Emerging Contaminants 10 (2024) 100275

Contents lists available at ScienceDirect

Emerging Contaminants

journal homepage: www.keaipublishing.com/en/journals/emerging-contaminants

# Gastric carboxylesterases of the edible crab *Cancer pagurus* (Crustacea, Decapoda) can hydrolyze biodegradable plastics

Lukas Miksch*, Lars Gutow, Reinhard Saborowski

*Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570, Bremerhaven, Germany*

A R T I C L E I N F O

*Article history:*
Received 1 September 2023
Received in revised form
5 October 2023
Accepted 5 October 2023
Available online 6 October 2023

*Keywords:*
Biodegradable plastics
Microplastics
Crustaceans
Ingestion
Enzymes
Enzymatic degradation

A B S T R A C T

A promising strategy to counteract the progressing plastic pollution of the environment can involve the replacement of persistent plastics with biodegradable materials. Biodegradable polymers are enzymatically degradable by various hydrolytic enzymes. However, these materials can reach the environment in the same way as conventional plastics. Therefore, they are accessible to terrestrial, freshwater, and marine biota. Once ingested by marine organisms, highly active enzymes in their digestive tracts may break down biodegradable compounds. We incubated microparticles of five different biodegradable plastics, based on polylactictic acid (PLA), polybutylene succinate (PBS), polybutylene adipate terephthalate (PBAT) and polyhydroxybutyrate-*co*-valerate (PHBV), *in-vitro* with the gastric fluid of the edible crab *Cancer pagurus* and evaluated the hydrolysis rates by pH Stat titration. A plastic blend of PLA with PBAT showed the highest hydrolysis rate. The enzymes in the gastric fluid of crabs were separated by anion exchange chromatography. Fractions with carboxylesterase activity were identified using fluorescent methylumbelliferyl (MUF)-derivatives. Pooled fractions with high carboxylesterase activity also hydrolyzed a PLA/PBAT plastic blend. Carboxylesterases showed molecular masses of 40–45 kDa as determined by native gel electrophoresis (SDS-PAGE). Our study demonstrated that digestive carboxylesterases in the gastric fluid of *C. pagurus* exhibit a high potential for hydrolyzing certain biodegradable plastics. Since esterases are common in the digestive tract of organisms, it seems likely that other invertebrates possess the ability to hydrolyze biodegradable plastics.
© 2023 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

## 1. Introduction

Plastics are synthetic polymers that are omnipresent in our daily life due to their variety of advantageous properties, such as light weight, low production cost and chemical resistance. In 2020, 367 million tons of plastics were produced worldwide [1]. High fabrication output and excessive use of plastic products challenges communal life and environmental care, creating an unprecedented amount of persistent waste. Unmanaged and mismanaged plastic waste enters the environment and accumulates in terrestrial and aquatic ecosystems [2]. Conventional plastics are highly resistant to degradation, but will eventually break down into smaller fragments through UV exposure and mechanical forcing by wind and wave action [3]. This continuous fragmentation leads to the generation of tiny particles, referred to as microplastics, at a size smaller than 5 mm [4]; microplastics are now ubiquitous in all ecosystems [5]. Due to their small size and widespread distribution in the water column and sediments, they are ingested by a variety of organisms and can induce deleterious effects on a cellular (oxidative stress, inflammation), individual (reduced growth and fecundity) and ecosystem level (altered population structure) [6–8].

To counteract environmental pollution by persistent synthetic polymers, interest in developing biodegradable plastics has increased. According to the definition by the International Union of Pure and Applied Chemistry [9], biodegradation is the breakdown of a substance catalyzed by enzymes. Depending on the duration it takes for a plastic to reach a certain degree of degradation, several standards for biodegradation under specific conditions or environments were defined by international institutions such as OECD, ASTM, and DIN-Certco (e.g. Refs. [10–12]). Biodegradable materials can be based on fossil resources, but can also derive from extant biomass. A common biodegradable polymer that is currently made

* Corresponding author.
*E-mail address:* lukas.miksch@awi.de (L. Miksch).
Peer review under responsibility of KeAi Communications Co., Ltd.

https://doi.org/10.1016/j.emcon.2023.100275
2405-6650/© 2023 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

starting from fossil resources is polybutylene adipate terephthalate (PBAT), while polymers that are both bio-based and biodegradable are e.g. polylactic acid (PLA), polybutylene succinate (PBS), and polyhydroxybutyrate-*co*-valerate (PHBV).

Irrespective of the feedstock, the biodegradation process of synthetic biodegradable polymers is similar to that of natural polymers such as cellulose or chitin [13]. Hydrolytic enzymes, capable of cleaving the polymer chains, play a key role in this process [14]. In the marine environment, extracellular enzymes are released by microbes and other organisms that form biofilms on the surface of the plastic [15]. These hydrolytic enzymes comprise various peptidases and esterases, some of which have been demonstrated to hydrolyze biodegradable plastics *in-vitro* (e.g. Refs. [13,16,17]). Although some biodegradable plastics are degraded by microbial enzymes, the degradation rates of most of these materials are very low under marine conditions [18,19]. As a result, microplastics originating from biodegradable materials may also accumulate in the ocean, potentially constituting a new form of emerging pollutants [20].

Progressive mechanical and chemical fragmentation of biodegradable plastic items may contribute to the pool of microplastics in the environment. Numerous species have been found to ingest microplastics, including fishes [21], mollusks [22], and crustaceans [23]. Higher crustaceans, such as crabs and lobsters, possess a well-defined digestive system with a voluminous stomach, which contains a variety of highly active digestive enzymes derived from the midgut gland, *syn.* hepatopancreas [24]. The digestive enzymes mainly comprise hydrolases, which can break down ester, peptide, and glycosidic bonds [25]. Upon ingestion, conventional and biodegradable microparticles are exposed to these enzymes. Accordingly, we address whether enzymes in the digestive tract of crabs can hydrolyze biodegradable plastics.

In this study, we investigated the potential of the edible crab *Cancer pagurus* (Decapoda, Brachyura) to hydrolyze biodegradable plastics based on PLA, PBS, PBAT and PHBV. We chose this crab species as a model organism because it is large enough to provide repeatedly sufficient amounts of gastric fluid for experimentation (up to one mL) by gastroscopy. Isolated gastric fluid was incubated *in-vitro* with different biodegradable plastics under controlled conditions. Enzymatic hydrolysis of the polymers was determined by pH-Stat titration [17]. Enzymes were separated and characterized by anion-exchange chromatography, native gel electrophoresis, and their substrate specificity.

## 2. Materials and methods

### 2.1. Chemicals

The NaOH-standard solution (cat. no. 5564732) for pH Stat titration was purchased from Omnilab (Bremen, Germany). 4-methylumbelliferone derivatives and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

### 2.2. Plastics

The test materials (Table 1) were provided by Arctic Biomaterials OY (ABM, Tampere, Finland) and NaturePlast (Mondeville, France). The materials are based on common bio-based and biodegradable polymers. The materials were supplied as granules ($3 \times 3$ mm, 25 mg), which were ground to a fine powder (<200 μm) with a cryogenic mill (SPEX SamplePrep, 6775 Freezer/Mill) as described by Miksch et al. (2022) [19]. A common conventional plastic, poly(methyl methacrylate) (PMMA), known for its high resistance against biodegradation, was used to compare biodegradable and non-biodegradable plastics. PMMA was manufactured and provided by Kunststoff- und Farben-Gesellschaft GmbH (KFG, Biebesheim, Germany) and supplied as a powder with particles <200 μm.

### 2.3. Origin and maintenance of crabs

Adult crabs (*Cancer pagurus* Linnaeus, 1758) were collected by beam trawling with the research vessel FK Uthörn in the North Sea near the island of Helgoland, Germany (54°09′54.7 N 7°52′06.2 E). Six animals, two females without eggs and four males, were immediately transferred to 50-L tanks with running seawater and shipped to the Alfred Wegener Institute in Bremerhaven (Germany). The body mass of the crabs varied between 300 and 1100 g. The animals were kept individually in 50-L aquaria in the laboratory, which were integrated into a 500-L recirculation seawater system. The temperature was $15 \pm 1$ °C and the light: dark cycle was 12:12 h. The crabs were fed three times per week with frozen shrimp or fish. Half of the seawater in the recirculation system was exchanged every other week. Water parameters (T, pH, $NH_4$, $NO_2^-$, and $NO_3^-$) were monitored once a week.

