

## Research Article

## Food spectrum of the Chinese mitten crab (*Eriocheir sinensis*): insights from the Lower River Rhine comparing stable isotope mixing models and genetic gut content analyses

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### Abstract

Originating from South East Asia the catadromous Chinese mitten crab (*Eriocheir sinensis* H. Milne Edwards, 1853) is listed as one of the 100 world's worst invasive species. In 1912 *E. sinensis* was first recorded in Central Europe. Nowadays the species is common in various European rivers and widespread in large rivers of the northern hemisphere. High densities of the crab can cause serious economic damage in water management and to the fishing industry. During their natural migration from the estuary upstream, subadult individuals are able to reach river sections several hundred kilometers away from the delta. The ecological impacts on community and food web structures of the Chinese mitten crab (CMC) are less often considered in scientific studies than their economic effects. The diet of CMC under natural conditions is rarely studied, but macrophytes are known to be an important food source of the crab. However, in the main stretch of the Lower Rhine macrophytes are broadly missing. Initial stable isotope analyses showed that CMC occupies a trophic level comparable to carnivorous fish in this system. Thus, a strong predatory impact of CMC on the fauna is likely. Here we use qualitative and quantitative approaches to investigate the diet of Chinese mitten crab to estimate their impact on the food web. Bulk stable isotope analyses of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of *E. sinensis* and potential food resources were conducted and genetic gut content analyses (GGCA) via group-specific primers for common macroinvertebrates of the River Rhine were used to determine prey organisms. While GGCA only rarely detected invertebrate prey and many plant fibres were visible in the stomachs and guts of the CMCs, stable isotope mixing model (simmr) analyses revealed a high contribution of some easily accessible macroinvertebrate species to the CMCs diet. This contradiction between the gut content findings and the simmr mixing model results indicate not only that animal material is more easily assimilated, but also that the CMC may have a strong impact on the benthic fauna of the Lower Rhine, which would have been underestimated if solely gut content analyses were used.

**Key words:** invasive species, diet, food web, simmr, trophic interactions, omnivory

### Introduction

The Chinese mitten crab (CMC), *Eriocheir sinensis* (H. Milne Edwards, 1853), the largest invertebrate species in the Rhine system, is listed as one of the

100 worst invasive alien species (Lowe et al. 2000). The natural distribution of the species ranges from the west coast of North Korea (approximately 40°N) to Hong Kong (approximately 22°N) (Panning 1939; Hymanson et al. 1999). The first record of *E. sinensis* for Europe was in 1912 from a tributary of the River Weser in northern Germany (Peters 1933), followed by a subsequent spread throughout northern Europe, as well as in large rivers of the northern hemisphere (Lowe et al. 2000). In all probability the CMC was introduced accidentally either because larval or juvenile stages were transported in the ballast water of ships or through the release of imported adult individuals for sale as food in Asian markets (Cohen and Carlton 1997; Hänfling et al. 2002; Herborg et al. 2007b; Low et al. 2013).

The CMC is a catadromous and euryhaline species spending most of its life cycle in freshwater but breeding as adults in saltwater (Cheng et al. 2018). The pelagic zoea larvae remain in marine conditions at high salinities, before the final larval stage, the megalopa, attains an increasingly benthic behaviour in the estuary (Anger 1991). After spending a few months within the estuaries, benthic juvenile crabs migrate to brackish or freshwaters where they mature and sometimes living as adults for several years before returning seawards to breed (Panning 1939; Rudnick et al. 2005). Daily upstream migration distances reported for juvenile crabs range from 1 to 1.5 km and for older crabs from 2 to 3 km, while downstream migration rates of up to 12 km daily have been reported for adult CMC in late autumn (Panning 1939).

The invasion of CMC is known to cause severe economic problems for the fishing industry not only because the crabs cause damages to fishing nets and weirs, but also prey upon fish captured in those nets (Peters and Hoppe 1938). Apart from that, CMC can cause severe ecological changes and economic damage due to their burrowing activities that cause erosion of riverbanks (Dutton and Conroy 1998; Dittel and Epifanio 2009; Harvey et al. 2019). Their potential ecological impact on community compositions due to their trophic ecology on the other hand is rarely studied (but see Clark et al. 1998; Rudnick and Resh 2005; Gollasch 2011; Zhang et al. 2019). Overall, the CMC is considered to be omnivorous. According to Hymanson et al. (1999) newly settled crabs feed predominantly on plant material (herbivorous) and switch to feeding on macroinvertebrates in omnivorous feeding habitats within a few months. Rudnick and Resh (2005) found that individuals in tidal influenced zones of the San Francisco Bay strongly relied on invertebrate food, while individuals at their freshwater sampling site showed equal dependence on plant and invertebrate food sources. Studies investigating foraging abilities of CMC from the River Thames showed that they are using several techniques to prey upon various animal prey (Mills et al. 2016), reduce densities of various macroinvertebrates within mesocosm experiments (Rosewarne et al. 2016), and are capable to affect the composition of the fish community by

predation on eggs of various fish species (Webster et al. 2015). However, analyses of the stable carbon and nitrogen isotope composition of five CMC individuals from the River Thames indicated a strong reliance on macrophytes (Rosewarne et al. 2016).

