Ecotoxicology (2013) 22:683–692
DOI 10.1007/s10646-013-1060-2

Simultaneous effects of two fungicides (copper and dimethomorph) on their phytoremediation using *Lemna minor*

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Accepted: 6 March 2013 / Published online: 16 March 2013
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Abstract Effects of two fungicides, copper and dimethomorph ((E,Z)4-[3-(4-chlorophenyl)-3-(3-4dimethoxyphenyl) acryloyl] morpholine) on *Lemna minor* growth and phytoremediation were evaluated. The toxicity of copper and dimethomorph alone and in combination, was assessed by growth inhibition of *L. minor* cultures after 96 and 168 h. Copper had a severe impact on growth (max. inhibition: 90 % at 1,000 μg L^{-1}) while dimethomorph (as pure ingredient or formulated as Forum) did not (inhibition <45 % at 1,000 μg L^{-1}) after 168 h of treatment. When both chemicals were combined, synergism was observed after 96 h of exposure to copper and Forum. However, this interaction was a simple additivity after 168 h. Additivity was also observed when the pure active ingredient (dimethomorph) replaced Forum in the mixture of copper and dimethomorph at 96 and 168 h. *L. minor* showed an excellent performance in removing copper from the medium since after 96 h, 36, 60, and 76 % removal were reached for 10, 20, and 30 μg L^{-1} of Cu respectively. Copper accumulated in the plants. The removal of copper increased with Forum concentration. After 96 h copper (10 μg L^{-1} initial concentration) elimination increased from 36.39 ± 5.86–60.70 ± 6.06 % when Forum concentration increased from 0 to 500 μg L^{-1}. Accumulation of copper in plants was also increased by Forum but not by the active ingredient alone. Depuration of Forum by *L. minor* varied between 10 and 40 % after 96 h and it was generally more efficient than that of the pure ingredient. This depuration decreased in the presence of copper possibly due to the metal toxicity.

Keywords Aquatic plants · Depuration · Metal–fungicide interactions · Synergism · Toxicity · Water quality

Part of this work was presented as at the 61st International Symposium on Crop Protection on May 19, 2009 in Gent, Belgium. As such, it was published in part as an extended summary in the proceedings of the conference.

Electronic supplementary material The online version of this article (doi:10.1007/s10646-013-1060-2) contains supplementary material, which is available to authorized users.

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Introduction

Copper has been widely used in the formulation of pesticides to control mildew and other fungal diseases in several cultures all over the world. In winegrowing areas, such as the champagne region (France), "Bordeaux mixture" has been used for over a century, resulting in the contamination of waters, sediments and soils (Besnard et al. 2001). Dimethomorph, a cinnamic acid derivative, is a widely used systemic anti-sporulant fungicide that protects plants from downy mildew in agriculture and viticulture by inhibiting cell wall formation of fungi (Albert et al. 1988). The biochemical mode of action of dimethomorph is still controversial (Gisi and Sierotzky 2008). During and after agricultural application, pesticides may enter adjacent water courses by spray drift, runoff, or leaching, and they can be harmful to aquatic organisms. In France, the highest recorded concentration of dimethomorph in surface water was 406 $\mu g\ L^{-1}$ (IFEN 2006). Pesticides or metals never occur alone in the environment but rather in combination. This is especially true in vineyards where copper has become ubiquitous in aquatic ecosystems and may be in contact with other pesticides in contaminated water. The interaction of copper with organic pesticides has been, and continues to be, an important subject of many environmental studies (Gatidou and Thomaidis 2007; Liu et al. 2007; Tilton et al. 2011). This interaction is very complex and may affect the toxicity and uptake of both the metal and the pesticide (Teisseire et al. 1998; Panemangalore and Bebe 2005).

To minimize the potential impact of this pollution, ponds collecting runoff water have become common practice downhill of Champagne vineyards. In these ponds natural dissipation of pesticides take place. Nevertheless, it is important to develop innovative technologies to accelerate the depuration of contaminated water in these ponds. In recent years, phytoremediation has gained attention and popularity as a cost-effective, environmentally friendly and efficient in situ technology for a variety of pollutants (Cunningham et al. 1995; Pilon-Smits 2005; Rai 2009), and among them, many pesticides (Schröder and Collins 2002; Dhir et al. 2009; Dosnon-Olette et al. 2010a). Several plants were shown to be efficient in removing pesticides from water, among them rooted macrophytes such as *Juncus effusus* and *Ludwigia peploides* (Bouldin et al. 2006), microalgae (Dosnon-Olette et al. 2010b) and also duckweed species. *Lemna gibba* and *L. minor* are the most often reported duckweed species for pollutant removal (Khellaf and Zerdaoui 2010; Reinhold et al. 2010). In *L. minor*, it was recently shown that dimethomorph removal depended on temperature, light, and concentration of the fungicide (Dosnon-Olette et al. 2010a). The simultaneous presence of other contaminants may also have an influence on phytoremediation.

