



Extracellular phosphoesterases of invertebrate origin: underrated actors in phosphorus recycling?

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Abstract

Extracellular enzymes contribute substantially to the remineralisation of organic matter in aquatic systems. Marine invertebrates release endogenous enzymes through activities such as sloppy feeding or egestion, but the significance of such processes is widely unexplored. We compared functional key properties such as activity, stability, and apparent molecular masses of extracellular phosphatases from faeces of the marine crustaceans *Idotea balthica*, *Palaemon varians*, and *Homarus gammarus* with those of their digestive organs. A fluorescent substrate was used to examine enzyme activity qualitatively on agarose plates and quantitatively in microplate assays. Apparent molecular masses and enzyme stability were examined by native substrate gel electrophoresis (NSGE). Active extracellular phosphatase were present in the faeces of all tested species. NSGE activity band patterns were similar in digestive tissue extracts and faeces extracts. The initial enzyme activity retained for about 2 days, but thereafter rapidly decreased. Activity band patterns of digestive organs and faeces of *I. balthica* remained consistent for up to 72 h. Antibiotic treatment did not reduce phosphatase activity in the faeces of *I. balthica* and *H. gammarus* but in the faeces *P. varians*. Because of the comparatively high activities in the faeces, the electrophoretic similarity between faeces and digestive organs, and the limited durability of the enzymes in faeces extracts, we conclude that the studied crustaceans release predominantly endogenous, rather than bacterial phosphatases through faeces. These can substantially contribute to the pool of active extracellular phosphatases and the recycling of phosphorus in aquatic systems. The wider physiological and ecological context is discussed.

Introduction

Phosphorus is an essential element for all life on earth. It fulfils many functions such as energy storage (ATP), structuring of cells (phospholipids in cell membranes), and the

formation of DNA (Paytan and McLaughlin 2007; White and Dyhrman 2013). Continental weathering and transport via rivers (fluvial) and air (aeolian) is the primary import of phosphorus to the oceans (Föllmi 1996). Biologically bound phosphorus is released as particulate or dissolved inorganic and organic phosphorus (Johannes 1964; Hargrave and Geen 1968). While the inorganic phosphorus compounds can be readily assimilated and incorporated by biota, the organic compounds need to be degraded first. Many organisms possess phosphoester hydrolases (phosphatases) that can hydrolyze organic phosphorus compounds and generate inorganic phosphorus (Clark et al. 1998; Štrojsová et al. 2003; Dyhrman and Ruttenberg 2006). The enzymatic degradation of organic phosphorus through alkaline phosphatase is regarded as the most important process for the remineralisation of dissolved organic phosphorus (Suzumura et al. 2012 and references therein).

Extracellular enzymes play important roles in aquatic nutrient cycles, as they are essential drivers of remineralisation processes (Baltar et al. 2010; Arnosti 2011). Dissolved enzymes contribute significantly to the pool of total enzyme

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activities in marine environments (Karner and Rassoulzadegan 1995; Pantoja and Lee 1999). Extracellular enzymes in marine systems comprise, among others, proteases, glucosidases, chitinases, lipases, and phosphatases (Hoppe 1983; Hoppe et al. 2002). Bacteria are able to express these enzymes and release them into the water (Martinez et al. 1996). In addition to the extracellular enzymes released by bacteria, Karner et al. (1994) and Bochdansky et al. (1995) showed that extracellular enzyme activity can be provided by phagotrophic nanoflagellates and copepod grazing activities (e.g., sloppy feeding).

It has been suggested, that marine invertebrates might release extracellular enzymes through major physiological processes such as excretion and moulting (Boavida and Heath 1984; Espie and Roff 1995; Oosterhuis et al. 2000). However, studies that focus on the enzymatic activity released through these processes are scarce and little is known about enzyme properties and their possible contribution to marine nutrient cycles.

The aim of the present study was to investigate the extracellular enzyme activity in the faeces of marine invertebrates, particularly crustaceans, and to examine whether these enzymes are of endogenous origin through comparison with activities of the digestive organs. Three crustacean species, the isopod *Idotea balthica* (Peracarida) and two decapod species, the shrimp, *Palaemon varians*, and the lobster, *Homarus gammarus*, were chosen for this study. The fluorogenic substrate 4-methylumbelliferyl phosphate (MUF-P) was used to investigate the activities of phosphatase enzymes by complementary biochemical methods: agarose-substrate plates were used to visualise enzymes liberated from the samples, microplate assays were conducted to quantify enzyme activities, and zymograms were prepared to compare the apparent molecular masses of activity band patterns. Phosphatase activity in faeces was also measured in antibiotic-treated samples to estimate the potential enzyme sources (bacterial vs. crustacean) and their relative contribution to the activities of species. Additionally, the stability of phosphatase enzymes from faeces was investigated. The following hypotheses were tested: (1) marine crustaceans release extracellular phosphatases through faeces.

(2) These phosphatases are of endogenous origin. (3) The phosphatases are stable under natural conditions and, thus, capable of contributing to marine nutrient cycling.

Materials and methods

Origin of animals

Marine isopods, *Idotea balthica*, were taken from an established mass culture at the Alfred-Wegener Institute (AWI) in Bremerhaven. The animals were maintained in a 50-L flow-through aquarium in natural seawater of 15 ± 1 °C and 30 ± 2 salinity. Twice a week, animals were fed with fresh bladder wrack, *Fucus vesiculosus*.

Atlantic ditch shrimps, *Palaemon varians*, were collected during summer with a hand-net from an urban pond in Bremerhaven (Holzhafen, $53^{\circ} 32' 08.4''$ N $8^{\circ} 35' 29.0''$ E). The animals were maintained in 10-L aquaria at 15 ± 1 °C and 15 ± 0.3 salinity. Animals were fed with commercial fish feed every other day (NovoVert, Neuhofen, Germany) and with freshly hatched *Artemia* nauplii (Great Salt Lake Artemia Cysts, Sanders, USA) once a week. The water was always exchanged the day after feeding.

Juvenile European lobsters, *Homarus gammarus*, (about 4 cm total length), were provided by the Lobster Rearing Facility at Helgoland (Dr. Isabel Schmalenbach). Upon arrival in Bremerhaven, the animals were kept separately in rectangular 10×10 cm compartments within a $40 \times 25 \times 20$ cm flow through aquarium supplied with natural seawater. The temperature was 15 ± 1 °C and the salinity 30 ± 2 . The animals were fed with frozen shrimps (abdominal muscle) twice a week. Pieces of *Fucus vesiculosus*, were also added, providing additional food and shelter.

Sample collection, dissection, and processing

A set of different analysis was carried out with samples of faeces and digestive organs of the three crustacean species (Table 1).

