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Effect of the toxin (microcystin) content of *Microcystis* on copepod grazing

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ABSTRACT

Although phytoplankton chemical defense may regulate plankton dynamics, demonstrating an ecologically relevant anti-grazer cue is challenging. Presented here is a novel approach to evaluate the quantitative effect of microcystin (MC), the most studied group of cyanobacterial metabolites, on grazing by the common copepod *Eudiaptomus gracilis*. A temperature-induced gradient in the intracellular MC concentration of three different *Microcystis* strains enabled the comparison of grazing pressure on cells of the same cyanobacterial strain producing different amounts of MC, in a diet with alternative food (*Chlamydomonas*). In all treatments, grazing pressure on *Microcystis* was inversely related to its MC-LR content, while selection for alternative prey was positively related to the MC-LR content of *Microcystis*. Moreover, grazing on *Chlamydomonas* also declined with increasing *Microcystis* MC-LR content, suggesting toxicity related inhibition of *E. gracilis*. The negative relation between cellular MC-LR concentration and feeding responses supported the anti-grazer hypothesis. Not all MC variants responded to temperature, and some were therefore not associated to grazing responses. Using an induced gradient in the concentration of a suspected phytoplankton defense metabolite to evaluate its quantitative relationship with grazing pressure offers an improved inference on the ecological roles of toxins. Results suggest that either MC-LR or a correlating trait may be inversely linked to the grazer pressure on *Microcystis*.

1. Introduction

In freshwater ecosystems cyanobacteria are the main phytoplankton group producing toxins, which degrade water quality and are harmful to a wide range of organisms including mammals (Miller et al., 2010). Yet, why they produce such toxic compounds is poorly understood (Rantala et al., 2004). Microcystins (MCs) are the most studied group of cyanobacterial toxins, and are synthesized by various cyanobacteria, such as *Microcystis*, *Anabaena* and *Planktothrix* (Funari and Testai, 2008). Although several intra- and extracellular roles have been suggested, these remain as putative mechanisms and the ecologic and evolutionary roles of MCs merit further attention (Kaebernick and Neilan, 2000; Welker and Döhren, 2006; Paerl and Otten, 2013).

Chemically mediated anti-grazer defense has been one of the earliest proposed roles for MCs (Kirk and Gilbert, 1992). MCs inhibit eukaryotic phosphatase enzyme activity (MacKintosh et al., 1990), but not cyanobacterial protein phosphatases (Shi et al., 1999). Zooplankton generally avoid MC producing cyanobacterial cells or experience acute toxicity upon ingestion (DeMott et al., 1991; Lürling, 2003). More substantial evidence concerning the anti-grazer function of cyanobacterial metabolites, however, is lacking.

Current evidence comes from experiments that compare zooplankton responses to MC producing (MC+) and lacking (MC−) cells of a cyanobacterial species, using either naturally occurring strains or mutant clones (Wilson et al., 2006). In this design, anti-grazer effects are considered likely if the MC− cells are ingested more than the MC+ cells. For example, copepods selectively avoided strains of cyanobacteria producing cyanotoxins while grazing more on non-producing strains of the same species (DeMott and Moxter, 1991; Ger et al., 2011). Likewise, *Daphnia magna* grazing was inhibited more by a MC+ *Microcystis* than its

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mutant MC− clone (Lürling, 2003). In contrast, *Daphnia galeata* grazed equally on the MC+ *Microcystis* and its MC− clone (Rohrlack et al., 1999). Clearly, MC is not a grazer deterrent for all zooplankton species.

Ecologically, anti-grazer cues are most relevant for zooplankton that can detect and avoid cellular cues before ingestion (Wolfe et al., 1997; Teegarden et al., 2001), which highlights the importance of looking at the interactions of selective grazers, especially those that dominate during toxic blooms (Pohnert et al., 2007). Yet, most of the information available for the role of MC is from studies with generalist grazers (*Daphnia*) that rarely co-exist with cyanobacterial blooms (Wilson et al., 2006; Ger et al., 2014). In contrast, copepods often co-exist with cyanobacteria by ingesting alternative food while avoiding the ingestion of a toxic dose (Bouvy et al., 2001; Koski et al., 2002; Ger and Panosso, 2014). In one of the few studies with copepods, *Eudiaptomus gracilis* used an unidentified lipophilic metabolite for avoiding the cyanobacteria *Planktothrix* (Kurmayer and Juttner, 1999). A more recent study with the same copepod species, however, indicated that MC might be a cue for avoiding *Microcystis* (Ger et al., 2011).

Given that a suspect anti-grazer cue must be detected before ingestion (Pohnert et al., 2007), the diffusion of surface bound chemicals accumulating on the cell surface may be a critical process (Wolfe et al., 1997). While the bulk of MC remains within cyanobacteria cells, the MC synthetase gene cluster includes an ABC transporter (*mcy*H) that encodes the export of MC, suggesting that biosynthesis and export is linked (Pearson et al., 2004). Yet, radioactively labeled MC provided no support for the active export of this metabolite (Rohrlack and Hyenstrand, 2007). Still, minor quantities of immunogold labeled MC were detected on the cell wall of *Microcystis* (Shi et al., 1995), and thus, it seems likely that grazers can detect surface bound cyanobacterial metabolites before ingestion.

