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Effects of the insecticide fipronil in freshwater model organisms and microbial and periphyton communities

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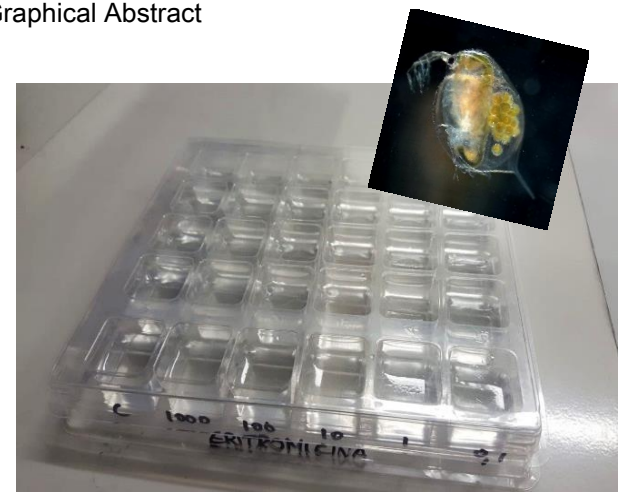
Abstract:	Fipronil is a broad-spectrum insecticide whose release in the environment damages many non-target organisms. This study evaluated the toxicity of fipronil at two biological levels using in vivo conditions and environmentally relevant concentrations: the first based on two model organisms (aquatic invertebrate Daphnia magna and the unicellular freshwater alga Chlamydomonas reinhardtii) and a second based on three natural communities (river periphyton and freshwater and soil microbial communities). The physicochemical properties of fipronil make it apparently unstable in the environment, so its behaviour was followed with high performance liquid chromatography (HPLC) under the different test conditions. The most sensitive organism to fipronil was D. magna, with median lethal dose (LC50) values from 0.07 to 0.38 mg/L (immobilisation test). Toxicity was not affected by the media used (MOPS or river water), but it increased with temperature. Fipronil produced effects on the photosynthetic activity of C. reinhardtii at 20°C in MOPS (EC50 = 2.44 mg/L). The freshwater periphyton presented higher sensitivity to fipronil (photosynthetic yield EC50 of 0.74 mg/l) in MOPS and there was a time-dependent effect (toxicity increased with time). Toxicity was less evident when periphyton and C. reinhardtii tests were performed in river water, where the solubility of fipronil is poor. Finally, the assessment of the metabolic profiles using Biolog EcoPlates showed that bacteria communities were minimally affected by fipronil. The genetic identification of these communities based on 16S rRNA gene sequencing revealed that many of the taxa are specialists in degrading high molecular weight compounds, including pesticides. This work allows us to better understand the impact of fipronil on the environment at different levels of the food chain and in different environmental conditions, a necessary point given its presence in the environment and the complex behaviour of this compound.
Response to Reviewers:	ANSWER REVIEWERS Reviewers/Editor comments: Reviewer #3: I am glad that the authors accepted my suggestions to make the text more concise and direct. I believe that all the questions that I sent to them have also been satisfactorily answered, and the explanations have been inserted in the text. Thus, I believe that the article can be accepted for publication. Autor Answer: Thank you very much