Table 1 Overview of methods and treatments applied (X) to species and sample type

Species	<i>Idothea balthica</i>		<i>Palaemon varians</i>		<i>Homarus gammarus</i>	
	Faeces	Tissue	Faeces	Tissue	Faeces	Tissue
Substrate agarose plates	X		X		X	
Phosphatase activity	X	X	X	X	X	X
Antibiotic/antimycotic treatments	X		X		X	
Native substrate gel electrophoresis	X	X	X	X	X	X
Stability of enzyme activity	X		X		X	
Stability of activity band patterns	X	X				

Faeces

The isopods, *Idotea balthica*, were maintained in plastic containers with 500 mL seawater at 16 °C prior to faeces collection. The animals were either kept individually (samples for activity measurements) or in groups of six per container (samples for gel electrophoresis, agar plates, and stability measurements). The animals were fed with *F. vesiculosus* for at least 4 h. The algae were removed, and the water of the container was exchanged with fresh seawater. Care was taken to remove food remains or already produced faecal pellets. Faeces were collected after 16 h of incubation.

The shrimps, *P. varians*, were also kept at 16 °C. They were fed with fish feed, that they usually consumed within 30 min. Therefore, the feeding time was reduced to one hour. After feeding, up to six shrimps were placed together in one 500-mL glass beaker filled with brackish water (salinity 15). To prevent coprophagia, a plastic grid with a mesh width of 5 × 5 mm was fixed about 2 cm above the bottom of the beaker. After 16 h, the faecal pellets were collected from the bottom of the beakers and prepared for further analysis.

The lobsters, *H. gammarus*, were not fed for three to four days before faecal pellet collection. On the day of collection, they were fed for at least 4 h with shrimps (abdominal muscle) and algae. The lobsters were placed separately in 1-L glass beakers, which were filled with natural filtered seawater. A grid was placed about 2 cm above the bottom. A small plastic halfpipe was inserted to provide shelter. After 16 h, the produced faecal pellets were removed from the beakers and stored on ice. In all experiments, the light/dark cycle was 16:8 h.

The faeces were transferred into weighed 1.5-mL reaction cups and allowed to settle for about 5 min. Excess water was removed with a pipette. If needed, faeces were pulse-centrifuged for a maximum of 1 min to accelerate pelleting. The wet weight of the faeces was determined with a high precision scale (RC 210S MC1, Sartorius AG, Göttingen, Germany).

Samples assigned for enzyme activity measurements were suspended in ten parts (w/v) of ultrapure water (Milli-Q), homogenised for two minutes with a micro pestle, and centrifuged for 10 min at 25,000g and 4 °C. The supernatants were used for further analysis.

Faecal pellet samples assigned for gel electrophoresis were suspended in 20 to 30 µL of diluted (1:2) sample buffer (0.375 mol L⁻¹ Tris/HCl, pH 8.8, 30% (v/v) glycerol, 4% (w/v) SDS, 0.2 mg mL⁻¹ bromophenol blue) and then homogenised and centrifuged as described before. The supernatants were directly loaded into the gel pockets. In between processing steps, samples were stored on ice.

Digestive organs

Digestive organs were used for enzyme activity measurements and gel electrophoresis. Prior to dissection, the animals were placed in a freezer (−80 °C) for 2 min to sedate them. Midgut glands, also referred to as hepatopancreas, were dissected from *P. varians* and *H. gammarus*. The digestive organs dissected from *I. balthica* included the midgut gland and the gut. Samples were extracted in ten parts (w/v) of ultrapure water (Milli-Q, Merck, Darmstadt, Germany), homogenised for 2 min with a micro pestle, and centrifuged for 10 min at 25,000g and 4 °C. Samples used for gel electrophoresis were diluted (1:2) with sample buffer (see 2.8) and directly loaded into the gel pockets.

Phosphoesterase assays

4-Methylumbelliferyl (MUF) derivatives provide a sensitive tool for enzyme activity detection. They have been used to measure enzymatic activity in water samples or small organisms (e.g., Hoppe 1993; Oosterhuis et al. 2000, Prim et al. 2003, Knotz et al. 2006). Here, we further refined this method to detect phosphoesterase activity the substrate 4-methylumbelliferyl phosphate (MUF-P, Sigma M8883) after electrophoretic separation in digestive organs and, particularly, the faeces of three crustacean species.

Visualisation of faecal pellet phosphatase activity on substrate agarose plates

Agarose plates were prepared with 1% agarose and 0.1 mmol L⁻¹ of the substrate MUF-P. The substrate was dissolved in dimethyl sulfoxide (DMSO, Sigma 34569). Agarose was mixed with either sterile filtered seawater (salinity 30 for *H. gammarus* and *I. balthica*) or sterile filtered brackish water (salinity 15 for *P. varians*) and heated in a water bath (>95 °C) until the agarose was completely dissolved. The agarose solution was cooled down to about 45 °C and mixed with the substrate/DMSO solution. The final DMSO concentration was 2%. After sufficient agitation, the agar was poured onto a petri dish (9.3 cm diameter, 0.7 cm height). The agar was left to polymerize for 5–10 min before faecal pellets were placed on the agar surface. Excess water was removed with a pipette. The plate was incubated for 10 min in the dark. Photographs of the agar plate were made with the Gel Doc™ XR and Gel Documentation System (Bio Rad Laboratories, Hercules, California, USA).

Enzyme assays

Phosphatase activity measurements were conducted with the Fluoroskan FL Microplate reader at 355 nm (ex.) and 460 nm (em.). The fluorescence was measured every 30 s for

20 min. The increase of the relative fluorescence per minute (RFU·min⁻¹) was the measure of enzyme activity. The activity was expressed as nmol·min⁻¹·g⁻¹. For the sake of legibility, this was further defined as mU·g⁻¹ (1 U = μmol·min⁻¹; nmol·min⁻¹ = 1 mU) throughout the study. A linear standard curve was prepared with 0–10 μmol·L⁻¹ of 4-methylumbelliferone (Sigma M1381). The buffer used for the enzyme assays was prepared with sterile filtered seawater (HEPES-SW buffer) for *I. balthica* and *H. gammarus*, or with sterile filtered brackish water (HEPES-BW buffer) for *P. varians*. Preceding studies on enzyme assays and conditions are presented in the Supporting information.

Phosphatase activity measurements

Phosphatase activity was measured in faeces extracts from five different specimens of *I. balthica* and *H. gammarus* and from five different incubation experiments of *P. varians*. Faecal pellets of a single *P. varians* were not sufficient to run a standard assay. Therefore, for each sample of *P. varians*, faeces of six animals were pooled and extracted. Additionally, phosphatase activity was measured in digestive tissue extracts from five different animals per species. MUF-P was used as substrate. Extracts (20 μL) were pipetted in triplicate into 96-well plates and mixed with 250 μL of buffer-substrate solution (2% DMSO, HEPES-SW buffer pH 8.2, MUF-P-substrate). The final substrate concentration was 0.1 mmol·L⁻¹.

Enzyme activities in antibiotic/antimycotic-treated samples

To test whether antibiotic/antimycotic treatment influence the enzyme activity in faeces, animals were incubated in antibiotic-treated seawater according to Donachie et al. (1995). Therefore, the antibiotics Ampicillin trihydrate (500 mg·L⁻¹; A-6140-25, Sigma) and Streptomycin sulfate (250 mg·L⁻¹; 35500, Serva), as well as the antimycotic Nystatin (20 mg·L⁻¹; J62486, Alfa Aesar (Thermo Fisher GmbH) were dissolved in sterile filtered seawater and sterile filtered brackish water, respectively. The animals were allowed to feed in the treated water for up to 5 h. Thereafter, they were transferred into clean antibiotic/antimycotic-treated water and kept there for 16 h.

