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Short communication

Histological effects and localization of dissolved microcystins LR and LW in the mayfly *Ecdyonurus angelieri* Thomas (Insecta, Ephemeroptera)

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ABSTRACT

The ability of microcystins (MCs), the main group of cyanotoxins, to affect the physiological processes and tissues of insects has received little attention. Fresh water dissolved MCs represent one of the main sources of cyanotoxins. In the experiment described herein, captured wild mayfly *Ecdyonurus angelieri* Thomas, 1968 larvae were exposed to 5 ppb of two distinct microcystins, MC-LR and MC-LW, in separate assays. Evidence of induced mortality, MCs bioaccumulation and severe histological damage affecting fat body and alterations in the tracheal system were evident. Our results reveal the acute sensitivity of the mayfly *E. angelieri* to MCS, which may serve as early indicators or cyanotoxins production and the quality of freshwater streams.

The direct uptake of dissolved cyanotoxins (CTs) from the environment has been widely reported from plants to animals, but not only in aquatic ecosystems since they also affect terrestrial organisms as well as humans (Carmichael, 1994; Huynh-Delerme et al., 2005; McElhiney and Lawton, 2005; Ferrão-Filho and Kozlowsky-Suzuki, 2011). Microcystins (MCs) are the best known group of CTs for the physiological alterations they cause in living organisms (Amorim and Vasconcelos, 1999; Magalhães et al., 2003; Ferrão-Filho and Kozlowsky-Suzuki, 2011), where they exert their main described effects through the inhibition of protein phosphatase 1 and 2A activities, which are widely involved in essential cell and tissue maintenance processes (Pinho et al., 2003; Vela et al., 2007). The relationship between exposure to MCs, as well as other CTs, and human health has been clearly established, being linked it to tissue alterations, the impairment of hormonal levels or even cancer onset (Pinho et al., 2003; Chen et al., 2005; Vela et al., 2007; Wang et al., 2012; Xu et al., 2012; Zhang et al., 2012), and leading to different strategies towards their environmental control and management.

Despite the fact that aquatic insects are the most important organisms in freshwater ecosystems in terms of species richness and abundance, present knowledge on MCs mainly concerns their distribution in food webs (Kotak et al., 1996; Tsuji et al., 2001) and their bioaccumulation and effects from zooplankton to vertebrates (Ferrão-Filho and Kozlowsky-Suzuki, 2011); but about insects there is only data from terrestrial species (Delaney and Wilkins, 1995; Hiripi et al., 1998). Several studies have shown the ability of administered MCs to promote histological disturbances in fish and mammals, but very little is known about their effect on invertebrates (Tencalla and Dietrich, 1997; Guzman and Solter, 2002; Pinho et al., 2003).

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Moreover, as Ferrão-Filho and Kozlowsky-Suzuki (2011) mention, no studies to date have addressed the bioaccumulation of CTs in insects.

Severe poisoning caused by MCs, observed from water invertebrates to terrestrial vertebrates, may result from (i) water contamination episodes involving cyanobacterial bloom, (ii) algal stress responses associated with hydric disturbances, (iii) the long-term bioaccumulation derived from filter-feeding in slightly contaminated water sources, or (iv) by feeding from life forms which previously accumulated them (Tencalla and Dietrich, 1997; Magalhães et al., 2003; Karjalainen et al., 2005; McElhiney and Lawton, 2005; Ferrão-Filho and Kozlowsky-Suzuki, 2011; Quiblier et al., 2013). Both processes occur simultaneously in the wild, greatly affecting the evolution and composition of ecological communities in lakes and streams (Aboal et al., 2000, 2002; Whyte et al., 2005). In this report, we exposed larvae of the mayfly *Ecdyonurus angelieri* Thomas, 1968 to two of the principal MCs (MC-LR and MC-LW) dissolved in water, to ascertain whether these toxins and their potential bioconcentration might trigger histological damage in this organism as described for other species.

