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The first millimetre – rearing juvenile freshwater pearl mussels (Margaritifera margaritifera L.) *in plastic boxes*

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ABSTRACT

1. The last remaining population of *Margaritifera margaritifera* in the Our River (Luxembourg, Europe) has come close to extinction. It consists predominantly of adult animals and will disappear within a few years without assistance. Juvenile mussels cannot survive in the river bed interstices, which are clogged by fine sediments, and rearing methods are needed to help them through this critical period.

2. The objectives of this study were to elucidate the best rearing conditions for juvenile mussels with respect to the food type and density of individuals in order to breed them in the laboratory to a size at which survival in the river is likely.

3. Different food mixtures (combinations of algae, detritus and crushed red bloodworms) were fed to juvenile mussels in plastic boxes containing 500 mL of river water during a period of 110 days. To understand fluctuations in the concentrations of nitrate, nitrite and ammonium between water exchanges, these ions were analysed over a period of 8 days.

4. The best rearing results were achieved with a combination of detritus and algae. This treatment resulted in a growth rate of 189% (up to 1.13 mm (SD ±0.30) per box) with a survival rate of 80% (101 dead mussels (SD ±163.71) per box). The optimum number of mussels per box was 200. When detritus was added to the boxes, levels of nitrite and ammonium were reduced by more than 50% compared with the initial value within 8 days. Without detritus, ion concentrations increased noticeably (ammonium >50%, nitrite >150%), probably explaining higher mortality rates.

5. Juvenile mussels have food reserves for less than 8 days after excystment and need additional food as soon as possible after dropping from the host fish.

6. Juvenile mussels showed the most favourable survival rate and growth rate if fed with a mixture of algae and detritus. Detritus functions not only as a food source but also as a biologically active compound which reduces harmful ions such as ammonium and nitrite in the boxes.

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KEY WORDS: river; stream; endangered species; protected species; invertebrates; sedimentation; pollution

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INTRODUCTION

Freshwater molluscs including mussels are an important component of aquatic ecosystems and their presence reflects the health of freshwater environments (Brim Box *et al.*, 2006). Although the life history and ecological importance of many freshwater mussels is still unknown, the taxon Bivalvia comprises the most endangered species among freshwater organisms (Neves *et al.*, 1997). Most of the freshwater bivalves in Europe are listed as endangered species (Bauer and Wächtler, 2001). Among them, the freshwater pearl mussel (*Margaritifera margaritifera* L.) is the most threatened species which shows a dramatic decline throughout its distribution range in Europe (Young *et al.*, 2001; Geist and Kuehn, 2005; Geist, 2010), and thus is listed on Annex II and Annex V of the Habitats Directive (Directive 92/43/EEC). In recent years freshwater pearl mussels were common in large parts of the Belgian and Luxembourgian massif of the Ardennes and of the German Eifel (Boettger, 1912). Today, these populations have drastically decreased or have become extinct because of a loss of suitable natural habitats, eutrophication, introduced predators, decline of fish stocks and sedimentation of the interstitial zone (Preston *et al.*, 2007; Gum *et al.*, 2011). Juveniles of *M. margaritifera* live for about 5 years completely buried in the substratum (Jones *et al.*, 2005), and it appears that sedimentation in addition to eutrophication is the main reason for the absence of young individuals in Luxembourg as at other sites (Geist and Auerswald, 2007).

To protect the remaining unionid mussel populations, four general conservation strategies have been used. These include (a) construction of protected areas, (b) transfer of adult mussels from rivers containing healthy populations to rivers with endangered populations, (c) release of large numbers of host fish infected with larvae (glochidia), and (d) cultivation of juvenile mussels for use in restocking programmes (Ziuganov *et al.*, 1994). In river systems where mussel populations are severely depleted or on the verge of extinction (which is the case in Luxembourg) Ziuganov *et al.* (1994), Preston *et al.* (2007) as well as Gum *et al.* (2011) have suggested that rearing of juvenile mussels for stock restoration is the only feasible conservation measure. Bolland *et al.* (2010) stated that the likelihood of cultured *M. margaritifera* surviving to adulthood is increased when mussels develop through their critical life stages under controlled conditions in a hatchery. Artificial rearing projects for *M. margaritifera* are currently being carried out in Great Britain, Northern Ireland, the Republic of Ireland, Germany, Czech Republic and Belgium (Schmidt and Vandré, 2010; Lima *et al.*, 2012). Spain and France have also started to culture *M. margaritifera* (Gum *et al.*, 2011) yet despite this research, many questions remain on the optimum breeding techniques.