The River Rhine is one of the European inland waterways with the highest number of alien species (Leuven et al. 2009), with first reports of CMC originating from 1931 (Kamps 1937; Tittizer et al. 2000; Herborg et al. 2007a). However, to our knowledge there are no published data on the abundance of CMC in the Rhine system so far. In an unpublished bachelor's thesis investigating the stable isotope composition of CMC in the Lower Rhine, near Cologne, the isotopic composition of CMC was comparable to known secondary consumers (Martin 2010), giving first hints that the crabs might act as secondary consumers within this system. However, more information on their trophic positioning and main food sources in the Lower Rhine is required. Hypothesising that the CMC mainly consume the dominant macroinvertebrate prey species, we investigated the feeding ecology of CMC in the Lower Rhine via a combination of stable isotope (SI) and genetic gut content analyses (GGCA). We analysed the stable carbon ( $^{13}\text{C}$ ) and nitrogen ( $^{15}\text{N}$ ) isotope composition of CMCs, crayfish, gobies and various macroinvertebrate taxa and genetically analysed the gut contents of *E. sinensis* using group-specific primers for different groups of macroinvertebrates to detect direct predation.

## Materials and methods

### *Study sites and sampling design*

The study was conducted in May 2013 within a stretch of the German section of the Lower Rhine, near the ecological research station of the Zoological Institute at the University of Cologne in Rees-Grietherbusch. We used fish traps connected to a fence leader ( $2 \times 7$  m), located near Rhine km 845 between  $51^{\circ}47'16.3''\text{N}$ ;  $6^{\circ}19'30.9''\text{E}$  and  $51^{\circ}47'18.6''\text{N}$ ;  $6^{\circ}19'32.6''\text{E}$ , to sample CMC as well as gobies and crayfish. Fish traps were emptied twice a day, once in the morning and once in the evening. Therefore, crabs were in the nets no longer than 8 hours during the day and 15–16 hours during the night, respectively. Collected individuals were taken to the laboratory and frozen at  $-20^{\circ}\text{C}$ . In the laboratory, the size of each individual was determined using an electric slide gauge by nearest 0.01 mm. The width of carapace was measured as a size indicator for the CMC, while for round gobies (*Neogobius melanostomus* Pallas, 1814) the length from the tip of the snout to the tip of the caudal fin (total length) and for crayfish the length from the tip of the rostrum to the end of the telson was measured. Additionally, the wet weight (accuracy 0.1 g) and sex was determined for individuals of *E. sinensis* and crayfish.

Samples of the benthic macroinvertebrate community were collected at the right bank of the river directly upstream (river kilometre 843.8, site 1) and downstream (river kilometre 846, site 2) of the fish traps, from a ship using a clamshell grab (each  $n = 5$ ), to determine dominant macroinvertebrate prey taxa. Out of each clamshell grab, two rocks were taken, of which each was considered as one semi-quantitative benthic sample ( $n = 10$ ). In the laboratory, macroinvertebrates were brushed from rocks, fixed in 96% ethanol, identified to the lowest taxonomic level possible and counted. Afterwards, to estimate surface area of the rocks, each rock was wrapped in aluminium foil: by weighing reference aluminium foil pieces, the function of foil weight to corresponding surface value was calculated (Rothmeier et al. 2022). Additionally, live specimens of potential macroinvertebrate prey were collected from stones out of an additional clamshell grab for stable isotope analyses (see below). Furthermore, periphyton samples ( $n = 5$  stones) were collected from additional rocks at each sampling site by brushing of a section of each stone into tubes using a nailbrush and used as potential basal resources, for stable isotope analyses (SIA). No macrophytes were available from the clamshell grabs, therefore, macrophytes were collected from near-shore locations manually. Collected macrophytes occurred only as suspended load because we observed no macrophytes growing near-shore in the main stretch of the Lower Rhine. Thereby, only three morphological distinct (not further determinable) macrophytes were collected in an amount that was considered adequate for stable isotope analysis. At each of the sampling sites temperature, pH, conductivity and dissolved oxygen concentration were determined (WTW MultiLine F/Set-3; see Supplementary material Table S1). Water-level data were obtained for nearest automatic gauge point (Rees; Elektronischer Wasserstraßen-Informationsservice ELWIS, Germany; see Table S1).

### *Gut content analyses*

A technique using group-specific primers for genetic gut content analyses amplifying only DNA regions of particular target groups while excluding non-target species (Jarman et al. 2004) was applied to test for recently ingested prey. For consumers, like CMC, that intensively comminute their food these analyses are more promising than visual gut content analyses because the latter tend to miss soft-bodied prey (e.g. Redd et al. 2008).

The gastrointestinal tract of each CMC ( $n = 25$ ) was dissected, separated into stomach and gut, and preserved in  $\geq 99.8\%$  ethanol (not denatured) for the genetic analyses. Prior to the extraction of DNA, stomach tube or intestinal wall of each sample was opened with a rip cut using a scalpel, and the content was transferred into a 2 ml reaction tube. Afterwards, DNA of each sample was extracted via a modified salt extraction protocol (Koester and Gergs 2014; section 1. within Koester and Gergs 2017). Five gut and

eleven stomach samples contained too much organic material to perform DNA extraction from the complete sample, which is why a subsample of each of those samples was used for DNA extraction and the following prey detection. Therefore, to each of those stomach and gut contents ethanol ( $\geq 99.8\%$ , not denatured) was added up to a total volume of 1 ml. Afterwards, whole samples were homogenised using a TissueLyser II bead mill (Qiagen) for approx. 2 min at 15 Hz. Then, depending on viscosity, 200–500  $\mu\text{l}$  of the respective ethanol homogenate were transferred into a fresh 2 ml reaction tube and dried at 60 °C until all the ethanol was evaporated before DNA extraction of those subsamples via the modified high salt extraction protocol.

