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Spillover but no spillback of two invasive parasitic copepods from invasive Pacific oysters (*Crassostrea gigas*) to native bivalve hosts

M. Anouk Goedknegt ⬤ · **Anne-Karin Schuster** · **Christian Buschbaum** · **René Gergs** ·
A. Sarina Jung · **Pieternella C. Luttikhuizen** · **Jaap van der Meer** · **Karin Troost** ·
K. Mathias Wegner · **David W. Thieltges**

Abstract Invasive species can cause indirect effects on native biota by modifying parasite-host interactions and disease occurrence in native species. This study investigated the role of the invasive Pacific oyster (*Crassostrea gigas*) in potential spillover (co-introduced parasites infect native hosts) and spillback (native or established parasites infect invasive hosts and re-infect native hosts) scenarios of recently introduced (*Mytilicola orientalis*) and previously established (*Mytilicola intestinalis*) marine parasitic copepods in two regions in northern Europe, the Dutch Delta and the Wadden Sea. By examining 3416 individuals of 11 potential host species from sympatric host populations, we found that the recently introduced parasite *M. orientalis* does not only infect its principal host, the invasive Pacific oyster (prevalence at infected sites 2–43 %, mean intensity 4.1 ± 0.6 SE), but also native blue mussels (*Mytilus edulis*; 3–63 %, 2.1 ± 0.2), common cockles (*Cerastoderma edule*; 2–13 %, 1.2 ± 0.3) and Baltic tellins (*Macoma balthica*; 6–7 %, 1.0 ± 0), confirming a spillover effect. Spillback effects were not observed as the previously established *M. intestinalis* was exclusively found in blue mussels (prevalence at infected locations 3–72 %, mean intensity 2.4 ± 0.3 SE). The high frequency of *M. orientalis* spillover, in particular to native mussels, suggests that Pacific oysters may cause strong parasite-mediated indirect impacts on native bivalve populations.

Keywords *Mytilicola orientalis* · *Mytilicola intestinalis* · *Mytilus edulis* · Parasite co-introduction · Invasive species · Wadden Sea

M. A. Goedknegt (✉) · A.-K. Schuster ·
A. S. Jung · P. C. Luttikhuizen · J. van der Meer ·
D. W. Thieltges
Department of Coastal Systems and Utrecht University,
NIOZ Royal Netherlands Institute for Sea Research,
P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands
e-mail: Anouk.Goedknegt@nioz.nl

A.-K. Schuster · R. Gergs
Institute of Environmental Sciences, University of
Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany

C. Buschbaum · K. M. Wegner
Helmholtz Centre for Polar and Marine Research, Alfred
Wegener Institute, Wadden Sea Station Sylt, Hafenstrasse
43, 25992 List/Sylt, Germany

K. Troost
IMARES Wageningen UR, P.O. Box 77,
4400 AB Yerseke, The Netherlands

Introduction

When species are introduced to new ecosystems they often cause a multitude of impacts on native biota (Davis 2009; McGeoch et al. 2010; Lockwood et al.

2013). Among the commonly identified direct impacts are resource competition with and predation on native species (Parker et al. 1999; Simberloff et al. 2013). However, invasive species can also exert indirect effects, e.g. by modifying native parasite-host inter-actions that can result in disease emergence in native species. The frequency of such effects is exemplified by the IUCN list of the '100 world's worst invasive alien species' (Lowe et al. 2000) which notes diseases as the cause for ecological and economic impacts for a quarter of the invasive species on the list (Hatcher et al. 2012).

Several mechanisms can lead to parasite-mediated effects on native biota (Goedknegt et al. 2016). A first underlying mechanism is the co-introduction of par-asites with invasive species which may spill over to naïve native host species (*parasite spillover*), which can lead to emerging diseases that potentially have knock-on effects on invaded ecosystems (Daszak et al. 2000; Prenter et al. 2004; Kelly et al. 2009). In marine systems this mechanism is relatively common as 73 % of parasite co-introductions with invasive species resulted in parasite spillover from invasive to native species, and almost half of these events led to mass mortalities of native host species (reviewed in Goed-knegt et al. 2016).

A second mechanism is that invasive species may modify established parasite-host interactions in the invaded region. For example, when invasive species function as an alternative host for native or previously established parasites, they may increase parasite population sizes and lead to increased infection levels in native host populations (*parasite spillback*; Kelly et al. 2009). The basic requirements for parasite spillback are that (1) the invasive host species must acquire a native or previously established parasite, (2) the invasive host species must be a competent host and amplify parasite populations, and (3) the parasite must spill back from the invasive to native host species (Kelly et al. 2009). In addition, when these three requirements are met, the relative density of invasive hosts to native hosts must be sufficiently high so that the invasive hosts can act as a reservoir of infection (Paterson et al. 2011, 2013). While there is ample evidence for the acquisition of native parasites by invasive host species (step 1 of parasite spillback; e.g. Miller and Vincent 2006; Georgiev et al. 2014; Sheath et al. 2015; Lewicki et al. 2015), there is little conclusive evidence for an actual amplification and/or

spillback to native species (but see Hershberger et al. 2010; Goedknegt et al. 2016). Finally, as an alternative mechanism to parasite spillback, incompetent invasive host species can act as a sink by diluting the native parasite population and thereby reducing the disease risk for native species (*transmission interference or parasite dilution*; Thieltges et al. 2009; Paterson et al. 2011; Poulin et al. 2011; Goedknegt et al. 2016).

Parasite spillover, spillback and dilution effects may also contribute to indirect impacts of one of the most prominent invaders in European marine ecosys-tems, the Pacific oyster (*Crassostrea gigas*). This species originates from East Asia and was imported for commercial cultivation worldwide (Arakawa 1990; Troost 2010), where it often spread from aquaculture facilities into the wild. Pacific oysters were also introduced from British Columbia to the Netherlands (1960s Dutch Delta; 1980s Southern Wadden Sea; Drinkwaard 1999; Troost 2010) and from the UK to Germany (1990s northern Wadden Sea; Reise 1998; Moehler et al. 2011). Currently, both of these genetically differentiated invasions (Moehler et al. 2011) established persistent populations in the Wadden Sea (Ruesink et al. 2005), raising conserva-tion concerns over competition with native blue mussels (*Mytilus edulis*; Troost 2010). Together with the Pacific oyster, the parasitic copepod *Mytilicola orientalis* was co-introduced to Europe in the 1960/70 s via oyster imports from cultures in British Columbia (His 1977), where it had been introduced in the 1930s (then still known as *Mytilicola ostreae*; Wilson 1938). This intestinal parasite has a direct life cycle with a free-living larval stage and was originally described by Mori (1935) from Pacific oysters in its native range in the Sea of Japan. In the Dutch Delta, *M. orientalis* has been observed since the 1990s (Stock 1993), whereas it has only recently been documented in the Wadden Sea (Elsner et al. 2011; Pogoda et al. 2012). The parasite was reported to show relatively low host specificity (Lauckner 1983; Grizel 1985) and has already spilled over to *M. edulis* and *Ostrea edulis* at European (French and Dutch) coasts (His 1977; Stock 1993). However, quantitative data on the host range of the introduced parasite in the different invaded regions are largely missing to date. In addition, information on the actual competency of different hosts for *M. orientalis* is absent. For example, the presence of egg-bearing females would be indica-tive of competent hosts. Likewise, the body sizes of

parasites in different host species could be indicative of the relative host competency, with larger parasite body sizes usually related to higher fecundity (Poulin and Morand 1997).