An important part of rearing young juveniles is the optimal feeding of mussels. Dietary studies have not been common for *M. margaritifera* and the nutritional requirements, specifically for juveniles, remain largely unknown. Therefore, the objectives of the present study were to raise juvenile mussels in river water and (a) to test different food mixtures (algae, detritus, crushed red bloodworms) by comparing growth rates and survival rates of juvenile mussels, (b) to determine the optimum number of mussels used to obtain healthy, well-grown individuals, and (c) to test the suitability of detritus in reducing the concentrations of harmful substances such as ammonium, nitrate and nitrite. Based on the results a protocol was suggested in order to optimize the rearing and feeding strategy of juvenile mussels in the laboratory, and to protect them during the most critical stage of their life. The ultimate aim of this work is to help restore the remaining populations of *M. margaritifera* in rivers where they are severely depleted or on the verge of extinction.

METHODS

Source of mussel larvae

In order to obtain freshly excysted mussels, brown trout (*Salmo trutta* L.) were infected with glochidia in August the year before the respective experiments commenced. The glochidia were collected from gravid female pearl mussels from the River Our population and were added to a 60 L tank containing 250 fish (0+, total length 8–9 cm). The water was stirred carefully every 10 min by hand for a period of 45 min. Subsequently, the infected fish

were held in a pond at the mussel rearing facility in Kalborn (Luxembourg) during winter. Seventy brown trout were randomly selected in spring and transferred to a 'juvenile mussel collection station'. The water in the fish tank of the collection station was heated over a 10-day period to 17 °C, and after approximately 2 weeks the glochidia developed into juvenile mussels with a length between 0.30 and 0.48 mm. These excysted and could be collected by sieves with a mesh size of 100 µm.

Feeding experiment

Young individuals of *M. margaritifera* were collected within a maximum period of 24 h after excystment from the host fish and transferred to plastic boxes (10 × 10 × 7 cm). Each box was loosely closed with a cover to allow air exchange and stored at a constant temperature in a conditioning cabinet (Grand cru, Liebherr, Germany) of 17–18 °C for a period of 110 days. In total, 500 young mussels were kept in 500 mL of river water (from R. Our, northern Luxembourg). The mussels were fed with different food mixtures once per week during water exchange that consisted of combinations of algae, detritus and/or crushed red bloodworms (Table 1). Group DAP contained 18 boxes, groups DA, DP and D(2A)P contained 10 boxes. The groups 'Combi' and AP contained five boxes each. The mussel group fed with the food mixture Combi was reared in 2011, 1 year later than the other groups; this experiment was based on the results obtained in 2010.

The algae comprised a mixture of Nanno3600 and Shellfishdiet1800 (Reed Mariculture Inc. Campbell, California, USA). Nanno3600 consists of *Nannochloropsis* sp. with a diameter of 1–2 µm, and Shellfishdiet1800 is a mixture of different algae (*Isochrysis* sp., *Pavlova* sp., *Thalossiosira weissflogii*, *Tetraselmis* sp.) with a diameter of 4–20 µm. Table 2 indicates the composition of Nanno3600 and Shellfishdiet1800 biomass (dry weight) according to Reed Mariculture Inc. Detritus was collected in a wet meadow in Wilwerdange, Luxembourg, to provide higher detritus concentrations than the river. This method was also described by Hruska (1999, 2001) and Lange (pers. comm. 2009). The collected detritus is a natural mixture of varying components depending on season, temperature, rainfall and plants in the collection area. Under normal conditions more than 50% of the algae in the detritus consists of diatoms, followed by green algae. The detritus also consists of organic material, zooplankton, large quantities of different bacteria and fungi as well as sediment comprising clay, silt and fine sand in variable ratios. Analysis of the detritus from the wet meadow in Wilwerdange (within the scope of the project 'Schutz und Erhalt der Flußperlmuschel in NRW', Germany) showed 1.1% dry weight, 48.9% ash content (from dry weight), a pH of 6.5 from the aquatic phase, a conductivity of 90 µS cm^{-1}, 225 000 mg kg^{-1} TOC, and a total nitrogen content of 11 400 mg kg^{-1} (measurements from July 2010). The amount of detritus used for different feeding groups is shown in Table 1. Fresh detritus was collected every 2–3 weeks and stored in the laboratory in a bucket aerated using a pump. The detritus was passed through a sieve of 180 µm mesh size directly before use.

Red bloodworms (Chironomidae, Tetra FreshDelica; Tetra GmbH, Melle, Germany) were crushed and filtered (mesh size 180 µm) and added

Table 1. Composition of the food mixtures used for maintaining young mussels

Group	Food mixture
AP	A: Algae, approximately 24 000 cells from Shellfishdiet1800 and 1 836 000 cells from Nanno3600 in 1 mL of river water and P: 'Protein-containing additive', 200 mg (wet weight) of crushed red bloodworms (Chironomidae) were added to one box of 500 mL river water after each water exchange.
DP	D: Detritus, 25 mL detritus was added to each box after each water exchange and P: 'Protein-containing additive', 200 mg (wet weight) of crushed red bloodworms (Chironomidae) was added to one box of 500 mL river water after water exchange.
DA	Detritus and Algae (see above)
DAP	Detritus, Algae and 'Protein-containing additive'
D(2A)P	Detritus, 2 x Algae and 'Protein-containing additive'
'Combi'	First 30 days: DA. Last 80 days: D(2A)

Table 2. Composition of Nanno3600 and Shellfishdiet1800 biomass (dry weight) as indicated by the manufacturer Reed Mariculture Inc.

