

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Evidence of low dose effects of the antidepressant fluoxetine and the fungicide prochloraz on the behavior of the keystone freshwater invertebrate *Gammarus pulex*[☆]

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ARTICLE INFO

Article history:

Received 12 January 2017

Received in revised form

18 July 2017

Accepted 26 July 2017

Keywords:

Gammarus pulex

Direct effects

Indirect effects

Swimming velocity

Feeding rate

ABSTRACT

In recent years, behavior-related endpoints have been proposed as rapid and reliable ecotoxicological tools for risk assessment. In particular, the use of detritivores to test the toxicity of pollutants through feeding is currently becoming a well-known method. Experiments combining feeding with other behavioral endpoints can provide relevant information about direct and indirect toxicological effects of chemicals. We carried out a feeding experiment with the shredder *Gammarus pulex* in order to detect indirect (through leaf conditioning) and direct effects (through water exposure) of two pollutants at environmentally relevant concentrations: the fungicide prochloraz (6 µg/L) and the antidepressant fluoxetine (100 ng/L). Prochloraz inhibited fungal growth on leaves, but it did not affect either the microbial breakdown rates or the C:N ratio of the leaves. Individuals of *G. pulex* that were fed with treated leaves presented lower consumption rates, not only those fed with prochloraz-treated leaves, but also those fed with fluoxetine-treated leaves, and those fed with the mixture-treated leaves. Mixed-effects models revealed that the swimming velocity of the amphipods after the experiment was modulated by the exposure to fluoxetine, and also by the exposure to prochloraz. We demonstrate that both the antidepressant and the fungicide may cause significant sublethal effects at low concentrations. The combination of behavioral endpoints together with the application of mixed models provided a useful tool for early detection of the effects of toxicity mixtures in freshwater ecosystems.

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1. Introduction

Most of the rivers of densely populated countries receive both diffuse (e.g., agricultural runoff) and point source (e.g., waste water treatment plants) pollution inputs, and thus are contaminated with complex mixtures of chemicals of different origin (Sumpster, 2009). Biota in running waters are exposed, to varying degrees, to this contamination.

Apart from well-known regulated pollutants, many studies have revealed the widespread occurrence of low level concentrations of different chemicals in the aquatic environment called 'emerging contaminants' (ECs) (Lapworth et al., 2012; Schwarzenbach et al., 2006). These compounds are not regulated and knowledge about

their effects on natural systems is scarce. One important group of ECs present in aquatic systems are Pharmaceutically Active Compounds (PhACs). The majority of PhACs are released to the environment in an active form which can potentially have an impact on the organisms that inhabit these areas (Bossus et al., 2014; Proia et al., 2013). Particularly, concerns regarding some antidepressants such as selective serotonin re-uptake inhibitors (SSRIs) have been increasing due to their demonstrated effects on biological activity at environmentally relevant concentrations, especially in crustaceans and mollusks (Campos et al., 2016; Demeestere et al., 2010; Ford and Fong, 2015; Guler and Ford, 2010; Johnson et al., 2007; Minagh et al., 2009; Styryshave et al., 2011). Fluoxetine is one of the most widely prescribed antidepressants (Bossus et al., 2014; Kaur et al., 2016) and it has been detected at tens and even hundreds of ng/L in freshwater systems (Giebułtowicz and Naięcz-Jawecki, 2014; González Alonso et al., 2010; Mennigen et al., 2011; Oakes et al., 2010; Pelli and Connaughton, 2015; Writer et al., 2013). This SSRI acts by blocking the plasma membrane serotonin

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transporter, and since most invertebrates use serotonin as neurotransmitter, it can have specific mode-of-action effects on a variety of different species of invertebrates (Johnson and Sumpter, 2014) at very low concentrations (ng/L). Apart from the SSRI specific mode-of-action, fluoxetine and its metabolite norfluoxetine can also interact with cytochrome P450s (CYP) enzymes (Hemeryck and Belpaire, 2002). CYP enzymes are an important superfamily of detoxification enzymes found in terrestrial and aquatic organisms ranging from bacteria to vertebrates. These enzymes metabolize a wide variety of substrates including endogenous molecules and xenobiotics (e.g., pesticides, drugs) (Burkina et al., 2015; Snyder, 2000).

