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ORIGINAL RESEARCH

Effects of nitrate on early life stages of *Astacus astacus* (Linnaeus, 1758) and *Procambarus virginalis* (Lyko, 2017)

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Abstract We examined the effects of nitrate on the embryonic development of two freshwater crayfish species, the indigenous noble crayfish (*Astacus astacus*) and the invasive marbled crayfish (*Procambarus virginalis*). Nitrate measurements show concentrations of 50 mg/L in surface waters of Germany, while concentrations in groundwater measure up to 100 mg/L. Performing a chronic exposure experiment, we exposed embryos of both species to nitrate concentrations of between 0.0 mg/L and 500 mg/L to estimate the influences of nitrate concentrations on survival, hatching development time, malformations and growth. We observed the first effects on survival at 14 mg/L LOEC (lowest observed effective concentration) nitrate for marbled crayfish. For noble crayfish, we estimated an EC_{50} value of 55.7 mg/L on hatching rate. Our results show that eutrophication of surface waters can negatively affect the embryonic development of freshwater crayfish with serious consequences on recruitment.

Keywords Marbled crayfish . Noble crayfish . Juveniles . Nitrate . Embryonic

Introduction

The negative effect of artificial water structures in combination with unnatural flow regimes on flora and fauna of European surface waters has been known for a long time (Rolls et al. 2013; Trinci et al. 2017). As a consequence, many species are severely threatened or have already become extinct (Crandall and Grave 2017; Thomsen et al. 2012). The regulations in the European water framework directive aim at improving these conditions (Birk et al. 2012).

The handling of the intensified pollution of waterbodies with nutrients from eutrophication or entering via non-point sources from farmland is more complex. Although there are some regulations for the maximum permissible nutrient inputs, e.g. the water framework directive and the habitat directive, these are rarely based on scientific data derived from natural communities. Toxicological investigations are therefore indispensable to the in-depth evaluation of the impact of nutrients.

Over the last few centuries and during the last 50 years in particular, the natural nitrogen cycle has been heavily influenced by human activities. Agriculture and industry introduce large amounts of nitrogen into surface and groundwater systems, mostly as ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) (Day, JR. 2013; Howarth 1988; Rabalais 2002; Wetzel 2001). In concentrations of up to 50 mg/L nitrate were measured in German surface waters (BMU 2016). Groundwater, contributing more than 70% to German drinking water supplies (upper limit: 50 mg/L), contained up to 100 mg/L nitrate (BMU 2016).
Despite their prevalence in European ecosystems, little is known about the effects of these pollutants on most species. This also holds true for “keystone species” and “ecosystem engineers” like freshwater crayfish. They belong to both of these groups due to their influence on nearly every trophic level and on their structural environment (Weinländer and Füreder 2016). Consequently, the habitat directive protects all

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native freshwater crayfish species in Europe.

Bohl 1989 showed that a nitrate concentration of more than 500 mg/L is harmful to adult noble crayfish (*Astacus astacus*). The impact on juveniles was not estimated for *A. astacus*. (Meade and Watts 1995) examined the influence of N-pollutants on juvenile Australian crayfish, *Cherax quadricarinatus*. They found that nitrate levels of up to 1000 mg/L had no effect on the survival and metabolic rates of this species, but there are no published studies estimating the influences of nitrate on the reproduction of these animals. However, the reproduction strategy of most crayfish species leads to a potentially long exposure time: female noble crayfish, for example, carry their eggs outside their body under their abdomen for many months. This relatively long timespan may lead to a high impact of pollutants on these early developmental stages. A higher sensitivity of juvenile stages to nitrogen compounds as compared to adults has also been found for the caddisfly *Hydropsyche* (Engels 1997) and for midge larvae of the genus *Chironomus* (Neumann et al. 2001) .