Larvae of *E. angelieri* collected from Tordera river (Barcelona, Spain), a silicic stream with an observable presence of benthic cyanobacteria and undetected MCs dissolved in water, were acclimated in aerated aquaria containing natural stream substrate, artificial mesh dishes and sterilized water at a rate of 25 individuals per experiment for two days. Commercial MC-LR and MC-LW (ALEXIS Biochemicals) were diluted in sterilized water at 5 ppb, based on the highest MCs levels registered by our group in water samples collected from streams where *E. angelieri* occurs naturally. The selected concentration corresponds MC-LR levels found in samples from the Muga river basin collected during summer and fall. Groups of 3 specimens were exposed separately to each MC for 3 h in Petri dishes with artificial substrate. Each assay involved 3 replicates and 3 controls. Upon exposure to both toxins, individuals initiated evasion behavior characterized by random movements across the flask. Such behavior was not observed in individuals under control conditions (no toxin). Eventually, in the case of the 5 ppb concentration, the exposed specimens died or ceased to move, with a greater mortality rate being observed for MC-LR (40% survival) than for MC-LW (60% survival) exposure, as shown in Fig. 1. Vertebrates and filter-feeding and grazer invertebrates usually show great resistance to CTs, acting as good bioaccumulators (Amorim and Vasconcelos, 1999; Magalhães et al., 2003), while non-filter-feeding invertebrates show greater sensitivity to dissolved MCs (Aboal et al., 2000; Whyte et al., 2005; Wiegand and Pflugmacher, 2005). In our case, it is clear that *E. angelieri* larvae showed acute susceptibility to these toxins, potentially making them a good candidate for the early detecting of MCs from benthic cyanobacteria mats as well as dissolved MCs transported from upstream reservoirs.

At the end of the treatments, three exposed and one control specimen from each treatment were fixed over night either in Bouin's solution or in methanol, for morphological and immunocytochemical analysis, respectively, dehydrated, embedded in paraffin and cut into 5 μm

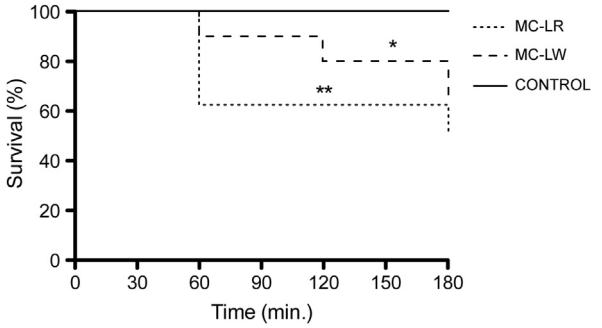

Fig. 1. Kaplan–Meier survival analysis for control, and MC-LR and MC-LW treatments. Acclimated *E. angelieri* larvae were exposed for 180 min to 5 ppb of MC-LR or MC-LW dissolved in water. Complete movement arrest was recorded as death. Asterisks denote different level of statistical significance.

sections along the sagittal plane for histological study. Microscopy examination of sections obtained from Bouin-fixed mayfly specimens and stained using hematoxylin-eosin allowed any morphological changes to be studied. As described below, we only found histological disturbances in the fat body (FB) and the tracheal system epithelium (TSE), especially in those specimens exposed to MC-LR.

The FB is a dispersed organ that partially fills the haemocelic cavity and surrounds other organs in close contact with the haemolymph, facilitating the exchange of macromolecules. The functions of this organ are not only related with the storage of proteins, lipids and carbohydrates, but also with the supply of specific molecules that control certain physiological processes (e.g. molting and egg maturation); it is also involved in detoxification and immunological functions (Oliveira and Cruz-Landim, 2003; Arrese and Soulages, 2010; Roma et al., 2010), so that the role of the FB resembles that of the liver in vertebrates. Although we could not confirm the involvement of phosphatase activity in these processes, such a role is very likely, as has been described for other arthropods (Ferrão-Filho and Kozlowsky-Suzuki, 2011). FB cells, mainly trophocytes (Oliveira and Cruz-Landim, 2003; Roma et al., 2010), are large rounded cells containing a central nucleus and different types of storage structures, such as lipid droplets (Fig. 2A). The TSE is a flattened monolayer epithelium derived from the tegument, which synthetizes the cuticle towards the lumen of tracheal tubes (Fig. 2B). The exposure of mayflies to MC-LR produced cell lysis and the necrosis of large areas of the FB (Fig. 2C), and also generated a large degree of disintegration of the TSE that made it impossible to identify tracheoles (Fig. 2D), while large tracheal tubes could only be detected from the remains of the tracheal cuticle. On the other hand, no relevant alterations in FB morphology could be observed in specimens exposed to MC-LW (Fig. 2E), while the TSE showed slight depigmentation (Fig. 2F). Of particular note was the strong effect that MC-LR had on the TSE, which could be explained by the high degree of interdependence between tracheoles and the FB in terms of tissue maintenance and structural support (Oliveira and Cruz-Landim, 2003).