Efficient phytoremediation relies on healthy plants. If pesticides are to be absorbed as it is proposed for the elimination of dimethomorph by *L. minor* (Dosnon-Olette et al. 2011) they may have an effect on the plants. Therefore, information on the toxicity of pesticides and their combination is needed before the aquatic plants may be used for phytoremediation. Many studies have shown that the simultaneous presence of copper can affect the toxicity of pesticides such as folpet, flumioxazin, and carbendazim in plants and animals (Teisseire et al. 1998; Frankart et al. 2002; Jonker et al. 2004). Additivity occurs when the effects of combined components can be estimated from the effects of the individual components. A departure from additivity can be observed due to synergistic or antagonistic interactions between the components in the mixture. For example, the three types of interactions (antagonism, synergism and additivity) were found for the combination of copper and flumioxazin or several antibotrytic fungicides affecting photosynthetic activity of *L. minor*.

Toxicity and removal of Forum® (a commercial formulation of dimethomorph) and copper were already studied separately (Olette et al. 2008) and it was found that *L. minor* was efficient in eliminating both compounds from their medium, however no information exist on the fate of these compounds when they co-exist in a medium as it is often the case in vineyard ponds. Therefore, the objective of the present study was to evaluate the capacity of *L. minor* to simultaneously remove dimethomorph and copper from its liquid medium. Toxicity of the combination of copper and dimethomorph (pure or formulated as Forum) on *L. minor* was also studied and related to phytoremediation efficiency.

Materials and methods

Plant material

Lemna minor was collected from ponds in the Ardennes, France and acclimated to laboratory conditions for at least 3 months. Before initiating the stock culture, the fronds were disinfected by quick immersion in 0.5 % ethanol then in aqueous solution of 1 % hypochloric sodium for 3 min and then rinsed with distilled water to remove attached microalgae. The stock cultures were placed in 100 mL-Erlenmeyer flasks containing 50 mL of sterile inorganic growth medium (in mg L^{-1}: $CaCl_2$, 11.1; KNO_3 202; $MgSO_4\ 7H_2O$, 49.6; KH_2PO_4, 50.3; K_2HPO_4, 27.8; $FeSO_4\ 7H_2O$, 6; K_2SO_4, 17.4; H_3BO_3, 5.72; $MnCl_2\ 4H_2O$, 2.82; $ZnSO_4$, 0.6; Na_2-EDTA, 10; $CuCl_2\ H_2O$, 0.008; $CoCl_2\ 6H_2O$, 0.054; $(NH_4)_6Mo_7O_{24}\ 4H_2O$, 0.043; Chollet 1993). Before the medium was autoclaved its pH was adjusted to 6.5 ± 0.1 using 0.1 M HCl.

The Erlenmeyer flasks containing *L. minor* fronds were then placed in controlled environment chamber at 22 ± 1 °C under a 16:8 h photoperiod (60 µmol PAR $m^{-2}\ s^{-1}$) and plants were subcultured every 7 days.

Chemicals

Copper sulfate was used as $CuSO_4\ 5H_2O$ (Merck, Fontenay sous Bois, France).

Dimethomorph ((E, Z) 4-[3-(4-chlorophenyl)-3-(3-4-dimethoxyphenyl) acryloyl] morpholine) was used as Forum® (150 g L^{-1} of active ingredient, BASF Agro, France) and as pure ingredient (97.5 %, BASF Agro, France). Given concentration of dimethomorph is that of the active ingredient (a.i.). The pKa and Log K_{ow} of the dimethomorph are respectively: −1.3 (T = 25 °C) and 2.63 (T = 20 °C).

Experimental procedures

Ten days after subculture, *L. minor* fronds (20 in number, 50 ± 0.10 mg FW fresh weight) were transferred to 100 mL-Erlenmeyer flasks containing 50 mL of growth medium to which copper and/or dimethomorph had been added from stock solutions (concentrations in stock solutions were: for Cu 50 mg L^{-1} in water, for Forum 150 g L^{-1}, and for pure dimethomorph 20 mg L^{-1} in water). The growth medium did not contain any EDTA when the experiments included Cu to avoid interactions between the ion and the chelator (Wang 1990). The experiments were conducted under static conditions; meaning the medium was not renewed and concentration of copper ion or dimethomorph (a.i.) was set at the beginning of experiments. It was 10, 20, and 30 µg L^{-1} for copper and 250, 500, and 1,000 µg L^{-1} (a.i.) for dimethomorph. These concentrations were chosen because they are environmentally representative concentrations and because they induced only moderate growth inhibition as found in preliminary experiments (Megateli et al. 2009a).

Controls were run without copper and dimethomorph.

Toxicity assessment

The effects of copper and dimethomorph (as a.i. or as Forum) on *L. minor* were determined by measuring growth inhibition according to Eq. (1):

$$I(\%) = \frac{\Delta Nt - \Delta N}{\Delta Nt} \times 100 \quad (1)$$

where ΔNt is the variation of the number of fronds observed in the control. ΔN is the variation of the number of fronds in the presence of copper and/or dimethomorph.