For a closely related congeneric copepod, the previously established *Mytilicola intestinalis*, more data are available for native hosts in European waters. This species has become notorious as the 'red worm disease' since it allegedly caused mass mortalities of its main host, the blue mussel, in the North Sea in the 1950s and 1960s (Korringa 1968), although there has been considerable debate about the actual lethality of the parasite (e.g. review by Lauckner 1983). *M. intestinalis* has a smaller geographic range than its congeneric *M. orientalis* and is only found along European coasts where it was first described in Mediterranean mussels (*Mytilus galloprovincialis*) in the Adriatic Sea (Steuer 1902). Yet, as it has continuously existed in the North Sea for 80 years (first observation in 1937 in Southampton, UK; Cole 1951), this established parasite provides a suitable model system to study spillback effects. Pacific oysters have been mentioned as potential hosts for *M. intestinalis* (Gotto 2004), making spillback via invasive oysters to native host species a likely scenario. Alternatively, if the Pacific oyster appears to be an incompetent host for

the parasite *M. intestinalis*, the invasive species potentially acts as a sink, making a dilution effect likely. However, similarly to *M. orientalis* spillover, no quantitative data are available to evaluate the likelihood of such events.

To provide such quantitative data, we investigated the role of the invasive Pacific oyster in potential spillover, spillback and dilution scenarios of the two parasitic copepods (the invasive *M. orientalis* and the previously established *M. intestinalis*) at several sites in two regions in northern Europe with different invasion histories; the Dutch Delta and the Wadden Sea (Fig. 1). By conducting an extensive field survey of sympatric populations of potential hosts, we focused on four specific questions: (1) can the parasitic copepod *M. orientalis*, which was co-introduced with Pacific oysters, spill over to other native molluscs next to the blue mussel?; (2) are differences in the invasion history of the Pacific oyster between the two regions (Dutch Delta and Wadden Sea) reflected in differences in *M. orientalis* infection in blue mussels and Pacific oysters in these regions?; (3) do hosts of *M. orientalis* differ in their suitability for the parasite?; (4) do invasive Pacific oysters become infected with the previously established copepod *M. intestinalis* and thus have the potential to cause spillback to native
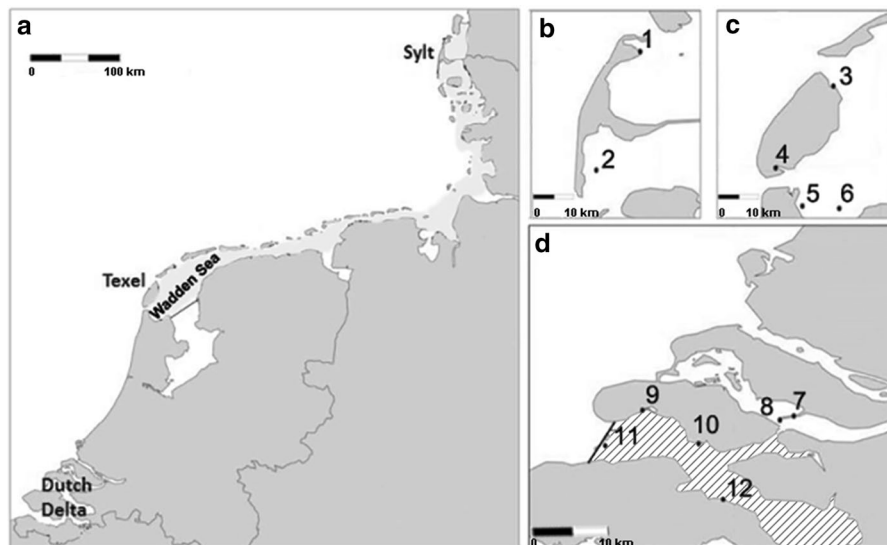

Fig. 1 Sampling locations with sympatric oyster and mussel populations within two regions (Wadden Sea and Dutch Delta) of the main sampling campaign in May 2012 (**a**), Wadden Sea-Sylt (**b**), Wadden Sea-Texel (**c**) and the Dutch Delta (**d**). The striped area indicates the Oosterschelde estuary where

additional samples were sourced (for more details see Materials and methods and Online Resource 2). Maps adapted from NOAA National Geophysical Data Centre. For exact coordinates of locations see Online Resource 1

mussel hosts, or does the Pacific oyster act as a potential sink for this parasite? Answering these questions allows for an evaluation of the overall risk of the invasive Pacific oyster to exhibit indirect impacts on invaded ecosystems by mediating parasite-host dynamics.

Materials and methods

Host sampling

Host samples originated from three sources: (1) Samples from May 2012 originating from 6 mixed oyster/mussel beds in the Wadden Sea (Fig. 1a–c) and 6 beds in the Dutch Delta (Fig. 1a,d; for coordinates of locations see Online Resource 1). In the first region, we focussed on two islands at both ends of the Wadden Sea to cover both invasion pathways of Pacific oysters (*C. gigas*): Sylt (north, n = 2 locations, Fig. 1a, b) and Texel (south, n = 4 locations, Fig. 1a, c). In the Dutch Delta (n = 6 locations, Fig. 1a, d), we concentrated on locations around the Oosterschelde estuary, the centre of oyster aquaculture in the Netherlands. At each location in the Wadden Sea and the Dutch Delta, at least 30 Pacific oysters (shell length 53.8–226.5 mm) and blue mussels (*M. edulis*; 15.5–65.5 mm) were collected by hand from mixed oyster/mussel beds. In addition, we collected other mollusc species in the surroundings (up to 500 m distance) of the mixed beds by sieving excavated sediment (see Table 1 for species list and shell lengths). (2) Due to the low availability of host species other than Pacific oysters and blue mussels in the Dutch Delta, we obtained samples from a monitoring program conducted by the research institute IMARES in this region in May 2012. These samples (mostly common cockles *C. edule*) were used in addition to samples we gathered ourselves from the Dutch Delta. Sampling took place within a grid (500 m distance between sampling points) in four areas in the Oosterschelde estuary (Van Zweeden et al. 2012; striped area in Fig. 1; Online Resource 2). At each sampling point, three samples were taken with a cockle shovel (Perdon and Troost 2012). Approximately 100 common cockles (11.5–46.0 mm) and all other bivalve species in each of the four areas were selected for dissection. (3) To ascertain the presence of previously found *M. orientalis* in common cockles (Marieke Feis, pers. comm.),

