REVIEW

Amphipod parasites may bias results of ecotoxicological research

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ABSTRACT: Amphipods are commonly used test organisms in ecotoxicological studies. Nevertheless, their naturally occurring parasites have mostly been neglected in these investigations, even though several groups of parasites can have a multitude of effects, e.g. on host survival, physiology, or behavior. In the present review, we summarize the knowledge on the effects of Microsporidia and Acanthocephala, 2 common and abundant groups of parasites in amphipods, on the outcome of ecotoxicological studies. Parasites can have significant effects on toxicological endpoints (e.g. mortality, biochemical markers) that are unexpected in some cases (e.g. down-regulation of heat shock protein 70 response in infected individuals). Therefore, parasites can bias the interpretation of results, for example if populations with different parasite profiles are compared, or if toxicological effects are masked by parasite effects. With the present review, we would like to encourage ecotoxicologists to consider parasites as an additional factor if field-collected test organisms are analyzed for biomarkers. Additionally, we suggest intensification of research activities on the effects of parasites in amphipods in connection with other stressors to disentangle parasite and pollution effects and to improve our understanding of parasite effects in this host taxon.

KEY WORDS: Test organism · Stress response · Accumulation · Pollution · Gammarid · LC_{50} · Microsporidia · Acanthocephala

1. INTRODUCTION

Parasites are abundant in all types of aquatic ecosystems and affect all groups of free-living animals in multiple ways (Marcogliese 2004, Kuris et al. 2008, Sures et al. 2017a). Parasites affect the well-being and physiological homeostasis of their hosts, which can lead to death of the infected host individual (Wallage et al. 2001, Sures 2006, Sures & Radszuweit 2007, Shirakashi & El-Matbouli 2009, Frank et al. 2011, 2013, Filipović Marijić et al. 2013). Nevertheless, parasites have usually been ignored in most ecotoxicological studies (Marcogliese & Pietrock 2011). In ecotoxicology, one approach is to relate the exposure to and uptake of pollutants with physiological responses of organisms. This discipline therefore addresses the fact that free-living biota is threatened by environmental stressors such as pollutants. These stressors may lead to changes in the physiology and therefore might have adverse effects on affected organisms. If these effects are measurable, they can be used for effect indication and are called biomarkers (Connon et al. 2012). In recent years, some working groups have raised awareness of combined effects of parasites and adverse environmental conditions on organisms used for field or laboratory experiments (Marcogliese 2004, Sures 2004, 2006, 2008a,b, Shirakashi & El-Matbouli 2010, Marcogliese & Pietrock 2011, Sures et al. 2017b).

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From the multitude of host–parasite systems that have been investigated to date concerning the effect of parasites on the biomarker response of the host (see review by Sures et al. 2017b and references therein), we selected amphipods as an example to illustrate the general problem of parasites (specifically Microsporidia and Acanthocephala) interfering with the effects of exposure to pollutants. Various species of amphipods are common test organisms in ecotoxicological studies, both in field and laboratory approaches, and have been used to assess the toxicity of a wide variety of substances (Kunz et al. 2010). Many factors can influence the stress response of amphipods as test organisms and can thereby confound the results of ecotoxicological studies. For example, the sensitivity of different amphipod species varies considerably. Experimental results have shown that congeneric species can differ in their response to toxicants (Alonso et al. 2010), but even different lineages of the same species can react differently under the same exposure conditions (Soucek et al. 2013). Furthermore, the stage of development (García et al. 2010, Charron et al. 2014), amphipod sex, and season can influence the outcome of ecotoxicological experiments (Sornom et al. 2010, Sroda & Cossu-Leguille 2011, Gismondi et al. 2012a, 2013). Also, species interactions such as inter- and intraspecific competition or predation can modulate the response of amphipods to chemical exposure (van den Brink et al. 2017), but parasites have only rarely been considered as an influencing factor in ecotoxicological studies (e.g. Gismondi et al. 2012b–f, Grabner et al. 2014, Chen et al. 2015).