For the above reasons we wanted to examine the influences of different nitrate concentrations on embryonic development and whether these effects are transferrable from our model organism to a native species of Germany. We used noble crayfish (*A. astacus*) as the model native species in this investigation. *A. astacus* is especially suited to our study because its natural habitat (lower sections of streams, lakes etc.) is often influenced by agricultural drainage and sewage (Skurdal and Taugbøl 2002). The second organism we studied was the marbled crayfish (*Procambarus virginalis*). One animal can produce up to 700 eggs every 8-9 weeks and due to its parthenogenetic reproduction strategy the offspring is genetically identical (Chucholl and Pfeiffer 2010; Vogt et al. 2004). These facts provide a predictable and continuous supply of clonal eggs, making this species a suitable model organism in the laboratory (Hossain et al. 2018; Vogt 2018).

In our hypothesis nitrate impacts survival until first moult, hatching and embryonic development and causes malformations and weight decrease. We investigated the effects on the embryonic development of *A. astacus* and *P. virginals* under controlled laboratory conditions. Survival rate was examined as response variable for potential effects on population size, hatching rate was recorded to reveal differences between hatching and survival. Weight decrease and indications of potentially occurring malformations were recorded to reveal critical concentrations.

Materials and methods

Adult marbled crayfish, the offspring of breeding individuals, were obtained through the aquatic trade and kept individually in 12 separate 25L aquaria in aerated tap water. The ambient temperature was 23 °C, light regime was L:D = 10:14 hours, and the animals were fed frozen midge larvae and peasad libitum. Under these conditions *P. virginalis* produces parthenogenetic eggs every 8-9 weeks in our laboratory.

Female and male noble crayfish were obtained from a hatchery in Schleswig-Holstein (Krebszucht Oeversee). They were kept together in three 600L tanks throughout reproduction at 8°C and a light regime of L:D = 10:14. The animals were inspected on a daily basis and eggs were separated from the females 72 hours after extrusion, whereupon egg incubation was started as described below.

The eggs were bred in 12-well multititer plates (Greiner bio-one, Kremsmünster, Austria), one egg per well. Each well was filled with 0,5 mL autoclaved and well aerated tap water and 0,5 mL deionized water with a specific concentration of nitrate. The seven stock solutions consisted of double the amount of the experimental concentration of nitrate (as $NaNO_3$) in double distilled water. Concentrations were chosen to cover a wide spectrum but considering naturally occurring concentrations. The concentrations tested and the number of replicates are shown in Table 1.

The plates were placed on a laboratory shaker rotating at low speed to simulate the abdominal movements of parental crayfish and to ensure a constant supply of oxygen for the eggs. The liquid in the wells was changed daily by removing the contents and pipetting 0,5 mL fresh, autoclaved water and 0,5 mL of the nitrate solution to maintain water quality and concentrations. Egg development was examined under a binocular microscope at 40x magnification three times a week. Within the scope of these observations developmental stages, mortality, hatching and occurrence of malformations were recorded. The developmental stage was gauged by the examples given in (Sandeman and Sandeman 1991) noble crayfish) and (Alwes and Scholtz

Table 1 Stock solutions, concentrations and number of replicates used in this study

Concentration stock solution (mg/L)	Volume stock solution (mL)	Volume deionized water (mL)	Experimental concentration nitrate (mg/L)	n marbled crayfish	n noble crayfish
1000	0,5	0,5	500	30	10
500	0,5	0,5	250	30	10
250	0,5	0,5	125	30	10
125	0,5	0,5	62,5	30	10
62,5	0,5	0,5	31,25	30	10
28	0,5	0,5	14	30	10
0	0,5	0,5	0	30	10