Another important group of pollutants widely distributed in aquatic systems due to the intensification of agriculture are pesticides, which have recently been identified as a threat for the biodiversity of freshwater invertebrates in Europe (Beketov et al., 2013). Fungicides, a group of pesticides mainly used to protect crops from fungal attacks, are widely used in many regions and, in particular, have been detected at high concentrations in aquatic systems in Europe (Belenguer et al., 2014; Bjergager et al., 2011; Deb et al., 2009). Prochloraz is a broad-spectrum imidazole fungicide that is widely used in Europe, South America, Asia and Australia as a pesticide in rice, oat, wheat, potato, tomato, garlic and citrus cultivation (Vinggaard et al., 2006). The imidazoles are fairly persistent, with half-lives of weeks to months, and, thus, can be detected in water samples from streams and rivers (Kahle et al., 2008; Tomlin, 2004). In fact, in the agricultural area of the Júcar (Spain), prochloraz has been detected at concentrations of 0.5 µg/L (Ccanccapa et al., 2015). The fungicide has also been detected in the Ebro, in the Llobregat, and in the Guadalquivir rivers (Spain) (Campo et al., 2013; Masiá et al., 2013), and in surface waters of other countries, such as France (Legrand et al., 1991), Italy (Urbatzka et al., 2007), or Canada (Jeannot et al., 2000). Prochloraz inhibits the growth of fungi by inhibiting a specific CYP enzyme: the cytochrome P450-monooxygenase lanosterol 14 α -demethylase; and, thus, the synthesis of ergosterol, the major fungal membrane sterol. Ergosterol regulates membrane fluidity, biogenesis and function, and, thus, its homeostasis is critical for fungal cells (Tyndall et al., 2016; Yang et al., 2015). In aquatic systems, the role of fungi in the microbial colonization or conditioning of leaf litter from terrestrial origin is very important, because they make these leaves more palatable and more nutritious for leaf-consuming detritivores, also called shredders (Bärlocher and Kendrick, 1973; M. A S Graça et al., 1993). Prochloraz not only can inhibit fungal growth, it can also affect other organisms by inhibiting other CYP enzymes, such as the detoxicative P450 activity in honey bees, and interact in the synthesis of testosterone in vertebrates at the ng/L level (Ankley et al., 2009; Dang et al., 2015; Schmuck et al., 2003). In particular, it has been found to inhibit activities of the enzymes cytochrome P450 c17Rhydroxylase, 17,20-lyase (CYP17), and aromatase (CYP19) in mammals and fish (Liu et al., 2011; Vinggaard et al., 2006). In invertebrates no direct effects have been reported at the ng/L or low µg/L range. The reported EC50 (50% Effective Concentration) for *Daphnia magna* is 4.3 mg/L, and the NOEC (Non Observed Effect Concentration) for *Chironomus riparius* is 0.8 mg/L (PPDB, 2013).

Pollutants can exert toxic effects in invertebrates through different pathways. If they are dissolved in water, they can directly affect the organisms through waterborne exposure (Feckler et al., 2016; Lebrun et al., 2011; Machado et al., 2013; Niyogi et al., 2014). They can also accumulate in different substrates, such as leaves or sediments, and cause toxic effects through ingestion (Burkhard et al., 2015; Ding et al., 2013; Mateo et al., 2016; Pacioglu et al., 2016). Moreover, they can cause other indirect effects, altering the activity of decomposers (i.e., fungi and bacteria) and, thus, altering the quality of the food for consumers (Bundschuh et al.,

2011; Zubrod et al., 2011). The use of toxicity tests based on single species responses (sublethal and lethal tests) has been widely proposed as a complementary tool in biological and chemical conventional surveys. Sublethal endpoints are more sensitive than mortality or community structural changes, and can be used as early warning indicators of toxic stress. In recent years, sublethal behavior-related endpoints have been proposed as rapid and reliable ecotoxicological tools for risk assessment (Melvin and Wilson, 2013; Michalec et al., 2013; Ren et al., 2015). In particular, the use of detritivores to detect specific effects of pollutants through feeding is currently becoming an extensively used method (e.g., Agatz et al., 2014; Bundschuh et al., 2009; Pacioglu et al., 2016; Willming and Maul, 2016). Feeding is a behavioral endpoint mechanistically linked to ecosystem functions and, therefore, an unequivocal ecologically meaningful response (Maltby et al., 2002b; Wallace and Webster, 1996). However, the application of feeding tests as early detection tools of toxicity in freshwater ecosystems is still scarce in the context of ecotoxicological assessment of chemicals. Experiments combining feeding and other behavioral endpoints, such as locomotive behavior, could provide relevant information about direct and indirect toxicological effects of chemicals (Arce-Funck et al., 2016; Denoël et al., 2013; Rodrigues et al., 2016).

In this study, a conditioning experiment followed by a feeding experiment were carried out using the freshwater shredder *Gammarus pulex* to detect the effects of the two pollutants previously described: the fungicide prochloraz and the antidepressant fluoxetine. *G. pulex* is an important generalist detritivore in many European freshwater systems, where it plays a key role in leaf litter degradation (Maltby et al., 2002a; Pinkster, 1972; Piscart et al., 2011). Previous studies have demonstrated that the feeding rate of this shredder is inhibited by different environmental contaminants (e.g., Ashauer et al., 2010; De Lange et al., 2009; Felten et al., 2008). We measured leaf consumption rate of the amphipod and its movement velocity following exposure. The objectives of the experiment were (i) to study the indirect effects of the pollutants on the amphipod consumption rate through leaf litter conditioning (dietary exposure), and (ii) to study the direct effects of fluoxetine on the animal due to the exposure to the pollutant in the water (waterborne exposure). We hypothesized that amphipod consumption on leaves conditioned with these pollutants would be reduced. On one hand, consumption rate would be lower on leaves conditioned with the fungicide because this limits fungal growth and makes leaves less palatable. On the other hand, as fluoxetine can affect the nervous system of the animal, effects on consumption and on velocity are expected if the animals are exposed to this antidepressant. We also expected some kind of interactive toxic effect of both compounds in the mixture, in relation to their shared potential to interact with CYP detoxification enzymes.