Combination of copper and dimethomorph

In order to determine the type of interaction between copper and dimethomorph, observed inhibition (I_{obs}) was compared to expected inhibition ($I_{\exp}$) estimated using Abott's formula (Gisi 1996). In this widely used model, the expected inhibition of the mixture, expressed as percent can be predicted as (Eq. 2):

$$I_{\exp} = I_A + I_B - (I_A \times I_B)/100 \quad (2)$$

where I_A and I_B are inhibitions observed for single chemicals (copper and dimethomorph).

The ratio of inhibition (*RI*) was calculated as follows for each pesticide combination (Eq. 3):

$$RI = I_{\mathrm{obs}}/I_{\exp} \quad (3)$$

Synergism or antagonism was evaluated by comparing the *RI* with 1. A $RI < 1$ indicated antagonism between the two contaminants while a $RI > 1$ means synergism. Antagonism means "less negative effects" on *L. minor* and synergism "more negative effects" on *L. minor* when combining the two contaminants in comparison to an isolated application of both fungicides and just adding the negative effects by mathematics.

Determination of concentrations of copper and dimethomorph

Plants were placed in contaminated media as described in the experimental procedure. Copper and dimethomorph concentrations were assessed after 96 and 168 h in samples of contaminated growth medium. The analysis of dimethomorph was performed by a high performance liquid chromatography (HPLC) system (Prostar system, Varian, Les Ulis, France). The molecule was separated using C18 reversed phase column (100 × 3 mm, Pursuit XRs 5, Varian, Les Ulis, France) and eluted isocratically with acetonitrile (60 %) and water (40 %) acidified with H_3PO_4 (0.1 %). Identification of the compound was confirmed by its UV spectrum and concentration determined according to an external calibration curve based on reference samples at 246 nm. Limits of quantification were <1 µg L^{-1} for copper and <10 µg L^{-1} for dimethomorph. The removal of the copper and dimethomorph was expressed as percentage of contaminant concentration decrease vs. nominal concentration in solution (Eq. 4):

$$R(\%) = \frac{C_o - C_t}{C_o} \times 100 \quad (4)$$

where C_0 and C_t represent the concentrations of copper or dimethomorph at the beginning (nominal) and at time *t*. Maximum deviation from nominal concentration was 3 % for dimethomorph and Cu.

To determine Cu contents of plants, ~0.1 g fronds were collected after 96 and 168 h, rinsed in distilled water, patted dry with a paper towel, weighed and digested in 4 mL of a 1:1 (vol/vol) mixture of 65 % HNO_3 and 30 % H_2O_2 (Drost et al. 2007). The extract was then diluted in 50 mL of 0.3 % HNO_3 and analyzed as described above and expressed in µg per gram fresh weight (FW) of plant material. Copper concentration was determined by direct injection into an atomic absorption spectrometer equipped with electrothermic atomization (SpectrAA Zeeman 220, Varian, Les Ulis, France). Equipment was operated with the spectrAA 2202.10 FS software.

Controls without plants were run in which copper and dimethomorph were included to check a possible spontaneous disappearance of the chemicals. Culture medium pH was measured before and after phytoremediation tests.

Statistical analyses

For toxicity tests, three independent experiments were run in which two replicates were performed for each concentration. For phytoremediation tests, independent experiments were repeated twice and each concentration was duplicated. Means ± standard deviations (SD) are shown. All statistical analyses were performed with SigmaStat 3.5 (Systat Software Inc, San Jose, CA, USA) for Windows. Significance of differences of samples was calculated by Student's *t* test. Results of testing were considered significant when calculated p values were ≤ 0.05.

Results

Effects of copper and dimethomorph on growth of *L. minor*

Growth of *L. minor* was significantly ($p < 0.05$) affected by copper. Indeed, after 96 h, inhibition ranged from 15.8 ± 5.3 to 34.9 ± 0.8 % (Tables 1, 2). Inhibition increased with duration of treatment. After 168 h, the maximum inhibition observed was 43.9 % in the presence of 30 μg L^{-1} of copper (Table 2). Comparable growth inhibition by dimethomorph (as a.i. or as Forum) required much higher concentrations than those of copper. After 96 h the maximum inhibition reached was 43.8 ± 5.3 % with 1,000 μg L^{-1} when applied as Forum (Table 1) and 19.1 ± 2.8 % when applied as pure ingredient (Table 2). After 168 h inhibition increased to 33.3 ± 3.8 % with 1,000 μg L^{-1} of the pure ingredient (Table 2) while it remained unchanged at 43.8 ± 11.0 % with Forum (Table 1).

