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State University of New York, United States
Michael Leonard Judge,
Manhattan College, United States

*CORRESPONDENCE
Reinhard Saborowski
Reinhard.Saborowski@awi.de

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Trophic ecophysiology of the native green shore crab, *Carcinus maenas*, and the invasive Asian shore crab, *Hemigrapsus sanguineus*, in the rocky intertidal of Helgoland (North Sea)

Reinhard Saborowski[1*], Patrick Bartolin[1,2], Marie Koch[1,2] and Simon Jungblut[1,2]

[1]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany,
[2]Marine Zoology, University of Bremen, Bremen, Germany

The brachyuran crabs *Carcinus maenas* and *Hemigrapsus sanguineus* belong to the most ´successful´ invaders along the oceans coasts. In 2009, *H. sanguineus* appeared at the rocky intertidal of the island of Helgoland in the North Sea, where it encounters the native Green shore crab, *C. maenas*. *H. sanguineus* established a self-sustaining population, approaching in numbers and biomass that of *C. maenas*. Both species are considered to be opportunistic omnivores with variable food preferences and, thus, are potential competitors for food. To evaluate the intrinsic properties of either species to utilize food, we analyzed their stomach content, the morphology of the gastric mills, which shred the ingested food, the activities of digestive enzymes during a seasonal cycle, and the stable isotope ratios. A huge share of the stomach contents was macerated and, thus, could not be identified. The shares of animal food and algae food were almost equal in *C. maenas* but algae food dominated over animal food in *H. sanguineus*. The gastric mill of *C. maenas* shows blunt medial tooth and rounded lateral teeth, which indicates efficient grinding of a carnivorous diet. In contrast, the gastric mill of *H. sanguineus* shows sharp ridges, which facilitate cutting of algal food. The activities of the proteolytic enzymes trypsin and leucine-aminopeptidase were almost equal in both species with slightly higher activities in *C. maenas* in summer. The activities of the carbohydrases laminarinase and amylase dominated in *H. sanguineus* during all seasons. Stable isotope ratios indicate a higher degree of carnivory in *C. maenas*. The morphological and biochemical features indicate that *C. maenas* is better suited to utilize animal food and *H. sanguineus* algal food. Upon scarcity of animal food or severe competition with *C. maenas*, *H. sanguineus* may be able to increase the amount of algal food and to utilize it efficiently.

KEYWORDS

invasive species, food competition, herbivory, carnivory, gastric mill, digestive enzymes

1 Introduction

Unintentional dispersal of marine species into foreign regions has drastically increased along with human trading and shipping activities. The proliferation of foreign species may entail various consequences for established ecosystem and is considered to be a serious threat to biodiversity in marine habitats (Grosholz, 2002). Introduced species may compete for space and food and, in the worst case, displace native residents (Bax et al., 2003). Among marine taxa, brachyuran crabs (Crustacea, Decapoda) are common invaders due to their high larval dispersion capability as well as their habitat preference and lifestyle in coastal regions. Well-documented cases are the almost global dispersion of the green shore crab, *Carcinus maenas* (Linnaeus, 1758), and the Asian shore crab, *Hemigrapsus sanguineus* (De Haan, 1835) (Epifanio, 2013; Jungblut et al., 2017; Young and Elliott, 2020).

The Asian shore crab *Hemigrapsus sanguineus* is a newcomer at the western European coasts where it encounters the native green shore crab. Originally, *H. sanguineus* inhabited the coasts of the northwest Pacific but started in the early 1990s to settle along the North American coast (Williams and McDermott, 1990; Blakeslee et al., 2017). In the late 1990s, *H. sanguineus* was introduced most likely via ballast water to the French harbor of Le Havre (Breton et al., 2002). From there, it rapidly dispersed along the European Atlantic coasts through the English Channel into the North Sea (Dauvin et al., 2009). In 2009, *H. sanguineus* appeared at the rocky intertidal of the island of Helgoland, North Sea, where it established a self-sustaining population, rapidly approaching in number and biomass that of the native *C. maenas* (Jungblut et al., 2017).

Both species show distinctive biological and ecological parallels. Similar to juvenile green shore crabs, *H. sanguineus* inhabit the intertidal zone where they are strongly affected by the tides. At low-tide, they hide beneath stones, kelp, or mussel beads to protect themselves from desiccation and predators. Therefore, foraging periods are limited to the high-tide which may additionally increase the competition for food. Based on respiration measurements, Jungblut et al. (2018a) estimated that the energy consumption of the *H. sanguineus* population in the Helgoland intertidal almost equals that of *C. maenas*.

Both species are considered to be opportunistic onmivorous (Ropes, 1968; Ledesma and O´Connor, 2001). However, *C. maenas* shows a strong tendency for carnivory (Crothers, 1967; Ropes, 1968), whereas the food preference of *H. sanguineus* appears to be more variable. Stomach content analysis of wild-caught *H. sanguineus* revealed predominantly algal food components (Lohrer and Whitlatch, 1997; McDermott, 1999; Tyrrell and Harris, 2000). Other studies report a balanced or carnivorous diet depending on the environmental food availability (Brousseau and Baglivo, 2005). Apparently, *H. sanguineus* shows high adaptive capacity towards variable food sources.

Given a strong overlap in habitat use and food preferences of both species, a strong competition for both factors appears unavoidable. Comparative studies are scarce and were almost exclusively carried out at the coasts of USA where both species are invasive. Competition experiments for food and shelter showed that *H. sanguineus* dominated over *C. maenas* (Jensen et al., 2002). However, these experiments were carried out in the laboratory and direct competition was promoted. In our case, *C. maenas* is native, facing the invading *H. sanguineus*, which reduced comparability with previous reports.

To circumvent behavioral and ecological influences, we focused our study on the intrinsic morphological and physiological capabilities of food utilization in *C. maenas* and *H. sanguineus*. First, stomach contents of both species from the intertidal of Helgoland were analyzed over a seasonal cycle to estimate the shares of animal and algal food. Then, we described the morphology of the gastric mills and discussed the functional features in view of food preferences (Giddins et al., 1986; Skilleter and Anderson, 1986; Heeren and Mitchell, 1997; Salindeho and Johnston, 2003; Allardyce and Linton, 2010). A set of digestive enzymes from the midgut gland was analyzed, including trypsin and leucine aminopeptidase representing the proteolytic potential, and laminarinase and amylase representing the potential for carbohydrate utilization. Finally, analysis of stable isotopes of C and N was included in the comparison of the trophic position between both species. The results will show the potential of either species to utilize specific food sources and will support interpretations about their trophic spectrum and, to a certain degree, their ecological performance.

