

# Trophic relationship between the invasive parasitic copepod *Mytilicola orientalis* and its native blue mussel (*Mytilus edulis*) host

## Research Article

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### Author for correspondence:

M. Anouk Goedknegt, E-mail: [Anouk.G Goedknegt@nioz.nl](mailto:Anouk.G Goedknegt@nioz.nl)

M. Anouk Goedknegt, David Shoesmith, A. Sarina Jung, Pieterella C. Luttkhuizen, Jaap van der Meer, Catharina J. M. Philippart, Henk W. van der Veer and David W. Thieltges

NIOZ Royal Netherlands Institute for Sea Research, Department of Coastal Systems, and Utrecht University, P.O. Box 59, 1790 AB Den Burg Texel, The Netherlands

### Abstract

Invasive parasites can spill over to new hosts in invaded ecosystems with often unpredictable trophic relationships in the newly arising parasite-host interactions. In European seas, the intestinal copepod *Mytilicola orientalis* was co-introduced with Pacific oysters (*Magallana gigas*) and spilled over to native blue mussels (*Mytilus edulis*), with negative impacts on the condition of infected mussels. However, whether the parasite feeds on host tissue and/or stomach contents is yet unknown. To answer this question, we performed a stable isotope analysis in which we included mussel host tissue and the primary food sources of the mussels, microphytobenthos (MPB) and particulate organic matter (POM). The copepods were slightly enriched in  $\delta^{15}\text{N}$  (mean  $\Delta^{15}\text{N} \pm \text{s.d.}; 1.22 \pm 0.58\text{‰}$ ) and  $\delta^{13}\text{C}$  ( $\Delta^{13}\text{C} 0.25 \pm 0.32\text{‰}$ ) with respect to their host. Stable isotope mixing models using a range of trophic fractionation factors indicated that host tissue was the main food resource with consistent additional contributions of MPB and POM. These results suggest that the trophic relationship of the invasive copepod with its mussel host is parasitic as well as commensalistic. Stable isotope studies such as this one may be a useful tool to unravel trophic relationships in new parasite-host associations in the course of invasions.

## Introduction

Predation and parasitism are important trophic interactions that shape ecological communities and food webs. The key differences between predators and parasites are their relative size compared with their victims (parasite < host and predator > prey), the number of victims made during a life-history stage (one for a parasite, but more than one for a predator; Lafferty and Kuris, 2002) and the duration of the interaction (very short in the case of predator-prey systems and much longer in the case of parasite-host relationships; Dubois *et al.* 2009). Additionally, in food webs, predators practically always have a higher trophic position than their prey, while the trophic position of parasites can be more complex. Firstly, parasites with complex life cycles involving multiple hosts may feed on different trophic levels across distinct life cycle stages, making it difficult to determine a single trophic level for all parasite life cycle stages (Lafferty *et al.* 2008). Secondly, some parasites feed on various host tissues and some may not feed directly on the host at all, but rather on the host's stomach contents or specific pre-digested biochemical compounds (Iken *et al.* 2001; Lafferty *et al.* 2008). Hence, some endoparasites living inside a host's intestine may not necessarily be true parasites living strictly on host tissue, but may rather live in a (partially) commensal relationship with their host.

To address the latter problem, traditionally an analysis of parasite stomach contents was used to confirm a parasite-host relationship, but recently stable isotope analysis (SIA) has been proven to be a valuable method to determine the trophic position of parasites (e.g. Pinnegar *et al.* 2001; Deudero *et al.* 2002; Dubois *et al.* 2009) and other consumers (e.g. Inger *et al.* 2006; Dubois *et al.* 2007). This method uses the differences ( $\Delta$ ) between isotopic ratios of naturally occurring stable isotopes of nitrogen ( $\delta^{15}\text{N}$ ) and carbon ( $\delta^{13}\text{C}$ ) between consumers and their diet to reconstruct trophic relationships (Post, 2002). The  $\delta^{13}\text{C}$  discrimination factor ( $\Delta^{13}\text{C}$ ) is used to determine the diet source of carbon (e.g. terrestrial *vs* marine primary producers; Hobson, 1986) with a standard discrimination factor of 1.0‰, while trophic enrichment ( $\Delta^{15}\text{N}$ ) is used to estimate the trophic position (Vander Zanden *et al.* 1997), in which a fixed value (also known as the trophic fractionation factor) of 3.4‰ is most commonly used to analyse relative species trophic levels (Minagawa and Wada, 1984; Vander Zanden *et al.* 1997; Post, 2002). However, studies which compare isotopic signatures of parasites with their hosts indicate that parasites do not always fit with the commonly accepted consumer-diet discrimination patterns seen in free-living species (e.g. Neilson and Brown, 1999; Iken *et al.* 2001; Power and Klein, 2004; O'Grady and Dearing, 2006; Xu *et al.* 2007; Dubois *et al.* 2009; Navarro *et al.* 2014; Behrmann-Godel and Yohannes, 2015).

