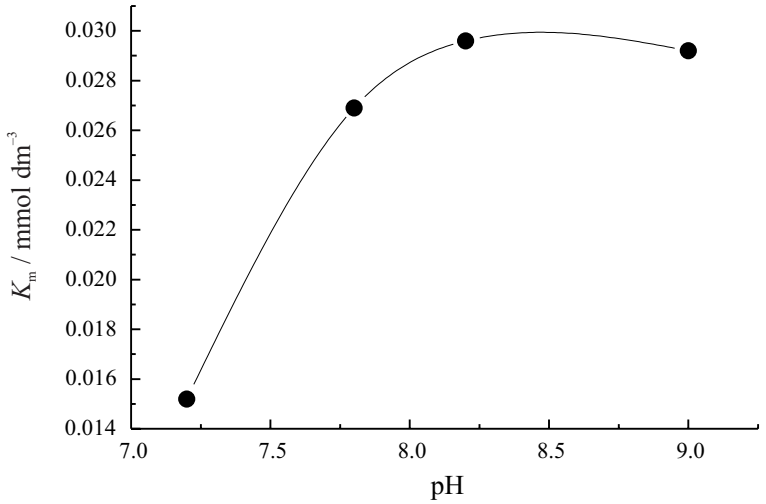

Figure 1. The K_m dependence of free alkaline phosphatase on the pH in 0.1 mol dm^{-3} Tris buffer.

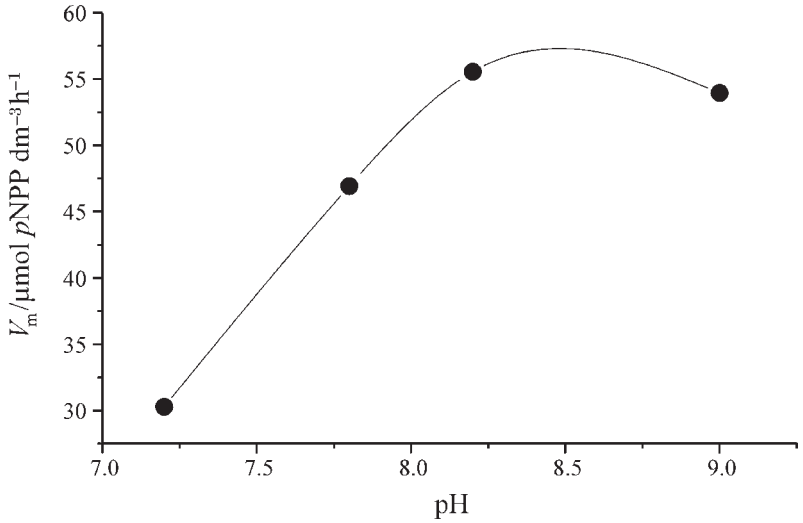

Figure 2. The V_m dependence of free alkaline phosphatase on the pH in 0.1 mol dm^{-3} Tris buffer.

and V_m values, measured in 0.1 mol dm^{-3} Tris, pH = 7.2 to 9, is presented in Figures 1 and 2, respectively. A change of the pH from 7.2 to 8.2 increases the V_m and K_m values two-fold. Further increase up to pH = 9.0 does not have a significant impact on the kinetic constants. The highest affinity for the substrate was recorded at pH = 7.2, and the highest V_m at pH = 8.2.

Effectors (Zn^{2+}, Mg^{2+}, and P_i)

The influence of divalent metal ions on the kinetic properties of soluble ALP in seawater samples was determined upon adding Zn^{2+} and Mg^{2+} ions (Table VI). Each experiment was conducted with a separately collected sample. An increase of the Mg^{2+} concentration by 2 mmol dm^{-3} causes a minor increase of the V_m value. On the other hand, the K_m is ten times higher than the value obtained without exogenous Mg^{2+}. Addition of 0.1 mmol dm^{-3} Zn^{2+} increases both the K_m and V_m values. The inhibitory effect of inorganic phosphate (0.1 and 0.2 mmol dm^{-3} P_i) on *p*NPP hydrolysis by ALP is reflected in the reciprocal rate to substrate concentration plot shown in Figure 3. The calculated kinetic constants, K_m and V_m, and their respective inhibition constants, K_i and K_{is}, are presented in Table VII. Inorganic phosphate effects the ALP activity, displaying an inhibition pattern of a mixed type with a significantly lower affinity for P_i than for *p*NPP in Tris buffer, pH = 8.2.