Functional efficiency of each DNA extract was tested using universal primers targeting the nuclear small subunit (18S) rDNA (NSF1419/20, NSR1642/16; Jarman et al. 2006) and the nuclear large subunit (28S) rDNA (LSU D1,D2 fw1 and LSU D1,D2 rev2 or LSU D1,D2 rev 4; Sonnenberg et al. 2007). These universal primer sets target the same rDNA regions as the group-specific primer sets. Therefore, the presence and functional efficiency of the used templates for the subsequent detection of prey was proven directly for the tested samples (e.g. section 2. within Koester and Gergs 2017). Each two stomach and two gut content samples failed to show positive results in terms of presence and functional efficiency of templates and were thus excluded from further analysis. Hence, 46 DNA extracts (stomach and gut content samples each  $n = 23$ ) were analysed using 17 group-specific primer sets for macroinvertebrate prey species established by Koester et al. (2013) and Koester et al. (2016); thereof 16 primer sets were already utilised for prey detection in our previous studies (Koester et al. 2016; Koester and Gergs 2017) and additionally the Gamae28S primer set specific for the Gammaridae family. Fish and crayfish are not covered by the genetic analyses, because adequate primers are not available and their development is time-intensive and costly. Single PCRs for each of the primer sets were conducted separately according to Koester et al. (2013): DNA extract (1  $\mu\text{l}$ ) was mixed with 9  $\mu\text{l}$  of a reaction mixture containing 0.5  $\mu\text{M}$  of each primer (labelled forward, Metabion International AG, Planegg/Steinkirchen, Germany and unmodified reverse primer, Eurofins MWG Operon, Ebersberg, Germany; modification see Table S1 Koester et al. 2016; Table 2 Koester and Gergs 2017; Gamae28Sf modification IRD700), 0.25 mM dNTPs, 1 $\times$  reaction buffer Y (20 mM Tris-HCl, pH 8.55, 16 mM  $(\text{NH}_4)_2\text{SO}_4$  and 2 mM  $\text{MgCl}_2$  final concentrations; Peqlab Biotechnologie GmbH, Erlangen, Germany) and 0.05 U Taq DNA polymerase (Peqlab Biotechnologie GmbH). We used the following standard PCR protocol: 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature for 30 s, 72 °C for 90 s and final extension at 72 °C for 10 min. Variations in the PCR protocol see Koester et al. (2013) or Koester and Gergs (2017). To prove the success of the PCR, we used one positive control per PCR with pure DNA from an appropriate target species and three negative

controls with different concentrations of pure DNA from CMC. PCR runs for which success of the PCR reaction could not be verified were discarded and repeated. Automated fragment analysis on an automated sequencer (CEQ8000 Genetic Analysis System, Beckmann Coulter, Fullerton, CA USA) was conducted in two batches (see Table S2) to separate PCR products using an internal size standard (GenomeLab™ DNA Size Standard Kit–400 and –600, respectively, Beckman Coulter) to determine fragment length (for a detailed description of the process see Koester and Gergs 2017). Identification of amplified fragments was performed using the software GeneMarker® (Version 1.95, SoftGenetics LLC®, State College, PA 16803).

### *Stable isotope analyses*

Bulk stable isotope analyses (SIA) were conducted for stable nitrogen and carbon isotopes. Stable isotope composition of each *E. sinensis* individual ( $n = 25$ ) and of crayfish individuals ( $n = 6$ ) were analysed using muscle tissue from the claws. From gobies ( $n = 6$ ) a piece of dorsal muscle (approximately 1 cm<sup>3</sup>) was used for SIA. Live specimens of potential macroinvertebrate prey species were kept individually in filtered water (filter mesh size 30 µm) over gauze for 24 h to facilitate gut clearance. Afterwards, only soft tissue from mussels and snails was used for the analyses of the stable isotopes, while whole organisms were analysed for all other macroinvertebrates. Single individuals were analysed wherever size allowed it. In case of Chironominae (3 individuals), *Jaera* sp. (3–5 individuals) and Tubificidae (2–3 individuals) individuals were pooled to provide sufficient material for SIA. Periphyton samples (sampling see above) for isotopic analyses from primary producers were further split into two subsamples for stable carbon and nitrogen measurements, respectively. The subsamples for stable carbon analyses were acidified (1 M HCL approx. 2 h) to remove carbonate and rinsed with VE-water, while the subsamples for the stable nitrogen analyses were used without acidification.

