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Combined effect of copper sulfate and water temperature on key freshwater trophic levels – Approaching potential climatic change scenarios

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ABSTRACT

This work relied on the use microcosms to evaluate the individual and the combined effects of different levels of copper sulfate (0.0, 0.013, 0.064 and 0.318 mg Cu L^{-1}) – a fungicide commonly exceeding allowable thresholds in agricultural areas – and a range of water temperature increase scenarios (15, 20 and 25 °C) on freshwater species belonging to different functional groups. Hence, the growth inhibition of primary producers (the microalgae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*), as well as the survival and feeding behavior of a shredder species (the Trichoptera *Schizopelex* sp.) were evaluated. The results revealed that copper was toxic to primary producers growth, as well as shredders growth and survival, being the growth of *L. minor* particularly affected. Higher water temperatures had generally enhanced the growth of primary producers under non-contaminated (microalgae and macrophytes) or low-contaminated (macrophytes) conditions. Despite the tendency for a more pronounced toxicity of copper under increasing water temperatures, a significant interaction between the two factors was only observed for microalgae. Since the test organisms represent relevant functional groups for sustaining freshwater systems functions, the present results may raise some concerns on the impacts caused by possible future climate change scenarios in aquatic habitats chronically exposed to the frequent or intensive use of the fungicide copper sulfate.

1. Introduction

Copper-based compounds are one of the chemicals most used in the European Union, namely in organic and conventional viticulture to control fungal diseases like downy and powdery mildew. In Europe, viticulture is responsible for copper consumptions between 1883 and 6842 t per year (EUROSTAT, 2007). The large amounts and wide variety of copper-based agrochemicals applied is likely to lead to the contamination of the neighboring water resources, hence affecting their quality in terms of chemical and ecological *status*, which represents a particular challenge for the successful implementation of the Water Framework Directive (EC, 2000).

Copper toxicity was already proved in several studies targeting primary producers (Oliveira-Filho et al., 2004; Khellaf and Zerdaoui, 2009; Zhao et al., 2015), cladocerans (Mastin and Rodgers, 2000; Oliveira-Filho et al., 2004), mussel larvae (Clearwater et al., 2014), shredders (Gama et al., 2014; Zubrod et al., 2014) and fish species (Oliveira-Filho et al., 2004; Haverroth et al., 2015). However, such studies usually fail to integrate other relevant stress factors and/or evaluate their combined impacts on biological responses.

For instance, climate change has been discussed as an additional threatening stress to freshwater resources normally impacted by agricultural diffuse pollution. According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2013), the annual temperature of Southern European/Mediterranean area is predicted to increase 1.7 (min – max: 0.7 – 3.1 °C, scenario RCP4.5) and 2.3 °C (0.6 – 4.0 °C, RCP4.5) up to the middle and end of the 21st century, respectively. In summer, the predictions indicate even bigger temperature increases, 2.2 (1.0 – 4.3 °C, RCP4.5) and 2.8 °C (1.2 – 5.5 °C, RCP4.5), respectively. These increments may lead to similar increases in the temperatures of surface water bodies. Overall, warming conditions are expected to result in shifts in the hydrologic cycle, depletion of dissolved oxygen levels and increase of the concentrations of nutrients and contaminants (Ficke et al., 2007; Whitehead et al., 2009; Kim et al., 2010; Mas-Martí et al., 2015). In biological terms, higher temperatures tend to accelerate the metabolism of organisms, affect

Abbreviations: ASGR, average specific growth rate; EC, effect concentration; GE, growth efficiency; LC, lethal concentration; LOEC, lowest observed effect concentration; RCR, relative consumption rate; RGR, relative growth rate; ROS, reactive oxygen species

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their respiration rates, growth and development time, together with an increased consumption, decomposition and excretion rates (e.g., Friberg et al., 2009; Dallas and Ross-Gillespie, 2015). Furthermore, a temperature rise affects the sensitivity of organisms to contaminants since, on one hand, the uptake of chemicals by organisms is enhanced, resulting in higher accumulation of toxic compounds. On the other hand, higher rates of chemical reactions may lead to the production of free radicals and biotransformation products that can be more toxic than the parent compounds (Murdoch et al., 2000; Ficke et al., 2007). At a higher ecological level, increased water temperatures are expected to change the geographic distribution of species, hence influencing the overall aquatic biodiversity (Heino et al., 2009; Zhao and Feng, 2015).

In the actual climate change context, it is of crucial importance to anticipate the impact of temperature per se and its effects in combination with other stressors, like copper contamination, in order to improve environmental management and protection strategies in agricultural areas. As such, we hypothesized that the combined action of copper toxicity and temperature variations within the previewed climate change scenarios may affect biological responses of organisms belonging to different trophic levels. To address this hypothesis, the present study relied on the use of microcosm experiments to assess: (i) the toxicity of a copper-based fungicide on three non-target freshwater species – two primary producers (the green algae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*), and one shredder species (the trichoptera *Schizopelex* sp.); (ii) the impact of increased water temperatures linked to climate change scenarios on the biological responses of these organisms; and (iii) the combined effect of these two stress factors on those key species from freshwater ecosystems.

