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Magali Lespilette, Gérard Bogé, Simone Richard, Jean-Louis Jamet. Origin and characteristics of the zooplankton phosphatase activity in a coastal ecosystem of the Mediterranean Sea (Toulon bay). Marine Environmental Research, 2007, 63 (5), pp.445. 10.1016/j.marenvres.2006.12.004 . hal-00501897

HAL Id: hal-00501897

<https://hal.science/hal-00501897>

Submitted on 13 Jul 2010

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Accepted Manuscript

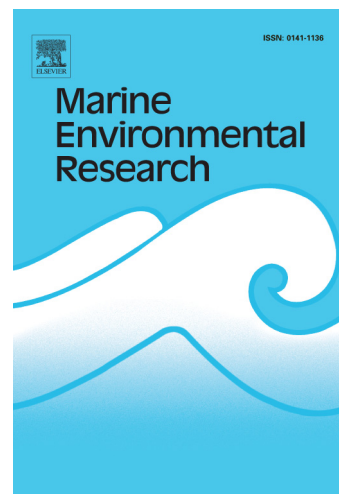
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PII: S0141-1136(06)00218-2
DOI: [10.1016/j.marenvres.2006.12.004](https://doi.org/10.1016/j.marenvres.2006.12.004)
Reference: MERE 3081

To appear in: *Marine Environmental Research*

Received Date: 17 March 2006
Revised Date: 4 December 2006
Accepted Date: 6 December 2006



Please cite this article as: Lespilette, M., Bogé, G., Richard, S., Jamet, J.L., Origin and characteristics of the zooplankton phosphatase activity in a coastal ecosystem of the Mediterranean Sea (Toulon bay), *Marine Environmental Research* (2006), doi: [10.1016/j.marenvres.2006.12.004](https://doi.org/10.1016/j.marenvres.2006.12.004)

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**Origin and characteristics of the zooplankton phosphatase activity
in a coastal ecosystem of the Mediterranean Sea (Toulon bay)**

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Abstract:

In Toulon Bay (France), very high phosphatase activities have been found in the zooplankton fraction $>90\mu\text{m}$. This work was intended to specify their origin. For that purpose, larvae, juvenile and adult Crustacea (Copepods: Calanoids, Cyclopoids, Branchiopods: Cladocera, and Cirripeds) were isolated. Their activities were measured using paranitrophenyl phosphate dissolved in sea water in order to calculate K_m (the enzyme half saturation concentration) and V_{max} (the reaction rate when the enzyme is saturated with substrate). V_{max} were referred to protein contents of the isolated organisms to calculate specific activities. For all zooplankton groups high and low affinity phosphatase activities were found. The low affinity enzyme was responsible for at least 70 % of the total phosphatase activity. Its specific activity was higher for larvae than for copepodites and adults. In Cirriped nauplii this activity was particularly high with values which were several hundred times higher than that in other Crustacea. These enzymes had optimum pH close to 8.4, magnesium requirement and were competitively inhibited by orthophosphate. Experiments with intact and lysed Cirriped nauplii confirmed that living organisms had only a weak external activity and showed that most of the activity of these larvae was primarily intracellular.

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Introduction:

Much of the hydrolysis of phosphate esters in the aquatic environment results from the presence of enzymes on the cell surface or in the periplasmic space of bacteria and phytoplankton (Ammerman, 1991, Ammerman and Glover, 2000). However, enzymatic activities are also secreted by bacteria and phytoplankton, or released into the medium following the death of planktonic organisms (Jansson et al, 1988; Hoppe, 2003; Nausch and Nausch, 2004). In phytoplankton, as in bacteria, phosphatases, particularly alkaline phosphatase, can be induced under conditions of phosphorus limitation; nevertheless, some forms of phosphatase activity are constitutive and their synthesis is independent of external phosphorylated compounds (Cembella et al., 1984).

Zooplankton are generally not regarded as an important contributor to phosphate ester hydrolysis. However, as early as 1951, Margalef speculated that soluble phosphatases could be produced by zooplankton (Boavida, 2005). Phosphatase secretion from zooplankton has been characterised notably in Daphnia, Bosmina, Holopedium, and Cyclops (Jansson et al., 1988) and may contribute to the overall phosphatase activity in fresh water. In sea water, we observed very high specific activities in homogenates of the >90 µm size class of the particulate material in Toulon Bay when the abundances of cirriped larvae were high (Gambin et al., 1999, Bogé et al., 2002; Jean et al., 2003; Bogé et al., 2006). This work was undertaken to give deeper insight into the origin of the zooplankton phosphatase activities originated from Toulon Bay. Phosphatases activities were investigated by measuring the biochemical constants, K_m and V_{max} . We first identified the taxonomic groups responsible for such activities. For that purpose, developmental stages of zooplankton groups (Copepodes : Calanoids, Cyclopoids, Branchiopodes : Cladocera, and Cirripeds) were isolated separately. So the influence of phytoplankton and detritus was excluded. A particular study was then carried out with Cirriped larvae to specify the localization of phosphatases. By using living larvae, it was possible to measure the internal, external and secreted activities. A study of its cation requirement, of its pH dependence and of the phosphorus inhibition has been also carried out.