Growth inhibition by copper-Forum mixtures increased with concentration of both contaminants. Furthermore, growth inhibition after exposure to the mixtures was higher than the sum of single inhibitions, indicating a synergism. For example after 96 h, inhibition caused by 10 μg L^{-1} of copper was 15.8 ± 5.3 %, that of 250 μg L^{1} of dimethomorph applied as Forum was 38.0 ± 10.6 % and of the inhibition after exposure to the mixture was 75.8 ± 11.0 % (Table 1). The synergism between the two compounds is best illustrated by values of ratios of inhibition (RI) greater than 1 (Table 3). These values ranged from 1.54 to 2.07 and 1.24 to 1.46 after 96 and 168 h, respectively. The highest RI value was observed after 96 h when copper concentration was low (10 μg L^{-1}) and Forum concentration was high (1,000 μg L^{-1}). In contrast, the lowest RI value was observed after 168 h for 30 μg L^{-1} of copper.

When dimethomorph was used as pure ingredient, RI values were close to 1. They ranged from 0.92 to 1.26 and 0.95 to 1.09 after 96 and 168 h respectively, indicating additivity between the two contaminants (Table 4).

Removal of copper and dimethomorph by *L. minor*.

In the control without Forum the removal of copper was comprised between 36.4 ± 5.9 and 70.5 ± 0.7 % after 96 h (Fig. 1). This elimination was between 41.9 ± 2.9–74.7 ± 1.2 % after 168 h. The removal of copper was higher when the initial concentration of the metal was higher. It could be noted that in the absence of plants, copper concentration also decreased in the medium however this decrease ranged between 13.4 ± 0.4 and 29.3 ± 0.6 % of initial concentration (see Fig. S1).

Table 1 Growth inhibition (%) of *Lemna minor* caused by mixture of copper (030 μg L^{-1}) and dimethomorph (0–1,000 μg L^{-1}) applied as Forum after 96 and 168 h of exposure

	Copper (μg L^{-1})	Dimethomorph (μg L^{-1})			
		0	250	500	1,000
96 h	0	0	38.0 ± 10.6#	45.5 ± 6.4#	43.8 ± 5.3#
	10	15.8 ± 5.3*	75.8 ± 11.0*#	77.3 ± 8.5*#	80.3 ± 11.0*#
	20	25.2 ± 7.1*	84.1 ± 9.6*#	85.6 ± 11.0*#	87.1 ± 6.1*#
	30	26.7 ± 1.4*	84.8 ± 7.7*#	93.2 ± 7.8*#	86.1 ± 7.1*#
168 h	0	0	43.5 ± 2.8#	45.3 ± 5.3#	43.8 ± 11.0#
	10	26.5 ± 5.2*	83.0 ± 11.6*#	84.2 ± 7.1*#	85.7 ± 10.5*#
	20	39.2 ± 3.5*	83.0 ± 6.0*#	85.4 ± 3.5*#	87.2 ± 12.5*#
	30	39.9 ± 6.1*	89.7 ± 7.1*#	92.7 ± 8.6*#	93.3 ± .7.5*#

Data are the mean ± SE of duplicates form three independent experiments

* Statistically significant different from the Cu control ($p < 0.05$)—rows

Statistically significant different from the Dimethomorph control ($p < 0.05$)—lines

Table 2 Growth inhibition (%) of *Lemna minor* caused by mixture of copper (0–30 μg L^{-1}) and dimethomorph (0–1,000 μg L^{-1}) applied as pure ingredient after 96 and 168 h of exposure

	Copper (μg L^{-1})	Dimethomorph (μg L^{-1})			
		0	250	500	1,000
96 h	0	0	12.4 ± 2.3$^{\#}$	15.2 ± 1.8$^{\#}$	19.1 ± 2.8$^{\#}$
	10	20.0 ± 2.8*	29.5 ± 2.2*	38.5 ± 0.7$^{*\#}$	44.3 ± 3.4$^{*\#}$
	20	29.9 ± 1.3*	35.6 ± 2.3*	41.3 ± 3.8$^{*\#}$	49.1 ± 4.3$^{*\#}$
	30	34.9 ± 0.8*	40.0 ± 2.8*	49.9 ± 1.6$^{*\#}$	53.0 ± 2.8$^{*\#}$
168 h	0	0	16.5 ± 1.4$^{\#}$	30.8 ± 3.1$^{\#}$	33.3 ± 3.8$^{\#}$
	10	30.2 ± 0.3*	45.5 ± 0.7$^{*\#}$	55.6 ± 0.6$^{*\#}$	57.6 ± 0.6$^{*\#}$
	20	43.8 ± 4.0*	50.1 ± 3.0*	60.3 ± 2.3$^{*\#}$	62.0 ± 1.4$^{*\#}$
	30	43.9 ± 1.5*	55.0 ± 2.8$^{*\#}$	65.1 ± 2.7$^{*\#}$	81.6 ± 3.6$^{*\#}$

Data are the mean ± SE of duplicates form 3 independent experiments

* Statistically significant different from the Cu control ($p < 0.05$)—rows

Statistically significant different from the Dimethomorph control ($p < 0.05$)—lines