two additional sample batches of this host species were taken in summer 2012, one from a tidal flat near an oyster bed in the south of Texel (Mokbaai; Online Resource 3) and one from extensive tidal flats southeast of Texel (Balgzand), the latter being aggregated samples from five transects of 1 km length each (Dekker and Beukema 2007; Online Resource 4). Host samples were stored in plastic bags for up to 1 week at 4 °C (known to prevent cross-infections of *Mytilicola*; Dethlefsen 1972). When longer storage was necessary, samples were frozen for no longer than 3 months at −20 °C.

Parasite sampling

Before dissections, we measured the maximum length of each host shell with vernier callipers to the nearest 0.1 mm. Subsequently, the shells were opened and the tissue was first searched for the presence of *Mytilicola* individuals under a magnification glass (magnification 3–8×). Following this, the molluscan stomach and intestine were squeezed between two glass slides (to improve visibility of small *Mytilicola* individuals) and searched again under a stereo microscope (magnification 10–30×). With these two methods we were able to retrieve larger juveniles (1.5–2.3 mm) and adults (>2.3 mm, sexually mature from 2.8 mm (males) and 4.6 mm (females)), following Gee and Davey (1986) and Dethlefsen (1985). If present, copepods were carefully removed from the tissue and stored in 95 % denatured ethanol.

Parasite identification

Species identity of all individuals was morphologically ascertained under a stereo microscope (magnification 10–30×) by using the shape of the dorsal appendages as main differential character (pointy in the introduced *M. orientalis* and blunt in the established *M. intestinalis*; Gotto 2004; Elsner et al. 2011). Since the reliability of morphological identification is currently under investigation (Goedknegt et al., in prep.), subsamples from both parasite species were also molecularly identified with a diagnostic RFLP (restriction fragment length polymorphism) assay developed from taxon specific primers based on Elsner et al. (2011; see Online Resource 5 for details). During the main sampling in May, 921 adult *Mytilicola* were recovered from Pacific oysters and blue

Table 1 Sample sizes and mean shell length (±SE) per host species, region and location from sympatric mussel and oyster populations in the Wadden Sea and the Dutch Delta in May 2012 (12 locations, see Fig. 1 and Online Resources)

Region	Location	Pacific oyster (Crassostrea gigas)	Blue mussel (Mytilus edulis)	Common cockle (Cerastoderma edule)	Baltic tellin (Macoma balthica)	American slipper limpet (Crepidula fornicata)	Sand gaper (Mya arenaria)	American jack knife clam (Ensis directus)	Peppery furrow shell (Scrobicularia plana)	Common periwinkle (Litorina llittorea)	European flat oyster (Ostrea edulis)	Manila clam (Ruditapes philippinarum)
Wadden Sea	1. Sylt North**	**1/50**	**24/30**	0/50	0/45	0/16	0/12	0/25	–	0/50	–	–
	2. Sylt South	**3/30**	**29/30**	0/48	0/50	0/51	–	–	0/1	0/50	–	–
	3. De Cocksdorp	**5/30**	**2/30**	0/63	0/42	–	0/44	–	0/8	0/50	–	–
	4. Mokbaai A	**9/30**	**6/30**	0/50	0/23	–	0/10	–	0/14	0/50	–	–
	5. Balgzand C	**13/30**	**8/30**	**11/85**	**1/14**	–	0/7	–	–	0/50	–	–
	6. Balgzand A	**7/30**	**20/30**	0/50	**1/16**	–	–	–	–	0/50	–	–
	Balgzand B (transects)*	–	–	**12/242**	–	–	–	–	–	–	–	–
	Mokbaai B*	–	–	**16/145**	–	–	–	–	–	–	–	–
Dutch Delta	7. Grevelingen 1	**7/30**	**10/30**	–	–	–	–	–	–	0/6	–	–
	8. Grevelingen 2	**4/30**	**22/30**	0/17	–	0/17	–	–	–	0/67	0/1	–
	9. Schelphoek	**4/30**	**20/30**	0/1	–	0/1	–	–	–	0/3	–	–
	10. Zeelandbrug	**5/30**	**17/30**	–	–	–	–	–	–	–	–	–
	11. OS. kering	**11/30**	**15/30**	–	–	–	–	–	–	–	–	–
	12. Kattendijke	**3/30**	**17/30**	0/114	0/28	–	–	–	–	0/19	–	0/11
	OS south*	–	0/3	**5/100**	0/84	–	0/47	–	0/3	–	–	0/8
	OS central*	–	**13/26**	0/127	0/71	0/5	0/10	0/1	0/2	0/6	–	0/3
	OS outlet*	–	0/2	0/103	0/72	–	0/11	–	–	0/1	–	–
	OS north*	–	–	0/102	0/116	–	0/11	–	–	0/19	–	0/5
	Total	**72/380**	**203/391**	**44/1297**	**2/561**	0/132	0/152	0/26	0/28	0/421	0/1	0/27
Shell	Mean (mm)	118.9	43.7	14.9	27.0	32.0	17.6	124.8	27.6	43.7	6.7	27.9
Length	SE (mm)	1.3	0.4	0.1	0.2	0.7	1.4	6.5	1.8	0.4	–	2.2

Sample batches in which *Mytilicola* individuals were found are stated in bold, with the number of infected hosts in the numerator and the number of dissected individuals in the denominator. Additional samples from the Dutch Delta from May 2012 (4 locations) and from the Wadden Sea in August/September 2012 (2 locations) are all marked with an asterisk (*). Two asterisks (**) mark the location 'Sylt north' where 20 extra Pacific oysters were dissected, in response to the first batch of 30 Pacific oysters that appeared to be uninfected (n = 50 in total). OS = Oosterschelde estuary

mussels. For oysters and mussels, a subsample of 269 *Mytilicola* individuals was used for molecular analysis, although for 12 individuals the analysis failed, leaving 257 parasites molecularly identified to species level. More individuals were examined from mussel hosts (n = 166, excl. 6 that failed to amplify) than from oyster hosts (n = 91, excl. 6 that failed to amplify), because blue mussels harboured two morphospecies of *Mytilicola* and the Pacific oyster only one. The subsamples consisted of randomly picked samples from the Dutch Delta and the Wadden Sea. For the two other host species found to be infected by *Mytilicola*, we molecularly checked all *Mytilicola* per host individual (common cockles, n = 53, excl. 3 that failed to amplify) and Baltic tellins (*Macoma balthica*, n = 2). For all copepods that were molecularly checked for their species identity we also measured their body length. This was done by taking a picture with a camera (AxioCam ICc3) attached to a stereo microscope (Zeiss V8 discovery) and measuring body length with the software package AxioVision.