Material and Methods :

Origin and isolation of zooplankton: Zooplankton were collected using a 90 μm net in May 2003 and June 2005, from Toulon Bay on the French Mediterranean Coast. Zooplankton were immediately brought to the laboratory, and were frozen at -20°C in natural seawater to immobilize the organisms and to facilitate their isolation. Zooplankton were then thawed at ambient temperature. We used a binocular lens and a dissecting needle with a wire loop ending to isolate and collect larval, juvenile and adult specimens of Copepoda (Calanoids and Cyclopoids), Branchiopods (Cladocera), Cirripeds and Malacostracea. About thirty individuals of each group were placed in Eppendorf tubes containing 250 μl distilled water. We observed that when the congelation lasted between 2 and 20 hours, most zooplankton were immobile, except for some Cirriped larvae that were still alive. Enzyme activities were measured on dead organisms isolated from zooplankton frozen during at least 24 hours, except for the last experiment for which living Cirriped larvae were needed. In that case, the larvae were patiently isolated without previous congelation.

Phosphatase activity: Batches of zooplankton were lysed by sonication at 2°C and the homogenates were used as enzyme sources. The substrate was paranitrophenyl phosphate (pNPP), dissolved in sea water which had been prefiltered through 0.45 μm pore-size filters. Paranitrophenol (PNP) produced during the reaction was detected at 410 nm during one hour (Reichardt et al., 1967). The experiments were carried out at 25°C . The biochemical constants K_m (the half saturation concentration) and V_{max} (the reaction rate when the enzyme is saturated with substrate) were calculated by means of an iteration program based on an analysis of Eadie Hofstee kinetics. Previous work showed that these kinetics are composed of two Michaelian components: a low affinity/high capacity mechanism and a high affinity/low capacity mechanism (Jean *et al.*, 2003, Bogé *et al.*, 2002, 2006). For each extract at least four substrate concentrations (0.01 to 10 mM, in geometrical progression) were tested and the V_{max} of each component was calculated.

Phosphatase activity is typically pH dependent (Hope, 2003). The pH dependence of Cirriped phosphatase activity was studied for a 15 mM substrate concentration prepared in prefiltered sea water. Glycine (50 mM) was used to buffer this medium at alkaline pH values (7.22 to 10.24).

Phosphatase activity is also influenced by specific cations (Mc Comb et al.,1979). The cation dependence of the Cirriped activity has been studied. Experiments were carried out with synthetic water made of distilled water adjusted to pH 8.4 with glycine (final concentration: 50 mM), and containing Mg^{2+} (10-100 mM), Ca^{2+} (5-20 mM), Na^+ (50-500 mM) and K^+ (5-20 mM) (all added as chloride salts) and pNPP (15 mM).

Orthophosphate competitively inhibits phosphatase activity at high phosphorus concentrations (Chrost, 1991). To investigate the influence of phosphorus on the zooplankton phosphatase activity, Cirriped larvae homogenates were used. The effect of 5 mM K_2HPO_4 on the hydrolysis of pNPP (0.083 mM to 6.7 mM) was studied. At this concentration, phosphorus precipitates in sea water. To avoid this precipitation and to demonstrate the relevance of phosphate inhibition for seawater, natural seawater was replaced by synthetic sea water, containing 500 mM NaCl, 50 mM $MgCl$, and 20 mM KCl, buffered at pH 8.4 with glycine.

An experiment was devoted to the study of the localization of the phosphatase activity in Cirriped larvae. Phosphatases are either internal or external enzymes (Jansson et al., 1988). The hydrolysis of substrates in the water is due to external enzyme activities whereas that of intracellular compounds results from internal enzymes. To characterize external enzyme activity, living Cirriped nauplii were placed in sea water with 15 mM pNPP. The PNP concentrations were then followed over one hour. The same experiment was carried out using larvae homogenates to characterise total phosphatase activities, including internal and external enzymes. We also looked for secreted phosphatase activity in water conditioned by larvae for 24 hours.

