Freshwater mysids in ecotoxicology

Testing and culturing freshwater mysid species under laboratory conditions

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Mysid shrimps such as the marine Americamysis bahia have become a subject of interest in ecotoxicological research as they appear to react rather more sensitive to exposure to plant protection products than previously tested freshwater macroinvertebrates. Although it is curious that in the freshwater risk assessment of plant protection products a marine species is used, the interesting question is whether its observed sensitivity can actually be a representation of a response that has not yet been identified by testing relevant freshwater macroinvertebrates. As the order of the mysid shrimps also contains several freshwater species, this project focussed on the development of a laboratory test with freshwater Mysids. For this purpose, holding and culturing conditions needed to be investigated first in order to keep the animals in the lab properly. This report is an account of the different attempts of culturing and holding the freshwater mysid Limnomysis benedenii in the laboratory of Wageningen Environmental Research.

Keywords: Freshwater Mysids, laboratory, culture methods, ecotoxicology

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Photo cover: Limnomysis benedeni (Dick Belgers)

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Summary

In the authorization process of plant protection products the environmental impact of the product is investigated on different ecosystem components to assess its potential risk. This assessment is constructed along several tiers and in the first tier ecotoxicological test data of the marine mysid *Americamysis bahia* is used. Although a relatively large amount of published toxicity data is available for the marine/estuarine *A. bahia*, relatively limited data are available on the sensitivity of other mysid species. A disadvantage of *A. bahia* as test organism is that its narrow salinity and temperature range limits its use in colder water or low-salinity testing. The European mysids *Neomysis integer* and *Limnomysis benedeni* might be a good alternative, since they are widely spread and usually present in high densities. However, before an organism can be appointed as a standard test organism for ecotoxicological testing, there is a longer list of requirements that should ideally be met:

- First, they have to be available whenever necessary, either through field collection or laboratory culturing. The latter is preferred, so that the organisms are already accustomed to laboratory conditions, and do not require an acclimatization phase. In case of field collection, it has to be possible to collect a sufficient number of individuals without causing damage to the natural population or environment, and it has to be easy to transport them from the field site to the laboratory. Easy transportation has as additional advantage that it enables cooperative studies, for instance, ring tests;
- Second, the type and quantity of food that the organisms require needs to be known and easily manageable;
- Third, the life cycle should be relatively short, to allow for reproduction and full life cycle testing;
- Finally, the ecological importance of the organism needs to be well understood, and ideally, the genetic composition is well defined.

The main aim of this study is, therefore, to obtain an understanding on the potential of whether the Mysis species *Neomysis integer* and *Lymnomysis benedeni* can be kept and cultured under laboratory conditions. As information on *N. integer* was more abundant available than for *L. benedeni* we focused the experiments on the latter species. Multiple experiments were performed to determine the appropriate test medium, medium refresh rate, mode of aeration, and diet, and eventually provide the first insights on what a standard toxicity test will entail.

1 Mysid shrimps in regulatory ecotoxicology

1.1 Why Mysids?

In the authorization process of plant protection products the environmental impact of the product is investigated on different ecosystem components to assess its potential risk. In addition to agricultural use of plant protection products and the resulting potential exposure of edge-of-field surface waters, also potential exposure via effluents can be a routing enabling contact of aquatic organisms to these products. For the aquatic environment, several standard test species have been selected in order to get a first indication of sensitivity

Mysids shrimps are relatively small crustaceans, often referred to as opossum shrimp due to the females brood pouch containing the unhatched eggs (Figure 1). Mysid shrimps have been put forward as suitable aquatic test organisms in ecotoxicology in the U.S. Where the U.S Environmental Protection Agency (US EPA) and the American Society for Testing and Materials (ASTM) have both adopted the American subtropical *Americamysis bahia* as a standard test organism to evaluate the potential risks of exposure to chemicals in coastal and marine environments.

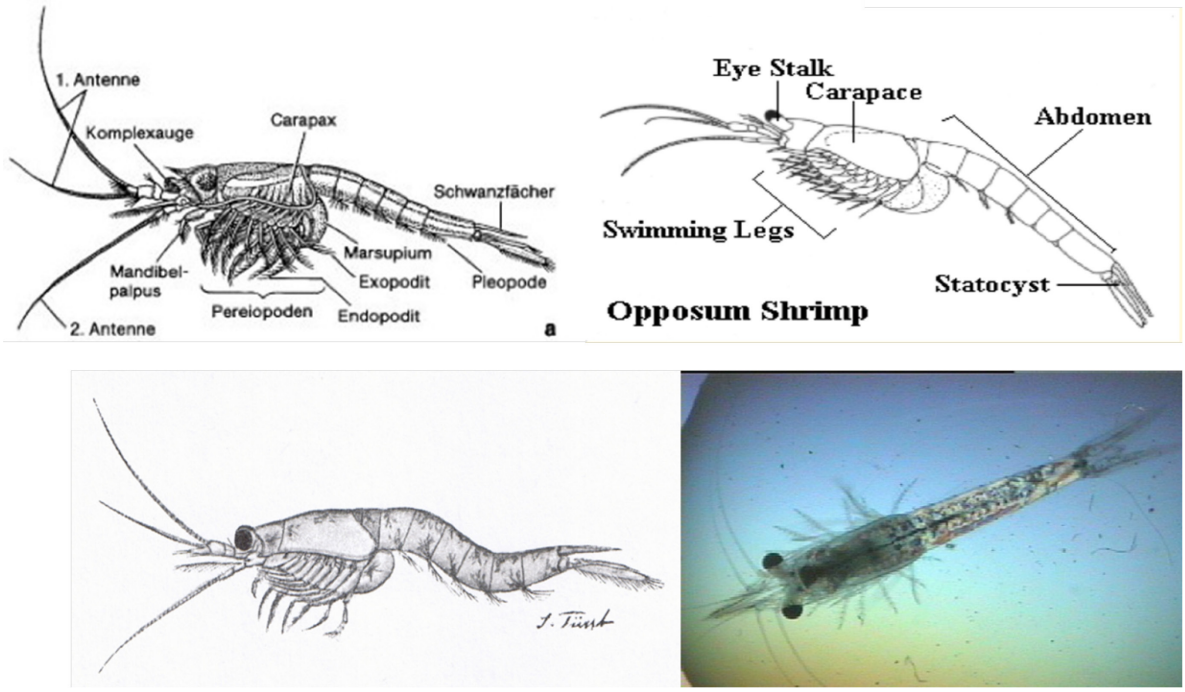

Figure 1 *Above: Morphological characteristics of mysid (or opossum) shrimps. Below: Drawing and photograph of Neomysis integer.*

Currently, in Europe the mysid shrimp *Americamysis bahia* is considered a suitable second tier-1 arthropod test species (besides the cladoceran *Daphnia* sp.) in the prospective aquatic effect assessment for insecticides in edge-of-field surface water. However, freshwater species (e.g. *Chironomus* sp.) are preferred (EFSA PPR, 2013).

Although a relatively large amount of published toxicity data is available for the marine/estuarine A. bahia, relatively limited data are available on the sensitivity of other mysid species. A disadvantage of A. bahia as test organism is that its narrow salinity and temperature range limits its use in colder water or low-salinity testing (Verslycke et al., 2004c). The European mysids *Neomysis integer* and

Limnomysis benedeni might be a good alternative, since they are widely spread and usually present in high densities (Audzijonyte et al., 2009). However, before an organism can be appointed as a standard test organism for ecotoxicological testing, there is a longer list of requirements that should ideally be met (Reish, 1973; Hutchinson et al., 1995). First, they have to be available whenever necessary, either through field collection or laboratory culturing. The latter is preferred, so that the organisms are already accustomed to laboratory conditions, and do not require an acclimatization phase (Reish, 1973). In case of field collection, it has to be possible to collect a sufficient number of individuals without causing damage to the natural population or environment, and it has to be easy to transport them from the field site to the laboratory. Easy transportation has as additional advantage that it enables cooperative studies, for instance, ring tests. Second, the type and quantity of food that the organisms require needs to be known and easily manageable. Third, the life cycle should be relatively short, to allow for reproduction and full life cycle testing. Finally, the ecological importance of the organism needs to be well understood, and ideally, the genetic composition is well defined.

The European mysid *Neomysis integer* might be a good alternative as test species in prospective effect assessment for insecticides (Verslycke et al., 2004b). It is relatively small species up to 17 mm in length and it is found in very shallow water in both high and low-salinity habitats. It is a filter feeder and the female broods her eggs in a brood pouch beneath her cephalothorax. It is often the commonest mysid shrimp in the low-salinity upper parts of estuaries and is also present in lakes (Bremer and Vijverberg, 1982). *Limnomysis benedeni* also meets most of these demands: being widely spread throughout Europe (Audzijonyte et al., 2009), having a relatively short life cycle, and comprising a crucial part of the food link between phyto- and zooplankton and larger fish species. In contrast to most mysid shrimp, however, *L. benedeni* occurs only in freshwater.

However, so far, a laboratory culture usable for standard ecotoxicological testing has not been established yet, primarily due to uncertainties regarding the exact diet of *L. benedeni*. Hanselmann and colleagues (Hanselmann et al., 2013) studied the food preference of adult *L. benedeni* collected from the field and from laboratory feeding experiments by analyzing their stomach contents. They found that *L. bendeni* is an omnivore and feeds through filtering and grazing on both pelagic and benthic sources.

The main aim of this study is, therefore, to obtain an understanding on the potential of whether the Mysis species *Neomysis integer* and *Lymnomysis benedeni* can be kept and cultured under laboratory conditions. As information on *N. integer* was more abundant available than for *L. benedeni* we focused the experiments on the latter species. Multiple experiments were performed to determine the appropriate test medium, mode of aeration, and diet, and eventually provide the first insights on what a standard toxicity test will entail.

1.2 *Neomysis integer*

1.2.1 Geographical distribution and environmental conditions of its habitat

N. integer is one of the most common mysids around the coasts of Europe (see Figure 2), typically occurring in high numbers in estuarine, brackish environments but also occurring in freshwater ecosystems which in recent geological history were connected to the sea (Bremer and Vijverberg, 1982) such as low salinity waters in the delta area of The Netherlands. *N. interger* is euryhaline (<0.5 to >25‰) and mainly occurs in cold water (<20°C) (Verslycke et al. 2004a). According to Vilas et al. (César Vilas et al., 2006) *N. integer* is reported to have an efficient osmoregulatory capacity up to a salinity of 32‰ and an oxygen consumption rate that is independent of salinity. Nevertheless, the field distribution of *N. integer* is mainly at salinities below 12‰.They hypothesised that the greater euryhalinity of *N. integer* makes it possible for this species to avoid competition with other mysid species in temperate estuaries by inhabiting the more stressful oligohaline zone.
Verslycke & Janssen (Verslycke and Janssen, 2002) report that temperature, salinity and dissolved oxygen had no significant effect on the energy metabolism within the tested range, illustrating its adaptation to occur in estuarine and brackish water environments.

According to Remerie et al. (Remerie et al., 2009) the within-population variability in genetic diversity is low, whereas a significant (moderate to high) divergence was observed between estuarine and brackish water populations. Unfortunately, these authors did not analyse *N. integer* populations occurring in freshwater habitats.