2. Material and methods

2.1. Conditioning experiment

2.1.1. Leaf conditioning

Alnus glutinosa leaves were collected after abscission in late autumn of 2014 in New Forest Park (N 50°53'01.1", W 1°31'42.9"). Leaves were conditioned with pre-sieved (0.5 mm-mesh-size sieve) stream water for 14 days in 1 L glass tanks under continuous aeration. Each tank contained from 10 to 12 leaves. The conditions of exposure were 12:12 LD cycle at 15 °C, conditions similar to the autumn conditions of the region, and the leaves were conditioned under four different treatments: (i) control (water of the river, C), (ii) 6 µg/L of Prochloraz, equivalent to the LC50 (50% Lethal Concentration) values reported for algae (PPDB, 2013), (P), (iii) 100 ng/L of Fluoxetine (F) and (iv) a mixture of both compounds, 6 µg/L of

Prochloraz and 100 ng/L of Fluoxetine, (M). Water was renewed every 3 days.

2.1.2. Water analyses

Fluoxetine (CAS no. 56296-78-7) and prochloraz (CAS no. 67747-09-5) were obtained from Sigma–Aldrich® (St. Louis, MO, USA).

Water concentrations of prochloraz and fluoxetine were analytically verified by liquid chromatography tandem mass spectrometry (LC–MS/MS) before and after water renewal (Table S2). LC analyses were performed using an Agilent (Waldbronn, Germany) Model 1260 binary pump equipped with an autosampler, and a Kinetex column (100 × 2.1 mm i.d., 2.6 μm) (Phenomenex, Torrance, CA, USA) was used. Gradient elution was carried out with water–0.1% formic acid (solvent A) and acetonitrile–0.1% formic acid (solvent B) at a constant flow-rate of 500 μl min⁻¹. A linear gradient profile with the following proportions (v/v) of solvent B was applied (t(min), %B): (0, 10), (5, 50), (10, 100), (12, 100), (12.1, 10), (16, 10). The column was thermostated at 40 °C. A linear ion trap quadrupole LC–MS/MS 4000 QTRAP mass spectrometer (ABSciex, Concord, ON, Canada) was used to obtain the MS and MS/MS data. All the analyses were performed using a Turbo V ion source in positive ion mode with the following settings: capillary voltage +5500 V, nebulizer gas (N₂) 50 (arbitrary units), curtain gas (N₂) 20 (arbitrary units), collision gas (N₂) 10 (arbitrary units), declustering potential (DP) +40V, focusing potential –200 V, entrance potential 10 V, drying gas (N₂) heated to 500 °C at 20 (arbitrary units). The declustering potential (DP) and the collision energy (CE) were optimized for each compound in infusion experiments. Individual standard solutions (10 ng μl⁻¹) dissolved in 80:20 mobile phase (A: B) were infused at a constant flow-rate of 5 μl min⁻¹ into the mass spectrometer using a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). Quantitation was done in MRM mode with the following transitions: 376.06/307.90 for prochloraz: during 50 msec and 310.3/44.3 for fluoxetine during 50 msec. Quantification was done by the standard addition method spiking blank river samples at different concentrations between 0.02 and 6 ng/ml. Good correlation coefficients (R² > 0.99) were obtained for both compounds with accuracies between 80 and 120% after adjusting the 1/x² weighting for both compounds as recommended by Kiser and Dolan (2004). Detection limits in river water for fluoxetine were 2 ng/l and 200 ng/l for prochloraz.

2.1.3. Leaf analyses

13-mm leaf discs were cut with a cork borer after the 14d of conditioning. The biomass of leaves remaining after the 14d was estimated as the difference between initial and final ash-free dry mass. Five leaf discs of the senescent leaves collected and of the leaves after the exposure to the different treatments were oven-dried (70 °C, 72 h) and weighted. After that, they were burnt in a muffle furnace (500 °C, 4h) and weighted to calculate the ash-free dry weight (AFDW).

Three litter discs from each treatment were dried (60 °C, 72 h) and pulverised with a mortar and pestle. C and N analyses were performed with EA Flash 1112 ThermoFinnigan Scientific Analyzer using vanadium pentoxide as oxidation catalyzer.

Ergosterol was measured as an indicator of fungal biomass in freeze-dried leaf discs after the 14 days of conditioning for each treatment. It was extracted from lyophilized leaf discs using KOH methanol (0.14 M at 80 °C for 30 min), and then separated by solid-phase extraction (Waters Sep-Pack® Vac RC, 500 mg, tC18 cartridges, Waters Corp., Milford, MA, USA) (Gessner and Schmitt, 1996). Ergosterol was quantified using a JASCO LC-2000 series HPLC system (JASCO, Tokyo, Japan). Separation was achieved by a Gemini-NX 5 μm c18, 250 × 4.6 mm analytical column

(Phenomenex, Torrance, CA, USA). The mobile phase was 100% methanol at a flow rate of 1200 iL·min⁻¹. Chromatographic data were acquired and processed with ChromNAV Software (JASCO). Fungal biomass was estimated based on an ergosterol content of 5.5 mg g⁻¹ fungal biomass (Gessner and Chauvet, 1993; Mille-Lindblom et al., 2004).

