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Living in polluted waters: a meta-analysis of the effects of nitrate and interactions with other environmental stressors on freshwater taxa

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Capsule:

Nitrate pollution reduces the activity, growth, and survival of aquatic taxa and, when combined with other stressors, affects survival in a non-additive manner.

Keywords: Nitrate pollution, Fertilisers, Multiple Stressors, Ecotoxicology, Environmental change.

Abstract

Nutrient effluents from urban and agricultural inputs have resulted in high concentrations of nitrate in freshwater ecosystems. Exposure to nitrate can be particularly threatening to aquatic organisms, but a quantitative synthesis of the overall effects on amphibians, amphipods and fish is currently unavailable. Moreover, in disturbed ecosystems, organisms are unlikely to face a single stressor in isolation, and interactions among environmental stressors can enhance the negative effects of nitrate on organisms. Here, the effects of elevated nitrate on activity level, deformity rates, hatching success, growth and survival of three taxonomic groups of aquatically respiring organisms are documented. Effect sizes were extracted from 68 studies and analysed using meta-analytical techniques. The influence of nitrate on life-stages was also assessed. A factorial meta-analysis was conducted to examine the effect of nitrate and its interaction with other ecological stressors on organismal survival. Overall, the impacts of nitrate are biased towards amphibians (46 studies) and fish (13 studies), and less is known about amphipods (five studies). We found that exposure to nitrate translates to a 79% decrease in activity, a 29% decrease in growth, and reduces survival by 62%. Nitrate exposure also increases developmental deformities but does not affect hatching success. Nitrate exposure was found to influence all life-stages except embryos. Differences in the sensitivity of nitrate among taxonomic groups tended to be negligible. The factorial meta-analysis (14 amphibians and two amphipod studies) showed that nitrate in combination with other stressors affects survival in a non-additive manner. Our results indicate that nitrate can have strong effects on aquatic organisms. Moreover, nitrate can interact with other environmental stressors leading to compounding, negative effects on survival and highlights the need for research and management to take a holistic approach in conserving freshwater biodiversity in the face of ongoing global change.

1. Introduction

Nutrient effluents into freshwater ecosystems have increased considerably in recent decades as human demands for food and energy exponentially increase (Díaz-Álvarez et al., 2018). In particular, the extensive use of nitrogen has caused a cascade of environmental impacts, and nitrogen pollution is now regarded as one of the greatest threats to biodiversity worldwide (Díaz-Álvarez et al., 2018; Payne et al., 2017) causing severe impacts on terrestrial and aquatic ecosystems and their biota (Camargo and Alonso, 2006; Galloway et al., 2008).

Various anthropogenic activities (e.g. agricultural fertilisers, urban, sewage and mine runoff) have increased the amounts nutrients (nitrogen and phosphorus) entering freshwater habitats (Jenkins, 2003). Excess nitrogen and phosphate runoff into freshwater can result in mass-scale eutrophication events and, in turn, threaten aquatic fauna (Dodds and Smith, 2016). In particular, nitrate accumulation in freshwater is one of the most predominant threats facing aquatic organisms on worldwide scale (Jenkinson, 2001). As the final stage of the nitrification process, nitrate is the most common and abundant form of inorganic nitrogen in freshwater ecosystems. Nitrate occurs at naturally low background levels (~ 0 – 2 mg L^{-1} NO_3^-), but excessive nitrate pollution has resulted in elevated concentrations at around 10 – 100 times above baseline levels (Galloway et al., 2004). Nitrate pollution is particularly prominent in areas of high fertiliser use and urban development which can cause surface water concentrations to increase to 25 – 100 mg L^{-1} NO_3^- (Camargo et al., 2005). Similarly, considerable amounts of nutrients are discharged from aquaculture facilities that impact surrounding environments (Cao et al., 2007; Verdegem, 2013). Nitrate concentrations may remain elevated for prolonged periods of time and pose severe impacts on aquatic fauna.

In spite of environmental concerns, nitrate is often considered as non-toxic and this is due in part to a lack of understanding of the effects of nitrate on aquatic organisms.

Aquatically respiring organisms, including amphibians, amphipods, and fish may be particularly vulnerable to elevated nitrate concentrations because nitrate can be taken up passively via the gills (Jensen, 1996). Once inside the body nitrate can cause significant physiological and behavioural alterations (Camargo et al., 2005; Guillette Jr. and Edwards, 2005). At the whole animal level, nitrate can reduce growth (McGurk et al., 2006) and activity levels (Alonzo and Camargo, 2013), decrease reproductive outputs (Alonzo and Camargo, 2013; Soucek and Dickinson, 2016), increase the incidence of developmental deformities (Krishnamurthy et al., 2008) and ultimately reduce survival (Hamer et al., 2004; McGurk et al., 2006) of aquatic organisms. Nitrate also causes significant disruptions to oxygen delivery mechanisms (e.g. affecting haemoglobin concentrations, Monsees et al., 2017), thyroid gland and thyroid hormone synthesis (Guillette Jr. and Edwards, 2005), and can cause epithelial necrosis in vital organs (Grabda et al., 1974; Romano and Zeng, 2007). Aquatically respiring organisms however vary in their sensitivity to nitrate. For instance, amphipods are often considered to be relatively tolerant of waterborne nitrate under laboratory toxicity tests (i.e. higher lethal concentrations) (Benítez-Mora et al., 2014; Soucek and Dickinson, 2012), while amphibians may be more sensitive (Camargo et al., 2005). Variations in biological responses to nitrate have been attributed to differences in nitrate uptake rates (Williams et al., 2008), body size (Hamlin 2006) and life-history stage (Ortiz-Santaliestra et al., 2006), environmental context (i.e. source population, previous exposure; Edwards et al., 2006), and is dependent on nitrate concentration and duration of exposure (Camargo et al., 2005).

Along with increased nitrate pollution, freshwater species also have to contend with a myriad of co-occurring ‘stressors’ (Jenkins, 2003; Ormerod et al., 2010). Environmental ‘stressors’ refers to novel and/or extreme environmental changes that have arisen as a result of anthropogenic global change (Christensen et al., 2006; Jackson et al., 2016). Environmental stressors can be both natural (e.g. abrupt fluctuations in temperature) or of anthropogenic origin (e.g. agricultural pollutants) and include biotic (e.g. introduced predators) and abiotic variables (e.g. acidic pH). There is a current need to model organismal responses to multiple, interacting stressors since stressor interactions are not always linear and multiple stressors often exacerbate or confound existing problems (Todgham and Stillman, 2013). Stressor interactions can often result in "ecological surprises" where the effects of one stressor are enhanced by the presence of a second stressor, even when one stressor is not considered a major threat in a particular system (Christensen et al., 2006). As such, two interacting stressors can have an effect which is greater (synergistic) or lesser (antagonistic) than the sum of the two stressors in isolation (additive; Folt et al., 1999). Simultaneous exposure to nitrate and other environmental stressors may, therefore, enhance mortality rates, or alternatively, increase organismal tolerance of elevated nitrate levels. For instance, nitrate (20 mg L^{-1}) and the pesticide malathion (250 μg L^{-1}) alone reduced survival by 20% and 14%, respectively, in the American toad (*Anaxyrus americanus*); however, when combined, survival was reduced synergistically to 48% (Krishnamurthy and Smith, 2010). Conversely, the presence of a predator (*Gambusia affimis*) did not enhance mortality rates in *A. americanus* tadpoles exposed to elevated nitrate (Smith and Dibble, 2012).

