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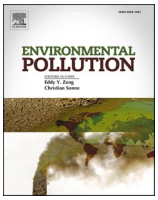

Fishing in troubled waters: Limited stress response to natural and synthetic microparticles in brown shrimp (*Crangon crangon*)☆

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ABSTRACT

Marine invertebrates inhabiting estuaries and coastal areas are exposed to natural suspended particulate matter (SPM) like clay or diatom shells but also to anthropogenic particles like microplastics. SPM concentrations may reach 1 g per liter and more, comprising hundreds of millions of items in the size range of less than 100 μm. Suspension feeders and deposit feeders involuntarily ingest these particles along with their food. We investigated whether natural and anthropogenic microparticles at concentrations of 20 mg L^{-1}, which correspond to natural environmental SPM concentrations in coastal marine waters, are ingested by the brown shrimp *Crangon crangon* and whether these particles induce an oxidative stress response in digestive gland tissue. Shrimp were exposed to clay, silica, TiO_2, polyvinyl chloride (PVC), or polylactide microplastics (PLA) for 6, 12, 24, and 48 h, respectively. The activities of the anti-oxidative enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) were measured. All five particle types were ingested by the shrimp along with food. The presence of the particles in the shrimp stomach was verified by scanning electron microscopy. The activities of the anti-oxidative enzymes did not vary between animals exposed to different types of microparticles and control animals that did not receive particles. The temporal activity differed between the three enzymes. The lack of a specific biochemical response may reflect an adaptation of *C. crangon* to life in an environment where frequent ingestion of non-digestible microparticles is unavoidable and continuous maintenance of inducible biochemical defense would be energetically costly. Habitat characteristics as well as natural feeding habits may be important factors to consider in the interpretation of hazard and species-specific risk assessment.

1. Introduction

Microplastic pollution is an urging issue in environmental, toxicological, and medical research. There is hardly any habitat on earth, which is not burdened with plastic items and particulate and fibrous microplastics. Even remotest regions and habitats, such as the deep-seafloor or polar snow are contaminated with microplastics (e.g. Woodall et al., 2014; Bergmann et al., 2017, 2019; Courtene-Jones et al., 2020; Cunningham et al., 2020). Marine organisms are particularly exposed to microplastics in estuarine and coastal regions where they face high loads of anthropogenic pollution through rivers, urban agglomerations, or marine traffic (Browne et al., 2010). Direct discharge of e.g. textile fibers (e.g. Sait et al., 2021) as well as fragmentation of larger plastic items by weathering and wave action at the shore make microplastics available for a wide range of organisms (Claessens et al., 2011; Kalogerakis et al., 2017; Elfimova et al., 2018).

Intensive research effort is being made on the bioavailability of plastic particles, their interaction with organisms, and their diverse ecotoxicological effects (Gautam et al., 2020). Ingestion of microplastics may entail various adverse effects, ranging from behavioral changes over nutritional deficiency to reduced fertility (Anbumani and Kakkar, 2018; de Sá et al., 2018). At the cellular level, microplastics can affect metabolic pathways and cause cellular oxidative stress (e.g. Jeong et al., 2016, 2017; Yu et al., 2018). Oxidative stress may trigger various adverse effects such as metabolic imbalance, neurotoxicity, and cytotoxicity (Lushchak, 2011).

Besides anthropogenic discharge, coastal waters and shallow estuaries carry substantial loads of suspended particulate matter (SPM) in the micro- and nano size range, such as minerals and organic particles (Xhoffer et al., 1992; Jago et al., 1993; D'Sa et al., 2007). Because of tidal mixing, wave action, river discharge, and seasonal plankton blooms, the amount of SPM in the shallow North Sea may exceed

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Table 1
Quantities of suspended particulate matter in the North Sea.

Location			Suspended particulate matter		Size range µm	Reference
			$mg \cdot L^{-1}$	$n \cdot L^{-1}$		
Elbe estuary	North Sea	Germany		$(2.7–8.0) \cdot 10^7$	5–12	Martens (1978)
				$(12.0–32.6) \cdot 10^5$	11–31	
				$(5.4–12.7) \cdot 10^4$	30–80	
Wadden Sea	North Sea	Germany		$(0.7–3.8) \cdot 10^7$	5–12	Martens (1978)
				$(4.9–17.5) \cdot 10^5$	11–31	
				$(2.4–10.2) \cdot 10^4$	30–80	
N Frisian coastal water	North Sea	Germany		$(0.1–2.4) \cdot 10^7$	5–12	Martens (1978)
				$(0.5–1.7) \cdot 10^5$	11–31	
				$(0.4–2.9) \cdot 10^4$	30–80	
Bight	S North Sea	Belgium	9–592		<62.5	Fettweis and Van den Eynde, 2003
Coastal zone	S North Sea	Belgium	<10 - <20[a]		400 - <600[a]	Fettweis et al. (2006)
			<20 - >100[b]		<20 - >120[b]	
			<200–300[c]		350–600[c]	
Elbe estuary	North Sea	Germany	20–320			Kappenberg and Fanger (2007)
Coastal zone	S North Sea	Belgium	2.1–989			Fettweis et al. (2007)
Southern North Sea		The Netherlands	1–85			Eleveld et al. (2008)
E Frisian Wadden Sea		Germany	0.2–160		2.5–500	Badewien et al. (2009)
N Frisian Wadden Sea		Germany	130–320		63–200	Hache et al. (2020)
Weser estuary	S North Sea	Germany	13.5–54.0		>0.7	Roscher et al. (2021)
Weser estuary	North Sea	Germany	14.4		>100	Current study
		Germany	10.8		10–100	

[a] Sampled in June 2003.
[b] Sampled in September 2003.
[c] Sampled in March 2003.

concentrations of 1 g per liter (Table 1). For idealized spherical particles made of clay (density 2.6 g cm^{-3}) with a diameter of 10 μm, the observed concentrations for the North Sea would correspond to more than 700 million particles in 1 L of seawater. Epibenthic organisms are constantly in close contact with primarily sandy to muddy sediments of the German Bight. In the Wadden Sea the particles are mostly smaller than 250 μm (Becker et al., 1992; Gade et al., 2008). The SPM concentrations in the estuaries of the rivers Weser and Elbe in the SE North Sea, may exceed 50 mg L^{-1} and 300 mg L^{-1}, respectively (Kappenberg and Fanger, 2007; Roscher et al., 2021) and may still account for 20 mg L^{-1} off the island of Scharhörn, about 20 km off the mouth of the Elbe river (Kappenberg and Fanger, 2007). Similar morphological features (e.g. size, shape and colour) make the distinction between natural and synthetic microparticles difficult without a proper spectroscopic analysis (Korez et al., 2019). However, regardless of the origin both types of microparticles may be ingested by organisms and potentially induce similar effects (Ogonowski et al., 2018).