1.2.2 Developmental stages

The morphology in the embryonic development of *N. integer* was classified by Janssen et al. (Janssen et al., 2007) in 3 sub stages (Figure 3). The early embryos or stage I larvae (a-b) are spherical or sub-spherical and stage I ends with the hatching from the egg membrane. Stage II larvae (c-d) are dorsally bent and have a comma-like appearance. In subsequent phases an initiation of segmentation becomes visible (d), an extension of the body with elongation of the appendages (e), an optical rudiment (f), a clear segmentation and lateral chromatophores (g) and finally the optical lobes (h) can be observed. The naupliar stage II terminates with the moulting of the cuticle. The post-naupliar stage III larvae (i-j) have stalked eyes, a developed telson and uropods, however, without lith in the statocyst. Stage III terminates in a moult leading to free-living young juveniles (k) that are, except for the sexual characteristics, morphologically similar to the adults. More detailed information is found in Fockedey et al (Fockedey et al., 2006).

In a study focussing on intra-marsupial development of *N. integer*, Fockedey et al. (Fockedey et al., 2006) showed that the survival and hatching success are highly dependent on the salinity conditions, while the development time is strongly affected by temperature. High temperatures (21°C) shorten the development time in comparison with low temperatures (11°C) from 22 to 10 days, but have an opposite effect on survival. Optimal salinity for in vitro embryonic/larval development of *N. integer* is 14-17‰. The salinity range at which the embryos and larvae develop is more restricted than the salinity range at which the female mysids can survive. Living in lower or higher salinities thus implies suboptimal conditions for the juvenile recruitment to the population, unless the species can actively regulate the concentration of its marsupial fluid. Since populations of *N. integer* are able to survive in

low-saline freshwater lakes it seems that *N. integer* is able to actively regulate the salinity within the marsupium. It is suggested that the marsupial salinity can be actively regulated by redirecting urine from the antennary excretory gland into the brood pouch. In addition, genetically different populations of *N. integer* may differ in the way their intra-marsupial development is affected by salinity and temperature (Fockedey et al., 2006, and literature cited therein).

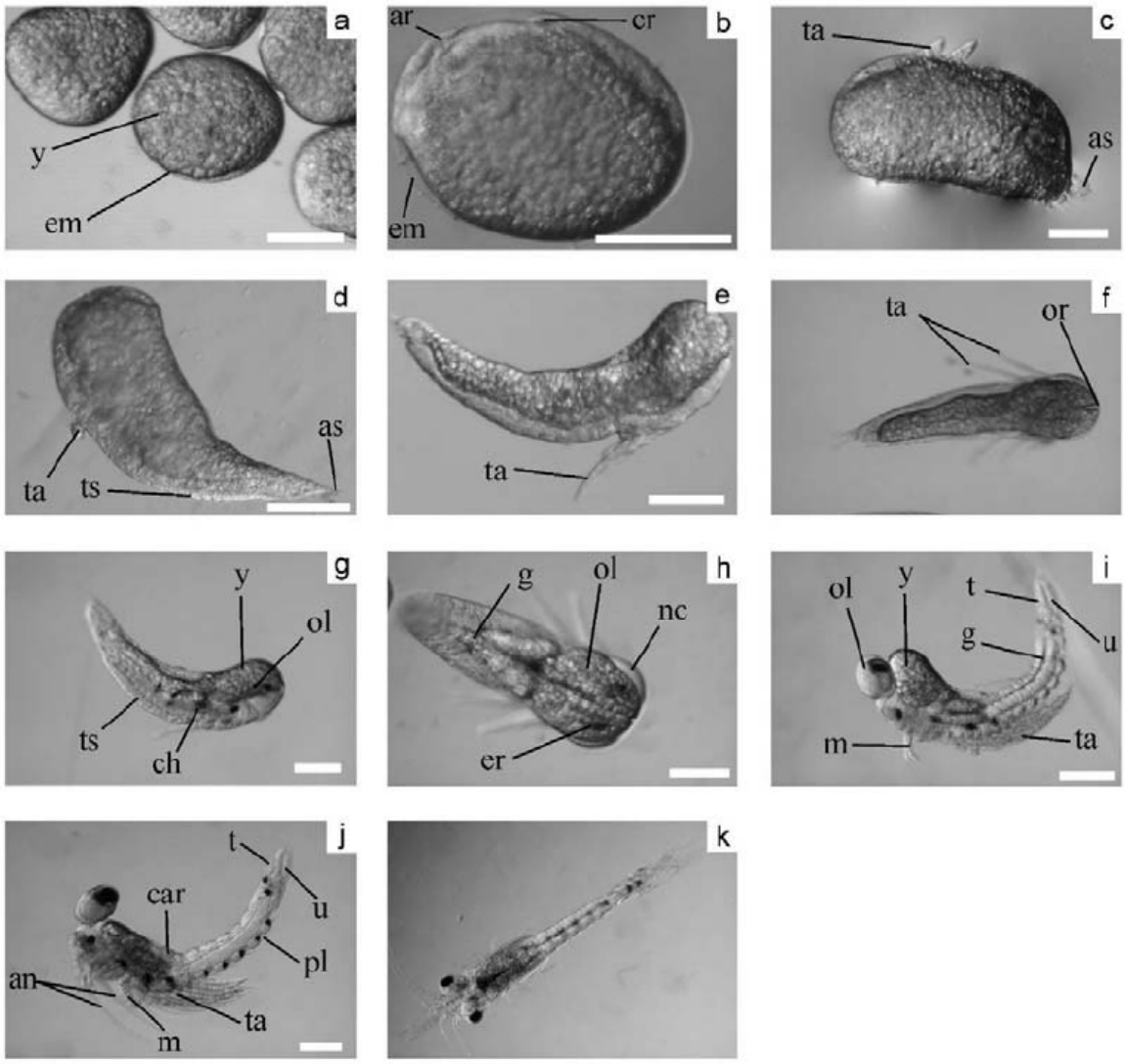

Figure 3 *Embryonic and larval stages of Neomysis integer: stage I (a-b), stage II (c-h), stage III (i-j), juvenile (k).*
The sale bar id 250 µm. Figure from Janssen et al (2007), see also Fokedey et al. (2006). an=antennae; ar=abdominal rudiment; car=carapace; c=cuticle; cr=cephalic rudiment; ch=chromatophore; er=eye rudiment; em=egg membrane; g=gut; m=mouth; nc=naupliar citicle; or=optic rudiment; ol=optic lobe; pl=pleopodes; t=telson; ta=thoracic appendages; ts=thoracic segmentation; u=uropods; y=yolk.

Fockedey et al. (Fockedey et al., 2005), studies individual post-marsupial growth (size, intermoult period, growth factor) in *N. integer* from first day neonates until adulthood at relevant temperature-salinity conditions. Three salinities were tested (5, 15 and 30 ‰) at 15 and 20°C, and two more temperatures (8 and 25°C) were tested at a salinity of 5‰. The size of *N. integer* increased with decreasing temperature and increasing salinity. Salinity had a stronger effect than temperature on the duration of maturation. Higher temperatures caused smaller intermoult periods but had no effect on the growth increment, while salinity effects were less straightforward and dependent on the water temperature. Survival and growth of *N. integer* was possible within the tested range of temperatures (8-25°C) and salinities (5-30‰), but maturity was only possible in a smaller range of 15-25 °C and

5-15‰. Within this range, the size at sexual differentiation was constant, but the size at maturity increased with decreasing temperature and increasing salinity.
In culture experiments conducted at 20°C individual females were found to produce up to 5 consecutive broods (C.R. Janssen unpublished in Mees et al., 1994).

Astthorsson & Ralph (Astthorsson and Ralph, 1984) studied growth and moulting of *N. integer* in laboratory tests. The maximum time which a mysid was kept alive in the experimental beakers was 121 d. During this time the mysid moulted 12 times and increased in length from 3.4 to 12.3 mm. The mysids that lived longest at 16°C lived for 82d, during which time it moulted 15 times and increased in length from 3.2 to 11.6 mm. A faster growth rate at 16°C is only achieved through more frequent moulting, not through a greater size increase at each moult. After the same number of moults, individuals growing at 9°C and 16°C were always of the same length (Astthorsson and Ralph, 1984).

Winkler and Greve (Winkler and Greve, 2002) conducted laboratory studies on growth, moulting and reproduction of *N. integer*. Maturation occurred after 15 to 16 moults and an age of 110d at 10°C. At 15°C, the mean age was 45d and mean length was 8 mm at maturity. Up to this stage they had moulted 9 to 10 times. In post-larval and juvenile individuals of *N. integer* (3 to 7 mm), growth strongly depended on temperature as shown by high temperature coefficients (Q_{10}=4.0 to 7.4). Temperature had a strong effect on marsupial development from the egg to the post-larval stage in *N. integer*. At 10°C the mean incubation tome (difference between time of laying eggs and the date of release of the juveniles from the marsupium) was nearly double that of 15°C. A mean of 19±6 juveniles were released at 10°C and 22±15 at 15°C, with the highest number of juveniles (64) being hatches at 15°C and only half the number at 10°C. According to Winkler and Greve (Winkler and Greve, 2002) the pronounced effect of temperature on *N. integer* compared to other mysids would seem to be of advantage in maintaining populations in its natural environment: fast growth at the higher temperatures in late spring may enhance the chance of survival for post-larval individuals, as a larger size potentially eases predation pressure. The fact that *N. integer* had a relatively short generation time, relatively high numbers of individuals per brood and several successive ovipositions, reflects an r-strategy.

1.2.3 Life-cycle characteristics

Two generations per year were found in northern areas of Europe such as in the Ythan (Astthorsson and Ralph, 1984), the coastal waters of the Balthic Sea (Rudstam and Hansson, 1990) and in Danish brackish lakes (Aaser et al., 1995). In the Ythan estuary (UK), the mysids that form the over-winter generation are born over a 2 to 3 month period and their size range in the field is always wide. The summer generation consists predominantly of individuals born over a relatively short period between the middle of May and early June (Astthorsson and Ralph, 1984). Density and biomass of *N. integer* in Lake Ferring (a hypertrophic brackish lake in Denmark) was high; maximum 0.8 ind./L corresponding to 1250 ind./m^2 and a maximum biomass of 1.1 g DW/m^2 (Aaser et al., 1995). The high mysid density in Lake Ferring may reflect the hypertrophic character of the lake and the low predation pressure by fish.