A number of reviews have sought to provide a generalised synthesis of the effects of nitrate on aquatic organisms (Camargo and Alonso, 2006; Camargo et al., 2005; Guillette Jr. and Edwards, 2005; Mann et al., 2009). These reviews provide valuable toxicological information by summarising the nitrate lethal concentration (e.g. LC_{50}; the nitrate concentration that kills half of the population), the ecosystem scale effects of nitrogen pollution and the role of nitrate on endocrine disruption to a variety of freshwater taxa. However, these reviews consider few response variables (e.g. survival), comparisons are restricted to single species, and often report much higher concentrations than would be measured in the field. Literature reviews are also limited in that they lack statistical power and are therefore unable to provide a quantitative assessment of the impacts to species. In comparison, meta-analyses allow for an objective and transparent evaluation of data. Meta-analyses are particularly effective in that they allow for a quantitative evaluation of effect sizes, integrate data from multiple sources (Foley et al., 2018; Gurevitch and Hedges, 1993), allow for the assessment of how effect sizes differ between pre-defined groups (e.g. life-stages, taxa) and can account for additional sources of variation (e.g. methodological difference such as length of exposure, experimental set-up) among studies.

In this study, we quantified variation in biological responses to waterborne nitrate exposure by conducting a meta-analysis. First, we aimed to examine the impact of elevated nitrate on key fitness related traits, including activity, deformity rates, hatching success, growth, and survival in three groups of aquatically respiring freshwater organisms (aquatic amphibians, amphipods and fish). We also considered how effect size estimates are impacted on by methodological difference such life-stage (embryo – adult stages), source population (i.e. wild versus captive sources) and nitrate salt (ammonium nitrate, sodium nitrate). Second, we examined how survival is affected by nitrate and its interaction with other environmental stressors.

2. Methods

2.1 Selection Criteria

Databases (Web of ScienceTM Core Collection and Scopus) were searched to identify appropriate literature following the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) model (Fig. S1; Moher et al., 2009). Literature searches were conducted using the following search strings: (“nitrate”) AND (“activity*” OR “deform*” OR “growth*” OR “hatch*” OR “surviv*”) AND (“amphibian* OR “arthropod” OR “fish” OR “larva*” OR “fry” OR “egg”). A total of 1729 titles were initially screened for relevancy and

1102 were screened by abstract. We read the full text of 424 articles, including 13 from the bibliographies of prominent reviews (Camargo and Alonso, 2006; Camargo et al., 2005; Guillette Jr. and Edwards, 2005; Mann et al., 2009). Data retention criteria were established that included studies having: 1) experimentally manipulated the water nitrate concentration; 2) using controls that were not exposed to nitrate; 3) stated concentrations and type of nitrate salt used (i.e. ammonium nitrate, sodium nitrate or potassium nitrate); 4) exposure period clearly stated; and 5) extractable means, variances, and sample sizes for both control and treatment groups. If standard errors (SE) were reported, values were transformed to standard deviations (SD) following the equation: $\mathrm{SD} = \mathrm{SE} \times \sqrt{\mathrm{N}}$. When data were presented graphically, Image J (v. 1.51k, National Institute of Health, USA, http://imagej.nih.gov.ij) was used to extract means and variances. Forward and backward searches through reference lists were performed to examine for additional data. Studies were collected for analysis until 2 August 2019 and a total of 68 studies were included.

Data were extracted for the effects of nitrate on five response variables including activity (percentage of time spent active), deformity rate (percentage of developmental deformities over time), growth rate (change in mass over time, g; change in length over time, cm), hatching success (percentage of eggs hatched), and survival (survival percentage at the end of the exposure period). Nominal nitrate concentrations were also extracted from every study. We used nominal nitrate concentrations instead of average measured concentrations because average concentrations are not always presented in the main text of published papers. Further, we extracted other meta-data from papers including taxonomic group (amphibian, amphipod, fish), life-stage (embryo, larvae, juvenile, adult), origin of source population (wild or captive), nitrate salt used (ammonium nitrate, potassium nitrate, sodium nitrate), exposure duration and experimental temperature (°C). If experiments reported the response of nitrate over time (e.g. for survival) only the final time point was used in the analyses. In cases where the published study reported data for more than one species, population or nitrate concentration, each outcome was included in the analysis. Although the inclusion of multiple measures from the same study could decrease the independence of some of the data points, this allowed us to explore the impacts of nitrate across a wider range of concentrations, response variables and species.

2.2 Effect sizes

Effects sizes were calculated as the *ln*-transformed response ratio (*LnRR*),

$$LnRR = ln\left(\frac{\bar{X}_E}{\bar{X}_C}\right) = ln(\bar{X}_E) - ln(\bar{X}_C),$$

where $\bar{X}_E$ and $\bar{X}_C$ are the mean responses from the experimental and the control groups, respectively. The variance (v) of each *ln*-transformed response ratio was also calculated,

$$v = \frac{(SD_E)^2}{N_E\,\bar{X}_E^2} + \frac{(SD_C)^2}{N_C\,\bar{X}_C^2},$$

where *SD* and *N* are the standard deviation and sample size of the experimental and the control groups (Hedges et al., 1999). Log response ratios and variances were calculated using the *escalc* function of the *metafor* package (v. 2.1-0; Viechtbauer, 2010) in R (v. 3.6.11 https://www.r-project.org/) using the R studio interface (v. 1.1.463). A negative *ln*-transformed response ratio is interpreted as having a negative effect on the response variable, while a positive response ratio represents a positive effect. We chose log response ratios over other methods because response ratios calculate the proportional change between the control and the treatment groups and positive and negative deviations from the control have equal weight. Log response ratios are also robust to small sample sizes, less sensitive to differences in units, and normalises data that are not normally distributed (Lajeunesse, 2011; Viechtbauer, 2010). For interpretation, log response ratios were converted into a measure of percent change ($\%\Delta = \exp((\mathit{LnRR})-1)*100$).

2.3 Single-stressor effects of nitrate

Meta-analytic multilevel linear mixed-effects models were run in order to calculate the overall effect size for each of the response variables (activity, deformity rate, growth rate, hatching success and survival), using the *rma.mv* function of the *metaphor* package. All models were fit using restricted maximum likelihood estimation (REML). Model estimates were considered significant if their 95% confidence intervals did not overlap with zero. Our data contained multiple levels of non-independence (Noble et al., 2017), including data that shared a common control and phylogenetic dependencies. We accounted for these dependencies by: first, including study ID and phylogeny (modelled with a phylogenetic relatedness correlation matrix) as random effects under the *random* argument of the *rma.mv* function; second, we constructed sampling variance–covariance matrices of our random effects. For studies that shared a common control, we calculated variance–covariance matrices using the *covariance_commonControl* function of the *metagear* package (v. 0.4) following the methods described in Lajeunesse (2016). Phylogenetic dependencies were modelled by constructing a phylogenetic tree (Fig. S2) of the 58 species within our dataset. The phylogenetic tree was constructed using the *tnrs_match_names* function in the R package *rotl* (v. 3.0.1; Michonneau et al., 2016), which uses the Open Tree Taxonomy database (Hinchliff et al., 2015). Branch lengths were computed using the default settings of the *compute.brlen* function in the R package *ape* (v. 5.3; Prandis and Schliep, 2019). The *vcv* function was then used to create a phylogenetic covariance matrix. We specified correlation matrices corresponding to our random effects (study ID and phylogeny) via the *R* argument of the *rma.mv* function (Lajeunesse, 2016) within all models. All models conform to the assumptions of a linear model (normality and heterogeneity).

