The effects of dietary *Microcystis aeruginosa* and microcystin on the copepods of the upper San Francisco Estuary

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SUMMARY

1. Increasing blooms of *Microcystis aeruginosa* have unknown impacts on the copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi*, which are the dominant zooplankters and key prey species for endangered larval fish in the upper San Francisco Estuary.
2. Laboratory feeding experiments were designed to measure the effect of *Microcystis* on copepod survival and to distinguish the effects of toxicity and nutrition. In a series of survival tests, copepods were fed a mixed diet of algae plus one of two strains of *Microcystis*, either producing (MC+) or lacking microcystin (MC−).
3. *Microcystis* significantly reduced survival even when it was a small proportion of the diet, indicating that toxicity was the major cause of mortality. Contrary to expectation, however, the MC+ strain did not result in higher mortality, suggesting that non-MC metabolites of *Microcystis* can be toxic to copepods.
4. Across treatments, survival of *P. forbesi* was greater than that of *E. affinis*, although the two copepods responded differently to both the ratio and the strain of *Microcystis* in their food. Survival of *P. forbesi* was greater on the MC+ strain and was inversely proportional to the ratio of dietary *Microcystis* (MC+ or MC−). In contrast, survival of *E. affinis* declined similarly across treatments and was not related to the proportion or strain of dietary *Microcystis*. Results indicate that the copepod *P. forbesi* can coexist with *Microcystis* while the other copepod *E. affinis* cannot.
5. Regardless of species, dietary *Microcystis* caused significant mortality to copepods, and it may cause adverse impacts to the potentially food-limited zooplankton community of the San Francisco Estuary. These impacts may not be related to the cellular MC concentration because *Microcystis* contains other metabolites that negatively affect copepods.

Keywords: copepod, harmful algal bloom, microcystin, *Microcystis*, San Francisco Estuary

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Introduction

Global increases in the extent of bloom-forming cyanobacteria have attracted growing attention from both management and scientific interests because of potential negative impacts on water quality and food web structure (Kirk & Gilbert, 1992; Chorus &

Bartram, 1999). *Microcystis* is a common bloom-forming cyanobacterium that can dominate nutrient-enriched freshwaters including upper reaches of estuaries, such as the San Francisco Estuary (Paerl, 1988; Lehman *et al.*, 2008).

In blooms, *Microcystis* can change the food web because it is a poor quality food for zooplankton, reducing their fitness and restricting the trophic transfer of carbon (Paerl, 1988; Christoffersen, 1996; Chorus & Bartram, 1999). Negative impacts on zooplankton occur in response to a combination of factors including: (i) secondary metabolites with toxic effects, such as microcystins (MCs), (ii) nutritional inadequacy and (iii) poor digestibility or feeding inhibition (Fulton & Paerl, 1987; DeMott & Moxter, 1991; Lurling, 2003). However, not all zooplankton are affected equally (Christoffersen, 1996; Wilson, Sarnelle & Tillmanns, 2006). Species-specific adaptations in zooplankton lead to variation in the observed responses to *Microcystis* blooms (Kirk & Gilbert, 1992; Rohrlack *et al.*, 1999; Gustafsson & Hansson, 2004). Although many studies have evaluated the interaction between zooplankton and *Microcystis*, most focus on cladocerans while impacts to copepods remain poorly understood (reviewed in Wilson *et al.*, 2006).

Controlled manipulations of diets in the laboratory can provide valuable evidence of the mechanisms involved. A major problem in predicting the impacts of *Microcystis*, however, has been because of the use of oversimplified experimental conditions, such as pure or single strain diets (presence/absence experiments), a disproportionate focus on daphniids as the test organism, and species/strain-specific responses, preventing the interpretation of impacts on other zooplankton or under field conditions (reviewed in Wilson *et al.*, 2006). As a result, the effect of *Microcystis* blooms on copepods and the dynamics of food webs in estuaries dominated by copepods are poorly understood.

Copepods are an important link between primary production and fish, and commonly dominate the mesozooplankton of estuaries (Turriff, Runge & Cembella, 1995; Sommer *et al.*, 2007). Calanoid copepods, particularly *Eurytemora affinis* (Poppe, 1880) and *Pseudodiaptomus forbesi* (Poppe & Richard, 1890), are the principal food source of the endangered larval and pelagic fish in the San Francisco Estuary, where the abundance of pelagic organisms, including copepods

and nutritious phytoplankton, has declined to values not seen before (Sommer *et al.*, 2007). Increasing blooms of *Microcystis* in the freshwater and low salinity zone of the estuary raise concerns about the further depletion of high-quality fish food (Muller-Solger, Jassby & Muller-Navarra, 2002; Lehman *et al.*, 2005, 2008). Understanding the impacts of *Microcystis* on the dominant copepods of the San Francisco Estuary has become highly significant in the management of its declining fish populations (Sommer *et al.*, 2007).