Statistical analysis

All statistical analyses were performed using the statistical software package R (R Development Core Team 2015). Model assumptions for all analyses were confirmed using diagnostic model plots (Zuur et al. 2010).

Spillover of M. orientalis

For all host species that were infected with the recently introduced *M. orientalis*, we calculated the overall prevalence of infection by dividing the total number of infected hosts by the total number of sampled hosts.

Regional differences in M. orientalis infections

For the 12 sympatric populations of mussels and oysters (main sampling campaign May 2012), we tested whether prevalences and intensities of *M. orientalis* were, relative to the Dutch Delta, on average higher in the Wadden Sea, where the parasite is more recently introduced and where native hosts are still relatively naïve to *Mytilicola* exposure. We tested this by the use of linear mixed models (LMMs) with a binomial (lme4 package; Bates et al. 2015), and a negative binomial (glmmADMB package; Fournier et al. 2012; Skaug et al. 2014) distribution to model infection prevalence

and intensity, respectively. In these models we used region (Wadden Sea or Dutch Delta), host species (blue mussels or Pacific oysters) and the interaction between these factors as fixed factors, and site nested within region as a random factor. For model selection we used backward elimination of non-significant fixed effects that explained little variation.

Additionally, we investigated expected relationships between prevalence and mean intensity of the introduced *M. orientalis* in the invasive Pacific oyster and in the native blue mussel with linear regressions.

Host competency and effect of host shell length on M. orientalis

For all hosts that were infected with the introduced parasitic copepod *M. orientalis*, we used linear models to investigate the relationships between body length of the molecularly identified *M. orientalis,* the sex of the parasite, host species and shell length. A post-hoc Tukey test was applied to test whether parasite body length differs between host species. Additionally, the relationship between the intensity of all (molecular and morphological identification) introduced *M. orientalis* individuals and host shell length was investigated with a GLM with a negative binomial distribution (MASS package, Venables and Ripley 2002).

Spillback of M. intestinalis

In blue mussels, we tested whether there was a difference in *M. intestinalis* prevalence and intensity between both regions with LMMs with binomial (lme4 package; Bates et al. 2015), and negative binomial distributions (glmmADMB package; Fournier et al. 2012; Skaug et al. 2014), respectively. In these models we used region (Wadden Sea or Dutch Delta) as fixed factor and location nested within region as random factor. Model selection was performed by using backward elimination of non-significant fixed effects that explained little variation.

Results

Parasite sampling

In total, we investigated 3416 individual hosts from 11 different host species for the presence of both

Mytilicola species (for sample sizes, numbers of infected individuals and average shell length per species see Table 1). In these 3416 host individuals we found a total of 1020 parasites of both *Mytilicola* species.

Parasite identification

For all the *Mytilicola* individuals that could be morphologically (sex and morphospecies) and molecularly identified (307 of the 327 individuals originating from the four principal host species that showed infections, i.e. Pacific oysters (*C. gigas*), blue mussels (*M. edulis*), common cockles (*C. edule*) and Baltic tellins (*M. balthica*), molecular results revealed that 90.0 % of females (n = 144 out of 160 females) and 78.9 % of males (115/147) were correctly identified to species level with morphological methods.

Spillover of *M. orientalis*

The introduced parasite *M. orientalis* (molecularly identified) was found in Pacific oysters (overall prevalence 18.9 %), blue mussels (31.3 %), common cockles (3.3 %) and Baltic tellins (0.4 %); (for site-specific infection levels see Table 1). No other mollusc species investigated (*Crepidula fornicata, Mya arenaria, Ensis directus, Scrobicularia plana, Littorina littorea, Ostrea edulis, Ruditapes philippinarum*) was infected with the invasive copepod.

Differences in *M. orientalis* infections between host species and regions

While Pacific oysters and blue mussels were infected with the introduced *M. orientalis* at almost all locations, the other two host species were only infected at a limited number of locations in the Wadden Sea: 4 out of 15 locations in common cockles and 2 out of 11 locations in Baltic tellins (Table 1). However, there were probably five infected common cockles in the frozen aggregated samples from the Dutch Delta ('OS south'; prevalence 5 %; mean intensity 1 ± 0), but morphological and molecular identification of the parasites was not possible due to frost damage. The range of prevalences of *M. orientalis* observed over all locations differed among the four host species (Pacific oysters 2–43 %, blue mussels 3–63 %, common cockles 0–13 %, and Baltic

tellins 0–7 %) as well as the mean intensities (Pacific oysters; 4.1 ± 0.6 SE, blue mussels; 2.8 ± 0.2, common cockles; 1.2 ± 0.1 and Baltic tellins; 1.0 ± 0).

Within the 12 locations with sympatric populations of Pacific oysters and blue mussels, prevalence of *M. orientalis* in Pacific oysters (mean prevalence $\pm$ SE, 19.9 ± 0.03 %) and blue mussels (24.0 ± 0.05 %) did not significantly differ between the two host species (LMM; $\chi^2 = 1.925$, $P = 0.165$), or between the two regions ($\chi^2 = 0.045$, $P = 0.832$; Fig. 2a; Table 2). There was also no significant interaction term between host species and region ($\chi^2 = 0.022$, $P = 0.883$; mean prevalence oysters: Dutch Delta 18.9 %, Wadden Sea 20.9 %; mussels Dutch Delta 22.8 %, Wadden Sea 25.2 %). Intensity of *M. orientalis* also did not differ between regions (LMM; $\chi^2 = 0.062$, $P = 0.803$), but it differed between host species, being, averaged over the two regions, almost twice as high in Pacific oysters (mean $\pm$ SE, 4.1 ± 0.6) compared to blue mussels (2.4 ± 0.3; $\chi^2 = 21.744$, $P < 0.001$; Fig. 2b; Table 2). No significant interaction term between regions and host species was detected ($\chi^2 = 0.272$, $P = 0.602$; mean intensity $\pm$ SE oysters Dutch Delta 4.0 ± 0.6, Wadden Sea 4.2 ± 1.0; mussels Dutch Delta 2.0 ± 0.3, Wadden Sea 2.2 ± 0.4).