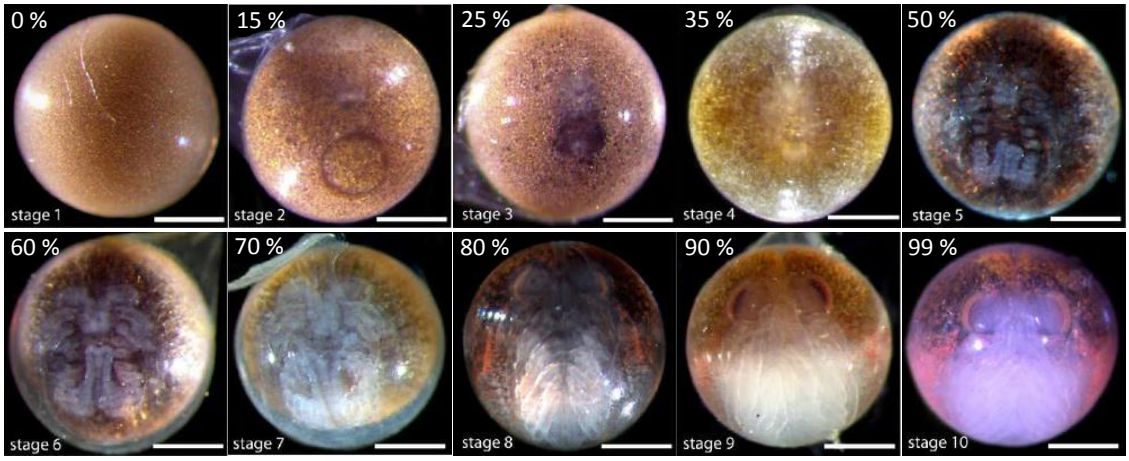

Fig. 1 Developmental stages of unfixed embryos exemplifying the ten characteristic stages with percentages of total development until hatching. Scale bar=500 µm. Stage 1: the egg without external signs of the cleavage process. Stage 2: the beginning of gastrulation results in the formation of a circular blastopore of about 300 µm in diameter. Stage 3: the caudal papilla adjacent to the closing blastopore and the two optical lobes are visible as whitish regions. The closing blastopore appears as a contracting dark patch posterior to the germ disc. Embryo at the end of stage 3: the recruitment of cell material of the naupliar anlagen is advanced and the blastopore is now closed. Beneath the posterior half of the germ, the restructuring yolk appears as a dark diffuse cloud. Embryo at the end of stage 4: the dark patch has disappeared and the yolk is reorganized. The caudal papilla is bent ventrally from now on. Stage 5: the caudal papilla is flexed and its tip reaches the margin of the second maxillary buds. Embryo at the beginning of stage 6. The tip of the caudal papilla reaches the segment of the second maxillae. Stage 6: the tip of the caudal papilla now reaches the posterior margin of the mandibles. Stage 7: the thoracopods are arranged like a basket. Stage 8: the pereiopods cover the caudal papilla, but do not meet at its midline. The retinal pigmentation begins as yellowish stripes below the outer eye margin. The orange chromatophores of the carapace appear. Stage 9: the tips of the chelae of the first pereiopods nearly overlap the rostrum between the eyes. The advanced retinal pigmentation has turned the yellowish stripes into distinct brown retinal layers. Stage 10: the prehatchling (transformed Alwes and Scholtz 2006).

2006), marbled crayfish). Both graduations were transferred to percentages to generate direct comparability (Fig. 1).

We calculated the hatching rate for all individuals that had reached the development stage of 95%, leading to the exclusion of survival data until this stage. To asses delay in hatching we recorded it for every individual separately. Therefore, from the development stage of 90 % every egg was observed on a daily basis. Furthermore, we also photographed the malformed embryos. These kinds of malformations were first observed in this study. They were detectable from the stage of at least 75% embryonic development onwards. We defined a malformation as less than 70% coverage of the ventral side embryo by extremities, compared to control groups, while showing a visible heartbeat.

The experiments were carried out as blind studies to avoid observer-biased results. They were terminated after the first moult of juvenile crayfish.

