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The interactive effects of microcystin and nitrite on life-history parameters of the cladoceran *Daphnia obtusa*

Zhou Yang[a,b,*], Fuhui Xiang[a], Ewan J.A. Minter[c], Kai Lü[a], Yafen Chen[b], David J.S. Montagnes[c]

[a] Jiangsu Province Key Laboratory for Biodiversity and Biotechnology, School of Biological Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, China
[b] State Key Laboratory for Lake Science and Environment, Nanjing Institute of Geography and Limnology, the Chinese Academy of Sciences, 73 East Beijing Road, Nanjing 210008, China
[c] Institute of Integrative Biology, Biosciences Building, University of Liverpool, Liverpool L69 7ZB, UK

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ABSTRACT

Elevated nitrite and microcystin concentrations co-occur during degradation of *Microcystis* blooms and are toxic to aquatic organisms. We studied the relative and combined effects of these on *Daphnia obtusa* life-history. Nitrite and microcystin-LR treatments were: 0, 1, 3 mg L^{-1} and 0, 10, 100, 300 μg L^{-1}, respectively. Experiments were factorial with 12 treatment combinations. Incubations were 15 d and recorded: moult number; time to first batch of eggs; time to first clutch; size at first batch of eggs; size at first clutch; number of clutches per female; number of offspring per clutch; total offspring per female. Interactive effects of the toxins occurred for time to first batch of eggs and time to first clutch. The remaining traits were negatively affected by nitrite: a significant decrease occurred in number of offspring per clutch and total number of offspring per mother (both decreased by ~50%); total clutches per mother; number of moults; mother size at first clutch; and first appearance of eggs (primarily at the highest nitrite concentration). We support the literature, recognising nitrite is toxic, and although *Microcystis* is toxic to zooplankton, the main threat is not from dissolved microcystin but from degradative products such as nitrite.

1. Introduction

The toxic cyanobacterium *Microcystis* is globally distributed in freshwaters and forms extensive blooms that disrupt aquatic ecosystems worldwide, especially in eutrophic systems [1,2]. As blooms may poison livestock, natural communities, and even humans [3–7], understanding the potential impact of *Microcystis* on planktonic assemblages is of considerable importance. For instance, it is now well established that grazing of *Microcystis* by zooplankton can result in acute and chronic toxicity [8–14]. There is, however, another fundamental issue associated with algal blooms: their demise and the subsequent physical and bacterial degradation of the organic materials result in post-bloom toxicity. This toxicity is not through grazing but through association with the ambient environment.

Here, we focused on a condition that may seriously impact fresh waters [15], the relative and combined effects of two main toxins that arise as end products of *Microcystis* blooms: dissolved nitrite, a by-product of cellular degradation, and dissolved microcystin-LR, a compound occurring within cells that is released as cells lyse [16]. These two toxins may impact zooplankton populations in the region of the blooms, as they will persist for days and may remain at low levels over weeks [17]. Thus, to assess the chronic, short-term impact on biota, we examined the effect of these dissolved compounds, at relatively high levels, over a short period, fifteen days, on the key zooplankton, *Daphnia obtusa*. More specifically, to address the impact of the toxins within lakes that are presently influenced by *Microcystis*, we focused on a *Daphnia* clone that had been isolated from a eutrophic lake where *Microcystis* blooms regularly occur every year [18,19]. In this way, we examine the issue of the relative need for concern regarding these toxins in regions where they already are problematic.

It is well accepted that high nitrite levels are physiologically deleterious to aquatic animals [20], resulting in a host of problems to aquatic communities [21]. However, toxicological studies have tended to focus on nitrite acute toxicity, with chronic studies being directed towards commercially important species of fish [22–24]. High nitrite levels sometimes found in lakes may have sublethal effects rather than being lethal. Here, we examine sublethal impacts of nitrite on *D. obtusa* reproduction and compare them to the impacts of microcystin-LR.

* Corresponding author at: School of Biological Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, China. Tel.: +86 25 85891671; fax: +86 25 85891526.
E-mail address: yangzhou@njnu.edu.cn (Z. Yang).