Amphipods host a variety of pathogens such as viruses, bacteria, and uni- and multicellular parasites (e.g. Ovcharenko et al. 2008, Bojko et al. 2013, 2017, Grabner et al. 2015, Grabner 2017), among which acanthocephalans and microsporidians have been studied in more detail, also concerning their effects on metabolism and stress response of their host (e.g. Sures & Radszuweit 2007, Gismondi et al. 2012b–e, Grabner et al. 2014, Chen et al. 2015).

Microsporidians are microparasites that belong to the group Opisthosporidia, a sister group of the Fungi (Karpov et al. 2014). Depending on the microsporidian species, they can develop in various host tissues where they form spores that are infective for the next host (horizontal transmission). Some microsporidians are transmitted vertically from the mother to the offspring (Dunn & Smith 2001, Smith 2009). Acanthocephalans, or 'thorny-headed worms,' are a group of helminth parasites with complex life cycles including an invertebrate and a vertebrate host (Sures 2014).

Arthropods, such as crustaceans or insects, serve as intermediate hosts, whereas all classes of vertebrates are known as final hosts, in which reproduction occurs in the intestine (Fig. 1). In some species, additional paratenic hosts can exist (Taraschewski 2000, Kennedy 2006, Sures 2014). Compared to microsporidians, acanthocephalans grow to a large size, when developing from the acanthor, over the acanthella to the cystacanth larva in the body cavity of their invertebrate host (Taraschewski 2000).

We selected Microsporidia and Acanthocephala for the purpose of the present review to illustrate the possible impact of parasites on the outcome of ecotoxicological studies using amphipods as test organisms, thereby increasing the awareness that neglecting parasites in ecotoxicological studies can distort or disguise the actual exposure effects.

2. PARASITE EFFECTS ON THE AMPHIPOD HOST

In this section, we summarize the most important impacts of microsporidians and acanthocephalans on their hosts, which could also bias the outcome of ecotoxicological studies.
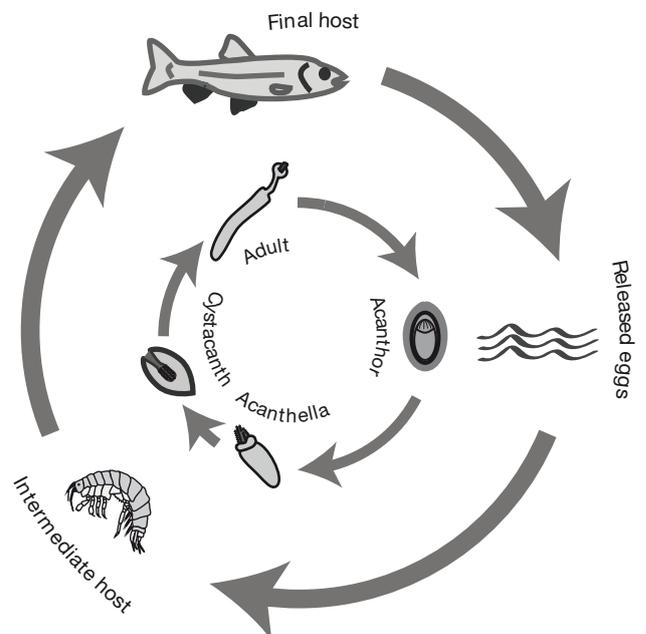

Fig. 1. Life cycle of an aquatic acanthocephalan of the genus *Pomphorhynchus*. The adult worm lives in the intestine of the final host. Females and males mate and eggs are produced that are released with the feces. The eggs are consumed by the intermediate host and the acanthella and later the cystacanth larvae develop. The final host becomes infected by consuming the infected intermediate host. Illustration by Dr. Nadine Ruchter; reproduced from Sures (2014; used by permission)