All samples were dried for 24 h at 60 °C, powdered, and weighed into tin capsules (0.2–1.0 mg for animal material and 1.6–6.0 mg for periphyton, and 2.0–3.5 mg for macrophytes; IVA Analysentechnik e. K., Meerbusch, Germany). Twenty-five replicates of *E. sinensis* as well as six replicates from other groups of macroinvertebrates, and gobies, and crayfish and the primary producers (i.e. periphyton and each of the three macrophytes) were analysed (exceptions are given at the appropriate place in the results section). Isotope ratios were measured using a Delta Advantage Isotope Ratio Mass Spectrometer connected to a Flash HT elemental analyser (Thermo Finnigan) and are presented as  $\delta$ -values in units of per mille (‰) relative to the international reference standards Vienna PeeDee belemnite for carbon and atmospheric N<sub>2</sub> for nitrogen. Calibration of  $\delta$ -values was done using the standards IAEA-CH3 for carbon and IAEA-NO3 for nitrogen. Repeated analyses of internal lab standards (casein) resulted in a typical measurement precision of  $\pm 0.12$  ‰ for carbon and  $\pm 0.05$  ‰ for nitrogen.

### *Statistical analyses*

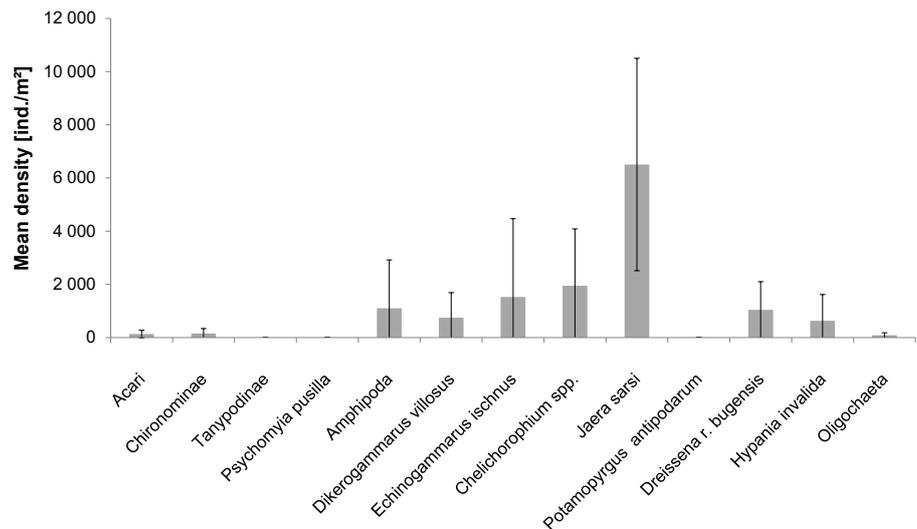
Statistical analyses were performed using the statistical software R (R Core Team 2017). We used PERMANOVA, which analyses multivariate data on the basis of any distance measure using permutations (Anderson 2001; McArdle and Anderson 2001), to test for differences of the benthic community between the two sampling sites. Therefore, Bray-Curtis similarities, which compare ranked similarities for differences between defined groups, using square root transformed abundances were analysed. Dominant macroinvertebrates were then determined using dominance ranking following Engelmann (1978).

To compare CMC, gobies and crayfish we tested for significant differences in their stable isotope composition ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) using a Wilcoxon-Mann-Whitney rank sum test. The contribution of different animal and plant food sources to the diet of CMC was determined using the simmr mixing model (version 0.4.1 Parnell 2019) implemented in R software, which is based on a Bayesian framework that estimates probability distributions of resource contributions to a consumer diet and also takes into account variability of the included resources (Parnell et al. 2010, 2013). We used trophic discrimination factors (TDF) and uncertainties published by McCutchan et al. (2003) for invertebrate diets analysed whole (i.e.  $0.3 \pm 0.14\text{‰}$  for  $\delta^{13}\text{C}$  and  $2.1 \pm 0.21\text{‰}$  for  $\delta^{15}\text{N}$ ), and in case of mussels, gobies and crayfish for muscle analysed (i.e.  $1.3 \pm 0.30\text{‰}$  for  $\delta^{13}\text{C}$  and  $2.9 \pm 0.32\text{‰}$  for  $\delta^{15}\text{N}$ ), as well as for vascular plants (i.e.  $0.4 \pm 0.28\text{‰}$  for  $\delta^{13}\text{C}$  and  $2.4 \pm 0.42\text{‰}$  for  $\delta^{15}\text{N}$ ) and for periphyton according to acidification treatment (i.e. acidified  $-0.2 \pm 0.21\text{‰}$  for  $\delta^{13}\text{C}$  and no treatment  $2.4 \pm 0.24\text{‰}$  for  $\delta^{15}\text{N}$ ). Combining resources was done a-posteriori as suggested by Parnell (2019), in the way that resources which most likely can't be differentiated by the mixing model, as indicated by lying in a similar region of the isospace plot and/or high correlations (negative or positive), were combined with regard to ecological relevant background; meaning plant resources were only combined within the categories autochthonous and allochthonous production and animal resources were mainly combined using their feeding types (for R script see Appendix 1). For further details on isotope mixing and mixing models the reader is referred to e.g. Parnell et al. (2013); Semmens et al. (2013); Phillips et al. (2014).