Table 3 Ratios of inhibition (*RI*) of *Lemna minor* growth caused by mixture of copper (0–30 μg L^{-1}) and dimethomorph (0–1,000 μg L^{-1}) applied as Forum after 96 and 168 h of exposure

Copper (μg L^{-1})	Dimethomorph (μg L^{-1})					
	250		500		1,000	
	96 h	168 h	96 h	168 h	96 h	168 h
10	1.58 ± 0.30	1.38 ± 0.22	1.74 ± 0.13	1.46 ± 0.20	2.07 ± 0.55	1.46 ± 0.17
20	1.57 ± 0.27	1.33 ± 0.11	1.55 ± 0.21	1.38 ± 0.17	1.93 ± 0.39	1.41 ± 0.18
30	1.55 ± 0.22	1.24 ± 0.11	1.54 ± 0.15	1.28 ± 0.17	1.84 ± 0.43	1.32 ± 0.18

Table 4 Ratios of inhibition (RI) of *Lemna minor* growth caused by mixture of copper (0–30 μg L^{-1}) and dimethomorph (0–1,000 μg L^{-1}) applied as pure ingredient after 96 and 168 h of exposure

Copper (μg L^{-1})	Dimethomorph (μg L^{-1})					
	250		500		1,000	
	96 h	168 h	96 h	168 h	96 h	168 h
10	0.99 ± 0.15	1.09 ± 0.16	1.20 ± 0.14	1.07 ± 0.04	1.26 ± 0.16	1.08 ± 0.05
20	0.92 ± 0.01	0.95 ± 0.08	1.02 ± 0.04	0.99 ± 0.08	1.13 ± 0.03	0.99 ± 0.08
30	0.93 ± 0.07	1.03 ± 0.00	1.11 ± 0.06	1.06 ± 0.05	1.12 ± 0.07	1.10 ± 0.07

When dimethomorph was added as Forum, a significant increase of copper removal was observed, especially with 10 μg L^{-1} of copper where the removal of copper increased from 36.4 ± 5.9 % without dimethomorph to 64.8 ± 1.4 % when dimethomorph concentration reached 1,000 μg L^{-1} (Fig. 1). There was little difference in copper removal between 96 and 168 h incubation; it seemed that copper removal was already maximum after 96 h (Fig. 1).

The effect of Forum was no longer significant for 30 μg L^{-1} of copper, removal being almost at its maximum for all Forum concentrations.

The amount of copper recovered in plants was between 2.30 ± 0.44 and 2.90 ± 0.38 μg g FW^{-1} being slightly higher for lower initial concentrations (Fig. 2). Plant Cu depended on the concentration of DMM in the medium only when the fungicide was applied as Forum. The amount of copper inside the plants increased with the concentration dimethomorph applied as Forum in the medium. This was especially remarkable for *L. minor* treated with 10 μg L^{-1} Cu for which the highest concentration of dimethomorph resulted in a 56 % increase in the amount of Cu in the fronds.

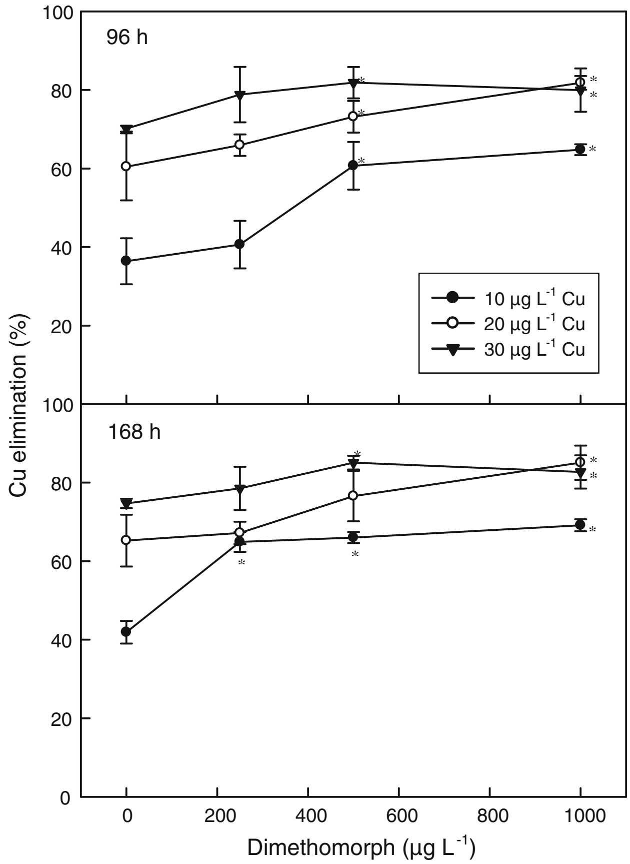

Fig. 1 Effect of dimethomorph applied as Forum on the elimination of copper by *Lemna minor* after 96 and 168 h exposure. Elimination is expressed as % of initial concentration of copper in the medium, which was 10, 20, or 30 µg L^{-1}. *significantly different from control ($p < 0.05$)