2.2. Feeding experiment

G. pulex individuals were collected by 500 μm-mesh hand-net from the second order stream River Ems, in Westbourne (N 50°51'34.8", W 0°55'45.8") in late November 2014. According to historical data of the U.K. Environment Agency (<http://apps.environment-agency.gov.uk>), in the stretch from Emsworth to Westbourne the River Ems belongs to a very good water quality status (grade A). After a week of acclimation in lab conditions and 24 h of starvation, animals were sorted and adult males with no visual sign of infection by acanthocephalan were isolated. Males and females were identified by the presence or absence of female brood plates or male genital papilla (Green Etxabe et al., 2015). *G. pulex* were photographed, and the dorsal length (DL) of their first thoracic segment measured (software: ImageJ v.1.45s, National Institutes of Health, USA; 0.001 mm accuracy). Individuals ranging between 0.7 mm and 1.2 mm in size of the first segment were used for the experiment.

During the shredding experiment the amphipods were kept individually in 100 mL containers filled with 80 mL of filtered water of the river at 10 °C under a 12:12 LD cycle and fed with the leaves previously conditioned under the four different treatments (C, P, F and M; n = 26 for each treatment). For each one of these feeding treatments, 13 individuals were kept with river water (Control, C) and 13 individuals were exposed to 100 ng/L of fluoxetine dissolved in water from the river (F) for 14 days (Fig. 1) to test the direct effects of the antidepressant. Water without or with fluoxetine was renewed every 3 days. In this case the concentrations were not analytically verified, but we expect the same behavior as in the conditioning phase. Molting and mortality were recorded along the 14d experiment, and excrements were collected each day with a Pasteur pipette to avoid their consumption. The different treatments were named using the first letter of the leaves-conditioning

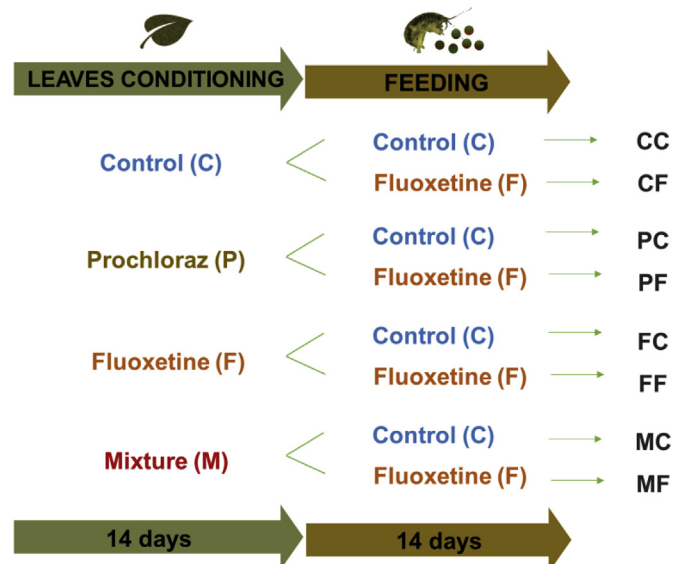


Fig. 1. Experiment design and nomenclature of each treatment.

treatment followed by the first letter of the feeding treatment (e.g., PF for Prochloraz in the leaves conditioning, and Fluoxetine waterborne exposure in the feeding experiment) (Fig. 1).

Leaf consumption was estimated from the 13-mm alder leaf discs as the difference between initial (freshly fallen leaves) and final ash-free dry mass minus the mean microbial consumption, divided by the final invertebrate biomass and by the time (Feio and Graça, 2000; Zubrod et al., 2010). A total of six discs were offered to each animal along the experiment, and after a week, the remnant material was recovered, oven-dried and weighted to estimate final leaf AFDW. Invertebrates were weighted at the end of the experiment to express consumption per mg of animal. Leaf consumption was not measured in microcosms where invertebrates died. Mass losses due to factors other than consumption (e.g., microbial consumption) were estimated in six discs incubated in the same conditions, and for each treatment, with no shredders. Consumption rates were expressed as mg of leaf AFDW per mg dry weight of *G. pulex* per day.

Velocity assays were performed after the 14 d of exposure to each condition using DanioVision™ (Noldus Information Technology, Wageningen, The Netherlands) and its software EthoVision® XT (v 8.1). Animals were put in 12-wells plates and placed within the DanioVision hardware for 2 min to allow settling prior to recording. The velocity (mm/s) measurements of the amphipods were recorded every 0.1 s during 4 cycles of 2 min dark and 2 min light, for a total period of 16 min (see Fig. S1 for an example of the data obtained).