Furthermore, when investigating the relationship between prevalence and intensity of *M. orientalis* in Pacific oysters and in blue mussels at the 12 sympatric populations (Table 3), we found no significant relationship for prevalence (linear regression; $P = 0.868$; Fig. 2a), but a positive relationship for intensity ($R^2 = 0.36$; $df = 10$, $P < 0.05$, Fig. 2b). In addition, the relationship between prevalence and intensity was significant for mussels (linear regression; $R^2 = 0.78$; $df = 10$, $P < 0.001$, see Online Resource 6a), but not for oysters ($P = 0.527$, see Online Resource 6b).

Host competency and effect of host shell length on *M. orientalis*

In general, sexually mature *M. orientalis* females (>4.6 mm) were found in all four host species, suggesting them to be generally competent hosts for *M. orientalis*. In the three host species where both parasite sexes were present (Pacific oysters, blue mussels, common cockles), females were always significantly larger than males (linear model $F_{1,194} = 485.90$, $P < 0.001$). In addition, there was a significant difference in parasite body size among the

Fig. 2 a Prevalence of *M. orientalis* in blue mussels (*M. edulis*) against prevalence of *M. orientalis* in Pacific oysters (*C. gigas*) and **b** mean intensity of *M. orientalis* in blue mussels against the mean intensity of *M. orientalis* in Pacific oysters. The black line (y = 0.5 x) indicates that the intensities in Pacific oysters are about twice as high as in blue mussels. *Circles* show values for the Dutch Delta, whereas *triangles* show the values for the Wadden Sea
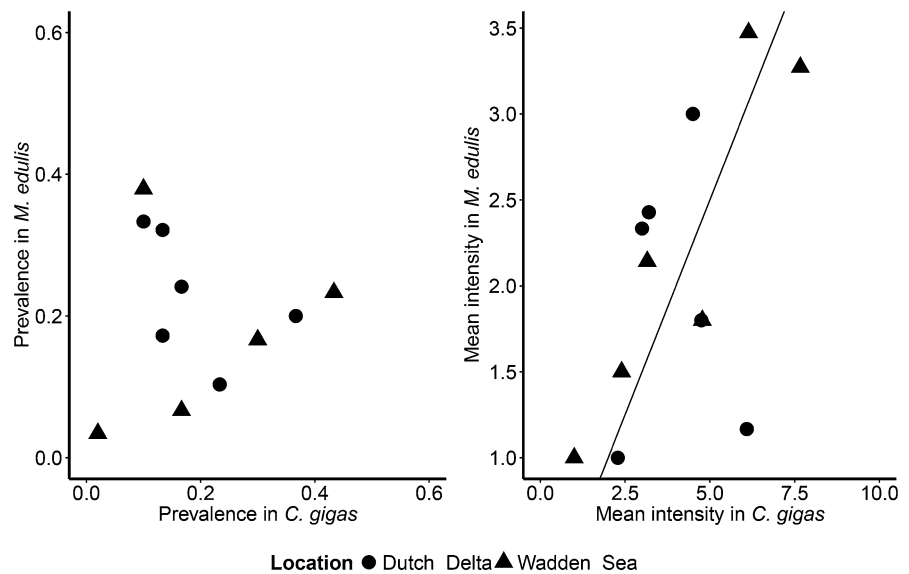
Table 2 Results of linear mixed models explaining variation in *M. orientalis* prevalence and intensity in bivalves. Model selection was performed by backwards elimination of non-significant variables and values shown are from the point at which each variable was removed from the model

	Variables	Coefficient	SE	χ^2	*P*
Prevalence model					
Fixed effects	Intercept	−1.457	0.190		
	Host species	0.231	0.264	1.925	0.165
	Region	−0.119	0.470	0.045	0.832
	Host species * Region	0.055	0.371	0.022	0.883
Random effects	Variables	Variance	SD		
	Intercept	0.000	0.000		
	Location (nested in Region)	0.864	0.929		
Intensity model					
Fixed effects	Intercept	1.439	0.132		
	Host species	−0.685	0.183	21.74	**<0.001**
	Location	−0.004	0.216	0.062	0.803
	Host species * Region	0.134	0.258	0.272	0.602
Random effects	Variables	Variance	SD		
	Intercept	0.012	0.109		
	Location (nested in Region)	0.067	0.258		

three host species ($F_{2,194} = 25.34$, $P < 0.001$), with the largest parasites in Pacific oysters, followed by blue mussels and common cockles (Tukey-test; Pacific oysters – common cockles, $P < 0.001$; Pacific oysters – blue mussels, $P < 0.05$; blue mussels – common cockles, $P < 0.001$). Only in blue mussels there was a significant relationship between host shell length and body length of *M. orientalis* individuals (linear regression, $R^2 = 0.86$, $F_{1,78} = 22.67$, $P < 0.001$), but not in the two other host species (Pacific oysters $F_{1,84} = 3.18$, $P = 0.078$; common cockles; $F_{1,32} = 0.195$, $P = 0.662$; Fig. 3).

Furthermore, the relationship between *M. orientalis* intensity and host shell length differed among host

Table 3 Prevalences and mean intensities of the parasite *M. orientalis* in 12 sympatric populations of blue mussels (*M. edulis*) and Pacific oysters (*C. gigas*) in May 2012. OS = Oosterschelde estuary

Region	Sampling site	Prevalence in oysters	Prevalence in mussels	Mean intensity in oysters	Mean intensity in mussels
Wadden Sea	1. Sylt North	0.02	0.03	1.0	1.0
	2. Sylt South	0.10	0.38	7.7	3.3
	3. De Cocksdorp	0.17	0.07	2.4	1.5
	4. Mokbaai A	0.30	0.17	4.8	1.8
	5. Balgzand C	0.43	0.23	3.2	2.1
	6. Balgzand A	0.23	0.63	6.1	3.5
Dutch Delta	7. Grevelingen 1	0.23	0.10	2.3	1.0
	8. Grevelingen 2	0.13	0.32	4.5	3.0
	9. Schelphoek	0.13	0.38	4.8	3.3
	10. Zeelandbrug	0.17	0.17	3.2	1.8
	11. OS. Kering	0.37	0.20	6.1	1.2
	12. Kattendijke	0.10	0.33	3.0	2.3

species. While for blue mussels (GLM; $R^2 = 0.11$, $\chi^2 = 10.91$, $P < 0.001$) and common cockles ($R^2 = 0.24$, $\chi^2 = 13.12$, $P < 0.001$) there was a weak but significant positive relationship, it was absent in Pacific oysters ($\chi^2 = 0.03$, $P = 0.857$, Fig. 4). The relationships between host shell length and parasite length, and host shell length and parasite intensity were not tested for Baltic tellins because of the small sample size for this species ($n = 2$).