Parker & West (1979), Bremer & Vijverberg (1982) and Mees et al. (1994) all concluded that *N. integer* produced 3 generations per year in the IJselmeer (former Zuiderzee), in the west coast lochs of Scotland, in Lake Veere (SW Netherlands), in the Dutch Frisian lakes, and in a lough in western Ireland, respectively. In the brackish part of the Westerschelde estuary *N. integer* showed 3 peaks in the seasonal pattern in density and biomass: a relatively small, yet distinct, peak in early March (30 ind./m2; 60 mg AFDW/m^2), and 2 peaks in late spring (160 ind./m^2; 225 mg AFDW/m^2) and in summer (140 ind./m^2, 125 mg AFDW/m^2). This suggests that 3 cohorts (generations) were produced per year. The overwintering generation lived from autumn until the following spring. The spring generation was born in early spring and lived for about 3 months, while the summer generation lived from summer until early winter. The three cohorts showed marked differences in their biology. The overwintering population showed a larger brood size and a larger size at maturity. Individuals belonging to the other two cohorts generally grew faster, produced less young per female, and attained maturity at a smaller size. Females generally lived longer, grew faster and consequently became larger than males. The spring cohort accounted for almost half of the annual production

(= 0.3 g AFDW/m^2/year). Despite its longer life span, the overwintering generation generated a quarter of the annual production only. The species does not reproduce when water temperature is lower than 10°C (Mees et al. 1994). The annual production of *N. integer* in the Westerschelde estuary was 0.3 g AFDW/m^2/year)

For a freshwater population Bremer & Vijverberg (1982) estimated the annual production of *N. integer* to be 0.01 g DW/m^2/year. Bremer & Vijverberg (1982) also report that in freshwater Frisian lakes reproduction of *N. integer* completely stopped during winter and that in Slotermeer the maximum population density in two different years ranged between 6 and 110 ind./m^2. Vijverberg (unpublished data), however, observed maximum densities of 1200 and 1600 ind./m^2 in the Bergumermeer during 1976 and 1977. The high variability in maximum population density between years might find its cause in adverse winter conditions (low oxygen levels under ice covered with snow) and fish predation. Densities of young fish varies considerably from year to year in Frisian lakes (Bremer & Vijverberg, 1982). According to Bremer & Vijverberg (1982), brood size in *N. integer* is positively correlated with salinity; the higher the chloride content, the larger the brood size. Why most populations of *N. integer* are found in salinity ranges that correlate with relatively small brood sizes is unclear. Mees et al. (1994), suggest a trade-off for sub-optimal brood sizes with competitive advantages of living in low-salinity. In shallow freshwater Frisian lakes, the size at which females matured varied during the course of the year. In May, females of the overwintering generation were mature when they reached 13 mm, in early spring the length of the smallest female was 12 mm, and only 10 mm in August-September. At the end of October reproduction stopped and the percentage of females with brood became zero for all size classes (Bremer & Vijverberg, 1982).

In shallow freshwater Frisian lakes, the sex ratio, expressed as the percentage of females in relation to total adults, ranged between 40 and 60%, and the mean value was close to 50% (Bremer & Vijverberg, 1982). Mating takes place at night and coincides with the moulting of the female (Fockedey et al, 2006 and literature cited).

1.2.4 Feeding behaviour

According to Lucas (1936) and Tattersall & Tattersall (1951) *N. integer* is an efficient filter feeder, grazing on organic detritus and/or planktonic diatoms and only feeds on zooplankton when concentrations of other suspended food items are too low. Other studies describe *N. integer* as an omnivore consuming detritus, algae, diatoms, rotifers, copepods, amphipods, other crustaceans, carrion, fragments of leaves and of macroalgae, spores and seeds, terrigenous materials and insect larvae (Kinne, 1955; Mauchline, 1971; Jansen, 1985). The growth efficiency has been shown to be highest with animal food and lowest with detritus and, generally, the gut passage time of mysids has been reported to be in de order of 30-90 min (Zagursky & Feller, 1985). *N. integer* possesses cellulase enzymes (Zagursky & Feller, 1985) so the species can theoretically digest macrophyte detritus.
In the Elbe, Westerschelde and Gironde estuaries, *N. integer* was found to be an omnivore which mainly utilised mesozooplankton and detritus carbon pools. The mysids feed in the hyperbenthic layer of the water column and do not scrape the bottom while foraging. The quality of the diet did not differ between the sexes or between different developmental stages, although smaller individuals consumed fewer items. In all three estuaries the diet was dominated by Copepoda Calanoida and was supplemented with Rotifera and Cladocera. Phytoplankton and benthic organisms, though present in the stomachs, were negligible. Macrophyte detritus and amorphous material (originating from suspended sediment flocs) were very abundant food items. *N. integer* consumed filamentous algae rather than solitary phytoplankton cells (Fockedey & Mees, 1999).

In the Guadalquivir estuary (SW Spain) *N. integer* is an opportunistic omnivore, feeding mainly on mesozooplankton and on members of the detrital-microbial loop, shifting prey seasonally according to availability. Most important animal prey items for *N. integer* were zooplankton species from the oligohaline zone, such as rotifers, cladocerans, copepods and juveniles of the mysid *Mesopodopsis slabberi*. In addition a great quantity of detrital items were observed in the stomachs of *N. integer* (Vilas et al. 2008).

In the northern Baltic sea, *N. integer* was observed to be omnivorous, feeding on various food items ranging from dead organic material to different zooplankton, phytoplankton prey and algal filaments. Almost equal amounts of animal and plant particles were found in the stomachs of *N. integer*. This species, which swims in shoals near the sediment outside macrophyte beds, can easily filter upwelling sediment particles. Compared to other mysid species in the Baltic, *N. integer* consumed more detritus (Lehtiniemi & Nordström, 2008).
In a hypertrophic brackish lake in Denmark a qualitative analysis of *N. integer* gut contents showed the presence of copepods, cyanobacteria, chlorophytes and diatoms, as well as various zooplankton such as ciliates and rotifers. Fragments of vascular plants, pollen and detritus were also detected (Aaser et al. 1995).

In freshwater lakes in Friesland (NL), most of the food items eaten by *N. integer* during the day were algae, but their share in the biomass of the total gut content was very small. More than 95% of the gut content was of animal origin, mainly *Bosmina coregoni*, *Bosmina longirostris* and cyclopoid copepods, or detritus. These were also the dominant taxa in the lake. Rotifers were eaten as well, but were of less importance in terms of biomass. *N. integer* showed no distinct size selective predation on *Bosmina*. In addition, the size of the consumed *Bosmina* was independent of the size of *N. integer*. Feeding intensity reached a maximum at sunset. It was still high during the first part of the night but low in the morning just after sunrise. *N. integer* showed a strong negative selection of the filamentous blue-green alga *Oscillatoria* and a strong positive selection of the filamentous green alga *Planctonema*. There was some diurnal variation in the zooplankton eaten. At night *N. integer* consumed more *Bosmina*, while copepods were more often eaten during the day. This is probably related to the vertical migration pattern of *N. integer* and cyclopoid copepods, which both tend to concentrate near the bottom during the day (Bremer & Vijverberg, 1982).

1.2.5 Ecology and position in the food-web

Aaser et al. (1995) suggested that *N. integer* enhances eutrophication in nutrient-rich brackish lakes, i.e. *N. integer* predation on zooplankton reduces grazing pressure on the phytoplankton. In addition, a more direct stimulation of phytoplankton growth in these lakes may find its cause in excretion of nutrient to the water column when processing food collected at the sediment surface.

In the Frisian freshwater lakes *N. integer* may be an important food organism for fish, especially for the 0^+ fish (young-of-the-year) during the second half of their growing season (Bremer & Vijverberg, 1982). In Dutch Frisian freshwater lakes significantly higher population densities were observed in habitats with a sand substrate covered by a mud layer than on mudless sand and peat substrates. In addition, in these lakes *N. integer* showed a marked variation in diurnal distribution. At noon practically all animals were living at the bottom and this was still so at sunset, while at midnight the animals were more or less evenly distributed over the bottom and the whole water column. At sunrise a substantial part of the population was still present in the water column (Bremer & Vijverberg, 1982). This diurnal migration behaviour might be explained by avoiding fish predation.

Jeppesen et al. (1994) found an inverse relationship between the density of fish and that of *N. integer* and argued that the predation pressure of *N. integer* on zooplankton is particularly high in hypertrophic brackish lakes because fish biomass is low and dominated by sticklebacks, which are inefficient predators of *N. integer*.

The importance of fish as predator of *N. integer* is also evidenced by fish manipulation experiments undertaken in Lake Wolderweijd, The Netherlands, where removal of 75% of the planktivorous fish biomass resulted in major increases in *N. integer* density (Meijer et al. 1994).

1.2.6 Laboratory culturing

Verslycke et al.(2003) describe the following culturing procedure for *N. integer*. After collecting organisms from a brackish aquatic ecosystem, they were transferred to 60 L plastic shallow holding tanks (1.5 x 0.4 m) with a water depth of 10 cm. Culture medium was filtered (1.2 µm) natural seawater, diluted with aerated tap water to a salinity of 5‰. The laboratory culture was maintained

under a 12-h light:12-h dark photoperiod. Holding tanks were aerated continuously and water quality was maintained using a gravel bottom filter and through renewal of 50% of the culture medium every week. Ammonia and ammonium concentrations were checked twice a week. Cultures were fed ad libitum twice daily with 24 to 48-h old *Artemia* nauplii to prevent adult mysids from cannibalizing their young. A feeding rate of 150 nauplii/mysid/day was applied. Hatching of the cysts of *Artemia* was performed in 1-L, cylinder-conical vessels under vigorous aeration and continuous illumination at 25°C. Culture density was kept at 20 mysids/L. Due to their epibenthic behaviour, the number of mysids per bottom volume may be more limiting than the number of mysids per unit water volume. Verslycke et al. (2014b) describe the following laboratory maintenance procedure for field collected *N. integer* to conduct acute toxicity tests. An initial population collected in a shallow brackish aquatic ecosystem (Galgeneel) near Antwerp, Belgium, was, after a short acclimatization period, transferred to 200-L glass aquaria equipped with a circulating under-gravel filter. The culture medium was artificial seawater (Instant Ocean, Aquarium Systems, Sarrebourg, France) diluted with aerated, deionized tap water to a final salinity of 5‰. A 14:10-h light:dark photoperiod was used during culturing, and water temperature was maintained at 15°C. Cultures were fed daily with 24- to 48-h old nauplii of *Artemia* ad libitum. Hatching of the cysts of Artemia was performed in 1-L, cylinder-conical vessels under vigorous aeration and continuous illumination at 25°C. The under-gravel filter was replaced every 6 months.

Fockedey et al. (2005) furthermore describe that gravid females were transferred at regular intervals to 10-L aerated static incubators, in which the culture medium was renewed every day for 50%. In these incubators, animals were fed twice a day with 24-48h old Artemia nauplii ad libitum and were checked daily for the release of juveniles from the marsupium. These juveniles were separated from the adult females using a netted brood chamber to prevent the adults from cannibalizing their young. Fockedey (personal observations) found that subadult *N. integer* (5-9 mm) show a growth limitation when fed with less than 200 nauplii per mysid per day. The ratios given in the individual growth experiments conducted by Fockedey et al. (2005) were 1.7 to 6.7 times higher than given in the stock culture and assured a good growth without excessive accumulation of left-over food in the containers. The laboratory growth curves of individual mysids indicate that, although the natural diet of *N. integer* is complex, the laboratory diet of *Artemia* sp. nauplii was presumably meeting its nutritional requirements for long periods of time and that the mysids were healthy and actively growing under favourable conditions (Astthorsson & Ralph, 1984).

During laboratory experiments conducted by Winkler & Greve (2002), *N. integer* was fed with a surplus of newly hatched (1 to 3 d old) *Artemia* sp. and frozen pieces of mysids. The experimental studies on growth were carried out in basins connected to a brackish-water circulation system, whereby the water was pumped up to the first basin and flowed sequentially through the following basins, cascading into a sand and gravel filter and subsequently flowing back to the reservoir aquarium.

1.2.7 Ecotoxicology

N. integer was most frequently used as test organism in acute toxicity tests. Nevertheless, several measures of reproductive performance can be used to assess sublethal responses in chronic toxicity tests, including sexual maturity, the time to first brood release, the time required for egg development, fecundity, brood success, and alterations in reproductive characteristics in populations (Verslycke et al 2004a).