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Unlike nitrite, many studies have explicitly indicated chronic effects of microcystin-LR on *Daphnia* [25–30]. Although direct ingestion of *Microcystis* cells by *Daphnia* may be the main means by which toxins are transferred to animals [26], dissolved microcystins, often reaching high levels in lakes [16,31], have been proposed as a major cause of reduced growth and increased mortality in *Daphnia*, and in particular the variant microcystin-LR may exert strong toxic effects on *Daphnia* [25,32,33]. However, tolerance levels are highly variable between *Daphnia* species, populations, and even clones, suggesting that local adaptation to toxins occurs [34–37]. Consequently, as indicated above, we have focused on *Daphnia* that have been exposed to *Microcystis*, rather than using potentially more sensitive strains from pristine waters.

Of equal importance, to our study is a recognition that nitrite and microcystin-LR will co-occur and may have synergistic toxic effects. The combined effects of multiple toxins on organisms can reveal a complex picture of additive, synergistic, or even antagonistic effects, and *Daphnia* have been used to elucidate such processes [38,39]. For instance, microcystin-LR and a common pesticide, carbaryl, produce an unpredictable, synergistic toxic effect on *Daphnia pulicaria* [40]. To date, however, there has been no examination of synergism between nitrite and microcystin-LR. Clearly, given that these two toxins will co-occur in lakes (e.g., in certain areas of Lake Taihu after the collapse of heavy *Microcystis* blooms, concentrations of nitrite and microcystin can reach 2.5 mg L^{-1} and 10–15 μg L^{-1}, respectively. Yang, personal communication), it is important to assess the extent to which synergism is a concern.

Our goal was thus to examine the relative chronic effects of nitrite and microcystin-LR, independently and in combination. Specifically, we tested the following hypotheses: (i) dissolved nitrite and microcystin-LR will have similar toxic impact on reproduction of *Daphnia* isolated from a lake that experiences *Microcystis* blooms and (ii) there will be synergism between these two toxins that is detrimental to *Daphnia* reproduction. We tested these hypotheses by examining a range of reproductive attributes over a range of toxin levels. In fact, our data rejected both hypotheses, revealing that nitrite was by far the most important toxin, synergism rarely occurred, but when synergism did occur, increased microcystin concentration appeared to shorten time to maturation.

2. Materials and methods

2.1. Test organism

A clone of *D. obtusa*, isolated from Lake Taihu, China (119°53′45″–120°36′15″E, 30°55′42″–31°33′50″N), was maintained under constant conditions for >6 months prior to this study. Zooplankton were cultured in 200 mL beakers and fed the chlorophyte *Scenedesmus obliquus*, at 25 °C, under fluorescent light at 40 μmol photons m^{-2} s^{-1}, with a light–dark period of 12:12 h. All experiments were also conducted under these conditions.

2.2. Experimental design

New-born (<24 h-old) *D. obtusa* taken from a single mother (F_0) in a stock culture were isolated and grown individually in 50 mL beakers and fed daily on *S. obliquus* (5.0×10^4 cells mL^{-1}). Experimental lines (F_1, F_2) were conditioned for maternal effects under constant conditions for two generations prior to experiments, with each new generation arising from randomly chosen individuals from the third clutch of the previous generation.

Experimental animals (F_3) were placed randomly in 50-mL beakers. Each beaker contained one experimental animal to avoid density effects [41], and each treatment was replicated ($n = 4$). Based on our preliminary experiments of acute toxicity, nominal nitrite concentrations (NO_2-N) and purified microcystin-LR con-