In this study, we analyse the trophic relationship between the invasive endoparasite *Mytilicola orientalis* and its new host in European seas, the native blue mussel *Mytilus edulis*

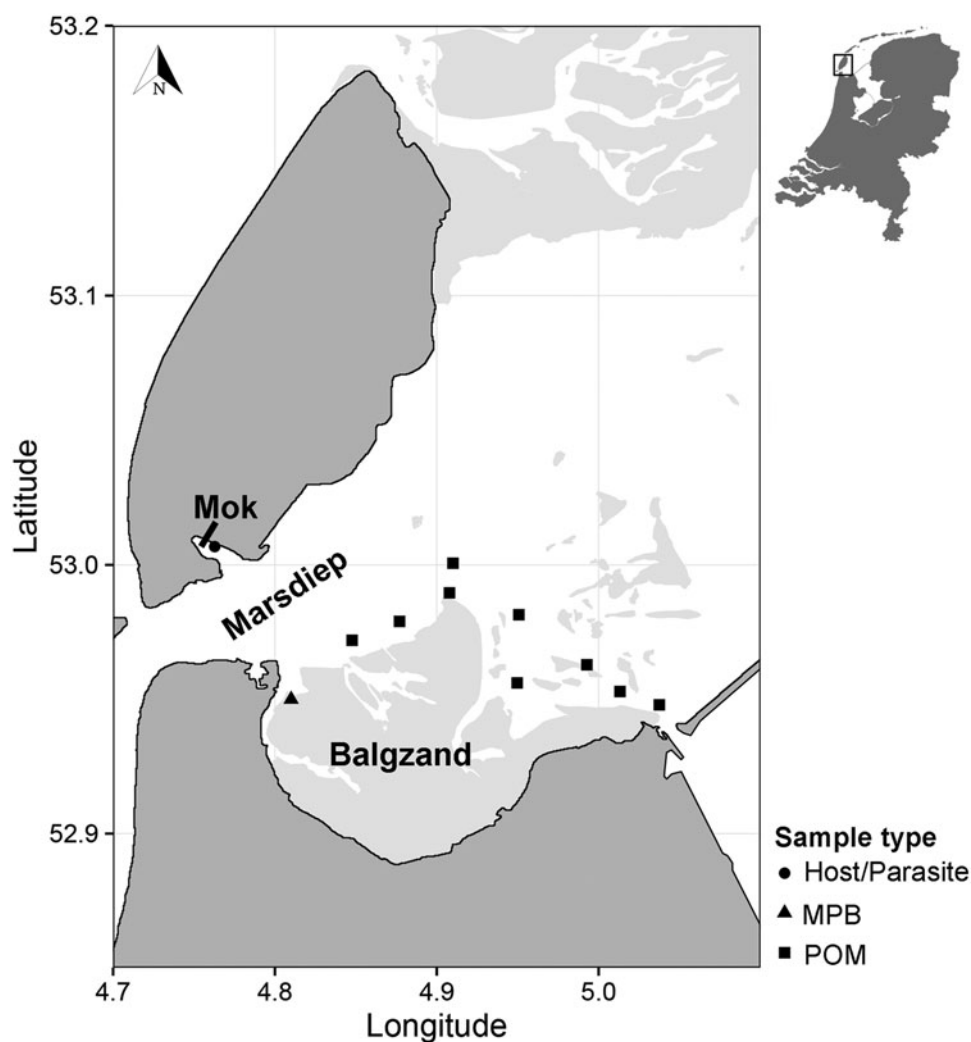
in the Dutch Wadden Sea. This parasitic copepod has been recently co-introduced with aquaculture imports of the invasive Pacific oyster (*Magallana* (previously *Crassostrea*) *gigas*) (Elsner *et al.* 2011) and is known to spill over to native bivalves such as blue mussels and to a lesser extent to common cockles (*Cerastoderma edulis*) and Baltic tellins (*Limecola* (formerly *Macoma*) *balthica*; Goedknecht *et al.* 2017a). *Mytilicola orientalis* was first described in the Sea of Japan (Mori, 1935) and has a direct life cycle with a short non-feeding free-living stage, after which it lives in the intestines of its host. Here, the parasite is either feeding directly on the host tissue or indirectly on host gut content, resulting in a reduction in body condition of infected blue mussels (M. A. Goedknecht, unpublished results). As the exact diet source of the parasite is yet unknown, we performed a SIA to clarify the trophic relationship between the parasite *M. orientalis* and its new blue mussel host. Field samples of mussel hosts and parasites were analysed as well as the two principal food sources of mussels, being particulate organic matter (POM) and microphytobenthos (MPB, Dubois *et al.* 2007). This approach allowed us to determine the relative contributions of host tissue and host food to the diet of the invasive copepod and to identify the trophic relationship of this new parasite-host association that has resulted from the recent co-introduction of the copepods with their oyster hosts.

## Material and methods

### Collection of samples

Suspended POM samples ( $n = 17$ ) were collected on 2 and 4 July 2013 at nine locations in the subtidal Marsdiep channel (Wadden Sea, The Netherlands, Fig. 1). At high tide, water from this channel feeds a small intertidal bay in the south of the island of Texel (Mok, The Netherlands) and therefore we assumed that POM originating from this channel is a major food source for blue mussels (*Mytilus edulis*) living in the bay where we sourced the mussels and parasites (*Mytilicola orientalis*) for the SIA (Fig. 1; see below). At each sampling point, water samples were collected with a Niskin bottle from approximately 1 m below the water surface. Samples were then sieved through a 200  $\mu\text{m}$  mesh to exclude larger zooplankton from the sample and subsequently filtered onto pre-combusted 25 mm GF/F filters using a 25 mm filter cartridge mounted on a 60 mL syringe. Between 80 and 250 mL of water was filtered depending on the amount of suspended matter in the water column. Filters were then stored at  $-20^\circ\text{C}$  until further analysis.