While a pure diet of *Microcystis* reduces fitness and survival in zooplankton, it is not likely to occur in nature (DeMott, Zhang & Carmichael, 1991; Kumar, 2003; Wilson *et al.*, 2006). Unlike daphniids, copepods can ingest nutritious particles selectively and avoid those that are unwanted or toxic (DeMott *et al.*, 1991; Kleppel, 1993; Burns & Hegarty, 1994; Engstrom *et al.*, 2000; Panosso *et al.*, 2003). Even daphniids show different responses to *Microcystis* when offered in mixed versus pure diets (Rohrlack *et al.*, 1999; Lurling, 2003). Therefore, in studying interactions between zooplankton and *Microcystis*, we need to experiment with diets consisting of mixtures of alternative, more palatable, food (Wilson *et al.*, 2006).

Microcystis toxicity comes from well-known toxins, such as MC, but also from other, poorly understood and often unidentified, secondary metabolites (Lurling, 2003; Rohrlack *et al.*, 2004; Wiegand & Pflugmacher, 2005; Wilson *et al.*, 2006). Though the significance of metabolites other than MC is becoming clear, the relative toxicity to zooplankton of MC versus non-MC metabolites remains unresolved (DeMott *et al.*, 1991; Kurmayer & Juttner, 1999; Lurling, 2003; Rohrlack *et al.*, 2004). It is possible to quantify the effect of MC in the zooplankton diet using *Microcystis* strains of identical morphology (assuming similar nutritional profile) but different MC cellular content. This approach has been used successfully to distinguish the toxicity of MC versus non-MC metabolites in daphniids (Rohrlack *et al.*, 1999; Lurling, 2003). When offered in a mixed diet, strains of *Microcystis* containing (MC+) and lacking (MC−) both caused mortality, showing the toxic effects of non-MC metabolites on daphniids (Lurling, 2003). The current study was designed to compare the effects of *Microcystis* and its toxic metabolites on the dominant copepods of the San Francisco Estuary.

Our objective was to evaluate the impact of *Microcystis* in the diet on the survival of the dominant

copepods. To distinguish between the effects of toxic metabolites and nutritional deficiency of *Microcystis* on copepods, mixed diets containing nutritious algae and *Microcystis* (MC+ or MC−) were offered to copepods in laboratory survival tests. Experiments were designed to compare the survival of *E. affinis* and *P. forbesi* as a function of dietary *Microcystis* (MC+ versus MC−) and food quantity. We hypothesised that (i) both strains of *Microcystis* would negatively affect survival, though the response might be species specific, (ii) survival with the MC+ strain in the diet would be lowest and (iii) survival would be inversely proportional to the proportion of *Microcystis* in the diet.

Methods

Microcystis aeruginosa strains

Cultures of two different strains of *M. aeruginosa* (Kutzing, 1846) were maintained for the feeding experiments in a modified ASM-1 medium (Reynolds & Jaworski, 1978). MC producing (MC+, UTEX 2385) and MC absent (MC−, UTEX 2386) strains of *Microcystis* were obtained from the University of Texas Culture Collection (Austin, TX, U.S.A.). Exponentially growing axenic batch cultures were maintained by dilution with freshly prepared cultured medium to keep cell density between 1×10^6 and 3×10^6 cells mL^{-1}, in gently bubbled 500-mL glass flasks, under a 16 : 8 L : D cycle under 30 µmol quanta m^{-2} s^{-1}, in a temperature-controlled room (22 ± 1 °C). Under these conditions, both strains were uni- and bi-cellular, the average growth rate of *Microcystis* was 0.32 (±0.04) day^{-1} and cell diameter was approximately 4 µm (±0.7) for both strains. We assumed that the only difference between the two strains used was MC content and that each strain had a comparable nutritional profile and digestibility.

Instant algae (IA)

Commercially available and highly nutritious pure phytoplankton food composed of *Nannochloropsis* and *Pavlova*, rich in fatty acids, were kept fresh at 4 °C (Reed Mariculture, Campbell, CA, U.S.A.). Because IA is in a fresh but non-living state, cells have a constant density. Cell diameter was approximately 2 µm for *Nannochloropsis* and 4 µm for *Pavlova*.

Copepod cultures

Copepods were collected from Rio Vista and Suisun Marsh in the San Francisco Estuary using a 174-µm zooplankton tow net. *Pseudodiaptomus forbesi* was collected in June 2007 and *E. affinis* in April 2007 (respective periods of dominance), and returned to the laboratory within 2 h of collection. Gravid females were selected and rinsed three times with salinity-adjusted culture medium before transferring them to the culture. We used reconstituted water (modified by NaCl to increase salinity) as the culture medium (Horning & Weber, 1985). Cultures were grown in aerated 4-L beakers placed in an environmental water bath, at 24 ± 1 °C for *P. forbesi* and 20 ± 1 °C for *E. affinis*. Water quality in beakers, including dissolved oxygen (>8 mg L^{-1}), pH (8.2 ± 0.1), water hardness (100 mg L^{-1}), salinity (5.0 ppt) and ammonia (<1 µg L^{-1}), was monitored weekly (Hach, Loveland, CO, U.S.A.). For optimal culture and population growth under laboratory conditions, a previously determined amount of the IA mixture (an equal biovolume of *Nannochloropsis* and *Pavlova*) was given as food at 400 and 500 µg C L^{-1} day^{-1} for *E. affinis* and *P. forbesi*, respectively. Approximately, 60% of the total culture medium was replaced weekly with aerated and temperature-adjusted medium. Copepod culture density was monitored weekly, maintained between 50 and 100 adults L^{-1}, and diluted as necessary to ensure exponential population growth and minimise overcrowding. The systems were maintained under a natural photoperiod (16L : 8D) and covered with a semitransparent black tarpaulin. The brood cultures were acclimated to these conditions for a minimum of 4 months before the feeding experiments.