	Nanno3600	Shellfishdiet1800
Proteins	58.6%	52.0%
Lipids	14.5%	16.1%
Carbohydrates	20.0%	22.0%
Ash	5.9%	9.9%

after every water exchange as suggested by Lange (pers. comm. 2009) (Table 1).

River water and food mixtures were changed once per week and the mussels were photographed to determine their length, using the computer software ImageJ (http://rsbweb.nih.gov/ij/). Dead animals were removed after visual inspection and counted.

Another 200 juvenile mussels were collected within a maximum period of 24 h after excystment. Two plastic boxes with 100 mussels each were kept in 500 mL of river water at a constant temperature of 17–18 °C in a conditioning cabinet without artificial feeding. The young mussels were observed and the mortality and growth rate of the juveniles were determined after each week to measure the survival rate in river water without additional food. In order to reduce the number of juvenile mussels that were expected to be unable to develop normally without feeding, the number of individuals was reduced to 100 instead of 500.

Density-dependent growth

Following the optimization of the feeding conditions, the optimum number of mussels per box was determined in order to obtain healthy and well-grown individuals. Therefore, juvenile mussels were collected no later than 24 h after excystment. Fifteen 500 mL plastic boxes were filled with water from the River Our. The plastic boxes were divided into three subsets, each with five boxes. Two hundred juveniles were placed in each box in the first subset, 300 juveniles in each box in the second and 400 juveniles in each box in the third. Owing to the high risk of fungal infections occurring in boxes with 500 mussels per 500 mL during the feeding experiments this density was not used again for this experiment. Each box was placed in a conditioning cabinet and kept at a constant temperature of 17–18 °C for a period of 110 days. The mussels were fed with the food mixture Combi (Table 1). River water and food were changed weekly and the mussels were photographed to determine their length using ImageJ. Dead animals were removed after visual inspection and counted.

Water analyses

The water used for rearing juvenile mussels was analysed with a spectrophotometer (Spectroflex 6100, WTW, Weilheim, Germany) to determine the concentrations of ammonium, nitrate and nitrite. Water samples from the rearing boxes were carefully removed from a depth of approximately 3-4 cm with a pipette directly before analysis. Samples were subjected to a nitrate test (9713), nitrite test (14776) and an ammonium test (14752) (all Merck, Darmstadt, Germany) with the ions measured as NO_3^-, NO_2^- and NH_4^+. Daily analyses of each box (each water sample was measured twice) were carried out during an 8-day period (maximum time between water exchanges) for four different groups. Group A contained four boxes with 500 mL river water, group B contained four boxes with 500 mL river water and 25 mL detritus, group C contained four boxes with 500 mL river water and 500 juvenile mussels, group D contained four boxes with 500 mL river water, 25 mL detritus and 500 juvenile mussels. If boxes contained mussels they were fed with algae directly before the first measurement (approximately 48 000 cells from Shellfishdiet1800 and 3 772 000 cells from Nanno3600 per mL of river water).

Statistics

All statistical analyses were carried out with SPSS Statistics (IBM, Chicago, IL, USA).

For comparison of the length of mussels (fed with different food mixtures), Q-Q plots and box plots showed that the data on length were not normally distributed. Thus, log-transformed data were used and a linear mixed model was developed with the different types of food as the independent variable, and the length as the observed variable. Following significant ANOVA Fisher F-tests ($P < 0.001$), individual groups were compared and adjustments (for an overall alpha error of 5%) for multiple comparisons were made using the Bonferroni correction.

To compare the death rate of the mussels (fed with different food mixtures), data were log-transformed to achieve normal distribution. Another linear mixed model was designed with dead mussels per box as the dependent, observed variable, and 'type of food' as the independent variable (fixed factor). Following a significant Fisher F-test ($P < 0.001$), Bonferroni post hoc tests were carried out.

To compare the relationship between the length and the number of mussels per box a linear mixed model was developed with 'length' as the dependent variable and 'number of mussels per box (200, 300, 400)' as the independent variable (fixed factor). As the Fisher F-test was highly significant ($P < 0.001$), individual post hoc tests (Fisher protected LSD tests) were carried out.

For the water analysis, data were log-transformed and a multivariate model was created with NH_4^+, NO_3^- and NO_2^- as observed variables and the contents added to the river water (mussels, algae and detritus) as fixed factors.