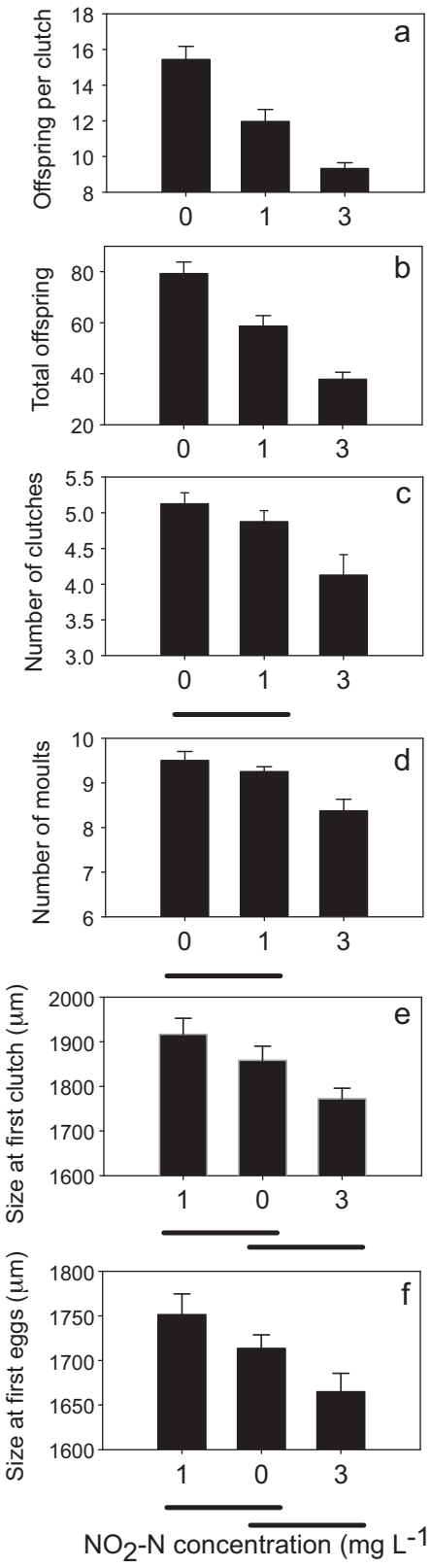

Fig. 1. Effect of nitrite concentration (mg L^{-1}) on (a) mean number of offspring per clutch, (b) mean total number of offspring per mother, (c) mean number of clutches per mother, (d) mean number of moults per mother, (e) mean mother size at first clutch (μm), and (f) mean mother size at appearance of eggs in the brood pouch (μm). Note some *x*-axes are not sequential, and data from all microcystin-LR concentrations are pooled as there was no significant effect. Error bars indicate 1 SE, and black bars beneath panels indicate groups not significantly different from one another.

centrations (Express, Beijing, China) treatments were: 0, 1, 3 mg L^{-1} and 0, 10, 100, 300 μg L^{-1}, respectively. These microcystin concentrations were chosen not only to determine the effect of existing levels in many lakes but also to assess levels that may occur in the future; in fact, dissolved microcystin in the environment can reach 1800 μg L^{-1} or higher in some waters, immediately after the collapse of a highly toxic bloom [16]. Experiments followed a fully factorial design; i.e., there were 12 treatment combinations. To maintain constant nitrite and microcystin concentrations, test solutions were replaced every two days. Experimental conditions were identical to those described above for culturing, and *S. obliquus* concentration was maintained at 5.0×10^4 cells mL^{-1}.

Experimental incubations were 15 d, during which survival and moulting (i.e., shed carapaces) were monitored daily. Body length was measured from above the eye to the base of the tail spine. Offspring production was measured daily; once counted these were removed. Also recorded were: the time to first batch of eggs appearing in the brood pouch; the time to first clutch; the size at first batch of eggs; the size at first clutch; the number of clutches per female; the number of offspring in each clutch; and the total number of offspring per female.

2.3. Statistical analysis

Treatment effects were assessed by ANOVA. Firstly, two-way ANOVA was applied to assess for interaction. When interaction existed, treatment effects were examined at all levels by one-way ANOVA. When no interaction occurred data were pooled, appropriately, and one-way ANOVA was performed. ANOVA was followed by Tukey's multiple range test ($\alpha = 0.05$). In the rare cases where data were not normal or homoscedastic, we applied to the robustness of ANOVA [42]. Data are presented as means ± 1 SE, with underscoring to indicate lack of significant differences.

3. Results

Of the eight traits examined, interactive effects of microcystin and nitrite occurred for only two: (i) the time to first batch of eggs appearing in the brood pouch and (ii) time to first clutch. Surprisingly, in both cases microcystin appeared to have a small, positive influence on these life history traits (Table 1, and Figs. 1–3). The remaining six traits were negatively affected by nitrite concentration but not by dissolved microcystin.