2.3. Statistical analyses

2.3.1. Conditioning phase

AFDW, fungal biomass and C:N data were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test), and subsequently log-transformed. Two-way ANOVA followed by Dunnett's post hoc tests were performed to detect differences between leaves treatments (Prochloraz, Fluoxetine, and the interaction among both) and control. Statistical analyses were conducted using SPSS® Statistics v.20.0.0 software (IBM®).

2.3.2. Feeding phase

Consumption rates and weights of the amphipods were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). Three-way ANOVA followed by a Dunnett's post hoc tests were performed using SPSS® Statistics v.20.0.0 software (IBM®) to detect differences between treatments (Prochloraz, Fluoxetine and their interaction during the conditioning phase, and Fluoxetine during the feeding phase) and control.

Molting and survival were analyzed using χ^2 proportion tests with continuity correction using R v.3.2.3 (R Core team, 2015). Two-sided χ^2 proportion test were used to test differences in molting rates respect to the control, and a one-sided χ^2 proportion test were used to test if survival in the different treatments was lower respect to the control.

Generalized linear mixed-effects models (GLMM) were conducted on the velocity of each amphipod during the light periods of the DanioVision behavioral assays to test for the effects of the different treatments (Leaves treatment –prochloraz and/or fluoxetine–, and Water treatment –fluoxetine) and leaves consumption, both considered fixed effects, in the swimming velocity of *G. pulex* after the 14 d feeding experiment. The models were fitted including Subject ($n = 86$) as a random effect on the intercept to account for the repeated measures (within the same individual or subject). GLMMs have been described as the best tool for analyzing non-normal data that involve random effects, without the need for transforming them (Bolker et al., 2009; Lo and

Andrews, 2015). Models were fit by maximum likelihood (ML, Laplace approximation). The gamma distribution was chosen, which is appropriate for continuous, non-negative and heteroscedastic data with unimodal skewed distributions (Eberhardt et al., 1976; Flack et al., 2016; Ng et al., 2016), and a log link was used due to problems with convergence during the iteration process. This approach allowed us to use the mean values of velocity calculated every 10 s for the 4 light periods (Time 1, Time 2, Time 3, and Time 4) to test the effects of the different treatments in locomotive behavior. The R script is available in the Supplementary Material (Table S1). The dataset included a total of 3456 observations, 48 for each individual. GLMMs were conducted using the *glmmADMB* package (Bolker et al., 2012) for R v.3.2.3 (R Core team, 2015).

The significance of the fixed effect terms was assessed by starting with the full factorial model and then simplifying by removing non-significant terms identified using likelihood ratio tests. The likelihood ratio test was used to assess whether the implemented models significantly improved the fit to the data and, together with Akaike's Information Criterion (AIC), to select the most parsimonious models.

Marginal R squared (the variance explained by fixed effects), and conditional R squared (the variance explained by both fixed and random effects) values were calculated following Nakagawa and Schielzeth (2016, 2013).

3. Results

3.1. Conditioning experiment

3.1.1. Water analyses

At the beginning of the experiment the mean concentration of prochloraz in water was 6.08 ± 0.5 $\mu\text{g/L}$ (mean \pm SD) (treatments P and M), and the mean concentration of fluoxetine was 101 ± 5 ng/L (treatments F and M). Before water renewal, the concentration of prochloraz was 8.92 ± 4.72 $\mu\text{g/L}$ (treatment P), and the concentration of fluoxetine was 19.5 ± 2.5 ng/L (treatment F). For the M treatment, concentrations before water renewal were 5.06 ± 2.2 $\mu\text{g/L}$ for prochloraz, and 29 ± 7 ng/L for fluoxetine. In addition, prochloraz was detected in the water of the river (C) at very low concentrations (0.02 $\mu\text{g/L}$).

3.1.2. Leaf quantity and quality

The amount of leaf litter remaining after the 14 d conditioning period ranged from 62% for the prochloraz treatment to 82% for the fluoxetine treatment. Non-significant differences were detected among treatments (Fig. S1A).

The final fungal biomass after the 14 d was 134 ± 59 mg/g AFDW for the control, 61 ± 11 mg/g for the prochloraz treatment, 98 ± 22 mg/g AFDW for the fluoxetine treatment and 104 ± 40 mg/g for the mixture treatment. Significant differences were found among the control and the prochloraz treatment (ANOVA, $F_{1,16} = 4.55$, $p < 0.05$) (Fig. 2), no significant differences were found among the control and the fluoxetine treatment, and the control and the mixture treatment. There was a significant interaction of both compounds in the mixture (ANOVA, $F_{1,16} = 4.82$, $p < 0.05$).

C:N molar ratio at the end of the 14 d of colonization was 16.3 ± 2 for the control, 14.5 ± 0.4 for the fluoxetine treatment, 15.7 ± 0.6 mg/g for the prochloraz treatment and 14.9 ± 0.7 mg/g for the mixture treatments. No significant differences between treatments were detected (Fig. S1B).