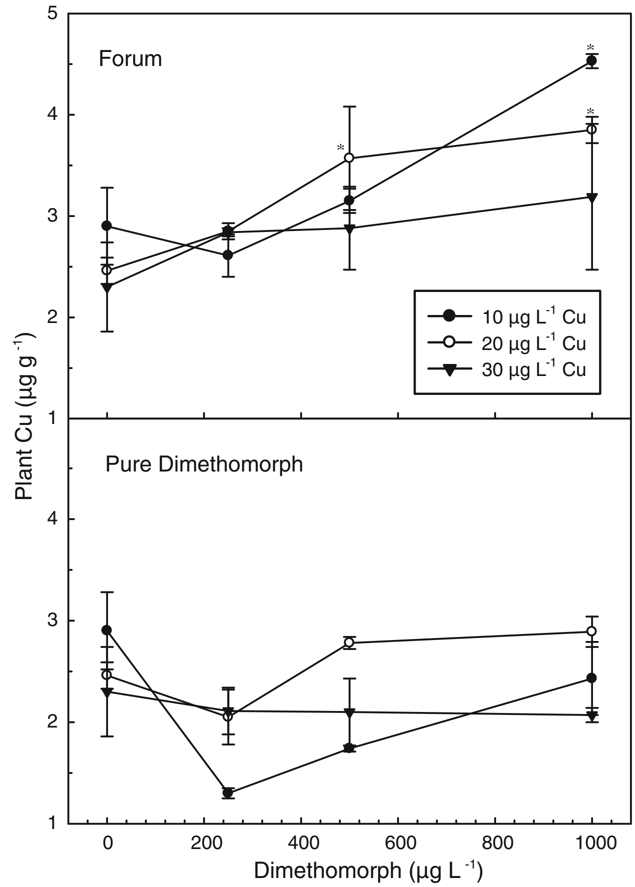

Fig. 2 Effect of dimethomorph applied as pure ingredient or formulated as Forum on the accumulation of copper by *Lemna minor* after 96 h. *significantly different from control ($p < 0.05$)

Without plant, dimethomorph concentration in culture media remained relatively stable during the 168 h of experiment and elimination never exceeded 5 % (see Fig. S2). When plants were present, absolute removal of dimethomorph increased with initial concentration of the fungicide from 100 to 150 µg L^{-1} for Forum and from 75 to 100 µg L^{-1} for the pure ingredient as initial concentration increased from 250 to 1,000 µg L^{-1}. In contrast, the relative removal decreased with initial concentration (Fig. 3). It was comprised between 10 and 45 % and it depended on the initial concentration of the fungicide, the time of incubation, and the mode of application. Indeed, after 96 h relative removal of dimethomorph applied as Forum was between 40 and 15 % while it was only 10 % in the case of pure dimethomorph (Fig. 3a, b).

As copper concentration in the medium increased from 0 to 30 µg L^{-1} removal of dimethomorph applied as Forum decreased slightly. For example after 96 h, for an initial dimethomorph concentration of 250 µg L^{-1} of dimethomorph, the absolute removal from the medium was down to 85 from 100 µg L^{-1}, which in relative amount represented a decrease from 40 to 34 % (Fig. 3a). After 168 h, the effect of copper on Forum removal became significant for 250 µg L^{-1} (from 43 % down to 36 % with 30 µg L^{-1} Cu, $p = 0.039$) and 500 µg L^{-1} (33 down to 13 % with 30 µg L^{-1} Cu, $p = 0.01$) forum (Fig. 3c).

As far as the pure ingredient is concerned, the negative effect of copper on dimethomorph removal by plants was observed for 250 and 500 µg L^{-1}. Removal decreased from 30 to 17 % ($p < 0.05$) and 19 to 10 % ($p < 0.05$) as copper concentration increased from 0 to 30 µg L^{-1} after 96 h (Fig. 3b). Likewise, the effect of copper became more important after 168 h and removal decreased from 34 to 16 % and from 26 to 11 % (Fig. 3d). At the highest concentration of dimethomorph (1,000 µg L^{-1}), effect of copper on removal was not significant (10–7 and 11–8 % for 96 and 168 h).

Discussion

Copper is an essential nutrient for plant growth and development, and is normally present in plant tissue