Statistical methods

All statistical analyses were performed using R version 3.2. (R Core Team 2015). Differences in hatching-rate, weight and number of malformations between different groups were tested for normality and equal variances prior to analysis. If both were evident, a one-way ANOVA was performed and subsequently a Tukey post-hoc test was performed. For non-parametric data, a Kruskal-Wallis test was used. The EC_{50} and LC_{50} values were first corrected according to Abotts and then estimated utilizing the trimmed Spearman Karber method. We were only able to estimate EC_{50} values for hatching rate due to lower effects on malformation and weight. Survival rates were analysed using the Kaplan-Meier survival analysis of Gehan Breslow and the groups were compared via the Holm-Sidak method. The embryonic development was analysed via linear regressions. Due to good correlation values (>0,8) the linear regressions were compared with an ancova (Analysis of covariances). Pictures were analysed in GIMP 2.8 (version 2.8, Fa. the Gimp Team).

Results

Survival rate

The survival rate of both species was reduced with increasing nitrate concentrations. However, significantly lower rates occur even from low concentrations starting at 14 mg/L ($p<0.021$) in the case of the marbled crayfish (Fig. 2 a). For noble crayfish the first negative effects were observed at concentrations of 62.5 mg/L. ($p=0.0178$; Fig. 2 b). Notably, all embryos that were exposed to concentrations of 125 mg/L or higher died before hatching. Additionally, only two individuals survived until the first moult, both originated from the control group (Fig. 2).

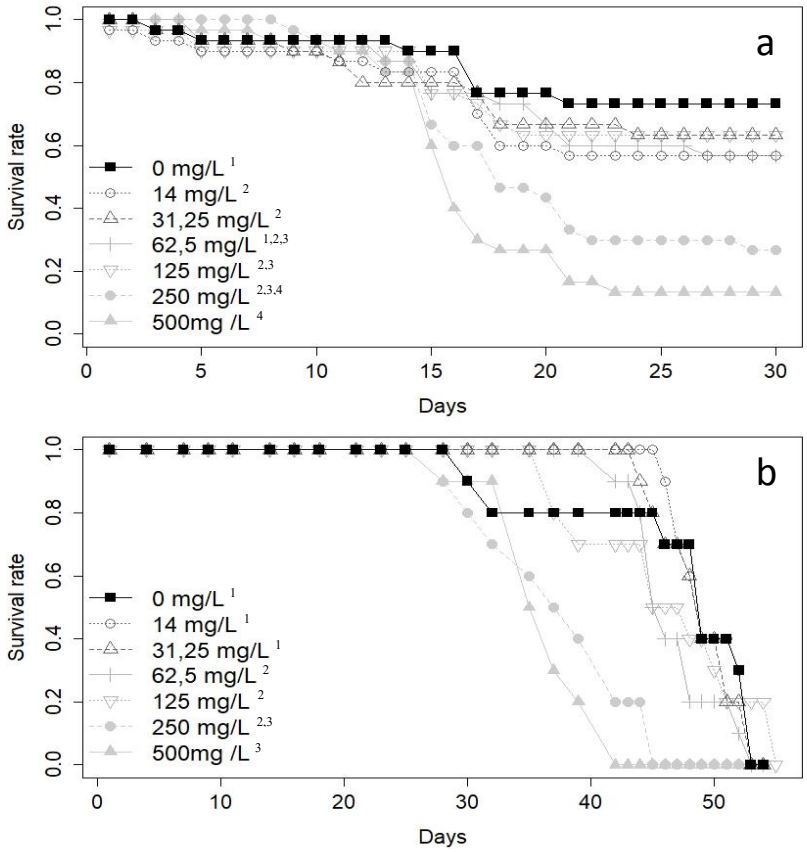

Fig. 2 Survival rates of marbled crayfish (a) and noble crayfish (b) under exposure to different nitrate concentrations. Note the different time scales on the x-axis / ordinate. Numbers in Legend represent statistical differences calculated with Kaplan-Meier survival analysis of Gehan Breslow.