Proteins: To evaluate specific phosphatase activities, protein concentrations of the plankton extracts were determined according to Lowry's method (1951). Specific activity was defined as the ratio between enzyme activity and protein concentration.

Statistics: At least three batches of zooplankton were used for each taxonomic group (10 experiments for Calanoids, 3 experiments for cirriped larvae, and 5 for the other taxonomic groups). Standard errors are presented. Non parametric tests were used for V_{max} intergroup comparisons (Mann Whitney).

Results:

Structure of the zooplankton community in Toulon Bay:

In June 2005, the zooplankton community averaged 19000 individuals per m³. Nearly 89 % of the zooplankton were crustaceans (Fig. 1). Copepoda was the largest group. Cyclopoids, particularly *Oithona nana*, were the most numerous Copepoda. Several developmental stages were found: only 34 % of the zooplankton community and 39% of the Copepoda group was adult; 43 % of total zooplankton and 48 % of Copepoda were copepodites, 23% of total zooplankton and 13 % of Copepoda were larvae. The Cirriped larvae were almost all nauplii and made up only 1 % of the zooplankton community. Eleven % of the community were larvae that were not Crustacea. Less than 1% of total zooplankton were Decapodes zoe.

Interspecies comparisons: Phosphatase activities with high and low affinities were found for all zooplankton groups. The Km for the low affinity activity was between 0.3 and 1 mM. For the high affinity activity, it was below 0.02 mM. At least 70 % of the phosphatase activity (expressed as Vmax) was supported by low affinity enzymes (Fig. 2). The contribution of this low affinity component to the overall phosphatase activity (expressed as Vmax) was more important in Zoe (Decapods: ZDec) and nauplii (Cyclopoids: Ncycl and Cirripeds: NCir), than in adults (Cyclopoids: ACycl and Calanoids: ACal) and copepodites (Calanoids: CCala). For Cirriped nauplii (NCir), more than 99 % of the phosphatase activity was due to low affinity enzyme. Conversely, the contribution of high affinity activity was predominant in Copepod adults (Cyclopoids: ACycl, and Calanoids: ACal) and copepodites (Calanoids: CCala). The relative proportion of the phosphatase activity due to the high affinity enzymes was substantially lower in Branchiopods (Cladocera) than in Copepods.

The Vmax of the low affinity enzyme showed considerable differences between developmental stages (Fig. 3). In Copepods (Cyclopoids and Calanoids), Vmax values were relatively low for adults and copepodites (ACycl, ACal and CCala), but were approximately 50 times higher for nauplii (NCycl) than for adults. However, there was substantial variability between batches for this group. Significantly higher activities existed in Branchiopod (Cladocera) adults than in Copepoda ($p<0.05$). But the most exceptional activities were found for Cirriped nauplii. Their

specific phosphatase activity was approximately 70 times higher than for Copepoda nauplii and more than 1000 times higher than for adult Copepoda. The differences with the other taxonomic groups were highly significant ($p < 0.05$).

The V_{max} of the high affinity enzyme was always lower than the V_{max} of the low affinity component (Fig. 4). Copepod larvae and copepodites (Calanoid copepodites: CCal and Cyclopoid nauplii: NCycl) in addition to Cirriped nauplii (Cirriped nauplii: NCir) had the highest activities (not statistically significant except for Cirriped nauplii with $p < 0.05$). However, for Cirriped, the high values of the low affinity activity made the accurate determination of the high affinity activity more difficult.

Analysis of the phosphatase activity of Cirriped nauplii: We characterised the phosphatase activity of Cirriped nauplii, including its dependence on pH, cations and phosphorus, and its cellular localisation..

- pH dependence: pNPP hydrolysis by the larvae had a pH optimum of 8.4, close to that of sea water (Fig. 5). At pH 7 this activity was still significant since it accounted for approximately 40 % of its maximum value. This is also the case at pH 9.5 where this percentage was 27%, whereas, the activity was completely inhibited at pH 10.

- Role of cations: Phosphatase activity was inhibited by almost 90 % when the substrate was prepared in fresh water instead of in seawater. In synthetic water made of 50 mM Mg^{2+} , 20 mM Ca^{2+} , 500 mM Na^+ and 50 mM K^+ , phosphatase activity was nearly 70 % of its seawater value. Individually, these ions stimulated the activity unequally. With magnesium (up to 50 mM) the activity reached nearly 40 % of its level in sea water. Sodium also stimulated the activity, but to a lesser extent. For 500 mM Na the activity was only 20 % of its level in sea water. The other ions like calcium and potassium had more limited effects (Fig. 6).