Natural particles are ingested by various deposit and filter feeders. The lugworm, *Arenicola marina*, ingests on average 4.7 g sediment per day (Jacobsen, 1967) with a preferred grain size between 100 and 1000 μm (Andresen and Kristensen, 2002). Suspension feeding bivalves, which possess eu-latero-frontal cirri, completely retain particles above 1 μm. Bivalves lacking these cirri show a decreased retention efficiency for particles smaller than 7 μm (Møhlenberg and Riisgård, 1978). Similarly, brown shrimp, *Crangon crangon*, contain sand grain and mud in their stomach (Ehrenbaum, 1890; Plagmann, 1939; Oh et al., 2001). Korez et al. (2020) found several hundred sand grains and shell fragments, mostly smaller than 200 μm, in freshly captured shrimp. Laboratory studies by Schmidt et al. (2021) showed that sand grains were not ingested intentionally but rather incidentally as a side effect of feeding. It is, however, yet unknown whether natural and synthetic microparticles cause similar effects in shrimp species.

We studied the effects of ingested natural and synthetic particles on the brown shrimp, *Crangon crangon*. The small epibenthic caridean decapod measures up to 60 mm in length and has a lifespan of up to three years. This species is highly abundant in the estuaries and coastal areas of the southern North Sea (Tiews, 1970; Boddeke et al., 1986; Oh et al., 1999; Campos and van der Veer, 2008), making the organisms a valuable shrimp fishery target. The species is exceptionally well adapted to changing environmental conditions of the southern North Sea, where changes in temperature, salinity, and food availability may be extreme (Oh et al., 1999; Hünerlage et al., 2019 and references therein). In the North Sea, the shrimp are exposed to both natural and anthropogenic microparticles (Devriese et al., 2015). Given the huge number of natural particles, microplastics (MP) constitute only a minor fraction of the overall microparticle load brown shrimp are exposed to in their natural environment. MP occur in habitats of *C. crangon* along the Belgian, Dutch, and German North Sea coast at concentrations of up to 1190 items per kg dry sediment and 0.25 items per liter seawater (Claessens et al., 2011; Lorenz et al., 2019). In the Weser Estuary, the sampling site for *C. crangon* in this study, up to 2.5 MP per liter of seawater were collected (Roscher et al., 2021), which is substantially less than the concentration of natural particles. Environmental concentrations of TiO_2 in the North Sea are not known (Baker et al., 2014). However, considerable amounts of TiO_2 have been disposed of at the Dutch coast in the late 1980s with adverse consequences for the local meiofauna (Smol et al., 1991).

To demonstrate the high particle load in the natural habitat of *C. crangon*, we quantified the seston concentration in the Weser estuary where our study organisms were sampled. In laboratory experiments, we exposed brown shrimp for up to two days to suspensions of natural particles (clay and silica), and anthropogenic contaminants (titanium dioxide, polyvinyl chloride, and polylactic acid) at concentrations of 20 mg L^{-1} each. After exposure, we determined the cellular anti-oxidative parameters superoxide dismutase, glutathione peroxidase, and glutathione reductase.

We tested the following hypotheses: (1) *Crangon crangon* ingest natural and synthetic particles. (2) The ingestion of microparticles induces an antioxidant response in digestive tissue of the shrimp. (3) Natural and synthetic particles induce differential antioxidant responses. (4) The antioxidant response to ingested microparticles varies with exposure time.

2. Material and methods

2.1. Collection and maintenance of brown shrimp

Brown shrimp (*Crangon crangon*) were collected in August 2019 by beam trawling with the research vessel FK Uthörn in the Weser estuary, SE North Sea (53.8000N, 8.1700E). The hauls lasted for 10 min maximum. The shrimp were immediately sorted from the catch, transferred into 50-L basins with running seawater, and shipped to the Alfred Wegener Institute in Bremerhaven, Germany. There, the shrimp were maintained for one week in a 100-L recirculating seawater system at 15 °C. The seawater system was equipped with a bio-filter and a protein skimmer. The seawater quality was controlled, and the medium was exchanged when critical levels of nitrite (>0.2 mg·L^{-1}), nitrate (>50 mg·L^{-1}), and ammonium (>0.4 mg·L^{-1}) were approached. Every second day, the shrimp were fed *ad libitum* with frozen abdominal muscle tissue from shrimp (*C. crangon*).

2.2. Extraction of suspended particulate matter from the Weser estuary

Water samples were collected in April 2019 in the Weser estuary (53.5317N, 8.5748E) with a bucket. In the laboratory, the water was left to sediment for 5 days in the dark and at 4 °C. The supernatant was decanted, and the remaining particles were resuspended with demineralized water. After another 24 h-sedimentation the particle-free supernatant was removed again and the resuspended seston was vacuum-filtered onto a 100-μm nylon gauze. The filtrate was again passed through a 10-μm nylon gauze. The fractions >100 μm and 10–100 μm, respectively, were rinsed onto a glass Petri dish, dried in an oven (40 °C, 24 h), and weighed.

2.3. Preparation of particle suspension

The shrimp were exposed to natural and synthetic microparticles (Table 2). According to the manufacturer, the microplastics did not contain additives. Stock suspensions with clay, silica, titanium dioxide (TiO_2), polyvinyl chloride (PVC), and Poly(L)-lactide plastic (PLA), respectively, were prepared in MiliQ at concentrations of 20 mg·mL^{-1}. The final microparticle concentration in the aquaria was 20 mg·L^{-1} with only a single type of microparticle in each aquarium. The concentration corresponded to natural seston concentrations in the German Bight (Kappenberg and Fanger, 2007). Both conventional and biodegradable plastics were selected because of their frequent commercial application

Table 2
Features of the microparticles used in the exposure experiments.