Demonstrating chemical anti-grazer defense is limited by the ability to ensure that algal cells differ only in the production of a single metabolite (Pohnert et al., 2007). Indeed, MC is one of the many classes of metabolites produced by cyanobacteria (Wiegand and Pflugmacher, 2005), some of which, such as protease inhibitors like microviridin, can be toxic if ingested by zooplankton (Rohrlack et al., 2004; Schwarzenberger et al., 2012). Such metabolites, as well as lipophilic compounds produced by all cyanobacteria may also act as grazing deterrents (Kurmayer and Juttner, 1999). Moreover, different strains of the same species, and even cells of genetically modified cyanobacteria clones may involve unaccounted compensatory dynamics in the production of these metabolites (Repka et al., 2004). These strain or clone-specific dynamics of cyanobacterial metabolites complicate efforts to isolate the role of MC in bioassays with cells producing or not producing MC (Welker and Döhren, 2006; Ger et al., 2010b).

In the current study, a novel approach is proposed to evaluate the quantitative effect of MC on grazer deterrence without having to use different cyanobacterial strains. The traditional method comparing grazing pressure on cells of different cyanobacterial strains or clones has evaluated the qualitative effects of MC based on its presence. Instead, it is also possible to compare grazing pressure on cells of the same cyanobacterial strain producing different amounts of MC. If a toxin such as MC is indeed a grazing deterrent, a higher cellular concentration should result in stronger avoidance for a given strain of cyanobacteria, assuming that MC concentration on the cell surface layer is proportional to intracellular concentration.

Temperature is known to have a significant effect on the production and cellular concentration of MC. Initial reports of temperature effects on cyanotoxin production showed an inverse relationship between toxicity and temperature in *Microcystis aeruginosa* (Westhuizen and Eloff, 1985). Later studies with *Microcystis* and also *Planktothrix agardhii* indicated lower concentration of MC for cultures grown at elevated temperatures (Sivonen, 1990; Tonk et al., 2008). Thus, culture temperature may be used to control the cellular concentration of MC of a given *Microcystis* strain, which can then be used in subsequent grazing experiments.

A temperature induced gradient in the MC concentration of three different *Microcystis* strains was used to test the hypothesis that increased cellular concentration of MCs would result in stronger avoidance by the selective copepod *Eudiaptomus gracilis*. This is a common copepod species that coexists with MC producing cyanobacteria in nature (Kurmayer and Juttner, 1999). Using mixed diets including a nutritious food matched with the toxic food is critical for understanding mechanisms affecting selective grazers (Colin and Dam, 2002; Pohnert et al., 2007). Accordingly, single and mixed food treatments containing a readily ingested green alga (*Chlamydomonas*) and *Microcystis* were used in an effective setup for studying copepod grazing behavior (Ger et al., 2011). It was predicted that copepod ingestion of *Microcystis* would be inversely related to the cellular MC concentration and copepod selective avoidance of *Microcystis* would be positively related to its MC content.

2. Methods

2.1. Organisms cultured

Three different strains of *Microcystis aeruginosa* (CYA 140, PCC 7941, SAG 1785 – hereafter CYA, PCC, and SAG, respectively) were maintained at 17, 25, or 32 °C each in separate temperature controlled incubators (Sanyo Gallenkamp Orbital Incubator, Loughborough, United Kingdom). All cultures were grown in WC medium, exposed to a 14:10 h (light:dark) cycle of 50 μmol photons m^{-2} s^{-1} light intensity, maintained as semi-continuous cultures in 500 mL glass flasks (culture volume 250 mL), and kept in the exponential growth phase by routine dilution to maintain cell density between 2×10^5–2×10^7 cell mL^{-1}. The maximum cell density reached under the conditions of this experiment was 4×10^7 cell mL^{-1}. Mean cell size of *Microcystis* varied from 4 to 5 μm, occurring as an equal mix of single and double spherical cells, and was similar for all strains and temperatures. Only cultures in the exponential growth phase were used for the grazing experiments.

Cultures of *Cryptomonas pyrenoidifera* (NIVA 2/81) and *Chlamydomonas reinhartii* (NIVA-CHL 13) were maintained in WC media at 25 °C, with a light intensity of 50 μmol photons m^{-2} s^{-1} in a photoperiod of 12:12 h (light:dark) in semi-continuous cultures, which were kept at exponential growth by routine dilution. *Cryptomonas* (ellipsoid cells ~ 6 μm × 12 μm) was used as the food source for copepod cultures, while *Chlamydomonas* (spherical cells ~ 9 μm) was used in the grazing experiments to minimize the size difference with *Microcystis* and for better distinction of pigments (see below). Phytoplankton cell density was measured by microscopic cell counts calibrated against light absorbance at 800 nm. Phytoplankton specific chlorophyll-a concentration was determined using the PHYTO-PAM fluorometer (Heinz Walz GmbH Effeltrich, Germany), which was calibrated for all food types including *Microcystis* grown at different temperatures in experimental media. Phytoplankton carbon biomass was estimated using previously determined relationships between cell volume and carbon content (Rocha and Duncan, 1985).

Cultures of the calanoid copepod *Eudiaptomus gracilis* originated from wild caught gravid females in Lake Rauwbraken (The Netherlands). This lake has been free of cyanobacterial blooms since 2008, and no blooms were observed during sampling. Copepods were collected with a sub-surface tow using a 55 μm