### 2.4. Extraction of the gastric fluid

The gastric fluid of the crabs was extracted every two weeks by inserting a small PTFE tube (1 mm inner diameter) connected to a syringe (1 mL) through the esophagus into the gastric chamber of the crab. Digestive fluids were then slowly aspirated. Sampling was performed 24–48 h after feeding. The gastric fluid from the individuals was transferred into 2-mL reaction tubes and centrifuged for 10 min at 20,000 g and 4 °C to remove suspended solids. Parts of the supernatants (50 μL) were transferred into new reaction tubes for individual electrophoresis. The remaining supernatants were pooled and aliquoted into portions of 100 or 500 μL and stored at −80 °C until further use. The pooling was done to provide sufficient gastric fluid for further experiments such as liquid chromatography.

### 2.5. Enzyme screening and protein concentration of the gastric fluid

Semi-quantitative ApiZym strips (BioMerieux, Marcy l'Etoile, France) were used to identify 19 hydrolytic enzymes in the gastric fluid. Pooled gastric fluid (65 μL) was dispensed into each of the

**Table 1**
Specification sheets of the used polymers.

| Designation | Base polymer | Producer | Application | Biodegradability |
|---|---|---|---|---|
| C-PLA | PLA/PBS | ABM | Cutlery | No information |
| RP-PLA | PLA/PBS | ABM | Rigid packaging | Manufactured from compostable plastic (EN 13432) |
| AMF-PLA | PLA/PBAT | NaturePlast | Mulch films | No information |
| SP-PBS | PBS | NaturePlast | Soft packaging | Industrially compostable (NF EN 13432:2000) |
| T-PHBV | PHBV | NaturePlast | Toys | Industrially compostable (ASTM D6400) |
| PMMA | PMMA | KFG | Industry | Not biodegradable |

microcupules of the ApiZym strip. The strips were placed in the supplied tray, covered with the lid, and incubated in the dark at room temperature. After 24 h, ZYM A and ZYM B reagents were added to the microcupules to initiate the chemical color reaction. The results were visually compared with a control (no color development) and ranked by numbers as per the instructions of the manufacturer according to the strength of the color from low intensity (1) to high intensity (5) and intermediates. No visible color development was graded as 0. The protein concentration in the gastric fluid was determined after Bradford (1976) [26], using bovine serum albumin as a reagent standard.

### 2.6. pH-Stat titration

pH-Stat titration was carried out with the automatic titrator TitroLine 7000 (SI Analytics GmbH, Mainz, Germany). The titration unit had a 20-mL exchangeable head and a 1 mm diameter PTFE tube as the titration tip. The unit was connected to a magnetic stirrer (TM 235), a pH-electrode model A 162 2 M DIN ID, and a circulation thermostat (Lauda, Lauda-Königshofen, Germany). The reaction vial was a 20-mL glass vial placed in a custom-made thermostat jacket to maintain a constant temperature [17].

pH-Stat titration was performed after Miksch et al. (2021) [17]. Briefly, suspensions of polymer microparticles (3 mg $mL^{-1}$) were prepared in a solution of 32 ppt sea salt (Seequasal, Münster, Germany) in deionized water (referred to as artificial seawater). The suspensions were stirred in a glass beaker with a magnetic stirring bar at 800 rpm for 16 h before aliquots of 10 mL were subjected to pH stat titration. Pooled gastric fluid (80 μL) was added to the reaction vial with a 100-μL micro-syringe (Model 710 N, Hamilton Bonaduz AG, Bonaduz, Switzerland). The pH was kept constant at 8.2 by titration of 10 mmol $L^{-1}$ NaOH-solution. The addition of NaOH-solution was recorded every minute for 60 min before and after enzyme addition. The hydrolysis rate was calculated from the linearly increasing consumption of NaOH over time. The hydrolysis of the polymers was measured at 15 °C and 30 °C. The electrode was calibrated every day before use. Routine measurements were carried out in triplicate.

### 2.7. Fractionation of gastric fluid

Pooled gastric fluid was first desalted and rebuffered over Sephadex™ NAP-5 gel filtration columns (GE Healthcare, Little Chalfont, United Kingdom) and diluted (1:2 v/v) with 0.1 mol $L^{-1}$ Tris-HCl buffer (pH 7.5). Thereafter, 1 mL of the sample was injected into the LC-system (NGC Chromatography System, BioRad) equipped with an anion exchange column (UNO Q1 R, 1.7 × 35 mm, BioRad). The proteins in the gastric fluids were eluted by increasing the concentration of NaCl from 0 to 1 mol $L^{-1}$ in the buffer (0.1 mol $L^{-1}$ Tris-HCl, pH 7.5) with a flow rate of 1 mL $min^{-1}$. The absorbance of the eluate was detected at 280 nm. The gastric fluid was separated into 65 fractions of 1 mL each, which were immediately monitored for esterase enzyme activity in a 96-well microplate. Fractions with high enzyme activity were frozen and stored at −80 °C for further usage. To determine whether the potential to hydrolyze biodegradable plastic was maintained after anionic exchange separation, fractions with high carboxylesterase activity were pooled and subjected to ultrafiltration (Vivaspin 15R, Sartorius, Göttingen, Germany). Twelve mL of enzyme-containing fractions were spun at 3000 g and 4 °C for 60 min, reducing the volume to about 300 μL. The concentrated fractions were then incubated with plastic suspensions and the hydrolysis rates were measured by pH Stat titration as described above.

### 2.8. Enzyme activity assay

Esterase and lipase activities in the 65 gastric fluid fractions were assayed since previous studies report the highest hydrolysis rates of these plastics by lipolytic enzymes [19]. The hydrolysis of fatty acid esters and the resulting increase in fluorescence was interpreted as esterase or lipase activity [27,28]. 4-methylumbelliferone derivatives from esters of fatty acids (MUF-butyrate, MUF-heptanoate, MUF-oleate) were dissolved in dimethyl sulfoxide (DMSO) and then diluted with 40 mmol $L^{-1}$ Britton-Robinson buffer, pH 5 to 9 [29] to obtain stock solutions of 0.1 mmol $L^{-1}$ substrate concentration and 2% DMSO. Stock solution (250 μL) was pipetted into a 96-well microplate, adding 50 μL of sample to each well. The plate was shaken for 10 s in a microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Corporation, USA) before measuring the fluorescence over 5 min in 20 intervals of 15 s at room temperature ($\lambda_{ex}$ 355 nm, $\lambda_{em}$ 460 nm). The results were recorded by the Ascent Software for Fluoroskan Ascent FL. Standard curves with 0–35 μmol $L^{-1}$ 4-methylumbelliferone (MUF, Sigma cat.no M1381) were prepared at pH 5 to 9, as the fluorescence signal varies with pH.

The use of SDS in protein separation may affect enzyme activity and thus impair the visibility of active protein bands. To determine the effect of SDS on enzymatic activity, the same stock solutions with MUF-derivatives dissolved in DMSO were prepared, but only with Britton-Robinson buffer at pH 7. 0.12% SDS was then added to the stock solution of each substrate (MUF-butyrate, MUF-heptanoate, MUF-oleate) and vortexed. The stock solution (250 μL) was pipetted into a 96-well plate and 50 μL of sample was added to obtain a final concentration of 0.1% SDS in each well. The fluorescence was measured in the same way as described above.

### 2.9. SDS-PAGE

Samples of individual and pooled gastric fluid and selected fractions after LC separation were separated by native SDS-PAGE (12% acrylamide) in a vertical Hoefer SE 250 electrophoresis device. Fractions were diluted 1:2 with sample buffer containing 2% SDS and bromophenol blue. The samples were not treated with mercaptoethanol, nor were they heated. Polyacrylamide gels were loaded with 10 μL of sample per lane and 2.5 μL of molecular weight markers (Page Ruler Plus Prestained Protein Ladder, Thermo Fisher Scientific, cat. no. 26619) in each leftmost and rightmost lane. Electrophoresis was conducted at a constant current of 15 mA per gel and a maximum voltage of 300 V at 4 °C.

After electrophoretic separation, the gels were washed in demineralized water for 10 min and then in 50 mmol $L^{-1}$ Tris-HCl buffer (pH 7) for 10 min. Thereafter, the gels were incubated in 100 mL of fluorogenic MUF-substrate solution (0.1 mmol $L^{-1}$) for another 10 min. The gels were photographed under a BioRad Gel DOC EZ Imager (BioRad, Hercules, California) to identify active protein bands that hydrolyzed the substrates. The gels were then rewashed for 10 min in demineralized water, before Coomassie-staining overnight and after de-staining with an aqueous ethanol and phosphoric acid solution. Blue-dyed protein bands were identified and compared with fluorogenic protein bands.