Native substrate gel electrophoresis

Enzymes were separated by native sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were loaded with 10 μL of extracts of faeces or digestive tissue, which were mixed with the bromophenol blue sample buffer. The extracts were neither treated with mercaptoethanol nor heated to avoid degradation of the

enzymes. Gel electrophoresis was run at 300 V and 15 mA (2 gels = 30 mA) in a SE 260 Mini-Vertical unit and an EPS 301 power supply (GE Healthcare, Chicago, Illinois, USA) until the migration front (bromophenol blue) reached the bottom of the gel (~1 h). Subsequently, the gel was washed for 30 min in demineralised water and incubated for 30 min in 0.1 mmol·L⁻¹ MUF-P substrate solution. If a non-fluorescent molecular weight marker was used, the gels were additionally stained overnight in a solution of Coomassie Brilliant Blue. Gels were photographed with a Gel Doc™ EZ device. The fluorescence of the gel pictures was presented using the colour scale “spectrum” (Image Lab software). The molecular mass of the bands was calculated using the relative migration distance (R_f = retardation factor).

Stability measurements

Enzyme activities

The determination of enzyme stability demanded high amounts of faecal pellets. Therefore, isopod and shrimp faeces were collected from six separate incubations in 1-L glass beakers, each containing six *I. balthica* and six *P. varians*, respectively. The faeces of either species were pooled to obtain three replicates. Three samples of lobster faeces from six individuals were also collected. The samples were homogenised in 400–450 μL sterile filtered natural seawater or brackish water, respectively. The enzyme activities were measured immediately after the extracts were prepared (T_0) and then again after 4, 24, 48, 96 and 192 h. In between, the samples were stored in the dark at 16 °C. The assays and the measurements were conducted as described before.

Activity band patterns

To investigate whether aged faeces showed changes in the activity band patterns, extracts were incubated for up to 72 h. Samples taken at different incubation times were subjected to native electrophoresis. For the time series, 5–6 *I. balthica* were placed in a single container (25 containers total) and left to feed for 4 h. Thereafter, animals from each container were transferred into new containers with fresh seawater and left to egest for 2 h. Next, the animals were separated from the faeces produced during the 2 h of egestion. The faeces remained in the containers at 16 °C in the dark and samples were taken after 2, 12, 24, 48 and 72 h. The samples were stored at –80 °C until electrophoresis. Digestive organs were dissected, transferred into reaction tubes, and incubated for up to 72 h at 16 °C. Extracts were prepared as described before and mixed with 20–30 μL of sample buffer. Electrophoresis was carried out as described before.

Statistics

The raw data were assembled for analysis with Excel (Microsoft Corporation, Washington, USA). Statistical analyses were conducted with R-studio (RStudio Inc., Boston, USA). Graphs were prepared using the ggplot package of R-studio.

The homogeneity of variance of data sets was tested with the Bartlett's test and normal distribution with the Shapiro–Wilkinson test. If data were not normally distributed and/or lacked variance homogeneity, the non-parametric Kruskal–Wallis test was used to test for significant differences within data sets. For the post hoc pairwise comparisons (Dunn's Test) of phosphatase activity between species, the Benjamini–Hochberg correction was used to adjust the p values and the H_0 hypothesis was rejected if $p \leq \alpha/2$. To identify significant differences between the antibiotic-treated samples and the untreated samples, the Student's t Test was applied whenever data were normally distributed, and variances were equal. Otherwise, the Mann–Whitney U test was applied.

The time series was conducted with three replicates for faeces extracts of each species. First, the highest activity of any time-point of each replicate was set to 100%. The other activities were calculated in relation to the highest value (%). To calculate the time-point where 50% of the initial enzyme activity remained (similar to effective dose values ED_{50}) and to display enzyme stability over time, a dose response model based on the Boltzmann function was applied:

$$f(x) = a + \frac{b}{1 + e^{\frac{x-m}{s}}}$$

The dose response models were calculated with the “drm” function from the package “drc v2.2–12”. The “ ED_{50} ” values were calculated with the “drc v3.01” from the same package. (a = minimum of the fit, b = maximum of the fit, m = point of inflection, 50% level of the function, s = slope at the point of inflection).

Results

Visualisation of phosphatase activity on substrate-agarose plates

Faecal pellets collected from *I. balthica* and placed on the seawater MUF-P agar yielded a strong fluorescence around the pellets (Fig. 1a, b). The faecal pellets of *P. varians* produced a faint but also clearly visible fluorescent signal on the agar substrate plate prepared with brackish water (Fig. 1c, d). The faecal pellets of *H. gammarus* produced an intensive fluorescent signal on the seawater MUF-P agar (Fig. 1e, f).