Cell size, density and algal biomass determination

Algal biomass concentration (µg C mL^{-1}) for the IA mixture and *Microcystis* was determined by a calibration curve of total carbon and biovolume. A serial dilution of algal biovolumes were filtered on pre-combusted 25-mm GF/C (Whatman, Kent, U.K.), desiccated at 50 °C, and stored at −80 °C until the total carbon was measured by a gas chromatogram (UC Davis Stable Isotope Facility, U.S.A.). *Microcystis* cell size and density was determined using an inverted compound epifluo-rescent microscope (2007 Zeiss Axio Observer A1, Thornwood, NY, U.S.A), counted in a Haemocytometer

(Hausler Scientific, Horsham, PA, U.S.A.) and calibrated against light absorbance at 800 nm.

Survival test

We used a series of survival tests in which dietary treatments included *Microcystis* while controls did not. The treatment diets consisted of a *Microcystis* – IA mixture, with the proportion of *Microcystis* at 0, 10, 25, 50 or 100% of total food (by carbon), using either the MC+ or MC− strain of *Microcystis*, plus IA, to a total food concentration of 400 (*E. affinis*) and 500 (*P. forbesi*) µg C L^{-1} day^{-1}. The control diets were comprised of IA only at the same amount as in the associated treatment of IA and *Microcystis* mixture, which corresponded to 100, 90, 75, 50 and 0% (starvation) of the total food. This design allowed the distinction of impacts because of food quantity (total nutrition available) versus quality (MC and non-MC toxicity) by comparing survival over a range of a given diets with and without *Microcystis*. This design also enabled the assessment of the impact of alternative food sources when *Microcystis* was present in food.

Experiments took place in triplicates over 11 days in 1-L glass beakers, starting with 10 copepods per beaker and maintained under identical conditions described for the brood cultures. Animals were obtained by growing a new cohort of adults from nauplii in the brood cultures. Experimental copepods were then collected from the pool of >400 copepods in the new cohort by gently filtering in a Petri dish for each set of experiments. To minimise the chance of age-related mortality and stress over the 11 days, only active C$_5$ copepodites and young adults were randomly selected. Survival and activity were monitored daily using a light table and a dissecting microscope when necessary. Copepods were considered dead only if they showed no sign of movement after disturbance under a dissecting microscope; dead copepods were removed as soon as recorded.

MC verification of strains and treatments

Microcystis cultures were sampled for MC analysis during the experiment, to ensure the MC+ and MC− strains were uncontaminated and to measure MC concentration of the MC+ strain culture. In addition, the MC+ experimental beakers were sub-sampled at day 7 of the survival test for total and dissolved MC analysis to ensure that mortality was not because of any dissolved MCs. A commercially available MC – specific competitive ELISA assay with a detection range of 0.16–2.5 µg L^{-1} MC-LR was used for MC analysis (Envirologix, Portland, ME, U.S.A.). *Microcystis* cultures were sampled and kept frozen until analysis, upon which they were thawed to room temperature, sonicated, diluted and measured for MC concentration using a microplate reader at 450 nm. Dissolved MC in treatments was measured 7 days during the experiment, from whole water samples by separating algal cells on a GF/F glass fibre filter (Whatman Corp) and comparing MC concentration in the filtrate with an unfiltered sample. Additionally, a standard polymerase chain reaction (PCR) using published primers designed for the conserved *Microcystis*-specific 16S ribosomal DNA (rDNA) region and amplification of the MIC and MC synthetase genes *mcy*B and *mcy*D was used to verify the identity of the two strains of *Microcystis*. Ability to produce MC is based on the presence or absence of the synthetase genes (Ouellette & Wilhelm, 2003). Conventional PCR was conducted to verify the presence of *Microcystis* and the MC synthetase genes *mcy*B and D using specific PCR primer sets targeting 16S rDNA and conditions described in Ouellette & Wilhelm (2003). The *Microcystis* strains were processed for genomic DNA extraction using a modified phenol–chloroform procedure (Sambrook & Russell, 2001).

Qualitative confirmation of Microcystis ingestion

To verify ingestion, the presence of *Microcystis* cells in copepod guts was determined in a separate feeding experiment. Copepods were starved for 5 h to evacuate gut contents before the experiment, which took place under the same conditions as the survival tests. Copepod density was kept at 10 adults in a 1-L beaker with clean media, and then fed the same treatment diets (MC+ and MC−) described earlier. Following a 30-min feeding period, animals were gently filtered, rinsed three times to remove any attached *Microcystis* cells and narcotised immediately with carbonated water. For each treatment diet, 10 copepods were dissected to expose the gut under a dissecting microscope. Guts were observed for phycocyanin fluorescence (a pigment specific to cyanobacteria), as a qualitative indication of *Microcystis* cells in copepod guts, using an epifluorescent microscope.