### 2.10. Statistics

Statistical analysis and graphs were done with the software GraphPad Prism version 7.05 for Windows ( GraphPad Software, La Jolla California USA, www.graphpad.com). The significance level of all statistical analyses was $\alpha = 0.05$. The hydrolysis rates of the different compounds by the gastric fluid were compared by a 1-factorial analysis of variance (ANOVA) ($n = 3$). Before the ANOVA,

the data were tested for heteroscedasticity by the Brown-Forsythe test. The carboxylesterase activities of the fractions with and without the addition of SDS were compared with a paired *t*-test.

## 3. Results

### 3.1. The gastric fluid of Cancer pagurus

Depending on the size of the individual crabs, between 200 μL and 1 mL of gastric fluid could be obtained from each crab. The fluid showed an intense color, varying from bright orange to dark olive brown. The gastric fluid was slightly acidic, with pH values ranging from 6.0 to 6.4. The protein content measured by the Bradford method accounted on average for 8.62 ± 2.74 μg $\mu L^{-1}$. The enzyme screening with ApiZym® showed intensive color development and thus high activity for 16 of the 19 examined enzymes (Table 2). The only enzymes with low intensity were cysteine arylamidase, valine arylamidase, and α-chymotrypsin.

### 3.2. Hydrolysis of plastics by the gastric fluid

pH-Stat titration of plastics with the gastric fluid of *C. pagurus* showed significantly different hydrolysis rates for the different polymers (ANOVA: $F_{(2,6)} = 78.55$, $p < 0.01$). The conventional petroleum-based plastic PMMA showed no degradation. The same was observed for the putative biodegradable plastics C-PLA and RP-PLA. SP-PBS and T-PHBV showed low hydrolysis rates of 0.26 ± 0.25 and 0.72 ± 0.63 nmol $min^{-1}$, respectively. The hydrolysis rate was highest for AMF-PLA with 8.01 ± 1.32 nmol $min^{-1}$ (Fig. 1).

### 3.3. Chromatographic separation of gastric fluids

Anion exchange chromatography resulted in fractionation of the gastric fluid into 65 separate fractions of 1 mL each. The best separation was achieved using a low gradient with increasing NaCl concentrations from 0 to 0.6 mol $L^{-1}$ over the first 40 min. The remaining proteins on the column were eluted at higher NaCl concentrations of up to 1 mol $L^{-1}$. Six protein peaks emerged in the chromatogram of the gastric fluid. The most intense absorbance at 280 nm was observed between fractions 25 and 55, comprising five of the highest absorbance peaks. The highest absorbance was measured in fraction 36. High absorbance was also present in fractions 46/47, 50/51, and 63 (Fig. 2a).

### 3.4. Enzyme activities

#### 3.4.1. Screening for carboxylesterases

Enzymes in the separate fractions of the gastric fluid could hydrolyze short-chain, medium-chain, and long-chain carboxylesters (Fig. 2b). MUF-butyrate (short-chain, C4) was mainly hydrolyzed by fractions 29 to 51. The activity peaked in fractions 30 (1.21 mU·$mL^{-1}$), 37 (4.06 mU·$mL^{-1}$), 41 (2.0 mU·$mL^{-1}$), and 45 (2.43 mU·$mL^{-1}$). Hydrolysis of MUF-heptanoate (medium-chain, C7) appeared in the same fractions as hydrolysis of MUF-butyrate, but with different intensities. Fraction 45 showed the highest activity of 8.56 mU·$mL^{-1}$, followed by fraction 30 (5.18 mU·$mL^{-1}$) and fraction 37 (3.92 mU·$mL^{-1}$). The lowest activity peak appeared in fraction 41 with 1.15 mU·$mL^{-1}$. Hydrolysis of MUF-oleate (long-chain, C18) showed only one distinct activity peak over fractions 44 and 45 (29.3 and 28.3 mU·$mL^{-1}$, respectively). The fractions showing carboxylesterase activity peaks are denoted as $E_{(I)}$ to $E_{(IV)}$, as displayed in Fig. 2b.

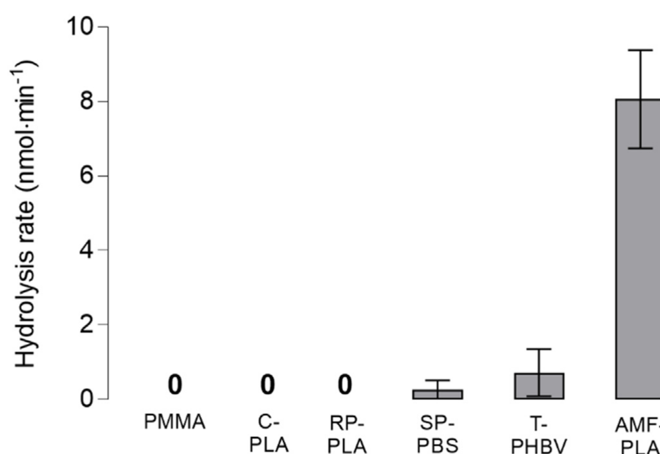


**Fig. 1.** Hydrolytic degradation of five bio-based plastics and one petroleum-based plastic (PMMA) by the gastric fluid of *Cancer pagurus* measured by pH-Stat titration at 15 °C, pH 8.2 and 3.2 ppt salinity (means ± SD, n = 3). A zero indicates no detected hydrolysis.

**Table 2**
Semi-quantitative assay of enzyme activities in the gastric fluid of *Cancer pagurus* ranked 0–5 according to color intensity in ApiZym® strips.

| Enzyme | Intensity | Enzyme | Intensity |
|---|---|---|---|
| Control | 0 | Acid phosphatase | 5 |
| Alkaline Phosphatase | 5 | Naphthol-AS-BI-phosphatase | 5 |
| Esterase (C4) | 4 | α-Galactosidase | 3 |
| Esterase/Lipase (C8) | 4 | β-Galactosidase | 5 |
| Lipase (C14) | 4 | β-Glucuronidase | 5 |
| Leucine arylamidase | 5 | α-Glucosidase | 5 |
| Valine arylamidase | 1 | β- Glucosidase | 5 |
| Cysteine arylamidase | 0 | *N*-acetyl-β-D-glucosaminidase | 5 |
| Trypsin | 5 | α-Mannosidase | 4 |
| α-Chymotrypsin | 1 | α-Fucosidase | 4 |

#### 3.4.2. pH profiles

Carboxylesterase activities of $E_{(I)}$ to $E_{(IV)}$ showed specific pH profiles between pH 5 and 9.

$E_{(I)}$ (Fraction 30): Hydrolysis of MUF-butyrate increased from 0.74 mU·$mL^{-1}$ at pH 5 to 2.45 mU·$mL^{-1}$ at pH 7. At pH 8, the activity decreased again and ceased at pH 9. Similarly, hydrolysis of MUF-heptanoate increased from pH 5 (1.25 mU·$mL^{-1}$) to pH 7 (10.29 mU·$mL^{-1}$) and pH 8 (10.62 mU·$mL^{-1}$) and decreased at pH 9 (5.56 mU·$mL^{-1}$). No hydrolysis of MUF-oleate was present in $E_I$ (Fig. 3a).

$E_{(II)}$ (Fraction 37): Highest hydrolysis rates of MUF-butyrate appeared at pH 5 to pH 8 (7.54–8.1 mU·$mL^{-1}$). The activity decreased to 2.53 mU·$mL^{-1}$ at pH 9. Hydrolysis of MUF-heptanoate showed a broad pH maximum between pH 5 to pH 8 and a slight decrease of activity at pH 9 (4.55 mU·$mL^{-1}$). No hydrolysis of MUF-oleate was detected (Fig. 3b).

$E_{(III)}$ (Fraction 41): Hydrolysis of MUF-butyrate increased from pH 5 (3.3 mU·$mL^{-1}$) to pH 6 (6.71 mU·$mL^{-1}$) and decreased continuously toward pH 9, where it reached 1.94 mU·$mL^{-1}$. The hydrolysis rate of MUF-heptanoate was lower than that of MUF-butyrate but less variable over the investigated pH range. The activities ranged from 1.84 mU·$mL^{-1}$ at pH 5 to 3.58 mU·$mL^{-1}$ at pH 8. As in $E_{(I)}$ and $E_{(II)}$, no hydrolysis of MUF-oleate was detected (Fig. 3c).