Microphytobenthos (MPB;  $n = 4$  samples within an area of 50  $\text{m}^2$ ; Fig. 1) was sampled in the beginning of July 2013 at an intertidal area south of the Marsdiep (Balgzand, Wadden Sea, The Netherlands, Fig. 1) by collecting sediment from diatom



**Fig. 1.** Sampling locations of suspended POM, MPB, hosts (*Mytilus edulis*) and parasites (*Mytilicola orientalis*). On the MPB location, four samples were taken in an area of approximately 50  $\text{m}^2$ . POM locations were sampled once (2 locations), twice (6 locations) or three times (1 location), adding to a total of 17 samples. MPB = microphytobenthos; POM = particulate organic matter.

mats into plastic bottles that were put on ice and brought to the research facility. Extraction of microphytobenthic diatoms in the laboratory was done by following the method of Riera and Richard (1996), slightly modified by Herlory *et al.* (2007). The sediment was spread in a tray, covered by three layers of nylon mesh ( $2 \times 100 \mu\text{m}$ ,  $1 \times 50 \mu\text{m}$ ) that was kept moist by repeatedly spraying filtered seawater on top. The samples were then left in a temperature-regulated room overnight at  $20^\circ\text{C}$ . The next morning, the algae were washed into a beaker with filtered seawater. This solution was centrifuged (10 min at  $10^3 \text{G}$ ) and the remaining pellet was collected and stored at  $-20^\circ\text{C}$ .

Blue mussel and parasite samples were collected about 3 months later than the POM and MPB samples (26 September 2013), to cover the minimum time it takes for the diet to be incorporated into consumer tissue (Dubois *et al.* 2007; Phillips *et al.* 2014). Mussels ( $n = 150$ ) were collected from a mixed oyster and mussel bed located in the Mok (Fig. 1) and checked for presence of *M. orientalis* parasites under a magnification glass (magnification 3–8 $\times$ ). Mussels infected with at least two female parasites ( $n = 28$  mussels), which can be more than twice as large as males (Mori, 1935), were selected for the analysis, as a minimum of 0.4 mg dry weight of each pooled parasite and corresponding mussel sample (the adductor muscle of the mussel) were required for the SIA. In these selected mussels, the mean *M. orientalis* intensity ( $\pm$ s.d.) was  $3.6 \pm 1.8$  and ranged between 2 and 9 copepods, with an average ( $\pm$ s.d.) ratio of  $0.78 \pm 0.21$  females per infected mussel. Both parasite and matched mussel samples (each  $n = 28$ ) were then stored at  $-20^\circ\text{C}$ .

### Stable isotope analysis

Prior to the SIA, all samples were freeze-dried for 48 h at  $-60^\circ\text{C}$  to remove water content. Additionally, as *M. orientalis* is a crustacean, parasite samples were treated with 1 M HCl to remove inorganic carbonate and dried for another 24 h at  $60^\circ\text{C}$ . Isotope ratios of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  in all samples were determined with a Thermo Scientific Delta V Advantage Isotope Ratio Mass Spectrometer equipped with a Flash 2000 Organic Element Analyser at the Royal Netherlands Institute for Sea Research, Texel, The Netherlands. In addition, mean total organic carbon (TOC) and mean total nitrogen (TN) content and the carbon-to-nitrogen ratio (C:N) were determined for hosts and parasites, but due to logistical constraints this was not possible for the POM and MPB samples.

The standard reference materials acetanilide (s.d.:  $\delta^{15}\text{N}$  0.3‰,  $\delta^{13}\text{C}$  0.1‰) and urea ( $\delta^{15}\text{N}$  0.2‰,  $\delta^{13}\text{C}$  0.1‰) were respectively used as a correction and control of the isotope ratios found in the samples. Isotope ratios of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were then expressed as permille (‰) differences from a standard reference material using the formula  $X = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$ , with  $R$  being the ratio between the heavy and light isotopes of nitrogen ( $^{15}\text{N}:^{14}\text{N}$ ) and carbon ( $^{13}\text{C}:^{12}\text{C}$ ). The reference material used for  $^{15}\text{N}$  was atmospheric nitrogen  $\text{N}_2$  and for  $^{13}\text{C}$  Vienna Pee Dee-Belemnite Limestone (vPDB).

### Statistical analysis

Normality and homoscedasticity of the data were checked with histograms, qqplots and boxplots (Zuur *et al.* 2010). Subsequently, differences in isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) among the trophic groups (POM, MPB, hosts, parasites) were analysed with analysis of variance (ANOVA) and post-hoc Tukey tests. Furthermore, comparisons and relationships between stable isotope data of parasites and corresponding hosts ( $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$ ) and parasite intensity within the host were made using paired Student's  $t$ -tests and Pearson correlations, respectively. All statistical analyses

were performed in the statistical software environment R (R Development Core Team, 2015).

### Isotope mixing models

The relative contribution of diet sources in the consumers' diet can be determined by the use of stable isotope mixing models (i.e. Phillips and Gregg, 2003; Inger *et al.* 2006). In this study, we used an isotope mixing model to determine the relative contributions of host tissue (blue mussel) and host gut content (represented by POM and MPB) to the diet of the parasitic copepod *M. orientalis*. The package *simmr* (Parnell, 2016) was used to solve mixing equations for stable isotopic data within a Bayesian framework in R (R Development Core Team, 2015). This package allows the use of multiple diet sources with adjustable source specific trophic fractionation factors. In the mixing model, individual  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of the parasite samples were used as the consumer data. Diet source data included the mean ( $\pm$ s.d.)  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of the sources POM, MPB and blue mussel, and were corrected for trophic fractionation. This correction for trophic fractionation was done in two different ways: first, we used the standard trophic fractionation factors of 3.4‰ for  $\delta^{15}\text{N}$  and 1.0‰ for  $\delta^{13}\text{C}$  for all diet sources (Minagawa and Wada, 1984; Vander Zanden *et al.* 1997; Post, 2002), as controlled diet studies and thus taxon-specific fractionation factors are not (yet) available for the parasite. Second, we varied the trophic fractionation values used for  $\delta^{15}\text{N}$  between 1 and 4‰ to determine how much the estimated relative contribution of all diet sources changed with the fractionation factor. This second approach served as a sensitivity analysis to account for the unknown 'real' trophic fractionation factor of the parasites (see Discussion for more details). Finally, we ran a third mixing model approach where we used mussel, POM and MPB data from four seasons from the long-term monitoring at our sampling site to identify whether seasonal changes of mussel, POM and MPB isotope signals would change our results.