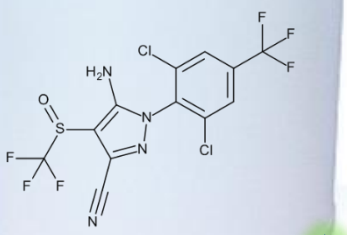
*Graphical Abstract



Daphnia Magna

Acute toxicity

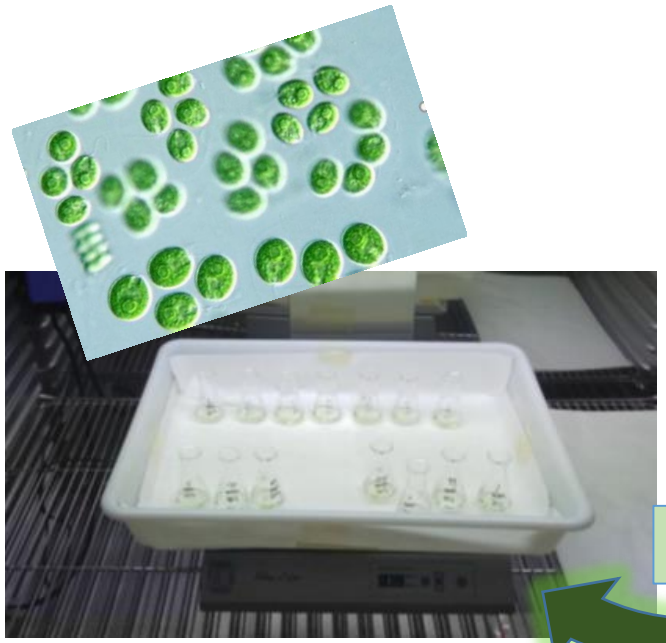
Fipronil



Periphyton communities

River microbial community

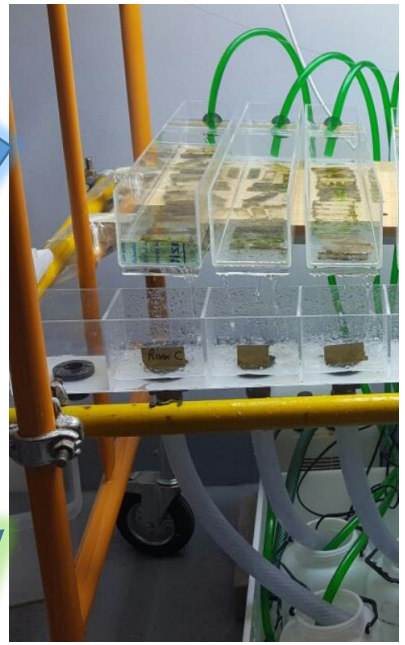
Soil microbial community



Chlamydomonas reinhardtii

Photosyntesis yield

Acute toxicity



Community-level physiological profiling: Biolog Ecoplate

*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

HIGHLIGHTS

- Fipronil produces the greatest effect on *D.magna* which increases with temperature
- Fipronil affects the photosynthetic yield of *C. reinhardtii* at 20ºC
- The freshwater periphyton are sensitive to fipronil in a time-dependent manner
- River water medium decreases fipronil toxicity to *C. reinhardtii* and periphyton
- Metabolism of aquatic and soil bacteria communities is little affected by fipronil

Effects of the insecticide fipronil in freshwater model organisms and microbial and periphyton communities

ABSTRACT

Fipronil is a broad-spectrum insecticide whose release in the environment damages many non-target organisms. This study evaluated the toxicity of fipronil at two biological levels using *in vivo* conditions and environmentally relevant concentrations: the first based on two model organisms (aquatic invertebrate *Daphnia magna* and the unicellular freshwater alga *Chlamydomonas reinhardtii*) and a second based on three natural communities (river periphyton and freshwater and soil microbial communities). The physicochemical properties of fipronil make it apparently unstable in the environment, so its behaviour was followed with high performance liquid chromatography (HPLC) under the different test conditions. The most sensitive organism to fipronil was *D. magna*, with median lethal dose (LC_{50}) values from 0.07 to 0.38 mg/L (immobilisation test). Toxicity was not affected by the media used (MOPS or river water), but it increased with temperature. Fipronil produced effects on the photosynthetic activity of *C. reinhardtii* at 20°C in MOPS (EC_{50} = 2.44 mg/L). The freshwater periphyton presented higher sensitivity to fipronil (photosynthetic yield EC_{50} of 0.74 mg/l) in MOPS and there was a time-dependent effect (toxicity increased with time). Toxicity was less evident when periphyton and C. *reinhardtii* tests were performed in river water, where the solubility of fipronil is poor. Finally, the assessment of the metabolic profiles using Biolog EcoPlates showed that bacteria communities were minimally affected by fipronil. The genetic identification of these communities based on 16S rRNA gene sequencing revealed that many of the taxa are specialists in degrading high molecular weight compounds, including pesticides. This work allows us to better understand the impact of fipronil on the environment at different levels of the food chain and in different environmental conditions, a necessary point given its presence in the environment and the complex behaviour of this compound.