2.1. Microsporidia

Infections with microsporidians are common in amphipods from all types of aquatic ecosystems (Bulnheim 1975, Krebes et al. 2010, Stentiford et al. 2013, Grabner et al. 2015, Weigand et al. 2016, Bącela-Spychalska et al. 2018), including species with horizontal transmission from one individual to the next via infective spores, and species that are transmitted vertically from the ovaries of the females directly to the offspring (Terry et al. 2004). In invertebrates, microsporidians can be found in various tissues such as muscle, digestive tract, connective tissue, and gonads, depending on the microsporidian species (Stentiford & Dunn 2014). The effects of microsporidian infections can range from asymptomatic to highly pathogenic (Stentiford & Dunn 2014), but even microsporidians that do not have significant effects on adult amphipods, even under stressful conditions, can impair early life stages (Kelly et al. 2003). Therefore, if endpoints such as growth rate are used for toxicological experiments, microsporidians could affect the results. Furthermore, microsporidians can influence experiments with different temperature settings, as their rate of replication increases at higher temperature (Dunn et al. 2006). This, in turn, can affect the results of toxicological endpoints such as biomarker responses (Grabner et al. 2014). Some microsporidians are also able to modify the behavior of their amphipod host (Bącela-Spychalska et al. 2014), which can be particularly relevant in assays where the activity of the test organism is measured (Kunz et al. 2010). The most immediate effect of microsporidian infections is an increase in mortality of infected hosts. In some cases, only males are affected, leading to a distorted sex ratio in vertically transmitted species (see below), and in horizontally transmitted species mortality can be caused by massive multiplication of spores, e.g. in muscle tissue (Dunn & Smith 2001, Smith 2009, Bącela-Spychalska et al. 2014, Stentiford & Dunn 2014, Bojko et al. 2018). Parasite-induced mortality will clearly confound the conclusions drawn from toxicological experiments, especially when parasite load and prevalence differ between different test populations or over time.

A particularly interesting trait of many microsporidian species is their effect on the ratio of male and female hosts in a population by selectively high virulence in male individuals ('male-killing') or by modifying host sex, resulting in intersex individuals. This behavior is mostly related to vertical transmission, when the parasite relies on the direct transfer

from the mother to the offspring (Dunn & Smith 2001, Smith 2009). Deviations from the expected sex ratio have been described several times from amphipods infected with vertically transmitted microsporidian species (e.g. Dunn et al. 1995, Terry et al. 1998, 2004, Grilo & Rosa 2017). The mechanism of the parasite-induced sex reversal is related to the impairment of the androgenic gland in the infected amphipods (Rodgers-Gray et al. 2004, Jahnke et al. 2013). In relation to ecotoxicological questions, such changes in the manifestation of host sex due to microsporidian infections is particularly relevant in studies addressing chemicals that act on the endocrine system (endocrine disruptors, EDs). To date, several studies have investigated the effect of EDs on amphipods (reviewed by Kunz et al. 2010, Grilo & Rosa 2017), but additional infection with microsporidians as a confounding factor was only rarely considered (Ford et al. 2006, Gismondi et al. 2017). Accordingly, it is generally accepted that the phenomenon of intersex in crustaceans can be induced by various factors, including chemicals and parasites, but the proximate cause for intersex individuals is often not clear (Ford et al. 2004, Ford & Fernandes 2005, Hyne 2011, Ford 2012, Short et al. 2012a,b).

2.2. Acanthocephala

Acanthocephalans are metazoan parasites that are trophically transmitted from prey to predator (Fig. 1). Many of the known acanthocephalan species occur as adults in the intestines of aquatic hosts such as fish, while intermediate hosts are often amphipods (Sures 2014). Young larvae (usually called acanthella) inside the hemocoel of the crustacean intermediate host develop into the cystacanth stage, which is the infective stage for the final host. Acanthocephalan larvae in amphipods affect their host in various ways. First, the growth of the cystacanth stage requires considerable amounts of energy from the host, which leads to parasite-related changes in the host energy metabolism. For example, a decrease in protein and lipid content and an increase in glycogen content was consistently measured in amphipods infected with acanthocephalans (Plaistow et al. 2001, Gismondi et al. 2012b,c, Chen et al. 2015). Also, the acanthocephalan cystacanths are able to modify the food source used by infected amphipods as well as their feeding activity (McCahon et al. 1988, Pascoe et al. 1995, Médoc et al. 2011, Dianne et al. 2014, Labaude et al. 2017). Another effect of acanthocephalan infections that might confound results of eco-