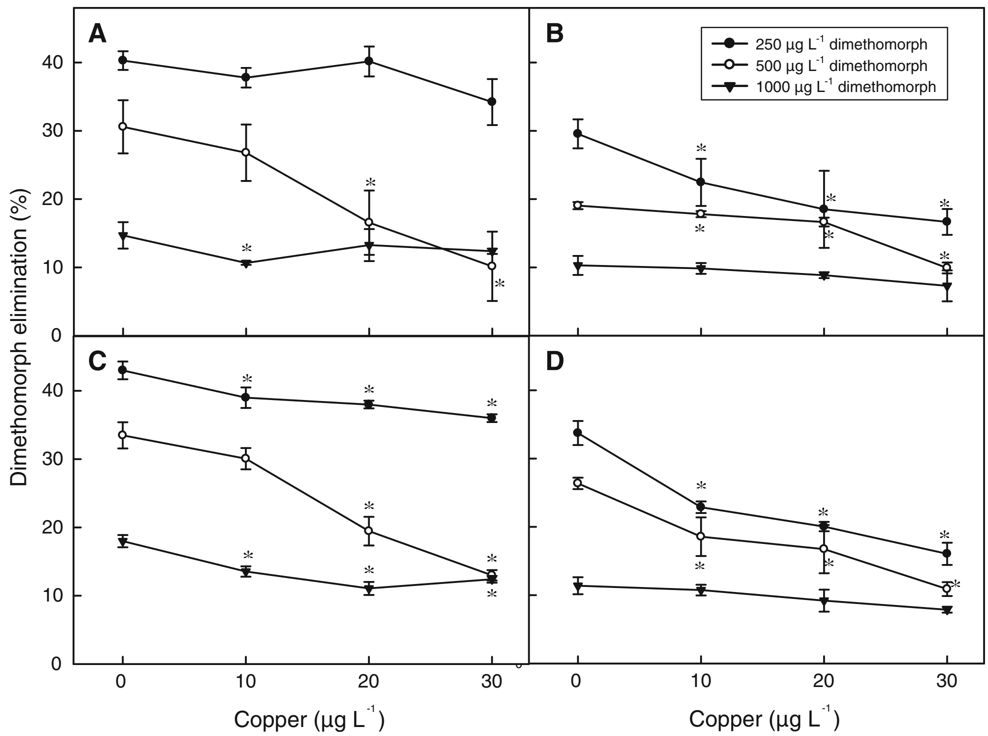

Fig. 3 Effect of copper on the elimination of dimethomorph by *Lemna minor* after 96 (a and b) and 168 h exposure (c and d). Elimination is expressed as % of initial dimethomorph (a.i.) concentration in the medium. Dimethomorph was applied either formulated as Forum (**a**, **c**) or as pure ingredient (**b**, **d**). *significantly different from control ($p < 0.05$)

(Marschner 1995) and the presence of low concentrations of copper in duckweed medium may stimulate its growth. However, excessive Cu accumulation in plant tissues can be toxic, affecting several physiological and biochemical processes, such as reduction of photosynthetic activity (Frankart et al. 2002; Olette et al. 2008) and growth (Weckx and Clijsters 1996; Teisseire et al. 1999; Babu et al. 2003; Megateli et al. 2009b). In our study, growth inhibition by copper depended on concentration and duration of exposure. It was significantly inhibited after 96 h at 10 µg L^{-1} and after 168 h, growth inhibition was even higher.

In contrast, dimethomorph applied alone (as pure a.i. or as Forum) appeared to have low toxicity towards duckweed since 50 % inhibition could not be reached after the longest incubation time and the highest concentration. The low toxicity of dimethomorph for plants could have been expected since fungicides are designed to be applied on plants without harm. Furthermore this plant seems to have a low sensitivity to various fungicides (Teisseire et al. 1998; Verdisson et al. 2001). A low effect of Forum on photosynthetic activity of *L. minor* was also described (Dosnon-Olette et al. 2010a). Nevertheless, the maximum concentration of this fungicide reported by IFEN (2006) in surface waters shows that it might impair aquatic plant growth in ponds in case it is still associated to the additives that are included in Forum. Indeed, our results showed that dimethomorph was more toxic to *L. minor* when applied as Forum than as pure ingredient, particularly after 96 h. Pesticide formulations may contain additives such as carrier, solvents, emulsifiers, and stabilizers, all of which may have some effect on toxicity (Walker et al. 2006). The difference of toxicity between the two modes of application of the fungicide was lower after 168 h than after 96 h, suggesting that the additives included in the formulation have only helped the active ingredient to enter the plant faster. However, the list of additives in Forum is not available, which makes it difficult to conclude at this point.

As highlighted by the ratios of inhibition, the mixture of copper and dimethomorph induced two types of interactions: synergism and additivity. Synergism was observed with formulated dimethomorph while additivity was observed with pure ingredient. Synergism increased when Forum concentration was higher possibly because penetration of copper was accelerated by adjuvants of the formulated fungicide as could be observed in *L. minor* fronds (Fig. 2). Indeed, the adjuvants may cause dissolution of leaf cuticles, which in turn favors copper penetration into plant cells, and induce higher copper phytotoxicity (Zabkiewicz 2000). This is also confirmed by the fact that pure active ingredient did not yield any synergism.

Synergism that might present a threat for *L. minor* in ponds was also higher when copper concentration and incubation time were low. Indeed, with higher copper concentration and longer exposure synergism tented towards additivity probably due to the high toxicity of the mixture approaching 100 %.