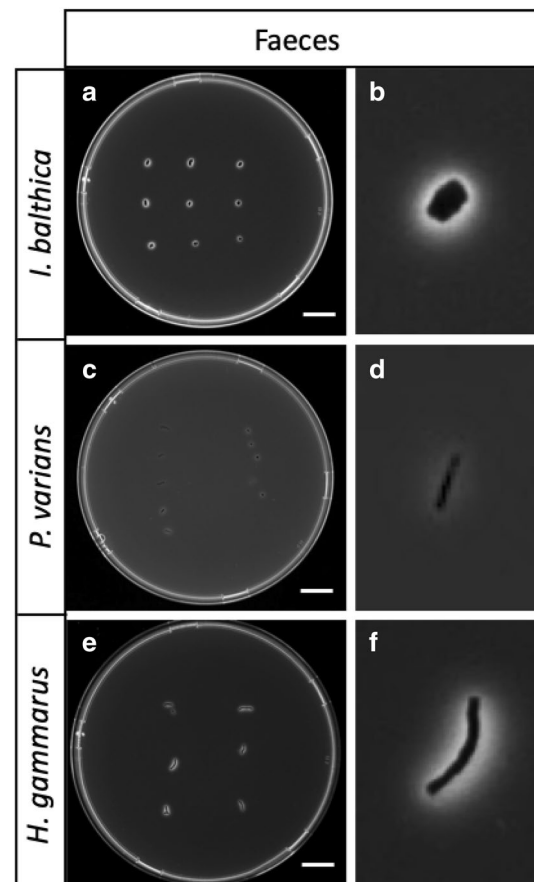


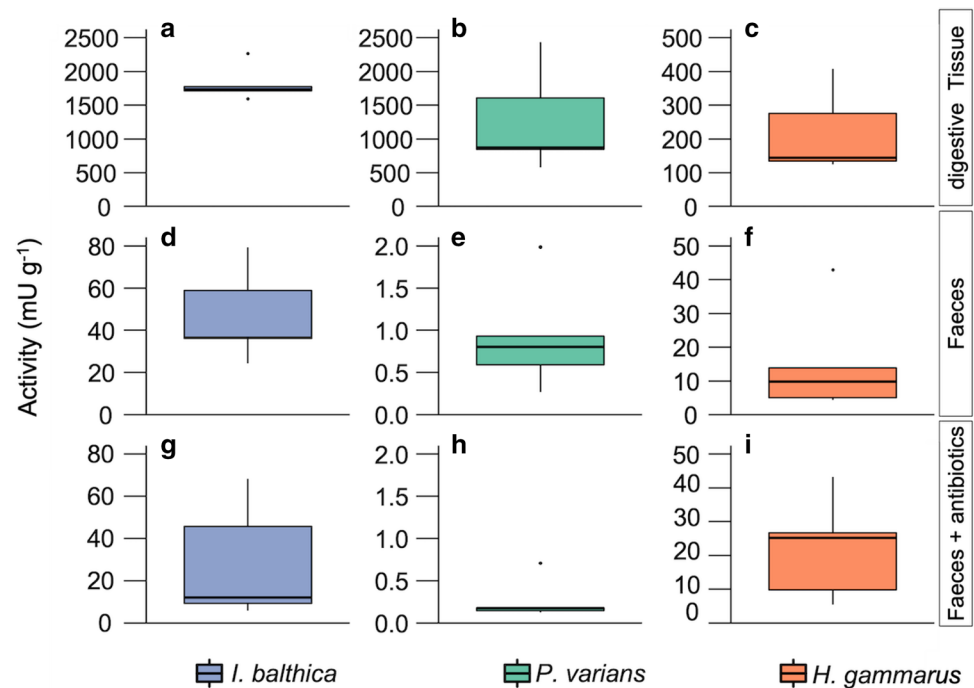
Fig. 1 Visualisation of phosphatase activity diffusing from faeces of the three crustacean species *Idotea balthica* (a, b) *Palaemon varians* (c, d), and *Homarus gammarus* (e, f). The white scale bar indicates a width of 10 mm. Pictures on the right without scale are not zoomed in but electronically enlarged

Phosphatase activity measurements

Phosphatase activities in digestive tissue extracts of *I. balthica* varied strongly between specimens. Average activity was $1816 \pm 260 \text{ mU}\cdot\text{g}^{-1}$. The maximum activity was $2266 \text{ mU}\cdot\text{g}^{-1}$ and the minimum activity was $1594 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2a). The phosphatase activities in digestive tissue extracts of *P. varians* also differed between samples. It varied between 577 and $2431 \text{ mU}\cdot\text{g}^{-1}$. The mean activity was $1268 \pm 755 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2b). The average phosphatase activity in the digestive tissue extracts of *H. gammarus* was $226 \pm 158 \text{ mU}\cdot\text{g}^{-1}$. The lowest activity detected was $125 \text{ mU}\cdot\text{g}^{-1}$ and the highest activity was $408 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2c).

The average phosphatase activity in faeces of *I. balthica* was $47.0 \pm 21.9 \text{ mU}\cdot\text{g}^{-1}$. The maximum activity was $79.2 \text{ mU}\cdot\text{g}^{-1}$ and the minimum activity was $24.4 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2d). The phosphatase activity in the faeces represented about 2.5% of the phosphatase activity in the

Fig. 2 Boxplots showing phosphatase activity in digestive tissues (a–c), faeces (d–f), and faeces (g–i) after antibiotic/antimycotic treatment of *Idotea balthica*, *Palaemon varians* and *Homarus gammarus* ($n=5$)



digestive tissue of *I. balthica*. Phosphatase activity in faeces extracts of *P. varians* also differed between samples (faeces of six animals were pooled for analysis). The activities varied between 0.27 and 1.99 $\text{mU}\cdot\text{g}^{-1}$. The calculated mean activity was $0.92 \pm 0.65 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2e). The average phosphatase activity still present in the faeces made up 0.07% of the phosphatase activity in the digestive tissue of *P. varians*. The average phosphatase activity in faeces extracts of *H. gammarus* was $15.2 \pm 16.0 \text{ mU}\cdot\text{g}^{-1}$. Here, activities also varied between specimens. The lowest activity detected was $4.4 \text{ mU}\cdot\text{g}^{-1}$ and the highest activity was $42.9 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2f). The average phosphatase activity still present in the faeces made up 6.7% of the phosphatase activity in the digestive tissue of *H. gammarus*.

Statistical analysis of enzyme activity measurements

Phosphatase activity in digestive tissue extracts was significantly different between *I. balthica* and *H. gammarus* ($p=0.009$) but not between *I. balthica* and *P. varians* ($p=0.145$) or *P. varians* and *H. gammarus* ($p=0.051$).

Statistical analysis revealed a significant difference between the phosphatase activity in faeces extracts of *I. balthica* and *H. gammarus* ($p=0.001$) but not between *P. varians* and *H. gammarus* ($p=0.036$) and between *I. balthica* and *P. varians* ($p=0.089$).

Enzyme activities in faeces after antibiotic/antimycotic treatment

Average phosphatase activity in faeces extracts of antibiotic/antimycotic-treated *I. balthica* was $28.3 \pm 0.9 \text{ mU}\cdot\text{g}^{-1}$. Maximum activity was $68.3 \text{ mU}\cdot\text{g}^{-1}$ and minimum activity was $5.8 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2g). Phosphatase activities in faeces extracts of antibiotic/antimycotic-treated *P. varians* also differed between samples (faeces of six animals were pooled for analysis). It varied between 0.12 and $0.71 \text{ mU}\cdot\text{g}^{-1}$. The mean activity was $0.27 \pm 0.25 \text{ U}$ (Fig. 2h). Average phosphatase activities in faeces of antibiotic/antimycotic-treated *H. gammarus* was $22.1 \pm 15.1 \text{ mU}\cdot\text{g}^{-1}$. Lowest activity was $5.4 \text{ mU}\cdot\text{g}^{-1}$ and highest activity was $43.2 \text{ mU}\cdot\text{g}^{-1}$ (Fig. 2i).

Statistical analysis of enzyme activities after antibiotic treatment

Phosphatase activities in faeces extracts of *I. balthica* were on average lower ($28.3 \text{ mU}\cdot\text{g}^{-1}$) after antibiotic/antimycotic-treatment than in untreated samples ($47.0 \text{ mU}\cdot\text{g}^{-1}$). Statistical analysis indicated that the phosphatase activity of the two groups was not significantly different ($p=0.27$).

The comparison of phosphatase activities in untreated and antibiotic/antimycotic-treated faeces of *P. varians* showed a higher mean phosphatase activity in the untreated samples ($0.92 \text{ mU}\cdot\text{g}^{-1}$) than in faeces after antibiotic treatment ($0.27 \text{ mU}\cdot\text{g}^{-1}$). Statistical tests indicated that the

phosphatase activities of the two groups were significantly different ($p=0.03$).