Verslycke et al. (2003) studied the combined effects of six metals on *N. integer*. Acute 96-h toxicity tests were performed with mercury, copper, cadmium, nickel, zinc and lead, as single compounds and as mixture of all six metals. The 96-h LC50's for the single chemicals at a salinity of 5 ‰ ranged from 6.9 to 1140 µg/L, with the following toxicity ranking: Hg>Cd>Cu>Zi>Ni>Pb. Increasing the salinity from 5 to 25‰ resulted in lower toxicity and lower concentrations of the free ion for nearly all metals. This salinity effect was strongest for cadmium and lead and could be attributed to complexation with chloride ions. The 96-h LC50 for mercury was the same for both salinities. Also the toxicity of the mixture, which could best be explained by additive effects, clearly decreased with increasing salinity until 15‰. The mean 96-h LC50 values of *N. integer* at 25‰ for mercury, cadmium, zinc, nickel and

lead are higher than the reported literature values for *Americamysis bahia* tested at 30‰, but those for copper are markedly lower.

Verslycke et al. (2004b) conducted 96-h toxicity tests with *N. integer* and a diverse set of chemicals suspected of having an endocrine-disrupting mode of action, including the pesticides (synthetic insect juvenile hormone analogs) methoprene (96-h LC50 = 0.32 mg/L) and fenoxycarb (96-h LC50 = 0.53 mg/L). They demonstrated that energy and testosterone metabolism could be detected at concentrations below acute toxicity values. Consequently, endocrine-disruptive activity in *N. integer* can be detected after short-term exposure.

Roast et al. (1999) demonstrated that in flow-through and semi-static acute toxicity tests, *N. integer* was more sensitive to the insecticide chlorpyrifos (96-h LC50 = 0.13 µg/L) than to the insecticide dimethoate (96-h LC50 = 540 µg/L). Juvenile *N. integer* were equally tolerant to chlorpyrofos as adult mysids (96-h LC50 = 0.19 µg/L).

Disruption of swimming and position maintenance behaviour has been investigated in laboratory studies with *N. integer* exposed to sublethal concentrations of the organophosphorus insecticide chlorpyrifos. The observed disruption in chlorpyrofos-exposed mysids was probably due to the inhibitory action on acetylchlinesterase (Roast et al. 2000). Impairment of N. integer swimming behaviour by cadmium exposure was studied by Roast et al. (2001). They showed that cadmium concentrations may disrupt mysid swimming behaviour at concentrations considerably lower than lethal concentrations, demonstrating the sensitivity of swimming behaviour as possible sublethal endpoint.

Janssen et al. (2007) report for the herbicide atrazine and *N. integer* a 96h LC50 of 48 µg/L and for TBT 0.164 µg/L.

The developed in vitro technique by Fockedey et al. (2006) may be used for testing effects of (endocrine) disrupting chemicals on the intra-marsupial development of N. integer. Survival, hatching success and development time appeared to be adequate endpoints, while size and growth increment of the embryos/larvae seemed to be unsuitable.

1.3 *Limnomysis benedeni*

1.3.1 Geographical distribution and environmental conditions of its habitat

Another current widely distributed freshwater mysid species in Europe is the Caspian slender shrimp *Limnomysis benedeni*. The species is native to the Ponto-Caspian region but has colonized much of the European freshwater territories during the last decades. In the 1960s, the species was very popular as a food organism to boost fish populations in various reservoirs and so colonized much of eastern and central Europe (Ioffe, 1968; Ketelaars, 2004). In the 1990s, the species was able to spread even further through the Danube and Rhine rivers, thanks to the excavation of the Main-Danube canal (see also Figure 3; Wittmann, 2007). Interestingly, *L. benedeni* is one of the few freshwater mysids of Central Europe (Mauchline 1980) as the vast majority of mysid species are found in brackish estuaries or in the oceans (Porter et al. 2008). The preferred habitat of *L. benedeni* is the littoral, where it lives nectobenthically, i.e. associated with the ground or large structures (Hanselmann, 2012). The wide occurrence, and therefore availability, of *L. benedeni* potentially makes it a suitable freshwater mysid species to assess the sensitivity of this group of organisms.

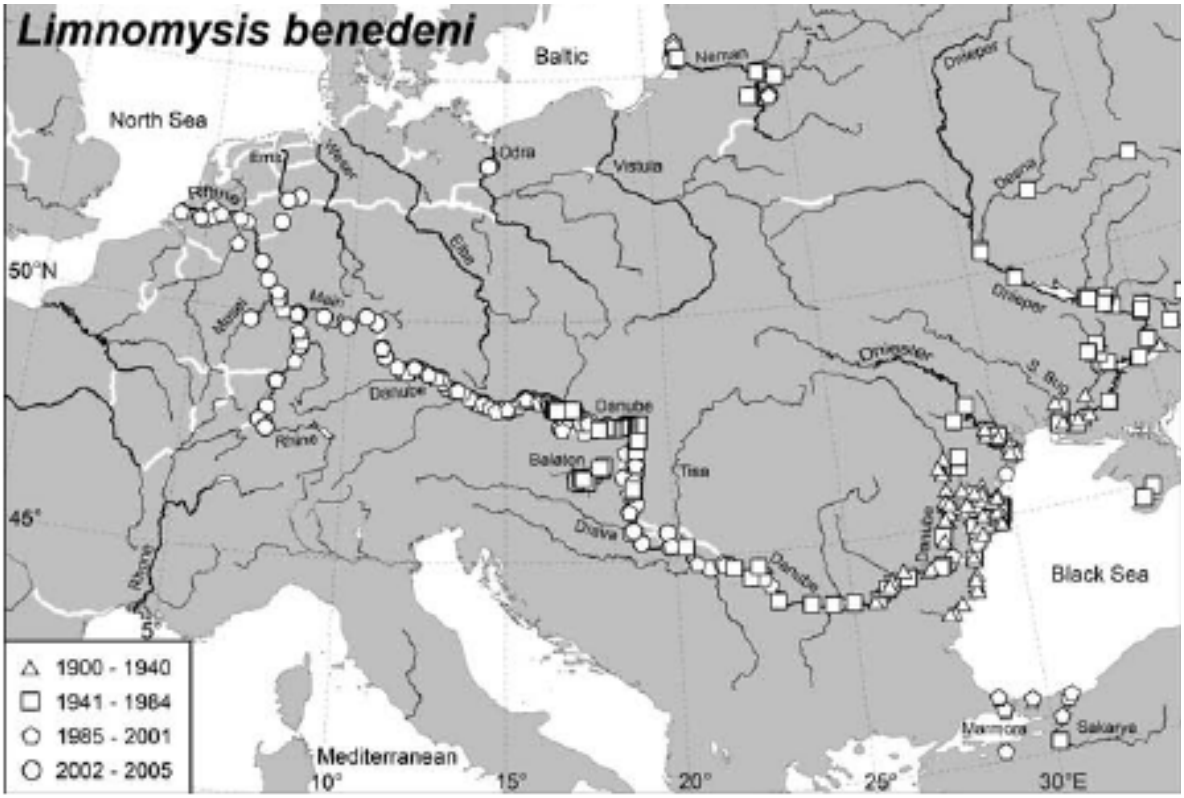

Figure 4 *Distribution of Limnomysis benedeni in the Rhine-Danube system and other waters (Wittmann, 2007).*

1.3.2 Developmental stages

As with other mysids, *L. benedeni* larvae develop inside the female marsupium, a brood pouch formed by two pairs of oostegites (Westheide & Rieger 1996) and the development time is highly dependent on water temperature (Hanselmann et al., 2011a). Females are not able to store spermatophores; therefore, the eggs for each clutch have to be newly fertilized. Egg fertilization, embryonic development, and all larval moults occur inside the marsupium. Therein, the larvae gain all energy and nutrients from the yolk; the mother provides only oxygen by pumping fresh water into the marsupium. The last larval moult takes place directly after the larvae are released into the water. The juveniles start to feed actively during this last larval stage (Mauchline 1973, Wittmann 1981a, Wittmann 1984).

1.3.3 Life-cycle characteristics

Reproduction occurs in the warmer season from April to September, which leads to a life-cycle shift of the adults. In the winter generation, the adults grow large and reproduce only once with a large clutch in early spring; the summer generation reproduces continuously with a smaller size at maturity, and smaller body and clutch sizes (Hanselmann et al., 2011b).

1.3.4 Feeding behaviour

Food source and temperature are two factors that play a major role in the growth and survival of juvenile mysis (Domingues et al., 2001; Lussier et al., 1988). *L. benedeni* is both a grazer and a filter feeder, feeding both on pelagic algae and benthos. The feeding mainly involves small organic matter such as algae (seston), biofilm, and small benthic food sources such as pseudofaeces of *Dreissena polymorpha* (Gergs et al., 2008; Hanselmann, 2013). Although animal food seems to be more nutritional, it is not the preferred food type (Fink et al., 2012; Gergs et al. 2008).

1.3.5 Ecology and position in the food-web

Although *L. benedeni* was able to feed on Daphnia in laboratory experiments, they preferred the green algae *S. obliquus* when offered a choice (Fink et al., 2012). This implies that *L. benedeni* does not have a structuring effect on the zooplankton distribution which was supported by field observations (Lesutiene et al. 2005). In the field, *L. benedeni* feeds on small organic matter like algae (seston), biofilm, detritus, and biodeposited material from D. polymorpha or other benthic food sources up to a maximum diameter of about 200 µm. It seems that they do not compete for small zooplankton with other species, for example juvenile fish like *Perca fluviatilis* (D. Schleuter & Eckmann 2008) or predatory zooplankton like *Bythotrephes longimanus* (Straile & Hälbich 2000). As a consequence, *L. benedeni* does not have the impact on zooplankton communities that other mysid shrimps have.

The importance of fish as predator of *L. benedeni* is also evidenced by a mesocosm experiment that excluded fish predators, resulting a shift in size-class distribution towards adults where the field population was dominated by juveniles (Hanselmann et al., 2011b). Stomach analyses of fish collected in the field showed that *L. benedeni* was preyed upon by juvenile *Perca fluviatilis*, which indeed fed size selectively on larger mysids.

1.3.6 Laboratory culturing

Only a few records have been found of Limnomysis in a laboratory setting using artificial/commercial foods (Abmann et al., 2009;Swinkels, 2019). The study by Swinkels (2019) obtained mysis survival and reproduction of adult *L. benedeni* using Trouvit fishmeal pellets. In contrast to the natural food sources, this mainly consists of animal derived proteins. The study by Abmann et al. (2009) utilized TetraPleco tablets instead. This food comprised a plant derived fish food product. Unfortunately, this study did not record survival or any other data of the individuals kept with this food source.
A study by Hanselmann and co-workers (Hanselmann et al., 2011a) showed that the highest survival rate of adult individuals occurred around 16℃, and observed that at higher temperatures, accelerated life cycles took place at the expense of shorter life spans. Similar results have been reported for other mysis species (e.g. Domingues et al., 1999; Fockedey, 2006; Punchihewa, 2020).