Statistical analysis

The effect of diet type on the survival of each copepod species was evaluated by a parametric survival analysis which modelled survival with a Weibull distribution curve (JMP version 7.0; SAS, Cary, NC, U.S.A). Individual probability of mortality or survival (expected proportion of the population dead or surviving) for each treatment and species was calculated by a test of deviance based on the mean number of surviving copepods on the last day of the experiment. Then, the effect of diet type (e.g. *Microcystis* versus no *Microcystis*) and copepod species on survival was calculated to compare differences in the effects of the treatments and controls. Finally, the *Microcystis* only treatments were further broken down to strain (MC+ or MC−) and ratio of dietary *Microcystis* (% *Microcystis*) to analyse differences in the effect of these parameters on copepod survival. Time to 50% mortality was analysed by linear regression of individual replicate survival responses and calculating when 50% mortality occurred on average per treatment. All differences noted are significant at $P = 0.05$ unless otherwise mentioned.

Results

Survival test

Both dietary composition and quantity had very pronounced effects on copepod survival. The presence and proportion of *Microcystis* drastically reduced survival in all treatments for either copepod species, regardless of *Microcystis* strain and MC content (Fig. 1). In contrast, survival with the control diets was significantly higher than corresponding *Microcystis* treatments (Fig. 1; $P < 0.001$).

Reducing the concentration of IA in the controls without *Microcystis* reduced survival. At the end of the experiment, 100% IA had the highest survival (85% ± 3.4), while survival in the 50% IA treatment was lower (75% ± 2.3 for *E. affinis*, 60% ± 8.2 for *P. forbesi*) (Fig. 1). Starved copepods (i.e. those fed 0% IA and 0% *Microcystis*) survived for several days, reaching 100% mortality after 7–9 days for both species, though *P. forbesi* had slightly longer average persistence than *E. affinis*.

Compared to *E. affinis*, *P. forbesi* was more sensitive to differences between the *Microcystis* strains (MC+ versus MC−) and had a higher survival rate on a MC+

diet (Fig. 1). Differences in survival of *P. forbesi* between the two different *Microcystis* strains took several days to emerge. Both strains (MC+ or MC−) caused comparable mortality until day 6, and thereafter *P. forbesi* mortality on the MC− diet was greater than that on the MC+ diet (Fig. 1). Towards the end of the experiment, *P. forbesi* mortality began to decline in most of the MC+ diets, but not in the MC− diets (Fig. 1). For treatments with <50% *Microcystis*, the MC− diet killed more *P. forbesi* than the MC+ diet, while these diets caused similar mortality for *E. affinis* (Fig. 1).

Survival analysis

The increased proportion of *Microcystis* in the diet caused higher mortality and increased the probability of mortality (Fig. 2). The effect test showed that diet type had a significant effect on survival and that differences in survival were not because of copepod species (Table 1a). When comparing results among the treatments (Table 1b), differences in survival were caused by the *Microcystis* strains (MC) and the ratio of *Microcystis* in food (% *Microcystis*). There was a significant difference in how each species responded to the MC+ versus the MC− strains, as well as the ratio of *Microcystis* in food, as shown by the interaction terms (species × MC) and (species × % *Microcystis*), respectively, in Table 1b.

Survival/mortality probability tests confirmed that copepods responded differently, both to the individual strains (MC+ versus MC−) and to the ratio of *Microcystis* in their food. *Pseudodiaptomus forbesi* was more likely to survive on the MC+ diet compared to an MC− diet when the diet contained <50% *Microcystis* (Fig. 2). In contrast, differences in the *Microcystis* strain had no significant effect on *E. affinis* survival at the end of the experiment. Additionally, *P. forbesi* was more sensitive to changes in the *Microcystis* content of its diet, as increased *Microcystis* resulted in higher mortality. In contrast, increasing *Microcystis* did not significantly change the probability of mortality for *E. affinis* (Fig. 2). Rather, the presence of *Microcystis* was more important in reducing *E. affinis* survival probability by the end of the experiment.