The mean phosphatase activity in faeces of *H. gammarus* was higher in the antibiotic treated faeces ($22.1 \text{ mU}\cdot\text{g}^{-1}$) than in untreated samples ($15.2 \text{ mU}\cdot\text{g}^{-1}$). Statistical analysis showed no significant differences between the two groups ($p=0.51$).

Native substrate gel electrophoresis

The gels stained with MUF-phosphate displayed an activity band of apparently 56 kDa in digestive tissue and faeces extracts of *I. balthica*. Additionally, a band of 34 kDa appeared in all three extracts (Fig. 3a). Comparison of *P. varians* faeces extracts (F) with digestive tissue (DT) extracts resulted in activity bands at 125 kDa, 40 kDa and 33 kDa, which were visible in extracts of both DT and F (Fig. 3b). Faeces extracts (F) and digestive tissue extracts (DT) of *H. gammarus* revealed the same activity band at 46 kDa (Fig. 3c).

Stability of enzymes

The phosphatase activity in faeces extracts of *I. balthica* remained at initial high levels for 48 h but declined sharply thereafter. After 192 h, only about 4% of maximum activity remained. The ED_{50} was 91.6 h (3.8 days) (Fig. 4a). The phosphatase activity in faeces extracts of *P. varians* showed initial high values for 24 h, and thereafter a strong decline. The calculated ED_{50} was 50.3 h (2.1 days). After 96 h, no enzyme activity remained (Fig. 4b). The phosphatase activity in faeces extracts of *H. gammarus* decreased continuously from the beginning. The calculated ED_{50} was 64.2 h (2.7 days). After 192 h, enzyme activity was $14.5 \pm 10.3\%$ of the highest activity (Fig. 4c).

The stability time series conducted with digestive tissue extracts (Fig. 5a) and faeces extracts (Fig. 5b) of *I. balthica* showed the same band pattern from 2 to 72 h of incubation. Intensities of activity bands in digestive tissue extracts remained high until the end of the experiment (Fig. 5a). The intensity of the fluorescent signal of the faeces decreased after 12 h. A faint activity remained until the end of the incubation after 72 h (Fig. 5b).

Discussion

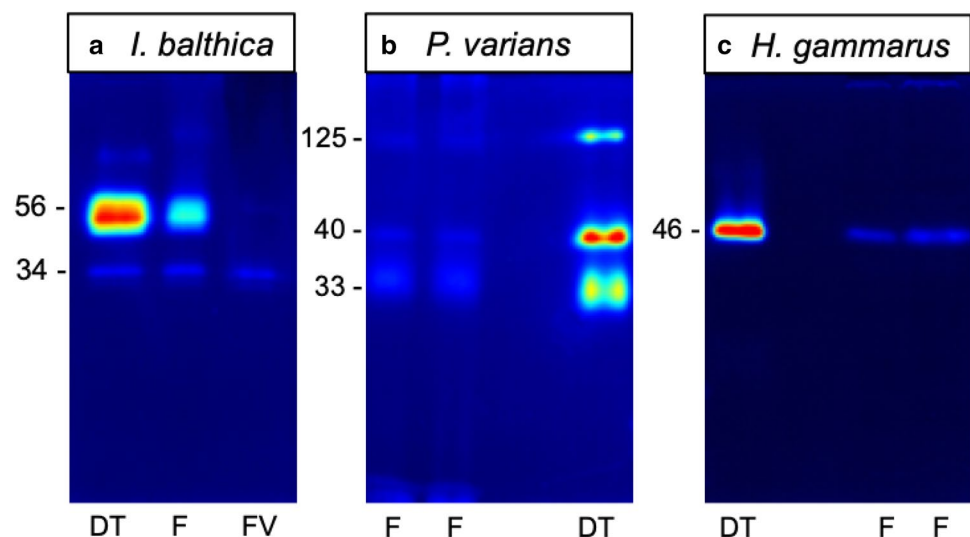
Marked phosphatase activity was present in the faeces of the isopods and the two decapod species. Though microorganisms, e.g., bacteria, are omnipresent in nature and exude their enzymes, our study provides strong indications that the phosphatase in crustacean faeces are primarily of endogenous, i.e., crustacean, origin.

Functional properties

Phosphoesterases (phosphatases) are defined by their catalytic activity and certain structural and molecular properties like the active centre and specific domains (e.g., Millán 2006). Their molecular mass and their isoform pattern may however vary between taxa and, thus, can serve as a distinctive feature between crustacean and bacterial phosphatases.

The phosphatase activity bands visible in digestive tissue extracts of all three species were also present in the faeces extracts. This supports the hypothesis that the phosphatases found in the faeces are consistent with the digestive enzymes of the respective species. Extracts prepared with faeces and digestive tissue from *H. gammarus* showed a single phosphatase activity band, which very likely represented the endogenous phosphatase synthesised in the

Fig. 3 Zymograms of phosphatases stained with MUF-P. **a** *Idotea balthica*; **b** *Palaemon varians*, and **c** *Homarus gammarus*. Extracts of digestive tissues (DT), faeces (F), and the alga *Fucus vesiculosus* (FV). The apparent molecular weights in kDa are indicated on the left side of each panel



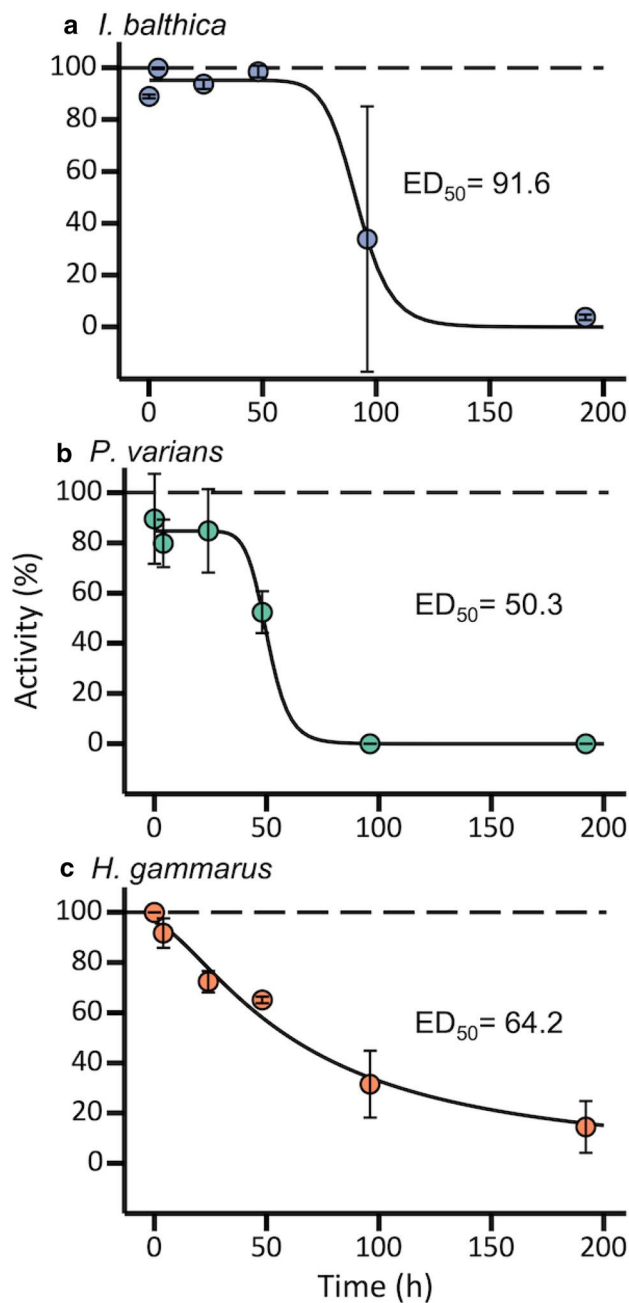


Fig. 4 Stability of phosphatase activity in extracts of faeces of **a** *Idotea balthica*, **b** *Palaemon varians*, and **c** *Homarus gammarus*. Activities measured at six time points are expressed in relation (%) of maximum activity (100%) indicated by the dashed lines