Type	Origin	Size (μm)	Form	Colour
Clay (Kaolin, Aluminum silicate hydroxide, K7375, Sigma-Aldrich)	natural	<20[a]	disk-like	white
Silica (Celpure® P65, Sigma-Aldrich)	natural	<500[a]	diverse	orange
TiO_2 (204,757, Sigma-Aldrich)	synthetic	<3	drops	white
PVC (9002-86-2, PyroPowders)	synthetic[b]	<60	spherical	white
PLA (764,698, Sigma-Aldrich)	synthetic[b]	<2	disk-like	white

[a] Can mechanically disintegrate into smaller microparticles.
[b] Inert microparticles according to the manufacturer; TiO_2: titanium dioxide, PVC: polyvinyl chloride, PLA: polylactide.

(Folino et al., 2020; PlasticsEurope, 2020).

2.4. Morphology of particles

About one mg of natural particles obtained from the Weser estuary and of each type of microparticles used in the exposure experiment were spread onto stubs with double-sided adhesive tape and sputter-coated with gold-palladium. The particles were investigated with a scanning electron microscope (SEM, Quanta 3D 200, FEI) to illustrate the shape and the size of the particles.

2.5. Experimental setup

Two days prior to exposure, the shrimp were transferred to a temperature-controlled room (11 °C) with indirect illumination and a 12/12 h light-dark cycle. The shrimp were maintained individually in 500-mL glass jars filled with 400 ± 15 mL filtered (0.2 μm pore size) seawater and connected to an aeration system. The water was exchanged daily during this acclimation period. To motivate the uptake of the food in the subsequent exposure period, the animals were starved during the acclimation period.

After the acclimation period, the shrimp were gently transferred into clean jars filled with the respective microparticle suspension. Shrimp exposed to microparticles had continuous access to food (*C. crangon* muscle, 300–400 mg) throughout the entire experimental period. Previous experiments by Schmidt et al. (2021) showed that indigestible microparticles are primarily taken up by *C. crangon* together with the regular food. Accordingly, the shrimp were fed in the exposure experiments to facilitate the uptake of the particles. Specimens without food and without microparticles (starved) and specimens with food but without microparticles (fed) served as controls, to allow for disentangling the potential effects of uptake of microparticles and food and the uptake of food alone. The shrimp were exposed for 6, 12, 24, and 48 h, respectively. In total, 336 shrimp were randomly allocated to the respective particle types and incubation periods yielding an initial number of 12 replicates per treatment. Five shrimp died during the exposure, resulting in a total mortality of 1.5%. One shrimp each died after exposure to silica for 12 h and 48 h. Three shrimp died after 48 h-exposure to TiO_2. Accordingly, eleven shrimp were separately exposed to silica for 12 and 48 h, and nine shrimp were exposed to TiO_2 for 48 h. All other treatments were replicated twelve times.

After exposure, the specimens were sedated on ice and rinsed with demineralized water to remove adhering particles from the body surface. The body length and the body mass were recorded. The shrimp were chilled on ice, briefly frozen at −80 °C, and dissected as described in Korez et al. (2020). The pleon was cut off and the cephalothorax opened by two dorsolateral incisions. The dorsal part of the cephalothorax was lifted, and the entire midgut gland was withdrawn. The midgut gland was placed in a 1.5-mL reaction cup, weighed and shock frozen in liquid nitrogen. The samples were stored at −80 °C until further analysis.

2.6. Biochemical analyses

Superoxide dismutase (SOD) was analysed after Livingstone et al. (1992) with slight modifications. The midgut gland tissue was homogenized with a stainless-steel micro-pestle in a ratio of 1:6 to 1:12 (w/v) of homogenizing buffer (20 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 7.6) and centrifuged for 15 min at 15,000 g and 4 °C. The supernatant was used for SOD analysis.

The SOD activity was measured spectrophotometrically in a 1.5-mL cuvette at room temperature. The cuvette contained 870 μL measuring buffer (43 mmol L^{-1} K_2HPO_4, 43 mmol L^{-1} KH_2PO_4, 0.1 mmol L^{-1} EDTA, pH 7.68), 100 μL cytochrome *c* (100 mmol L^{-1}), 10 μL xanthine (5 mmol L^{-1} in 0.1 mol L^{-1} NaOH), 10 μL sample, and 10 μL xanthine oxidase (XOD, 0.3 mU μL^{-1} in 2 mol L^{-1} $(NH_4)_2SO_4$). The reagents were thoroughly mixed with a small plastic spatula and the increase of absorbance at 550 nm was measured for 2 min. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. The sample volume or XOD volume were adjusted accordingly. The SOD activity was normalized for the wet weight (ww) of the midgut gland ($U \cdot g_{ww}^{-1}$).

Glutathione peroxidase (GPx) was assayed after Flohé and Günzler (1984). The tissue was homogenized in 120 μL buffer (100 mmol L^{-1} potassium phosphate with 2 mmol L^{-1} Na_2-EDTA, pH 7) in a ratio of 1:6 (w/v) and centrifuged for 15 min at 15,000 g and 4 °C.

The activity of GPx was measured spectrophotometrically in 96-well microplates. The wells were filled with 30 μL reduced glutathione (GSH, 10 mmol L^{-1} in MilliQ), 30 μL β-nicotinamide adenine dinucleotide (NADPH, 1.5 mmol L^{-1} in 0.1% $NaHCO_3$), 30 μL glutathione reductase (GR, 24 U mL^{-1} in buffer), and 30 μL of sample or blank, before buffer was added to the final volume of 270 μL. The H_2O_2 independent reaction (blank reaction) was measured at 340 nm for 1.5 min. Subsequently, 30 μL of 70% t-butyl hydroperoxide (12 mmol L^{-1}) was added to the wells, and the measurement was repeated. The oxidation of NADPH was calculated from the linear regression slope, using the extinction coefficient (6.2 mL μmol^{-1} cm^{-1}). The GPx-dependent reaction was calculated by subtracting the hydroperoxide independent NADPH consumption rate from the hydroperoxide dependent NADPH consumption rate and expressed as U mL^{-1}. The GR activity was normalized for the wet weight (ww) of the midgut gland ($U\ g_{ww}^{-1}$).