From all multilevel linear mixed-effects models, we calculated total heterogeneity (*Q*) in effect sizes, where a significant *Q* statistic indicates significant heterogeneity within the mean effect size. To explain some of this heterogeneity, we ran meta-regression models to test for the influence of moderators (covariates) on the magnitude of the effect of nitrate on response variables. Moderators considered in the analysis included: taxonomic group (amphibians, amphipods and fish), life-stage (embryo, larvae, juvenile or adult), source population (captive or wild) and nitrate salt (ammonium nitrate, potassium nitrate or sodium nitrate). Nitrate concentration, exposure duration and experimental temperature were included as continuous moderators. These moderators were chosen because they can potentially influence mean effect sizes. Model selection were performed using the *glmulti* function of the *glmulti* package (Calcagno, 2014). Model selection was completed following the information-theoretic approach (Burnham and Anderson, 2002) and model evaluation was corrected for small sample sizes (AICc). Best models were determined by calculating AICc differences (ΔAICc) and AIC weights (w_i).

2.4 Multiple-stressor effects of nitrate

For the factorial meta-analysis, we identified studies that employed a factorial design to test the interaction between nitrate in combination with other environmental stressors. Due to the scarcity of data on interacting stressors, only survival data were extracted for the factorial meta-analysis. Data were extracted from each study, including the isolated effects of nitrate; the isolated effect of exposure to other stressors (i.e. stressor two); the effect of the observed interaction (nitrate × stressor two); and data from the controls (no nitrate or other factors). As for the previous analyses, we calculated *LnRR* as a measure of effect size and corresponding sampling variance. In cases where the published study reported data for more than one species, population or nitrate concentration, each outcome was included in the analysis.

Multilevel mixed-effects models (using the *rma.mv* function of *metafor*) were run to calculate overall effect sizes for the effects of (1) the isolated effect of nitrate; (2) the isolated effect of exposure to other stressors (i.e. stressor two); (3) the effect of the observed interaction (nitrate × stressor two). As for the previous analyses, we accounted for dependent effect sizes by including study ID and phylogeny as random effects and constructed sampling variance–covariance matrices of our random effects. Predicted interactions were calculated in order to classify the observed interactions using a multiplicative null model (Darling and Côté, 2008):

$$LnRR_{(multiplicative)} = ln\frac{Nitrate + Stressor\ 2 - Nitrate \times Stressor\ 2}{Control}$$

A multiplicative model was chosen for the survival data because survival is a common response that represents probabilistic event and corrects for the fact that individuals killed by one stressor cannot be killed by another and bounds the combined mortality estimates to a maximum of 100% (Côté et al., 2016). The mean effect size of the observed percent survival was compared to the calculated predicted survival for the multiplicative model. The observed stressor interaction was classified as antagonistic if the effect size estimate was lower than the lower 95% confidence limit of the multiplicative survival model. Conversely, if the observed stressor interaction was greater than the upper 95% confidence limit, it was classified as a synergistic interaction. If the observed interaction overlapped with the predicted 95% confidence limit, the experiment was classified as an additive interaction.

2.5 Publication bias

To test for publication bias (i.e. publishing only results that show a significant finding) we used contour enhanced funnel plots of observed effect sizes against standard errors (Egger et al., 1997), via the funnel function in *metafor*. Secondly, we calculated Rosenberg's fail-safe number which represents the number of papers that would need to be published with non-significant results to change the mean effect size to a non-significant result (Rosenberg, 2005). Fail-safe numbers are considered robust to publication bias when larger than 5*n* + 10 (where *n* = the number of studies already included in the meta-analysis) (Rosenberg, 2005).

3. Results

A total of 68 studies met our selection criteria and were included in the meta-analysis (Table S1, Fig. S1), however, not all studies provided data for all response variables nor taxonomic groups (Table 1). For the factorial meta-analysis, 16 studies met our selection criteria (Table S2) and included data only on amphibians (14 studies) and amphipods (two studies).

3.1 Activity

Activity decreased with nitrate exposure, with a mean effect size of -0.77 *lnRR* in our multivariate effects model (z = -4.52, P < 0.001; Fig. 1). This value translated to a 79.45% (± 15.71% *SE*) reduction in activity. There was significant heterogeneity in the multivariate mixed-effects model (Q = 22.87, P < 0.001), indicating that additional variance between studies may be explained by moderators. Activity levels were impacted in amphibians but not in fish exposed to elevated nitrate (Fig. 2), while no studies on amphipods were available. The choice of experimental nitrate salt had a significant impact on effect size estimate (Fig. 3A), where the use of potassium nitrate tended to have no effect. Larval (z = -10.13, P < 0.001), but not adult (z = -1.46, P = 0.23), exposure significantly affected activity. The use of wild over captive-raised animals tended to result in stronger, negative effect sizes. Nitrate concentration and exposure duration were negatively correlated with activity (Table 2; Fig. S3, S4), but there was no influence of experimental temperature.

3.2 Deformity rate

Exposure to nitrate increased developmental deformity rates by an average of 184.62% (± 52.35% *SE*; z = -2.48, P < 0.001; Fig. 1). There was significant heterogeneity in the multivariate mixed-effects model (Q = 59.32, P < 0.001). In the best fitting model (Table

S3), mean effect sizes and 95% confidence intervals for group moderators suggest that nitrate exposure increases developmental deformities in both amphibians and fish (Fig. 2). Both larval ($z = -12.09$, $P < 0.001$) and embryonic ($z = -2.29$, $P = 0.02$) exposure to nitrate increased deformity rates (Fig. 3B). Moreover, nitrate exposure duration was correlated with increased deformity rates but not exposure concentration or experimental temperature (Table 2; Fig. S3, S4).

3.3 Growth

On average, exposure to nitrate reduced growth (*LnRR* = -0.26; Fig. 1) by 28.65% (± 6.7% *SE*; $z = -3.91$, $P < 0.001$). Significant heterogeneity was detected in the multivariate model for growth ($Q = 56.18$, $P < 0.001$). For the moderators (Table S3), nitrate exposure significantly reduced the growth of amphibians, amphipods, and fish. Larval and juvenile exposure to elevated nitrate reduces growth performance (Fig. 3C), but adult and embryonic exposure did not impact on growth rates. The use of sodium nitrate, but not ammonium nitrate or potassium nitrate, impacted on growth performance. The growth performance of captive and wild-caught individuals tends to be impacted by nitrate exposure to a similar degree. Nitrate concentration, exposure duration and experimental temperature were all correlated with growth reduction (Table 2; Fig. S3, S4).

3.4 Hatching success

Although hatching success tended to decrease (1.51% mean decrease, ± 6.07% *SE*) with exposure to nitrate across all studies, the overall effect was not statistically significant ($z = -0.26$, $P = 0.79$; Fig. 1). There was also no significant heterogeneity for hatching success model ($Q = 1.39$, $P = 1$). Hatching success was also unaffected by any of the moderator variables (Fig. 2; Table 2).

3.5 Survival

Across all studies, exposure to nitrate had a negative effect on survival (*LnRR* = -0.53; Fig. 1) and translated to a 62.50% (± 17.23 % *SE*) decrease in survival (z = -3.35, P < 0.001). The mean effect size for survival was significantly heterogeneous (Q = 433.13, P < 0.01). For the moderator variables, exposure to nitrate reduces survival of all three taxonomic groups (Fig. 2). The use of ammonium nitrate and sodium nitrate tended to affect survival by a similar margin, but is not affected by the use of potassium nitrate (Fig. 3D). Nitrate exposure impacts the survival of all life-stages except for embryos. Moreover, the use of wild and captive-raised animals had a significant impact on survival. There was also a significant relationship between nitrate concentration, exposure duration and experimental temperature on survival (Table 2; Fig. S3, S4).