## Results

### All trophic groups

The four trophic groups (POM, MPB, mussel, parasite) differed significantly in  $\delta^{15}\text{N}$  (ANOVA;  $F_{3,73} = 588.16$ ,  $P < 0.001$ ) and  $\delta^{13}\text{C}$  ( $F_{3,73} = 200.41$ ,  $P < 0.001$ ). Values of  $\delta^{15}\text{N}$  were highest for the parasitic copepod and lowest for POM, while for  $\delta^{13}\text{C}$  MPB and POM had the highest and lowest values, respectively (Table 1; Fig. 2).

### Parasites and hosts

Parasitic copepods were significantly enriched in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  with respect to their host, the blue mussel (Student's paired  $t$ -test;  $\delta^{15}\text{N}$ :  $t = 11.178$ ,  $df = 27$ ,  $P < 0.001$ ;  $\delta^{13}\text{C}$ :  $t = 4.071$ ,  $df = 27$ ,  $P < 0.001$ ; for means see Table 1; for raw data see Goedknecht *et al.* 2017b). However, the levels of enrichment were relatively small (mean  $\pm$  s.d.;  $1.22 \pm 0.58$ ‰ for  $\delta^{15}\text{N}$  and  $0.25 \pm 0.32$ ‰ for  $\delta^{13}\text{C}$ ; Fig. 2). This minor enrichment of the parasite in relation to its host was not reflected in the differences in mean TN and TOC content in both tissues (Student's  $t$ -test; TN (%):  $t = -1.361$ ,  $df = 27$ ,  $P = 0.185$ ; TOC (%):  $t = -0.741$ ,  $df = 27$ ,  $P = 0.465$ ; for means see Table 1).

Furthermore, there was a significant positive correlation for  $\delta^{13}\text{C}$  between host and parasite (Pearson correlation,  $r = 0.63$ ,  $P < 0.001$ ; Fig. 3a), but this relationship did not exist for  $\delta^{15}\text{N}$  ( $r = -0.13$ ,  $P = 0.509$ ; Fig. 3b). Consequently, parasite enrichment ( $\Delta^{15}\text{N}$ : parasite  $\delta^{15}\text{N}$  – mussel  $\delta^{15}\text{N}$ ) scaled negatively with mussel

**Table 1.** Mean values ( $\pm$ s.d.) of  $\delta^{15}\text{N}$  (‰) and  $\delta^{13}\text{C}$  (‰) for suspended particulate organic matter (POM), microphytobenthos (MPB), the blue mussel host (*Mytilus edulis*) and its intestinal parasite *Mytilicola orientalis*

Value	POM (n = 17)	MPB (n = 4)	Host <i>Mytilus edulis</i> (n = 28)	Parasite <i>Mytilicola orientalis</i> (n = 28)
$\delta^{15}\text{N}$ (‰)	5.97 $\pm$ 0.88	9.44 $\pm$ 0.36	11.42 $\pm$ 0.38	12.64 $\pm$ 0.39
$\delta^{13}\text{C}$ (‰)	-21.86 $\pm$ 1.16	-15.67 $\pm$ 0.18	-18.08 $\pm$ 0.34	-17.83 $\pm$ 0.40
TN (%)	NA	NA	7.99 $\pm$ 0.90	7.76 $\pm$ 0.97
TOC (%)	NA	NA	35.92 $\pm$ 3.68	35.18 $\pm$ 5.42
C:N	NA	NA	4.51 $\pm$ 0.32	4.53 $\pm$ 0.19

Total nitrogen content (TN %), total carbon content (TOC %) and C:N ratios are only given for parasite and host.

$\delta^{15}\text{N}$  mussel (Pearson correlation,  $r = -0.75$ ,  $P < 0.001$ ; Fig. 4), while this relationship was not significant for  $\Delta^{13}\text{C}$  enrichment ( $r = -0.29$ ,  $P = 0.130$ ). In addition, there was no relationship between the enrichment of the parasite ( $\Delta^{15}\text{N}$ : parasite  $\delta^{15}\text{N}$  - mussel  $\delta^{15}\text{N}$ ) and the C:N ratio of the mussel (Pearson correlation,  $r = 0.06$ ,  $P = 0.743$ ). Finally, in our dataset parasite intensity in infected hosts did not affect the  $\delta^{15}\text{N}$  ( $r = 0.35$ ,  $P = 0.064$ ) nor  $\delta^{13}\text{C}$  ( $r = 0.07$ ,  $P = 0.708$ ) signals of the parasites and neither those of the hosts ( $\delta^{15}\text{N}$ :  $r = -0.07$ ,  $P = 0.719$ ,  $\delta^{13}\text{C}$ :  $r = -0.19$ ,  $P = 0.334$ ).