$E_{(IV)}$ (Fraction 45) showed the highest hydrolysis rate of all MUF derivatives. Hydrolysis of MUF-butyrate was highest at pH 5 (38.62 mU·$mL^{-1}$) and decreased continuously toward pH 8 (2.52 mU·$mL^{-1}$). No hydrolysis was present at pH 9. Similarly, MUF-heptanoate showed the highest degradation at pH 5 (58.85 mU·$mL^{-1}$) with a continuous decrease at higher pH. No activity

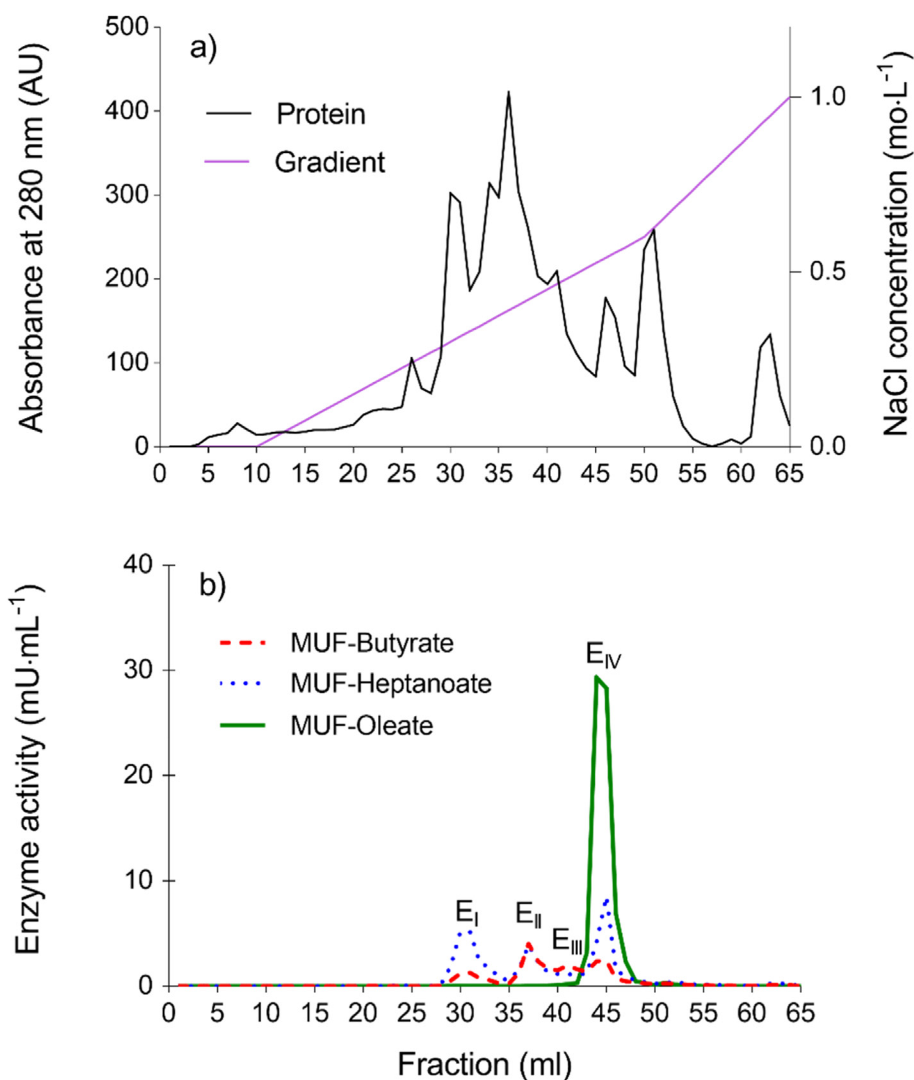


**Fig. 2.** a) LC chromatogram of gastric fluids from *Cancer pagurus*. The consecutive fractions of 1 mL each at a flow rate of 1 mL $min^{-1}$ are given on the x-axis. The left y-axis shows the absorbance at 280 nm (protein) in arbitrary units (AU). The purple line represents the steadily increasing NaCl concentration in the buffer over time (right y-axis). b) Enzyme activity ($mU \cdot mL^{-1}$) of esterases and lipases of each fraction measured as hydrolysis of the specific fluorogenic substrates at pH 7. Four maxima with elevated esterase/lipase activity ($E_{(I)} - E_{(IV)}$) were identified.

was present at pH 9. Hydrolysis of MUF-oleate increased from pH 5 (13.32 $mU \cdot mL^{-1}$) to pH 6 (24.69 $mU \cdot mL^{-1}$) and decreased at higher pH towards 4.73 $mU \cdot mL^{-1}$ at pH 8. No activity remained at pH 9 (Fig. 3d).

#### 3.4.3. Effect of SDS on enzyme activities

The presence of 0.1% SDS significantly reduced the esterase activity in the gastric fluid and, thus, the hydrolysis rates of all tested substrates (Paired *t*-test: $p < 0.01$). The activities of the fractions were not equally affected by SDS.

While the activity in $E_{(I)}$ dropped by 34.7% for MUF-butyrate and 55.1% for MUF-heptanoate, the activity in $E_{(II)}$ dropped by over 90% to residual activities of 3.4% and 7.4%, respectively (Supplementary Material, Fig. S1a).

The hydrolysis rate of MUF-butyrate by $E_{(III)}$ dropped by 63.8%, whereas the hydrolysis of MUF-heptanoate ceased altogether (0.4% residual activity – Supplementary Material, Fig. S1b). The hydrolysis rate of all three substrates by $E_{(IV)}$ extinguished almost completely (0.2–2.3% residual activity - Supplementary Material, Fig. S1c) with 0.1% SDS.

### 3.5. Hydrolysis of AMF-PLA by gastric fluid fractions

The fractions around $E_{(I)}$ to $E_{(IV)}$ with high carboxylesterase activity were pooled and will be denoted in the following as Pool $E_{(I)}$ to Pool $E_{(III + IV)}$ (Fig. 4). $E_{(III)}$ and $E_{(IV)}$ were combined to one Pool $E_{(III + IV)}$, because the esterase peak in $E_{(III)}$ was missing in some samples. Proteins in Pool $E_{(I)}$ (Fractions 29–32), Pool $E_{(II)}$ (Fractions 36–38), and Pool $E_{(III + IV)}$ (Fractions 41–47) were concentrated, and their potential to hydrolyze AMF-PLA was tested. As a negative control, fractions without esterase activity were pooled, concentrated, and tested (Fraction 60–65).

All pooled enzyme fractions showing carboxylesterase activity were able to hydrolyze the AMF-PLA microparticles (Fig. 4). Pool $E_{(I)}$ showed the highest hydrolysis rate (8.1 ± 4.4 nmol $min^{-1}$), followed by Pool $E_{(III + IV)}$ (5.4 ± 1.3 $nmol \cdot min^{-1}$). Pool $E_{(II)}$ showed the lowest hydrolysis rate (1.4 ± 1.3 nmol $min^{-1}$). The negative control showed no hydrolysis rate.

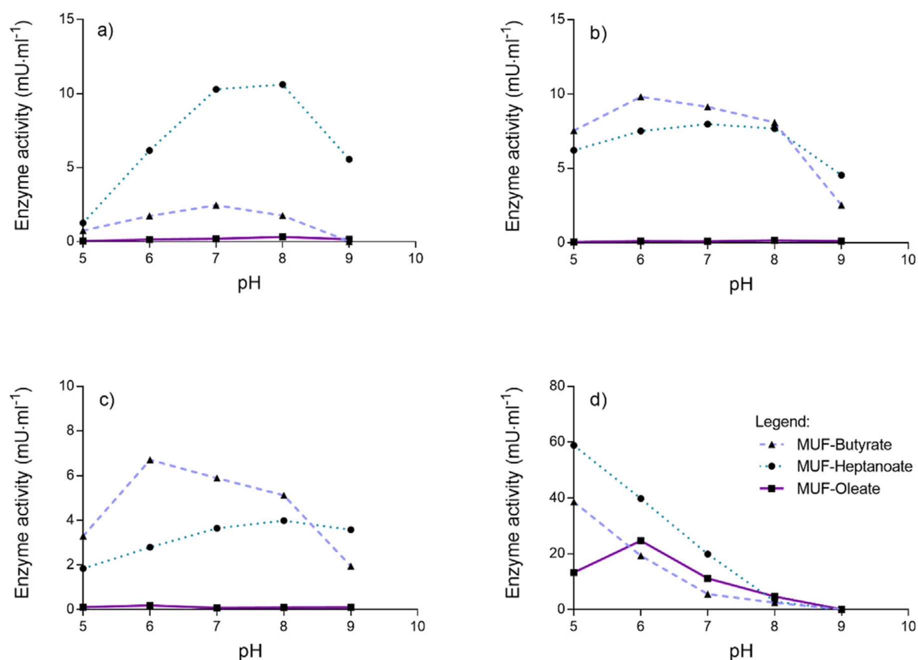


**Fig. 3.** Esterase activities of a) $E_{(I)}$, b) $E_{(II)}$, c), $E_{(III)}$ and d) $E_{(IV)}$, measured with MUF-butyrate, MUF-heptanoate and MUF-oleate at pH 5 to pH 9.