3.6 Factorial meta-analysis

The factorial meta-analysis showed that exposure to nitrate alone reduced survival (*LnRR* = -0.29; z = -13.01, P = < 0.001), as did exposure to other abiotic or biotic stressors (*LnRR* = -0.13; z = -4.17, P < 0.0001; Fig. 4A). Survival was further reduced by the interaction between nitrate and other stressors (*LnRR* = -0.43, z = -26.59, P < 0.001; Fig. 4A). Calculations of the multiplicative model (*Ln*RR = -0.52, P < 0.001) were also significant. Simultaneous exposure to nitrate and some pollutants (Malathion, glyphosate) and abiotic variables (dissolved oxygen) showed highly negative effect sizes, while nitrate in combination with predators and elevated salinity, for example, showed no effect (Fig. 4B). Based on specific effect size comparisons (64 effect sizes from 16 studies), we identified 27 antagonistic, 16 synergistic and 21 additive interactions, or alternatively, 21 additive and 43 non-additive interactions.

3.7 *Publication bias*

Rosenberg's fail-safe numbers were robust (ranging from 3254 to 4068573) for all response variables suggesting that many non-significant results are required to alter the conclusions. Contour enhanced funnel plots are presented in Fig. S5, and visual inspection of the funnel plots suggest that there is no evidence of publication bias. For the factorial meta-analysis, the fail-safe number for the factorial meta-analysis were also robust (1402 – 7894). Collectively, the results suggest that there was no publication bias for any of our response variables, although it cannot be ruled due to the low number of studies included in some of the response variables.

4. Discussion

The meta-analysis conducted in this study provides quantitative evidence that exposure to nitrate poses consistent, negative effects on aquatically respiring organisms living in freshwater. The magnitude of the effects varied, with the strongest effects exerted on organismal deformities, growth and survival. Fish and amphibian embryos appear resilient to the effects of nitrate, having no effect on hatching success. In addition, we found that there is generally an antagonistic interaction when nitrate is combined with a second environmental stressor. The large magnitude of the effects on various response variables suggests that nitrate exposure may have cascading consequences on freshwater ecosystems. Our analyses highlight the need for a broader evaluation of the effects of nitrate on aquatically respiring organisms as most of the data have centred on growth and survival estimates, and do not consider other relevant fitness correlates (e.g. activity, deformity rates). Further, a broader taxonomic evaluation of the effects of nitrate beyond amphibians is warranted.

Overall Effects of Nitrate

Growth, deformity rate and survival were among the most sensitive processes to waterborne nitrate exposure. On average, exposure to nitrate reduces growth by 29.2% and increases the incidence of developmental deformities by 184% relative to controls. Further, nitrate exposure decreased survival by 62.5%. Our results are in keeping with previous reviews of the effects of nitrate, which conclude that elevated nitrate exposure reduced regular growth, altered developmental trajectories and lowered survival rates (Camargo and Alonso, 2006; Camargo et al., 2005; Mann et al., 2009) of aquatically respiring organisms. The negative effects of nitrate can be attributed to disruptions of a number of physiological processes such as increased energy expenditure (Gomez Isaza et al., 2018), increased methaemoglobin concentrations (Grabda et al., 1974; Monsees et al., 2017) and nitrate accumulation in the tissues (Cheng et al., 2002; Romano and Zeng, 2007). Nitrate also alters the functioning of the thyroid gland (Guillette Jr. and Edwards, 2005) which can interfere with regular growth, development, and survival of freshwater organisms. Declines in growth and development caused by nitrate exposure may pose additional fitness constraints including: increased predation risk (Englund and Krupa, 2000; Schlosser, 1988), reduced competitive abilities (Englund and Krupa, 2000; Krishnamurthy et al., 2006), and delayed reproductive maturity and output in later life (Alonzo and Camargo, 2013; Gibbons and McCarthy, 1986; Kolm, 2002).

Environmental stressors that alter a species' ability to move through its environment have the potential to have broad-scale implications and can affect many species and life-history stages (Killen et al., 2013). Our results show that nitrate acts to alter activity and behaviours of species. However, the scarcity of data (ten studies) evaluating the impacts of nitrate on whole-animal activity and the distinct taxonomic bias towards amphibians limits the potential for generalisable trends. That been said, nitrate influences organismal behaviour by increasing the percentage of inactive periods (Ilha and Schiesari, 2014), reducing foraging-related behaviours (Krishnamurthy et al., 2006; Xu and Oldham, 1997) and reducing mate guarding behaviours (Pandey et al., 2011). Animals exposed to nitrate are often characteristically lethargic – a possible consequence of reduced oxygen carrying capacity (Gomez Isaza et al., 2018; Monsees et al., 2017). Nitrate-induced alterations to an organism's behaviour can reduce the amount of time spent feeding, guarding territories and seeking out potential mates lowering overall fitness. Lethargy may also increase susceptibility to predation (Scott and Sloman, 2004), however, it is noteworthy that nitrate does not affect fast muscles movements associated with predator escape responses (Secondi et al., 2013). Our analyses indicate that across a variety of experimental conditions, nitrate adversely affects organismal activity, and that the effects are not limited to specific behavioural responses or species.

A positive correlation was found between nitrate concentration and the mean effect size of activity rates, growth and survival. This result was expected as higher nitrate concentrations increase the passive uptake of nitrate into the body (Freitag et al., 2015). However, nitrate dose-response curves are not always linear, and as such, low nitrate concentrations can have an effect that is greater than would be expected. For example, exposure to increasing nitrate caused a non-monotonic effect on plasma testosterone levels in juvenile Atlantic salmon (*Salmo salar*) (Freitag et al., 2015). Further, prolonged exposure to modest nitrate concentrations (80 – 100 mg L^{-1} NO_3^-) can be toxic to a variety of freshwater species (Davidson et al., 2014; Ortiz-Santaliestra et al., 2010; Soucek and Dickinson, 2016). Current nitrate concentration guidelines are based on limited datasets (e.g. lethal concentrations of nitrate, LC_{50}) and are set at approximately 50 mg L^{-1} NO_3^- (Canadian Council of Ministers of the Environment, 2012; Environment Australia, 2000; European Commission, 2018) and at 44.3 mg L^{-1} NO_3^- by the United States Environmental Protection

Agency (EPA, 2002). Current nitrate concentration guidelines are likely to provide protection to some but not all species given the variability in the effect size estimates reported here (Fig. S3). Some response variable are also more sensitive to nitrate exposure (e.g. activity) and management guidelines should aim to incorporate a broader range of available data.

Exposure duration tended to exacerbate the negative effects of nitrate. Freshwater organisms appear resilient to short-term nitrate exposures, as shown by numerous acute exposure experiments (Hecnar, 1995; Romano and Zeng, 2007; Soucek and Dickinson, 2012). However, long-term nitrate exposure may result in the accumulation of nitrate in the plasma and, in turn, impact on physiological processes. To date, experimental studies examining the chronic effects of nitrate are scarce and so the results of this meta-analysis likely underestimates species susceptibility. For example, a 35-day exposure to 6.45 mg L^{-1} NO_3^- caused significant reductions in the growth performance of green frog tadpoles (Smith et al., 2013), indicating that even low nitrate concentrations can pose negative effects under chronic exposures. Further, a time series experiment showed significant reductions in the aerobic scope of blueclaw crayfish (*Cherax destructor*) appear after five-days of exposure to 50 and 100 mg L^{-1} NO_3^- (Gomez Isaza et al., 2018) and suggests that prolonged elevations in nitrate are the most threatening to aquatic fauna. The results presented here call for research to investigate the short and long-term effects of nitrate in order to adequately predict the susceptibility of species to elevated nitrate concentrations.