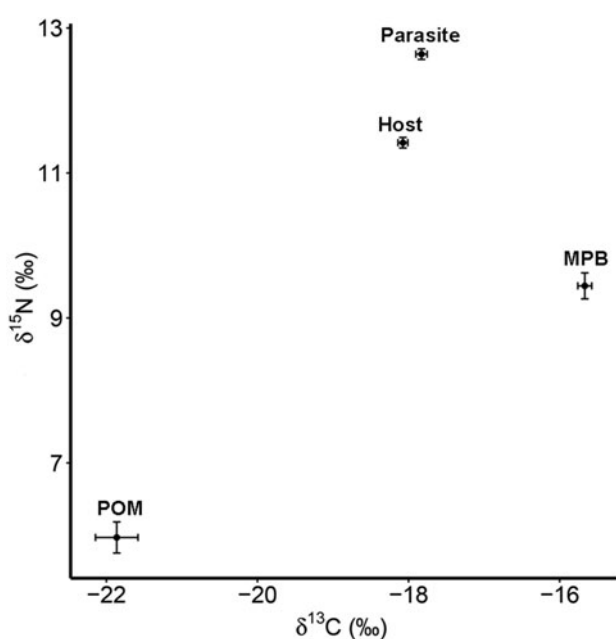
### Isotope mixing models

In the first mixing model, we used the standard trophic fractionation factor of 3.4‰ for  $\delta^{15}\text{N}$  and the discrimination factor of 1.0‰ for  $\delta^{13}\text{C}$  for all diet sources (POM, MPB, mussel host). Results of this model showed that mussel tissue was the main contributor to the parasites' diet (95% confidence interval; 42–52%), with lower contributions by POM (30–35%) and MPB (15–25%). When we varied the  $\delta^{15}\text{N}$  trophic fractionation factors in the second run of the mixing models, the relative contributions of all diet sources changed (Table 2; Fig. 5) but for fractionation factors between 0 and 3.4‰ for  $\delta^{15}\text{N}$  this did not affect the dominance of mussel host tissue in the parasites' diet. Only for a

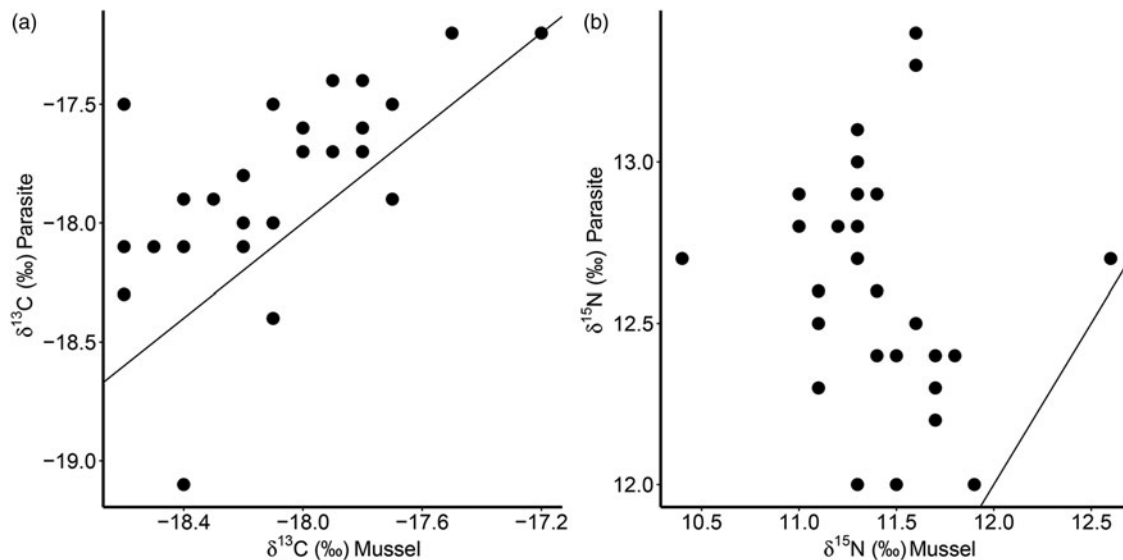
fractionation factor of 4‰ for  $\delta^{15}\text{N}$ , the model showed higher proportions of POM (37–42%) and MPB (26–37%) in the diet of the parasite relative to blue mussel tissue (24–35%; Table 2; Fig. 5). Finally, using isotope values of mussels, POM and MPB from four seasons/months at our sampling site (March, June, September and December 2014) in the main mixing model (3.4‰ for  $\delta^{15}\text{N}$  and 1.0‰ for  $\delta^{13}\text{C}$ ) did not change the results qualitatively (Supplementary Table S1).

### Discussion

Our SIA showed that the intestinal parasitic copepod *Mytilicola orientalis* is enriched in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  with respect to its blue mussel (*Mytilus edulis*) host. Yet, for both isotopes, the observed enrichment of the parasite compared with its host (1.2‰ for  $\Delta^{15}\text{N}$  and 0.25‰ for  $\Delta^{13}\text{C}$ ) was considerably lower than the standard trophic fractionation factor of 3.4‰ for  $\Delta^{15}\text{N}$  and the standard discrimination factor of about 1‰ for  $\Delta^{13}\text{C}$ , which are commonly used to distinguish between trophic levels (e.g. Minagawa and Wada, 1984; Vander Zanden *et al.* 1997; Post, 2002). Given that these values are also appropriate for the parasites, this would indicate that this intestinal parasite does not only feed on host tissue, but also on host gut content, suggesting a complex mix of a parasitic and commensal relationship in this new parasite-host association. Such a mixed diet was also indicated by the results of the stable isotope mixing modelling, a statistical method that is increasingly used by ecologists (reviewed by Phillips *et al.* 2014). Generally, the results of our mixing models (using standard fractionation factors) demonstrated that host tissue dominated with suspended POM and microphytobenthos contributing to the parasites' diet. However, alternatively, the relatively small signals of enrichment may not result from mixed diet contributions but could also suggest parasite specific lower trophic fractionation patterns. Indeed, smaller than standard enrichment patterns have previously been found in other parasite-host systems (O'Grady and Dearing, 2006; Dubois *et al.* 2009; Yurlova *et al.* 2014; Behrmann-Godel and Yohannes, 2015; Demopoulos and Sikkil, 2015), including parasites with a strict parasitic way of life such as trematodes (Dubois *et al.* 2009). To investigate the effect of potentially lower and higher than usual trophic fractionation factors, we conducted a second run of mixing models using a variation of trophic fractionation values (0–4‰). These models showed that mussels were still the dominant food source in all but the highest trophic fractionation value (4‰). In addition, the positive correlation in carbon signatures between parasite and host suggests that the host represents a major carbon source for the parasitic copepod. All these results confirm that the parasite has, at least to a large extent, a parasitic trophic relationship with its host. This would also explain the negative effect of the parasite on host body condition, which has been previously



**Fig. 2.** Isotope values of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  with standard errors for suspended POM, MPB, the blue mussel host (*Mytilus edulis*) and its intestinal parasite *Mytilicola orientalis*. MPB = microphytobenthos; POM = particulate organic matter.