RESULTS

Feeding experiment

The survival rate of juvenile mussels was highest for animals fed with Combi (Figure 1). Statistical analyses showed that the survival rate of 80% (101 dead mussels (SD ±164) per box) was significantly different from the food mixtures DP and D(2A)P. The growth of juvenile mussels that were fed with different food mixtures is displayed in Figure 2. Mussels receiving a double concentration of algae (D(2A)P and Combi) grew the most: the greatest length was found for D(2A)P (205.3% or up to 1.16 mm per box (SD ±0.25)), followed by Combi (189.0% or 1.13 mm per box (SD ±0.30), while the two treatments were not significantly different from each other. DAP showed no significant difference when compared with the food mixtures AP and DA. Mussels fed without algae (DP) grew the least (79.5% or up to 0.70 mm (SD ±0.07)) and were significantly smaller than all other groups ($P < 0.05$).

If mussels were kept in river water without additional food the survival rate was 1.5% after 8 days and 100% mortality occurred after 28 days (Figure 3). The growth rate determined for the only individual surviving for 2 weeks (from initially 200 organisms) was determined as 18.0%.

Density-dependent growth

The number of mussels (200, 300 or 400) maintained in a box did not influence the survival rate of juvenile *M. margaritifera*. The survival rate for 200 mussels per box was 96.0%, for 300

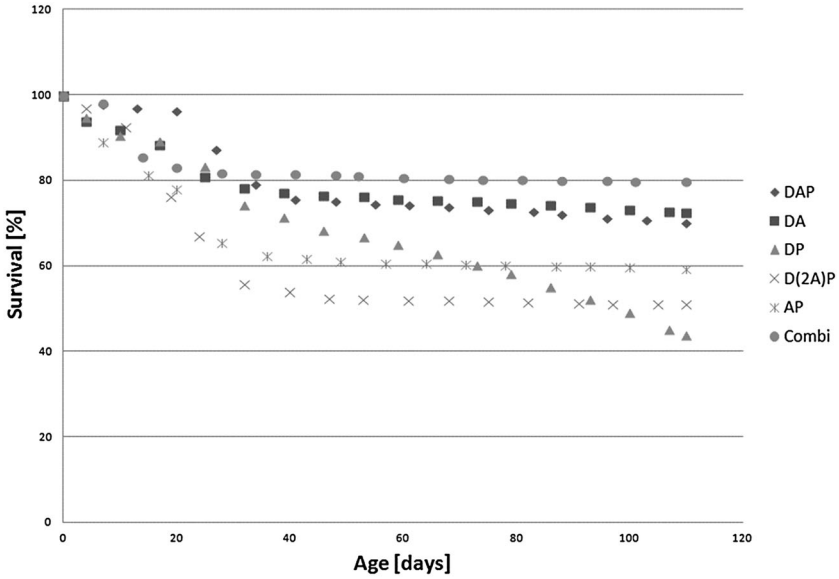

Figure 1. Survival (%) of juvenile *Margaritifera margaritifera* fed with different food mixtures over a period of 110 days. Food mixtures are composed of different elements: A: algae, 2A: 2 × algae, D: detritus, P: crushed red bloodworms (protein-containing additive). Combi: first 30 days: DA, last 80 days: D(2A).

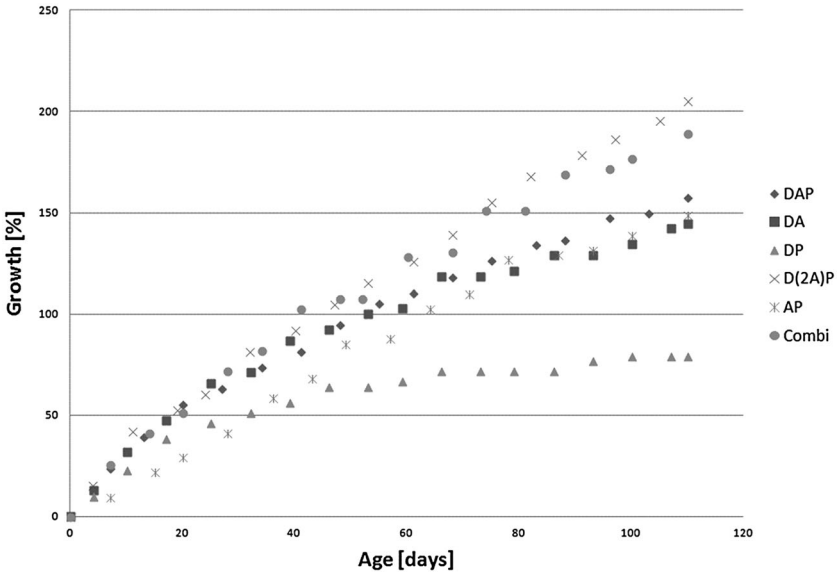

Figure 2. Growth after excystment (length in %) of juvenile *Margaritifera margaritifera* fed with different food mixtures over a period of 110 days. Food mixtures are composed of different elements: A: algae, 2A: 2 × algae, D: detritus, P: crushed red bloodworms (protein-containing additive). Combi: first 30 days: DA, last 80 days: D(2A).

mussels per box 95.7% and for 400 mussels per box 97.5%. However, if 200 mussels were maintained in a box, their growth rate (Figure 4) was significantly higher (1.13 mm or 197.4 %) than in boxes with 300 (1.07 mm or 181.6 %) or 400 individuals (0.96 mm or 152.6 %), and all boxes differed significantly from each other with respect to the size of mussels ($P < 0.05$).