Mean survival time

The time to 50% mortality (TL 50) was inversely proportional to the % *Microcystis* in diets, but varied significantly with copepod species and *Microcystis*
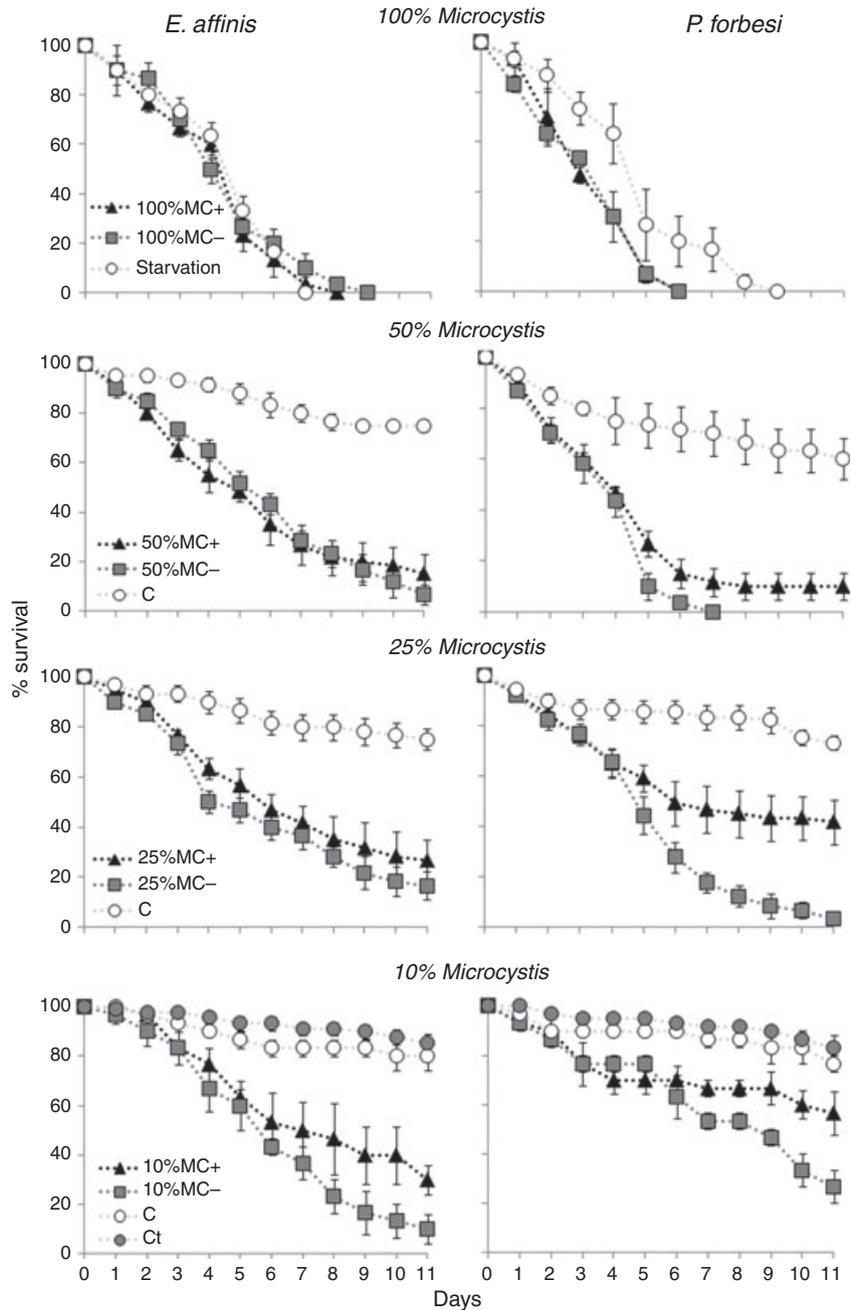
Fig. 1 Survival of *Eurytemora affinis* and *Pseudodiaptomus forbesi* on diets containing a mixture of instant algae (IA) and *Microcystis* (MC+ or MC−), at various proportions of the total food, compared to survival on corresponding control diets containing only IA at the same IA concentration as in the corresponding mixture of IA and *Microcystis*. C_T shows survival on the total amount of control diet (100% IA). Each line represents the mean of a minimum of three replicates (±SE).

strain (Fig. 3). Among the two copepod species, TL-50 was similar when the ratio of dietary *Microcystis* was high (50 and 100%), but diverged at lower ratios of dietary *Microcystis* (10 and 25%) (Fig. 3). Two patterns emerged at these lower *Microcystis* treatments. First, on average, *P. forbesi* lived longer than *E. affinis* when *Microcystis* was in the diet. Second, the variation in *Microcystis* strains caused a significant difference in *P. forbesi* survival time compared to *E. affinis* (Fig. 3).

The IA control diets (50, 75, 90 and 100%) all had TL-50 >15 days. Starved copepods had a mean survival time of 3.99 ± 0.04 (*E. affinis*) and 4.23 ± 0.6 (*P. forbesi*) days.

Verification of Microcystis strains, dissolved MC and ingestion

Cross-contamination between the MC+ and MC− strains of *Microcystis* was not observed in the
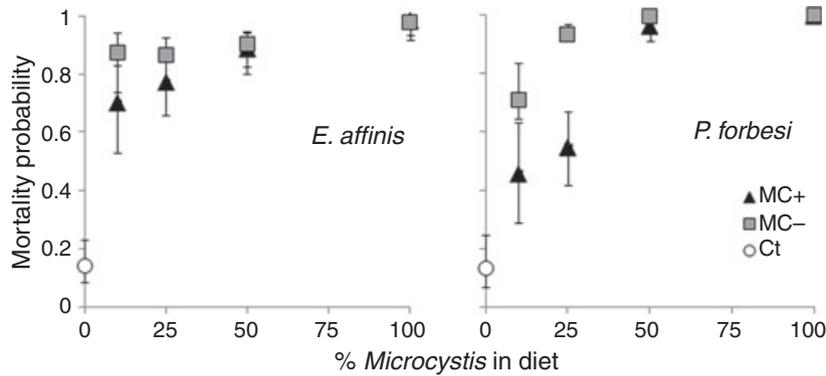
Fig. 2 Probability of copepod mortality in relation to the proportion of *Microcystis* (MC+ or MC−) in the diet. Values are based on survival estimates of the parametric model (test of deviance), for the final day of feeding experiment (day 11). For comparison, C_T refers to mortality on the total amount of control diet with no *Microcystis* (100% instant algae). Bars indicate 95% CI.