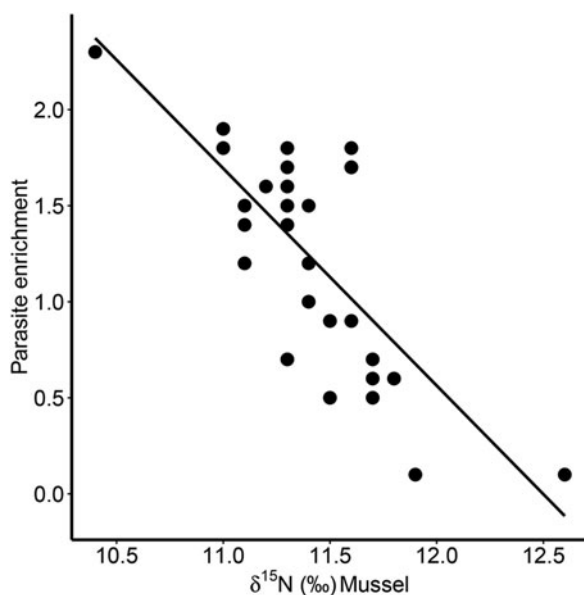
**Fig. 3.** Relationships of  $\delta^{13}\text{C}$  (a) and  $\delta^{15}\text{N}$  (b) for the native mussel host *Mytilus edulis* and the invasive parasite *Mytilicola orientalis*. Each dot represents values for one individual host-parasite pair. Black lines indicate the 1:1 relationship.

observed in controlled laboratory experiments (M. A. Goedknecht, unpublished results). However, in all the scenarios of the mixing models developed in our study, host tissue (*M. edulis*; proportions of 24–99%) was never the only resource of *M. orientalis* but host gut content, represented by suspended POM (0–42%) and microphytobenthos (MPB: 0–37%), consistently contributed to the parasite's diet. This suggests that the trophic relationship of the parasite with its new host is also partly commensalistic. The exact contributions of the diet under different environmental conditions as well as the resulting diet specific trophic fractionation factors remain to be experimentally studied (although this will be logistically challenging, see below).

Our findings differ from the results of a SIA of a congeneric species of *M. orientalis*, the copepod *M. intestinalis*, which also lives in the intestine of *M. edulis*. Gresty and Quarmby (1991) found  $\delta^{15}\text{N}$  values of the parasite that were, on average, 2.8‰ higher than for the blue mussel and suggested a parasitic trophic

relationship between the parasite and its host. In their study, infected mussels (collection season unknown) were kept in aquaria that were filled with estuarine water and mussels were fed with the diatom *Phaeodactylum trycornutum* 2–3 weeks prior to dissection, after which the mussel intestine was used in the SIA analysis. Methodological differences may underlie the diverging trophic fractionation factors in the two parasite species but it is also possible that the feeding behaviour of both congeneric copepods is different. Although *M. intestinalis* may not directly feed on host tissue but rather on sloughed-off cells of the intestine or on mucus produced by the host (Gresty and Quarmby, 1991), it may still mainly feed (indirectly) on its mussel host. In contrast, the much lower trophic enrichment ( $\Delta^{15}\text{N}$ ) of 1.2‰ observed in *M. orientalis* in our study might suggest a more complex mix of a parasitic and commensal relationship between this parasite and its new host. A direct comparison of the two parasites in future experimental stable isotope studies would be interesting and could help to identify potential differences in diet composition of the two related parasite species.

In the present study, mussel diet sources were sampled at other sites (Marsdiep and Balgzand) than mussels and parasites (Mok). However, during flood the three areas are tightly connected, when water from the North Sea is feeding the intertidal areas of Balgzand and Mok via the same deep channel, the Marsdiep (Postma, 1954; Duran-Matute *et al.* 2014). Therefore, we assume that POM originating from this channel is incorporated in the mussel and parasite tissue 2–3 months later (Dubois *et al.* 2007; Phillips *et al.* 2014). MPB samples were collected at the same time as the POM samples, but on a sampling site from a seasonal isotope monitoring study located on the tidal flats of Balgzand, on the opposite side of the channel feeding the Mok, where hosts with parasites were sampled. For the stable isotope mixing models, we considered the samples from Balgzand to be representative for the MPB available to the mussels. However, MPB is known to occur in higher abundances in the Mok than at Balgzand ( $4\text{ g C m}^{-2}$ ; Borsje, 2006) and both areas are under the influence of different freshwater sources. The exact impact of these discharges on the mussels' diet is yet unknown. Potential differences in the isotopic composition of MPB between the areas may introduce bias in our analyses, but given the relatively small range of isotope signals observed in MPB on local scales as observed in a recent large-scale isotope study along the entire Dutch Wadden Sea (Christiansen *et al.*



**Fig. 4.** Relationship between parasite enrichment ( $\Delta^{15}\text{N} = \text{parasite } \delta^{15}\text{N} - \text{mussel } \delta^{15}\text{N}$ ) of the invasive parasite *Mytilicola orientalis* and the  $\delta^{15}\text{N}$  of the blue mussel host *Mytilus edulis*. Each dot represents values for one individual host-parasite pair.