Sublethal effects

By recording the hatching rate, we were able to show that concentrations of 62,5 mg/L and higher lowered the number of hatching individuals for both species (noble crayfish p=<0,001; marbled crayfish p= 0,035) (Fig. 3). We were able to calculate half-maximal effective concentration (EC_{50}) of both species for better comparability of effects with other potentially harmful substances. For noble crayfish the EC_{50} value was 55.7 ± 1.9 mg/L after 45 days. For marbled crayfish, EC_{50} was calculated to be 349.2 ± 2.1 mg/L after 15 days.

Malformations (as depicted in Fig. 4) were only observed for noble crayfish (Fig. 5). Here the first higher malformation numbers occurred at concentrations of 31.25 mg/L., accounted by the low number of noble crayfish embryos. Statistical analyses were only calculated for marbled crayfish, without significant differences. All individuals exhibiting malformations died before hatching.

To compare the developmental stages of the embryos, we calculated and compared linear regressions of the stages depending on time. However, significant effects based on nitrate concentration could neither be detected for marbled crayfish, nor for noble crayfish (Fig. 6).

The comparison of weight distributions did not show significant differences. The data are given in the supplemental material.

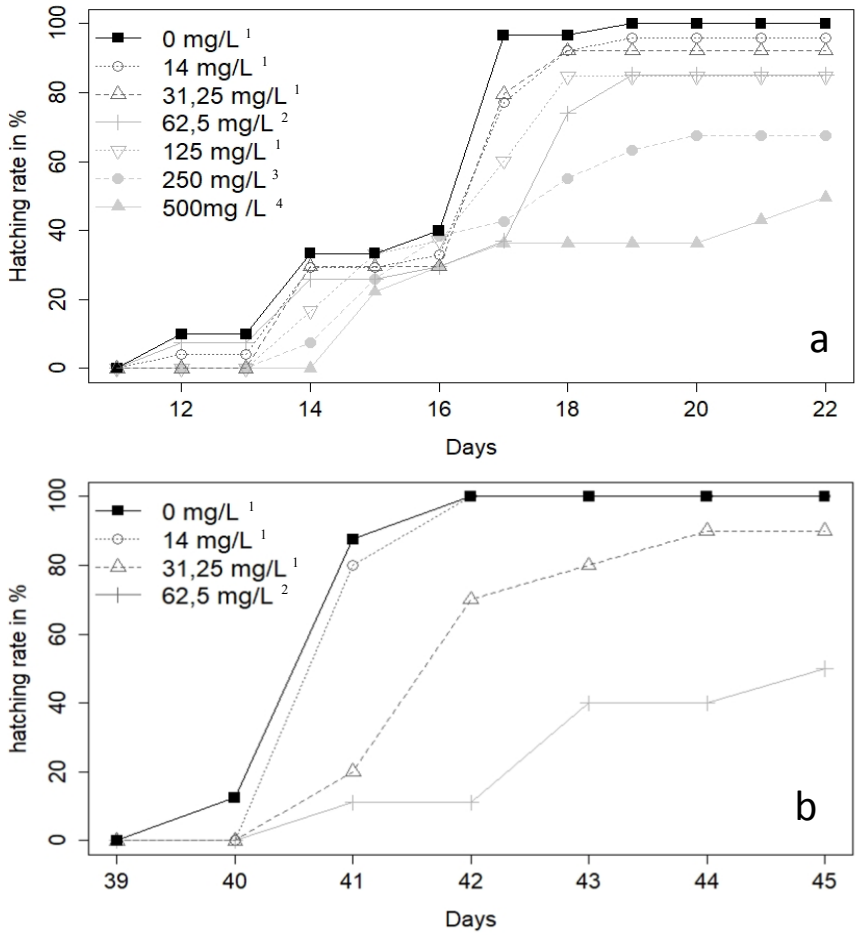

Fig. 3 Hatching rates of marbled crayfish (a) and noble crayfish (b) under exposure to different nitrate concentrations. Numbers in Legend represent statistical differences calculated with one way ANOVA and Tukey post-hoc test to estimate differences in means of hatched animals over time.