Table 1 Summary results for the parametric survival fit (test of deviance) and differences in the effect of (a) species and diet (between all diets, controls and *Microcystis*) on copepod survival probability; results show the factorial interaction of species (*Pseudodiaptomus forbesi* and *Eurytemora affinis*) with each diet treatment and (b) the effect of species, MC presence and % *Microcystis* on copepod survival probability (excludes control diets); results show the factorial interaction of species (*P. forbesi* and *E. affinis*) with MC presence (MC+ or MC− strain) and % of *Microcystis* in the diet (10, 25, 50 and 100%)

Source	No. of parameters	d.f.	χ^2	Prob > χ^2
(a)				
Species	1	1	0.928	0.335
Diet	12	12	730.193	<0.001
Species × diet	12	12	42.383	<0.001
(b)				
Species	1	1	0.833	0.361
MC	1	1	22.372	<0.001
% *Microcystis*	3	3	141.288	<0.001
Species × MC	1	1	8.917	0.003
Species × % *Microcystis*	3	3	30.938	<0.001
MC × % *Microcystis*	3	3	13.236	0.004

MC, microcystin.

treatment diets during the trials as verified by periodic measurements of MC-LR concentration with an ELISA. Furthermore, PCR confirmed the presence of *Microcystis*-specific 16S rDNA genes in both *Microcystis* strain but, as expected, the MC synthetase genes *mcy*B and *mcy*D were detected only in the MC+ strain. Mean cell-bound MC-LR concentration in the 100% MC+ treatment was 1.62 µg L^{-1} (±0.18, $n = 7$) and 348 µg L^{-1} (±49, $n = 8$) in the MC+ *Microcystis* culture. This corresponds to an estimated 4.87 µg mgC^{-1} (±0.98, $n = 7$) of MC-LR per *Microcystis* biomass. The mean cell density in the 100% *Microcystis* treatment was 93 000 cells mL^{-1} (±1458, $n = 14$). Dissolved MC-LR was not detected in the treatment beakers

during the experiment. Copepods ingested *Microcystis* in all treatments as observed qualitatively from the fluorescence of phycocyanin.

Discussion

Our results provide strong evidence that the mortality of both *P. forbesi* and *E. affinis* was because of dietary *Microcystis*. As predicted, an increase in the ratio of *Microcystis* in the diet reduced copepod survival within days, and even the smallest addition to the diet (10%) significantly reduced copepod survival. High survival on the control diet suggests that mortality was driven by toxicity from *Microcystis* and that background mortality was low. Similar toxic effects of dietary *Microcystis* on zooplankton have often been observed (DeMott *et al.*, 1991; Kirk & Gilbert, 1992; Wilson *et al.*, 2006). What is unique to this study is the contrast between the two copepod species in their response to the dietary ratio and strains of *Microcystis* and, more surprisingly, the greater tolerance of *P. forbesi* for the MC+ strain relative to the MC− strain.

The hypothesis that MC+ cells would cause higher mortality was not supported, as the MC− strain caused similar (*E. affinis*) or higher (*P. forbesi*) mortality. *Microcystis* produces several non-MC metabolites that are toxic when ingested and probably resulted in the harmful effects found here. Examples include lipopolysaccharides, which reduce the efficiency of detoxification, microviridin, which reduces protease activity, and various other unidentified toxins (Kurmayer & Juttner, 1999; Rohrlack *et al.*, 2004; Wiegand & Pflugmacher, 2005). As shown with other zooplankton, our results highlight the potential toxicity of non-MC metabolites to copepods (Wilson *et al.*, 2006).
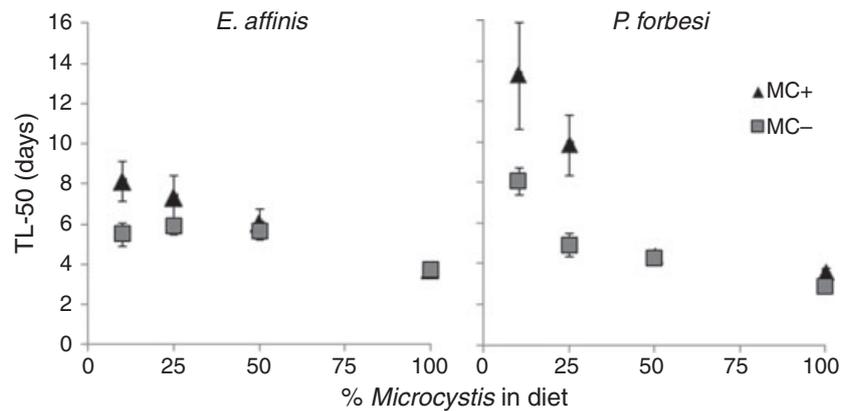
Fig. 3 Mean time to mortality (TL-50) of each treatment and copepod species as a function of the ratio of *Microcystis* (MC+ or MC−) in diet (±SE).