Life-History Stages

Differences among life stages have been demonstrated in response to nitrate (Ortiz-Santaliestra et al., 2006), a result consistent with our findings. We found that nitrate impacted on all life-stage, but effect sizes tended to be greatest in larval and juvenile exposed animals. This result may indicate that nitrate acts similarly regardless of life-stage (e.g. thyroid gland, metabolism, toxic accumulation) or that aquatic organisms may have a generally low capacity for the detoxification and/or active excretion of nitrate. Embryonic exposure to nitrate appears benign, with studies reporting no net effect on hatching success. In addition, nitrate exposure during embryonic development has been found to have negligible impacts on hatchling length (Lou et al., 2016; Ortiz-Santaliestra et al., 2011a), days to hatching (Ortiz-Santaliestra et al., 2011a; Ortiz-Santaliestra et al., 2011c), or developmental stage at hatching (Ortiz-Santaliestra et al., 2011a; Ortiz-Santaliestra et al., 2011c) further suggesting that nitrate does not pose a threat to this early life stage. It is possible that embryos are protected from the effects of nitrate by the jelly layer of fish and amphibian eggs being impermeable to nitrate. Additionally, the incomplete development of organ systems may act to protect embryos from the negative effects of nitrate. For example, the incomplete formation of the thyroid gland may protect embryos from endocrine disruption (Ortiz-Santaliestra et al., 2006), while the incomplete formation of the digestive system and/or the reduced abundance of symbiotic gut bacteria which transform nitrate into nitrite may decrease the formation of methaemoglobin in embryos exposed to nitrate (Hecnar, 1995; Huey and Beitinger 1982)**.** Our results indicate that adult exposure to nitrate influenced few of our effect size estimates, but this could be due to the scarcity of data available on large bodied, mature animals. Indeed, adult exposure to nitrate is known to interfere with sexual cues (Secondi et al., 2013), secondary sexual traits (Secondi et al., 2009), and reduces male and female fecundity (Alonzo and Camargo, 2013; Kellock et al., 2018) and therefore nitrate exposure may impact adult life-stages in ways not considered in this analysis (e.g. reproductive rates). Future research would benefit from investigating the long-term effects of nitrate across multiple life stages and elucidate on possible defences against elevated nitrate.

Taxonomic Grouping

Differences in the sensitivity of nitrate among taxonomic groups tended to be negligible, with negative impact recorded among all three groups. Fish tended to be most resilient of nitrate exposure, while effect size estimate were greater in amphibians and amphipods. Moreover, there is a high degree of variation between and within taxonomic groups. Between taxa, concentrations as low as 20 mg L^{-1} NO_3^- can pose adverse effects to some invertebrates (Alonzo and Camargo, 2013; Soucek and Dickinson, 2016), fish (McGurk et al., 2006; Stormer et al., 1996), and amphibians (Baker and Waights, 1993; Ortiz-Santaliestra et al., 2010), while others are relatively tolerant of high concentrations (> 500 mg L^{-1}) (e.g. Benítez-Mora et al., 2014; Monsees et al., 2017; Soucek and Dickinson, 2012; Wang et al., 2015). Within species differences in nitrate sensitivity have also been documented, such as population (Johansson et al., 2001), sex (Kellock et al., 2018) and body size differences (Hamlin, 2006). However, the fact that some taxonomic groups (amphipods) were under-represented or completely absent in some analyses may contribute to the general lack of an observed pattern. Thorough investigations of the effects of water-borne nitrate on non-amphibian groups are required to gain a better understanding of how nitrate pollution may impact other taxa. Management practices likely need to consider species-specific nitrate tolerances and base environment guidelines on the most sensitive species living in an ecosystem.

Nitrate salt

Experimental design can alter effect sizes, for example the choice of experimental nitrate salt can have differential effects on aquatic taxa (Schuytema and Nebeker, 1999b), influencing the conclusions of scientist and policy makers. Nitrate concentrations are manipulated by dissolving nitrate salts in water, most commonly using ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3) or sodium nitrate ($NaNO_3$). The use of $NaNO_3$ and KNO_3 are often justified due to the relatively non-toxic ionic species of Na^+ and K^+, as compared to NH_4NO_3 (Schuytema and Nebeker, 1999a, b). However, $NaNO_3$ and KNO_3 can alter ionic composition/ratios of the test treatments and result in osmo-ionoregulatory disturbances (Hrubec et al., 1996; Romano and Zeng, 2007). NH_4NO_3 is expected to be most toxic and result in larger effect sizes due to the interaction with the ammonium ion, known to be toxic to aquatic organisms even at low concentrations (Schuytema and Nebeker, 1999a). Indeed, our data indicate that exposure to NH_4NO_3 tended to have greater effect sizes on most response variables (activity, deformity rate and survival) than $NaNO_3$ and KNO_3, but did not affect growth. It is worth noting that no study included in the analysis exposed fish or crustaceans to NH_4NO_3 and few studies have used KNO_3. As such, it is difficult to determine the effects NH_4NO_3 and KNO_3 to aquatic taxa and warrants future investigation given their extensive use as agriculture fertilisers (Simplício et al., 2017).

Nitrate and Interactions

Multi-stressor studies are one of the largest knowledge deficiencies to ecological conservation (Christensen et al., 2006), and this knowledge gap is reflected in the factorial meta-analysis. Only sixteen studies were included in the factorial meta-analysis, and a strong taxonomic bias towards amphibians was apparent (fourteen studies). Other freshwater taxa (fish and amphipods) were underrepresented or completely absent from the factorial meta-analysis. Given that taxonomic differences to nitrate exposure are limited, the results from the factorial meta-analysis may inform potential responses of other freshwater taxa. However, considering the myriad of stressors acting on freshwater ecosystems, there is need for a broader taxonomic evaluation of this research question.

The factorial meta-analysis revealed that exposure to nitrate in combination with an additional stressor tends to affect survival in an antagonistic manner (i.e. less than the sum or the product of the two stressors). Additional stressors included both abiotic (e.g. pH, salinity, UV-B) and biotic (e.g. predators) variables that are likely to occur in tandem in anthropogenically-disturbed environments. The results reported here are consistent with those of a previous meta-analysis which found that the net effect of stressor interaction are most frequently antagonistic (~41%) in freshwater ecosystems (Jackson et al., 2016). Although often seen as a "best case scenario", antagonistic interactions are still concerning as the combined effects of two stressors are stronger than their individual effects and the management of two antagonistic stressors would require, in part, the relief of both stressors.