## **Results**

### *Benthic community*

Overall, 13 macroinvertebrate taxa were found in our study (Figure 1), of which only 3 were present in all 10 semi-quantitative samples from the clamshell grabs (*Jaera sarsi*, *Chelicorophium* spp. and *Echinogammarus ischnus*; see Table S1). PERMANOVA revealed no significant differences



**Figure 1.** Mean densities and standard deviations of the 13 macroinvertebrate taxa found in the semi-quantitative benthic samples from both sampling sites.

between the benthic communities of the two sampling sites ( $p > 0.5$ ). Dominance ranking according to Engelmann (1978), revealed that the following benthic macroinvertebrates were dominant *J. sarsi* (29%), *E. ischnus* (21%), *Chelicorophium* spp. (15%), and unidentifiable Amphipoda (13%; see Table S1).

The captured individuals of CMC covered different phase of the life cycle of this species, ranging from young stages (CW: 30 to 40 mm) up to mature specimens (CW: > 60 mm, Table S2); the latter being shortly before their downstream migration to the breeding areas near the Rhine delta, where they develop sexual maturity.

#### *Gut content analyses*

Visual analyses – Stomach and gut fullness was assessed separately for 25 individuals of CMC. The stomach of 36% were more than 80% full, 8% were 51 to 80% full, and 56% were only filled 50% or less. The gut of 80% of inspected individuals was filled 50% or less, while 4% were more than 80% full, and 16% were 51 to 80% full (details for each individual are given in Table S2). Upon opening the stomach tube or respectively intestinal wall, many plant fibres were visible in all of the samples.

Genetic analyses – Analyses were performed for stomach and gut contents separately. The presence and functional efficiency of DNA templates were assured for each 23 out of 25 stomach and gut content by both universal primer sets prior to the genetic analyses via group-specific primer sets. Overall, only 2 stomach content samples of the 23 individual CMC sampled contained macroinvertebrate prey from the 17 taxa we tested for with the genetic analysis (see Table S2). DNA belonging to the mayfly taxa *Caenis/Ephoron* sp. and the isopod *Jaera sarsi* was detected in one of the stomach contents, while in the other one, only chironomid DNA

was detected (see Table S2). We did not detect DNA of any of the 17 tested taxa in the 23 analysed CMC gut content samples.

### *Stable isotope analyses*

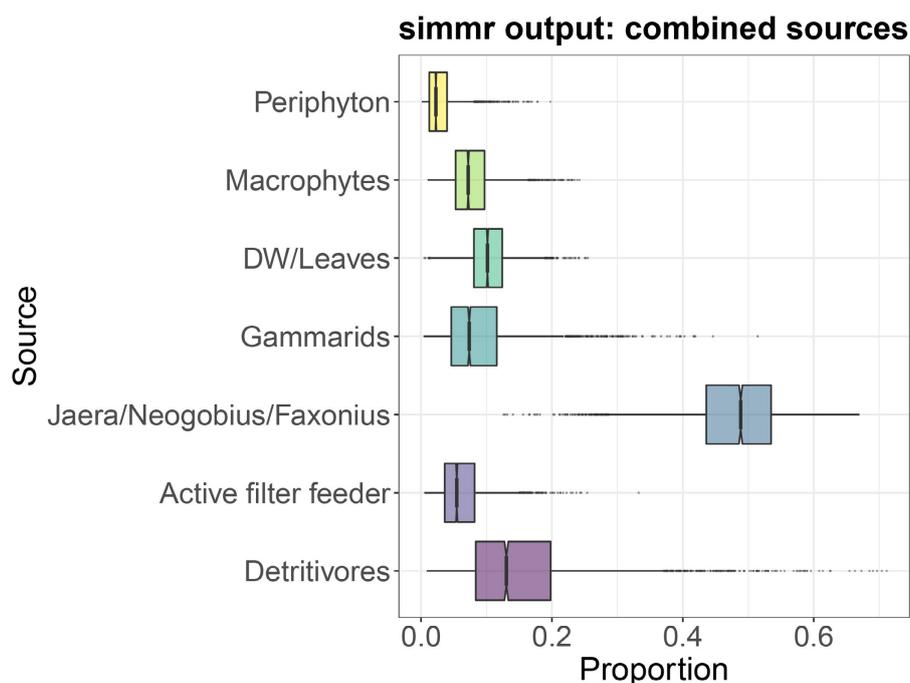
The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of CMC in the Lower Rhine ranged from 13.43 to 15.88‰ ( $14.67 \pm 0.62\text{‰}$ ) and  $-30.60$  to  $-26.43\text{‰}$  ( $27.36 \pm 0.81\text{‰}$ ) respectively, and were comparable to those of the invasive invertivorous round goby *Neogobius melanostomus* ( $15.03 \pm 0.19\text{‰}$  and  $-26.91 \pm 0.21\text{‰}$ ,  $p > 0.08$ ). While  $\delta^{15}\text{N}$  values of CMC were also comparable to those of the alien omnivorous crayfish *Faxonius limosus* ( $15.21 \pm 1.43\text{‰}$ ,  $p > 0.5$ ), the latter was significantly  $^{13}\text{C}$ -depleted relative to CMC ( $\delta^{13}\text{C}$  of *F. limosus*:  $-28.22 \pm 0.59\text{‰}$ ,  $p = 0.002$ ).