2 Materials and methods

2.1 Origin of samples

Specimens of *Carcinus maenas* (Linnaeus 1758) and *Hemigrapsus sanguineus* (De Haan 1853) were collected in April, June, August, and October 2016 in the intertidal of the island of Helgoland at a spot called “Kringel” (54°10´37N 7°53´07E). This area is characterized by boulders of sandstone and coarse gravel. Sampling was performed one hour after the first low tide at daytime. The animals were collected from under stones and macroalgae and from crevices. The crabs were placed in buckets, covered with moist macroalgae, and carried to the laboratories of the Marine Station Helgoland. In the lab, the catch was sorted according to species and sex and kept in aerated seawater, awaiting subsequent measurement and dissection.

2.2 Dissection of midgut gland and stomach content analysis

The crabs (2.1 to 4.5 g) were sedated on ice for about 10 min before the carapace was rapidly pulled off and the crabs instantly died. The midgut gland was removed, transferred into weighed reaction cups, shock frozen in liquid nitrogen, and transferred on dry ice to Bremerhaven for biochemical analysis. The esophagus was cut close to the mouth opening and the chitinous stomach capsule was withdrawn as a whole with fine surgical forceps. The stomach was dissected and preserved in 70% ethanol for visual stomach content analysis. The stomach was cut ventrally, the content was removed with forceps, and transferred into 5-mL reaction cups. The fullness of the stomach was graded into five categories from empty (0%) to half-filled (50%) and fully filled (100%) with intermediate grades of 25% and 75%, respectively. Ten stomachs were used per species and per season and the values were averaged. The stomach content often consisted of an agglutinated mash. To disperse the mash, 3 mL of ethanol were added and the sample was vortexed or placed for a few seconds in an ultrasonic bath. The dispersed stomach content was transferred into a small petri dish and inspected under a stereo microscope. A definite identification of the stomach content was not possible due to the very small size of the items and the advanced digestion process. Obvious body parts of marine invertebrates as well as chitin residues, mussel shell fragments, or polychaete spines were classified as ´animal´. Greenish and brownish components, derived from green algae and brown algae, were classified as ´alga´. Mashed material without distinct color was classified as ´not identified (n.i.)´.

2.3 Morphology of the gastric mill

Scanning-electron-microscopy (SEM) was used to illustrate the morphology and ultrastructure of the gastric mill. The dissected stomach capsules were ventrally cut and opened under a stereo microscope. The stomach content was carefully rinsed out with a syringe. Subsequently, the entire gastric mill or individual lateral and median teeth of the gastric mill were dissected and prepared for SEM. The preparations were dehydrated in an ethanol series of 2 × 15 min in 50% ethanol, 2 × 15 min in 70% ethanol, 2 × 15 min in 90% ethanol. After air-drying overnight in a desiccator, the samples were mounted on SEM stubs with double-sided carbon tape. The stubs were sputter coated with gold-palladium and the samples were inspected and photographed under the SEM (FEI, Quanta FEG 200).

2.4 Biochemical analysis

2.4.1 Preparation of tissue extracts

Frozen midgut gland tissue (about 65 mg) was transferred into 2-mL reaction cups and 500 µL of demineralized water was added. The tissue was thoroughly homogenized with a micro-pestle. Demineralized water was added to adjust a tissue concentration of 50 mg·mL^{-1}. The homogenates were centrifuged for 15 min at 13,000 g and 4°C. Aliquots (250 µL) of the aqueous enzyme extract were transferred into new 1.5-mL reaction cups and stored at -80°C.

2.4.2 Semi-quantitative enzyme screening

Samples taken in April were screened for a set of 19 enzymes with the commercial ApiZym test kit (BioMerieux, Nürtingen, Germany) as per the manufacturer´s instructions. Sixty µL of midgut gland extract (50 mg·mL^{-1}) were given into each of the test wells and incubated in darkness for 4 hours at room temperature. The dye reaction was initiated by the addition of ZymA and ZymB reagents. After 10 min, the intensity of the dye reaction was visually determined and classified from ´0´= no activity to ´5´= full activity.

2.4.3 Enzyme assays

The activities of endo- and exopeptidases were determined photometrically after Saborowski et al. (2004) and Saborowski et al. (2006), respectively. Trypsin activity (E.C. 3.4.21.4) was assayed with the chromogenic substrate L-BAPA (N$^{\alpha}$-benzoyl-L-arginin-4-nitroanilid-hydrochloride, Applichem A5030). Fifty µL of the extract were pipetted into a glass semi-micro cuvette and 930 µL of Tris/HCl-buffer (0.1 mol·L^{-1}, pH 7.0) were added. The cuvettes were first incubated for 5 min at room temperature (Jena Analytic Specord 200, TempControl). Subsequently, the enzymatic reaction was started with 20 µL of the substrate solution (50 mmol·L^{-1} in dimethylsulfoxide, DMSO). The final substrate concentration in the reaction mixture was 1 mmol·L^{-1}. The increase of absorbance at 405 nm was recorded for another 5 min. Enzyme activity was expressed as U·g$^{-1}_{FM}$ (= µmol·min^{-1}·g$^{-1}_{FM}$) using the extinction coefficient ϵ_{405} = 10.2 L·mmol^{-1}·cm^{-1}.

Leucine aminopeptidase (LeuAP) was determined with the substrate L-leucine-p-nitroanilide (Sigma, L-9125). The substrate was dissolved in dimethylsulfoxide (DMSO) and applied at a final concentration of 1 mmol·L^{-1} in the reaction mixture. The activity was expressed as U·g$^{-1}_{FM}$ (= µmol·min^{-1}·g$^{-1}_{FM}$) using an extinction coefficient of 9.9 L·mmol^{-1}·cm^{-1}.

The enzymatic degradation of laminarin and starch were determined by the liberation of reducing sugars from natural substrates. Laminarinase (endo-β-1,3-glucanase) was assayed after Linton and Greenaway (2004) with slight modifications. The reaction mixture contained 20 µL of the tissue extract, 130 µL Na-acetate buffer (0.1 mol·L^{-1}, pH 5.5) and 50 µL of a laminarin solution (1% (w/v) in a. dest.). The sample blank of each sample contained 20 µl of the respective tissue extract and 180 µl Na-acetate buffer and the substrate blank was prepared with 50 µL of laminarin solution. The reaction mixtures were incubated for 10 min in a thermomixer at room temperature and permanent agitation (300 rpm). Thereafter, the reaction was stopped by addition of 50 µL HCl (0.3 mol·L^{-1}), incubated for another 10 min, and neutralized by addition of 10 µL K_2CO_3-solution (2.5 mol·L^{-1}). Subsequently, the reaction mixture was assayed for reducing sugars with the tetrazolium blue method after Jue and Lipke (1985). Fifty µL of the tests, the blanks, and glucose standard (0.22 to 1.1 mmol·L^{-1}) were transferred into new reaction cups, mixed with 1 mL of the tetrazolium dye reagent (see below), and incubated for 3 min at 100°C in a water bath. Subsequently, the reaction cups were cooled in an ice-water bath to stop the dye-reaction. The absorbance of the tests, blanks, and the glucose standards were read at 660 nm. The tetrazolium dye reagent consisted of equal parts of a tetrazolium blue chloride solution (0.2% w/v) in 0.1 mol·L^{-1} NaOH and a potassium sodium tartrate solution (0.5 mol·L^{-1}). Amylase activity was assayed as described for laminarinase but with soluble starch as the substrate.