3.2. Feeding experiment

Survival rates were all above 70% (Table 1). Molting rates ranged

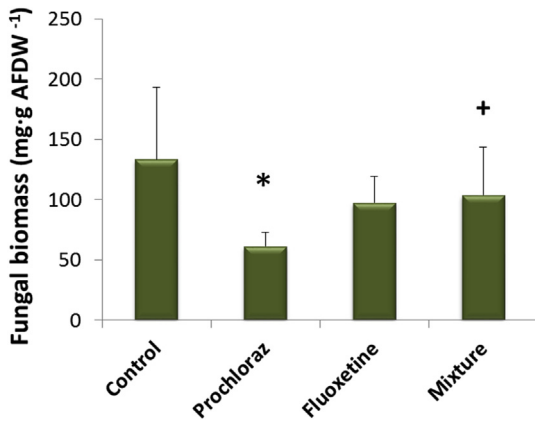


Fig. 2. Leaf fungal biomass measured at the end of the conditioning experiment (mean \pm SD). Asterisk indicates significant differences among leaf treatment (Prochloraz) and control (* $p < 0.05$). A significant interaction between prochloraz and fluoxetine was detected (Mixture) (+ $p < 0.05$).

between 10% and 67%. The amphipods fed with leaves conditioned under the prochloraz treatment, including the mixture treatment (PC, PF, MC, MF), presented a percentage of individuals that molted below 42%. The MF treatment presented the lowest molting rates, of 10%. No significant differences were found in molting (two-sided χ^2 proportion test, $p > 0.05$) or survival (one-sided χ^2 proportion test, $p > 0.05$).

Leaf consumption rates ranged between 0 and 0.49 mg AFDW $\text{mg}^{-1} \cdot \text{day}^{-1}$. Significant differences were detected among those amphipods fed with control leaves and those fed with prochloraz treated leaves (ANOVA, $F_{1,83} = 4.45$, $p < 0.05$), and fluoxetine treated leaves (ANOVA, $F_{1,83} = 11.46$, $p < 0.001$). In addition, there was a significant interaction (i.e., non additive effects) of both compounds in the mixture (ANOVA, $F_{1,83} = 18.67$, $p < 0.001$). No differences were detected due to the Water treatments (ANOVA, $F_{1,83} = 1.11$, $p = 0.30$) (Fig. 3).

No differences in the weights of the amphipods due to the feeding of prochloraz treated leaves (ANOVA, $F_{1,83} = 1.37$, $p = 0.25$), feeding of fluoxetine treated leaves (ANOVA, $F_{1,83} = 0.26$, $p = 0.61$), feeding of the mixture treated leaves (ANOVA, $F_{1,83} = 2.79$, $p = 0.10$), or waterborne exposure to fluoxetine (ANOVA, $F_{1,83} = 0.04$, $p = 0.84$) were found at the end of the experiment.

The mean values of velocity in the first cycle of treatment in the DanioVision chamber under light conditions for the control individuals were 5.6 ± 1.7 mm/s. The generalized mixed-effects model indicated that, under control conditions, the swimming velocity of *G. pulex* was positively influenced by leaf consumption and negatively influenced by time of exposure in the chamber (Table 2). Under the different treatment conditions both, fluoxetine and prochloraz (CF, FC, FF, PC, PF, MC, and MF) altered the swimming velocity of the amphipods with respect to the control.

Table 1
Survival and molting rates for the different treatments after the 14 d feeding experiment.

Treatment	% survival	% molting
CC	100	62
CF	85	45
FC	92	67
FF	92	58
PC	77	40
PF	100	38
MC	92	41
MF	77	10

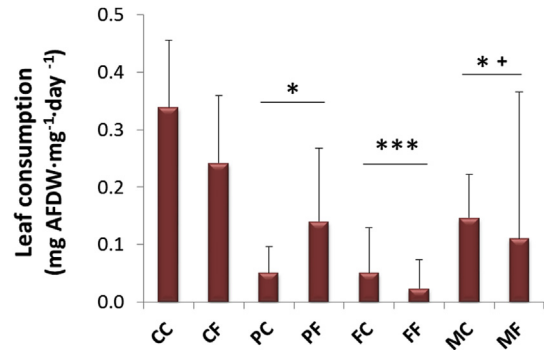


Fig. 3. *G. pulex* consumption of *A. glutinosa* leaves at the end of 14 d feeding experiment (mean \pm SD). Asterisks indicate significant differences among leaf treatments and control (* $p < 0.05$; *** $p < 0.001$). No significant differences were found between water treatment and control. A significant interaction between prochloraz and fluoxetine was detected (M treatment) (+ $p < 0.05$).

Specifically, the direct –waterborne exposure–to fluoxetine and the exposure during the leaf litter conditioning to fluoxetine or to prochloraz enhanced the velocity of the amphipods. In addition, there was a significant interaction between prochloraz and fluoxetine (conditioning phase), which modified the individual effects of the compounds, to the point that the individuals exposed to the mixture of both compounds (MC, MF), instead of having an increased swimming velocity (i.e., additive effects) respect to the individual treatments (FC, FF, PC, and PF), presented a lower swimming velocity (Table 2).