Despite an overall prevalence of antagonistic interactions, our results show that the strength of the interaction varies depending on the stressors involved. Interactions among nitrate and other pollutants all had significant effects on survival. This finding is of particular significance as species living in anthropogenically disturbed environmental are increasing subject to novel chemical mixtures which often have unknown effects. Species may not be adapted- or have a poor capacity (e.g. detoxification or excretion mechanisms) to cope with man-made substances such as atrazine and Malathion (Hayes et al., 2006; Thrupp et al., 2018) and underscore the overall negative effects on survival reported here. In contrast, survival was not affected when nitrate exposure was combined with an abiotic variable. The notable exceptions were interactive effects of nitrate with aquatic oxygen levels and with low pH. The interaction between nitrate and dissolved oxygen levels resulted in the greatest effect size estimate on organismal survival (Ortiz-Santaliestra and Marco, 2015), likely because both stressors impact on oxygen availability. On the other hand, exposure to low pH may have facilitated the uptake of nitrate, as has been demonstrated for pollutants (Çoĝun and Kargin, 2004), thereby increasing the concentration of nitrate with the body and impacting survival. However, nitrate – pH interactions have been tested using extreme low pH conditions (pH = 5.0; Gomez Isaza et al., 2018; Hatch and Blaustein, 2000) and more intermediate pH values might show other responses (Meade and Perrone, 1980). Interaction between nitrate and biotic variables (e.g. predator exposure), which account for ~20% of the data included in the factorial meta-analysis, tended to report no interactive effect on survival likely because they exert their influence on different physiological traits (Ortiz-Santaliestra et al., 2011b;

Romansic et al., 2006; Smith and Dibble, 2012; Smith et al., 2013). Overall, the strength and direction of stressor interactions depend on various other factors, including exposure duration, exposure intensity, and order of exposure, highlighting the uncertainty surrounding the impacts of multiple environmental stressors on species. As such, future research and meta-analyses should consider the impacts of nitrate in combination with other stressors which act on similar physiological mechanisms or exacerbate nitrate uptake and consider their interactive effects on wide range of response variables including predator escape responses, behaviour, and reproductive output to adequately gauge a species susceptibility to multiple stressors.

5. Conclusion

Increased nitrate pollution as a result of anthropogenic global change represents a major issue for freshwater species. This study provides quantitative evidence that aquatic exposure to elevated nitrate concentrations negatively affects numerous behavioural, morphological and physiological traits. Further, we show that the effects of nitrate are not restricted to a particular life-history stage or taxon, suggesting that elevated nitrate concentrations may have cascading consequences on populations and communities living in nitrate-polluted freshwater environments. Sensitive species may be displaced from nitrate-polluted environments if nitrate inputs are not curbed, leading to the homogenisation of ecological communities. Our research highlights a heavy taxonomic bias towards amphibians and call for a broader evaluation of the effects of nitrate on various species, taxa and life-stages. Moreover, we have shown that there is a predominance for antagonistic interactions between nitrate and other ecological stressors in freshwater ecosystems, although, further research on stressors that act on the same physiological mechanism should be prioritised as they represent the greatest likelihood for "ecological surprises". Together, the results of the meta-analysis provide compelling and consistent evidence that aquatically respiring organisms are negatively impacted by exposure to nitrate and indicates the ability of nitrate to cause broad-scale ramifications within freshwater ecosystems.

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Figure Captions:

Figure 1. Overall effects on different response variables of freshwater taxa in response to waterborne nitrate exposure. The mean effect size (*LnRR*) and 95% confidence intervals are shown for separate analyses of activity, deformity rate, growth, hatching success, and survival. Sample sizes included in each analysis are shown in parenthesis. The zero (dotted) line indicates no effect, and mean effect sizes are considered significant when 95% confidence intervals do not overlap with the zero line. Activity, deformity rate, growth and survival are significantly reduced by nitrate exposure, denoted by asterisks (*$P < 0.01$, ** < 0.001), but not hatching success.

Figure 2. Taxonomic variation in effect sizes in response to waterborne nitrate exposure. The mean effect size (*LnRR*) and 95% confidence intervals for Activity, Deformity rate, Growth, Hatching Success, and Survival are shown. The horizontal zero (dotted) line indicates no effect, and mean effect sizes are considered significant when 95% confidence intervals do not overlap with the zero line. Statistical significance is indicated by an asterisk (*$P < 0.05$, ** < 0.01). Sample sizes included in each analysis are shown in parenthesis. No data means that there are too few studies ($n < 4$) for comparison. Note the different scales on the *y*-axis of the graphs.

Figure 3. Mean effect size (*LnRR*) of nitrate compound and life-history stage in response to nitrate exposure in freshwater taxa. Response variables include Activity, Deformity rate, Growth, and Survival. Data presented as mean ± 95% confidence intervals. The zero (dotted) line indicates no effect, and mean effect size is considered significant when 95% confidence intervals do not cross overlap with the zero line. Statistical significance is indicated by an asterisk (*$P < 0.05$). Differences between groups are considered significant if confidence intervals do not overlap. Sample sizes included in each analysis are shown in parenthesis. No data (N.D.) means that there are too few studies ($n < 4$) for comparison.

Figure 4. Effects of nitrate, additional stressors (stressor 2) and their interaction on the survival of freshwater taxa. The mean effect size (*LnRR*) and 95% confidence intervals are shown for (A) nitrate-exposure only, stressor-2: average effect size of exposure to another stressor only, nitrate × stressor 2: the interaction between nitrate and additional stressors, and for the predicted interaction based on the multiplicative model. (B) Effect sizes for the interactions between nitrate and series of biotic and abiotic stressors. Statistical significance is indicated by an asterisk (*$P < 0.05$). Differences between groups are considered significant if confidence intervals do not overlap.

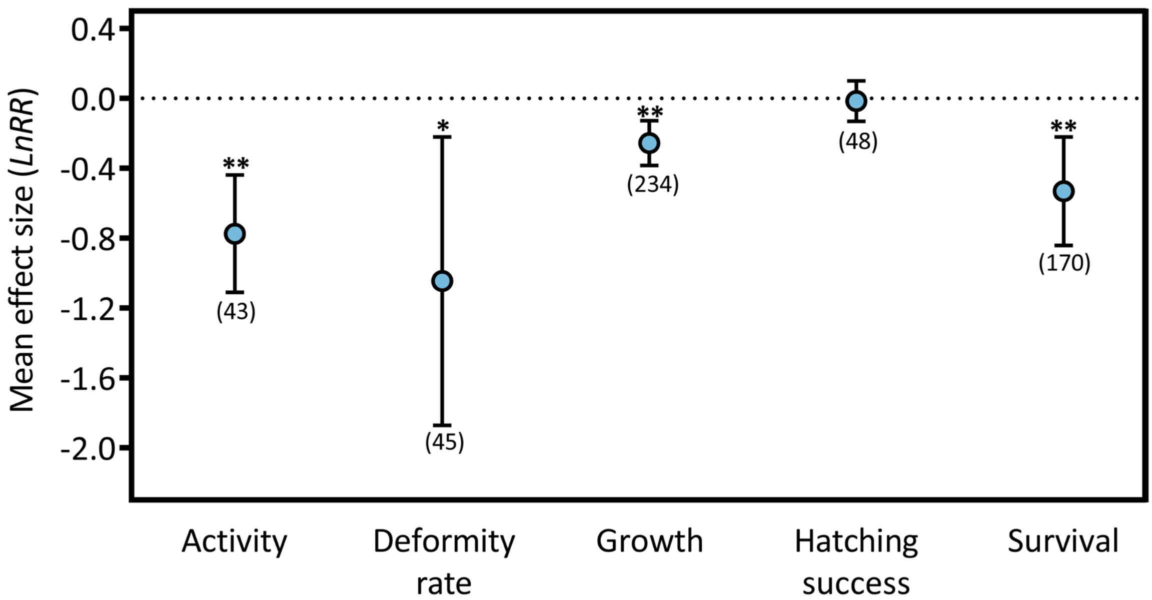
Table 1. Summary count of studies and data point included for each response varibale in the mata-analysis. Data counts are broken down for each taxonomic groups.