- Role of phosphorus: In the synthetic medium, the V_{max} of Cirriped larvae phosphatase activity was approximately 25 % lower than in sea water without any significant change in its K_m . The K_m was typically 0.3 mM in the absence of phosphate and 0.6 mM in its presence; the

V_{max} remained unchanged (Fig. 7). This K_m effect indicates that phosphorus competitively inhibited the Cirriped larvae activity.

-Localization of phosphatase activity: Very high phosphatase activities were found when larvae homogenates were used (Fig. 8), whereas intact larvae gave very low pNP concentrations. Very low phosphatase activities were found in the water where larvae remained for 24 hours. These results indicate that phosphatase activity associated with Cirriped larvae was essentially intracellular.

Discussion:

We investigated the role of marine zooplankton in the production of enzymes involved in the metabolism of phosphorylated compounds in Toulon Bay on the French Mediterranean Coast. These compounds are of major importance for plankton growth and for the control of cellular activities.

We confirmed that in size fractionation experiments (Jean et al. 2003, Bogé et al. 2006, Gambin et al. 1999, Jamet and Bogé, 1998) the hydrolysis of phosphate esters by zooplankton could be described by two Michaelian mechanisms with distinct K_m and V_{max} . This work specifies that, on the basis of their V_{max} , the contribution of the low affinity activity was always more significant than that of the high affinity activity, particularly in larvae. The high affinity activities have been revealed with adults as well as larvae. They could be active in the local hydrolysis of low phosphoric ester concentrations, as found in the natural medium. Previous results have suggested that bacteria may contribute to the phosphatase activity associated with the $>90\ \mu m$ size class fraction, which is mostly composed of zooplankton. But they cannot explain the high levels of the low affinity activity (Gambin et al., 1999; Jean et al., 2002).

The low affinity activity is probably responsible for the hydrolysis of intracellular substrates. Indeed, this activity cannot be detected extracellularly with intact Cirriped nauplii as the enzyme source (Figure 8). We also showed that this low affinity activity fluctuated greatly according to the developmental stages of the zooplankton. In adults and juveniles (copepodites), phosphatase levels were generally low, notably in Cyclopoids, Cladocera and Calanoids. Phosphatase activity in larvae was more variable, but was generally higher as observed for Copepoda nauplii (Figure 3). In Toulon Bay, nauplii were only approximately 13 % of the total number of Copepods during the sampling period (Figure 1).

Since the phosphatases are internal activities it is thus possible that zooplankton lose a major part of their enzyme activity after the freezing treatment. To test this possibility additional experiments have been carried out. Copepods were isolated from fresh and pre-frozen zooplankton. The results indicated that the activities of individuals isolated from pre-frozen zooplankton were lower, but the differences with fresh zooplankton were not significant due to natural intra-species variation. Cirriped nauplii are much more tolerant to freezing, and their cell membranes are probably better at withstanding freezing or thawing. The possible under estimate

of the activity of pre-frozen copepod does not change the conclusion that the activity of the cirriped larvae was considerably higher.

The role of these high phosphatase activities remains obscure. Physiologically, phosphatases could contribute to the synthesis or to the use of nutritional or energy reserves. In nauplii the mean P content is higher than in adults which could be related with higher phosphatase activities (Carillo et al., 2001). Nauplii have several stages during which molting and growth take place. Naupliar stage I derives energy from remaining egg yolk reserves, while stages II through VI derive their energy from consumed phytoplankton. In egg yolk, glycolipoproteins are an important source of proteins, lipids, and carbohydrates (De Chaffoy de Courelles and Kondo, 1980). Phosphate has been found covalently bound on these proteins in insects and crustaceans (Allerton and Perlmann, 1965; Fialho et al., 2002; De Chaffoy de Courelles and Kondo, 1980). In Crustacea, the degradation of these reserves is greatest during the nauplii stage due to enhanced lysosomal hydrolytic activities (Perona and Vallejo, 1985). In *Artemia*, these activities include cathepsin B acid ribonuclease, acid deoxyribonuclease, acid phosphatase, acid phosphodiesterase, β -glucosidase, β -N-acetylgalactosaminidase and acid lipase (Perona and Vallejo, 1985). Nucleic acids, mono and poly-phosphate esters are also possible sources of organic phosphorus for plankton (Benitez-Nelson, 2000). Phosphatases (acid and alkaline), and nucleotidases catalyze the hydrolysis of these compounds (Hope, 2003). These activities catalyze also pNPP hydrolysis. The most striking observation of this work was the high specific phosphatase activity associated with Cirriped larvae, several hundred times higher than for other taxons. In Toulon Bay, Cirriped nauplii accounted for less than 1 % of the total zooplankton community. Higher abundances were generally found in May, June and August, but they never exceed 400 individuals per m³ (Jean et al. 2003). During these periods the phosphatase activity of zooplankton homogenates peaks such that the contribution of zooplankton to the total particulate activity reached 80% or more (Gambin et al., 1999). On the other hand when these larvae are absent, the activity of the zooplankton falls by more than 90 % (Jean et al. 2003).