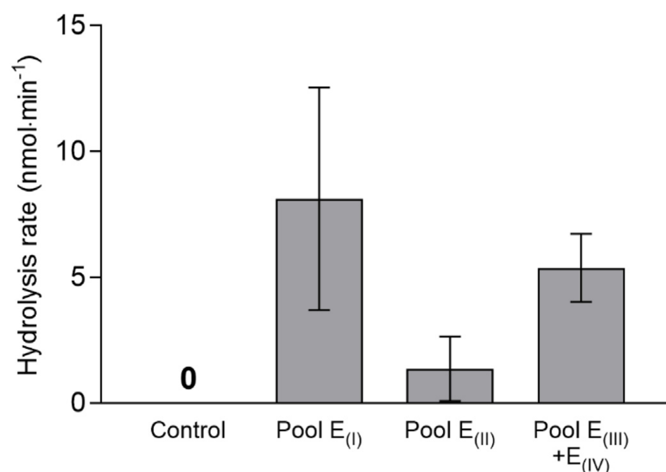


**Fig. 4.** Hydrolytic degradation of AMF-PLA by the pooled fractions with esterase activity of the gastric fluid of *Cancer pagurus* measured by pH-Stat titration at 30 °C, pH 8.2 and 32 ppt salinity (means ± SD, n = 3). A zero indicates no measured hydrolysis in the negative control.

### 3.6. Electrophoretic separation of proteins

SDS-PAGE with subsequent Coomassie staining showed different protein bands in the four fractions. Incubation with MUF-substrates showed clear activity bands in $E_{(I)}$ and $E_{(III)}$.

$E_{(I)}$ showed a faint activity band at around 45 kDa (Fig. 5a, Lane 1). Another faint band, which appeared at the top of the gel, is considered an artefact because of the unrealistic high molecular mass (~250 kDa), which may have resulted from protein agglutinations. A strong activity band appeared after incubation with MUF-heptanoate at around 45 kDa and a faint signal at 40 kDa (Fig. 5a, Lane 2). No activity bands were visible after incubation with MUF-oleate (Fig. 5a, Lane 3).

$E_{(III)}$ showed clear activity bands at 40 kDa for MUF-butyrate and MUF-heptanoate (Fig. 5c, Lane 1–2). MUF-butyrate also showed a faint activity band at 35 kDa. Similar to $E_{(I)}$, $E_{(III)}$ showed no activity bands after incubation with MUF-oleate (Fig. 5c, Lane 3).

$E_{(II)}$ and $E_{(IV)}$ showed only faint activity bands after incubation with MUF substrates. For visualization, the positions of these bands are indicated by white ticks (Fig. 5b, d).

In summary, several AMF-PLA-degrading carboxylesterases were identified in the gastric fluid of the edible crab *Cancer pagurus*. They exhibit different characteristics such as pH optimum, inhibition by SDS and substrate specificity, and their molecular weights range around 40 kDa (Table 3).

## 4. Discussion

Crustaceans are economically important as marine food resources and are ecologically crucial in many habitats and food webs. Crustaceans of the order Decapoda show various feeding habitats, covering a wide range of feeding strategies that implicate the uptake of microplastic particles, whether as predator, herbivore, omnivore, or filter feeder [30]. Microplastics were found in the gastrointestinal tracts and stomachs of various decapod species collected in natural environments [31–34].

Adaptation to different ecological niches and the associated food sources yielded a broad range of digestive hydrolytic enzymes [24]. Besides a large number of proteinases, the gastric fluids of decapods contain glucosidases, esterases, and lipases [24], which were also confirmed for *Cancer pagurus* by ApiZym-screening. This variety of highly active digestive enzymes allows decapods to utilize natural polymers efficiently, including chitin and cellulose ([35] and literature cited therein). Accordingly, it appears reasonable that gastric enzymes may also be able to hydrolyze biodegradable plastics. The edible crab, *C. pagurus*, served in our study as a model species. It allowed us to withdraw gastric fluid and investigate *in-vitro* the enzymatic processes that appear within the crab's stomach.

The *in-vitro* degradation experiments were conducted at 15 °C, the upper-temperature range of *Cancer pagurus* in its preferred sublittoral habitat [36,37]. Among the five plastics incubated with gastric fluid, AMF-PLA was hydrolyzed at the highest rate. AMF-PLA

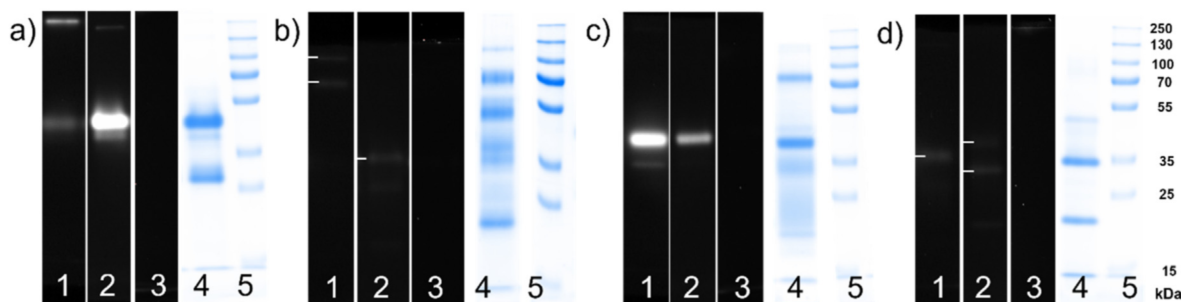


**Fig. 5.** Snippets of polyacrylamide gels with proteins separated by SDS-PAGE of a) $E_I$, b) $E_{II}$, c) $E_{III}$ and d) $E_{IV}$. The gels were incubated with MUF-butyrate (Lane 1), MUF-heptanoate (Lane 2), MUF-oleate (Lane 3) and Coomassie (Lane 4). Lane 5 shows the molecular weight marker. White ticks in b) and d) indicate the position of faint activity bands.

**Table 3**
Characteristics of the carboxylesterases present in the fractions $E_{(I)}$ to $E_{(IV)}$. n.d. = not defined.

| Fractions | Substrate | pH optimum | Inhibition by SDS (%) | Molecular weights (kDa) |
|---|---|---|---|---|
| $E_{(I)}$ | Butyrate (C4) | 7 | 35 | 45 |
| | Heptanoate (C7) | 8 | 55 | 45, 40 |
| | Oleate (C18) | n.d. | n.d. | n.d. |
| $E_{(II)}$ | Butyrate (C4) | 6 | 97 | n.d. |
| | Heptanoate (C7) | 7 | 93 | n.d. |
| | Oleate (C18) | n.d. | n.d. | n.d. |
| $E_{(III)}$ | Butyrate (C4) | 6 | 64 | 40, 35 |
| | Heptanoate (C7) | 8 | >99 | 40 |
| | Oleate (C18) | n.d. | n.d. | n.d. |
| $E_{(IV)}$ | Butyrate (C4) | (<) 5 | 98 | n.d. |
| | Heptanoate (7) | (<) 5 | >99 | n.d. |
| | Oleate (C18) | 6 | >99 | n.d. |

is a biobased biodegradable plastic blend consisting of PLA and PBAT polymer components. PLA is an aliphatic polyester that can be degraded by several hydrolytic enzymes, predominantly proteases [17,38,39]. PBAT is an aromatic aliphatic polyester, which can be degraded by esterases and lipases [40,41]. The enzyme screening of the gastric fluid of *C. pagurus* via ApiZym strips revealed high activities of both proteolytic and lipolytic enzymes. Accordingly, either of these enzyme classes in the gastric fluid can be responsible for the observed hydrolysis of AMF-PLA. However, previous studies have shown that AMF-PLA was preferably degraded by lipase from yeast *Candida antarctica* under the same *in-vitro* conditions as in the present study [19]. No hydrolysis was observed by a protease from *Bacillus licheniformis* suggesting that the lipolytic enzymes, displaying esterase activity, rather than the proteolytic enzymes in the gastric fluid of *C. pagurus* are responsible for the degradation of the material. Accordingly, we focused on the investigations on gastric enzymes with esterase/lipase activity.