4. Discussion

Toxicity tests with individual compounds have been routinely performed to test for particular effects of a large diversity of compounds in different aquatic species since the mid-twentieth century. However, pollutants are present in the environment in complex mixtures (Altenburger et al., 2015; Tijani et al., 2016). Despite wide knowledge about the particular effects of pollutants in different species, little is known about the effects of mixtures of compounds at environmentally relevant concentrations. Antidepressants and fungicides are some of the compounds widely

Table 2

Selected generalized mixed-effects model for the swimming velocity of the amphipods under light cycles, after the 14d feeding experiment. The coefficient estimates, standard error (S.E.), t-values and p-values for fixed effects are shown. Subject was considered random effect.

Predictor	GLMM			
	Coefficient estimates (β)	S.E.	t-value	p-value
Intercept	1.153	0.174	6.62	<0.001***
Leaves treatment				
Fluoxetine (F)	0.535	0.2	2.67	<0.01**
Prochloraz (P)	0.471	0.195	2.41	<0.05*
Fluoxetine: Prochloraz (M)	-0.904	0.274	-3.3	<0.001***
Water treatment				
Fluoxetine (F)	0.271	0.124	2.18	<0.05*
Consumption	0.148	0.039	4.5	<0.001***
Time	-0.044	0.004	-9.9	<0.001***
Water treatment (F): Consumption	-0.092	0.0381	-2.41	<0.05*
R^2 marginal	12%			
R^2 conditional	43%			

detected in aquatic systems worldwide, especially in developed countries. Different studies provide evidence of the low dose effects of chronic direct exposure to fluoxetine in the behavior of several species (vertebrates and invertebrates) (Bossus et al., 2014; Campos et al., 2016; De Lange et al., 2009; H. J. De Lange et al., 2006a,b; Fong and Ford, 2014; Rivetti et al., 2015). In contrast, the effects of prochloraz on animals are still not very well known. There are some studies that report evidence of synergic interactions of this fungicide with other compounds (Bjergager et al., 2011; Cedergreen et al., 2006), but at higher concentrations than the one used in our study. Both compounds, prochloraz and fluoxetine, have been detected at low levels (ng/l, or µg/l) in surface waters (Cancappa et al., 2015; González Alonso et al., 2010; Kolpin et al., 2002; Kuzmanović et al., 2015; Metcalfe et al., 2003; Writer et al., 2013). In this study, we have detected behavioral effects (alterations in feeding and swimming velocity) of the exposure to low concentrations of the fungicide prochloraz (6 µg/L) and the antidepressant fluoxetine (100 ng/L) *G. pulex* after 14 days of experiment.

4.1. Effects on leaf conditioning

Although the exposure of the two compounds did not affect the breakdown rates of leaf litter, ergosterol concentration was lower in the prochloraz treatment with respect to the control. Ergosterol is an essential membrane sterol for fungi, and it is used as an indicator of fungal growth. Prochloraz affects the fungal community by inhibiting the cytochrome P450-monooxygenase lanosterol 14 α -demethylase, which is involved in the synthesis of ergosterol (Kahle et al., 2008; Lupetti, 2002). The low solubility and high hydrophobicity of prochloraz (Rütters et al., 1999), together with its weak basicity and lipophilic properties ($\log K_{ow} = 4.12$), could facilitate its interaction and adsorption to leaf surface and penetration into the leaf tissue (Farha et al., 2016; Lichiheb et al., 2016), and also facilitate the contact and interaction with the fungi, and the enzyme, leading to the consequent inhibition of fungal growth. However, these effects were not detected in the mixture treatment. The simultaneous exposure of fluoxetine and prochloraz may have led to the interaction of both compounds, decreasing the antifungal action of prochloraz.

Very few studies report the effects of fluoxetine or other SSRIs in natural microbial communities. Fluoxetine is known to have antibacterial activity against some groups of bacteria but at high concentrations (mg/L) (Munoz-Bellido et al., 2000). Toxicological tests on green algae report NOEC of <0.6 µg/L, EC50 values (48 h) of 24 µg/L, and LC50 of 2 mg/L (Fent et al., 2006; Oakes et al., 2010), which are values higher than the concentration used in our study (100 ng/L). In fact, we did not detect effects of fluoxetine in either leaf litter decomposition or fungal colonization.

4.2. Effects on *G. pulex*

Significant differences were detected among those amphipods fed with Control leaves (C) and those fed with prochloraz-treated leaves (P), but also with fluoxetine-treated leaves (F) and the mixture of both (M). The lower consumption rates of the individuals fed with P leaves could be related with the lower fungal colonization of these leaves. However, despite the lower quantity of fungi in the leaf tissues, the quality (C:N) of these leaves was not significantly different than the quality of the other leaves. Thus, indirect effects of prochloraz through leaf litter conditioning are not clear. Selective feeding (Arsuffi and Suberkropp, 1989; Bundschuh et al., 2009), feeding reduction (Agatz and Brown, 2014; Blockwell et al., 1998; Forrow and Maltby, 2000; Wilding and Maltby, 2006), and avoidance behavior (Bundschuh et al., 2011; De Lange et al., 2006a,b) in *G. pulex* have been described. Feeding

preference has been related with changes in the fungal community and thus in leaf quality (Forrow and Maltby, 2000), but it also can be related with the presence of chemicals and/or toxicity (Bundschuh et al., 2011; Hahn and Schulz, 2007; Zubrod et al., 2015). Notwithstanding, its specific mechanisms are unknown. Low consumption rates of the treated-leaves due to the detection of the compounds adsorbed in the leaves, and, thus, a repelling behavior of the amphipods cannot be discarded. Further research is needed in this direction.