	Activity		Deformity rate		Growth		Hatching success		Survival	
	Studies	Points	Studies	Points	Studies	Points	Studies	Points	Studies	Points
Amphipods	0	0	0	0	3	24	0	0	5	37
Amphibians	9	39	5	32	31	145	2	33	23	75
Fish	1	4	2	13	12	65	2	15	7	58
Total	10	43	7	45	46	234	4	48	35	170

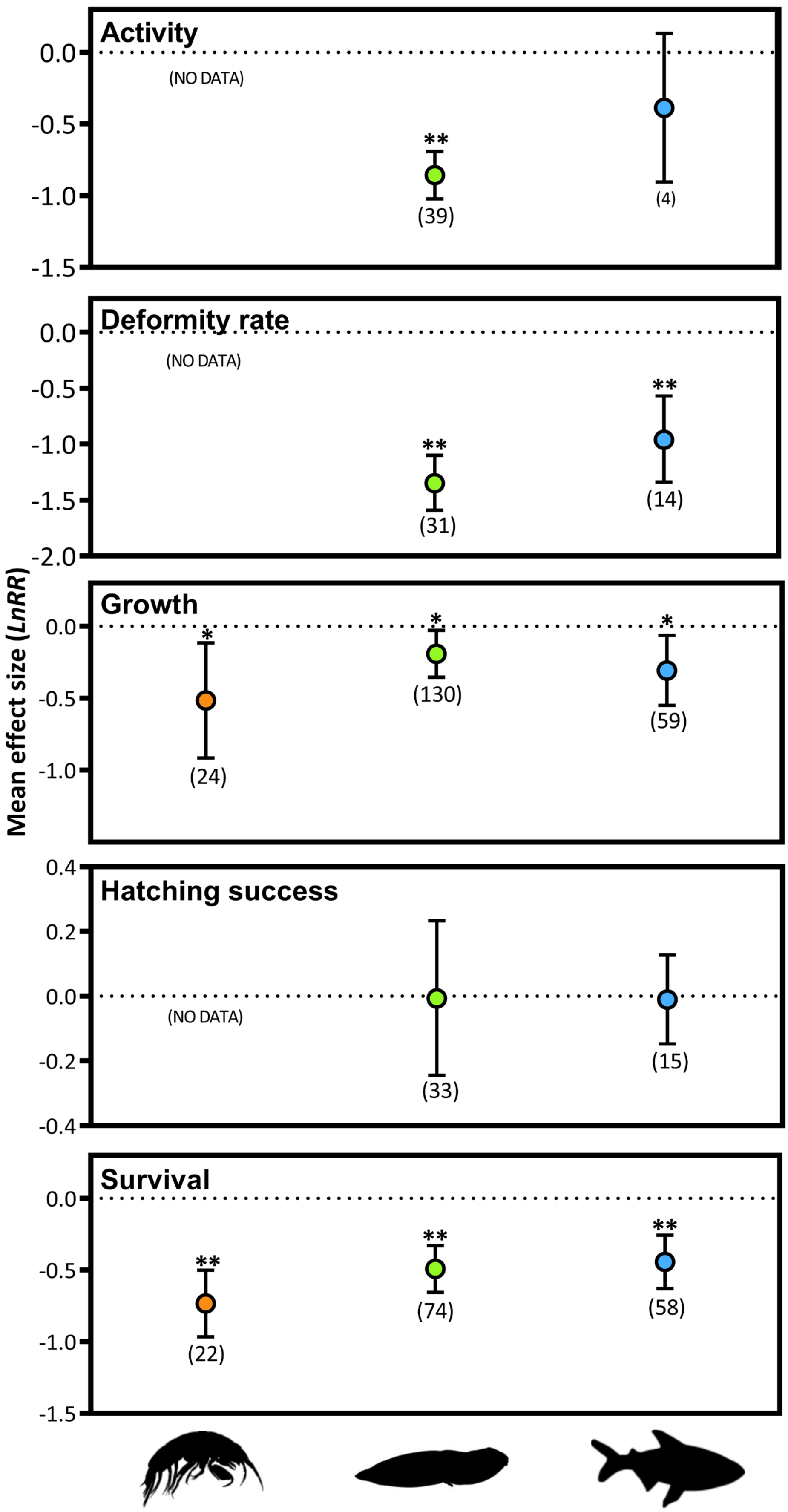
Table 2. Relationship between nitrate concentration and experimental temperature on effect size (*LnRR*) for each response variable (activity, deformity rate, growth, hatching success and survival). Data represents continuous moderators considered in the mixed effects meta-analyses. Significant results are highlighted in bold.

Response Variable	**Continuous moderator**	**Slope**	**z statistic**	***P***
Activity	Concentration	-0.27	-2.21	**< 0.01**
	Duration	-0.72	-4.32	**< 0.001**
	Temperature	-0.0001	-0.26	0.58
Deformity rate	Concentration	-0.16	-0.79	0.43
	Duration	-1.72	-2.78	**< 0.01**
	Temperature	0.01	0.13	0.89
Growth	Concentration	-0.23	-29.82	**< 0.001**
	Duration	-0.33	-4.64	**< 0.001**
	Temperature	0.005	0.56	0.57
Hatching Success	Concentration	-0.02	-0.26	0.79
	Duration	0.01	0.05	0.95
	Temperature	0.003	0.19	0.84
Survival	Concentration	-0.15	-4.36	**< 0.001**
	Duration	-0.09	-3.82	**< 0.001**
	Temperature	-0.01	-3.97	**<0.001**

Figure 1.