Water analyses

Levels of aqueous nitrate, nitrite and ammonium (measured as NO_3^-, NO_2^- and NH_4^+ in % of initial concentrations) for the maintenance of conditions tested are summarized in Figure 5. As a general trend it can be seen that river water with detritus added (Figure 5(b)) showed more or less constant nitrate levels and even decreasing concentrations of ammonium and nitrite during the experimental period, with nitrite being reduced from 0.14 to 0.06 mg L^{-1} and ammonium from 0.63 to 0.18 mg L^{-1}. If mussels were fed with algae and detritus (Figure 5(d)), ammonium was reduced from 0.63 to 0.18 mg L^{-1} and nitrite from 0.14 to 0.06 mg L^{-1}. The concentration of nitrate increased slightly from 9.95 to 11.70 mg L^{-1}.

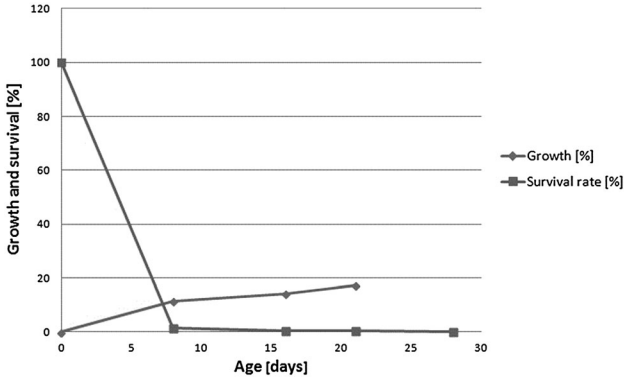

Figure 3. Survival (%) and growth after excystment (length) of juvenile *Margaritifera margaritifera* kept in river water (fed without additional food).

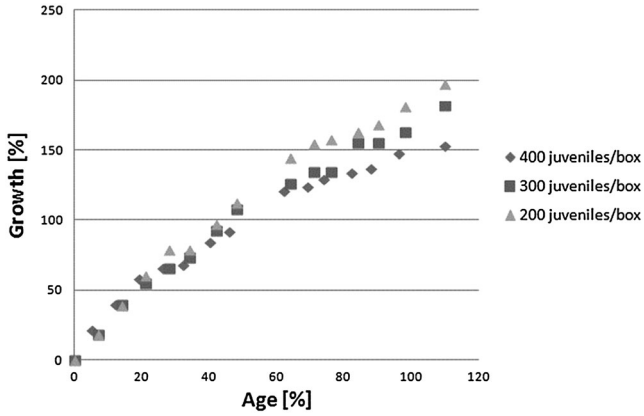

Figure 4. Growth after excystment (length in %) of juvenile *Margaritifera margaritifera* depending on the number of mussels per 500 mL plastic box over a period of 110 days.