The fertilized eggs of Cirripeds are brooded within the shell of adults. When they change into nauplius larvae, they are released into the water as free-swimming plankton. After several moult stages, they become cyprid larvae. These larvae are enclosed within a carapace and possess antennae and numerous appendages. They also have detectors that can recognise suitable solid substrata. During settlement, glands of the antennae secrete a special cement to attach the larvae

to a rock (Anderson, 1994; Foster, 1987). During these larvae nauplii stages, reserves, consisting of lipids and proteins, are also synthesized in abundance. These reserves are necessary for later stages, particularly for the cyprid stage which does not feed during settlement. In Balanus amphitrite, the concentration of a specific protein, the cyprid major protein (CMP), accumulates in the haemocoel, increases during the naupliar stages and decreases with aging of cyprids and during the early juvenile period (Satuito et al., 1996; Shimizu et al., 1996). This protein appears to function as a storage protein during settlement of cyprids as well as during metamorphosis to juveniles. It shares similarities with the egg-yolk phosphoprotein, vitellin. Incidentally, this protein may explain the unusual freeze resistance of these larvae, which can survive several hours at -20°C (personal observation). The high phosphatase activities found in Cirriped larvae of Toulon Bay could thus play an important role in the dephosphorylation of this protein or of other phosphorylated compounds during the fasting period.

These internal activities can also influence the regeneration of phosphate. It is generally accepted that less than 20 % of ingested phosphorus is used for growth. The remainder is excreted in the medium as soluble reactive phosphorus and organic phosphorus (Valiela, 1995). In Swedish west coast, Bamstedt (1985) showed, that excretion rates of inorganic phosphate and dissolved organic phosphorus by 19 zooplanktonic species averaged respectively $2.22\text{ nmol mg protein}^{-1} \cdot \text{h}^{-1}$ and $0.8\text{ nmol mg protein}^{-1} \cdot \text{h}^{-1}$ in spring. Taking into account the protein contents of barnacle nauplii, the quantities rejected per individual nauplius would approximate respectively $0.01\text{ nmol} \cdot \text{h}^{-1}$ and $0.0036\text{ nmol} \cdot \text{h}^{-1}$. But due to the very high specific phosphatase activities, the excretion of inorganic phosphate is probably higher, except in cypris which do not feed. These intracellular activities could also contribute to the hydrolysis of phosphorylated compounds of sea water when enzymes are released in sea water from dead cells (Jansson et al., 1988). In the case of Cirriped nauplii, the activity was approximately $200\text{ nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ in May-June and the dissolved activity was $450\text{ nmol} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ during the same period (Bogé et al., 2006). So it is thus possible that part of this dissolved activity comes from dead larvae.

Some characteristics of this activity have been also specified in this work. This activity had a pH optimum close to 8.4. It was strongly stimulated by the sea water components (Figure 6) and it was inhibited by high orthophosphate concentrations (Figure 7). These results are in agreement with previous data obtained on homogenates from particulate material of the $> 90\text{ }\mu\text{m}$ size class (Gambin et al. 1999). This work brings additional information concerning the role of sea water

cations. Magnesium was most effective, which suggests that the enzyme could be alkaline phosphatase, its activity being generally controlled by this cation (McComb et al., 1979, Jansson and Al, 1988). Sodium also stimulated the phosphatase activity of the larvae (Figure 6) but high concentrations were required (up to 500 mM). So it is possible that this effect comes from the high tonicity that these high concentrations generate (McComb and al. 1979).

In conclusion, this work showed that zooplankton of Toulon bay has phosphatase activities with low and high affinities; that these activities are higher in larvae and in particular in Cirriped larvae where they could be of great importance for their metabolism and for the regeneration of phosphate.

Acknowledgments

This work was supported by Region Provence-Alpes-Côte-d'Azur and Communauté d'Agglomérations Toulon-Provence-Méditerranée. The authors are grateful to Laetitia De Souza and Typhaine Vettard for their efficient technical assistance.