2. Materials and methods

2.1. Stress factors – temperature regimes & copper concentration and analysis

Three constant temperature regimes were established: 15, 20 and 25 °C. The lowest temperature was the temperature of the stream water during the field sampling (cf. Section 2.2.3), corresponding to the baseline scenario. The two higher temperatures are the result from two successive temperature increases of 5 °C, in line with the maximum temperature increases expected for the Southern European/Mediterranean region. The range of temperatures tested covers the current temperatures of Portuguese freshwater systems [8 – 12 °C in Winter (Pascoal et al., 2005; Ferreira et al., 2010), 16 – 21 °C in Spring (Pascoal and Cássio, 2004)] and of the large rivers and lakes in Europe [9 – 21 °C (EEA, 2012)], as well as their future temperatures according to the regional climate change projections.

Copper (as $CuSO_4 \cdot 5H_2O$; Merck) toxicity was assessed at three nominal concentrations: 0.0, 0.013, 0.064 and 0.318 mg Cu L^{-1}, being the stock solution prepared in distilled water. The highest concentration was selected according to the highest concentration of copper detected in Portuguese surface water bodies (0.318 mg Cu L^{-1}, SNIR, 2015), while the other two concentrations corresponded to two successive dilutions by applying a factor of 5. The obtained range of concentrations is representative of the copper levels reported for surface waters from different countries in the vicinity of agricultural lands (0.01 – 0.06 mg L^{-1}, Arbneshi et al., 2008; 0.01 – 0.117 mg L^{-1}, Fernández-Calviño et al., 2008). The copper sulfate stock solutions were maintained at 4 °C for no longer than 72 h until proceeding with the spiking of leaves and water.

The effective copper concentrations were determined immediately before the test by UV–Vis spectrophotometry (adapted from APHA (1992) and Ramadan et al. (2009)). This involved adding trisodium citrate buffer and the complexing agent 4-(2-pyridylazo)-resorcinol (PAR) to three aliquots of each stock solution, and then the copper levels were measured at 539 nm. The nominal copper concentrations of 0.013, 0.064 and 0.318 mg L^{-1} corresponded, on average, to effective concentrations of 0.021, 0.054 and 0.322 mg L^{-1}, respectively. No copper was detected in the stream water used in the experiments.

2.2. Culture of organisms, sampling and processing of biological material

2.2.1. Microalgae culture and immobilization

Unialgal cultures of *R. subcapitata* were maintained in the laboratory in sterile Woods Hole MBL (Marine Biological Laboratory) medium (Stein, 1973). The microalgae were harvested at the exponential growth phase (5 – 7 days old) and inoculated into fresh medium to initiate new microalgae cultures. Microalgae were normally reared at 20 ± 2 °C and a 16 ^{L}: 8^{D} photoperiod. A batch of microalgae cultures were acclimated to continuous light and to the other test temperatures (i.e., 15 and 25 °C), following the acclimatization procedure described for the trichopterans in sub-section 2.2.3.

Microalgae cells were immobilized in a calcium alginate matrix following the procedures outlined in Moreira dos Santos et al. (2002) and Marques et al. (2011). Briefly, for each testing temperature, an aliquot of the temperature-acclimated culture (at the exponential growth phase) was centrifuged and resuspended in MBL. A certain volume of this suspension was added to a solution of sodium alginate 1.3% (w/v) as to obtain an initial cell density of 10^6 cells mL^{-1}. After approximately 30 min of agitation, drops of such suspension of alginate and algal cells were incorporated into a solution of calcium chloride 2% (w/v). After 60 min of agitation to allow the formation and hardening of alginate beads, they were washed in distilled water and kept in 20x diluted MBL medium at 4 °C in the dark, for no longer than 48 h until use in the test.

2.2.2. Macrophytes culture

Colonies of *Lemna minor* were maintained in Erlenmeyers with Steinberg medium (OECD, 2006a), under 20 ± 2 °C and continuous light. The cultures were renewed weekly. Some of them were however acclimated to 15 °C and 25 °C before conducting the microcosm experiments, as previously mentioned (cf. Section 2.2.1).

2.2.3. Sampling and acclimatization of trichopterans

In late winter and beginning of spring season, *Schizopelex* sp. larvae of similar size (ubiquitous trichoptera species in low order Portuguese streams) were collected from the spring of a second order stream (Rio de Castelões, in the Mondego basin) in the Caramulo Mountain, North-Central Portugal (N40°32'0.73" and W8°09'15.79"; 222 m above sea level).

The trichoptera larvae were randomly divided into three aquariums containing aerated stream water and streambed sediment (also collected during the field campaign). The water of the three aquariums was initially maintained at 15 °C (the temperature of the stream water), but two of them were gradually warmed up to 20 and 25 °C at rates of 0.3 and 0.7 °C *per* day, respectively, over a period of 15 days. During this 15-day acclimatization period, the larvae were fed with leaves from a mixture of plant species (cf. Section 2.2.4). The water and sediments in the aquariums were weekly renewed after pre-acclimatization.

2.2.4. Collection, conditioning and contamination of leaf disks

Undamaged chestnut leaves (*Castanea sativa*) were collected at abscission time (in winter) at the banks of the same stream. The chestnut leaves were air dried and stored in a dry and dark place. Later on, leaf disks with 12-mm diameter were obtained from these leaves with a cork borer and then introduced into 0.5 mm mesh bags (20 × 20 cm). These bags were taken to the same stream and immersed in a stream section (in late winter/beginning of spring) with a slow current to promote microbial colonization, thereby enhancing palatability of leaves to detritivores. The bags were recovered after one week of in situ microbial conditioning.

Upon arrival at the laboratory, the leaf disks were gently rinsed with distilled water to remove detritus, and randomly divided into four