2.4.4 Stable isotopes

Muscle tissue from the claws of either species was dissected, shock frozen in liquid nitrogen, and lyophilized for 24 hours. Samples of 1 to 2 mg dried muscle tissue were weighed out in tin capsules and send for analysis to Agroisolab GmbH (TÜV Rheinland Group, Jülich, Germany).

2.5 Statistical analysis

Enzyme activity data sets were tested for normal distribution with the Shapiro-Wilk normality test. Statistical comparison was done by 2-way-ANOVA considering the factors ´species´ and ´season´, followed by the Tukey´s multiple comparisons test. The significance level was $\alpha = 0.05$. These statistical analyses and the graphs were done with the software GraphPad Prism version 7.05 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Cluster analysis and principal component analysis (PCA) of enzyme activities were performed with the software Primer 7 (ver. 7.0.20) from Primer-e on normalized data. The resemblance measure was the Euclidean distance, and the cluster mode was group average. We also investigated the overall variability in isotopic values for both species. Two metrics were applied: a) the total area (TA), calculated from the convex hull surrounding the outer data points in a $\delta^{13}C/\delta^{15}N$ biplot and b) the standard ellipses area (SEA). These calculations were done with the package Stable Isotope Bayesian Ellipses in R (SIBER Vers. 2.1.8) (Jackson et al., 2011).

3 Results

3.1 Stomach content

The stomach contents varied considerably in both species over the seasons (Table 1). A huge share of the stomach content was macerated and, thus, could not be identified. The stomach fullness of *C. maenas* was on average slightly lower than that of *H. sanguineus*. In *C. maenas*, it accounted for about 38% in June and 56% in August. Animal items accounted for 17.5 to 31.7%. The amounts of algae items were quite high in April and June and very low in August and October (Table 1). The lowest average stomach fullness in *H. sanguineus* (44%) appeared in June and the highest of 63% in April. The stomachs of *H. sanguineus* contained 25.8 to 60% algae items and 7.5 and 28.8% animal items (Table 1). Different to *C. maenas*, the share of algal items remained high in August and October.

3.2 Morphology of the gastric mill

The stomachs of both species showed the typical structures of the brachyuran gastric mill (Figures 1A, B). It consists basically of one median and two lateral robust calcified structures which complete the maceration of ingested material by complex movements facilitating squeezing, cutting, and grinding. A median tooth extends dorsally from the posterior end of the urocadiac ossicle into the lumen of the cardia. The elaborated lateral teeth protrude laterally from the zygocardiac ossicle along the stomach wall.

In *C. maenas*, the gastric mill appears quite massive. The urocardiac ossicle is consistently broad, forming a parallel-edged plate (Figure 2A). The posterior tip forms the medial tooth bearing a head-like structure (protrusion) which is slightly smaller in width than the urocardiac ossicle. The posterior protrusion possesses a small lip-like bulge at the anterior edge. A pair of cusps, pointing out ventrally, is located anterio-lateral to the protrusion. The lateral teeth of *C. maenas* possess four massive cusps, decreasing in size from anterior to posterior and merging into a smaller crest-like structure (Figure 2C). The cusps of both lateral teeth form a pair of claws and a basket-like space in which the median tooth may move. In an about rectangular median directed position from the huge cusps, the lateral teeth possess a series of seven about equally-sized blunt ridges (Figure 2E). Theses ridges may form within the putative basket a complement for the median tooth in shredding food.

The gastric mill of *H. sanguineus* appears more elaborated. The median tooth arises from a waist urocardiac ossicle at the anterior side which extends to a broad structure at the posterior side bearing the quite massive and complex median tooth (Figure 2B). The median tooth consists of well-defined protrusion with two posterio-lateral arches. Anterior to the main protrusion, the median tooth possesses a wide and sharply contoured bulge which occupies the width of the median tooth. The lateral teeth of *H. sanguineus* show four cusps (Figure 2D). These cusps are less massive than those of *C. maenas* and, except the smaller posterior cusp, of about equal size. The cusps appear sharper and more edged than those of *C. maenas*. Another difference show the series of 14 ridges at the median side of the lateral teeth. These are located closer to the row of cusps, apparently forming a functional unit. The ridges show sharp edges with signs of wear from grinding at their flattened surfaces (Figure 2F).

TABLE 1 Relative values (%) of stomach fullness and shares of stomach content of *Carcinus maenas* and *Hemigrapsus sanguineus* from the rocky intertidal of Helgoland (North Sea).

	Carcinus maenas				*Hemigrapsus sanguineus*			
Season	Fullness	Animal	Algae	n.i.	Fullness	Animal	Algae	n. i.
April	47.9	28.3	29.6	42.1	62.5	7.5	60.0	32.5
June	37.5	17.9	53.8	28.3	43.8	11.7	25.8	62.5
August	56.3	17.5	3.3	79.2	60.4	13.3	40.4	46.3
October	39.6	31.7	4.6	63.8	56.3	28.8	28.3	42.9

The stomach fullness refers to the content of food in the stomach (0 – 100%). The share of food items (i.e. animal, algae, and not identified = n.i.) sum up to 100% of the food present in the stomach.