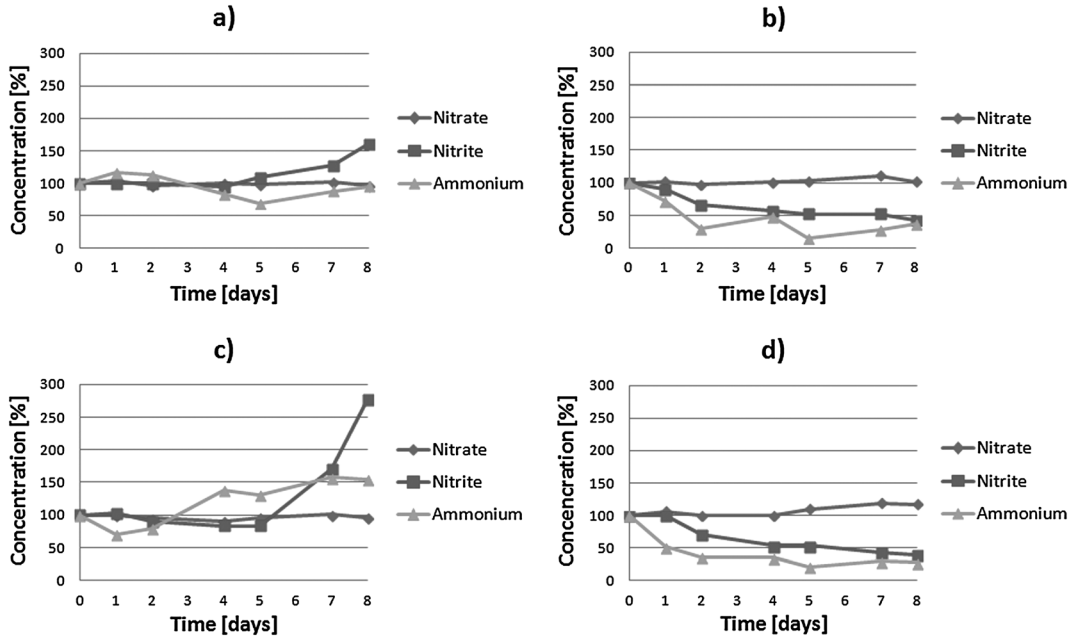

Figure 5. Levels of aqueous nitrate, nitrite and ammonium (in % of initial concentrations) in 500 mL plastic boxes with (a) river water, (b) river water and 25 mL detritus, (c) river water, mussels and algae (d) river water, mussels, algae and 25 mL detritus.

In contrast, increasing levels of nitrite and ammonium were found if mussels were fed with algae but without detritus (Figure 5(c)) and these were significantly different from the results on day 8 when detritus was present in the boxes. The concentration of nitrite altered noticeably from 0.16 to 0.43 mg L^{-1} and the ammonium concentration from 0.37 to 0.57 mg L^{-1}. Even if only water without mussels was monitored for 8 days (Figure 5(a)), an increase in nitrite was observed with ammonium levels being constant.

DISCUSSION

The results show that survival and growth rates of artificially reared juveniles of *M. margaritifera* were highest if mussels were maintained in a plastic box containing 500 mL of water with a weekly water exchange and being fed with a combination of detritus and algae, with a doubling of the algal concentration after the first 30 days (food mixture Combi). Similar conditions were described by Jones *et al.* (2004) who used plastic dishes (6 × 6 × 5 cm) in non-recirculating aquaculture systems containing fine sediment (<105 µm) to rear the endangered dromedary pearlymussel (*Dromus dromas* Lea). However, the survival rate of *D. dromas* after 1–2 weeks was only 29.7% (Jones *et al.*, 2004). Hruska (1999, 2001), who established the first successful rearing method for M. *margaritifera* in the 1980s also used small containers and fed detritus. After a few months of intensive care, mussels were placed into cages or containers installed in rivers or semi-natural flow channels. With this method it was possible to rear 30 000 mussels to an age of 3 years or older (Gum *et al.*, 2011). The method of Hruska was adapted by M. Lange (Germany) by adding animal protein to the containers (Gum *et al.*, 2011) to improve the amino acid supply. A few other strategies for rearing juvenile *M. margaritifera* have been used successfully, including mussel cages in the wild (Buddensiek, 1995), semi-natural stream channels with gravel (Preston *et al.*, 2007) and gravel containing salmonid hatching baskets transferred to indoor salmonid hatchery troughs (Hastie and Young, 2003; Skinner *et al.*, 2003). In North America, recirculation systems were used for rearing various freshwater mussel species (Gatenby *et al.*, 1996; Jones and Neves, 2002; Barnhart, 2006) but not for *M. margaritifera*. In these systems,

supplemental feeding of unicellular algae was found to be necessary (Barnhart, 2006). Growth and survival rates of juvenile freshwater mussels appear to be higher in flow-through systems when compared with recirculation systems, possibly due to dietary imbalance (Jones and Neves, 2002).

Owing to the high influx of sediment from the River Our, the aforementioned methods in cages and baskets would not be suitable for rearing young *M. margaritifera* at the facility in Kalborn, Luxembourg. All cages or baskets capable of holding juvenile *M. margaritifera* needed to have mesh sizes smaller than 0.3 mm as the juvenile size after excystment was approximately 0.3–0.4 mm. This mesh size would have become clogged very quickly and would have to be cleaned manually which would have increased the workload and disturbed juvenile mussels. In addition, a semi-natural stream channel with gravel was not suitable for the laboratory in Kalborn, as juvenile mussels would have become buried under sediments. Another method to rear mussels from sediment-rich rivers was therefore required. With this method of rearing juvenile mussels in plastic boxes in the laboratory it was possible to increase the likelihood of juvenile mussel survival. This method was both efficient and cost-effective as the boxes were easy to handle and compact. Intensive care was needed only once per week for water exchange and feeding, while the boxes required no further intervention in the meantime. During the water change, predators and food competitors such as *Daphnia* sp. could easily be removed with a pipette. According to Zimmerman *et al.* (2003) predation and competition by microfauna may be an important factor in the mortality of early juvenile freshwater mussels. For example, flatworms of the genus *Macrostomum* are known to be predators of newly metamorphosed juvenile mussels and different strategies have been developed to control their prevalence (Zimmerman *et al.*, 2003). Individuals of cladoceran species were often observed in the plastic boxes and removed using a pipette as they also feed on algae. During the water exchange it was also possible to remove mussels that were contaminated with fungi. The survival and growth rate could be determined easily by counting empty shells and by photographing and measuring shell length at various stages of development. One disadvantage of this method was that fungal contamination within a box could quickly spread among individuals because of the low volume of water (especially if 500 mussels were maintained in 500 mL water). Therefore cleaning the boxes with hot water or alcohol (70%) was occasionally required.