digestive tissues. The appearance of multiple phosphatase activity bands in *P. varians* and *I. balthica* extracts could represent isoenzymes, which can be expressed under certain dietary conditions (Guérin and Kerambrun 1982). Eguchi et al. (1972) found isoforms of alkaline phosphatase in the silkworm *Bombyx mori* and Lee and Chuang (1991) found three isoforms of alkaline phosphatase in the shrimp *Penaeus monodon*, one having a molecular mass of 43 kDa

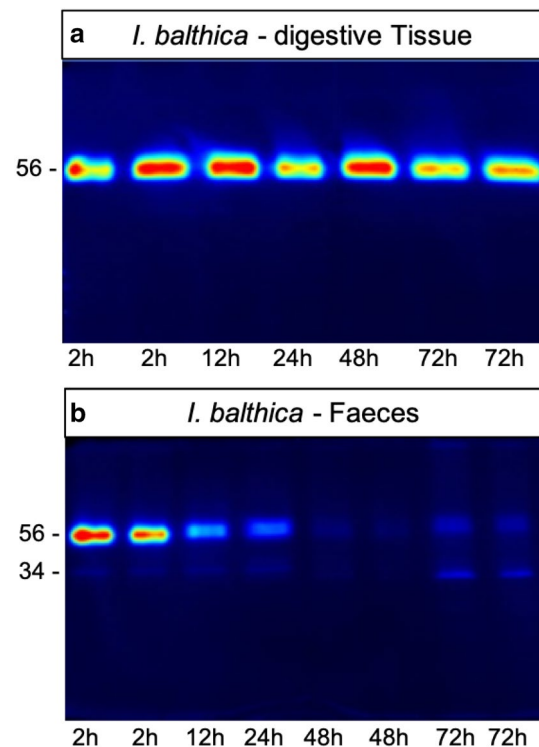


Fig. 5 Zymograms of extracts of **a** digestive tissue extracts; **b** faeces extracts of *I. balthica* stained with MUF-P which were incubated for 2, 12, 24, 48, and 72 h at 16 °C in the dark. The apparent molecular weights (in kDa) are indicated on the left side of the panels

and the other two of 31 kDa. These molecular masses are similar to those of phosphatases from the shrimp faeces and digestive tissue extracts (40 kDa and 33 kDa). Phosphatases with similar molecular masses were also detected in *H. gammarus* (46 kDa) and *I. balthica* (56 kDa and 34 kDa). However, the phosphatase activity band at 34 kDa found in the extracts of *I. balthica* was also present in an extract from the food *F. vesiculosus*. This band could represent a phosphatase expressed by *F. vesiculosus*, which was still detected in the digestive tract and the faeces of *I. balthica*, probably due to low assimilation capabilities. Multiple bands of both *I. balthica* and *P. varians* might indicate various isoforms or precursors of enzymes or active fragments as suggested by Vandooren et al. (2013). Alternatively, additional activity bands might also originate from bacteria, which were either attached to the food and ingested, or from commensal or symbiotic bacteria present in the digestive organs of the crustaceans.

Microbial sources

Exposure of the animals to the antibiotic/antimycotic mixture showed a significant reduction in phosphatase activity in the faeces of *P. varians* but not in those of *I. balthica*

and *H. gammarus*. Using the same substances, Donachie et al. (1995) showed that saprophytic bacteria as well as total bacteria counts strongly decreased in the hepatopancreas and stomach of Northern krill, *Meganyctiphanes norvegica*, already after 24 h. The authors also found a significant decrease of several digestive enzymes, coinciding with low bacterial numbers. In the present study, a similar incubation time (17–20 h) and antibiotic concentration were used, presumably reducing bacterial numbers as effectively as reported by Donachie et al. (1995). Accordingly, the interpretation of our results, that bacteria may contribute to the phosphatase production in the faeces of *P. varians* but not in *I. balthica* and *H. gammarus* appears suitable. However, it has to be considered, that reduction of bacteria numbers may not immediately entail a drop in enzyme activity because the released enzymes may remain stable for several days, as discussed below. Additional long-term experiments are required to better investigate the bacterial contribution on the phosphatase production of *P. varians*.

Information about the presence and the role of symbiotic bacteria in crustacean is scarce and inconsistent. Bacteria and other microorganisms are permanently ingested with the food and egested again with the faeces. However, it remains to be determined, when bacteria can be considered symbiotic in the narrower sense or just constitute the natural and temporal microbiome. According to Zimmer et al. (2001), semi-terrestrial isopods, *Ligilla pallasii*, contained high numbers of endosymbionts. Antibiotic treatment reduced bacteria numbers and decreased the digestibility of food. In contrast, the marine species *Idotea wosnesenskii* and *Gnori-mosphaeroma oregonensis* did not contain bacteria in their hepatopancreas and no decrease in approximate digestibility was observed after antibiotic treatment (Zimmer et al. 2001). Sleeter et al. (1978) discovered that no resident microflora was present in the digestive tract of marine wood boring isopod *Limnoria tripunctata*. The gut contents as well as faeces of this species were also only sparsely colonised by bacteria. In addition, the results of Guarino et al. (1994) gave no indication for hepatopancreatic bacteria in *I. balthica*. Mattila et al. (2014) found bacteria in the gut and in the midgut gland of *I. balthica*, but effects of antimicrobial treatment on the bacteria counts differed with the predominant food source of the isopods.

Antibiotic treatment did not reduce phosphatase activity in samples of *H. gammarus* and no studies are available which clearly indicate the presence of digestive symbionts (in the narrower sense) in *H. gammarus*. However, several studies found bacteria in the digestive systems of other marine decapods, among them *Nephrops norvegicus* (Meziti et al. 2010), which belongs to the same family as *H. gammarus*. The bacterial community in *N. norvegicus* varied with sampling time (season) and is probably related to temporal variation of food (Meziti et al. 2010).

