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Pentachlorophenol toxicity to a mixture of *Microcystis aeruginosa* and *Chlorella vulgaris* cultures

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ABSTRACT

Pentachlorophenol (PCP) is a priority pollutant due to its persistence and high toxicity. For the first time, PCP effects were investigated at laboratory scale on co-cultures of two ubiquitous freshwater phytoplankton species: the cyanobacterium *Microcystis aeruginosa* and the microalgae *Chlorella vulgaris*. The cells were exposed to environmental levels of PCP for 10 days in Fraquil culture medium, at nominal concentrations from 0.1 to 10,000 $\mu g\,L^{-1}$. Growth was assessed by area under growth curve (cell count vs. time). The phytoplankton community structure can be changed as a consequence of a PCP contamination. Low $\mu g\,L^{-1}$ levels of PCP are advantageous to *M. aeruginosa*. This is the first report of the promoting effect of PCP on the growth of aquatic cyanobacteria, using mixtures with microalgae. As a result of the direct toxic effects of high PCP concentrations on *M. aeruginosa*, *C. vulgaris* cell count increased given that in biological controls *M. aeruginosa* inhibited the *C. vulgaris* growth. At 16.7 $mg\,L^{-1}$, PCP already had direct toxic effects also on the microalga. The pH of culture medium tended to decrease with increasing PCP concentrations, which was mostly related to the growth inhibition of cyanobacterium caused by PCP. The PCP concentration was stable in the co-cultures, which differed from what has been observed in monocultures of the same two species. Short-term laboratory assays with two phytoplankton species gives important information on the species interactions, namely possible direct and indirect effects of a toxicant, and must be considered in ecotoxicity studies regarding environmental extrapolations.

1. Introduction

Pentachlorophenol (PCP), a broad-spectrum pesticide, is considered a priority pollutant since it has a very long half-life in the environment and is harmful at very low concentrations (Tao et al., 2012; Xing et al., 2012). It can be an endocrine disruptor and inflict high toxicity to all kinds of organisms (Hong et al., 2010). The deprotonated form of PCP dominates at high pH (pKa 4.7) (Muir and Eduljee, 1999) and exhibits lower toxicity (Xing et al., 2012).

In order to predict potential adverse effects of PCP on an aquatic ecosystem, the knowledge of the level and the mechanisms of toxicity to aquatic organisms is important (Hong et al., 2010). The European Union legislation has set 1 $\mu g\,L^{-1}$ of PCP as the maximum admissible concentration (MAC) in inland and other surface waters (De Morais et al., 2012). The PCP concentrations in environmental freshwaters are usually very low, in the $ng\,L^{-1}$ level, but highly variable, from $ng\,L^{-1}$ to low $mg\,L^{-1}$ level (De Morais et al., 2012). Therefore, the freshwater phytoplankton might be exposed to different PCP concentrations, possibly leading to toxic responses.

In a previous work, the fate and effects of PCP at environmental levels on unialgal cultures of the toxic and bloom-forming cyanobacterium *Microcystis aeruginosa* and the green microalga *Chlorella vulgaris* were studied (De Morais et al., 2014). The results demonstrated different PCP toxicity profiles and removal abilities of the studied species. It is necessary to confirm if the behaviour of *M. aeruginosa* and *C. vulgaris* when exposed to PCP in mixed cultures (i.e., co-cultures) is similar to that of the single species tested in monoculture experiments.

Laboratory monoculture bioassays with phytoplankton species (De Morais et al., 2014; Gokcen, 1998; Hadjoudja et al., 2009; Leboulanger et al., 2001; Ma et al., 2006; Mostafa and Helling, 2002; Olivier et al., 2003; Tao et al., 2010; Tikoo et al., 1997, 1996) are useful to facilitate the achievement of results under well-controlled

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conditions, with high sensitivity and reproducibility. However, they lack environmental realism because microalgae species do not occur in isolation but as part of complex communities. Consequently, the plausible interspecific biological interactions (e.g., competition and mortality) are not considered in single species toxicity tests (Franklin et al., 2004). Therefore, not surprisingly, ecotoxicity studies using co-cultures of phytoplankton species are becoming common (Burrell et al., 1985; Chang et al., 2012; El-Bestawy et al., 2007; El-Dib et al., 2000; Erickson and Hawkins, 1980; Franklin et al., 2004; Jin et al., 2009; Lürling and Roessink, 2006; Willis et al., 2004).

Before investigating the effects of PCP in co-culture bioassays, however, it is necessary to study the somehow unpredictable interspecific interactions in the biological controls. For example, when *M. aeruginosa* and microalgae (other than *C. vulgaris*) were mixed, different responses have been observed, with the cyanobacterium being inhibited (Lürling and Roessink, 2006), stimulated (Franklin et al., 2004) or having even more complex responses (Chang et al., 2012). In a study with non-spiked co-cultures containing *C. vulgaris* and other phytoplankton species, *C. vulgaris* inhibited the microalga *Ankistrodesmus braunii* (Burrell et al., 1985). Concerning the selected species for the current study, in a recent work the microalga *C. vulgaris* was exposed to cyanobacterial cell extracts (from *M. aeruginosa* and also *Aphanizomenon ovalisporum*), leading to variable growth responses by the microalga depending on the cell extract used and the cyanotoxins present (Campos et al., 2013). Nevertheless, a true mixture of cultures of the two selected species has never been studied before.