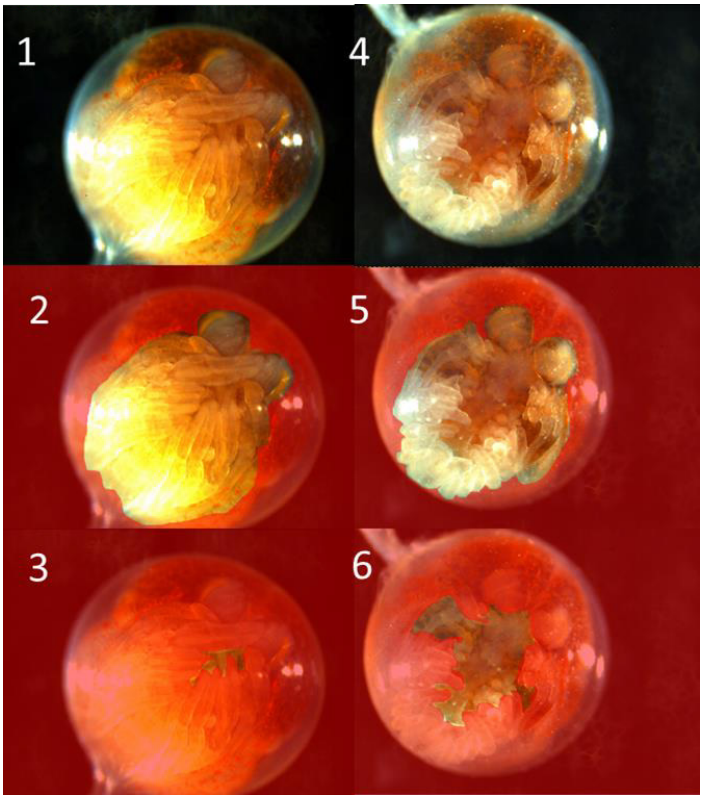

Fig. 4 Comparison of one normal developed A. astacus embryo of the control group (left) and one with malformations of the highest treatment group (right) at 95% development. In 1 and 4 the embryo is shown as seen under the binocular microscope, in 2 and 5 the visual body of the embryo is highlighted and in 3 and 6 the uncovered part of the ventral side is highlighted showing the underdeveloped extremities

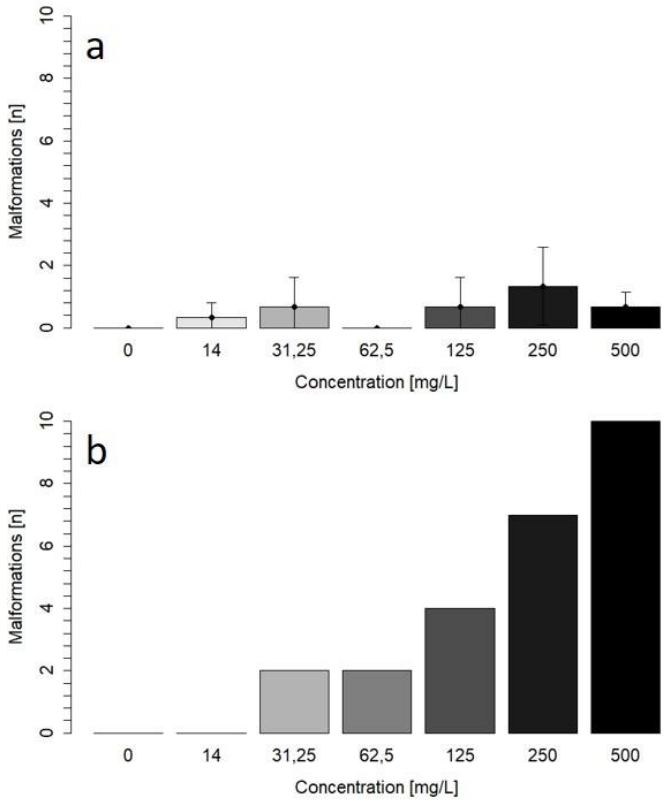

Fig. 5 Number of malformations of marbled crayfish (a) and noble crayfish (b) under exposure to different nitrate-concentrations. Statistical analysis with one way ANOVA and Tukey post-hoc test could not provide evidence of significant differences.