1.3.7 Ecotoxicology

No information could be retrieved on the use of *L. benedeni* in ecotoxicological studies.
In order to explore the suitability of Limnomysis for use in ecotoxicological studies we first need to keep the animals alive in the laboratory. To investigate this, we performed multiple experiments to determine the appropriate test medium, mode of aeration, and diet of *L. benedeni*, and eventually provide the first insights on what a standard toxicity test with *L. benedeni* will entail.
For the proposed test method to the suitable, a survival rate in control treatments (e.g. untreated test systems) of ≥ 80% would be preferred.

2 Materials and methods

We performed a total of four experiments to get a better understanding on the optimum culturing conditions of *L. benedeni*. For reasons of clarity, we first describe the general experimental conditions that were maintained throughout the experiments, or were subject to testing (section 2.1). Next, we briefly describe the four experiments that were performed.

2.1 General experimental conditions

2.1.1 Field collection and biological endpoints

Approximately 500 specimen of *L. benedeni* were collected with a dip net (500 µm) from a pond located on the campus of Wageningen University (the Netherlands). In the laboratory, the test animals were sorted from the bycatch, and were kept in aerated 10-liter buckets with pondwater. Test mysids were checked and selected in the laboratory, one hour prior to initiation of the experiment. In case of reproduction tests, the sex was determined under a microscope according to the characteristics described in the supporting information (Annex 1; Figures S1 – S3).

During the experiments, the endpoints survival, growth, and reproduction were monitored in different combinations. Survival and reproduction were easy to quantify, by respectively measuring the number of surviving organisms and counting the number of offspring. Growth was measured using two metrics: length and weight. Length was determined by photographing the individuals together with a calibration rod under a microscope. The photographs were subsequently analyzed by the software ImageJ. The exact size of the calibration rod is known, enabling length measurements of the organisms. Organism weights were determined by placing individuals in a pre-weighed aluminum container, drying them for 12 hours at 105°C, and reweighing the aluminum containers (now with the dried organisms) at room temperature. Although length could be determined at multiple points throughout an experiment (by taking photographs), weights could only be determined after termination of an experiment. Therefore, a sample of 10-20 individuals was taken at the start of an experiment, to serve as the average length and/or weight at t=0.

2.1.2 Test medium, aeration, and abiotic parameters

Regarding the test medium, three different types of test media were tested throughout the different experiments. The first was pond water, collected from the same pond the test organisms were collected from, and filtered through a 35µm phytoplankton net. The second was groundwater, collected from the Sinderhoeve test facility (Renkum, the Netherlands). The third was RT medium, an artificial medium that is frequently used in standard toxicity tests see Tollrian, 1993 for the exact composition. All test media were aerated and left to acclimatize in the lab for at least 24 hours at the test temperature.

Regarding aeration during the test duration, two modes of aeration were tested: by means of a stainless steel aeration rod, and by means of inserting a fresh 10 cm *Elodea nuttallii* shoot.
During the experiments, test media were refreshed either once or three times per week. Shortly before and directly after refreshment, the pH, dissolved oxygen (DO, mg/L), electrical conductivity (EC µS/cm), and temperature (°C) were measured. Stainless steel meshes were introduced into each test system to serve as substrate.

2.1.3 Diet

During the experiments, three forms of fish food pellets (commercially available) were tested. The first was called Novofect (JBL), and 1 tablet (≈0.3493 gram) was added per refreshment round. Novofect consists of vegetable products, grain, algae, fish and fish products, mollusks and crustaceans, vegetables, vegetable protein extracts and yeast. The second fish food was called Royal Caviar (Bern Aqua), and 3 mg/L/d was added to the test systems. Royal Caviar is used in shrimp farming as a substitute for live food, and consists of marine fish species, krill, fish roe, soy lectin, yeast, microalgae, fish gelatin, squid and vegetable fat. The third and last fish food we tested was called Trouvit (Skretting), and 1 pellet (≈0,0136 gram) was added per refreshment round. Trouvit consists mainly of animal proteins.

2.2 Experiments

2.2.1 Experiment 1: Determine optimal culturing conditions

The aim of the first experiment was to find the optimum combination of an initial set of test conditions. In total, three food types (Novofect, Royal Caviar and Trouvit), two media (pondwater and groundwater), and two modes of aeration (aeration rod and *E. nuttallii*) were tested, resulting in twelve treatments (3x2x2). Each treatment was replicated four times, resulting in 48 jars (see Figure 5).

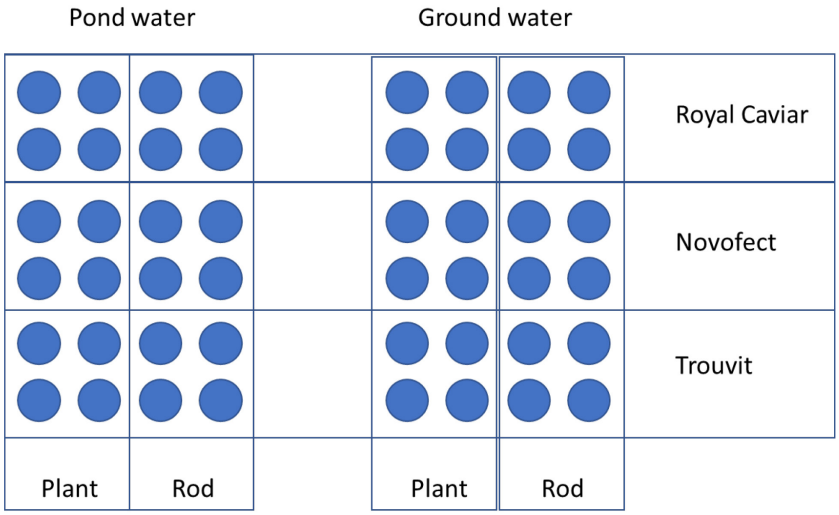

Figure 5 *Schematic set-up of Experiment 1.*

Each jar contained 1L of test medium, 10 individuals, a mode of aeration, and a metal mesh to serve as substrate. The jars were incubated in a water bath at a temperature of 15°C with a light-regime of 16 hours light and 8 hours dark. The treatments with an aeration rod were incubated under a light intensity of 20 µmol S/m^3, whilst the treatments with *E. nuttallii* were incubated under a light intensity of 100 µmol S/m^3. This was done to stimulate photosynthesis, and thereby oxygen production, in the treatments with *E. nuttallii*.

The total duration of the experiment was 28 days, and the media were refreshed three times per week. Survival was monitored during each refreshment, whilst length and weight of the test individuals were only measured after termination of the experiment. At the start of the experiment, 20 additional *L. benedeni* specimen were collected for a characterization of initial weight and length measurements.

2.2.2 Experiment 2: Fine-tune optimal culturing condition

The aim of the second experiment was to i) validate the results of the first experiment, and ii) further improve them where possible. Therefore, the treatments that gave the best results in the first experiment were re-assessed in the second experiment and were supplemented with some new treatments that were expected to improve the overall experimental set-up. In total, two food types (Royal Caviar and Trouvit), two media (pondwater and RT medium), and two refreshment schemes (one and three times per week) were tested. The RT medium was only tested with the three times per week refreshment scheme, resulting in a total of six treatments (Figure 6). Again, each treatment was replicated four times, and 10 individuals were kept in each jar in 1L of test medium, along with a metal mesh to serve as substrate.

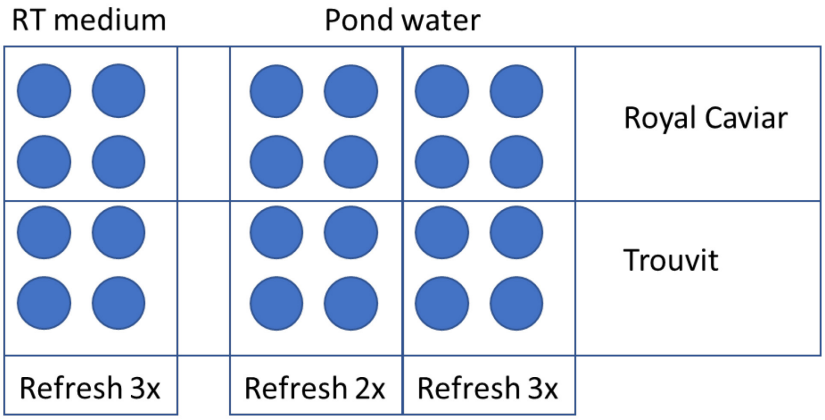

Figure 6 *Schematic set-up of Experiment 2.*

As mode of aeration, *E. nuttallii* was used, since this mode of aeration resulted in higher survival rates in the first experiment compared to the use of an aeration rod. The jars were incubated under the same temperature and light-regimes that were used in the first experiment. Since now all treatments contained *E. nuttallii*, the light intensity was set to 100 µmol S/m^3 for all treatments.
The total duration of the experiment was 28 days. Survival was monitored during each refreshment. At the end of the experiment, surviving females were collected and kept under identical conditions to assess the number of potential offspring they could produce.

2.2.3 Experiment 3: Rearing of juveniles in jars

The aim of the third experiment was to test the survival of juveniles at two temperatures (15°C and 20°C). Each treatment (temperature) was only replicated 2 times, due to a limited availability of juveniles (Figure 7). As in previous experiments, 10 individuals were kept in 1L of test medium, along with a metal mesh to serve as substrate. As test medium, mode of aeration and food, respectively pondwater, *E. nuttalli* and Trouvit were selected due to their superior performance in the previous experiments.

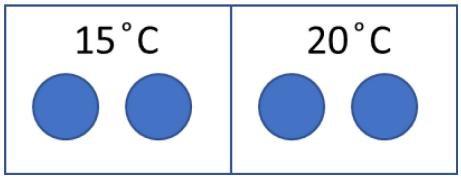

Figure 7 *Schematic set-up of Experiment 3.*

The jars were incubated under the same light-regime and -intensity as in experiment 2. The temperature under which the jars were incubated varied according to treatment. The experiment lasted 58 days, during which both survival and length were determined once a week.

2.2.4 Experiment 4: Rearing of juveniles in an aquarium

The aim of the fourth and final experiment was to determine the life cycle duration of *L. benedeni* under laboratory conditions. For this, 150 individuals were kept in an aquarium of 45L. Pondwater was used as test medium, and the individuals were fed by adding five Trouvit pellets, three times per week. Aeration was provided, both by means of both an aeration stone and some branches of *E. nuttallii*. The aquarium was incubated at 22°C (which was room temperature in the laboratory at that time). The experiment lasted 60 days, during which both survival and reproduction were monitored once a week.

3 Results

3.1 Experiment 1: Determine optimal culturing conditions

In the jars with an aeration rod, survival after 28 days was below 80% for all of the treatments (Figure 8a). In the jars with *E. nuttallii*, survival after 28 days was above 80% in 3 of the 6 treatments (Figure 8b). In the treatments with *E. nuttallii* and Novofect, mortality was immediately (t=2) 100%, and these treatments were therefore removed from the analysis.

In general, the treatments with pondwater performed better than the treatments with groundwater, and differences between Royal Caviar and Trouvit were small, both in terms of survival (Figure 8), growth (Annex 1; Figure S4 & S5).