Generally, PCP inhibits or is rather neutral to mixtures of phytoplankton species (Burrell et al., 1985; El-Dib et al., 2000; Erickson and Hawkins, 1980; Willis et al., 2004), although the response is both concentration- and species-dependent. For example, PCP stimulated the growth of the microalga *Cryptomonas* spp. in a seasonal plankton mesocosms experiment (Willis et al., 2004), so each case must be studied separately. In the abovementioned studies, cyanobacteria were absent (Burrell et al., 1985; Willis et al., 2004) or present as a group of phytoplankton without identification to the species level (El-Dib et al., 2000; Erickson and Hawkins, 1980).

Therefore, the objective of this work was to study the fate and effects of environmental levels of PCP on a mixture of two common phytoplankton species: *M. aeruginosa* and *C. vulgaris*. For the first time, the interactions between the two species and the effects of PCP on their population dynamics within co-cultures were investigated.

2. Materials and methods

2.1. Materials and decontamination

All material was cleaned and sterilized as described before (De Morais et al., 2014). Experiments were carried out in sterile Fraquil culture medium (Morel et al., 1975). Solutions of pentachlorophenol (98%, Aldrich, Steinheim, Germany) for spiking of the culture media, were prepared in dimethylsulfoxide (Riedel-de Haën, Seelze, Germany) and stored in a freezer in the dark. Working standard solutions were obtained daily by dilution of a stock solution, with concentration about 2 mg mL^{-1}, with ethanol (Panreac, Barcelona, Spain). All sample manipulations were carried out aseptically in a Class 100 laminar flow hood, in a clean room with Class 100 filtered air.

2.2. Cultures of M. aeruginosa and C. vulgaris

One unicyanobacterial culture of *Microcystis aeruginosa*, strain LEGE 05195, previously isolated from the Torrão reservoir in the Tâmega River (northern Portugal), and the unialgal strain *Chlorella vulgaris* ACOI 879 (synonym strain LEGE Z-001) were used in the exposure experiments. The selected isolates were kept in Z8 medium (Saker et al., 2005) at LEGE culture collection and, thus, prior to the experiments they were acclimated and cultured in Fraquil medium (Morel et al., 1975). In all the experiments, the cultures were incubated in a controlled-environment cabinet at $22 \pm 1\,^{\circ}C$ with a light intensity of 18 $\mu E\,m^{-2}\,s^{-1}$ (16 h light/8 h darkness) provided by a cool white fluorescent lamp.

2.3. Growth inhibition tests

For the toxicity studies, a mixture of *M. aeruginosa* and *C. vulgaris*, in exponential growth phase, was incubated for 10 days in the presence of PCP at six different nominal concentration levels: 0.1, 1, 10, 100, 1000 and 10,000 $\mu g\,L^{-1}$ with three replicates for each concentration. In the beginning of the experiment, the cell counts (CC) for *M. aeruginosa* (CC_M) and *C. vulgaris* (CC_C) were $59.1 \pm 14.9 \times 10^4$ cells mL^{-1} ($n = 36$) and $23.4 \pm 5.6 \times 10^4$ cells mL^{-1} ($n = 36$), respectively.

To determine the population growth, the cells of *M. aeruginosa* and *C. vulgaris* were counted each 2 days. The CC were determined by transferring 10 μL of sample, after stirring of the culture, to a haemocytometer (Marienfeld, Germany) and observing under an optical microscope (Nikon, Japan) with a magnification of 400×. The optical density at 750 nm (OD_{750}) was measured simultaneously as an additional quality control. A chemical control (Fraquil medium with PCP) was performed in duplicate for each concentration level. Four biological controls (Fraquil medium with both species), two cyanobacteria controls (Fraquil with *M. aeruginosa*) and two microalgae controls (Fraquil with *C. vulgaris*), were incubated simultaneously with the test flasks for each two consecutively higher nominal concentrations of PCP (Figs. 1 and 2). The pH of all cultures was determined in the last day of the incubations.

2.4. PCP measurements

PCP in the filtered cultures (1.2 μm glass fibre filters, Whatman®) was measured in the first and last days of the experiment, using previously developed solid phase microextraction method, combined with gas chromatography with electron capture detection (De Morais et al., 2011, 2012). The measured concentrations are summarized in Table 1. The initial concentrations of PCP during the abiotic experiments were calculated as the average of the duplicates of the chemical controls and were used for further treatment of the data. The recovery of PCP in Fraquil medium was calculated, in duplicate, as $92 \pm 1\%$; therefore, PCP concentrations were not corrected for recovery.

2.5. Data treatment

The area under curve (AUC) was calculated from the curves of the CC vs. incubation time. The effect of a single species on the other, in the absence of PCP, was evaluated using a comparison of AUCs for co-cultures and pure (i.e., mono-) cultures. The effect of PCP was studied by means of AUCs for co-cultures in the absence and in the presence of PCP. In all cases, the comparison of the average AUCs was performed using the Student's *t*-test.

The average growth rates (GR) for all experiments were calculated using the difference between $CC_{(t)}$, for each period of incubation time, *t* (days 6, 8 and 10), for *M. aeruginosa* and *C. vulgaris* and the corresponding counts at the beginning of the experiment ($CC_{(0)}$) divided by *t*: $GR_{(t)} = (CC_{(t)} - CC_{(0)})/t$.