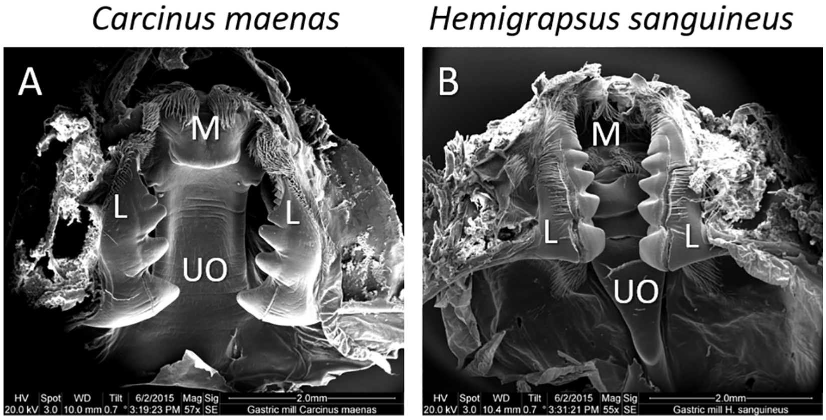

FIGURE 1

Scanning electron micrograph of the gastric mill of **(A)** *Carcinus maenas* and **(B)** *Hemigrapsus sanguines*. Overall impression (ventral view) of the gastric mill with the median (M) and lateral (L) teeth and the urocardiac ossicle (UO).

3.3 Digestive enzymes

The ApiZym enzyme screening revealed high activities of most enzymes in the midgut glands of both species (Table 2). Only the esterase (C4), lipase (C14), cysteine arylamidase, and α-galactosidase showed low activities. The substrate for chymotrypsin is not sensitive to hydrolysis by crustacean chymotrypsin as already previously noted (Saborowski, pers. obs.).

The trypsin activities of individual *C. maenas* ranged from 0.20 to 1.44 $U \cdot mg_{FM}^{-1}$. The lowest average activity of 0.37 ± 0.04 $U \cdot mg_{FM}^{-1}$ appeared in October and the highest of 0.77 ± 0.18 $U \cdot mg_{FM}^{-1}$ in August (Figure 3A). The span of trypsin activities in *H. sanguineus* was in the same range from 0.22 to 1.64 $U \cdot mg_{FM}^{-1}$. Minimum average trypsin activities appeared in April (0.40 ± 0.08 $U \cdot mg_{FM}^{-1}$) and the maximum in October (0.54 ± 0.23 $U \cdot mg_{FM}^{-1}$, Figure 3A). Two-way ANOVA showed no significant effect of season, no difference between species, and no interaction between season and species (Table 3).

The activities of the exopeptidase (leucine-AP) showed strong variation in *C. maenas* and ranged from 0.09 to 2.33 $U \cdot mg_{FM}^{-1}$. The seasonal minimum appeared in August (0.45 ± 0.14 $U \cdot mg_{FM}^{-1}$) and the maximum in June (0.79 ± 0.34 $U \cdot mg_{FM}^{-1}$, Figure 3B). The activities of leucine-AP in *H. sanguineus* were in the same range from 0.09 to 0.71 $U \cdot mg_{FM}^{-1}$. Minimum average activities appeared in August (0.26 ± 0.03 $U \cdot mg_{FM}^{-1}$) and the maximum in April (0.55 ± 0.07 $U \cdot mg_{FM}^{-1}$, Figure 3B). Similar to trypsin, the leucine-aminopeptidase showed no significant variation between season and between species (Table 3).

The ability of laminarin-degradation (laminarinase, endo-1,3-β-glucanase) was lower in *C. maenas* than in *H. sanguineus*. In many specimens, the activity was below the detection limit. So it ranged from 0 to 2.51 $U \cdot mg_{FM}^{-1}$. The lowest seasonal average appeared in August, showing no activity and the maximum appeared in June (0.92 ± 0.42 $U \cdot mg_{FM}^{-1}$). The laminarinase activity in *H. sanguineus* was significantly higher than in *C. maenas* and quite similar between seasons with lowest average values of 6.73 ± 1.11 $U \cdot mg_{FM}^{-1}$ in April and 6.96 ± 1.36 $U \cdot mg_{FM}^{-1}$ in June (Figure 3C). Two-way ANOVA showed no effect of season but a significant difference between species. There was no interaction between season and species (Table 3).

Amylase activities of *C. maenas* ranged from 0 to 2.95 $U \cdot mg_{FM}^{-1}$. The lowest average activities appeared in October (0 $U \cdot mg_{FM}^{-1}$) and the highest in June (0.91 ± 0.58 $U \cdot mg_{FM}^{-1}$). *H. sanguineus* showed amylase activities from 0 to 8.32 $U \cdot mg_{FM}^{-1}$. The average activities decreased continuously from April (2.24 ± 0.84 $U \cdot mg_{FM}^{-1}$) to October (0.30 ± 0.30 $U \cdot mg_{FM}^{-1}$, Figure 3D). The statistical analysis showed a significant effect of season, species, and interaction (Table 3).

The principal component analysis (PCA, Figure 4) revealed that 38.5% of the variation were covered by PC 1 which was primarily determined by laminariase and amylase. PC2 (27.3%) was determined by leucine aminopeptidase and PC3 (23.6%) by trypsin. The cluster analysis (dendrogram) of enzyme activities shows a clear separation between *C. maenas* and *H. sanguineus*. Only 3 of 24 specimens were grouped among the other species (Figure 5).

3.4 Stable isotopes

There were no statistically significant differences in the N- and C-isotope ratios between females and males within either species (unpaired t-tests, two-tailed p-values > 0.05, n = 5). Therefore, data of females and males of either species were combined to one data set (n = 10, Figure 6). The mean stable isotope value of $\delta^{13}C$ was significantly higher ($p < 0.0025$) in *C. maenas* (-14.6 ± 0.3) than in *H. sanguineus* (-15.4 ± 0.6). Similarly, the $\delta^{15}N$-value was significantly higher ($p < 0.0001$) in *C. maenas* (16.9 ± 0.3) than in *H. sanguineus* (15.6 ± 0.5). The average difference in the $\delta^{13}C$-values between species was 0.75 and that in the $\delta^{15}N$ values was 1.24. The convex hull total areas (TA) was 0.56 for *C. maenas* and 1.7 for *H. sanguineus*. The TAs of both species did not overlap