The health of the juvenile mussels in this experiment was strongly correlated with the dietary regime. When using river water with no additional food for juvenile mussels, only one out of 200 mussels had survived after a 2 week period. These results show that juvenile mussels have food reserves for less than 8 days after excystment and need additional food as soon as possible after dropping from the host fish.

The nutrient composition including algal species and optimum size for feeding *M. margaritifera* is unknown. Yeager *et al.* (1994) suggested that the primary food source for juveniles may change even within the first 2 weeks after metamorphosis, hence different feeding strategies would be required for different stages of growth. When juvenile mussels fall from the host fish they directly start to pedal-feed (i.e. ciliary tracts on the foot of the juveniles transport food particles to the labial palps) on algae and organic matter (Geist and Auerswald, 2007). According to Hastie and Young (2003) the change to filter feeding represents a critical period for survival in captive breeding programmes and the early juvenile stages are very vulnerable to disturbance and have very specific substrate requirements. These problems (narrow substrate requirements, unknown and changing food requirements) were avoided by mixing different food sources such as different species and sizes of algae, and the use of natural detritus. A mixture of algae generally results in higher growth and survival rates in bivalve aquaculture compared with single algal species (Romberger and Epifanio, 1981; Brown *et al.*, 1997). For this study, commercially available algae were preferred for the experiment because the culture of different algal species is very time-consuming and therefore expensive. In the present study, algae appeared to be essential for the development of juvenile mussels as their growth was associated with the concentration of algae and the survival

rate was dependent on the presence of algae. The absence of algae in mussel food could have been responsible for the substantial mortality after the first month in the mixture containing only detritus and crushed red bloodworms. All other mussel groups that were fed with algae showed virtually no mortality or only a low death rate after this first month period.

Various combinations of algal species have already been used to culture juveniles of other unionid mussels (Hudson and Isom, 1984) and because of their apparent importance in the diet of unionids, algal species such as *Nannochloropsis* sp. have been introduced into the design of recirculating culture systems as the primary food source (Gatenby *et al*., 1996; O'Beirn *et al*., 1998; Barnhart, 2006).

In addition to algae, 25 mL of detritus was added to each box as Way (1989) and Gatenby (2000) reported that algae and detritus are the main dietary components for mussels. The detritus used was a mixture of organic material, algae, zooplankton, fungi and bacteria but also sediment consisting of clay, silt and fine sand. Jones *et al*. (2005) recommended adding a sediment substrate to increase the survival and growth of two juvenile freshwater mussel species (*Epioblasma capsaeformis* Lea and *Villosa iris* Lea) during culture. In addition, naturally occurring silt is known to increase the growth of other bivalves. For example the growth of *Mytilus edulis* (L.) juveniles fed with algae was improved by 30–70% when 5 mg L^{-1} of natural silt was added (Kiørboe *et al*., 1981). Soil clays possess the property of adsorbing potassium, calcium and magnesium, creating a storehouse of certain elements and nutrients (Kelley, 1942). Furthermore, compounds such as amino acids and sugars can adsorb onto the surface of soil particles. Thus, juveniles that ingest fine sediments associated with algae could acquire minerals and nutrients essential for optimal growth and survival (Weiss, 1969). Although it is known that other aquatic invertebrates can show a better growth and survival rate when offered animal detritus (Yee and Juliano 2006; Yee *et al*., 2007) the addition of crushed red bloodworms to juvenile *M. margaritifera* showed no effect on either growth or survival rate in the present study. Moreover, the least favourable results with respect to survival and growth rates were obtained with a food mixture consisting of detritus and crushed red bloodworms only. This shows that algae are an essential part of the food composition and that detritus should not be used as the primary food for juvenile freshwater pearl mussels.

Mussels that were fed with the food mixture D(2A)P showed the highest growth rate but the survival rate was low. The majority of these mussels died within the first 30 days which might indicate that the high concentration of algae and potential decomposition products was harmful to the mussels. This suggests that it is better to feed a lower concentration of algae during the first month as was confirmed by the good survival rate (80%) of the mussels fed with the food mixture Combi (Table 1) in which a low concentration of algae was given during the first month and a higher concentration thereafter. The D(2A)P and Combi feeding regimes resulted in a mussel length >1 mm (1.16 mm and 1.13 mm respectively) during a short growing season of 110 days, which was favourable compared with the results obtained from other studies. For example, juvenile *M. margaritifera* in sheet cages from rivers in Germany and Scotland reached mean sizes from 1.2 to 1.8 mm after two growing seasons (Buddensiek, 1991). Hruska (1999) reported that mussels increased by a factor of 2.5 during the first growing season, when kept at a rearing station with optimized temperature and food supply. The size of 1 mm is regarded as the minimum total shell length for survival during the first winter (Lange and Selheim, 2011) and Buddensiek (1995) observed that whether or not juveniles survived the first winter in mussel cages in the wild was dependent on their size. Mussels < 700 µm died and only large animals > 900 µm had a 50% chance of survival during the cold winter months. When mussels reach a size > 1 mm it is possible to use flow-through rearing systems – for example, mussel cages with larger mesh size to avoid clogging, or aquaria with sand in which mussels are visible and can be relocated. Specific studies are needed for mussels larger than 1 mm that are kept in boxes as the volume of water and the amount of food must be adapted to an increased mussel size and food consumption.