Apart from inhibiting the cytochrome P450-monooxygenase lanosterol 14 α -demethylase, prochloraz, and also fluoxetine, can inhibit activities of other CYP enzymes. Recent studies have demonstrated that crustaceans do have CYP enzymes, and that these CYPs are involved in different functions, including detoxification of organic compounds, such as pesticides or pharmaceuticals (Chang and Thiel, 2015; Han et al., 2015; Trapp et al., 2014). For example, Dam et al. (2008) found that CYPs expression in crabs fluctuated over the molt cycle, with low expression during premolt and maximum during late postmolt, suggesting that premolt crabs would be more susceptible to organic pollution than postmolt crabs. The exposure to the fungicide and to the SSRI could have affected CYPs expression and, thus, could have impaired the detoxification mechanisms and made the amphipods more vulnerable. In our study, the prochloraz-treatments (PC, PF, MC, and MF) presented low molting rates, especially the mixture treatment (MF), but no significant differences respect to control were detected, possibly due to a low statistical power related with the sample size.

All the treatments altered the swimming velocity of *G. pulex*. The exposure to fluoxetine increased the velocity, and the simultaneous exposure to fluoxetine in water and to leaves conditioned with fluoxetine caused additive effects. Previous studies have detected altered swimming behavior in amphipods at very low concentrations (1–100 ng/L) (Bossus et al., 2014; Guler and Ford, 2010) and in cladocerans (Campos et al., 2016, 2012; Rivetti et al., 2015). Human targets for antidepressants are also present across vertebrates and 50–75% of them are found in crustaceans (Gunnarsson et al., 2008; Rivetti et al., 2015). Particularly in this group, serotonin regulates neurosecretory organs that release neurohormones that control several functions: reproduction, growth, maturation, immune function, metabolism, behavior and color physiology (Fong and Ford, 2014). As the concentration tested in our study was very low, the effects are likely to be related with a specific neurological response. The rationale of the increased velocities due to the exposure to the pollutants during the leaf conditioning phase is not so obvious. On one hand, the consumption rates of those amphipods fed with treated leaves were lower respect to control amphipods; and, on the other hand, the mixed-effects model revealed that the lower the consumption rates of the amphipods, the higher the swimming velocity during the light exposure. Combining the reported effects in the feeding rates with the effects in the locomotion, the low consumption rates of the gammarids fed with treated leaves could explain their increased velocities due to a general response to stress. In relation to the stress caused by the exposure to prochloraz, the main exposure route to the fungicide was at first assumed to be through leaf ingestion, but, taking into account its physicochemical properties, other exposure route cannot be discarded. A feasible explanation could be that prochloraz could have been adsorb onto the leaf tissue and a part of it could have been released during the feeding experiment and could have affected the gammarids.

In addition to these individual effects of the antidepressant and the fungicide, the mixed-effects models revealed that the simultaneous exposure of both compounds lead to opposite effects than the individual exposure: a remarkable decrease in the swimming

velocity. As has been previously mentioned, the combined exposure to both compounds (M treatment) did not affect the fungal colonization of the leaves. All these results suggest that there is some kind of chemical interaction and/or an interaction in their mode of action (e.g., interaction with the CYP detoxification system) that could modify their individual or particular toxic effects.

In summary, low dose effects of prochloraz and fluoxetine in *G. pulex* molting, feeding behavior, and locomotive behavior were detected at low concentrations. The results of the current study reinforce three main ideas: (i) the importance of including low-dose toxicity testing in environmental risk assessment, especially for those substances with potential specific toxicity (e.g., SSRIs); (ii) the exposure to contaminants may lead to unexpected direct or indirect toxic effects and thus should be further considered in risk assessment; and finally, (iii) that behavioral responses (feeding and locomotion) seem to be reliable and appropriate tools to detect sub-lethal impacts on individual organisms with potential relevance at higher organizational levels (i.e. community and ecosystem).

Acknowledgements

The present work was funded by the EU Seventh Programme through the Globaqua project (grant agreement No 603629). We want to thank all those who assisted us in the field and helped us to obtain data: Neil Fuller, Meritxell Abril, Pablo Rodríguez, Patricia Rodrigo and Olga Jáuregui (CCIT-UB). Núria De Castro-Català holds a predoctoral grant from the University of Barcelona. Alex Ford is supported by the EU Interreg program - Peptide Research Network of Excellence (PeReNE).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.07.088>.

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