For the mixing model analysis, a-posteriori combining of animal resources was done in case of detritivores, i.e. Chironomids, Oligochaeta and Polychaeta (each  $n = 5$ ), active filter feeders, i.e. *Dreissena rostriformis* ( $n = 6$ ) and *Chelicorophium* spp. ( $n = 9$ ), and gammarids, i.e. *Dikerogammarus villosus* and *E. ischnus* (each  $n = 6$ ) with regard to feeding types. Furthermore, the invasive isopod *J. sarsi*, the invasive round goby *N. melanostomus* and alien crayfish *F. limosus* (each  $n = 6$ ) were a-posteriori combined as the resource group *Jaera/Neogobius/Faxonius*. In case of plant resources, a-posteriori combining was performed for the allochthon resources dead wood and leaves (each  $n = 3$ ; DW/Leaves), as well as for three taxa of aquatic macrophytes which were not determined further (each  $n = 3$ ). Information regarding the stable isotope values of the different source taxa and the trophic discrimination factors used for the mixing model analysis are given in Table S3. Carbon and nitrogen values of the CMC individuals were intermediate between those potential dietary resources (see Figure S1; individual values of each CMC are given in Table S2).

According to the simmr mixing model, with a mean contribution to the diet of 47.9% (28.4–60.6%; 95% credibility interval) *Jaera/Neogobius/Faxonius* is the main resource group utilised by CMC, followed by detritivores (mean contribution 15.8%; 3.6–45.1%; 95% credibility interval; Figure 2, all values are given in Table S4). The mean contribution of allochthon plant material, i.e. dead wood and leaves (DW/Leaves), to the diet of CMC was 10.3% (3.7–17.8%; 95% credibility interval), while autochthon plant material contributed on average between 3% (periphyton, 0.4–9.2%; 95% credibility interval) and 8% (macrophytes, 2.7–17.2%; 95% credibility interval). Therefore, animal resources (overall approx. 79% mean diet proportion) most likely contributed more to the diet of the CMC than plant resources (overall approx. 21% mean diet proportion).

## **Discussion**

In a BSc degree study of the isotopic composition of CMC in the Lower Rhine, CMC showed a trophic level comparable to the carnivorous bighead



**Figure 2.** simmr output boxplot showing the relative contribution of seven a-posteriori combined resources (for details see result section) to the diet of Chinese mitten crab, *Eriocheir sinensis*, in the Lower Rhine. Boxes represent the 50% credible interval, vertical constrictions within the boxes indicate the mean values and horizontal lines display the 95% credible interval.

goby, *Neogobius kessleri* (Martin 2010). Likewise, in our study, we found similar  $\delta^{15}\text{N}$  values for the CMC and invertivorous round gobies, as well as for alien omnivorous crayfish, suggesting a comparable trophic function of the three species. However, visual inspection of the stomachs and guts of the CMC and the low frequency of positive macroinvertebrate prey DNA detection therein rather suggested that CMC strongly rely on plant material as food source. On the contrary, the stable isotope mixing model clearly indicated a stronger reliance of the CMC on animal matter as food source than on plant material.

Overall, the number of studies analysing the diet of CMCs from field samples is very low (for a summary of the seven studies available see Table 1). The majority of those seven studies used only one method to analyse the CMC's diet (4 studies used only visual inspection of stomachs or guts, and 1 study used only stable isotope analyses), while only three of those studies combined gut content (short-term) and stable isotope (long-term) analyses, like we did in the present study. Comparable to our results, using gut content analyses, the majority of studies revealed a larger contribution of detritus and/or plant material to the CMC's diet than that of animal matter, while stable isotope analyses usually revealed a stronger reliance of the CMC's on animal matter (Table 1). While such a discrepancy might be solely due to the fact that any gut content analyses only provide a snapshot of recently ingested food, whereas stable isotope analyses provide a time-integrated measure of food usage, this appears rather unlikely especially in a macrophyte dominated system like the ponds in China studied by Mao et al.

**Table 1.** Studies analysing the diet of Chinese mitten crab from individuals caught in the field. Given are the locations, sampling seasons, analysis method, number of individuals analysed (n) and the main diet of CMC for each reference as well as information whether sites investigated were freshwater, tidally influenced or brackish water.