TABLE VI

Influence of Mg^{2+} and Zn^{2+} ions on the kinetic constants of free ALP in seawater samples[a]

Metal ion concentration / mmol dm^{-3}		K_m / mmol dm^{-3}	V_m / μmol *p*NPP dm^{-3} h^{-1}
Mg^{2+}	–	0.0083 ± 0.0009	17.50 ± 0.54
	2.0	0.0835 ± 0.0038	21.80 ± 0.93
Zn^{2+}	–	0.0090 ± 0.0013	2.64 ± 0.21
	0.1	0.0188 ± 0.0017	3.33 ± 0.14

[a] The samples were collected in the Kaštela Bay on 27.06.1998 and 07.07.1998, respectively.

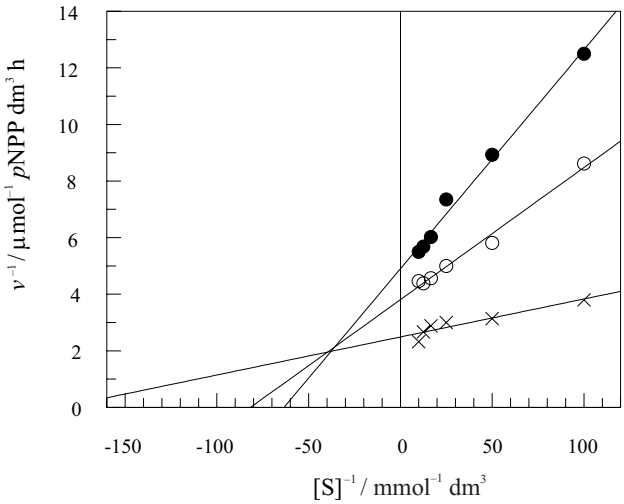

Figure 3. Inhibition of free alkaline phosphatase by inorganic phosphate. The reaction was monitored: (x) in the absence of inhibitor, (○) in the presence of 0.1 mmol dm^{-3} P_i, and (●) 0.2 mmol dm^{-3} P_i. The reaction mixture was prepared in 0.1 mol dm^{-3} Tris buffer, pH = 8.2.

DISCUSSION

Currently, the activity and kinetic properties of ALP in water environment are a subject of extensive investigation. In the absence of a standard method, a wide variety of data obtained under different reaction conditions

TABLE VII

Kinetic constants of free ALP from seawater determined in the presence of variable concentrations of inorganic phosphate[a]

$[P_i]$ / mmol dm^{-3}	K_m / mmol dm^{-3}	V_m / μmol *p*NPP dm^{-3} h^{-1}	K_i / mmol dm^{-3}	K_{is} / mmol dm^{-3}
–	0.0062 ± 0.0015	25.14 ± 1.38	–	–
0.1	0.0109 ± 0.0012	15.41 ± 0.35	0.1319	0.1568
0.2	0.0178 ± 0.0024	13.19 ± 0.51	0.1069	0.2179

[a] The sample was collected on 20.09.1998 in the Kaštela Bay.

is available. A satisfactory description of phosphatase activity in water media, especially while monitoring the change in activity against the environmental factors, requires precise information on the enzyme stability in water. It has been established that both free and cell-bound ALP from seawater are rapidly inactivated in our samples. In unfiltered samples, the activity initially increases, most likely due to excretion of the intercellular phosphatases, followed by a rapid reduction. Because of significant enzyme inactivation, the reaction should be initiated immediately after sampling and the released product measured following an optimal incubation period, which is generally unfeasible during fieldwork. Termination of the reaction by adding trichloracetic acid provides satisfactory results, however only in seawater samples containing the soluble ALP fraction.