Durability of enzymes

Our results on enzyme durability (activity measurements and zymograms) are in line with previous studies. Steen and Arnosti (2011) showed that natural free enzymes (β -glucosidase and phosphatase) lost very little activity over the course of 36 h. Their measurements were, however, terminated after 36 h. In the present study, phosphatase enzymes had half-lives (ED_{50}) of at least 50 h (~2 days) and maximum of almost 92 h (~4 days), corresponding to the finding of Steen and Arnosti (2011). The actual durability in the present study could have been even longer because the faeces were egested up to 16 h prior to collection and processing.

The decrease of phosphatase enzyme activities in the faeces extracts of all investigated species is a considerable argument against significant microbial enzyme production. It can be assumed, that the extracts are rich in various nutrients and, thus, provide suitable conditions for microbial growth, moreover at a fostering temperature of 16 °C. Accordingly, a prospering microbial community should be expected which increase enzyme activity or, at least, maintain it at initial levels. The observed decrease of activities could only then be explained by the collapse of the microbial community, which, in turn, would also undermine concepts of marine microbial growth and activity in marine systems. More likely, the decrease is due to the continuous degradation of phosphatases by proteolytic enzymes or other physicochemical effects.

Faeces extracts may also contain high amounts of proteases, which are capable of hydrolysing phosphatase enzymes and, thus, reducing their lifetime (Steen and Arnosti 2011). Saborowski et al. (2004) measured proteolytic activity in pure gastric fluid of the Edible Crab *Cancer pagurus* over a longer time span. Chymotrypsin, trypsin and total protease activity were stable for as long as 127 days and, therefore, much longer than the phosphatase of the present study. The stability of these endopeptidases is due to mutations, which reduced the number of autoproteolytic cleavage sites at the surface of the enzyme molecule (Hehemann et al. 2008). The structural resistance of crustacean phosphatase against proteolysis are yet to be investigated. Other factors influencing the stability and activity of exoenzymes may be association with dissolved organic matter (DOM) (Keil and Kirchman 1994), extrapolymeric substances (Huston et al. 2004), or cells or particles (Ziervogel et al. 2007).

Sources of crustacean phosphatases

Digestion is a complex process in crustaceans (Saborowski 2015). Digestive enzymes are released from the midgut gland (also referred to as hepatopancreas) into the stomach, where they facilitate the initial steps of food utilization.

The resulting chyme is pressed back into the midgut gland through the pyloric filter, which separates both organs. Nutrients are absorbed in the midgut gland (Loizzi 1971; Vogt 1994). Undigested food mash soaked with remaining gastric fluid including active enzymes is finally excreted with the faecal pellets. Presumably, only a small amount of digestive enzymes is released with the undigested material. Enzyme production is energetically costly. Therefore, it would be adverse for the animal to lose high amounts of these enzymes through egestion (Allison et al. 2010).

The different ratios of activities between faeces and digestive organs among the studied species may indicate differences in the anatomy and/or physiology of the species. Several factors including sex (Strong and Daborn 1979), size and age (Lee and Lawrence 1985), as well as the moulting cycle and starvation during pre-moult (Klein et al. 1996; Muhlia-Almazán and García-Carreño 2002) are known to influence metabolism and might also lead to intraspecific differences of enzyme activities. These factors were not assessed in our study but cannot be excluded. However, animals in the late stages of pre-moult were omitted from enzyme analysis, because they do not feed and, thus, do not produce faecal pellets.

Intraspecific variation of phosphatase activity in the faeces might also be due to leakage of enzymes from the faeces after egestion and exposure to water. The faecal material is enveloped within porous peritrophic membranes (Peters 1992). The leakage of dissolved organic carbon from faeces has been observed within a few hours after egestion (Thor et al. 2003). Faeces were collected every 16 h in our study. Thus, their maximum age (time after egestion) is defined, but not the real age of each single faecal pellet. Therefore, faeces from some animals might have been exposed to water for a shorter period than others.

Variations between species might be due to the specific physiological properties. Dietary differences, for example, might cause modifications in gut retention time, assimilation efficiency, and digestive enzyme synthesis. Gutow et al. (2006) observed differences in digestive enzyme activities between the mainly herbivorous *I. balthica* and the more carnivorous *I. metallica*. Herbivorous species might also have short gut retention times (Kurmaly et al. 1989). Long gut retention times, i.e., low ingestion and egestion rates, can have a positive effect on the assimilation efficiency (Boehlert and Yoklavich 1984). It also has to be noted that the parts of the digestive tract used for extract preparation were not the same for all species. In the case of *I. balthica*, the midgut gland was used together with the gut because both organs contribute significantly to digestive processes. In the case of *P. varians* and *H. gammarus*, only the midgut glands were used, as they represent the major digestive organs. The guts of these species are tenuous and difficult to dissect. Variation of phosphatase activity in the faeces of different species

could be due to the differences in the rate of enzyme leakage. Lobster and shrimp faeces have an elongated tubular shape compared to the almost spherical shape of the isopod faeces. It is possible that enzyme leakage from the faecal pellets of the lobsters and shrimps is higher than from those of isopods due to a higher surface/volume ratio of their faeces.

Ecological implications

Extracellular enzyme activity has almost exclusively been attributed to bacteria (Hoppe et al. 1988; Chróst 1991; Sala and Güde 1996; Arnosti et al. 2014). Thus, degradation of organic matter by bacteria is considered to be the key process in remineralisation (Azam and Malfatti 2007; Pomeroy et al. 2007). There are, however, indications that remineralisation processes cannot be exclusively explained by bacterial activity (Giering et al. 2014, and references therein). Bohdansky et al. (1995) studied the influence of zooplankton-grazing on the concentration of free dissolved enzymes in the water and found a significant increase in the concentration of free dissolved enzymes whenever zooplankton was present. This increase was attributed to a substantial release of digestive enzymes derived from the zooplankton. Rigler (1961) found little difference between the enzymatic activities in an antibiotic-treated culture of *Daphnia magna* and an untreated culture of this species, which indicates that active enzymes derived from *D. magna*. Wynne and Gophen (1981) reported that the enzyme activities released by *D. magna* varied according to the food source and suggested that these may either be digestive enzymes released by *D. magna* or components of their food. The latter was ruled out by the study of Boavida and Heath (1984).

The findings of the present study strongly indicate that extracellular enzymes from invertebrate origin can significantly contribute to the extracellular enzyme pool in the marine environment. Jacobsen and Azam (1984) concluded that bacteria colonising faecal pellets may not be the major group responsible for carbon mineralisation in the upper water column. Bacteria have been shown to be scarce on freshly egested faecal pellets, but they colonised a significant part of the pellet surface after 24 h (bacteria per surface area) (Jacobsen and Azam 1984). Organic matter, however, rapidly leaks from the faecal pellets directly after egestion (Møller et al. 2003). This indicates that bacterial activity cannot be the cause for this organic matter release. Jumars et al. (1989) stated that endogenous digestive enzymes are excreted with faecal pellets and remain active. The released organic matter, or the enzymes themselves, represent a primary nutrient source for bacteria, which helps the bacteria to settle on particles. This process could also initialise the colonisation of these particles by bacteria, assuming the bacteria follow chemical cues in search for food (Vetter et al. 1998; Kjørboe and Jackson 2001).