In order to be able to quantify the contribution of *L. minor* in the elimination of copper, removal without plants was assessed in the medium and it was comprised between 13.4 ± 0.4 and 29.3 ± 0.6 % of initial concentration. This elimination was possibly due to precipitation as Cu salts and/or adsorption to the glass wall of Erlenmeyer flasks. In a phytoremediation experiment Kamal et al. (2004) indicated that an average of half of the Cu was removed from water through the formation and precipitation of $Cu_3(PO_4)_2$, the remaining portion being due to the presence of macrophytes.

In the presence of *L. minor* copper removal from the medium was important since after 96 h, it was between 36 and 70 % and it was comparable with other macrophytes (Kamal et al. 2004; Mishra and Tripathi 2008). In our study, it appeared that metal removal from the medium was due at least in part to an accumulation in plants since *L. minor* was able to accumulate more than 3 µg Cu per g FW (Fig. 2). It is also possible, that some of the metal adsorbed on the plant surface since it was found that dead dried *L. minor* was able to remove between 63 and 94 % of metal from water contaminated with 10 and 20 mg L^{-1} of Zn, Cu, and Cd (Miretzky et al. 2006).

The presence of Forum favored copper elimination. At low copper initial concentration (10 µg L^{-1}), as Forum concentration increased in the medium, copper elimination increased (e.g. from 36 to 65 % removal). This was not observed for the pure ingredient (not shown). Therefore, this increased removal was probably not due to the presence of the active fungicidal ingredient but rather to the adjuvants associated with the fungicide in Forum that might help the penetration of the metal into the plant (Walker et al. 2006), possibly by complexation of the metal (Hernández-Soriano et al. 2011). This increased absorption is supported by the increased copper content inside the plants (Fig. 2) and the higher toxicity of copper in the presence of Forum, which is an indirect indication of Cu uptake. The stimulation of copper removal by Forum was less important for higher copper initial concentrations, it is possible that the maximum removal had already been reached without Forum or that the toxicity of both compounds be too high (>80 % inhibition) making plant uptake impossible.

Percent removal of dimethomorph from the medium was lower than that of copper but it was comparable to other investigations using duckweed to remove pesticides from contaminated water (Böttcher and Schroll 2007; Olette et al. 2008). Removal was always higher for Forum than for the pure active ingredient and it depended on the initial concentration of the fungicide. In the case of Forum, removal of dimethomorph was not much higher after 168 h than after 96 h. In contrast, removal of pure dimethomorph continued to increase after 168 h suggesting the maximum elimination had not been achieved yet. It could be suggested that the additives present in the formulation of Forum only accelerated the elimination but not the maximum removal capacity of the plants. Organic pollutants are usually man-made, and xenobiotic to plants. As a consequence, there are no specific transporters for these compounds in plant membranes (Pilon-Smits 2005). Organic pollutants therefore tend to move into and within plant tissues, driven most of the time by simple diffusion, depending on their chemical properties. Among these, hydrophobicity is one of the most important properties explaining the removal potential of plants. Organic chemicals with an octanol–water partition coefficient ($\log K_{ow}$) between 0.5 and 3 are considered hydrophobic compounds able to move through the lipid bilayer of membranes, but still water-soluble enough to travel into the cell fluids (Cedergreen et al. 2005). Dimethomorph, with a log K_{ow} of 2.63, can enter plants rapidly, explaining why removal was almost maximum after 96 h.

The presence of copper generally induced a decrease in the removal of dimethomorph. For example after 96 h with 500 µg L^{-1} of Forum, elimination of the fungicide was down from 31 to 10 % as copper concentration increase from 0 to 30 µg L^{-1}. It was previously suggested that elimination of dimethomorph by *L. minor* was only important in metabolically active plants and possibly related to the degradation of the fungicide by Cyt P450 oxidation (Dosnon-Olette et al. 2011). Therefore, copper toxicity which is enhanced by the presence of Forum (Table 1) may be responsible for the lower degradation of the fungicide and in turn to its reduced elimination.

Conclusion

The data presented here confirms the toxicity of copper for *L. minor* while dimethomorph appeared less toxic for the plant (50 % growth inhibition was not reached even after 168 h with 1,000 µg L^{-1}). However, synergism was observed in presence of formulated dimethomorph pointing at the complexity of toxicity assessment in the environment, where several compounds may interact.

Despite the inhibition induced by copper and dimethomorph, removal of these two contaminants by *L. minor* took place. This plant, which is often used for toxicity tests, also appeared to be a good candidate for phytoremediation. This study showed that phytoremediation of a metal or a pesticide may be influenced by the presence of another contaminant in the medium, either positively or negatively.

Acknowledgments Senior author is grateful to PROFAS cooperation for providing financial support though the BAF (Bourse Algéro-Française) fellowship. Thanks to BASF France, Ecully, for the generous gift of dimethomorph active ingredient. This research is part of the AQUAL program, financed by the French Ministry for Research and the European Fund for Regional Development (FEDER). The authors acknowledge the help of Laurence Delahaut in copper analysis. The authors declare that they have no conflict of interest.

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