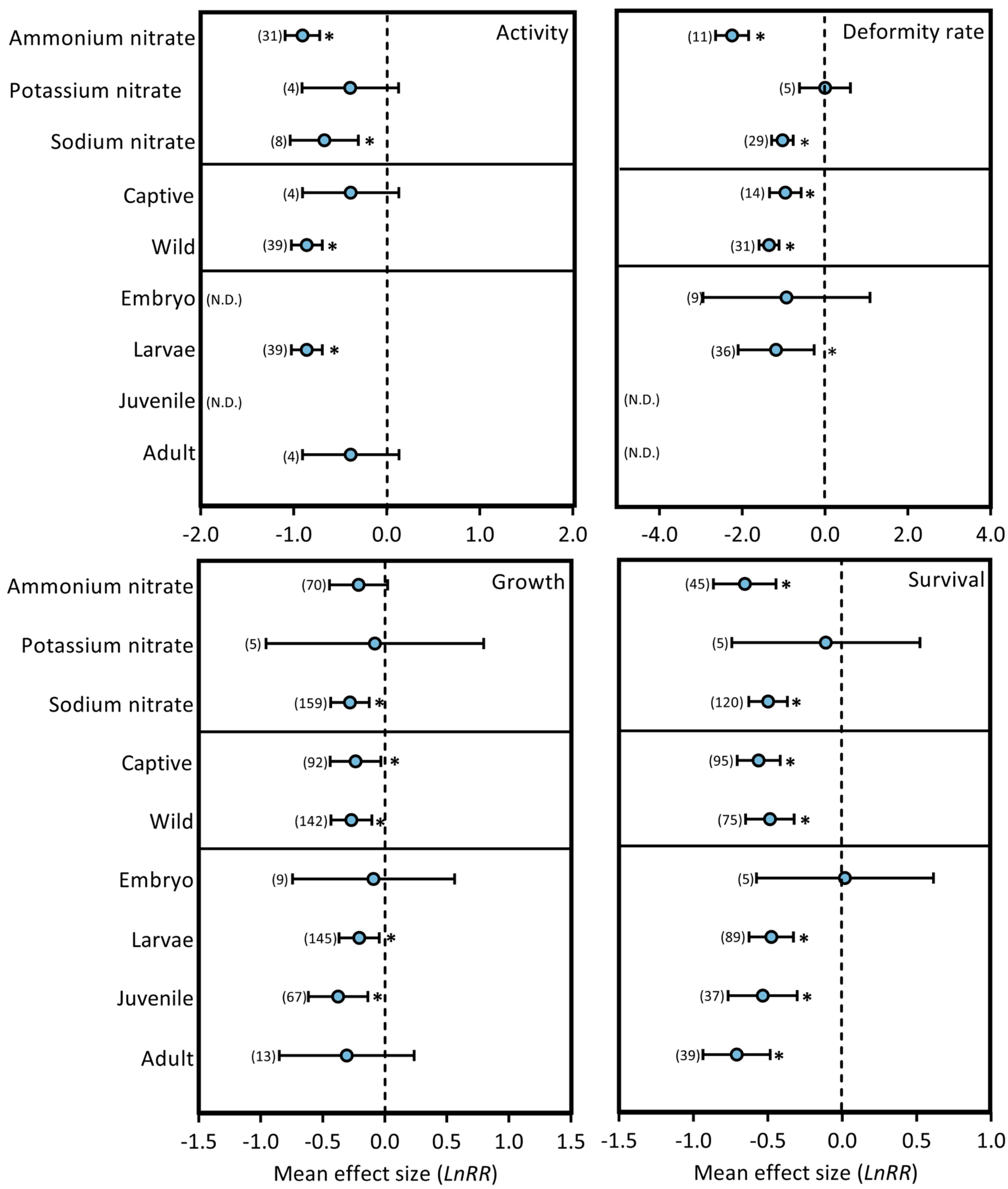

0.4
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
Mean effect size (LnRR)
**
(43)
*
(45)
**
(234)
(48)
**
(170)
Activity
Deformity rate
Growth
Hatching success
Survival

941 **Figure 2.**



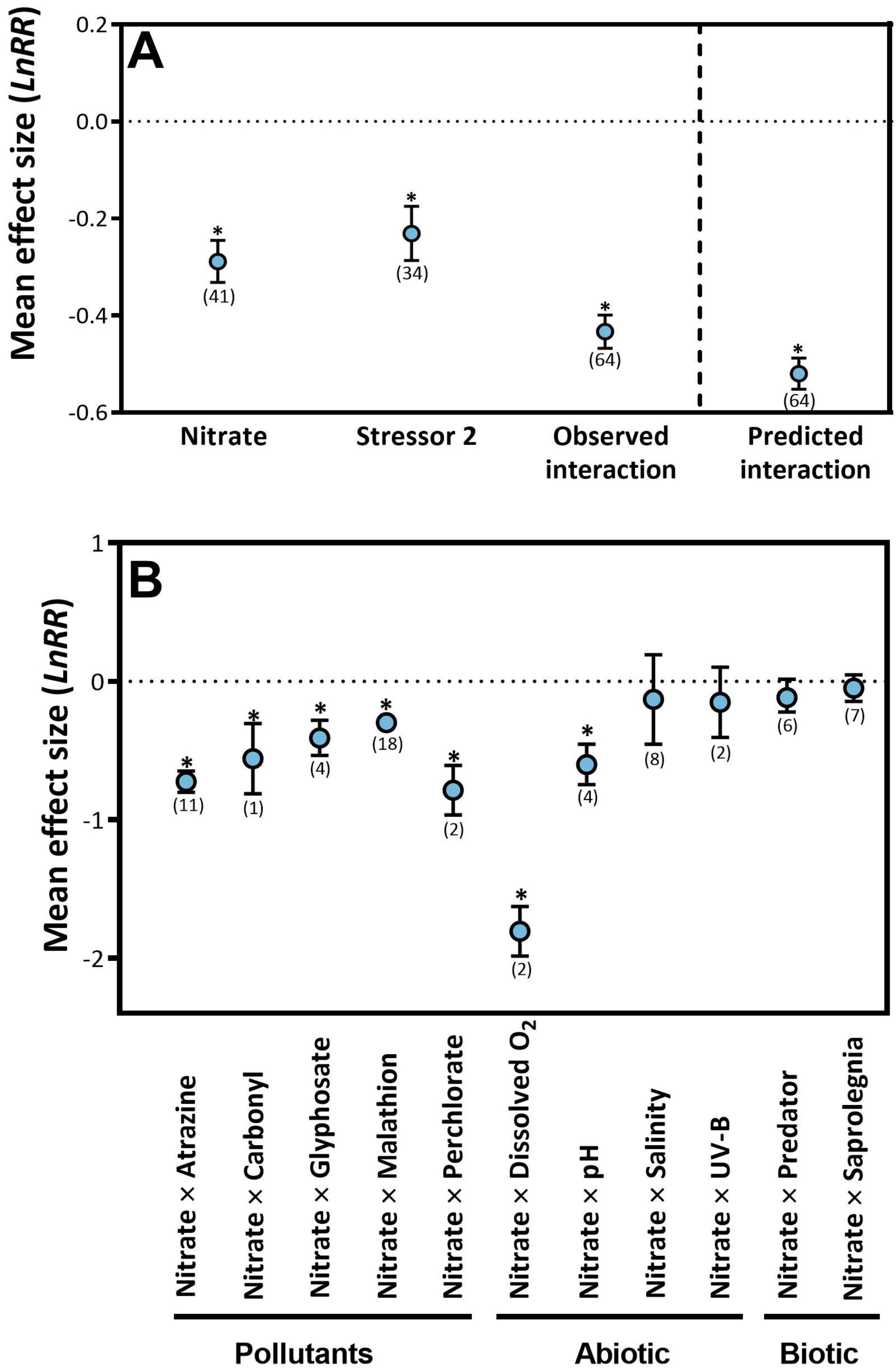
942

Figure 3.



Activity
Deformity rate
Growth
Survival
Ammonium nitrate
Potassium nitrate
Sodium nitrate
Captive
Wild
Embryo
Larvae
Juvenile
Adult
(31)
(4)
(8)
(4)
(39)
(N.D.)
(39)
(N.D.)
(4)
(11)
(5)
(29)
(14)
(31)
(9)
(36)
(N.D.)
(N.D.)
(70)
(5)
(159)
(92)
(142)
(9)
(145)
(67)
(13)
(45)
(5)
(120)
(95)
(75)
(5)
(89)
(37)
(39)
-2.0
-1.0
0.0
1.0
2.0
-4.0
-2.0
0.0
2.0
4.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
-1.5
-1.0
-0.5
0.0
0.5
1.0
Mean effect size (LnRR)
Mean effect size (LnRR)

Figure 4.


A
Mean effect size (LnRR)
0.2
0.0
-0.2
-0.4
-0.6
(41)
(34)
(64)
(64)
Nitrate
Stressor 2
Observed interaction
Predicted interaction
B
Mean effect size (LnRR)
1
0
-1
-2
(11)
(1)
(4)
(18)
(2)
(2)
(4)
(8)
(2)
(6)
(7)
Nitrate × Atrazine
Nitrate × Carbonyl
Nitrate × Glyphosate
Nitrate × Malathion
Nitrate × Perchlorate
Nitrate × Dissolved O2
Nitrate × pH
Nitrate × Salinity
Nitrate × UV-B
Nitrate × Predator
Nitrate × Saprolegnia
Pollutants
Abiotic
Biotic