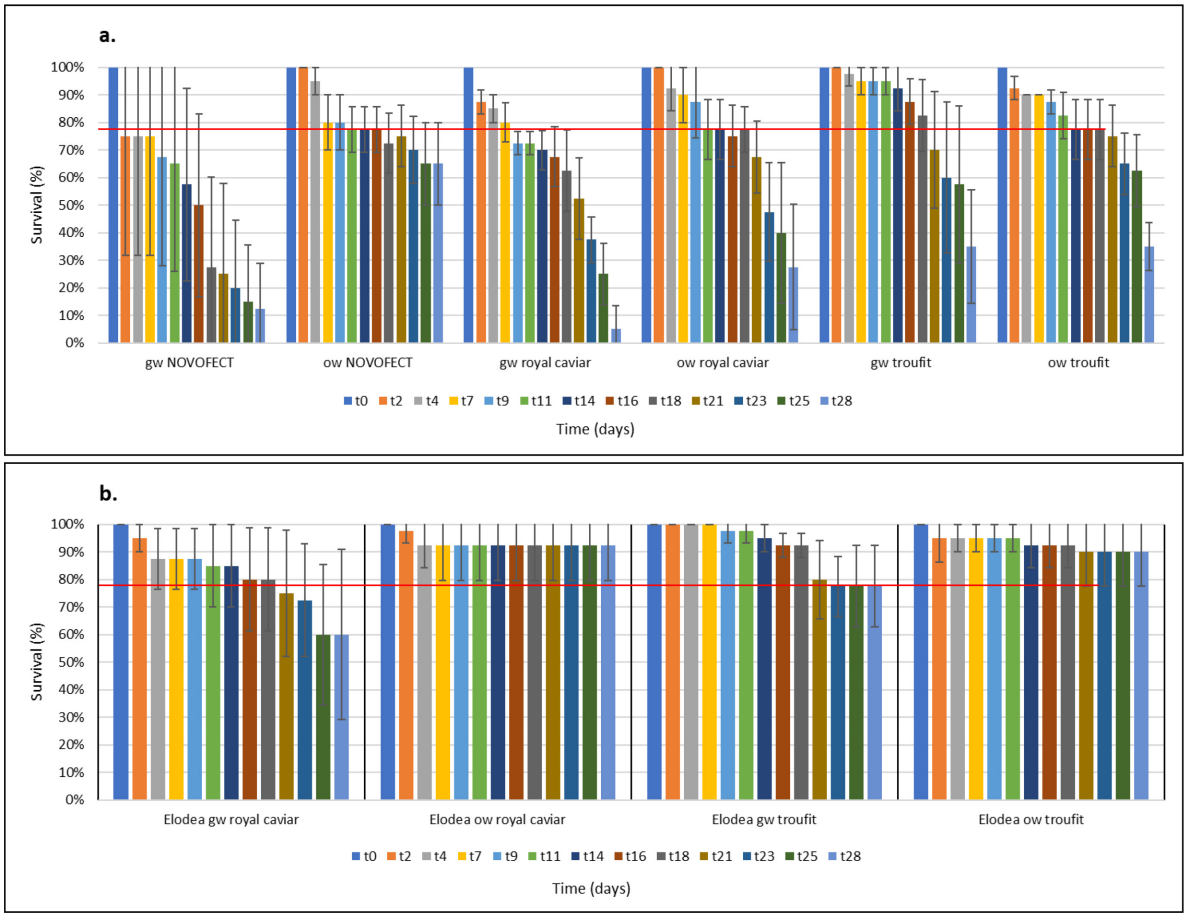

Figure 8 *Average survival (% ±SD) at each measured time point (t) for the treatments (a) with aeration, and (b) with Elodea. The red line indicates the required 80% survival. gw = groundwater; ow = pondwater.*

3.2 Experiment 2: Fine-tune optimal culturing conditions

In the second experiment, only the treatment with pondwater, Trouvit as food source with a refreshment rate of once per week, yielded average survival rates above 80%. In all the other treatments, average survival declined beneath 80% after 2 to 3 weeks. This decline was quite abrupt

in the treatments with surface water that were refreshed three times per week, and more gradual in the treatments with RT medium.

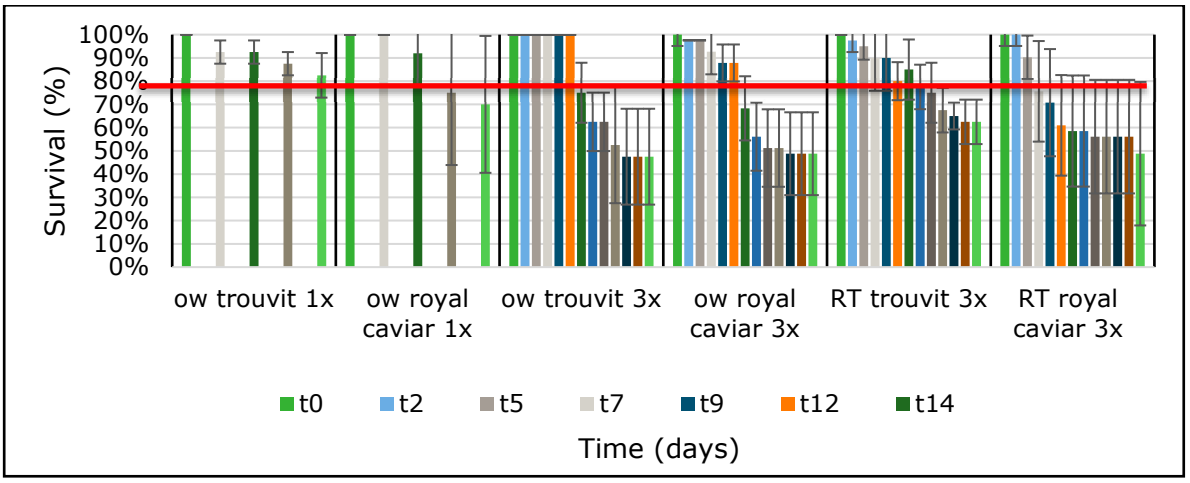

Figure 9 *Average survival (% ±SD) at each measured time point (t) for the different treatments. The red line indicates the required 80% survival. ow = pondwater, RT = RT medium.*

Reproduction occurred only in the four treatments containing surface water (Figure 10), while no reproduction occurred in the treatments with RT medium. Reproduction was higher when the systems were refreshed once a week (Figure 10b and d), compared to when they were refreshed three times per week (Figure 10a and c). Although differences in reproduction between the different food types were small, Trouvit outperformed Royal Caviar.

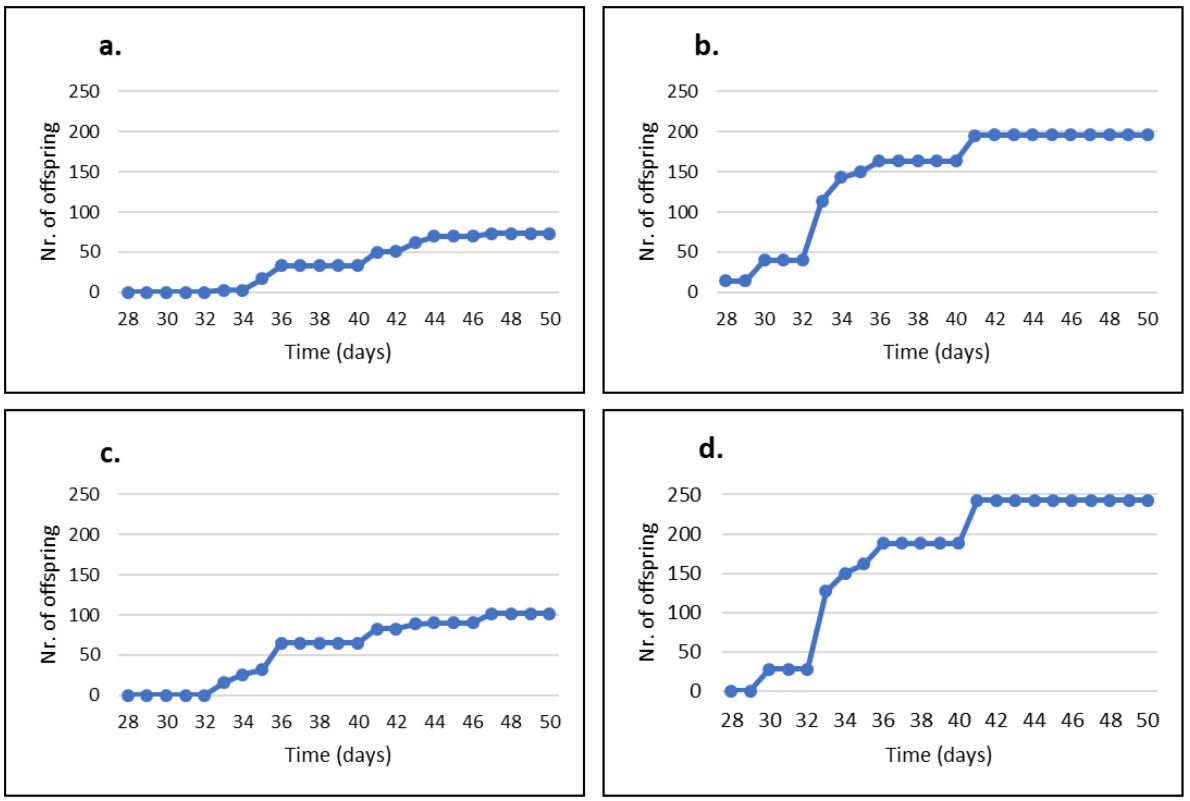

Figure 10 *Number of offspring over time for the treatments (a) OW - Royal Caviar – 3 refreshments, (b) OW - Royal Caviar – 1 refreshment, (c) OW – Trouvit – 3 refreshments, and (d) OW – Trouvit – 1 refreshment. In the RT treatments, no reproduction occurred.*

3.3 Experiment 3: Rearing of juveniles in jars

In the third experiment, the development of juvenile Limnomysis individuals were followed in time. From the graphs it becomes apparent that the survival was higher at 15°C (Figure 11a). Although growth in length was faster at 20°C, it was interesting to see that the final length reached by the individuals in both the 15 and 20°C treatment was similar (Figure 11b). Apparently, there is final length that the animals all reach but the speed at which they do this is temperature related indeed.