With increasing nitrite, there was a significant decrease in: (i) the number of offspring per clutch and (ii) the total number of offspring per mother (Fig. 1a and b); both traits decreased by ~50% from the lowest to highest nitrite concentration. Both (i) the total number of clutches per mother and (ii) the number of moults declined at only the highest nitrite concentration (3 mg L^{-1}) (Fig. 1c and d). Both (i) mother size at first clutch and (ii) the first appearance of eggs in brood pouch were significantly affected by nitrite; however these trends are not clear, as addition of low amounts of nitrite increased size in both cases (Fig. 1e and f).

As indicted above there were only two cases of significant interaction. Generally, the time to first eggs in the brood pouch was independent of nitrite concentration (Fig. 2a–d), while the effect of microcystin was dependent on the nitrite concentration (Fig. 2e–g). In the absence of nitrite, microcystin had no effect on time to first eggs in the brood pouch (Fig. 2e), but in the presence of nitrite, microcystin co-occurrence reduced the time required for eggs to develop (Fig. 2f). Nitrite increased the time to first brood; however the effects were neither consistent across all microcystin concentrations nor at all levels of nitrite concentration (Fig. 3a–d). At 0 mg L^{-1} nitrite, microcystin did not influence the time to first clutch (Fig. 3e), while at 1 mg L^{-1} nitrite, increasing microcystin concentrations decreased the time to first clutch (Fig. 3f), and at 3 mg L^{-1} nitrite the presence of any microcystin concentration decreased the time to first clutch (Fig. 3g).

Table 1
Results from two-way ANOVA for the factors nitrite and microcystin-LR (MC-LR) for each response variable.

Trait	Source of variation	df	*F*	*p*
Number of offspring per clutch	Nitrite	2	26.612	<0.001
	Microcystin-LR	3	1.323	0.282
	MC-LR × nitrite	6	1.131	0.364
Number of offspring per female	Nitrite	2	27.378	<0.001
	Microcystin-LR	3	1.36	0.270
	MC-LR × nitrite	6	0.43	0.854
Number of clutches	Nitrite	2	6.118	0.005
	Microcystin-LR	3	0.667	0.578
	MC-LR × nitrite	6	1.02	0.428
Number of moults	Nitrite	2	9.571	<0.001
	Microcystin-LR	3	0.810	0.497
	MC-LR × nitrite	6	1.810	0.125
Size at first clutch	Nitrite	2	5.415	0.009
	Microcystin-LR	3	0.619	0.607
	MC-LR × nitrite	6	0.979	0.454
Size at eggs in brood pouch	Nitrite	2	5.191	0.010
	Microcystin-LR	3	2.69	0.061
	MC-LR × nitrite	6	0.968	0.461
Time to eggs in brood pouch	Nitrite	2	2.483	0.098
	Microcystin-LR	3	19.461	<0.001
	MC-LR × nitrite	6	2.923	0.020
Time to first clutch	Nitrite	2	9.708	<0.001
	Microcystin-LR	3	13.8	<0.001
	MC-LR × nitrite	6	2.877	0.021

4. Discussion

Using the ecologically important, model (i.e., employed to investigate a wide range of biological issues) species *D. obtusa*, we investigated the impact of nitrite and microcystin-LR, both of which will be released into lake waters at the end of *Microcystis* blooms. Our main finding was that expected maximum levels of nitrite are substantially more detrimental to *D. obtusa* reproduction than expected maximum levels of microcystin. Furthermore, the interactive effects of microcystin and nitrite were rare, and when interaction did occur there was, surprisingly, a small reduction in development time (i.e., a potential benefit to population growth), due to increased microcystin in the presence of nitrite. To our knowledge, this is the first indication of apparent positive effects, as independently these two toxins are generally recognised as detrimental to *Daphina* and other animals [20,21,25,28]. Below, in more detail, we examine these toxins independently and then consider the implication of their interactive effects.