Location	freshwater/tidal zone/brackish water	season	analysis method	n	main diet of CMC	Reference
Stevens Creek, Coyote Creek, Guadalupe River (south San Francisco Bay tributaries), USA	tidally influenced (Stevens Creek and Guadalupe River); not tidally influenced (Coyote Creek)	late August until late October	visual foregut analyses	103	detritus (46%, ~67%, 35%), algae (~32%, 17%, ~38%), invertebrates (~6%, 0.9%, 2%);	Rogers (2000)
Guadalupe River, South Bay sloughs (south San Francisco Bay tributaries), USA		July	visual gut content analysis	150	mainly detritus; invertebrate parts only in 2% of the guts analysed	Rudnick et al. (2000)
Coyote Creek, USA (tributary feeding the south end of San Francisco Bay) – 3 sites	site 1: tidal inflow, salinities ranging from 1 to 10 ppt	summer, autumn 2001; spring 2002		75	invertebrates	
	site 2: just above zone with tidal inflow	autumn 2001, spring 2002	stable isotope analysis	18	intermediate between plants and invertebrates	
	site 3: freshwater	summer and autumn 2001		27	intermediate between plants and invertebrates	Rudnick and Resh (2005)
	site 1: tidal inflow, salinities ranging from 1 to 10 ppt	summer, autumn 2001; spring 2002	visual gut content analysis	15	~45% detritus, ~33% algae, ~3% invertebrates	
	site 2: just above zone with tidal inflow	autumn 2001, spring 2002		13	~57% detritus, ~18% algae, ~6% invertebrates	
River Odra estuary, Poland	brackish water		visual gut content analysis	50	~60% detritus, ~11% vascular plant, 15% inorganic material, ~10% animals	Czerniejewski et al. (2010)
River Stour, Suffolk, U.K.	immediately upstream of tidal limit	autumn 2012	stable isotope analysis	5	macrophytes	
			visual gut content analysis	5	~21% invertebrates, ~11% algae, ~17% macrophytes, ~7% detritus	Rosewarne et al. (2016)
Lake Gucheng, China	2 freshwater crab pond	summer 2013	stable isotope mixing model	24	>60% animal matter	
			visual gut content analysis	38	mainly macrophytes followed by forage fish, corn and mollusks	Mao et al. (2016)
Lake Gucheng and 6 farm ponds in Gaochun County, China	freshwater and freshwater crab ponds	summer and autumn 2014	stable isotope analysis	41	fish (lake ecosystem even >90%) and macrophytes (in ponds)	Huang et al. (2019)
Gulf of Gdansk, Poland	brackish water	May–December 2005	visual stomach analysis	49	56–69% animal matter, 11–22% plant material, 8–33% detritus	
Vistula Lagoon, Poland	brackish water	April–December 2006	visual stomach analysis	200	59–63% plant material, 24–28% animal matter, 13–14% detritus	Wójcik-Fudalewska et al. (2019)
Tagus Estuary, Portugal	brackish water	September 2013	visual stomach analysis	38	70–75% plant material, 25–31% animal matter	

(2016). Certainly, visual gut content analyses usually miss soft-bodied prey organisms (Redd et al. 2008) and thus probably overestimate the contribution of plant material. This is why genetic analyses are considered to be more promising, in particular when dealing with invertebrate consumers that intensively comminute their food before ingestion (e.g. Koester and Gergs

2017). However, in the present study gut and stomach contents were analysed genetically using 17 group-specific rDNA primers for macroinvertebrate prey. But, while those primers covered most of the common macroinvertebrate prey groups occurring in the River Rhine system, not all occurring macroinvertebrate and vertebrate species were covered by primers used for the genetic analyses, due to the fact that the development of adequate primers is time-intensive and costly, and were thus unavailable for this study. Apart from several taxa included in the resource group detritivores, the crayfish *F. limosus*, and no vertebrate food sources like *N. melanostomus* were covered by the genetic analysis. Therefore, the contribution of animal food to the diet of CMC might have been underestimated by our genetic analyses, in particular if the relevance of *Faxonius* and *Neogobius* for the contribution of the resource group *Jaera/Neogobius/Faxonius* (revealed by stable isotope analysis) was higher than that of *Jaera* sp. The latter assumption seems obvious because several studies showed that fish made the greatest contribution to CMC diets (e.g. Mao et al. 2016; Huang et al. 2019), and remains of fish (bones and spines) have also been found by studying gut contents of CMCs visually (e.g. Wójcik-Fudalewska et al. 2019).

However, the observed discrepancy might also be due to metabolic processes of CMCs, as, for instance, the assimilation efficiency is known to vary by food type and consumer species (e.g. Waldbauer 1968; Slansky and Scriber 1982; Eggert and Wallace 2007). Several macroinvertebrate species showed higher assimilation efficiency for animal than for plant material (e.g. Benke and Wallace 1980; Gutiérrez-Yurrita and Montes 2001; Gergs and Rothhaupt 2008). This might also be true for CMCs, possibly explaining that the high amount of plant diet in the gastro-intestinal-tract is not reflected in the contribution of that diet calculated from stable isotope compositions. Furthermore, while some authors found that macrophytes appear to contribute (almost) no energy towards tissue production (Ahlgren 2011), invertebrates might still gain energy for respiration and maintenance from macrophytes, whilst deriving much of the nutritional value from the microbial fauna and epiphyte of macrophytes (McClain et al. 1992; Jaschinski et al. 2011). Therefore, the consumption of macrophytes possibly serves as a vector to ingest other nutritionally higher value resources. Also, CMCs might derive necessary compounds from macrophytes, such as essential amino acids or fatty acids, which would not be uncovered by bulk-SIA used in the present study. Hence, to uncover such a reliance, further research, including additional methods, on assimilation efficiencies, dietary requirements and metabolic processes of CMCs is required.