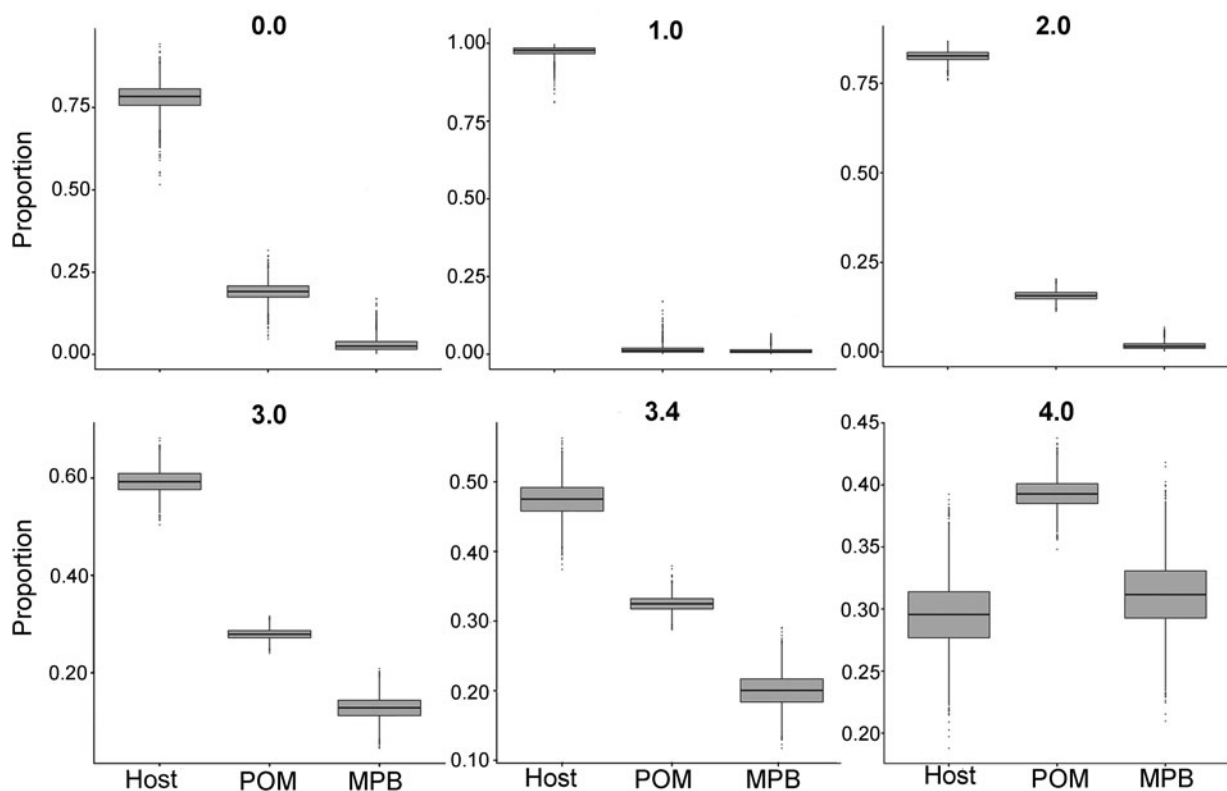
**Table 2.** Results of the sensitivity analysis of the mixing model

Trophic fractionation factor $\Delta^{15}\text{N}$ (‰)	Host <i>Mytilus edulis</i> (%)	MPB (%)	POM (%)	Order	Probability (%)
0.0	68–85	1–8	13–24	Host > POM > MPB	100
1.0	94–99	0–3	0–5	Host > POM > MPB Host > MPB > POM	61 39
2.0	79–86	0–4	13–18	Host > POM > MPB	100
3.0	55–64	8–17	26–30	Host > POM > MPB	100
3.4	42–52	15–25	30–35	Host > POM > MPB	100
4.0	24–35	26–37	37–42	POM > MPB > Host POM > Host > MPB	63 36

The trophic fractionation factor  $\Delta^{15}\text{N}$  was changed manually between 0.0–4.0‰ for all the three diet sources: host *Mytilus edulis*, microphytobenthos (MPB), particulate organic matter (POM). The discrimination fractionation factor  $\Delta^{13}\text{C}$  was in all scenarios set at  $1.0 \pm 0.0$ ‰. The table shows the diet proportions (95% confidence interval) of each of the three diet sources, the hierarchical order of diet proportions in the parasites' (*Mytilicola orientalis*) diet and the probability of this ordering.

2017; M. J. A. Christianen, pers. communications), we are confident that the spatial mismatch in sampling location is not adding a severe bias in MPB measurements. Besides spatial differences, seasonality may be another potential factor known to affect isotope signals over a wide range of trophic levels (Kang *et al.* 2006; Cabanellas-Reboredo *et al.* 2009; Ezgeta-Balić *et al.* 2014; de la Vega *et al.* 2016), and expected to affect  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  ratios of mussel diet sources, potentially confounding our mixing models. However, data from a seasonal isotope investigation in our study area from which the POM/MPB data originated, suggest a limited effect of seasonality on our results. Preliminary results of this seasonal study showed only small differences in isotope values between June and September for POM ( $\Delta\delta^{15}\text{N} = 0.01$ ;  $\Delta\delta^{13}\text{C} = 0.9$ ) and some larger differences for MPB ( $\Delta\delta^{15}\text{N} = 1.7$ ;  $\Delta\delta^{13}\text{C} = 1.6$ ; A. S. Jung, pers. communications). These results demonstrate that

the growing season of various phytoplankton species did not lead to strong changes in the isotopic signals of POM during the summer. For MPB on the other hand, the changes are larger and here a switch in microphytobenthos species composition may have caused a change of isotopic values during the summer. However, the seasonal change in isotopic values of these diet sources only affected the isotopic signal of the host *Mytilus edulis* to a small extent between June and September ( $\Delta\delta^{15}\text{N} = 0.1$ ;  $\Delta\delta^{13}\text{C} = 0.9$ ; A. S. Jung, pers. communications). As especially the  $\delta^{15}\text{N}$  values of mussels barely changed during the summer, we do not believe that seasonality effects are confounding our analyses, in particular with respect to our main focus of investigation, the trophic relationship of the parasite *M. orientalis* with its mussel host. Our inferences are further supported by stable isotope mixing models in which we used original data for POM and MPB from the isotope study of four