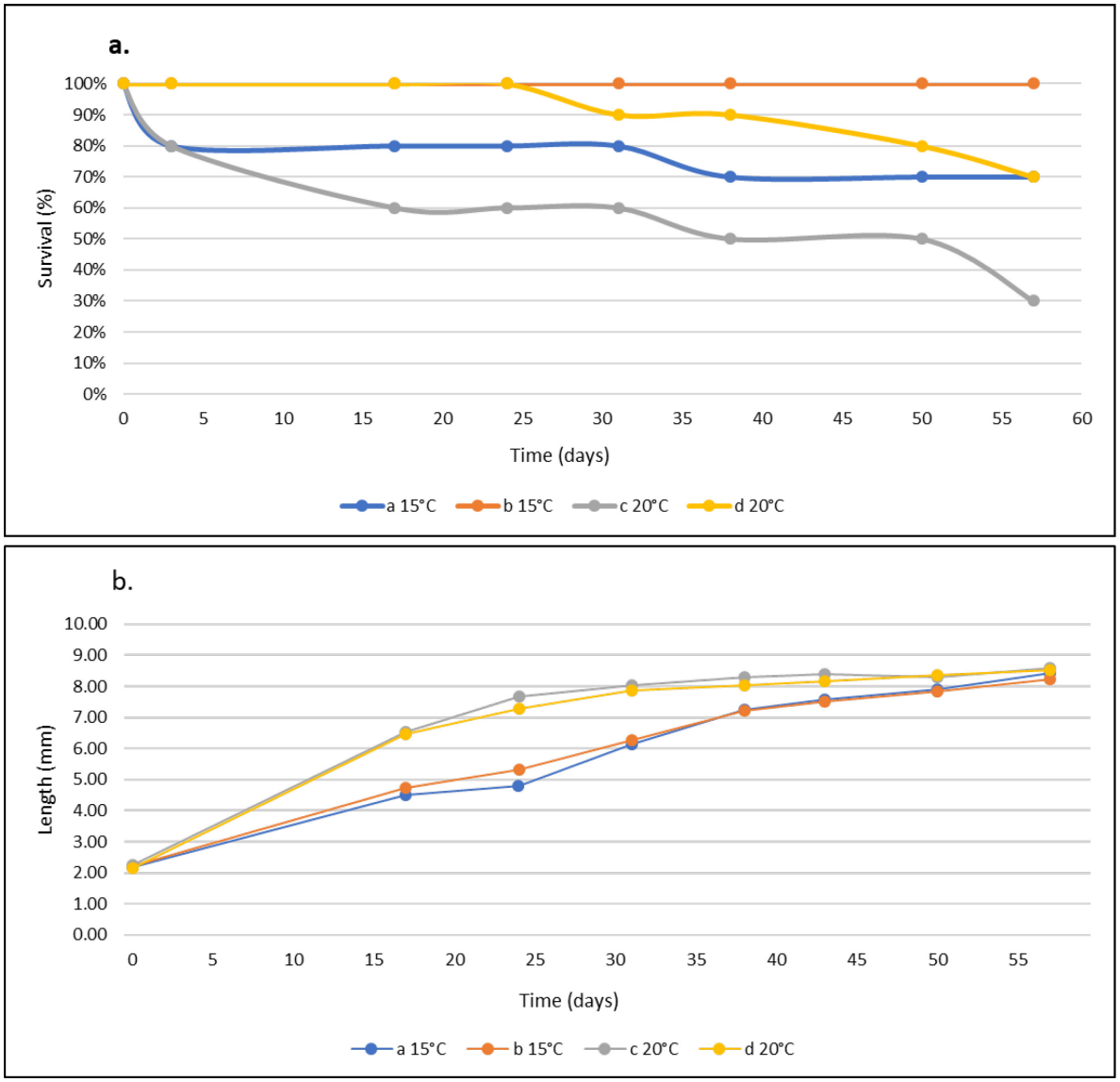

Figure 11 *(a) Survival of juveniles per replicate system and (b) average length of the juveniles per replicate system for the different temperature treatments.*

3.4 Experiment 4: Rearing of juveniles in an aquarium

In the fourth and last experiment, the initially inserted 150 juveniles, started to reproduce 28 days after starting the test. It must be said that these juveniles originated from the previous cultures and were not collected in the field. Interestingly, survival also started to decrease more rapidly after this day (Figure 12). Although for this culturing experiment there is no required level of survival other than that required to reproduce, we observed that survival declined under 80% after 30 days (Figure 12).

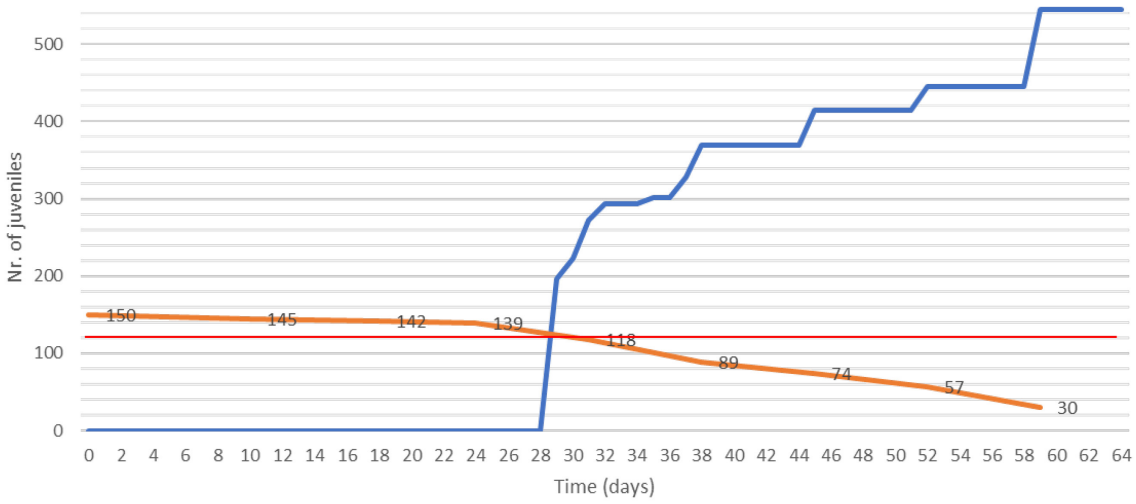

Figure 12 *Number of surviving juveniles (orange line) and number of offspring (blue line) over time. The red line indicates the 80% survival level.*

4 Discussion and conclusions

4.1 Observations on the laboratory culturing of *L. benedeni*

In the first experiment, a high mortality was observed in the treatments with *E. nuttallii* and Novofect. This was likely due to an oxygen deficiency (0.4 mg/L), caused by the apparently too large quantity in which Novofect was provided. In standardized tests with the mysid *A. bahia*, DO concentrations are maintained at a minimum of 6 mg/L (EPA, 1996). Below that threshold, occurring mortality may be due to an oxygen deficit.

In general, mortality rates were higher in the second experiment than in the first experiment. The exact reason(s) for this remains unknown, although insufficient or inappropriate acclimatization for the second test might be one of the explanations. Mysid species are relatively fragile organisms, and minor but abrupt changes in their environment might cause them stress. We postulate that this also explains why the mysids performed better while they were refreshed once per week compared to the three times per week refreshment treatments. Therefore, extra care should be taken with the acclimatization phase, ensuring gradual transition from field to lab conditions, especially regarding temperature, and food source.

The higher reproduction observed in the treatments that were refreshed only once per week compared to those refreshed three times per week could be an additional indicator of increased disturbance/stress due to increased handling of the organisms. However, this can also be related to the higher number of surviving individuals (females) in the once a week refreshment. Unfortunately, sex was only determined at the beginning of the experiment, and the number of females over time is therefore unknown.

Conclusion: The first two experiments revealed that the combination *E. nuttallii* and pondwater with either Trouvit or Royal Caviar as food source provided the best results and that refreshments should preferably be performed once a week.

4.2 Life-cycle characteristics

In the third experiment, we observed that both growth and mortality increased with an increasing temperature. This finding is confirmed by other studies as Hanselmann and colleagues (2011a) observed lower survival rates at 20 and 25°C compared to 15°C and determined that maximum survival was located around 16°C. They also found that development time decreased with increasing temperature, although the metric they used (time until all females released neonates) was different from the length measurements in our study.

In the fourth experiment, we found high survival rates at a relatively high temperature of 22°C. This is surprising, since other studies found decreasing survival rates starting from a temperature of 20°C (Hanselmann et al., 2011a). The high survival rates might be due to the known age of the individuals used in this experiment (1-3 days), and the fact that they were already acclimatized to laboratory conditions, since the individuals used in this experiment were the offspring from a field-collected population.

In general, we found that reproduction started when the laboratory-bred organisms were approximately 30 days old (experiment 2 and 4), and that mortality rates steeply increased around the same time. This relatively short life-cycle duration, together with the relatively large numbers per brood, reflects an r-strategy. The relationship between growth, mortality, and temperature found in

the third experiment and by Hanselmann and co-workers (Hanselmann et al., 2011a) is an indicator that temperature can be used as a way to manipulate the life-cycle duration of *L. benedeni*. This is also known for the mysid species *Neomysis integer*, whose life-cycle duration is halved with a 5°C increase from 10°C to 15°C (Winkler and Greve, 2002). This temperature-dependent life-cycle duration is a great way for *L. benedeni* to grow fast when circumstances are beneficial, and reduce growth when circumstances are hard. However, this also raises questions on a potential seasonal variation in sensitivity towards chemicals. Hanselmann and colleagues (Hanselmann et al., 2011a), for instance, found that time until reproduction depends on the season. These effects could potentially be resolved by maintaining a constant laboratory culture, as is for instance done for daphnid and algae species.

4.3 Suitability of *L. benedeni* in ecotoxicology

This study has demonstrated that culturing of *L. benedeni* under laboratory conditions is possible, and that the optimal experimental conditions as determined in this study can be used as a basis to construct protocols for standardized toxicity tests. Nevertheless, some remaining questions have to be answered to further standardize toxicity tests with *L. benedeni*, and to get a better understanding on the potential (seasonal) variability associated with them.

The first question that can be answered is: Can *L. benedeni* be cultured on a standardized test medium? Use of a standardized test medium would be a great improvement, since the exact composition of the test medium is known, enabling a better understanding of the fate of any chemical to be added to this in a ecotoxicological test and would allow for standardization across space and time. Our efforts with RT medium were unsuccessful, although further experiments are required to confirm the inappropriateness of RT medium. The use of other standardized test media, like Elendt M4 or M7 which are frequently used for *Daphnia* testing, should still be explored.

The second question that can be answered is: How large is the seasonal variation in sensitivity of *L. benedeni* towards chemical compounds? Since life history traits like development time, growth rates, and time until reproduction have shown to vary according to temperature or season, it is necessary to obtain an understanding on the range of this variation. This question will become irrelevant once it becomes possible to maintain a stable laboratory culture in which this seasonal variation is successfully eliminated.

Despite the remaining questions, *L. benedeni* has good potential to become part of the standard battery of test species. Other marine or estuarine mysid species have shown to be sensitive towards, for instance, endocrine-disrupters and insecticides looking at respectively energy metabolism (Verslycke et al., 2004a) and swimming behavior (Roast et al., 2000), and have demonstrated the suitability of mysid species as early warning indictor. We expect that especially the use of sub-lethal endpoints, primarily reproduction at start, will become more important in the future as many emerging chemicals result in chronic, sub-lethal exposure concentrations that might cause indirect effects at the population level. The use of a representative, freshwater mysid is therefore paramount to determine if the sensitivities observed in the marine and estuarine species also occur in fresh surface waters.

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Annex 1 Supporting Information

The male sex can be recognized by an elongated pleopod attached to the fourth segment of the abdomen (Figure S1). Females do not have this elongated pleopod.