The incubation time should be selected very carefully because, in addition to a relatively fast enzyme inactivation process, both enzyme and substrate are present at a very low concentration. The incubation period should be long enough to allow detectable amounts of the product to be released for accurate measurements, and yet short enough to avoid inactivation of the enzyme or significant reduction of the initial substrate concentration accompanied by a lower maximum velocity. In a sample characterised by high ALP activity, the substrate concentration decreases after a prolonged incubation, providing an apparent K_m that is higher than the true value. In a sample exhibiting high maximum velocity and high affinity, 5% of the lowest substrate concentration (0.01 mmol dm^{-3} *p*NPP) is used up within 12 hours from initiation. Therefore, when conducting measurements with samples of higher ALP activity, sample dilution or shorter incubation periods should be employed. However, upon dilution of a highly active sample, a reduction of the K_m was observed. It could be recommended to use an incubation period of 12 hours or less, and to adjust the sample dilution relative to

the expected activity. The concentration of the liberated product should then be extrapolated to an undiluted sample and an incubation period of 24 hours.

Buffers carrying hydroxyl groups participate in the reaction as phosphate acceptors, thereby influencing the kinetic constants of the respective enzyme system.[16–17] Although only Tris buffer supports the transphosphorylation reaction, there was no pronounced difference between the tested buffers with respect to either ALP affinity for *p*NPP or maximum velocity. Lack of significant variation with respect to buffer concentration allows the use of lower Tris concentrations than those usually (0.1 mol dm^{-3}) employed.

The relationship between ALP activity and pH varies with the buffer used and the enzyme under study. Enzymes from various plankton fractions exhibit different activity dependencies on the pH.[18] There is not enough data describing the relationship between pH and the kinetic properties of purified enzymes from various species of planktonic algae and bacteria. Information on the properties of enzyme mixtures occurring in different water sources is not complete either. Without such information it is not possible to recommend which buffer and pH should generally be most suitable for the measurement of ALP activity in water media. It is most appropriate to determine the kinetic constants under conditions providing the highest V_m value. Consequently, the optimal buffer for monitoring the soluble ALP fraction in our seawater samples was 0.1 mol dm^{-3} Tris, pH = 8.2, corresponding to the pH value of seawater at the sampling site.

The influence of potential activators or inhibitors, present at variable concentrations in water samples, has not been investigated to date. Data concerning the type and stoichiometry of metal ions bound to planktonic ALP and the influence of divalent metal ions on its catalytic properties is still missing. Addition of both 2 mmol dm^{-3} Mg^{2+} and 0.1 mmol dm^{-3} Zn^{2+} reduces the affinity of free phosphatases for *p*NPP, but has opposite effects on their catalytic activity. Inorganic phosphate, a competitive inhibitor of ALP activity, inhibits free seawater ALP, displaying a mixed type inhibition pattern. Since the inhibition constants are approximately a thousand times higher than the P_i concentration in seawater, the quantity of P_i present in the sample does not influence the determination of kinetic parameters.

All measurements were conducted at room temperature, providing uniform reaction conditions for each set of experiments. However, since the kinetic constants are temperature-dependent, a standardised method would require kinetic measurements to be performed at a precisely defined temperature.

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SAŽETAK

Aktivnost alkalne fosfataze u morskoj vodi: utjecaj reakcijskih uvjeta na kinetičke parametre alkalne fosfataze

Stjepan Orhanović i Maja Pavela-Vrančič

Alkalna fosfataza (ALP) izvanstanični je enzim koji hidrolizom fosfomonoestera osigurava anorganski fosfat (P_i) nuždan za održavanje staničnog metabolizma. Katalitička svojstva enzima u vodenoj okolini često se proučavaju radi opisivanja utjecaja alkalne fosfataze na kruženje fosfora u prirodnim vodama. Unatoč značenju i učestalom mjerenju aktivnosti alkalne fosfataze, standardna metoda još nije razvijena. Radi ispitivanja utjecaja reakcijskih uvjeta na kinetička svojstva alkalne fosfataze u morskoj vodi proučena je njezina stabilnost u uzorcima mora stabiliziranima kloroformom. Provjeren je i utjecaj tipa pufera, njegove koncentracije i pH, koncentracije metalnih iona, anorganskog fosfata i vremena inkubacije. Može se zaključiti da kinetička svojstva ALP značajno ovise o pH pufera, koncentraciji iona Mg^{2+} i Zn^{2+}, te o vremenu inkubacije. Stoga ti parametri moraju biti točno definirani u sklopu standardne metode za mjerenje aktivnosti alkalne fosfataze u vodenoj sredini.