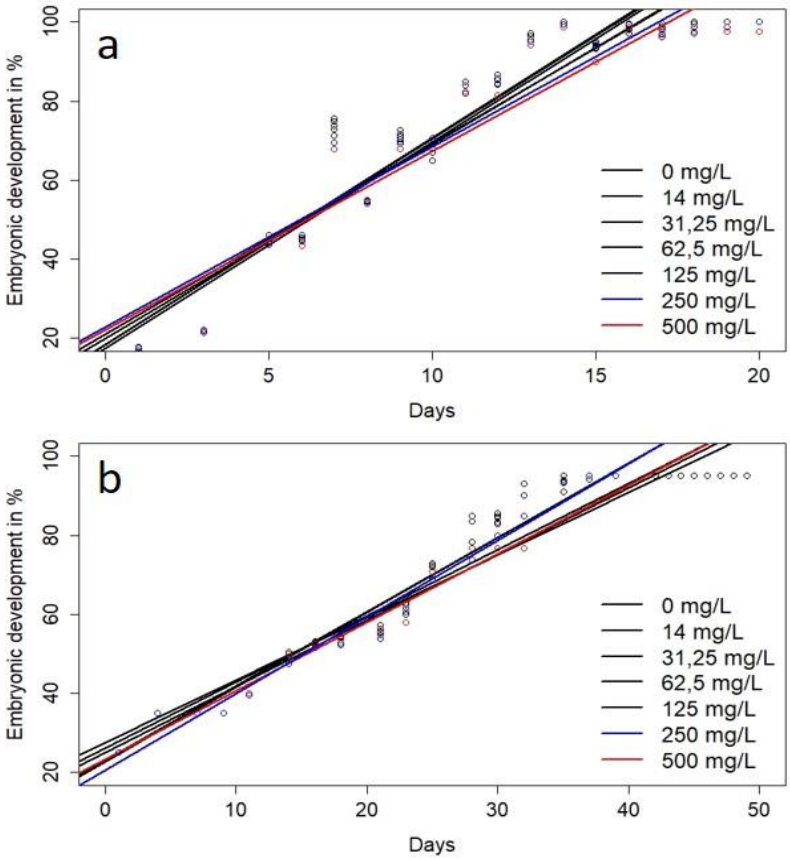

Fig. 6 Embryonic development of marbled crayfish (a) and noble crayfish (b) under exposure to different nitrate concentrations. ANCOVA analysis could not provide evidence of any significant differences.

Discussion

The results of this study elucidate that nitrate concentrations from 14 mg/L can have influences on crayfish survival. In relation to concentrations found in surface (50 mg/L) and ground waters (100 mg/L) in Germany (BMU 2016) it is therefore most likely that nitrate has an effect on the survival of crayfish embryos.

Beside survival- and hatching rates, sublethal effects such as malformations may strongly influence population dynamics. Such malformations may be the occurrence of conjoined twins as reported for decapod crustaceans (Alwes and Scholtz 2006; Harlioğlu 2002; Harzsch et al. 2000; Jara and Palacios 2001) but the underlying mechanisms remain largely unclear. However, in our study we observed a malformation of the extremities that had hitherto never been described (Fig. 1). Consequently, such malformations of the thoracopods of *decapoda* may serve as early indices of water pollution, especially as the number of malformations increased linearly with nitrate concentration.

Evaluation of other sublethal effects, such as development time and the individual body mass when hatching, delivered no significant proof for an impact of nitrate on these parameters. However, as shown in figure 5 we can observe a tendency to slower development with higher nitrate doses. Due to a lack of evidence about the effects on these parameters, further studies will be necessary in the future.