After anion exchange chromatography, the separate fractions were screened for their carboxylesterase activity using MUF-derivatives of different lengths. Four fractions ($E_{(I)}$ to $E_{(IV)}$) with distinct hydrolytic potential to cleave either MUF-butyrate (C4), MUF-heptanoate (C7), or MUF-oleate (C18) were identified. Short-, medium- or long-chain fatty ester acids were hydrolyzed differently by $E_{(I)}$ to $E_{(IV)}$, indicating the presence of several different esterases/lipases in the gastric fluid of *C. pagurus*. $E_{(I)}$ contains an esterase, which tends to cleave esters of medium-chain fatty acids, whereas enzymes in $E_{(II)}$ and $E_{(III)}$ are hydrolyzing both C4- and C7-chain length fatty acids at similar rates. This is different to lipases from other crustaceans like penaeids, which rather hydrolyze longer chain substrates [42–44]. Esterases in $E_{(IV)}$ showed higher specificity towards the long-chain substrate at pH 7, but were also capable of hydrolyzing short- and medium-chain substrates at high rates. A wide substrate acceptance is unusual for digestive lipases. However, Cherif et al. (2007) [45] observed a similar specificity in a digestive lipase from the European shore crab *Carcinus maenas* suggesting that lipases with a broad specificity are common in decapod crustaceans.

The fractions $E_{(I)}$ to $E_{(IV)}$ showed different pH optima. Although the pH in the gastric fluid of *C. pagurus* ranges from 6.0 to 6.4, the lipolytic activity in $E_{(I)}$ peaked at neutral and slightly alkaline pHs 7 and 8, respectively. A similar pH optimum was observed for the endopeptidase trypsin in the gastric fluid of *C. pagurus*, which was almost inactive at a pH below 6 but rose with increasing pH [46]. Unlike trypsin, carboxylesterases in $E_{(II)}$ and $E_{(III)}$ were active over the range of pH 5 to pH 9, albeit with decreasing activity towards the acidic and alkaline margins. Furthermore, different to trypsin of the gastric fluid, the esterase activity in $E_{(IV)}$ strongly decreased with increasing pH. The activity was highest at lower pH values of 5 and 6, which agrees with other studies of gastric lipase from crustaceans [47].

Fractions with high carboxylesterase activities were grouped in pools ($E_{(I)}$, $E_{(II)}$, $E_{(III)+(IV)}$) and tested again for AMF-PLA hydrolysis by pH-Stat titration. All pools, which previously exhibited lipolytic activity, i.e. cleavage of medium (C7) and long-chain (C18) carboxylesters, could also hydrolyze AMF-PLA. In contrast, the negative control lacking lipolytic activity showed no hydrolysis of AMF-PLA. The selected pool for the negative control included fractions, which were the last to elute from the anion exchange column and showed a high protein peak (Fig. 2a). These fractions possess high tryptic activity [48]. Trypsin is a protease that has been shown to hydrolyze PLA [38]. However, the trypsin in the gastric fluid of *C. pagurus* did not hydrolyze AMF-PLA. These results affirm the assumption, that the enzymes responsible for the degradation of the biodegradable plastic are lipases that hydrolyze the PBAT fraction of the compound, rather than proteases that may hydrolyze the PLA fraction. Furthermore, there seems to be a relationship between MUF-heptanoate and AMF-PLA hydrolysis. Fractions with higher activity towards MUF-heptanoate also showed higher

hydrolysis rates of AMF-PLA (Figs. 2b and 4). This indicates that lipases from the gastric fluid, which can cleave esters from medium-chain fatty acids, contribute most to the hydrolysis of PBAT-based plastics, at least at pH 8.2 and 32 ppt salinity.

Esterase activity was measured in all four fractions ($E_{(I)}$ to $E_{(IV)}$). However, the activity disappeared almost entirely in $E_{(II)}$ and $E_{(IV)}$ after separation by SDS-PAGE. The activity loss was caused by adding 2% SDS during sample preparation, confirmed by inhibiting esterase/lipase activity in single LC fractions after adding SDS (Supplementary Material, Fig. S1). Surfactants such as SDS can affect enzymes' molecular structure and activity [49,50]. At low concentrations, SDS forms complexes with enzymes. It inhibits lipase activity under simulated gastrointestinal conditions [51,52]. However, fractions $E_{(I)}$ and $E_{(III)}$ showed minor inhibition by SDS, resulting in bright activity bands on the gels.

The activity bands of lipolytic enzymes appeared at molecular masses of 40 and 45 kDa. Lipase in other crustaceans, such as *Penaeus vannamei*, showed a similar molecular mass of 44.8 kDa [44]. Lipases are a subclass of esterases with a unique mode of action. Unlike other enzymes, they catalyze reactions at solid-water interfaces by adsorption to the surface of water-insoluble substrates [53], which may promote the hydrolysis of microplastics. Lipases were studied in several other crustacean species, such as *Homarus americanus* [54], *C. maenas* [45], or *P. vannamei* [44]. Preliminary pH-Stat experiments showed that the gastric fluid of *H. americanus* could hydrolyze AMF-PLA to a similar extent as *C. pagurus* (L. Miksch, unpublished data). Therefore, screening additional species is required to obtain an overview of whether crustaceans and other invertebrates are generally capable of hydrolyzing ingested biodegradable plastics. However, not all species exposed to microplastic in the environment are large enough to provide enough gastric fluids for analysis. In those cases, molecular approaches and *de novo* peptide sequencing techniques would be suitable for comparing the sequences with known data from other crustacean species.

Hydrolysis rates of biodegradable plastics are lower than those of natural polymers, such as collagen [19]. Nevertheless, hydrolysis of biodegradable microplastics in the stomach of *C. pagurus* may have various consequences for the organism. The degradation of ingested bioplastic particles can facilitate the release of toxic additives [55,56]. Degradation products and intermediates may interact with digestive enzymes and inhibit their hydrolytic capacities, which, in turn, would hamper nutrient utilization from ingested food. The efficiency of the degradation process and, thus, the amount of degradation products also depends on the residence time of ingested particles within the digestive tract. Rapid egestion or regurgitation of microplastics, observed in some crustacean species [57,58], may prevent the microplastics from being hydrolyzed. Nonetheless, ingestion of biodegradable plastics may impact crustaceans differently than conventional microplastics. Whether this happens in a positive or negative way is a question for further research.

## 5. Conclusions

Biodegradable plastics have been proposed to be a promising alternative to conventional plastics due to their expected shorter residence time in the environment. However, the degradability of these plastics under marine conditions is under debate and there is no information about their fate upon ingestion by marine invertebrates. The gastric fluid of the edible crab *C. pagurus* contains several enzymes with carboxylesterase activity capable of hydrolyzing a plastic blend based on PLA/PBAT. Since carboxylesterases are common digestive enzymes, this study suggests that many marine invertebrates might be able to degrade biodegradable plastics. However, the nature of the degradation products and their biochemical effects in the intestine of animals is unknown and demands further research.

## Funding

This work was supported by the European Union's Horizon 2020 research and innovation program (grant agreement number 860407).

## Data availability statement

Datasets presented in this study are openly available in the PANGAEA online repository, https://doi.pangaea.de/10.1594/PANGAEA.956789.

## Ethics approval

☐ The authors declare that this study does not involve animals.
☐ The authors declare that this study involves animals but ethics approval is not needed for the following reason:
☒The authors declare that this study involves animals and the ethics approval statement is as follow:

According to the German law § 20 Abs. 1 Tierschutz-Versuchstierverordnung (TierSchVersV) the experiments conducted for this study were registered and approved by the relevant authority (Senatorin für Gesundheit, Frauen und Verbraucherschutz, Freie Hansestadt Bremen). Reference number: **21TSAU04**.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors acknowledge the support by the Open Access Publication Funds of Alfred-Wegener-Institut Helmholtz- Zentrum für Polar- und Meeresforschung.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.emcon.2023.100275.