#### *Ecological and food web consequences*

The results of our study suggest that dominant macroinvertebrates indeed play a more important role for the diet of CMCs than plant material, in

particular with regard to assimilated material which is the base for secondary tissue production. Nevertheless, crabs obviously also consume a large amount of plant material and thus act as omnivores in the Lower River Rhine. This is not only consistent with early reports describing CMC as an omnivore within invaded systems (Panning 1939; Hymanson et al. 1999), but also is suggested by other studies on the diet of the CMCs in the field (see Table 1), and stated likewise by the authors of several of those studies (Rudnick et al. 2000; Rudnick and Resh 2005; Rosewarne et al. 2016; Wójcik-Fudalewska et al. 2019). The relevance of omnivory for structuring food webs has often been neglected in ecology, but is receiving more attention nowadays as several studies show that omnivory is an important part of natural food webs (Polis 1991; Diehl 1993; Polis et al. 2004; Wootton 2017). Furthermore, the invasion of omnivorous species can cause complex modifications in species interactions (e.g. Verstijnen et al. 2019), which make the description of clear ecological consequences of such an invasion rather difficult in comparison to distinct top-down effects of predators. As consequence, ecological effects of the CMCs on indigenous communities as result of their feeding behaviour is part of an ongoing discussion (e.g. Rudnick and Resh 2005; Rosewarne et al. 2016).

In general, omnivores are capable to exploit a variety of food resources and, thus, can be expected to strongly interact with many other species with regard to resource consumption. Being omnivores, the CMCs can have the ability to affect benthic communities substantially directly by preying on macroinvertebrates and fish, but also indirectly through exploitative competition for plant resources with primary consumers and other omnivores. Both interactions might result in the same observable effect, e.g. reduced abundance or diversity of resident benthic invertebrates (e.g. Rosewarne et al. 2016). However, to make assumptions on how CMCs affect food webs, it is relevant to know if classical top-down effects of a predator or more complex interaction chains due to omnivorous feeding are the reason for those changes. Comparatively, omnivorous interactions have been observed as beneficial for food web and system stability, most likely because classical hierarchical structures like top-down or bottom-up effects become less relevant (e.g. Emmerson and Yearsley 2004; Gellner and McCann 2012). The extent to which an omnivore, such as the CMC, alters trophic cascades likely depends on its impact at each trophic level, because trophic cascades will more likely be diminished the more a predator also feeds omnivorously (Benkendorf and Whiteman 2021). In particular, high levels of omnivory may weaken single trophic interaction strength and thereby stabilise food webs (Brönmark et al. 1997; Granados et al. 2019).

In conclusion, of our findings from the Lower River Rhine are in line with the trophic annidation of the CMC (i.e. being an omnivorous species) from other invaded systems. However, different size classes of CMC in the Lower River Rhine seem to show a distinct assimilation of animal resources

and contributions of different resources can highly differ between studies (cp. Table 1). Comparing results of different studies suggests an overall opportunistic feeding strategy and high variability of foraging depending on environmental factors. To enable an assessment of relevant factors influencing to which degree CMC feeds on different resources, further research is needed. For instance, determining species-specific assimilation efficiency and trophic discrimination factors for different food types, within controlled feeding experiments, would considerably improve the conclusions drawn about their relevance for CMCs diet in the field. Aiming to gain knowledge on which processes are affected by its feeding behaviour, will also further contribute to understanding potential consequences for food web and community structures within invaded systems.

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### Authors' contributions

MK – sample design and methodology, investigation and data collection, data analyses and interpretation, writing – original draft, writing – review and editing; CF – investigation and data collection, data analyses and interpretation, writing – review and editing; GB – research conceptualization, investigation and data collection, writing – review and editing; RS – research conceptualization, investigation and data collection, data analyses and interpretation, writing – original draft; writing – review and editing.

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### Supplementary material

The following supplementary material is available for this article:

**Table S1.** Main abiotic environmental characteristics [water level, temperature, oxygen content, oxygen saturation, pH, conductivity and depth] of the two sampling sites of the Lower Rhine, as well as stone surface and density of macroinvertebrates [ind./m<sup>2</sup>] of each benthic sample.

**Table S2.** Sex (m = male; f = female), size, filling of the gut content, stable isotope signature ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) and results of the genetic gut content analyses of 25 Chinese mitten Crab individuals from the Lower Rhine. Filling of the gut content was classified in five categories, reflecting the degree of filling as follows: 1 < 45 %, 2 = 45-50 %, 3 = 51-80 %, 4 = > 80 %. Results of the tests with the primer sets are shown as not analysed (na), positive (+) or negative (-) for tested individuals (ID) derived from automated fragment analyses.

**Table S3.** Given are the names of the source taxa (s\_names) including the group names of a-posteriori combined source groups (red), the number of samples measured (n), their mean C:N ratio, their mean stable carbon (s\_mean d13C) and stable nitrogen (s\_mean d15N) values and the respective standard deviations (s\_SD d13C, s\_SD d15N). Additionally, mean values and standard deviations of the trophic discrimination factors from McCutchan et al. (2003) used for the respective source taxon are given.

**Table S4.** The contribution of a-posteriori combined resources to the diet of Chinese mitten crab (CMC) in the Lower Rhine calculated by the *simmr* mixing model. Given are the mean values and the credible intervals (CI) 2.5, 25, 50, 75 and 97.5%.

**Figure S1.** Isospace plot showing the isotopic composition of individual Chinese mitten crab, as well as mean values and standard deviations (already containing trophic discrimination factors) for resource groups within the  $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$ -space.

**Appendix 1.** R script of the complete analysis using the mixing model *simmr*.

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