toxicological studies is the increased salinity toler-ance of infected *Gammarus roeselii* (Piscart et al. 2007). Furthermore, Gismondi et al. (2012b) showed that *G. roeselii* infected with cystacanths of *Polymorphus minutus* might be more susceptible to toxicant-related effects than uninfected individuals due to the decreased concentration of reduced glutathione and reduced activity of γ-glutamylcysteine ligase in the tissue of infected hosts. Several other effects of acanthocephalans on the biochemistry of their amphipod host are described in the literature. For example, they are able to reduce the activity of the prophenoloxidase system, leading to an impairment of the function of the host immune system (Cornet et al. 2009). Acanthocephalan larvae can also have effects on the reproduction of their amphipod host by affecting the hormone levels (elevated estrogen and depressed testosterone levels) of infected males, which might affect reproductive success (Lewis et al. 2016). Particularly the reproduction of female amphipods can be severely impaired by acanthocephalans. Although cystacanths of the acanthocephalan *Pomphorhynchus laevis* reduced the fecundity of infected females, *P. minutus* completely castrated their female hosts (Bollache et al. 2002, Bailly et al. 2018). This could lead to false conclusions in ecotoxicological studies, for example when studying the effects of EDs in an infected amphipod population.

One of the best studied effects of acanthocephalan larvae on the physiology of the amphipod host is the modulation of serotonin metabolism that causes changes in host behavior (e.g. Perrot-Minnot et al. 2007, Helluy 2013). This behavioral manipulation increases the likelihood of trophic transmission of the parasite to the next host. To date it is not clear whether these metabolic changes also affect the response of the host to chemical exposure, but e.g. Sornom et al. (2012) showed that cadmium also affects the behavior of amphipods and can lead to changes in the anti-predator response, an effect that is similar to the manipulation by the parasite.

3. COMBINED EFFECTS OF POLLUTANTS AND PARASITES

Over the past 2 decades, there has been an increasing interest in the combined effects of parasites and pollutants on the health of organisms, leading to the establishment of 'Environmental Parasitology' as a discipline in parasitology (Sures et al. 2017b). Within this field, it has emerged that unpredictable or contradictory results occur if infected animals are used in ecotoxicological research without considering possible effects of parasites. In the following subsections, those ecotoxicological studies using amphipods as test organisms are summarized and include additional effects of either microsporidians, acanthocephalans, or both (see Tables 1–3).

3.1. Microsporidia

Even though microsporidian infections are rather the rule than the exception in amphipod populations, very few studies have addressed effects of chemical exposure in combination with infection status (Table 1). Ford et al. (2006) investigated the frequency of intersex individuals in the amphipod *Echinogammarus marinus* in relation to sites affected by industrial pollution and microsporidian infections. Intersex individuals were found more frequently at polluted sites, and the amphipods with intersex characteristics were also up to 16 times more likely to be infected with microsporidians; the final cause for the observed intersexuality seems to be multifactorial (Ford et al.

Table 1. Studies on combined effects of pollutants and microsporidians on amphipods. NI: no species identification; Ex: laboratory experiment; N: natural population; HSP: heat shock protein

Amphipod species	Parasite species	Exposure	Effects	Reference
Echinogammarus marinus	Microsporidia (NI)	Industrial pollution (N)	Higher rate of intersex gammarids at polluted sites; higher prevalence of microsporidians in intersex individuals	Ford et al. (2006)
Gammarus roeselii	*Dictyocoela muelleri* and *D. roeselum*	Cadmium (Ex)	Increased levels of lipid peroxidation, decrease of energy reserves and antitoxic defense in infected amphipods exposed to cadmium; no significant effects of infection without exposure	Gismondi et al. (2012d)
Gammarus fossarum	*Dictyocoela duebenum*	Cadmium (Ex)	Increased HSP70 levels in exposed and infected individuals	Chen et al. (2015)

2006). In an experimental study, 3-fold increased levels of cell damage (lipid peroxidation) were detected after cadmium exposure (2 µg l^{-1}) in the amphipod *Gammarus roeselii* infected with the microsporidians *Dictyocoela muelleri* and *D. roeselum* compared to a 2-fold increase due to the cadmium treatment alone. Furthermore, the energy reserves and several markers for the antitoxic defense capabilities showed a significantly decreased response in the microsporidian-infected amphipods exposed to cadmium, while the microsporidian infection treatment alone did not have significant effects (Gismondi et al. 2012d). A similar pattern was observed for the response of the heat shock protein 70 (HSP70) in *G. fossarum* infected with the microsporidian *D. duebenum*. About 2-fold increased HSP70 levels after cadmium exposure were only detected in the microsporidian-infected amphipods, not in the exposed but uninfected ones (Chen et al. 2015). All of these results show that microsporidians can affect the host response under exposure conditions, even if they are asymptomatic without an additional stressor. Moreover, it has to be noted that it is not possible to remove microsporidian stages from host tissues prior to the analysis of the desired markers. Therefore, the results always reflect a mixture of host and parasite proteins/enzymes.