1. INTRODUCTION

Fipronil is a widely used, broad-spectrum phenylpyrazole insecticide that can control many insects, including cockroaches, mosquitoes, locusts, termites, thrips, rootworms, ticks and fleas, at both larval and adult stages (Gunasekara, et al. 2007). Moreover, it is used as a plant protection product as well as a veterinary drug (reviewed by (Wang, et al. 2016). Fipronil has gained popularity throughout the world for pest management, including agricultural and urban environments due to its effectiveness against insects that are resistant to other agents, such as pyrethroids, organophosphates and carbamates (Bobe, Coste, and Cooper 1997).

Fipronil inhibits Gamma-Aminobutyric acid (GABA) receptor chloride channels, disrupting normal neuronal influx and resulting in the accumulation of GABA at synaptic junctions which causes hyperexcitation of the nervous system, severe paralysis and, finally, death (Wang, et al. 2016; Gunasekara, et al. 2007).

Fipronil is a chiral molecule; each enantiomer presents different toxicity (Qu, et al. 2014). It is unstable in the environment because it is affected by UV radiation (Mianjy and Niknafs 2013), pH (Ramesh and Balasubramanian 1999) and temperature (Ma, et al. 2012). It is biodegradable (Peret, et al. 2010; Hussain, et al. 2016), and some of its transformation products show enhanced toxicity against non-target organisms compared to the parent compound (Michel, et al. 2016). Reports have indicated that temperature influences the toxicity of insecticides on target organisms (Ma, et al. 2012), although the effects are irregular.

Due to its wide use, fipronil is currently present in soils and surface and ground waters. This distribution poses risks to the environment and provokes undesirable effects on non-target organisms as bees (Kiljanek, Niewladowska, and Posyniak 2016; Lourenco, et al. 2012; Sanchez-Bayo, et al. 2016), reptiles (Maute, et al. 2015; Peveling and Demba 2003), birds (Kitulagodage, et al. 2011), mammals (Szegedi, et al. 2005; Roques, et al.

2012; de Oliveira, et al. 2012; Khan, et al. 2015) and soil microflora (Ahemad and Khan 2011a).

The presence of fipronil in different aquatic environments has been reported around the world (Tousova, et al. 2017; Wu, et al. 2015; Maruya, et al. 2016; Toan, et al. 2013; Delgado-Moreno, et al. 2011). The fipronil concentration in freshwaters ranges from 0.5 (Michel, et al. 2016) up to 2000 ng/L (Ensminger, et al. 2013). Gan (Gan, et al. 2012) found a median concentration of 204−440 ng/L for fipronil and its derivatives in urban residential runoff. In addition, wastewater treatment plants have shown fipronil concentrations as large as 1388 ng/L (Sadaria, et al. 2017) and 12–31 ng/L (Supowit, et al. 2016). Fipronil has also been occasionally detected in drinking water (in 95% of collected samples), with median concentrations of 40 ng/L (Toan, et al. 2013) and in groundwater irrigated fields at a maximum concentrations of 3440 ng/L (da Silva, et al. 2011).

Based on these data, fipronil can reach aquatic environments (Garrison 2011), and direct or indirect effects on aquatic organisms can be expected. Fipronil toxicity has been assessed in different freshwater organisms (Gripp, et al. 2017; Schlenk, et al. 2001; Bedient, et al. 2005; Beggel, et al. 2012), including small aquatic invertebrates, such as oligochaetes (Liu, et al. 2012) and cladocerans (Hayasaka, et al. 2012b; Iwafune, et al. 2011). Less information can be found in the literature regarding the effect of fipronil on phytoplankton (Overmyer, et al. 2007; Qu, et al. 2014). All these studies using individual species have limited environmental relevance because they only partially show the impact on biological communities (Hayasaka et al., 2012a; Møhlenberg et al., 2001). Communities form groups or associations of different populations or species that occupy the same geographic area at the same time and that usually have the ability to resist (resistance) and return (resilience) to change, so they are realistic indicators of environmental impacts. Much less information can be found in the literature regarding the effect of fipronil on aquatic communities, despite the importance of such information for obtaining a realistic environmental impact assessment. As far as we know, the only

study available demonstrated the cumulative impacts of fipronil on aquatic communities – paddy mesocosms – with a significant decrease in the abundance of benthic organisms during both years in insecticide-treated fields (Hayasaka, et al. 2012a).