Glutathione reductase (GR) was determined after Carlberg and Mannervik (1985). Tissue samples were homogenized in 120 μL buffer as described above for GPx and further diluted in a ratio of 1:26 to 1:30

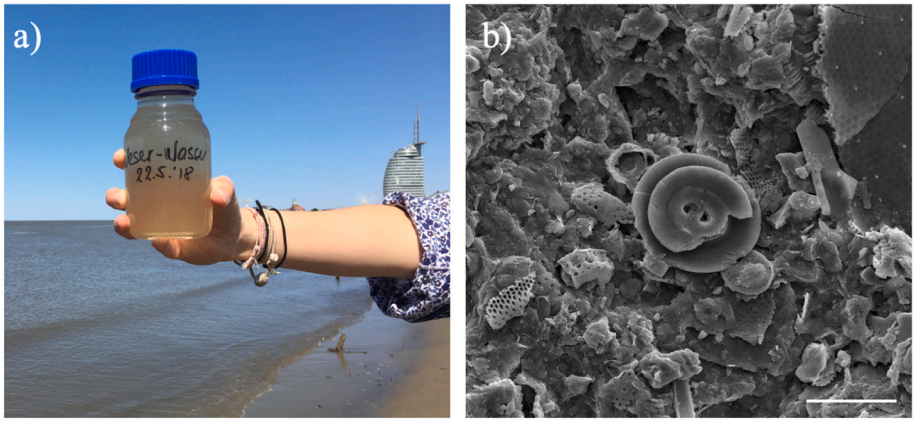

Fig. 1. Water from the Weser estuary (Bremerhaven, Germany). The water appeared a) turbid and b) contained broken diatom frustules and other unrecognised particles. Scanning electron micrograph. Scale: 10 μm.

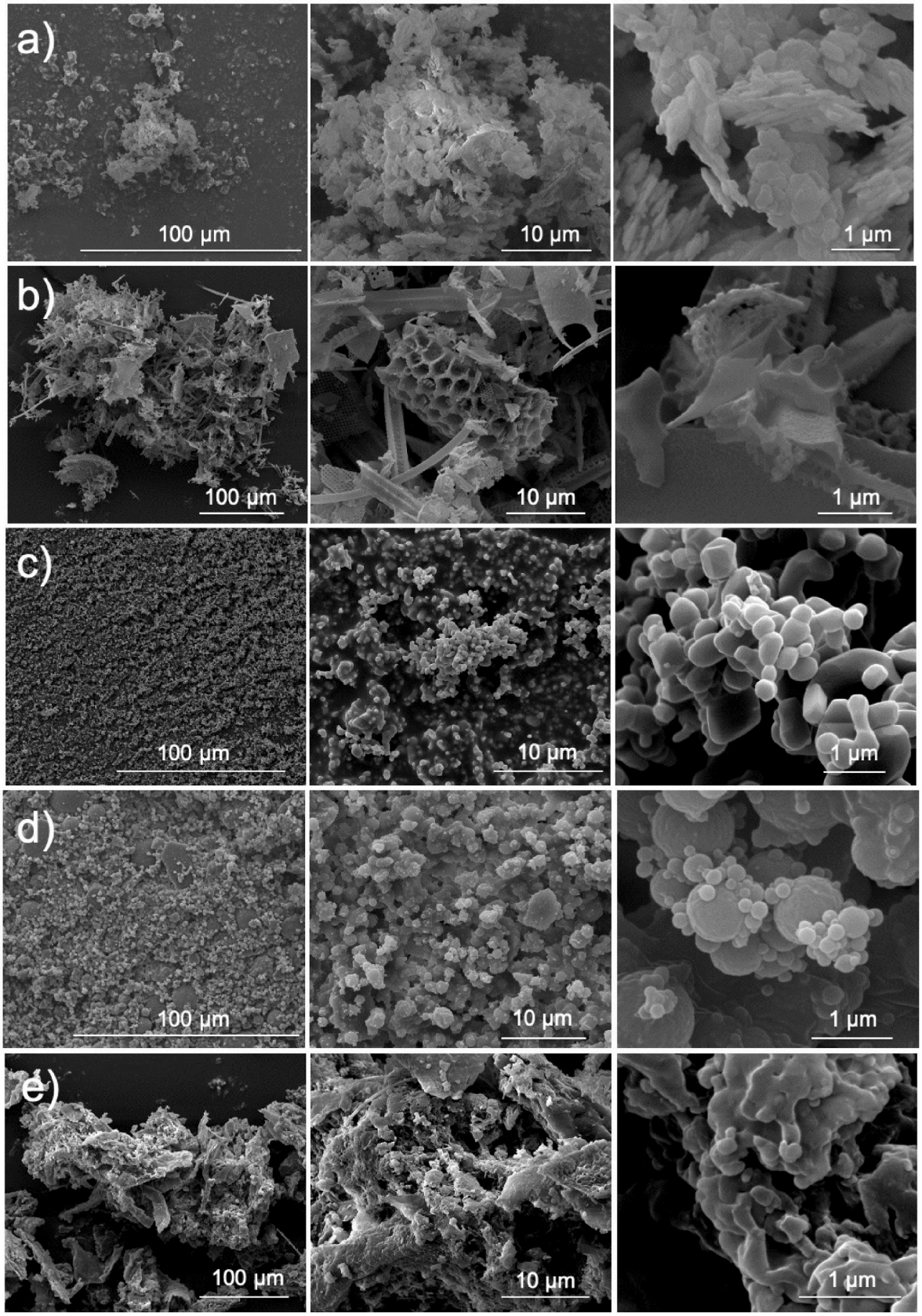

Fig. 2. Microparticles used in the present study. Natural particles: a) clay, b) silica; and anthropogenic particles: c) TiO_2, d) PVC, and e) PLA. Scanning electron microscopy.