Spillback of *M. intestinalis*

Of all investigated potential host species, including Pacific oysters ($n = 380$), we found only blue mussels to be infected with the previously established copepod *M. intestinalis*. The parasite was found in both regions in blue mussels ($n = 391$) with no significant difference in infestation (LLM; $\chi^2 = 1.588$, $P = 0.208$; mean prevalence Dutch Delta: 33.2 ± 5.9 SE %, Wadden Sea: 26.3 ± 14.1 %) and no significant difference in intensity (LLM; $\chi^2 = 2.844$, $P = 0.092$) between the two regions (mean intensity Dutch Delta 2.2 ± 0.3 SE, Wadden Sea 2.6 ± 0.6; Online Resource 7).

Discussion

Our extensive quantitative assessment of infections of more than 3400 potential hosts showed that the

parasitic copepod *M. orientalis*, which was co-introduced with the Pacific oyster (*C. gigas*), now also infects native blue mussels (*M. edulis*), common cockles (*C. edule*) and Baltic tellins (*M. balthica*) in European waters. Thereby this study adds to a growing amount of literature on parasite spillover from invasive to native species across terrestrial, freshwater and marine ecosystems worldwide (e.g. Warner 1968; Holdich and Reeve 1991; Bauer et al. 2002; Tompkins et al. 2002). *M. orientalis* has previously been reported to infect native blue mussels and European flat oysters (*O. edulis*; His 1979; Stock 1993). Unfortunately, our sample size of the European flat oyster was too low to confirm earlier observations of the parasite in this host species.

Our data indicate that blue mussels were infected with the introduced *M. orientalis* at a larger number of locations with much higher infection levels (prevalences and intensities) compared to the other two native host species, common cockles and Baltic tellins. Nevertheless, prevalences found in cockles and tellins should be high enough to sustain a parasite population, because in the native range in Japan, *M. orientalis* has similar prevalences in its native hosts and the local parasite populations are viable (M. Feis, unpublished data). Various reasons can explain the relatively low infection levels of introduced *M. orientalis* in native common cockles and Baltic tellins compared to native blue mussels. First of all, common cockles and Baltic tellins are not always positioned in
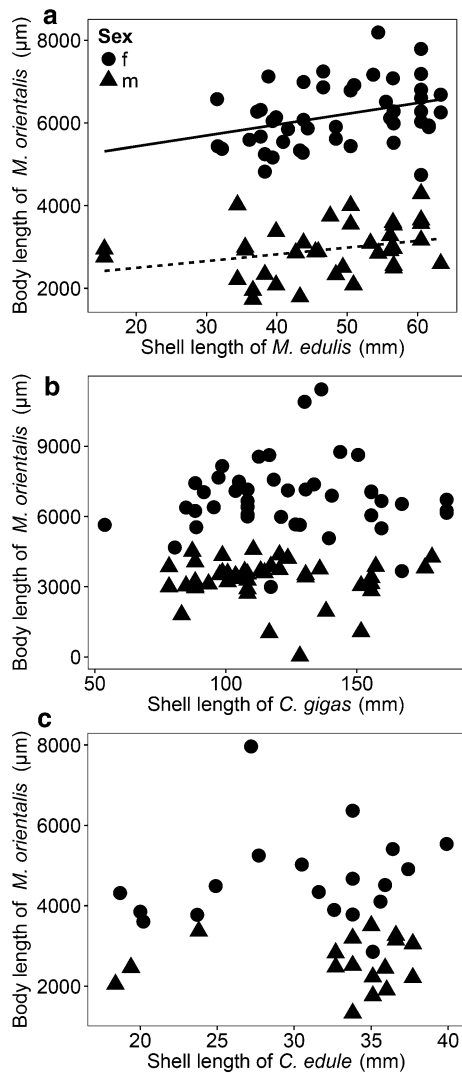
Fig. 3 Relationship between host shell length and body length of molecularly identified *M. orientalis* females and males for **a** blue mussels (*M. edulis*; females y = 4907.17 + 26.22 x, males y = 2168.34 + 26.22 x), **b** Pacific oysters (*C. gigas*) and **c** common cockles (*C. edule*)
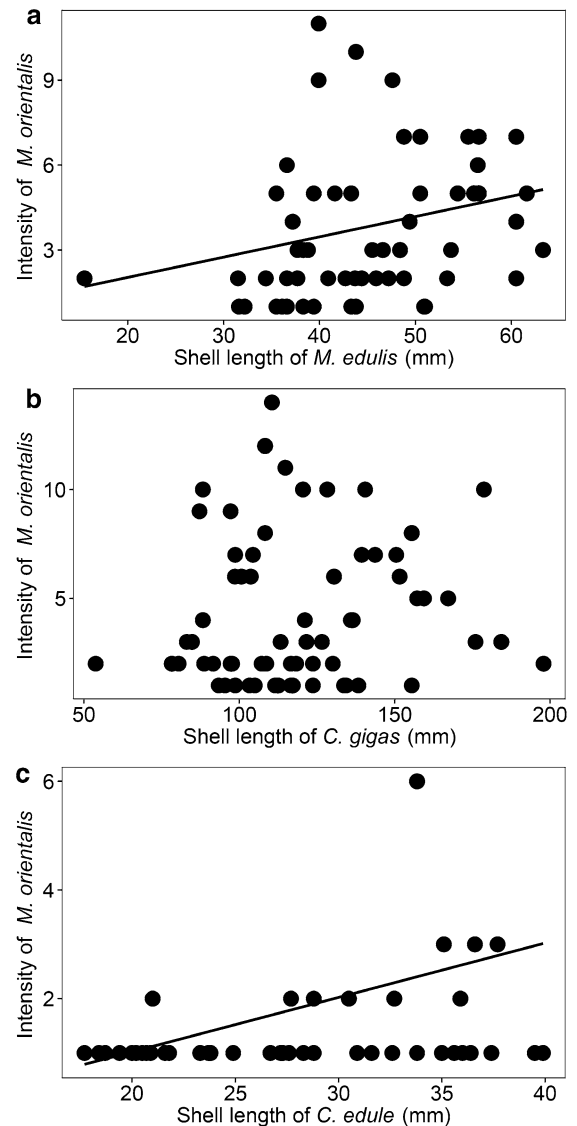