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Figure legends

Fig. 1 : Structure of the zooplankton community in Toulon Bay (Mean abundances in June 2005).

Fig. 2 : Contribution, in percent of the total activity, of the high (V2) and low (V1) affinity components to the phosphatase activity of isolated larvae (N: nauplii, Z : zoe), copepodites (C) and adults (A) from Cladocera (Clad), Cyclopoids (Cycl), Calanoids (Cal), Cirripeds (Cir) and Decapods (Dec) originated from Toulon Bay

Fig. 3 : The specific activities (in $\text{nmol h}^{-1} \mu\text{g}^{-1}$ protein, logarithmic scale) of the low affinity component of the phosphatase activity of isolated larvae (N: nauplii, Z : zoe), copepodites (C) and adults (A) from Cladocera (Clad), Cyclopoids (Cycl), Calanoids (Cal), Cirripeds (Cir) and Decapods (Dec) originated from Toulon Bay (Mean \pm Standard error).

Fig. 4 : The specific activities (in $\text{nmol h}^{-1} \mu\text{g}^{-1}$ protein) of the high affinity component of the phosphatase activity of isolated larvae (N: nauplii, Z : zoe), copepodites (C) and adults (A) from Cladocera (Clad), Cyclopoids (Cycl), Calanoids (Cal), Cirripeds (Cir) and Decapods (Dec) originated from Toulon Bay (Mean \pm Standard error).

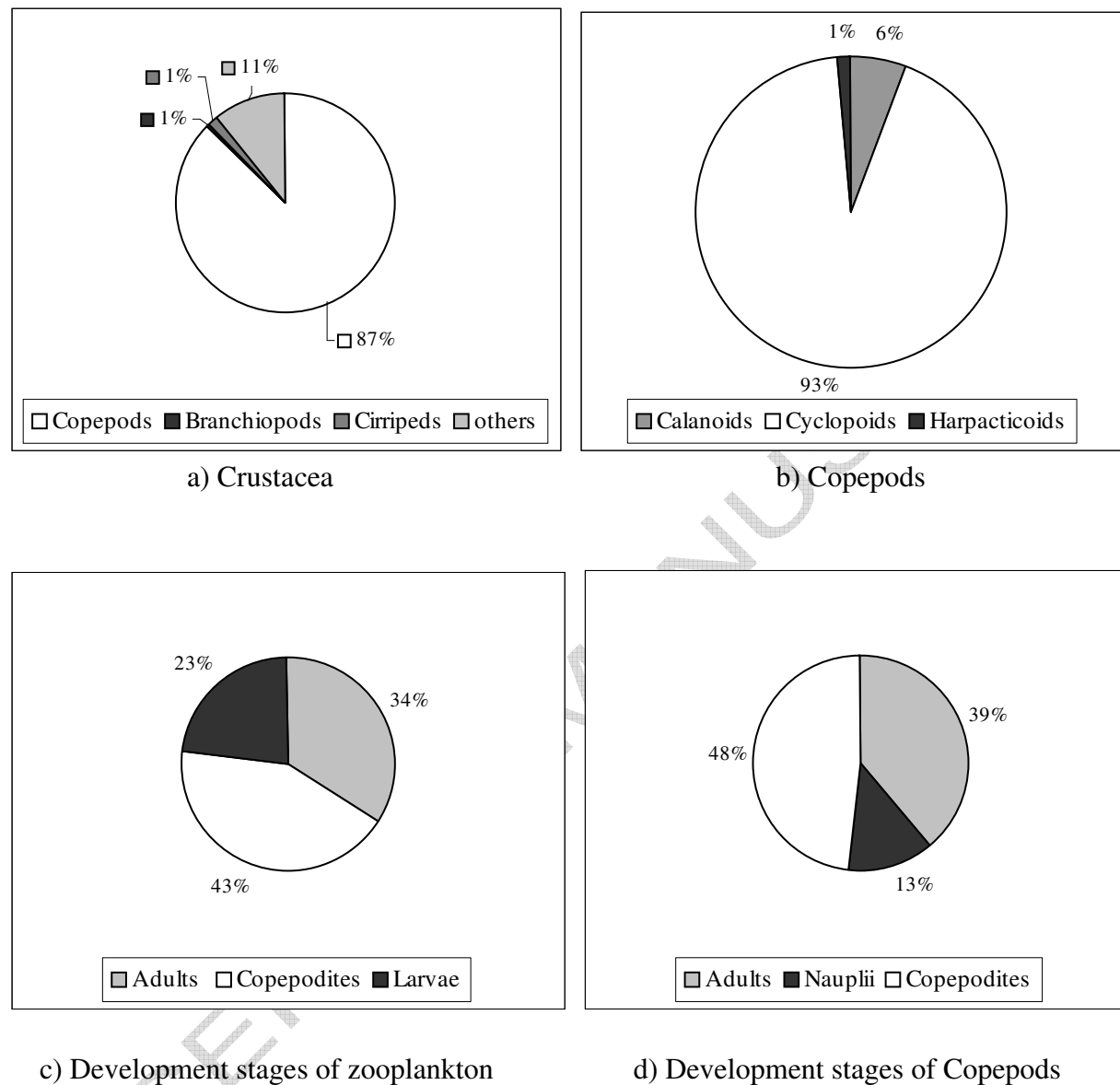
Fig. 5 : The pH dependence of the phosphatase activity of the Cirriped larvae (Specific activities are expressed in $\text{nmol h}^{-1} \mu\text{g}^{-1}$ protein) (Mean \pm Standard error).