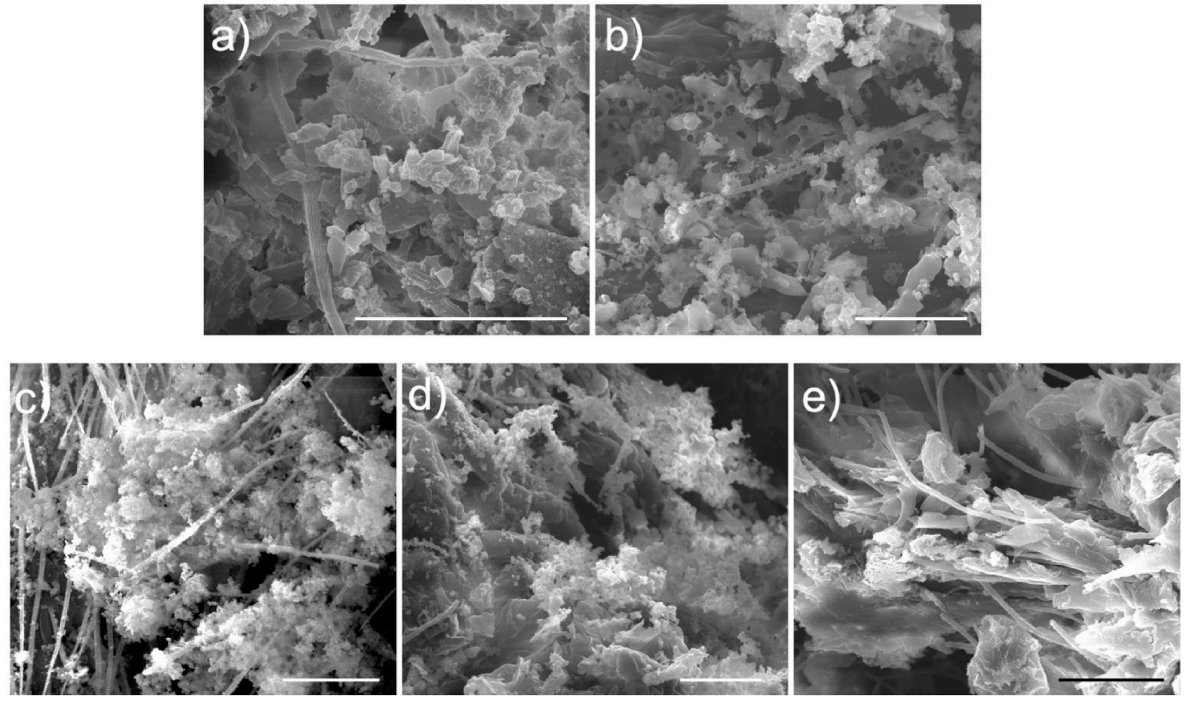

Fig. 3. SEM-micrographs of stomach content of the shrimp *Crangon crangon*, showing a) clay, b) silica, c) TiO_2, d) PVC, and e) PLA. Scales 50 μm.

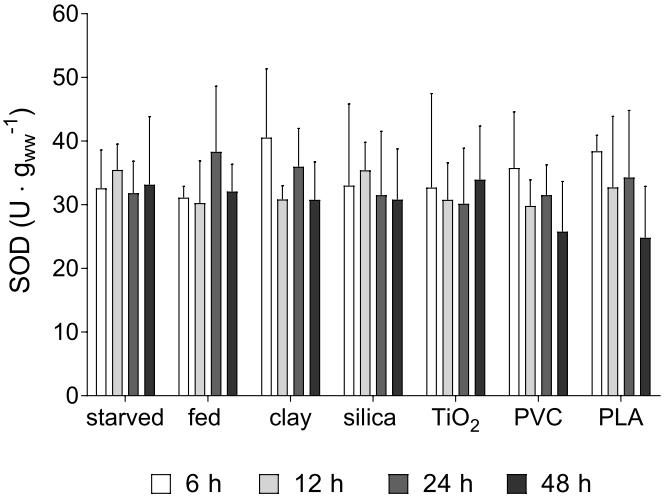

Fig. 4. Superoxide dismutase (SOD) activities in the midgut gland of *Crangon crangon* exposed to natural microparticles (clay, silica) and synthetic microparticles (TiO_2, PVC, PLA). Starved and fed animals served as controls (mean ± SD, n = 4–6). Two-way ANOVA showed no significant differences between organisms from different treatments.

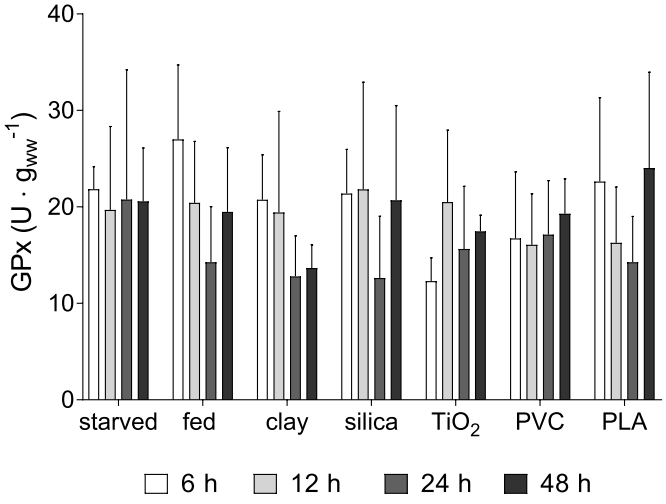

Fig. 5. Glutathione peroxidase (GPx) activities in the midgut gland of *Crangon crangon* exposed to natural (clay, silica) and synthetic microparticles (TiO_2, PVC, PLA). Starved and fed animals served as controls (mean ± SD, n = 4–6). The activity was decreased after 24-h exposure but recovered after 48 h.

(w/v).

The activity of GR was measured spectrophotometrically in 96-well microplates. Each well was filled with 105 μL MilliQ and 15 μL GSSG (20 mmol L^{-1}). 15 μL of sample (supernatant) or blank and 15 μL NADPH (2 mmol L^{-1} in 10 mmol L^{-1} Tris-HCl buffer, pH 7) were added before the reagents were mixed by addition of 150 μL buffer. A serial dilution of GR was used as standard. The GR activity was normalized for the wet weight (ww) of the midgut gland (mU $g_{ww}{}^{-1}$).