Fig. 4 Relationship between host shell length and *M. orientalis* intensity for **a** blue mussels (*M. edulis*; n = 177), **b** Pacific oysters (*C. gigas*; n = 191), and **c** common cockles (*C. edule*, n = 149). This analysis includes all morphologically and molecularly identified *M. orientalis* individuals

close vicinity of Pacific oysters compared to blue mussels which occupy the same habitat as oysters. Hence, the difference in distance between the source of infection (the Pacific oyster is the principal host of *M. orientalis*) could explain difference in infection levels between the three native species. However, larval stages of *M. orientalis* have a free-living phase and spend multiple days in the water column (based on *M. intestinalis*, Gee and Davey 1986; Goedknegt et al. in review), giving them the opportunity to disperse with currents. Consequently, the exposure to free-

living infective larvae might actually be similar for the three native host species and therefore the distance from infection source may not be the most plausible explanation for differences in infection levels between the three host species. Second, it could be that oysters and mussels attract larvae of *M. orientalis* from the plankton (e.g. by chemical cues), leading to an accumulation of infections in the main hosts. Thirdly, the endobenthic position and lower filtration rates of

cockles and tellins (compared to the epibenthic oysters and mussels; reviewed by Troost 2010) may result in lower encounter rates with infective larval stages of *M. orientalis.* This could also be the reason why introduced *M. orientalis* were not found in other potential host species which burrow deep in the sediment such as *M. arenaria, S. plana* and *E. directus,* although for these species sample size may have been too low for strong inference. Fourth, variation in infection levels of *M. orientalis* between the three native host species may be related to host size, with the small size of both species causing space constraint. Indeed, host shell length was a stronger driver of infection levels in common cockles compared to blue mussels. Finally, the difference in infection levels of *M. orientalis* in mussels, cockles and tellins could encompass the relatedness of these three host species to the Pacific oyster. Blue mussels (order: Mytilida) and Pacific oysters (order: Ostreida) both belong to the superorder Pteriomorphia, while common cockles and Baltic tellins both belong to the order Venerida and superorder Heteroconchia (Bouchet et al. 2010). Hence, blue mussels are more closely related to Pacific oysters than the other two host species, possibly explaining differences in host competency and infection levels. However, adult females carrying eggs were observed in all four hosts, suggesting a similar suitability of all host species for reproduction and growth but this remains to be investigated experimentally.

For the two principal hosts, blue mussels and Pacific oysters, the parasite *M. orientalis* displayed similar geographical distributions with comparable prevalences, but intensities were about twice as high in oysters as in mussels. This is not surprising given the generally smaller body size of blue mussels and their intestines compared to Pacific oysters, resulting in limited space for infections by the intestinal parasites in mussel hosts (mean body size—without egg sacks—of *M. orientalis* is approximately 4 mm in males and 10 mm in females; Grizel 1985). Such a space constraint may also underlie the observation that parasite body size was positively correlated with mussel shell length, while this was not the case for parasites in oysters. However, some care needs to be taken with interpreting these results, as parasites were conserved with different conservation methods which may affect body length measurements (e.g. Thorstad et al. 2007; Goedknegt et al. in prep). In general, mussel size only weakly explained *Mytilicola*

intensity in our data set so that other factors besides size may actually be stronger determinants of infection levels. One of these factors could be host competency which may differ between oysters and mussels. Nonetheless, egg-carrying females were observed both in mussels and oysters and first attempts to hatch eggs and raise copepodid larvae to infective stages originating from egg sacks of *M. orientalis* individuals in blue mussels have been successful, suggesting that blue mussels are a competent host for the parasite (Goedknegt et al. in review). Another potential factor driving infection levels of the invasive copepod may be competition with the previously established *M. intestinalis.* Yet, the relatively few cases of double infections observed were not more or less frequent than expected on a random basis. Therefore, based on this dataset, interspecific competitive exclusion between both parasite species within blue mussels seems to be unlikely. Nevertheless, experimental studies are needed to investigate the detailed modes of interaction between both parasite species.

Our data from 12 sympatric populations of blue mussels and Pacific oysters show that prevalences of the introduced copepod *M. orientalis* did not differ between the two regions within each host species. This suggests that the introduced copepod *M. orientalis* is already well established in the Wadden Sea, although the invasion of Pacific oysters of the Wadden Sea began 10–20 years later than that of the Dutch Delta. However, we observed relatively low infection levels of *M. orientalis* in northern populations of Pacific oysters in the Wadden Sea around the island of Sylt (see also Elsner et al. 2011). Whether this results from a later invasion of this region or differential susceptibility of the genetically distinct northern Pacific oyster populations (Moehler et al. 2011) remains to be investigated. In any case, the 12 sympatric populations showed a strong relationship between infection levels (intensity) in Pacific oysters and blue mussels, suggesting that *M. orientalis* infections in oysters may drive infection levels in mussels. However, mussels could also be an additional source of infection as copepodid stages raised from egg sacks of introduced *M. orientalis* in native blue mussels can experimentally infect conspecifics (Goedknegt et al. in review). The positive relationship between infection intensity of both main host species may also suggest that similar biotic and/or abiotic mechanisms drive infection patterns of both species. However, the

current dataset is too limited to investigate this further. Field studies investigating infections in both host species, including additional variables as host densities and environmental conditions, as well as controlled experiments will be needed to investigate the drivers of spillover dynamics of the introduced parasite *M. orientalis*.

The risk of parasite spillover may have severe consequences for the new native host species. Although early studies on the impacts of *Mytilicola* species on, for example, host filtration rates, oxygen consumption and condition index led to controversial results (reviewed by Lauckner 1983), later studies indicated negative effects on host condition (Theisen 1987; Kijewski et al. 2011; Camacho et al. 1997; Feis et al. 2016; Goedknegt et al. in review). Furthermore, both *Mytilicola* species are known to cause changes in cells of the gut epithelium of both blue mussels (*M. intestinalis;* Sparks 1962; Moore et al. 1978) and Pacific oysters (*M. orientalis*; reviewed by Lauckner 1983) that can lead to inflammations of the mucosa and sub-mucosa in the intestines (Watermann et al. 2008). If negative effects of the introduced copepod *M. orientalis* on native blue mussels exist, this may exaggerate the competition between the mussels and the invasive Pacific oysters and may play a role in the complex spatial and temporal population dynamics commonly observed on natural mussel beds (Folmer et al. 2014). Effects of *Mytilicola* species on common cockles and Baltic tellins have not been studied but seem likely given the fact that the copepods occupy the entire intestine of these relatively small species (observation by A-K. Schuster). However, experimental studies based on controlled infections of hosts are needed to be able to make further inferences about the direct impact of spillover of the introduced *M. orientalis* on native hosts.