## References

[1] Statista, Global plastic production 1950 - 2020. https://www.statista.com/statistics/282732/global-production-of-plastics-since-1950/, 2022. (Accessed 8 November 2022).
[2] W.C. Li, H.F. Tse, L. Fok, Plastic waste in the marine environment: a review of sources, occurrence and effects, Sci. Total Environ. 566–567 (2016) 333–349, https://doi.org/10.1016/j.scitotenv.2016.05.084.
[3] D.K.A. Barnes, F. Galgani, R.C. Thompson, M. Barlaz, Accumulation and fragmentation of plastic debris in global environments, Phil. Trans. Biol. Sci. 364 (1526) (2009) 1985–1998, https://doi.org/10.1098/rstb.2008.0205.
[4] A.L. Andrady, The plastic in microplastics: a review, Mar. Pollut. Bull. 119 (1) (2017) 12–22, https://doi.org/10.1016/j.marpolbul.2017.01.082.
[5] O.S. Alimi, J.F. Budarz, L.M. Hernandez, N. Tufenkji, Microplastics and nanoplastics in aquatic environments: aggregation, deposition, and enhanced contaminant transport, Environ. Sci. Technol. 52 (4) (2018) 1704–1724, https://doi.org/10.1021/acs.est.7b05559.
[6] E. Guzzetti, A. Sureda, S. Tejada, C. Faggio, Microplastic in marine organisms: environmental and toxicological effects, Environ. Toxicol. Pharmacol. 64 (2018) 164–171, https://doi.org/10.1016/j.etap.2018.10.009.
[7] T.S. Galloway, M. Cole, C. Lewis, Interactions of microplastic debris throughout