This method of rearing *M. margaritifera* in plastic boxes and feeding Shellfishdiet, Nanno and detritus has already been used successfully by other rearing facilities in Germany (Perlenbach) and Austria (Waldaist) and shows good results (Selheim and Scheder, 2012, pers. comm.). Also *Margaritifera falcata*, a species from North America, was found to survive and grow well when reared in boxes with a similar feeding regime (Barnhart, 2012, pers. comm.).

The optimum number of juvenile mussels in a 500 mL plastic box was 200. Although the number of mussels per box had no influence on the survival rate in this experiment, mussels from boxes with 300 or 400 individuals were significantly smaller than mussels from boxes containing 200 individuals, most likely because of food competition. The use of fewer than 200 mussels per box is not recommended because it is too space- and time-consuming, especially during the water exchange. On the other hand, the prevalence of fungal infections increased when 500 mussels were maintained together in one box.

Water chemistry is another important factor affecting the survival of juvenile mussels. A mortality of 100% can occur if a low level of pollution is present when mussels are leaving the host fish and try to establish themselves in the sediment (Skinner *et al.*, 2003). Accordingly, juvenile mussels are far less pollutant-tolerant than their adult counterparts. Nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) are potentially harmful and can lead to a low survival rate for juvenile *M. margaritifera*. The uptake of nitrate by aquatic animals is more limited than the uptake of nitrite or ammonium and is therefore less toxic (Jensen, 1996; Camargo and Alonso, 2006). It was reported that some mussel species close their valves when exposed to high ammonia levels, whereas other mussel species continue siphoning (Horne and McIntosh, 1979). It is unknown, which strategy is followed by juvenile freshwater pearl mussels, but even if they keep their valves closed this will prevent them from feeding and can result in starvation when ammonia levels are permanently high. Skinner *et al.* (2003) stated that it is generally accepted that pearl mussels need a high water quality with nitrate levels not exceeding 1.0 mg L^{-1} and phosphates < 0.03 mg L^{-1}. Toxicity tests with juvenile freshwater mussels (*Lampsilis siliquoidea*, *Lampsilis fasciola* and *Villosa iris*) showed chronic values for ammonia that ranged between 0.37 and 1.2 mg L^{-1} for survival and from 0.37 to 0.67 mg L^{-1} for growth (Wang *et al.*, 2007). Acute (96-h LC_{50}) toxicity tests with two freshwater unionid mussels showed median lethal NO_3 concentrations ranging from 357 mg L^{-1} for *Lampsilis siliquoidea* to 937 mg L^{-1}, and NO_2 concentrations of 177 mg L^{-1} for *Megalonaias nervosa* (Soucek and Dickinson, 2012). In the primary stage of nitrification, ammonium is oxidized by bacteria such as *Nitrosomonas* sp., which converts ammonia to nitrites. Other bacterial species, such as *Nitrobacter* sp., are responsible for oxidation of the nitrites into nitrates (Schlegel and Zaborosch, 1992). Without detritus, no nitrification occurred in the boxes with mussels and the nitrite and ammonium concentrations increased significantly. Furthermore, the tolerable levels of nitrite and ammonium were exceeded. If detritus (25 mL) was added to the plastic boxes, ammonium and nitrite levels were reduced by more than 50% compared with the initial value within 8 days which shows that nitrification occurred in these boxes. It can be assumed that juvenile *M. margaritifera* are very sensitive to these substances as others have stated that freshwater mussels (Unionidae) are one of the most sensitive faunal groups to organic enrichment (Simmons and Reed, 1973) and several studies indicate this in particular for ammonia (Wade, 1992; Augspurger *et al.*, 2003; Mummert *et al.*, 2003).

In conclusion, these experiments have shown that rearing juvenile *M. margaritifera* in 500 mL plastic boxes is an effective method for obtaining healthy mussels of 1 mm shell length or more within 110 days. We recommend a maximum of 200 (to 300) individuals per 500 mL river water, feeding a combination of algae and detritus within the first 30 days and doubling the concentration of algae thereafter to increase the growth and survival of the mussels. Furthermore, the addition of 25 mL of detritus per 500 ml river water led to a useful decrease of the potentially harmful ions ammonium and nitrite as detritus contains bacteria that are capable of nitrification.

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