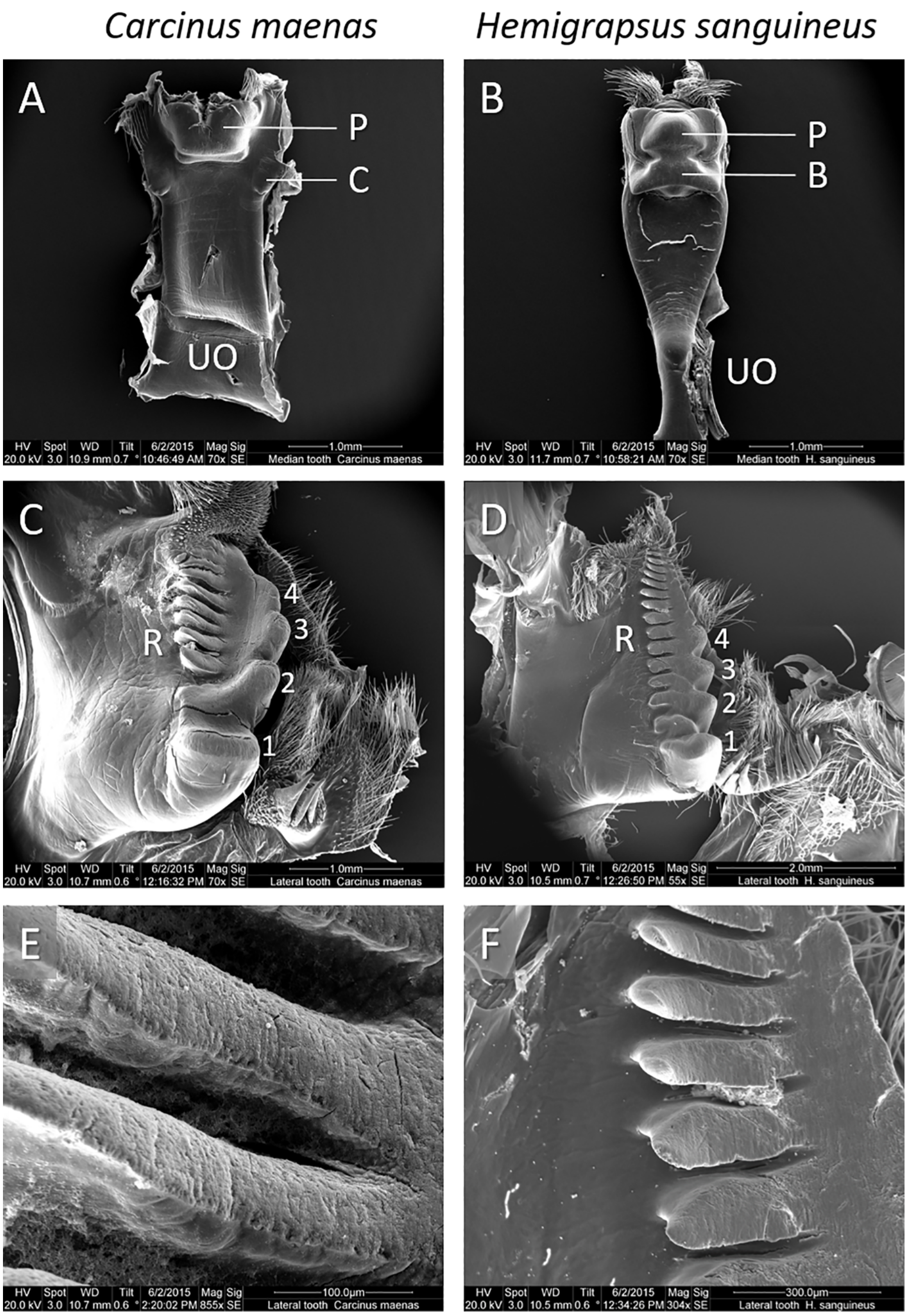

FIGURE 2
Details of the gastric mill of *C. maenas* (*C.m.*, left panel) and *H. sanguineus* (*H.s.*, right panel). The median teeth of **(A)** *C. m.* and **(B)** *H. s.* (UO: urocardiac ossicle, P: protrusion, C: cusps, B: bulge). The lateral teeth of either specie **(C, D)** showing massive cusps (1 – 4) and ridges (R). Magnification of **(E)** blunt ridges of *C.m.* and **(F)** sharp-edged ridges of *H.s.*

(Figure 6). The standard ellipses area (SEA) of *C. maenas* was 0.32 and that of *H. sanguineus* 0.80.

4 Discussion

Both species, the native *Carcinus maenas* and the invasive *Hemigrapsus sanguineus*, ingested animal as well as algal material, confirming omnivorous feeding. However, our studies revealed distinct intrinsic features, such as the morphology of the gastric mill and the activities of digestive enzymes indicating a predisposition for the prevalent food spectrum of either species.

4.1 Stomach content

The stomach contents of both species consisted of greenish algae and marine invertebrates, as deduced from fragments of bivalves, gastropods, polychaetes, and crustaceans. Carrion is probably ingested as well. Unfortunately, identification of food

TABLE 2 ApiZym semi-quantitative enzyme screening of midgut gland extracts of *Carcinus maenas* and *Hemigrapsus sanguineus* from the rocky intertidal of Helgoland (North Sea).

	Carcinus maenas	*Hemigrapsus sanguineus*
Enzyme	visual intensity	
Control	0	0
Esterases		
Esterase (C4)	0.8	0.4
Esterase/Lipase (C8)	3	3
Lipase (C14)	0.2	0.2
Peptidases		
Leucine arylamidase	4.4	5
Valine arylamidase	2.6	4.6
Cysteine Arylamidase	0.2	0.2
Trypsin	5	4
α-chymotrypsin*	0	0
Phosphatases		
Acid phosphatase	5	5
Alkaline Phosphatase	5	5
Naphtol-AS-Bi-phosphohydrolase	5	5
Glucosidases		
α-galactosidase	0.2	1.2
β-galactosidase	5	5
β-glucuronidase	5	5
α-glucosidase	5	4.8
β-glucosidase	3	4.8
N-acetyl-β-glucosaminidase	3.8	4.8
α-mannosidase	3	3
α-fucosidase	4.6	5

Enzyme activity was visually determined and classified from ´0´= no activity to ´5´= full activity. * not detectable with ApiZym substrate. Mean values of n = 5.

items was strongly hampered, because the stomach content was severely macerated and partially digested. Stomach fullness and appearance of the content varied strongly between individuals. Plant material was present in both species, but distinct differences were evident as well. Overall, *C. maenas* showed a higher share of animal food items than *H. sanguineus*. *H. sanguineus*, in turn, contained more algal items than *C. maenas*. Compared to the plant material, the amount of animal tissue may be underestimated because only the solid parts of animals remained recognizable in the stomach whereas the soft tissue is mostly macerated and not identifiable. Accordingly, it appears reasonable that a high share of unidentified material derived from animal soft tissue.