the marine ecosystem, Nat. Ecol. Evol. 1 (2017) 0116, https://doi.org/10.1038/s41559-017-0116.
[8] M. Pirsaheb, H. Hossini, P. Makhdoumi, Review of microplastic occurrence and toxicological effects in marine environment: experimental evidence of inflammation, Process Saf. Environ. Protect. 142 (2020) 1–14, https://doi.org/10.1016/j.psep.2020.05.050.
[9] IUPAC (the "Gold Book")., in: A.D. McNaught, A. Wilkinson (Eds.), Compendium of chemical terminology, second ed., Blackwell Scientific Publications, 1997 https://doi.org/10.1351/goldbook. Oxford (1997). Online version (2019) created by S. J. Chalk. ISBN 0-9678550-9-8
[10] OECD, in: Test No. 301: Ready Biodegradability, OECD Guidelines for the Testing of Chemicals, Section, vol. 3, OECD Publishing, Paris, France, 1992, p. 62, https://doi.org/10.1787/9789264070349-en.
[11] DIN, Water quality - Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer (ISO 9408:1999); German version EN ISO 9408:1999,, 22, https://dx.doi.org/10.31030/8506287, 1999.
[12] ASTM, ASTM D5864-11, Standard Test Method for determining Aerobic Aquatic Biodegradation of Lubricants or Their Components, 2017, p. 8, https://doi.org/10.1520/D5864-11.
[13] E.M.N. Polman, G.-J.M. Gruter, J.R. Parsons, A. Tietema, Comparison of the aerobic biodegradation of biopolymers and the corresponding bioplastics: a review, Sci. Total Environ. 753 (2021) 141953, https://doi.org/10.1016/j.scitotenv.2020.141953.
[14] B. Singh, N. Sharma, Mechanistic implications of plastic degradation, Polym. Degrad. Stabil. 93 (3) (2008) 561–584, https://doi.org/10.1016/j.polymdegradstab.2007.11.008.
[15] E. Marten, R.-J. Müller, W.-D. Deckwer, Studies on the enzymatic hydrolysis of polyesters. II. Aliphatic–aromatic copolyesters, Polym. Degrad. Stabil. 88 (3) (2005) 371–381, https://doi.org/10.1016/j.polymdegradstab.2004.12.001.
[16] S.S. Ali, T. Elsamahy, R. Al-Tohamy, D. Zhu, Y.A.-G. Mahmoud, E. Koutra, M.A. Metwally, M. Kornaros, J. Sun, Plastic wastes biodegradation: mechanisms, challenges and future prospects, Sci. Total Environ. 780 (2021) 146590, https://doi.org/10.1016/j.scitotenv.2021.146590.
[17] L. Miksch, L. Gutow, R. Saborowski, pH-Stat titration: a rapid assay for enzymatic degradability of bio-based polymers, Polymers 13 (6) (2021) 860, https://doi.org/10.3390/polym13060860.
[18] Y. Wang, C.J.E. Davey, K. van der Maas, R.-J. van Putten, A. Tietema, J.R. Parsons, G.-J.M. Gruter, Biodegradability of novel high $T_g$ poly(isosorbide-*co*-1,6-hexanediol) oxalate polyester in soil and marine environments, Sci. Total Environ. 815 (2022) 152781, https://doi.org/10.1016/j.scitotenv.2021.152781.
[19] L. Miksch, M. Koeck, L. Gutow, R. Saborowski, Bioplastics in the sea: rapid *in-vitro* evaluation of degradability and persistence at natural temperatures, Front. Mar. Sci. 9 (2022) 920293, https://doi.org/10.3389/fmars.2022.920293.
[20] E.D. Okoffo, C.M. Chan, C. Rauert, S. Kaserzon, K.V. Thomas, Identification and quantification of micro-bioplastics in environmental samples by pyrolysis–gas chromatography–mass spectrometry, Environ. Sci. Technol. 56 (19) (2022) 13774–13785, https://doi.org/10.1021/acs.est.2c04091.
[21] H. Jabeen, L. Su, J. Li, D. Yang, C. Tong, J. Mu, H. Shi, Microplastics and mesoplastics in fish from coastal and fresh waters of China, Environ. Pollut. 221 (2017) 141–149, https://doi.org/10.1016/j.envpol.2016.11.055.
[22] E. Scanes, H. Wood, P. Ross, Microplastics detected in haemolymph of the Sydney rock oyster *Saccostrea glomerata*, Mar. Pollut. Bull. 149 (2019) 110537, https://doi.org/10.1016/j.marpolbul.2019.110537.
[23] M. Martinelli, A. Gomiero, S. Guicciardi, E. Frapiccini, P. Strafella, S. Angelini, F. Domenichetti, A. Belardinelli, S. Colella, Preliminary results on the occurrence and anatomical distribution of microplastics in wild populations of *Nephrops norvegicus* from the Adriatic Sea, Environ. Pollut. 278 (2021) 116872, https://doi.org/10.1016/j.envpol.2021.116872.
[24] G. Vogt, Synthesis of digestive enzymes, food processing, and nutrient absorption in decapod crustaceans: a comparison to the mammalian model of digestion, Zoology 147 (2021) 125945, https://doi.org/10.1016/j.zool.2021.125945.
[25] R. Saborowski, Nutrition and digestion, in: E. Chang, M. Thiel (Eds.), Natural History of the Crustacea. Vol IV Physiological Regulation, Oxford University Press, New York, 2015, pp. 285–319.
[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[27] N. Prim, M. Sánchez, C. Ruiz, F.I.J. Pastor, P. Diaz, Use of methylumbelliferyl-derivative substrates for lipase activity characterization, J. Mol. Catal. B Enzym. 22 (5–6) (2003) 339–346, https://doi.org/10.1016/S1381-1177(03)00048-1.
[28] S. Knotz, M. Boersma, R. Saborowski, Microassays for a set of enzymes in individual small marine copepods, Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 145 (3) (2006) 406–411, https://doi.org/10.1016/j.cbpa.2006.07.019.
[29] H.T.S. Britton, R.A. Robinson, CXCVIII.-Universal buffer solutions and the dissociation constant of veronal, J. Chem. Soc. (1931) 1456–1462, https://doi.org/10.1039/JR9310001456.
[30] A.H. D'Costa, Microplastics in decapod crustaceans: accumulation, toxicity and impacts, a review, Sci. Total Environ. 832 (2022) 154963, https://doi.org/10.1016/j.scitotenv.2022.154963.
[31] G. Stasolla, G. Innocenti, B.S. Galil, On the diet of the invasive crab *Charybdis longicollis* Leene, 1938 (Brachyura: Portunidae) in the eastern Mediterranean Sea, Israel J. Ecol. Evol. 61 (3–4) (2015) 130–134, https://doi.org/10.1080/15659801.2015.1123362.
[32] S. Piarulli, S. Scapinello, P. Comandini, K. Magnusson, M. Granberg, J.X.W. Wong, G. Sciutto, S. Prati, R. Mazzeo, A.M. Booth, L. Airoldi, Microplastic in wild populations of the omnivorous crab *Carcinus aestuarii*: a review and a regional-scale test of extraction methods, including microfibres, Environ. Pollut. 251 (2019) 117–127, https://doi.org/10.1016/j.envpol.2019.04.092.
[33] Y. Li, Z. Liu, Y. Yang, Q. Jiang, D. Wu, Y. Huang, Y. Jiao, Q. Chen, Y. Huang, Y. Zhao, Effects of nanoplastics on energy metabolism in the oriental river prawn (*Macrobrachium nipponense*, Environ. Pollut. 268 (2021) 115890, https://doi.org/10.1016/j.envpol.2020.115890. Part A.
[34] H. Joyce, J. Frias, F. Kavanagh, R. Lynch, E. Pagter, J. White, R. Nash, Plastics, prawns, and patterns: microplastic loadings in *Nephrops norvegicus* and surrounding habitat in the North East Atlantic, Sci. Total Environ. 826 (2022) 154036, https://doi.org/10.1016/j.scitotenv.2022.154036.
[35] S.M. Linton, R. Saborowski, A.J. Shirley, J.A. Penny, Digestive enzymes of two brachyuran and two anomuran land crabs from Christmas Island, Indian Ocean, J. Comp. Physiol. B 184 (2014) 449–468, https://doi.org/10.1007/s00360-014-0815-2.
[36] M. Cuculescu, D. Hyde, K. Bowler, Thermal tolerance of two species of marine crab, *Cancer pagurus* and *Carcinus maenas*, J. Therm. Biol. 23 (2) (1998) 107–110, https://doi.org/10.1016/S0306-4565(98)00008-4.
[37] S. Bakke, S.I. Siikavuopio, J.S. Christiansen, Thermal behaviour of edible crab *Cancer pagurus* Linnaeus, 1758 in coastal Norway, Fauna Nor. 39 (1–2) (2019) 1–11, https://doi.org/10.5324/fn.v39i0.2738.
[38] H.-A. Lim, T. Raku, Y. Tokiwa, Hydrolysis of polyesters by serine proteases, Biotechnol. Lett. 27 (2005) 459–464, https://doi.org/10.1007/s10529-005-2217-8.
[39] F. Kawai, The biochemistry and molecular biology of xenobiotic polymer degradation by microorganisms, Biosci., Biotechnol., Biochem. 74 (9) (2010) 1743–1759, https://doi.org/10.1271/bbb.100394.
[40] P.W. Wallace, K. Haernvall, D. Ribitsch, S. Zitzenbacher, M. Schittmayer, G. Steinkellner, K. Gruber, G.M. Guebitz, R. Birner-Gruenberger, PpEst is a novel PBAT degrading polyesterase identified by proteomic screening of *Pseudomonas pseudoalcaligenes*, Appl. Microbiol. Biotechnol. 101 (2017) 2291–2303, https://doi.org/10.1007/s00253-016-7992-8.
[41] A. Kanwal, M. Zhang, F. Sharaf, C. Li, Enzymatic degradation of poly (butylene adipate co-terephthalate) (PBAT) copolymer using lipase B from *Candida antarctica* (CALB) and effect of PBAT on plant growth, Polym. Bull. 79 (2022) 9059–9073, https://doi.org/10.1007/s00289-021-03946-w.
[42] A. del Monte, H. Nolasco, A. Forrellat, C. Aragón, A. García, J. Díaz, O. Carrillo, Evidencias de la presencia de lipasas en el hepatopáncreas de *Litopenaeus schmitii*, AQUATIC 16 (2002) 207–222.
[43] A.F. Barrios, A. Del Monte, T.E. Lao, B.B. Castroconde, H.N. Soria, O.C. Farnés, Caracterización de lipasas en tres especies de camarones peneidos. Su importancia en la digestión, Memorias del Congreso Iberoamericano. España, Virtual de Acuicultura CIVA, Zaragoza, 2004.
[44] C. Rivera-Pérez, F.L. García-Carreño, R. Saborowski, Purification and biochemical characterization of digestive lipase in Whiteleg shrimp, Mar. Biotechnol. 13 (2011) 284–295, https://doi.org/10.1007/s10126-010-9298-7.
[45] S. Cherif, A. Fendri, N. Miled, H. Trabelsi, H. Mejdoub, Y. Gargouri, Crab digestive lipase acting at high temperature: purification and biochemical characterization, Biochimie 89 (8) (2007) 1012–1018, https://doi.org/10.1016/j.biochi.2007.02.005.
[46] R. Saborowski, G. Sahling, M.A. Navarrete del Toro, I. Walter, F.L. Garcıa-Carreno, Stability and effects of organic solvents on endopeptidases from the gastric fluid of the marine crab *Cancer pagurus*, J. Mol. Catal. B Enzym. 30 (3–4) (2004) 109–118, https://doi.org/10.1016/j.molcatb.2004.04.002.
[47] J.D. O'Connor, L.I. Gilbert, Aspects of lipid metabolism in crustaceans, Am. Zool. 8 (3) (1968) 529–539, https://doi.org/10.1093/icb/8.3.529.
[48] J.-H. Hehemann, L. Redecke, J. Murugaiyan, M. von Bergen, C. Betzel, R. Saborowski, Autoproteolytic stability of a trypsin from the marine crab *Cancer pagurus*, Biochem. Biophys. Res. Commun. 370 (4) (2008) 566–571, https://doi.org/10.1016/j.bbrc.2008.03.128.
[49] R. Miller, V.B. Fainerman, A.V. Makievski, J. Krägel, D.O. Grigoriev, V.N. Kazakov, O.V. Sinyachenko, Dynamics of protein and mixed protein/surfactant adsorption layers at the water/fluid interface, Adv. Colloid Interface Sci. 86 (1–2) (2000) 39–82, https://doi.org/10.1016/S0001-8686(00)00032-4.
[50] A.S. Antipova, M.G. Semenova, L.E. Belyakova, M.M. Il'in, On relationships between molecular structure, interaction and surface behavior in mixture: small-molecule surfactant+ protein, Colloids Surf. B Biointerfaces 21 (1–3) (2001) 217–230, https://doi.org/10.1016/S0927-7765(01)00174-6.
[51] V. Delorme, R. Dhouib, S. Canaan, F. Fotiadu, F. Carrière, J.-F. Cavalier, Effects of surfactants on lipase structure, activity, and inhibition, Pharmacol. Res. 28 (2011) 1831–1842, https://doi.org/10.1007/s11095-010-0362-9.
[52] Y. Li, D.J. McClements, Inhibition of lipase-catalyzed hydrolysis of emulsified triglyceride oils by low-molecular weight surfactants under simulated gastrointestinal conditions, Eur. J. Pharm. Biopharm. 79 (2) (2011) 423–431, https://doi.org/10.1016/j.ejpb.2011.03.019.
[53] F. Hasan, A.A. Shah, A. Hameed, Methods for detection and characterization of lipases: a comprehensive review, Biotechnol. Adv. 27 (6) (2009) 782–798, https://doi.org/10.1016/j.biotechadv.2009.06.001.
[54] H. Brockerhoff, R.J. Hoyle, P.C. Hwang, Digestive enzymes of the American

lobster (*Homarus americanus*), J. Fish. Res. Board Can. 27 (8) (1970) 1357–1370, https://doi.org/10.1139/f70-160.
[55] A.A. Koelmans, E. Besseling, A. Wegner, E.M. Foekema, Plastic as a carrier of POPs to aquatic organisms: a model analysis, Environ. Sci. Technol. 47 (14) (2013) 7812–7820, https://doi.org/10.1021/es401169n.
[56] B. Gewert, M. MacLeod, M. Breitholtz, Variability in toxicity of plastic leachates as a function of weathering and polymer type: a screening study with the copepod *Nitocra spinipes*, Biol. Bull. 240 (3) (2021) 191–199, https://doi.org/10.1086/714506.
[57] R. Saborowski, E. Paulischkis, L. Gutow, How to get rid of ingested microplastic fibers? A straightforward approach of the Atlantic ditch shrimp *Palaemon varians*, Environ. Pollut. Part B 254 (2019) 113068, https://doi.org/10.1016/j.envpol.2019.113068.
[58] Š. Korez, L. Gutow, R. Saborowski, Coping with the “dirt”: brown shrimp and the microplastic threat, Zoology 143 (2020) 125848, https://doi.org/10.1016/j.zool.2020.125848.