2.7. Data analysis

The data sets of the biochemical markers were screened for outliers (Grubb's test) at a significance level of $p = 0.05$. In total, 15 outliers or 3.0% of the values were omitted from 499 computed values.

The effects of the main factors "particle type" and "exposure time" and their interaction (particle type × exposure time) were tested separately for SOD, GPx and GR with a two-way ANOVA, followed by a Tukey's HSD post hoc test. The significance level was set to $p = 0.05$. The GPx data were square root transformed to achieve normal distribution (Shapiro-Wilk test, $p > 0.05$) and homoscedasticity (Levene's test, $p > 0.05$). No transformation was needed for the other data sets.

Data were analysed and visualized using GraphPad Prism 7.04 and R Studio (version 1.2.5019).

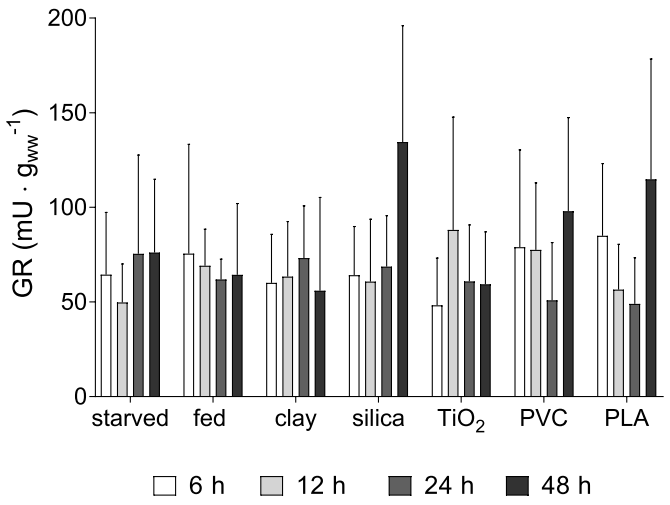

Fig. 6. Glutathione reductase (GR) activities in the midgut gland of the *Crangon crangon* exposed to natural (clay, silica) and synthetic microparticles (TiO_2, PVC, PLA). Starved and fed organisms served as control (mean ± SD, n = 5–6).

3. Results

3.1. Extraction of natural microparticles from Weser estuary

The water of the Weser estuary appeared turbid (Fig. 1a). One liter contained more than 25 mg of suspended particulate matter (>10 μm). The fraction >100 μm accounted for 57% (14.4 mg) and the fraction 10–100 μm for 43% (10.8 mg). The suspended particulate matter contained diatoms and various unrecognised natural material (Fig. 1b).

3.2. Characterization of the microparticles offered in exposure experiments

Most of the natural and synthetic microparticles were white. Only silica showed orange coloration. The shape of the microparticles ranged from discus-like, droplet-like, or spherical to unregular (Fig. 2). The microparticles were of different sizes, including particles smaller than 1 μm (Table 2). Especially the natural microparticles were prone to further fragmentation under low mechanical force (Š. Korez pers. obs.).

3.3. Ingestion of microparticles

Crangon crangon readily ingested the natural and synthetic microparticles offered under controlled laboratory conditions (Fig. 3). All types of particles were found in the stomachs of the shrimp mixed with the ingested regular food. The natural microparticles were identified in the mixture by their shape (Fig. 3a and b) whereas synthetic microparticles were recognised as fluff or agglomerates (Fig. 3c–e).

3.4. Superoxide dismutase (SOD)

Superoxide dismutase activities from 159 shrimp (94.6%) were analysed statistically. Five values were classified as outliers.

SOD activity of the control specimens ranged from 30.3 ± 6.6 U g_{ww}^{-1} to 34.0 ± 8.4 U g_{ww}^{-1} (Fig. 4). The highest SOD activity was measured in shrimp exposed to clay for 6 h (40.6 ± 10.8 U g_{ww}^{-1}), whereas the shrimp exposed to PLA for 48 h had the lowest activity (24.8 ± 8.1 U g_{ww}^{-1}).

SOD activities did not vary significantly with particle type ($F_{6,131}$ = 0.578, p = 0.7477) or exposure time ($F_{3,131}$ = 2.547, p = 0.0587), nor was the interaction between exposure time and particle type statistically significant ($F_{18,131}$ = 0.928, p = 0.5460).

3.5. Glutathione peroxidase (GPx)

In total, data from 168 shrimp were used for GPx analysis. Four data were identified as outliers (2.4%).

The GPx activity in the control specimens ranged between 14.3 ± 5.8 U g_{ww}^{-1} and 27.0 ± 7.7 U g_{ww}^{-1} (Fig. 5). Shrimp that were fed reached the highest activity (27.0 ± 7.7 U g_{ww}^{-1}) after 6 h. The lowest GPx activity was observed in shrimp exposed to TiO_2 for 6 h (12.3 ± 2.4 U g_{ww}^{-1}).

The GPx activity did not vary between shrimp that received different particle types ($F_{6,135}$ = 1.156, p = 0.3335). However, the GPx activity showed significant temporal fluctuation ($F_{3,135}$ = 4.871, p = 0.0030). The activity dropped significantly after 24 h but recovered after 48 h (Tukey's test, p < 0.05). There was no interaction between the main factors particles type and exposure time ($F_{18,135}$ = 1.239, p = 0.2397).

3.6. Glutathione reductase (GR)

The 167 extracts prepared for GR analysis were further analysed for GR activities. Six data points were identified as outliers (3.6%) and omitted from the statistical analysis.

The GR activities of the control organisms ranged between 49.8 ± 20.3 and 76.1 ± 38.7 mU g_{ww}^{-1} (Fig. 6). Shrimp exposed for 48 h to silica showed the highest GR activity (134.6 ± 61.5 mU g_{ww}^{-1}) and the specimens exposed for 6 h to TiO_2 the lowest (48.2 ± 25.0 mU $g_{w\ w}^{-1}$).