Our observations agree with previous studies. *C. maenas* from the Portuguese Mondego estuary showed opportunistic feeding behavior with local differences. Overall, the food of *C. maenas* was dominated by brown shrimp, *Crangon crangon*, the polychaete *Hediste diversicolor*, and fish or fish remains. Algae were present as well (Baeta et al., 2006). Green shore crab from Nova Scotia preferred bivalves over algae, gastropods, and crustaceans and showed seasonal differences (Elner, 1981). Field studies on *H. sanguineus* confirmed omnivorous feeding and reported a variety of algae and vascular plant remains in the stomachs (Ledesma and O´Connor, 2001). Griffen et al. (2012) reported that *H. sanguineus* from New England were omnivorous, consuming macroalgae and a variety of animal prey. The amount of 56.3% algal diet in April closely matches our results. Laboratory investigations, in turn, showed that *H. sanguineus* are opportunistic omnivores. The crabs showed well-developed predatory tendencies and a preference for animal food items over algae (Brousseau and Baglivo, 2005; Bleile and Thieltges, 2021) or no clear preference for mollusks or algae (Bourdeau and O´Connor, 2003). The laboratory studies may have biased the feeding behavior because carnivore diet was readily available. Brousseau and Baglivo (2005) suggested that starvation and competition for food can alter the food selection of *H. sanguineus*. Crabs that starved for 5 days consumed both food types (algal and animal) more often than those that starved for one day only, which preferred animal food. Moreover, increased crab density lead to increased diet spectrum, suggesting that *H. sanguineus* is prepared to switch to algal diet in case of food scarcity or competition for food.

Both species from Helgoland showed seasonal variation of their diet, which differed more in the amount of algae than in the amount of animal diet. *C. maenas* ingested a high amount of algae in spring but ceased feeding on algae in summer and autumn. This seems to reflect, on one hand, the high productivity and availability of algae in spring but, on the other hand, also the preference for animal food when the biomass increased during the seasonal course of succession (e.g. Munda and Markham, 1982; Janke, 1990 and references cited therein). Moreover, the palatability of young and fresh algae in spring may be better than that of older algae in summer and autumn. In *H. sanguineus*, we observed a very similar seasonal course of herbivory as Griffen et al. (2012) in crabs from New England with a decrease from April to June, an increase in August and again a decrease in October. The authors ascribed this to the seasonal occurrence and recruitment pulses of prey organisms, such as barnacles or bivalves, which are preferred by *H. sanguineus* upon appearance. Such behavior in food selection appears reasonable in the Helgoland intertidal as well.

4.2 Gastric mill

The gastric mill of decapod crustaceans facilitates the internal maceration of ingested food items and mix the food mash with gastric fluid from the midgut gland, initiating the first steps of extracellular digestion (Icely and Nott, 1992; Saborowski, 2015). The gastric mill is a complex calcified structure, basically consisting of two lateral and one median tooth movable against each other. The gastric mill of brachyuran decapods shows features which are characteristic for their preferred diet. A detailed description of the morphology of brachyuran decapods, the properties of the gastric

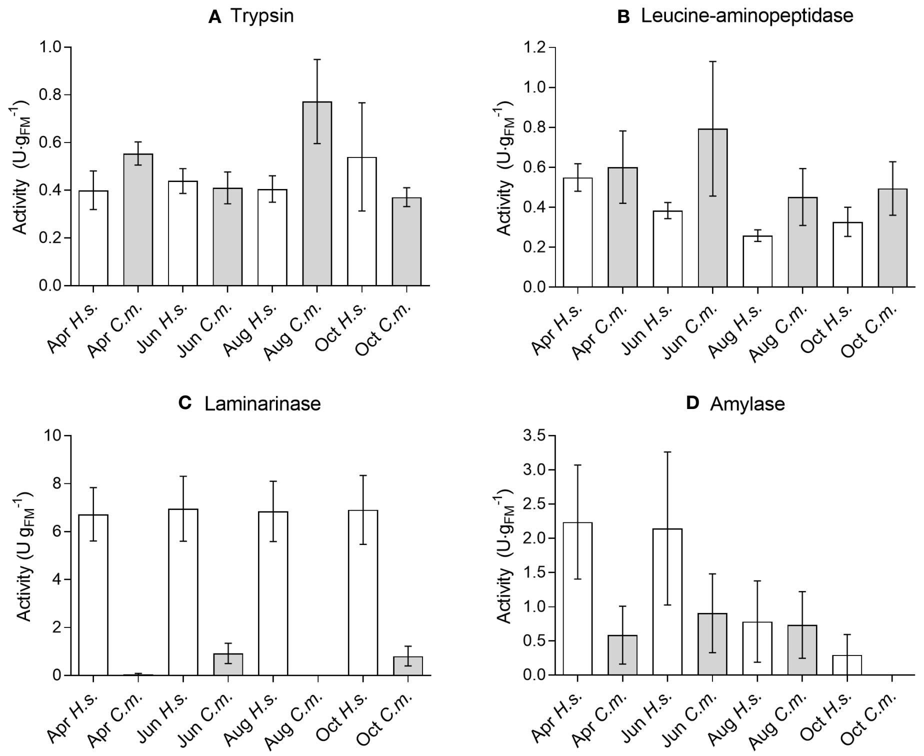

FIGURE 3
Seasonal variation of digestive enzyme activities **(A)** Trypsin, **(B)** Leucine-aminopeptidase, **(C)** Laminarinase, **(D)** Amylase in the midgut gland of *Carcinus maenas* (*C.m.*) and *Hemigrapsus sanguineus* (*H.s.*) samples in April (Apr), June (Jun), August (Aug), and October (Oct). Means ± SEM, n = 6-15.

TABLE 3 Two-way ANOVA – statistical parameters.

		SS(Type III)	DF	MS	F(DFn, DFd)	p-value
Trypsin	Interaction	0.4878	3	0.1626	F(3,41) = 2.129	0.1113
	Species	0.0796	1	0.0796	F(1,41) = 1.043	0.3132
	Season	0.1879	3	0.0626	F(3,41) = 0.82	0.4190
	Residual	3.131	41	0.0764		
Leucine-AP	Interaction	0.21	3	0.07	F(3,41) = 0.4829	0.6960
	Species	0.5157	1	0.5157	F(1,41) = 3.558	0.0663
	Season	0.5059	3	0.1686	F(3,41) = 1.164	0.3353
	Residual	5.941	41	0.1449		
Laminarinase	Interaction	1.682	3	0.5607	F(3,53) = 0.0652	0.9781
	Species	570.8	1	570.8	F(1,53) = 66.32	< **0.0001**
	Season	3.71	3	1.237	F(3,53) = 0.1437	0.9333
	Residual	456.1	53	8.606		
Amylase	Interaction	5.252	3	7.751	F(3,41) = 0.6574	0.5830
	Species	8.013	1	8.013	F(1,41) = 3.009	0.0903
	Season	14.96	3	4.985	F(3,41) = 1.872	0.1494
	Residual	109.2	41	2.663		

Sum of squares (SS), degrees of freedom (DF), mean squares (MS), F-ratio (F).
Bold p-values highlight significant relationships.