3.2. Acanthocephala

Several studies exist on the accumulation and effects of metals in amphipods parasitized with acanthocephalan larvae. The accumulation pattern seems to depend on the type of metal used for the exposure. While cadmium and lead are accumulated less in the larval parasites compared to the host tissues (Brown & Pascoe 1989, Siddall & Sures 1998, Gismondi et al. 2012f, Frank et al. 2013, Chen et al. 2015), palladium accumulation is up to 10 times higher in the cystacanth larvae compared to the amphipod host (Sures & Radszuweit 2007). Interestingly, the palladium concentration in the tissues of infected hosts (after removal of the parasite) was 2 times higher than the concentration in the uninfected amphipods (Sures & Radszuweit 2007). These results are somewhat contradictory to studies on metal accumulation patterns in adult acanthocephalans, which usually have higher levels of metals than their hosts (Sures & Siddall 2003, Nachev & Sures 2016, Sures et al. 2017b). Moreover, the very high metal accumulation in adult acanthocephalans may lead to reduced metal concentrations in host tissues (Sures & Siddall 1999, Sures et al. 2003, Filipović Marijić et al. 2014).

The results of several studies showed that the mortality rate of amphipods infected with acanthocephalan larvae (*Pomphorhynchus laevis* or *P. minutus*) increased about 2-fold (i.e. the lethal dose that killed 50 % of the test animals [LC$_{50}$] was more than 2 times lower) compared to uninfected amphipods at low cadmium concentrations (2.1–4 µg l^{-1}; McCahon et al. 1988, Frank et al. 2013). In contrast, at 6 µg l^{-1} cadmium or more, no difference in the mortality between uninfected and infected individuals was recorded (Brown & Pascoe 1989). A more than 2 times higher host mortality in *P. laevis*-infected vs. uninfected individuals was also observed in amphipods (*G. pulex*) in a natural stream after experimental exposure to aluminum and low pH (McCahon et al. 1989, McCahon & Poulton 1991). Interestingly, in the study of Sures & Radszuweit (2007), the mortality of *G. roeselii* was reduced in both palladium-unexposed (20 % lower mortality compared to uninfected individuals) and exposed groups (10 % lower mortality compared to uninfected individuals), when the animals were infected with larvae of *P. minutus*. Similar observations were made by Gismondi et al. (2012f) for the same parasite–host system. In their study, the mortality of infected male gammarids decreased 2-fold under cadmium exposure compared to uninfected individuals. In turn, infected females seemed to be 3 times more sensitive to cadmium. Possible explanations for these conflicting observations of either increased or decreased mortality of infected amphipods might be the metal used (palladium vs. cadmium), or the parasite–host system (*G. pulex*–*P. laevis* or *G. fossarum*–*P. minutus* vs. *G. roeselii*–*P. minutus*). Furthermore, different lineages of *P. minutus* exist in different amphipod species that might potentially differ in their virulence (Zittel et al. 2018).

A number of sublethal effects have been recorded for acanthocephalan-infected amphipods under chemical exposure. A behavioral effect due to cadmium exposure is the shortening of the separation time of amphipod precopula pairs. This effect was further enhanced in individuals infected with *P. laevis* (Poulton & Pascoe 1990). At the biochemical level, *P. minutus*-infected *G. roeselii* (only females tested) showed higher cell damage (2-fold increase of lipid peroxidation at 2 µg l^{-1} cadmium) and a concentration-dependent increase of reduced glutathione levels compared to uninfected animals after cadmium exposure (Gismondi et al. 2012c). In the same study, the metallothionein level increased with cadmium concentration, but there was no significant difference related to infection status. A similar metallothionein response

to cadmium was found by Frank et al. (2013) in *G. fossarum* infected with *P. minutus*.