The GR activity did not vary between shrimp that received different types of microparticles ($F_{6,133}$ = 0.766, p = 0.5978). The activity remained constant for 24 h but increased significantly after 48 h ($F_{3,133}$ = 3.278, p = 0.0231; Tukey's test, p < 0.05). The temporal development of the enzyme activity did not differ between animals that were exposed to different types of microparticles (interaction: $F_{18,133}$ = 1.465, p = 0.1127).

4. Discussion

To investigate cellular effects of different types of microparticles in *Crangon crangon* comparatively, all particle types (silt, diatom frustules, titanium dioxide, PVC, and PLA) were applied at common SPM concentrations of southern North Sea estuaries (20 mg L^{-1}). Seston concentrations reported from the estuaries of the rivers Weser and Elbe by Kappenberg and Fanger (2007) and Roscher et al. (2021) were confirmed in the present study. About 26 mg L^{-1} of SMP were found in the Weser estuary, with about 11 mg of particles smaller than 100 μm (Table 1). Accordingly, the particle concentrations applied in our experiments were well within the range of seston concentrations of the natural environment of *C. crangon*. The concentrations of natural particles are much higher in coastal waters of the North Sea than the concentrations of microplastics (Claessens et al., 2011; Lorenz et al., 2019). To evaluate the potential effects of microplastics requires, therefore, a comparison with the effects induced by natural particles (Ogonowski et al., 2018). Consequently, our comparative study contributes to the assessment of the risk arising from microplastics in the marine environment.

4.1. Ingestion of microparticles

Recent field and laboratory studies have demonstrated that the feeding habits of brown shrimp entailed ingestion of considerable amounts of sediment grains and other microparticles (Korez et al., 2020; Schmidt et al., 2021). Up to several hundred sediment grains and mussel shell fragments have been found in the stomachs of *C. crangon* (Korez et al., 2020), which is a result of rapid and indiscriminate feeding (Schmidt et al., 2021). Moreover, ingestion of sediment grains unavoidably adds smaller particles to the diet of the shrimp, such as diatom frustules, which attach to the surfaces of the grains (Schmidt et al., 2021). Ingestion of microplastics at a rate of more than one

particle per individual has previously been confirmed for brown shrimp (Devriese et al., 2015). Accordingly, a similarly unselective uptake of other anthropogenic microparticles, such as bio-based microplastics and TiO_2-microparticles, appears likely and could be confirmed in this study.

According to our first hypothesis, brown shrimp readily ingested natural as well as synthetic microparticles. Upon ingestion, all types of microparticles were visible in the stomachs in transmitted light. Additionally, the uptake of all types of microparticles by the shrimp in the experiments was confirmed by scanning electron microscopy.

The administered particles varied considerably in size. Particularly the natural microparticles, such as diatom frustules, easily fragment into smaller pieces already under low mechanical forcing (Š. Korez pers. obs.). Accordingly, progressive fragmentation is likely to happen during food mastication within the stomach increasing the number of smallest fragments, which can advance into the critical digestive organs. Fragmentation of synthetic microparticles into nanoparticles has been observed in the stomachs of krill, langoustine, and freshwater amphipods (Dawson et al., 2018a; Cau et al., 2020; Mateos-Cárdenas et al., 2020). Accordingly, characteristics and quantities of microparticles inside digestive organs of crustaceans and their potential cellular effects are difficult to predict from the composition of particles occurring in the environment.

4.2. Antioxidant responses

A large fraction of the microparticles used in our experiments were smaller than 1 μm. Particles in the sub-μm size-range can pass the pyloric filter of crustacean stomachs, enter the midgut gland tubuli, and translocate into the epithelial cells via endocytosis (Korez et al., 2020). The midgut gland is the principle digestive organ of crustaceans where the synthesis of digestive enzymes, nutrient resorption and cellular digestion takes place (Saborowski, 2015; Štrus et al., 2019). In the epithelial cells of the midgut gland, food particles are engulfed in phagocytotic vesicles (Hartenstein and Martinez, 2019). There, the synthesis of reactive oxygen species (ROS) can be induced as a defense mechanism against, for example, invading pathogens (Lushchak, 2011).

Model simulations as well as fluorescence microscopy confirmed that synthetic nanoparticles of up to a few micrometers pass through the immune cell membrane or invaginate into the immune cells via endocytosis where they disperse in the cytosol or accumulate in the phagosome (Zhang et al., 2009; Rossi et al., 2014; Pinsino et al., 2015; Greven et al., 2016; Soares et al., 2016). Following particle entry, the cellular oxidative activity increases (Greven et al., 2016), resulting in elevated concentrations of ROS (Xiong et al., 2011; Jeong et al., 2016). The radicals are sequentially counteracted by a cascade of enzymatically catalyzed antioxidant reactions (Lesser, 2006; Lushchak, 2011). The antioxidants measured in the present study were the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). Especially SOD and GPx are considered to constitute the first line of defense of ROS elimination. SOD catalyzes dismutation of $O_2^{\bullet -}$ to H_2O_2, which is further reduced to water by GPx. GPx is a part of a glutathione (GSH) dependent system. GSH prevents oxidation of essential cellular lipids and proteins. The level of GSH is regulated by GR, which recycles oxidized glutathione (GSSG) back into the reduced form GSH (Lesser, 2006; Prokić et al., 2019).

Elevated activities of antioxidants following the ingestion of synthetic microparticles have been shown in short-term experiments for a wide spectrum of marine invertebrates, including crustaceans. Activities of GPx, GR, SOD, and GST increased in the rotifer *Brachionus koreanus* and the copepod *Tigriopus japonicus* after administration of microbeads for up to 48 h (Jeong et al., 2016; Choi et al., 2020). Similarly, microplastic uptake induced a higher GSH content in the copepod *Paracyclopina nana* after 24 h (Jeong et al., 2017) and TiO_2 elevated activities of CAT, GPx and GST in *Daphnia magna* after 48 h exposure (Kim et al., 2010). In *Mytilus edulis*, Paul-Pont et al. (2016) reported decreased CAT and GR activities following seven-day exposure and an increase in SOD and GST activity following 14-day exposure. In the shrimp *Palaemon varians*, SOD activities increased after few hours of microbead exposure but decreases rapidly after a distinct peak in SOD activity after 4 h (Saborowski, unpublished data). The lack of an oxidative response in *C. crangon* suggests that the responses to microparticles can vary among closely related organisms.