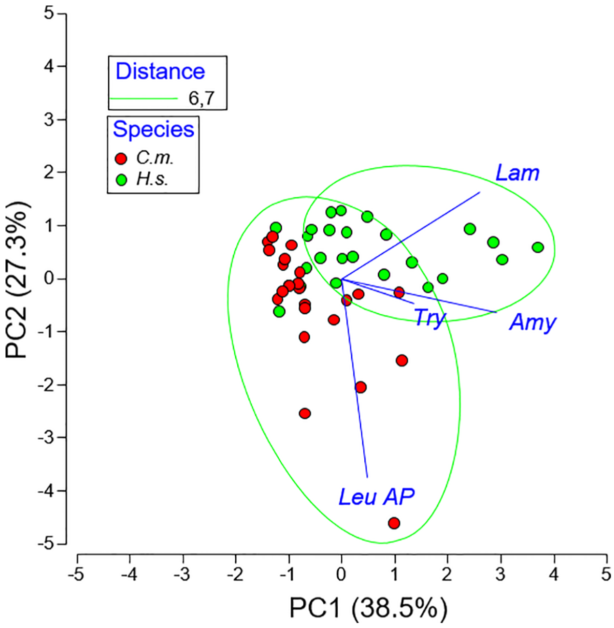

FIGURE 4
Principal Component Analysis (PCA) of four digestive enzyme activities of *Carcinus maenas* (*C.m.*) and *Hemigrapsus sanguineus* (*H.s.*).

mills of carnivorous, omnivorous, and herbivorous species, as well as their putative function is given elsewhere (e.g. Heinzel, 1988; Heinzel et al., 1993; Linton et al., 2009; Allardyce and Linton, 2010; Brösing, 2010; Allardyce and Linton, 2011).

Unlike in mammals, molar-like processes and rounded shapes of the gastric mill in crabs are indicative for the grinding of soft animal tissue as well as hard structures such as chitin or calcareous mollusk shells (Skilleter and Anderson, 1986). Characteristics of the gastric mills of herbivorous crabs are raised transverse ridges which facilitate cutting of fibrous food (Giddins et al., 1986). Both characteristics appear in omnivorous species, though with different manifestation (Salindeho and Johnston, 2003).

The gastric mills of *C. maenas* and *H. sanguineus* revealed differences. The gastric ossicles of *C. maenas* appear blunt and smooth. Such blunt and smooth surfaces are suitable to grind soft food items, which are typical for animal food. The huge cusp of the median tooth and the few ridges of the lateral teeth function like a mortar and pestle. Such structure coincide with carnivorous feeding

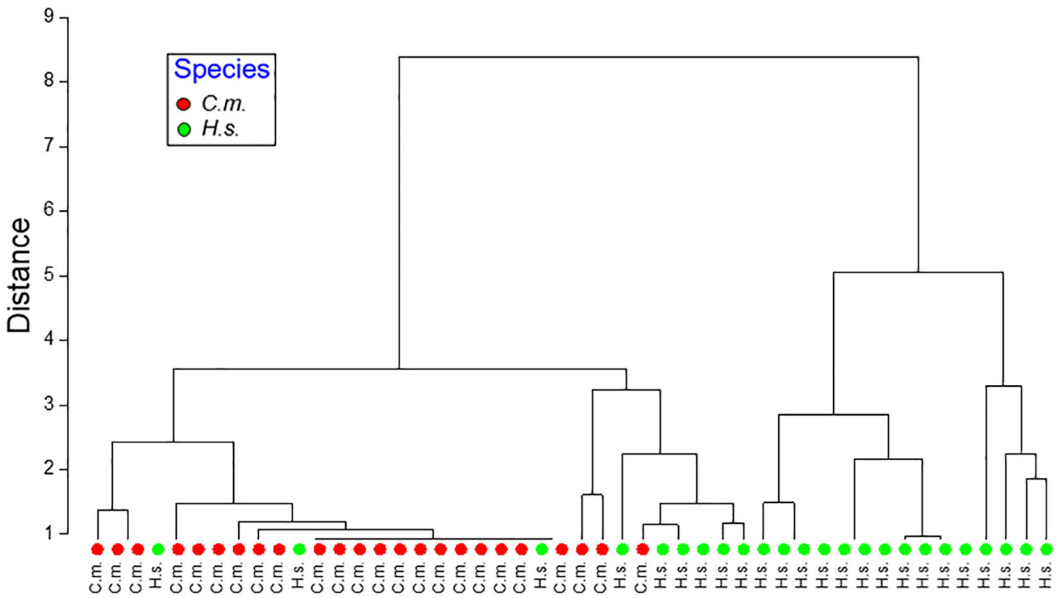

FIGURE 5
Hierarchical cluster analysis (Euclidian Distance) of digestive enzyme activities of *Carcinus maenas* (*C.m.*) and *Hemigrapsus sanguineus* (*H.s.*).

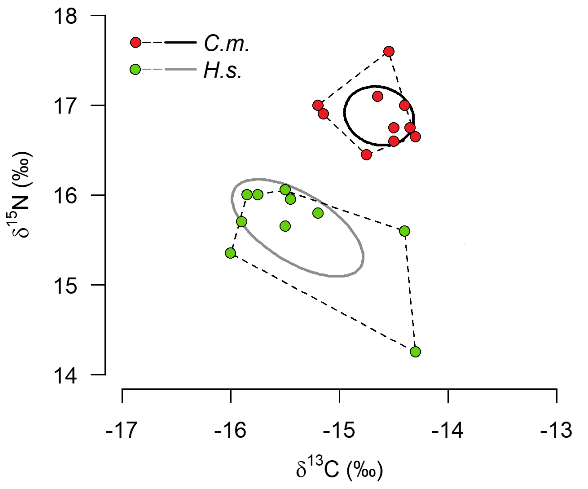

FIGURE 6
Isotope niche widths of *Carcinus maenas* (*C.m*) and *Hemigrapsus sanguineus* (*H.s.*). Display of Standard ellipses areas (SEA, solid lines) and total area from convex hull (TA, dotted lines) of 10 individulas of either species using Stable Isotope Bayesian Ellipses in R (SIBER).

and were correspondingly described in various carnivorous crustacean (Heeren and Mitchell, 1997; Salindeho and Johnston, 2003; Allardyce and Linton, 2010).

The gastric mill of *H. sanguineus* has a different appearance. It is characterized by well-defined sharp edges which form many cutting surfaces. As a result of the masticatory movement, the edges of the median tooth slide along those of the lateral teeth, capable of cutting fibrous material. Therefore, gastric mills with such structures are suitable to chop plant material and are generally ascribed to herbivorous or omnivorous crustacea (e.g. Giddins et al., 1986; Allardyce and Linton, 2010).