A general biochemical stress marker that was tested in several studies with acanthocephalan-infected amphipods is HSP70. Elevated levels of HSP70 were detected in *G. fossarum* infected with *P. minutus*, but the HSP70 level decreased more than 5-fold in infected individuals that were additionally exposed to cadmium (4 µg l^{-1}), compared to the cadmium exposure alone (Frank et al. 2013). In contrast to these findings, Chen et al. (2015) did not detect a significant HSP70 response in *G. fossarum* infected with *P. minutus* with and without cadmium exposure in a similar exposure experiment with the same cadmium concentration. The reason for these different results of the 2 studies is not clear but could be due to the effect of additional parasites. Frank et al. (2013) did not test for microsporidian infections in the amphipods, whereas Chen et al. (2015) grouped microsporidian-infected and uninfected individuals. Furthermore, there could be unknown factors like seasonal differences (Prato & Biandolino 2009) or differences between the populations of *G. fossarum* used for the experiments. Interestingly, *P. minutus*-infected *G. roeselii* seemed to cease their HSP70 production completely, even after palladium exposure or heat stress, while uninfected controls showed an HSP70 response to both stressors (Sures & Radszuweit 2007). Apparently there exists a wide variety of intermediate host reactions to acanthocephalan infections, depending on the host–parasite system, additional stressors, and other yet unknown factors (Table 2).

3.3. Effects of co-infections with microsporidians and acanthocephalans in the same host

In natural systems, organisms will not only be infected by single parasites, but will be affected by numerous pathogens at the same time. These can influence each other, leading to a highly complex response to chemical exposure. To our knowledge, only 2 studies have investigated the effects of simultaneous infection with microsporidians and acanthocephalans in combination with chemical exposure (Table 3).

Chen et al. (2015) investigated the effects of infection with the microsporidium *D. duebenum* and the acanthocephalan *P. minutus* in *G. fossarum* under cadmium exposure (4 µg l^{-1}). The results showed that glycogen concentration increased slightly in the group with both parasites (control and cadmium

exposure), while lipids and HSP70 did not show any significant effect of the interaction of the 2 parasites. Gismondi et al. (2012e) detected a ca. 2-fold increased cell damage (lipid peroxidation) in *G. roeselii* infected with *D. roeselum* and *P. minutus* exposed to cadmium (8 µg l^{-1}), while all other biomarkers tested were mostly influenced by microsporidian infection and cadmium exposure.

Generally, both studies indicated that microsporidians override the effects of acanthocephalans in the same host, but more studies are required to substantiate these findings.

4. CONSEQUENCES OF PARASITISM

A general rule concerning the physiological consequences of combined stressor effects of parasites and pollution for the host is so far not obvious. On the one hand, it is doubtless that a number of combined effects between parasites and pollution exist, which have to be addressed in ecotoxicology (Sures 2008b). On the other hand, these combined effects are of great importance for the determination of toxicity data of pollutants and associated safety limits under natural conditions. Most of the toxicity parameters such as LC$_{50}$ or lowest or no observed effect concentration data for amphipods were determined irrespective of the infection status of the test organisms (e.g. Williams et al. 1985, Felten et al. 2008). Therefore, the comparability between different studies with the same test species is limited, as no information on the respective parasite is available. To determine the effects of a substance in more detail and to study the mechanisms of toxicity, it would be necessary to clearly differentiate the effects of chemicals and potential parasites. Therefore, comparisons of the exposure of parasitized (single and co-infections with different parasite species) and unparasitized individuals would be essential.

4.1. Detection of parasite stages

To permit the comparison of infected and uninfected hosts, several requirements must be met. Firstly, the naturally occurring parasites have to be detected, and secondly, parasite-free individuals must be available. The latter is difficult, as prevalence levels are usually high in natural populations, at least for some parasites such as microsporidians (e.g. Grabner 2017). Sorting infected from uninfected individuals is comparatively easy in cases of late-

Table 2. Studies on combined effects of pollutants and acanthocephalans on amphipods. Ex: laboratory experiment; N: natural population; HSP: heat shock protein