Antioxidative responses were reported also in vertebrates. A dose-specific effect of 5-μm MP appeared in zebrafish, where SOD- and CAT-activity increased after three-week exposure (Lu et al., 2016). The alterations in activities of CAT and GST showed oxidative stress in embryos of zebrafish *Danio rerio* after 96 h exposure to TiO_2 (Clemente et al., 2014), while in juvenile carp *Cyprinus carpio* the activities of SOD and CAT increased with TiO_2 concentrations and exposure time (Hao et al., 2009).

Our hypothesis that the ingestion of microparticles induces an antioxidant response in the digestive epithelia of *C. crangon* was not supported by the results from our experiments. Similarly, our hypothesis that natural and synthetic particles induce differential antioxidant responses was not supported. No substantial antioxidant response was observed in *C. crangon* although the shrimp had ingested considerable amounts of either natural or synthetic microparticles. Moreover, the enzymatic activities were similar among individuals that received microparticles and control individuals that either starved or received regular food.

The SOD activity did not vary with exposure time. Contrarily, the activities of GPx and GR showed temporal variations. However, these variations were similar among individuals that received microparticles and control animals that either starved completely or did not have access to microparticles. Accordingly, the observed temporal variations are not a specific response to ingested microparticles and, therefore, do not clearly support our hypothesis that the antioxidant response to microparticle uptake varies with exposure time.

Similarly unexpected responses were observed in some crabs and mussels. Activities of SOD, GSH, and GPx increased when Chinese mitten crab, *Eriocheir sinensis*, were exposed to low MP concentration of 40 and 400 μg L^{-1} (Yu et al., 2018) but decreased at higher MP concentration. At 4000 and 40,000 μg L^{-1}, which are similar to the particle concentrations in our experiments, the activities of all three enzymes were even lower than the activities in control animals without particles. Simultaneously, levels of malondialdehyde (MDA) increased, indicating progressive oxidative stress and degradation of unsaturated fatty acids (Yu et al., 2018). The authors suggested that enzyme inhibition may be a consequence of metabolic cost, but they did not further elaborate on this idea. Activities of SOD and malondialdehyde were reduced in *Mytilus* spp. after long-term (42 weeks) exposure to polystyrene and polyvinylchloride microplastics (Hamm and Lenz, 2021). The mussel *M. edulis* showed decreased SOD, CAT, and GST activities, after exposure to biodegradable polyhydroxybutyrate microplastics (Magara et al., 2019). The authors attributed the decrease in activity to chemical-mediated damage of the enzymes. Similarly, Khalid et al. (2021), measuring CAT, GST and SOD activities, detected no antioxidant response in *M. edulis* following exposure to bio-based and biodegradable microplastics. Furthermore, Sıkdokur et al. (2020) report unchanged CAT activities in the gills and digestive gland of manila clam following seven-day exposure to MP. Similarly, Antarctic krill showed no evidence for oxidative stress, neurotoxicity or detoxifying activity when exposed to p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE), a common persistent organic pollutant (Dawson et al., 2018b).

Antioxidant enzymes may be affected by a variety of substances. Cu/Zn-SOD can be inactivated by high concentrations of hydrogen peroxide and other active oxygen species (Bray et al., 1974; Casano et al., 1997), chelators, or some metal ions (Ma et al., 2017). GPx can be inhibited by thiol-containing substances (Chaudiere et al., 1984; Ma et al., 2017). GR is vulnerable to plant polyphenols (Zhang et al., 1997), which might be ingested along with the food. However, it appears unlikely, that all three enzymes were inhibited simultaneously by effectors. We, therefore,

suggest that other, non-enzymatic antioxidant protection systems may have been involved in the antioxidant defense within the midgut gland of brown shrimp. Non-enzymatic antioxidants comprise various substances, including vitamins, polyphenols, carotenoids, and glutathione (Moussa et al., 2019). They are present in the cytosol, cell organelles, and phospholipid membranes and act as radical scavengers. Non-enzymatic antioxidants can be synthesized de novo in the cells or taken up along with the food. For example, dietary polyphenols are potent antioxidants (Han et al., 2007). When acquired from the food, non-enzymatic antioxidants may function as constitutive defense against the detrimental effects of reactive oxygen species. In species living in habitats with naturally high concentrations of microparticles, a constitutive defense that causes low metabolic cost may be an energetically more efficient strategy than an inducible enzymatic defense, which would require a constant synthesis of antioxidant enzymes. Unfortunately, due to the low amount of sample material (about 10 mg tissue per shrimp) we could not perform additional analyses on non-enzymatic antioxidants.

5. Conclusion

In their habitat, *C. crangon* are exposed to a great variety of particles, which differ in size, shape, and origin. The shrimp readily ingest natural and synthetic particles along with their food. However, they did not show oxidative stress responses in terms of increasing anti-oxidative enzyme activities. Given the complexity of the digestive tract in *C. crangon* (Korez et al., 2020), the microparticles may be retained by the pyloric filter in the stomach, preventing the entry of the particles into the midgut gland. Alternatively, *C. crangon* may be particularly tolerant towards the effects of ingested microparticles suggesting additional morphological and physiological adaption to life in the presence of high quantities of suspended and deposited particulate material. For example, the antioxidant protection of *C. crangon* may rely on other mechanisms, which may primarily comprise non-enzymatic antioxidants potentially acquired from the food. A constitutive antioxidant potential of the shrimp may be energetically less costly for a species, which is constantly exposed to high concentrations of microparticles, than an inducible enzymatic defense. Besides investigating other antioxidant protection systems, such as non-enzymatic antioxidants, comparative studies with other crustaceans and invertebrates from different habitats and with different feeding modes are needed, to evaluate the apparently high stress resistance of the brown shrimp towards microparticles.

CrediT author statement

Š.K.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition. L.G.: Conceptualization, Writing – review & editing, Funding acquisition, Supervision. R.S.: Conceptualization, Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

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.

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