Fig. 6 : The role of cations on the of the phosphatase activity of the Cirriped larvae (Synth water : synthetic water made of 50 mM Mg^{2+} , 20 mM Ca^{2+} , 500 mM Na^{+} and 50 mM K^{+}).

Fig. 7 : Effect of 5 mM K_2HPO_4 on the phosphatase activity of Cirriped larvae (Eadie Hofstee plots, and K_m and V_{max} values, SW- PO_4 : Sea Water without PO_4 , SyW- PO_4 : Synthetic Water without PO_4 , SyW+ PO_4 : Synthetic Water with PO_4 , Specific activities are expressed in $\text{nmol h}^{-1} \mu\text{g}^{-1}$ protein with standard errors).

Fig. 8 : Contribution, in per cent of the total activity, of external, internal and secreted activities

to the Cirriped larvae phosphatase.

Figures**Fig. 1**

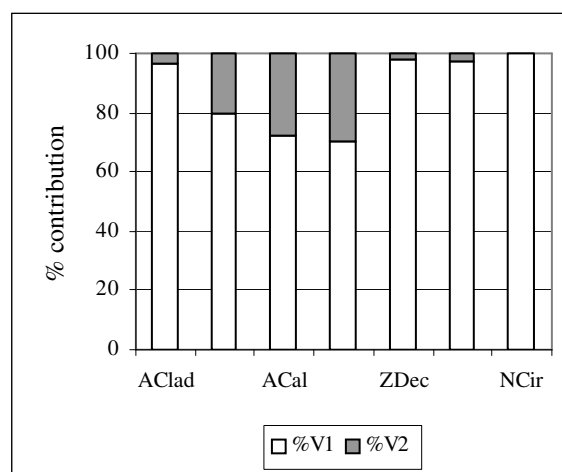


Fig. 2

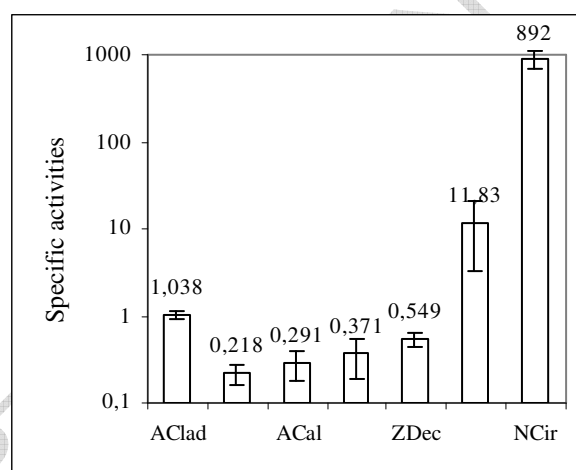


Fig. 3

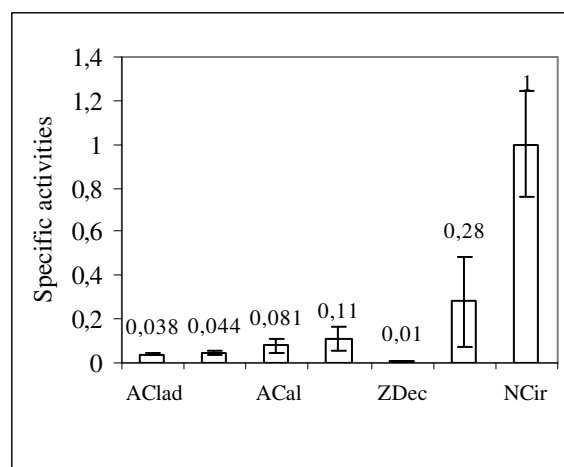


Fig. 4

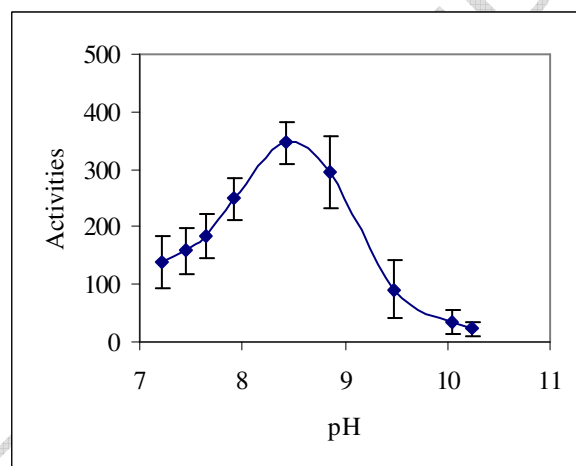


Fig.5

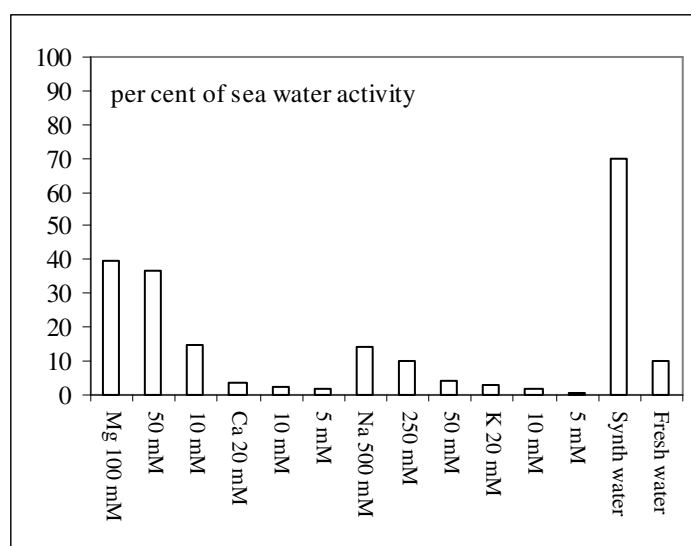
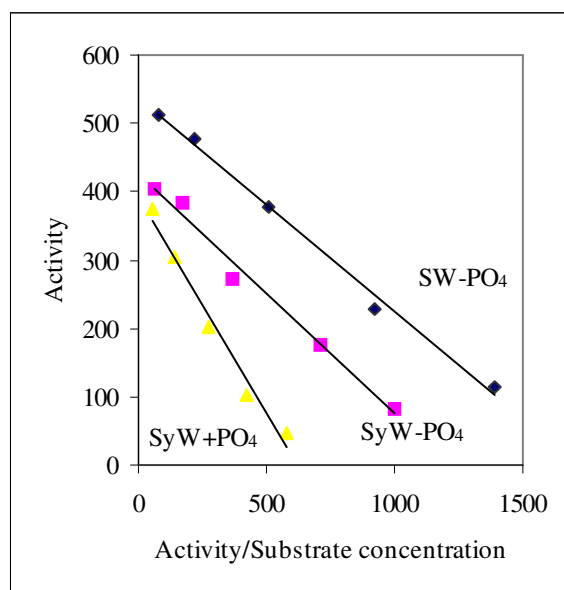


Fig. 6



	Km	Vm
SW-PO ₄	0,31 (0,02)	535 (15)
SyW-PO ₄	0,35 (0,02)	425 (15)
Sy+PO ₄	0,64 (0,054)	392 (30)

Fig. 7

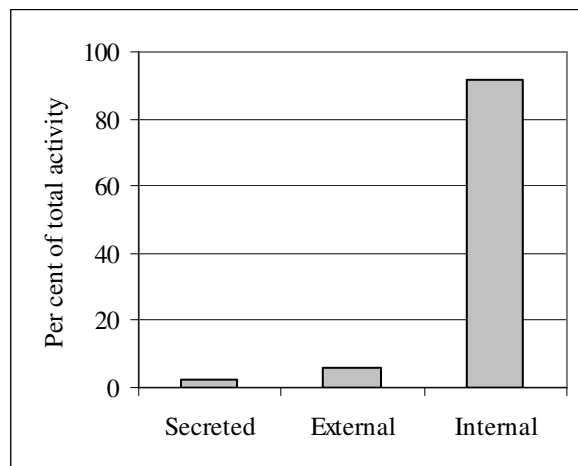


Fig. 8