Amphipod species	Parasite species	Exposure	Effects	Reference(s)
Gammarus pulex	*Pomphorhynchus laevis*	Cadmium (Ex)	Increased mortality of exposed and infected individuals	McCahon et al. (1988)
Gammarus pulex	*Pomphorhynchus laevis*	Cadmium (Ex)	Lower accumulation in cystacanths compared to host tissue; increased mortality of exposed and infected individuals	Brown & Pascoe (1989)
Gammarus pulex	*Pomphorhynchus laevis*	Cadmium (Ex)	Shortening of separation time of precopula pairs in parasitized	Poulton & Pascoe (1990)
Gammarus pulex	*Pomphorhynchus laevis*	Aluminum (N)	Increased mortality of exposed and infected individuals and exposed individuals compared to exposure alone	McCahon et al. (1989), McCahon & Poulton (1991)
Gammarus pulex	*Pomphorhynchus laevis*	Cadmium (Ex)	Shortening of separation time of precopula pairs in parasitizedand exposed individuals compared to exposure alone	Poulton & Pascoe (1990)
Gammarus pulex	*Pomphorhynchus laevis*	Lead (Ex)	Lower accumulation in cystacanth compared to host tissue	Siddall & Sures (1998)
Gammarus roeselii	*Polymorphus minutus*	Palladium (Ex)	Higher accumulation in cystacanth compared to host tissue and higher accumulation in tissue of infected hosts compared to uninfected; reduced mortality of infected individuals (exposed and unexposed); decreased HSP70 response in infected individuals (controls and exposed)	Sures & Radszuweit (2007)
Gammarus roeselii	*Polymorphus minutus*	Cadmium (Ex)	Lower accumulation in cystacanth compared to host tissue; mortality of infected and exposed males decreased compared to uninfected males, while mortality of infected and exposed females increased	Gismondi et al. (2012f)
Gammarus roeselii	*Polymorphus minutus*	Cadmium (Ex)	Increased levels of lipid peroxidation and reduced gluta-thione in infected and exposed individuals; metallothio-neins not affected by infection status	Gismondi et al. (2012e)
Gammarus fossarum	*Polymorphus minutus*	Cadmium (Ex)	Lower accumulation in cystacanth compared to host tissue; increased mortality of exposed and infected individuals; metallothioneins not affected by infection status; decreased HSP70 response in exposed and infected individuals compared to exposed	Frank et al. (2013)
Gammarus fossarum	*Polymorphus minutus*	Cadmium (Ex)	Lower accumulation in cystacanth compared to host tissue	Chen et al. (2015)

Table 3. Studies on combined effects of pollutants and co-infections of acanthocephalans and microsporidians on amphipods. Ex: laboratory experiment

Amphipod species	Parasite species	Exposure	Effects	Reference
Gammarus fossarum	*Polymorphus minutus + Dictyocoela duebenum*	Cadmium (Ex)	Increased glycogen concentration	Chen et al. (2015)
Gammarus roeselii	*Polymorphus minutus + Dictyocoela roeselum*	Cadmium (Ex)	Increased lipid peroxidation	Gismondi et al. (2012e)

stage acanthocephalan infections because mature cystacanths are visible from the outside in the body cavity of the living host. This is more problematic for early developmental stages of acanthocephalans or for microsporidians (and most other parasites) that cannot be detected without sacrificing the test organism. Therefore, experiments must be conducted first, and parasitized/unparasitized groups have to be determined afterwards by screening aliquots of host homogenates, e.g. by molecular methods (see for example Gismondi et al. 2012d, Chen et al. 2015). An initial screening of test populations for parasites can be conducted by microscopical examination of squash preparations or histological sections of host individuals. Furthermore, microsporidian infections can be detected by PCR using universal primers followed by sequencing of the PCR products, or by applying specific primers, if predetermined species

should be identified (Krebes et al. 2010, Grabner et al. 2015). In an analogous manner, early acanthocephalan infections can be detected by specific primers (Zittel et al. 2018).

Molecular methods allow fast screening of many specimens with high sensitivity. The major drawbacks of this method are that the exact location as well as the specific developmental stage of the parasite remain unknown. Tissue-specific analysis would be possible, but dissection is difficult and time consuming for small organisms such as amphipods, particularly if many individuals have to be screened. Pooling of samples can, in turn, reduce the workload for large-scale screening, particularly if next-generation sequencing techniques are applied. This approach has been used for other pathogens (e.g. Paparini et al. 2017, Batovska et al. 2018), but methods have yet to be developed for amphipod parasites.