**Fig. 5.** Comparison of proportions of diet sources in the intestinal parasite *Mytilicola orientalis* by using different mixing models with a varying fractionation factor  $\Delta^{15}\text{N}$  (0.0, 1.0, 2.0, 3.0, 3.4 and 4.0‰) for the blue mussel *Mytilus edulis* host, MPB and POM. In every model, the discrimination factor of  $\Delta^{13}\text{C}$  was set at  $1.0$ ‰. MPB = microphytobenthos; POM = particulate organic matter.

different seasons to investigate how this would change the results. These analyses showed that mussels remain the main food source for the parasites, independent of the season (see electronic appendix Table S1). However, over the course of a year, the relative contributions of POM, MPB and mussel to the parasites' diet may of course change with season and/or salinity and to what extent this actually happens should be a topic of future studies.

For isotope mixing models, the use of appropriate fractionation factors is essential (Phillips *et al.* 2014) but, as mentioned above, parasites may show enrichment patterns different from free-living species (O'Grady and Dearing, 2006; Dubois *et al.* 2009; Yurlova *et al.* 2014; Demopoulos and Sikkel, 2015) and we accounted for this by using mixing models with different fractionation factors (see above). However, further diversion from standard enrichment patterns may arise from the universal pattern that trophic fractionation factors of consumers are known to scale negatively with the isotope ratio of their resource (Caut *et al.* 2009; Hussey *et al.* 2014). The same negative scaling was observed in our data with the trophic enrichment in *M. orientalis* decreasing with host  $\delta^{15}\text{N}$ . Such a negative scaling relationship between resource  $\delta^{15}\text{N}$  and consumer trophic enrichment has also been observed within individual predators and their prey (Caut *et al.* 2009; Dennis *et al.* 2010) besides the general negative scaling relationship among species observed in comparative studies (Caut *et al.* 2009; Hussey *et al.* 2014). However, the underlying mechanisms of both scaling relationships are not well understood (Caut *et al.* 2009; Hussey *et al.* 2014). In the case of *M. orientalis*, the issue is further complicated by the fact that  $\delta^{13}\text{C}$  values of the parasite correlated positively, as expected for a trophic relationship, with those of their hosts, but that, surprisingly, this relationship did not exist for  $\delta^{15}\text{N}$ . Here, the variation in  $\delta^{15}\text{N}$  values among individual *M. orientalis* samples was larger than the variation among individual mussels, resulting inevitably in a negative scaling relationship between parasite trophic enrichment ( $\Delta^{15}\text{N}$ ) and  $\delta^{15}\text{N}$  values of hosts. This suggests that *M. orientalis* might be relatively decoupled from its host nitrogen sources. However, why this is the case we can only speculate. Ratios of stable isotopes may change between parasite and host due to differential digestion or fractionation during assimilation and metabolic processes. For example, the parasite could selectively use alternative or depleted nitrogen compounds stored within the mussels (Barrett, 1981), bacteria in the gut of the mussel could cause substantial changes in the nitrogen cycle within the host or specialized nitrogen turnover processes within the parasite could cause potential decoupling between host and parasite. Alternatively, our sample choice of selecting mostly larger females (which was necessary to obtain sufficient parasite tissue for the SIA analysis), could also have affected the relatively large variation in  $\delta^{15}\text{N}$  we have observed in parasite samples. Possibly, females exhibit different stable isotope composition than males due to differences in body size (growth rates) and feeding rates, as well as due to egg production by females. In addition, the natural variation in *M. orientalis* intensities in the selected hosts could have influenced the variation in nitrogen among parasites, but this correlation was not significant in our analyses. Controlled laboratory experiments may be needed to explore the exact mechanisms behind the stable isotope patterns observed in the new *M. orientalis*-mussel association. However, such experimental approaches with parasites are logistically challenging. In particular in the case of the parasitic copepod in our study, it will be difficult to experimentally disentangle the relative contributions of host mussel gut content (POM and MPB) and host tissue to the parasite's diet, as the parasite inhabits the gut of the mussel host and has access to both resources at the same time. Hence, if one feeds mussels with a different dietary source, the parasite will have access to the mussel diet and at the same time feed on

mussel tissue. Thus, the parasite acquires isotopes that are a mix of both food sources. Also conducting the usual diet switch studies with parasites is difficult, as the parasites cannot live without their hosts. Hence, letting parasites feed on algae without a host is impossible and due to host specificity changing hosts is also an issue. Such logistical complications due to the natural history of parasitic organisms are probably the reason why most isotope studies of parasites so far have not used any experimental approaches. However, these studies still show that also samples from the field can give some insight into how isotopes reflect trophic relationships of parasites and their hosts. In our case, the combination of careful interpretation of the data and sensitivity analyses using stable isotope mixing models allow for valid inferences in absence of experimental data.

In conclusion, our study indicates that the invasive parasite *M. orientalis* mainly feeds on tissue of its new mussel host, but, to a lesser extent, also on the gut content of mussels (represented by suspended particulate organic matter and microphytobenthos). This conclusion was also supported by stable isotope mixing models, which used various trophic fractionation values to account for potentially different isotope enrichment patterns in parasitic compared with free-living species. We propose that SIA combined with additional stable isotope mixing models promises to provide a useful tool to explore the trophic relationships of new parasite-host associations that result from the increasing co-introductions of parasites with their hosts into new ecosystems.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182017001779>.

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