Nitrite is toxic to most aquatic organisms, including arthropods and fish, with a range of physiological consequences [20,21]. Due to bacterial degradation of organic material maximum nitrite levels in lakes can be on the order of 2.5 mg L^{-1} (e.g., in certain areas of Lake Taihu at specific times). The toxic effects of nitrite are wide-ranging: it disrupts ion regulation and the respiratory system, causing oxidative stress [20], and it may have detrimental effects on the endocrine system [43] and excretory processes [44]. We indicate that sub-lethal nitrite levels negatively affect the reproduction of *D. obtusa*, causing reductions in offspring production and affecting maternal growth and size at maturity. Such chronic consequences of nitrite toxicity on cladocera are poorly understood, but tests on one other species, *Ceriodaphnia dubia*, reveal similar reproductive inhibition over a comparable nitrite range [45]. Thus, we support an increasing body of literature on the problems caused by

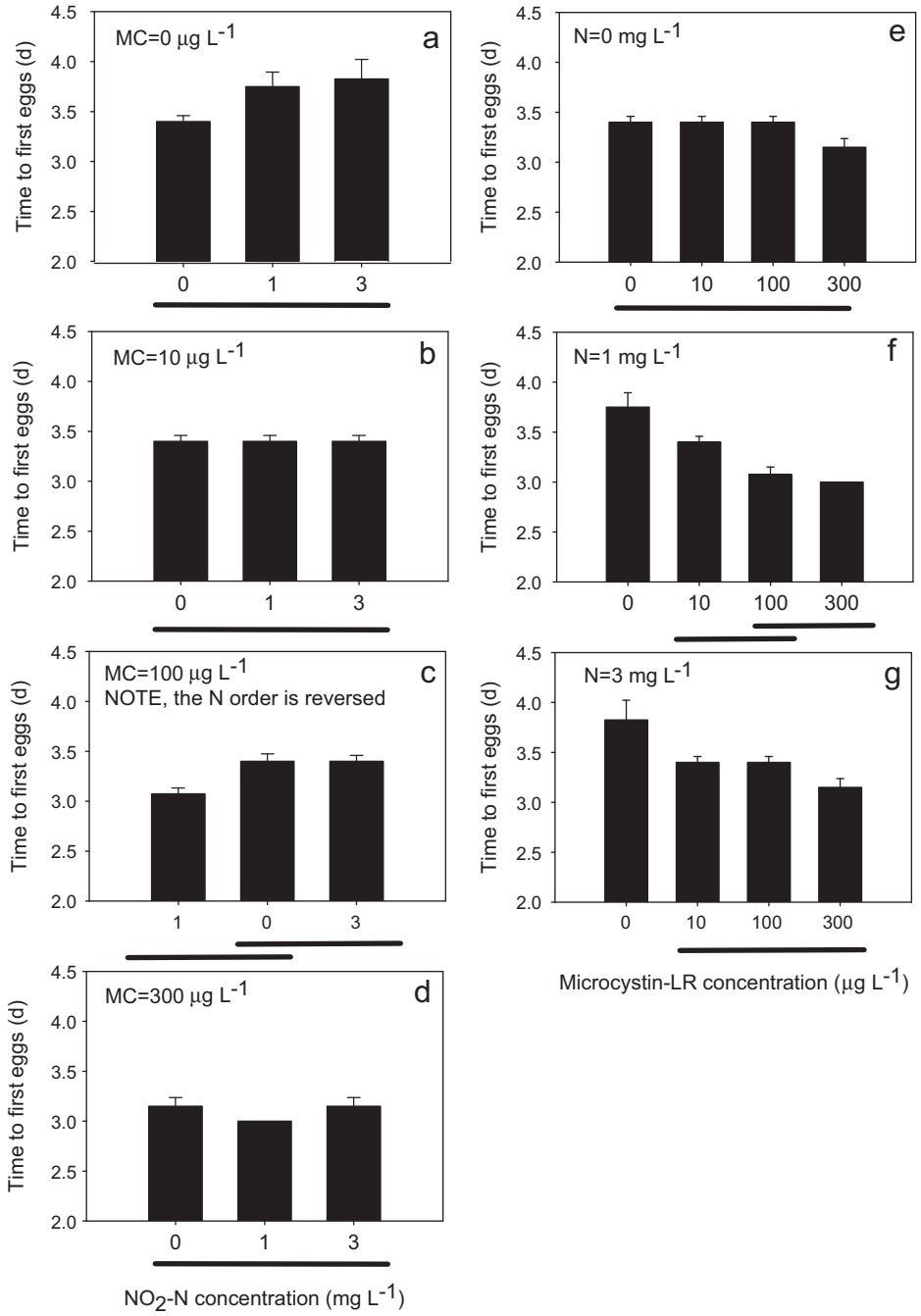

Fig. 2. Effect of microcystin-LR (a–d) under different nitrite concentrations and the effect of nitrite (e–g) under different microcystin-LR concentrations on mean time to first appearance of eggs in brood pouch. Error bars indicate 1 SE, and black bars beneath panels indicate groups not significantly different from one another.

sub-lethal nitrite concentrations and maintain that its generation and accumulation must be managed to protect aquatic ecosystems, including at sub-critical levels [23,24].