Histopathological methods are more time-consuming, but provide information about the location of the parasite, its morphology, the magnitude of infection (e.g. number or size of parasite stages), the host response (e.g. invading immune cells), and infection by 'unexpected' parasites that would not be targeted by molecular methods (e.g. Bojko et al. 2013, 2017).

4.2. Amphipod laboratory culture

A more elegant solution to investigate parasite effects separated from effects of pollutants would be parasite-free laboratory cultures of the test organism and controlled laboratory cycles of the desired parasite (e.g. *Gammarus pulex*; see McCahon & Pascoe 1988). Standardized laboratory cultures are available, for example, for the freshwater amphipod *Hyalella azteca* that is commonly used as a test organism (EPA 2000). Culture conditions have also been described for other amphipods (McCahon & Pascoe 1988), but those are normally not maintained on a routine basis in ecotoxicological laboratories. Furthermore, the parasite-free status of laboratory cultures is often not clear if they are not screened and if there is a regular replenishment with individuals from the field. Parasites with complex life cycles are comparatively easy to eliminate, as a second host would be required to complete the life cycle, but especially vertically transmitted microsporidians can be difficult to remove from laboratory cultures. To establish a microsporidian-free amphipod laboratory culture, gravid females must be collected from field populations with naturally low prevalence levels of microsporidians. Females must be kept separately

and must be removed and tested for microsporidian infection by PCR as soon as the offspring is released. Batches of offspring from microsporidian-free females can be fed with pre-conditioned leaves and bred further to build up a laboratory population. Juveniles of some amphipod species (e.g. *G. pulex*) additionally require adult feces as a food source (McCahon & Pascoe 1988). This can be problematic, as these feces can be the source of horizontally transmitted microsporidians. Therefore, batches of the used feces must be tested by PCR before using them as food source or they must come from a parasite-free stock.

4.3. Using naturally infected hosts for laboratory experiments

In many cases, it will be sufficient to use naturally infected amphipods. In this case, the diversity and prevalence of parasites can be assessed in advance in the respective host population. The observed prevalences can be used to estimate the required sample size for laboratory experiments, to assure that adequate numbers of infected and uninfected animals are used. After terminating the experiment, the test animals have to be screened for parasites and endpoints can be evaluated according to infection status. Additionally, individuals that show abnormal behavior or die during the experiments should be checked for parasite stages to determine if the observed effects are caused by the parasite (e.g. due to growth or multiplication). To this end, it is also possible to divide the specimens to be able to perform both PCR and histopathological analyses (Bojko et al. 2018). Moreover, studies with infected test organisms are important to estimate the response of naturally infected populations of amphipods to toxicants at a given site (even if not every single parasite is known) and provide a more realistic estimation of pollution effects compared to the use of uninfected individuals for ecotoxicological tests.

5. CONCLUSIONS

According to the present knowledge, it is obvious that the impact of parasites can confound the outcome of toxicological and ecotoxicological studies in various ways. Therefore, parasites in amphipods and other test organisms should be considered in ecotoxicological studies, as already suggested by several authors (Ford & Fernandes 2005, Sures 2008a, Ford

2012, Sures et al. 2017b). In this context, it would also be an important aim to relate the effect size of parasite impacts to the effects of other influencing factors, to obtain a quantitative estimate of the relevance of parasites on the outcome of ecotoxicological studies. Moreover, many parasite taxa can be found in amphipods besides microsporidians and acanthocephalans (Bojko et al. 2013, 2017). Therefore, research is required to elucidate their effects on the host, also in combination with other parasites and additional stressors. Because the effects of the interactions between host, parasite, and stressor differ in some cases, depending on the parasite–host system used, it would be desirable to conduct studies that compare the response of different parasite and host species. Finally, the mechanistic understanding of parasite effects on the stress response and detoxification mechanisms of the host should be improved. If possible, ecotoxicological tests should be conducted with uninfected individuals, but in most cases, we will have to work with parasitized amphipods. However, researchers should be aware of this phenomenon, test for parasites, and include possible parasite effects in their interpretation of the results.

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