Declining mortality of *P. forbesi* following sustained exposure to the MC+ *Microcystis* suggests that prolonged exposure may prompt an adaptation to this strain, either via physiological tolerance or a change in feeding behaviour. Species-specific tolerance has ecological significance, since it may explain how some zooplankton can dominate during blooms of toxic algae (Kirk & Gilbert, 1992; Work & Havens, 2003; Karjalainen *et al.*, 2007).

Previous exposure to *Microcystis* over evolutionary timescales may improve physiological tolerance (i.e. detoxification) in zooplankton (DeMott *et al.*, 1991), which may be naturally higher for some copepod species (Kumar, 2003). While increased tolerance of *Microcystis* in daphniids can also be triggered by short-term exposure, this ability varies among species and the relationship between evolutionary exposure (genotypic effects) and improved detoxification remains unknown (Gustafsson & Hansson, 2004; Sarnelle & Wilson, 2005). However, detoxification is a process that follows ingestion and probably not the mechanism by which *P. forbesi* was better able to tolerate MC+ *Microcystis*. For MC detoxification to occur, *P. forbesi* in the MC+ treatments would first have to ingest the *Microcystis* cells, thereby ingesting both MC and the non-MC metabolites. In such a case, we would expect to see higher survival for the copepods in the MC− treatments (relative to MC+), since MC detoxification causes added stress to zooplankton (Pflugmacher *et al.*, 1998).

Nevertheless, higher mortality in the MC− treatments confirms that the ingestion of non-MC metabolites is indeed lethal, excluding physiological tolerance of MC as the main cause for coexistence with MC+ *Microcystis*. A more likely mechanism is selective feeding, which is a common adaptation in copepods exposed to toxic cyanobacteria for extended periods (DeMott & Moxter, 1991; Kleppel, 1993; Koski, Engstrom & Viitasalo, 1999). Incidental mortality later in the experiment may be explained by accidental ingestion, as copepods can passively ingest *Microcystis* cells despite selective feeding (Panosso *et al.*, 2003). High mortality at the onset of exposure to the MC+ diet suggests an acclimation phase when *P. forbesi* optimises MC avoidance and subsequently improves its ability to survive in the presence of *Microcystis* by feeding selectively on the more palatable food particles.

Assuming that *P. forbesi* can reject harmful particles such as MC+ cells, why does it not also reject the fatal MC− cells with comparable success? Zooplankton have developed different means of detecting and avoiding cyanobacterial toxins, which are considered as evolved defences against zooplankton grazing (DeMott & Moxter, 1991; DeMott *et al.*, 1991). Although MCs have been shown to deter feeding in daphniids, some copepods rely on more general and unidentified lipophylic toxins to detect and avoid ingesting harmful cyanobacteria (Kurmayer & Juttner, 1999; Rohrlack *et al.*, 1999). Comparing different calanoid copepods exposed to the cyanobacteria *Nodularia*, the hepatotoxin nodularin was the grazing deterrent for *E. affinis*, which selectively fed on nodularin-free cyanobacteria (Engstrom *et al.*, 2000). In contrast, *Acartia* grazing was deterred by a more general cyanobacterial cue other than nodularin, as it selected against *Nodularia* regardless of its nodularin content. This is consistent with our results and shows that deterrence of copepod grazing on cyanobacteria is commonly mediated by species-specific chemosensory

cues. There is no real evidence in the current study showing that differences in the survival of *E. affinis* and *P. forbesi* exposed to each *Microcystis* strain is caused by differences in feeding selectivity. Nevertheless, based on the role of species-specific cues for copepod feeding selectivity, we speculate that MC may be the dominant feeding deterrent for *P. forbesi*, resulting in an ability only to avoid MC+ cells.

Higher survival of *P. forbesi* in the MC+ treatments is surprising and contrary to the presumption of most zooplankton–*Microcystis* interactions. Typically, the MC content of food either reduces or has no significant effect on survival (Rohrlack *et al.*, 1999; Lurling, 2003; Wilson *et al.*, 2006). Following this general prediction, and in contrast to *P. forbesi*, MC content of *Microcystis* had no significant effect on *E. affinis*, as both MC+ and MC− strains caused similar mortality. Despite such species-specific differences, our results indicate that the non-MC compounds in *Microcystis* cells can be the main factor causing negative effects on some copepods.

Eurytemora affinis and *P. forbesi* also responded differently to the proportion of *Microcystis* in their food, regardless of strain. In general, *P. forbesi* did better for a given *Microcystis* diet, and its survival improved with an increased proportion of good food. Calanoid copepods can show reduced ingestion for toxic cyanobacteria when alternative food is available, which probably explains the negative relationship between the ratio of dietary *Microcystis* and *P. forbesi* survival (DeMott & Moxter, 1991; Burns & Hegarty, 1994; Koski *et al.*, 1999). In contrast, *E. affinis* survival varied much less with the ratio of dietary *Microcystis*. This may indicate inefficient feeding selection by this species, which was also shown in the Baltic Sea (Engstrom *et al.*, 2000). *Pseudodiaptomus forbesi* may be better adapted to survive in the presence of *Microcystis* via feeding selection, and further investigation would help verify the mechanism.