These results indicate, that the gastric mills of both species are capable of processing carnivore as well as herbivore food, however with apparent preferences for carnivore diet in *C. maenas* and herbivore diet in *H. sanguineus*. Accordingly, these results support the previous observations of higher animal content in the stomach of *C. maenas* and more algae in *H. sanguineus*.

4.3 Biochemistry

The midgut gland (*syn*. hepatopancreas) is the principal organ of biochemical food utilization (comprehensively reviewed by Vogt, 2019). The relation between feeding mode and digestive enzyme activities in decapod crustaceans is ambiguous. Generally, it is accepted that the major enzymes represent the ingested food, meaning that carnivorous species possess high proteolytic activities and herbivorous species high carbohydrase activities. Omnivorous species show intermediate activities (e.g. Sather, 1969; Lee et al., 1984; Lovett and Felder, 1990; Johnston and Yellowlees, 1998; Figueiredo and Anderson, 2009). The preliminary screening of digestive enzymes, comprising esterases, peptidases, and glucosidases showed quite high activities in both species. For our inter-specific comparison, we chose two protein degrading enzymes and two carbohydrate degrading enzymes, representing the principal constituents of food organisms.

Animal food contains higher amounts of protein than algal food and, thus, is a valuable source for nitrogen and essential amino acids. For example, the crude protein content of the green algae *Ulva lactuca*, a potential food for both crab species, accounts for 23 to 26% of the dry mass (Bikker et al., 2016). Compared with this, the protein content of blue mussel (*Mytilus edulis*) flesh and that of *Macoma balthica* can exceed 60 to 70% of the dry mass (Dare and Edwards, 1975; Beukema and de Bruin, 1977).

The metabolic utilization of proteins is facilitated by proteases, *syn*. peptidases. Trypsin is a common endopeptidase in brachyuran crabs which cleaves proteins and peptides within the amino acid chain at the carboxyl side of arginine and lysine, thus generating peptides for further degradation (Muhlia-Almazán et al., 2008). Leucine-aminopeptidase is an exopeptidase, preferably liberating leucine at the *N*-terminus of peptides but often shows a wider substrate specificity (Matsui et al., 2006). Both enzymes were selected as representative for the concerted potential to utilize protein as the principal sources of dietary nitrogen and essential amino acids.

C. maenas as well as *H. sanguineus* show almost similar proteolytic activities which may indicate an equal ability to digest protein and, thus, supports their omnivorous feeding mode. Apparently, there is no need for *H. sanguineus* to increase proteolytic activity to compensate for nitrogen deficiency as a consequence of extended herbivorous feeding. Our findings are in agreement with Johnston and Freeman (2005) who found intermediate proteolytic activities in omnivorous species, indicative of their wide food spectrum. Although not statistically significant, the trypsin activity of *C. maenas* increased markedly in August, which coincides with the minimum of ingested algal material. It may be suggested that *C. maenas* increase their digestive efficiency to accumulate energy reserves.

Laminarinases (β-1,3-glucanase) are common digestive enzyme in many invertebrates where they hydrolyze the algal storage product laminarin (Piavaux, 1977). Likewise, they are present in marine, terrestrial, and fresh-water decapods (Johnston and Freeman, 2005; Linton et al., 2015). α-amylases facilitate the hydrolysis of α-1,4-glycosidic bound carbohydrates such as starch and glycolgen. They are common in herbivorous and omnivorous crustaceans but also show high activities in some carnivorous species (Rodríguez-Viera et al., 2016). In the marine environment, starch and starch-type polysaccharides are present as storage product of cellular and filamentous green algae including the order Ulvales (Love et al., 1963; Busi et al., 2013; Dominguez and Loret, 2019; Prabhu et al., 2019). Green algae like *Ulva* spp. or *Enteromorpha* spp. are common in the rocky intertidal of Helgoland and the greenish food items in the stomach of both species strongly suggest that these algae were ingested by both *C. maenas* and *H. sanguineus*.

The consistently higher laminarinase and amylase activities in *H. sanguineus* indicate that the respective carbohydrates present in algae will be better utilized by *H. sanguineus* than by *C. maenas*. Consequently, *H. sanguineus* can fall back on a rich algal food source and evade competition with other species for carnivore diet.

Other biochemical markers support the macroscopic and microscopic observations of the stomach contents. Jungblut et al. (2018b) showed in both species from Helgoland that trophic fatty acid indices for diatoms (Bacillariophyceae), green algae (Chlorophyta), and especially brown algae (Phaeophyceae) were higher in *H. sanguineus* than in *C. maenas*, suggesting a higher share of herbivorous feeding by the invader than by the native crab. Our stable isotope data do not allow for the assignment of the trophic level due to the lack of baseline data (Post, 2002). They are in the same range as reported for other crustacean species and different tissues (e.g. Rudnick and Resh, 2005; Bodin et al., 2007) but higher than stable isotope data previously reported for *C. maenas* and *H. sanguineus* (e.g. Watts et al., 2011; Wahyudi et al., 2013; Guo et al., 2016; Bordeyne et al., 2017). Nevertheless, the direct comparison clearly show higher δ^{15}N- and δ^{13}C-values in *C. maenas* than in *H. sanguineus*, indicating a higher degree of carnivory in *C. maenas*. Moreover, *C. maenas* occupied a smaller isotopic niche than *H. sanguineus*. The latter species appears to consume more carbon-depleted food (i.e., with more negative values). This may suggest that *H. sanguineus* consumes different algal species than *C. maenas*, which, in turn, may reduce competition for algal food in the rocky intertidal of Helgoland.

5 Conclusion

Our results draw a coherent picture about the trophic preference of both species and support previous studies about their feeding ecology. *C. maenas* shows distinct morphological and physiological adaptation for animal food and also seem to prefer it in the rocky intertidal of Helgoland. *H. sanguineus*, in contrast, shows clear morphological and biochemical adaptations for utilizing algal diet. If animal diets becomes scarce or competition with *C. maenas* or other species for animal food increases, *H. sanguineus* can efficiently utilize algal diet as well. Although both species occupy the same habitat, they are flexible to exploit different trophic niches and, thus, may widely coexist in their Helgoland habitat.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on animals in accordance with the local legislation and institutional requirements.

Author contributions

RS: conceptualization, data curation, writing – original draft preparation, reviewing and editing, supervision. PB: investigation, methodology, data curation, writing, reviewing and editing. MK: methodology, data curation, writing –reviewing and editing. SJ: conceptualization, data curation, writing – original draft preparation, reviewing and editing, supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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