Our other target compound, microcystin-LR, is a hepatotoxin produced by several cyanobacteria [46] that affects both vertebrates [47] and invertebrates [25,48]. Typically, maximum levels in lakes, such as in certain areas of Lake Taihu, in the late stages of a *Microcystis* bloom are on the order of 10–15 $\mu g\,L^{-1}$ [31], but they may reach higher levels in localised regions. Microcystin-LR has a broad range of toxic effects; at a molecular level it mainly inhibits protein phosphatase [49] which causes intracellular problems with cell growth, differentiation, and osmoregulation [46]. Microcystin can also cause oxidative stress in crustaceans [50] and is linked with immunosupression in fish [51]. Furthermore, microcystin-LR reduces *Daphnia* filtration rate, inhibiting feeding [27]. However, both chronic and acute toxicity of *Microcystis* blooms and toxins on *Daphnia* are variable, and unexpected levels of tolerance to microcystins by *Daphnia* occur [26,28,52]. For instance, Dao et al. [30] observed reduced neonate production and shortened time to maturity for *Daphnia magna* at 50 $\mu g\,L^{-1}$ of microcystin-LR. In contrast we noted no effect of microcystin (up to 300 $\mu g\,L^{-1}$) on offspring produced per clutch (a parameter akin to neonate production), and time to first eggs (a parameter akin to time to maturity) was invariant with microcystin levels, when nitrite was absent. Thus, as Dao et al. [30] note, it is difficult to make generalities regarding *Daphnia* from any one study. We can, at this point only suggest that our data indicate that typical post bloom levels of microcystin are insufficient to negatively impact the *D. obtusa* clone we used, which was