In addition to toxicity, *Microcystis* is also nutritionally inadequate for zooplankton (DeMott *et al.*, 1991; Christoffersen, 1996). Relatively high survival in the controls indicates that sufficient nutrition was provided by the IA part of the *Microcystis* – IA mixed diet. Hence, even if *Microcystis* nutritional deficiency was a significant issue, IA should have sustained survival at least as well as the controls. Since survival with *Microcystis* is significantly lower than the controls without *Microcystis*, the toxic effects of *Microcystis*

dominate any potential consequence of nutritional inadequacy. Toxic cyanobacteria can also harm zooplankton by inducing feeding inhibition (DeMott *et al.*, 1991; Rohrlack *et al.*, 1999; Lurling, 2003; Wiegand & Pflugmacher, 2005). However, this is unlikely for calanoid copepods that avoid lower quality food particles without reducing their ingestion rate on high-quality food (DeMott & Moxter, 1991; Kleppel, 1993; Koski *et al.*, 1999; Tackx *et al.*, 2003). Surviving copepods in *Microcystis* treatments were actively swimming, had full guts and continued to produce faecal pellets, indicating that copepods continued ingestion during the experiment. Both strains of *Microcystis* and the IA controls were uni- and bi-cellular, ruling out any potential effects from colonial morphology, which can also hinder ingestion (Fulton & Paerl, 1987; Ghadouani, Pinel-Alloul & Prepas, 2003).

Empirical evidence from the field for the toxic effects of cyanobacteria on zooplankton is less common and typically muted in comparison to laboratory interactions, where variables can be strictly controlled (Ferrao-Filho & Azevedo, 2003; Work & Havens, 2003). Ecosystem impacts of cyanobacteria at larger scales are more subtle because of stochastic variability, species-specific adaptations, colonial morphology, multiple trophic links and unpredictable food web dynamics (Kozlowski-Suzuki *et al.*, 2003; Rohrlack *et al.*, 2004; Wilson *et al.*, 2006; Beninca *et al.*, 2008). However, the impacts of cyanobacteria at the ecosystem scale have been demonstrated, and include the disruption of the benthic food web via bioaccumulation of toxins, shifts in zooplankton and phytoplankton species composition, and possible indirect effects shifting the pelagic community towards the microbial loop (Fulton & Paerl, 1987; Christoffersen, 1996; Karjalainen *et al.*, 2007).

We found strong evidence that dietary *Microcystis* reduces copepod survival even at low dietary ratios, suggesting possible direct impacts on copepods in the San Francisco Estuary where *Microcystis* seasonally dominates phytoplankton (Lehman *et al.*, 2005). During blooms in the estuary, *Microcystis* dominates the phytoplankton community and its density fluctuates, between about 0×10^3 and 2×10^3 cell mL^{-1} in the euphotic zone, depending on the location and time, though densities up to 2.2×10^6 mL^{-1} have been recorded (Lehman *et al.*, 2008). The average range monitored in the estuary corresponds with the

treatment diets in this study, at least in terms of cell density, as the highest concentration of *Microcystis* fed to copepods was 9.3×10^3 mL^{-1}. During blooms, persistent concentrations of *Microcystis* between 10^3 and 10^6 cell mL^{-1} are often observed in eutrophic waters globally, making the results of this study relevant to the zooplankton community of the San Francisco Estuary as well as other regions (Chorus & Bartram, 1999; Rinta-Kanto *et al.*, 2005; Costa *et al.*, 2006).

Nevertheless, as mentioned previously, *Microcystis* impacts measured in the laboratory may not always represent natural processes in the field. Depending on the intensity of the bloom and the availability of alternative food, including microbial sources such as ciliates, selectively feeding copepods may or may not persist in the presence of *Microcystis* (DeMott & Moxter, 1991; Work & Havens, 2003). Zooplankton in the San Francisco Estuary can be limited by low phytoplankton abundances (Jassby, Cloern & Cole, 2002; Muller-Solger *et al.*, 2002), and such food limitation can intensify the negative impacts of cyanobacteria on copepods by reducing the effectiveness of selective feeding (DeMott & Moxter, 1991; Engstrom *et al.*, 2000). Conversely, natural blooms of *Microcystis*, including in the estuary, are typically dominated by colonial forms (Lehman *et al.*, 2005; Wilson *et al.*, 2006), and copepods are probably more effective at selective feeding in the presence of colonies, when the particle size is larger (Tackx *et al.*, 2003). However, copepods in the San Francisco Estuary accumulate MC during *Microcystis* blooms, suggesting that ingestion in the field occurs, despite the dominance of colonies (Lehman *et al.*, 2008). For these reasons, although we found negative impacts of *Microcystis* on copepods, prediction of the ecological impacts must also be informed by coupled laboratory and *in-situ* field observations of ingestion and food limitation and evaluation of the ability of copepod species to detect and avoid harmful cells.

Acknowledgments

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