While the introduced *M. orientalis* showed spillover to a broad native host range, the previously established *M. intestinalis* was exclusively found in blue mussels. This is in contrast to the literature, where a wide range of mollusc host species has been reported for this copepod species in Europe: *M. edulis, M. galloprovincialis, Ruditapes decussatus, Modiolus barbatus, O. edulis, C. gigas, C. edule, Dosinia exoleta, C. fornicata* and *Venerupis corrugata* (Steuer 1902; Hepper 1953; Lauckner 1983; Aguirre-Macedo and Kennedy 1999; Gotto 2004; Dabouineau and Ponsero 2009). However, due to the difficulties of

morphological identification of the two species and potential unawareness of investigators of the additional introduction of *M. orientalis*, some of the host species noted in the literature may actually be false positives (Elsner et al. 2011). In our study area, previous studies have only reported the blue mussel, the European flat oyster *O. edulis* and the American slipper limpet *C. fornicata* as hosts for the previously established *M. intestinalis* (Stock 1965, 1993). The parasite was not found in the latter two host species during our sampling campaign, yet this could also be due to the small sample sizes of these hosts. Nowadays, infections seem to be exclusively restricted to blue mussels, also indicated by two recent studies in the Wadden Sea that found the previously established *M. intestinalis* only in mussels and not in Pacific oysters (Elsner et al. 2011; Pogoda et al. 2012). Since infections of the invasive Pacific oyster with the previously established *M. intestinalis* are the basic requirement for parasite spillback, our and other data from the literature suggest that the potential for spillback of this parasite species to the native fauna via Pacific oysters is extremely limited.

As a potential incompetent host for *M. intestinalis*, the Pacific oyster could still attract free-living infective stages and thereby act as a sink for the parasite. Results of experimental infections have shown that it is possible to experimentally infect a Pacific oyster with *M. intestinalis* copepodites, although the infection success was extremely low. In this experimental trial, 25 copepodites were added to each container with one oyster (n = 20 replicates) and after 133 days only one oyster was infected with one *M. intestinalis* parasite, with no remaining copepodites found in the water (M. Feis, unpublished data). This result suggests that the oyster could act as a potential diluter of *M. intestinalis* parasites, thereby reducing disease risk for native blue mussels. Therefore, it would be interesting to examine whether *M. intestinalis* infections in blue mussels have declined after the arrival of the Pacific oyster. Of all the locations investigated, qualitative historical data is only available for the northern German Wadden Sea (Sylt), where *M. intestinalis* prevalence was <5 % with a mean intensity of two parasites per infected mussel (Dethlefsen 1972), which is considerably lower than the 69 % prevalence and mean of 3.7 parasites per infected mussel found in our study (after the arrival of the Pacific oyster). This suggests that the Pacific oyster may not play a large

role in *M. intestinalis* dilution under natural conditions in the field, but controlled field experiments would help to further clarify this.

Similar to the introduced *M. orientalis,* we observed no difference in prevalence of previously established *M. intestinalis* between the two study regions, the Dutch Delta and the Wadden Sea. Hence, *M. intestinalis* seems to be well established in both regions. However, *M. intestinalis* intensity was slightly higher in the Wadden Sea. This resulted from the particularly high prevalences in blue mussels around the island of Sylt found in this study (69–72 %), that were also previously reported from the northern Wadden Sea (Thieltges et al. 2006; Elsner et al. 2011), while sites from the inner German Bight show intermediate infection levels (prevalence about 25 %; Pogoda et al. 2012). For the Texel region, previous data are, unfortunately, missing (Drinkwaard 1999) but our data tentatively suggest a north–south infection gradient of *M. intestinalis* in the Wadden Sea.

In this study, species identification of both *Mytilicola* species was based on morphological and (partial) molecular identification, the latter revealing an accuracy in morphological identification of 90.0 % for females and 78.9 % for males. Although morphological identification does not seem to be 100 % reliable, these inaccuracies do not affect our general conclusions due to the following: (1) all *M. orientalis* individuals from newly discovered hosts (Baltic tellins and common cockles) were molecularly identified, so that we could ascertain spillover from Pacific oysters; and (2) from the 91 *Mytilicola* individuals from Pacific oysters that were molecularly identified, none appeared to be the previously established *M. intestinalis*, which confirms low or no risk of parasite spillback mediated by the Pacific oyster. The exact prevalences and intensities of both *Mytilicola* species in blue mussels and Pacific oysters would probably slightly differ if all *Mytilicola* individuals had been identified with molecular methods. However, as these minor differences appeared to be unbiased, they are unlikely to affect the validity of the results obtained here.

In conclusion, our study revealed widespread spillover of *M. orientalis*, which was co-introduced with Pacific oysters, to native common cockles and Baltic tellins in addition to blue mussels, from which spillover was already reported (Elsner et al. 2011;

Pogoda et al. 2012). Second, our study showed that *M. orientalis* infections in Pacific oysters and blue mussels do not differ between the two study regions (the Dutch Delta and the Wadden Sea) despite their different invasion histories. However, within the Wadden Sea region there was a gradient with higher infection levels in the south (Texel) than in the north (Sylt), probably reflecting the ongoing invasion process from south to north. Finally, we showed that the risk for spillback of the previously established *M. intestinalis* via infections of Pacific oysters is very low as this copepod was absent from oysters. In contrast, the Pacific oyster may rather act as sink for the parasite, thereby reducing disease risk for native blue mussels. However, our data from sites with high prevalence of *M. intestinalis* infections suggest that dilution by invasive Pacific oysters might be a comparatively small effect for overall parasite population growth. The high occurrence of parasite spillover suggests that Pacific oysters may have strong indirect impacts on native ecosystems and future studies will be needed to investigate the effects of the introduced *M. orientalis* infections on native hosts. In addition, the exact biotic and abiotic drivers behind the spillover of *M. orientalis* from Pacific oysters to native hosts are thus far unknown and this remains a topic for future studies. To investigate these mechanisms on larger temporal and spatial scales, more extensive datasets are required that need to be analysed in an individual-based modelling framework (e.g. Wells 2015). Studying dynamics, drivers and effects of spillover of co-introduced parasites like *M. orientalis* will improve our understanding of the overall direct and indirect effects of invasive species, such as the Pacific oyster, in ecosystems worldwide.

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