Reasons for differences in marbled and noble crayfish regarding effective concentrations, survival rates, malformations and EC_{50} values can be explained in several different ways. One is the smaller size of eggs of marbled crayfish (Savolainen et al. 1996; Skurdal and Taugbøl 1994; Souty-Grosset 2006): the smaller the surface of an organism, the more it is influenced by toxins (Grinwis 2000). The different reproduction strategies of the species lead to different exposure times throughout embryonic development. Nevertheless, the longer the embryos are exposed to high nitrate concentrations, the higher the risk of negative effects (Annamalai and Arunachalam 2017; Cheng 2002). This observation can easily be transferred to wildlife

populations. Additionally, marbled crayfish have a high tolerance to environmental influences (Kaldre et al. 2012; Souty-Grosset 2006). The estimated EC_{50} values of this study support this statement. The EC_{50} values were about seven times higher for marbled crayfish at a three times shorter exploration time. These findings indicate that due to its resistance to environmental influences, studies of the model organism, the marbled crayfish, will always yield at least some different results to studies of the indigenous species, the noble crayfish.

Comparing data for the sensitivity of adult crayfish (Bohl 1989; Meade and Watts 1995) with data of embryonic and early juvenile stages, we were able to show a much higher sensitivity to nitrate during early life stages than in adult animals. The higher sensitivity of juvenile crayfish towards pollutants has also been shown by (Khan and Nugegoda 2007) for *Cherax destructor* and copper as stressor. They estimated an EC_{50} value of 1400 µg/L after 96 hours for adults, while determining 379 µg/L for juveniles. The work conducted by (Lindström-Seppä et al. 1983) on adult *Astacus astacus* can partially explain greater tolerance towards pollutants in adult crayfish. They reported that adult A. astacus contained considerable amounts of cytochrome P-450 and concluded that the tissues of these crayfish were capable of metabolizing foreign compounds at least to a certain extent. Even though we have known about cytochrome P-450 for a long time, we still do not know if juveniles have the same amounts of cytochrome P-450. If this was not the case, it could explain the greater susceptibility of juvenile crayfish to pollutants reported here (Naqvi et al. 1987). Another reason for the higher sensitivity of juvenile crayfish could be a trade-off effect as described by (Wölfle et al. 2009) in their study. The development and growth of the embryos require an immense amount of energy, so that resistance against pollutants is decreased (Bridges and Farrar 1997). Additionally, most of the defensive mechanisms of the crayfish against harmful pollutants develop during the embryonal time, so that early life stages are more susceptible to environmental influences (Le Moullac and Haffner 2000). The negative influence of nitrate on the reproduction of invertebrates has been described before. (Álonso and Camargo 2013) showed that the amount of the progeny of the snail *Potamopyrgus antipodarum* decreased at nitrate concentrations higher than 21.4 mg/L. The results of our study show that the estimated tolerance range up to 30 mg/L for adult freshwater crayfish (Bavarian State Office for the Environment 2007) is not suitable for embryos and therefore not for reproduction.

Effective concentrations found in our study are far surpassed by observations of nitrate in surface waters. This appears of great importance as the highest nitrate concentrations commonly occur between January and February (BMU 2016) which coincides with the time of embryonic development of European freshwater crayfish in their natural habitat. We conclude that currently found concentrations of nitrate in surface waters have the potential to influence embryonic development of European freshwater crayfish negatively. This has serious implications for conservation strategies and aquaculture, as nitrate may lower reproduction rates in natural waters with potentially severe effects on the recruitment, population establishment and population growth of these highly threatened species.

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Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

Competing interests The author(s) declare no competing interests.

Author Contributions JL, KL and AG conceived and designed the experiments. JL performed the experiments. JL analysed the data. JL, KL, AG and HB wrote the manuscript; all authors provided editorial advice.

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