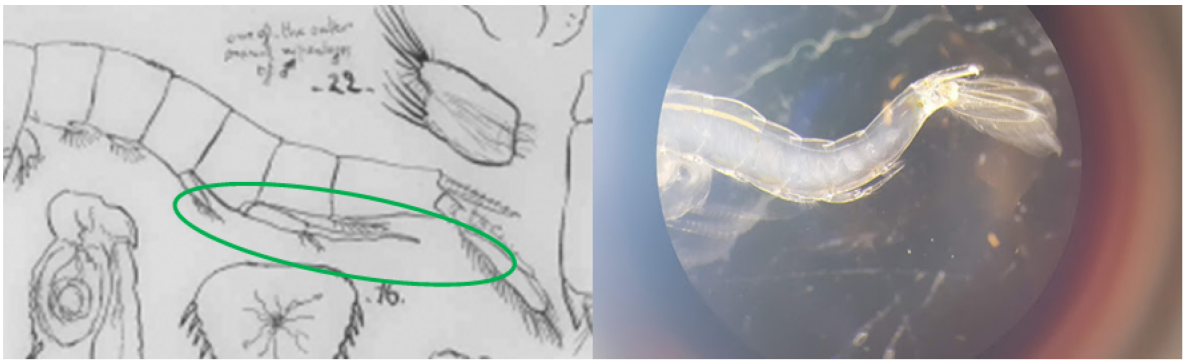

Figure S1 *These figures show the characteristics used to discriminate males from females. The green circle indicates the elongated pleopod located on the fourth segment of the male abdomen.*

The female sex can be recognised by the presence of a marsupium, which is a brood pouch that is usually well visible in adult specimen (Figure S2). The marsupium is located between the last pair of legs of the thorax. Males do not carry a marsupium.

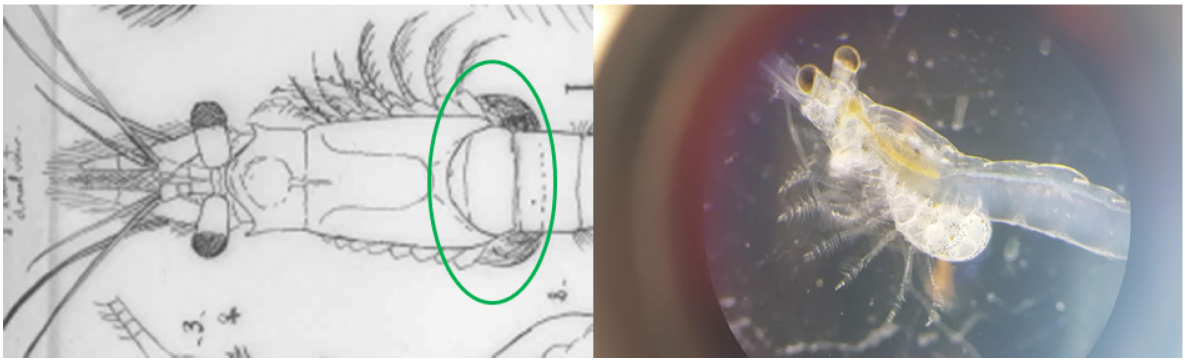

Figure S2 *These figures show the characteristics of the female sex of L. benedeni. The green circle indicates the marsupium*

If these two characteristics did not provide a conclusive sex, we looked at the antenna as an additional characteristic. Males have an additional foot on their antenna, whilst females do not have this (Figure S3).

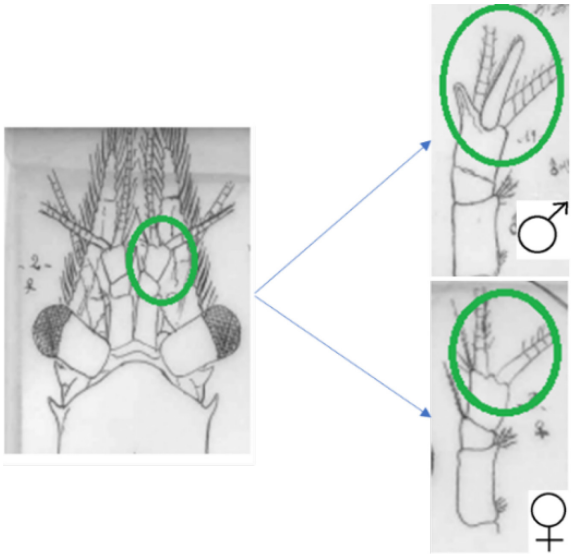

Figure S3 *The difference between the male and female antenna of L. benedeni. Males have an additional foot on their antenna.*

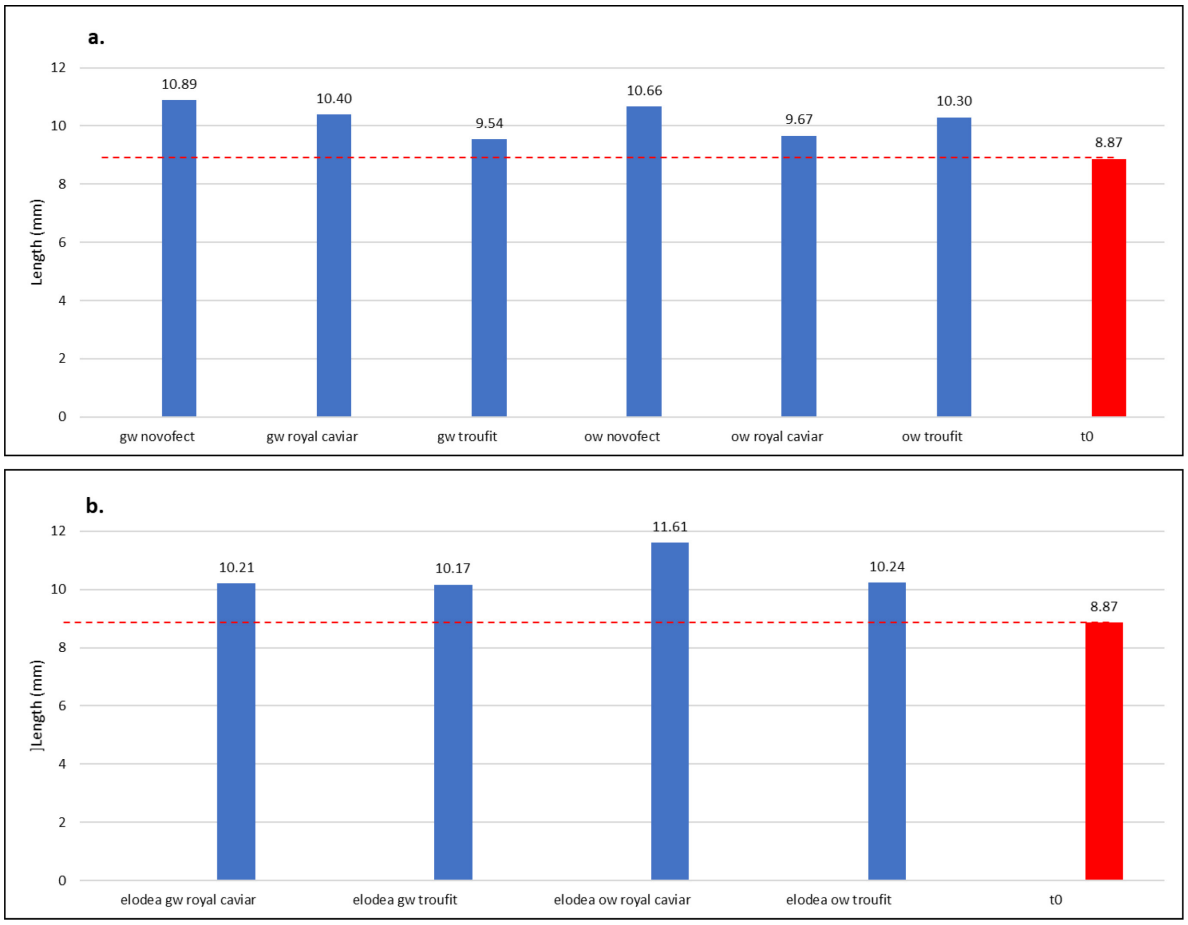

Figure S4 *Average length (mm) after 28 days for the treatments (a) with aeration and (b) with E. nuttallii. The red bar indicates the average length at the start of the experiment (t=0). The standard deviation is not indicated in these plots, since the number of measured individuals varies over the different treatments.*

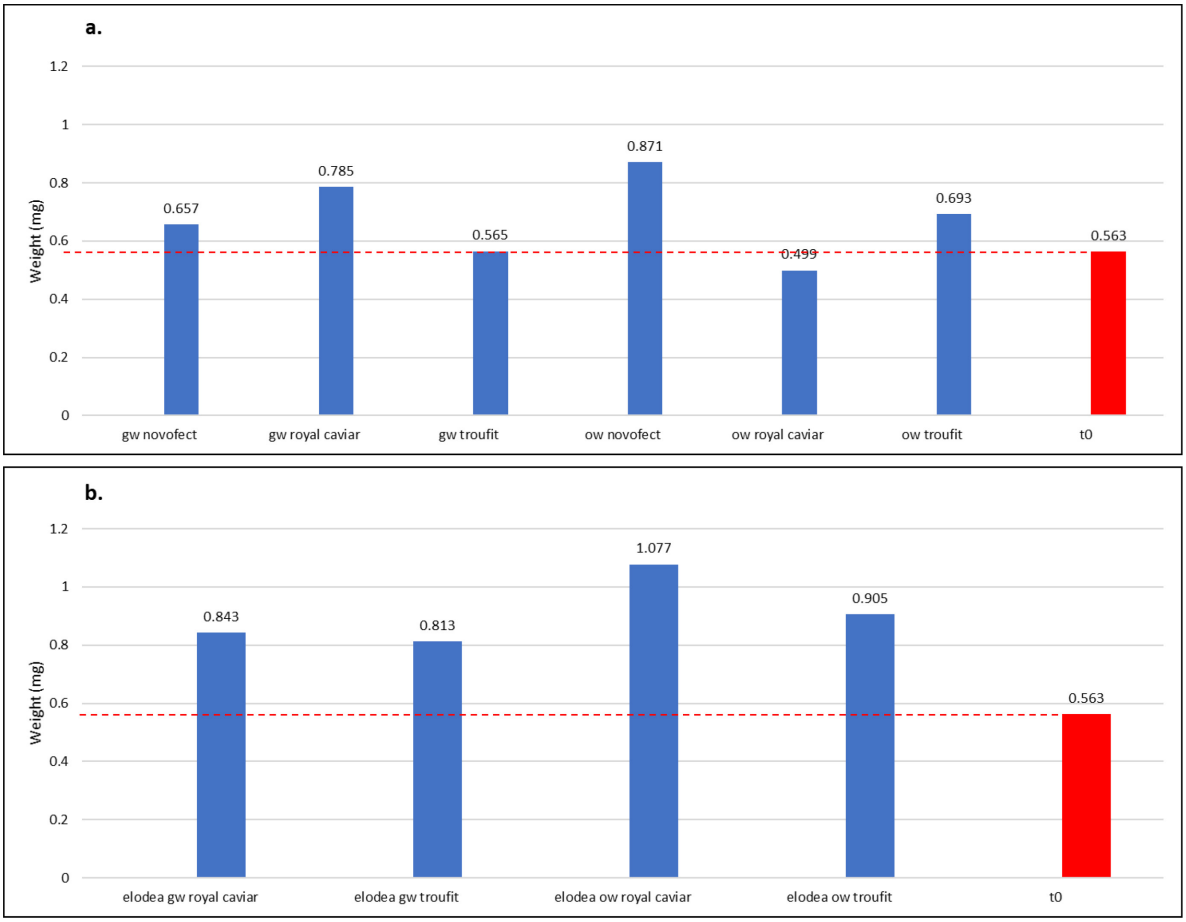

Figure S5 *Average weight (mg) after 28 days for the treatments (a) with aeration and (b) with E. nuttallii. The red bar indicates the average weight at the start of the experiment (t=0). The standard deviation is not indicated in these plots, since the number of measured individuals varies over the different treatments.*

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