In offshore surface waters or shallow coastal waters, the released nutrients, here phosphorus, can be directly utilised by primary producers like phytoplankton or macroalgae. In this way, herbivorous marine animals (e.g., *I. balthica*) could potentially directly fertilise their own food source (Boavida and Heath 1984). Epibenthic animals like *H. gammarus* and *P. varians* may contribute directly in fertilising the benthic flora and turnover rates of organic matter in shallow water sediments. The advantage for carnivorous species would be more indirect, but they still benefit if nutrients are instantly provided to primary producers, which form the basis of the food chain.

Our considerations can be easily transferred into the open water, where various pelagic species feed on phytoplankton and zooplankton, thereby releasing immense amounts of faecal pellets. Enzymes which are stable for several days could also, via particle flux, be taken down to deeper waters, potentially adding to the active enzyme pool in the deep sea and further participating in remineralisation processes (Baltar et al. 2010). Stability of cell free and particle-associated extracellular enzymes can have a great impact on the microbial loop and on turnover rates of organic matter in marine systems (Boudreau et al. 2008; Steen and Arnosti 2011). Faecal pellets of zooplankton sink up to 50 m per day (Urban-Rich 1999). Most enzymes examined in the present study still had activity after 8 days (half-lives were shorter). During this time, these enzymes could reach depths between 400 and 540 m (mesopelagic realm). The importance of enzymes originating from sinking particles in greater depths has been demonstrated by Koike and Nagata (1997), who suggested that sinking particles, rather than bacteria inhabiting these depths, were the source of alkaline phosphatase activity (APA) in the deep sea. This corresponds with the findings of Davis and Mahaffey (2017), showing that alkaline phosphatase activity was strongly concentrated in fast-sinking particles, which were zooplankton faecal pellets. Slow-sinking particles had low alkaline phosphatase activities and were mostly phytodetritus (from “sloppy feeding”). Phytodetritus probably derived from autotrophic biomass in surface layers. Therefore, it was hypothesised that zooplankton may have contributed to alkaline phosphatase (AP) present in the mid-water layer through the introduction of AP associated with faecal pellets (Davis et al 2019). “Sloppy feeding” contribution to APA was considered less likely because of low APA in slow-sinking phytodetritus. Generally, these findings show that sinking particles, and especially faecal pellets, might

be an important source of enzymes and that these enzymes are not necessarily derived from bacteria. The discussed literature, in concert with the results of our study, suggests a strong potential for the contribution of extracellular enzymes of invertebrate origin to marine nutrient cycles. Enzymes released with egesta from animals could initialise the bacterial colonisation of these particles through the release of dissolved organic matter (carbohydrates, amino acids, lipids, etc.) or dissolved inorganic matter, such as dissolved inorganic phosphorus or dissolved inorganic nitrogen. These solutes could act as chemical cues for the bacteria, facilitate bacterial production, and help sustain the microbial population in and on these particles. In this way, egesta would contribute to remineralisation processes and at the same time fuel microbial growth and, consequently microbial remineralisation abilities (Fig. 6). Thus, extracellular enzymes from marine invertebrates (or metazoan in general) would precede and fuel the processes attributed to the microbial loop.

Conclusions

The results support our first hypothesis, that crustaceans release active phosphatases with their faeces. Antibiotic treatment did not reduce phosphatase activities in the faeces of *I. balthica* and *H. gammarus* but in the faeces of *P. varians*. This may allow for the assumption that enzymes did not derive from bacteria in *I. balthica* and *H. gammarus* but partly in *P. varians*. However, a definite conclusion cannot be drawn from the present results but needs further validation. The enzymes were stable for several days, which was shown by the activity time series of all three species and the zymogram time series of *I. balthica*. Consequently, the hypothesis that crustacean extracellular enzymes are stable under natural conditions can be accepted. The fact that the enzymes were most active at the beginning of the time series and activity declined with increasing incubation time is a strong indication that numbers of bacteria might have been too low to establish a sustaining population, which produced significant amounts of phosphatase enzymes. This further supports our hypothesis that phosphatases were of endogenous, i.e., crustacean origin, capable of facilitating degradation and remineralisation processes, and potentially boosting microbial growth.

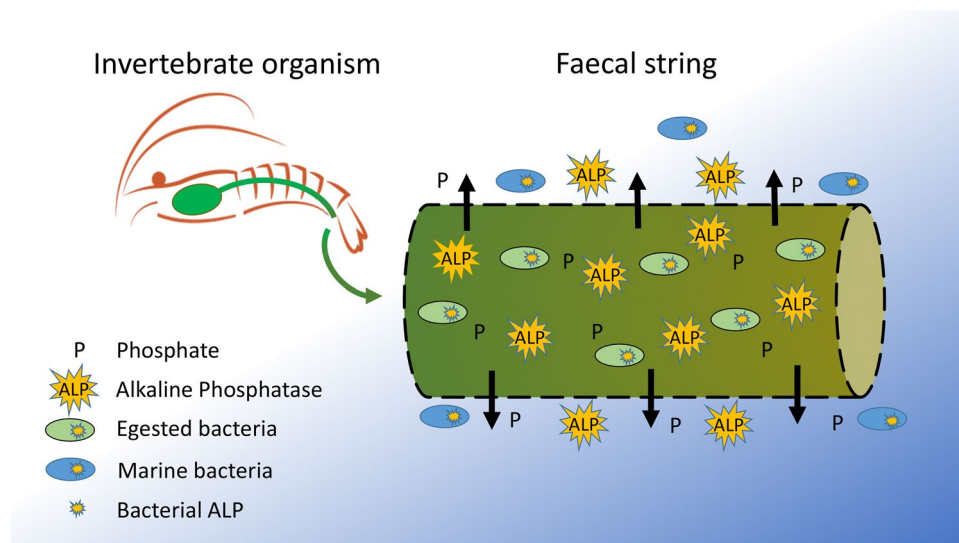


Fig. 6 Conceptual consideration on the generation, liberation, and biological interaction of extracellular phosphatases derived from invertebrate faeces. Animals ingest food (phyto- and zooplankton, macroalgae, etc.) and process it in the stomach and other digestive organs. The food is mixed with highly active digestive enzymes, which initiate the first steps of food digestion and nutrient utilization. Undigested material in the stomach contains remains of enzymes, here endogenous phosphatases, which are egested with the faeces.

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Author contributions IMB and RS designed the study. IMB performed the experiments, collected and processed the data. IMB and RS drafted and finalized the manuscript. Both authors read and approved the final version of the manuscript.

Data availability Data collected and analyzed within the current study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All international, national, and institutional guidelines for sampling of organisms for this study have been followed.

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The faeces are coated by a porous peritrophic membrane. It maintains the shape of the pellet but allows diffusion of molecules from the inside into the surrounding water. These molecules can be degraded nutrients as well as still active enzymes. Simultaneously, the pellets will contain bacteria and other microbes which were ingested together with the food. Diffusing nutrients may additionally fuel growth of external marine bacteria which attached at the surface of the faecal pellet

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