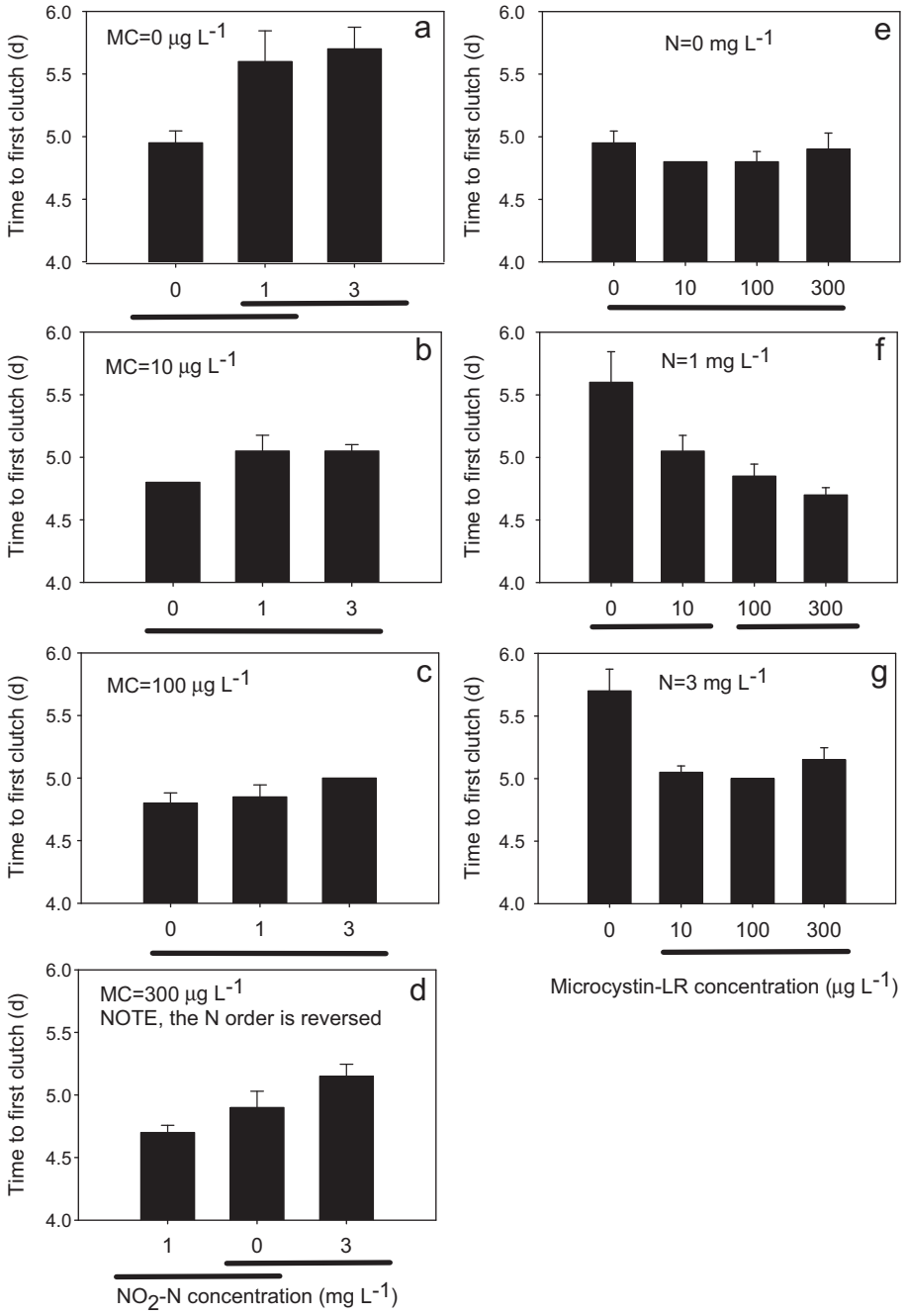

Fig. 3. Effect of microcystin-LR (a–d) under different nitrite concentrations and the effect of nitrite (e–g) under different microcystin-LR concentrations on mean time to first clutch. Error bars indicate 1 SE, and black bars beneath panels indicate groups not significantly different from one another.

isolated from a lake that regularly experiences *Microcystis* blooms [18,19]. Possibly, our isolate had become adapted to microcystin, as has occurred in other eutrophic lakes [34] and as has evidenced in a laboratory study [37].

Finally, we have shown that microcystin toxicity does not become more pronounced in the presence of the co-occurring toxin nitrite; if anything the combined effects would increase reproduction, as microcystin reduced the time to maturity and offspring production in treatments that included nitrite, despite nitrite itself increasing the developmental period. Thus, microcystin may reduce the harmful effects caused by nitrite and could be a factor in increasing tolerance to nitrite at concentrations typically found at the end of blooms. Clearly, such an unexpected results requires further assessment, and we encourage pursuit of the possible benefits of microcystin as an antagonist against nitrite toxicity.

5. Conclusions

We support the wide body of literature that recognises nitrite as a toxin and extend the few observations on sublethal toxicity. Furthermore, we argue that although *Microcystis* is clearly toxic to zooplankton, especially when it is grazed, the main threat to zooplankton presented by bacterial degradation and cell lyses may not be from the toxin microcystin-LR but from other degradative products, such as nitrite. The robustness of these findings requires evaluation, on other strains and species of cladocera, on *Daphnia* strains from pristine and *Microcystis*-impacted waters, and on other zooplankton. If, however, our observations are supported, it may be necessary to reassess concerns regarding the late stages of *Microcystis* toxicity on lake ecosystems.

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