Therefore, it is necessary to determine if the doses at which fipronil presents toxicity not only in individual organisms but also in natural communities are in the range of those that have been described for this insecticide in aquatic and edaphic environments.

Our hypothesis was that fipronil would not only present toxic effects in model organisms but would also be capable of affecting natural water and soil communities which would more realistically demonstrate the risk of this insecticide for the environment.

Therefore, in this study we evaluate the ecotoxicity of fipronil at two biological levels: first, two model organisms (aquatic invertebrate *Daphnia magna* and the unicellular freshwater alga *Chlamydomonas reinhardtii*) and second, three natural communities (river periphyton and freshwater and soil microbial communities). We utilise the following endpoints: acute toxicity tests (*D. magna*), photosynthetic yield reduction (*C. reinhardtii* and periphyton communities), and the ability of aquatic and soil microbial communities to degrade different carbon sources. To increase the environmental relevance of the assessment, the effect of temperature and different assay media is included as a modulating factor for fipronil toxicity.

2 Material and methods

2.1 Fipronil

Fipronil ($C_{12}H_4Cl_2F_6N_4OS$, CAS 120068-37-3) was purchased from Cymit Chemical S.L., with a minimum purity of 97.0% and a molecular weight of 437.14.

2.2. *D. magna* assay

D. *magna* (water flea) assays were performed in accordance with OECD 202 (2004) guidelines and following the standard operational procedures of the Daphtoxkit F™

111 magna (1996), purchased from Vidrafoc Daphtoxkit (number DM121219) and stored at
112 4°C.
113 A TOXKIT incubator (ECOTEST, model CH-0120D-AC/DC) was used for *D. magna* egg
114 incubation for 72 h at 20–22°C with 6,000 lx light. The neonates (22 h old) were pre-fed
115 with one vial of spirulina microalgae. After 2 h of feeding, D. magna were exposed to the
116 following fipronil concentrations: 10, 50, 100, 250 or 500 µg/L, to cover ranges described
117 in previous studies (Hayasaka, et al. 2012b; Iwafune, et al. 2011,) USEPA, 2000). There
118 were five replicates with five organisms per concentration. This procedure was replicated
119 for six plates, three of which contained synthetic freshwater provided by the Daphtoxkit
120 for the negative control and for dilutions. The other three plates used water collected
121 from the Gallego River, a tributary of the Ebro River (northeast Spain) in Villanueva de
122 Gállego (Zaragoza, Spain) on 16 July 2016. The water samples were transported to the
123 laboratory in less than 15 min and stored at 4°C until use. The physicochemical
124 characteristics of this river water are provided in Table 1. The pH was adjusted to a range
125 between 7 and 8 in all cases, using NaOH. Plates were incubated from 18 to 22°C,
126 according to OECD 202 (2004) guidelines. However, to study the effects of temperature
127 on fipronil toxicity, plates with both synthetic and River Gallego water were incubated in
128 complete darkness for 24 h at 18, 23 or 25°C. After a 24-h exposure, immobilised
129 individuals (unable to swim for 15 s after gentle agitation of the test vial) were checked
130 and counted. The results were calculated as the EC_{50} (chemical concentration resulting
131 in 50% immobilisation).
132
133 **2.3 *C. reinhardtii* assay**
134 Unicellular green algae *C. reinhardtii* (CC125) were cultivated in standard growth
135 medium (Szivak, Behra, and Sigg 2009); the pH was adjusted to 7.5. Algae were
136 maintained in the exponential growth phase and under controlled conditions (90 rpm,
137 130 µE photosynthetically active radiation (PAR) m^{-2} s^{-1} between 23 and 26°C. The
138 exposure medium comprised river water collected on 29 September 2017 from the

Gallego River (Table 1) or standard buffer solution, namely 0.01 nM 3-(N-morpholino)-propanesulfonic acid (MOPS) (CAS 1132-61-2; purity ≥ 99%; Merck). The water samples were transported to the laboratory in less than 15 min and stored at 4°C until use. Before testing, the water samples were kept under agitation with strong magnetic stirring to ensure oxygenation. In addition, the following measures were made *in situ* at the river: conductivity (2680 µS/cm), dissolved oxygen (4.4 mg/L O_2 and % sat, 52.4 mg/L O_2), pH (7.6) and water temperature (22.3ºC).

Later, algae were centrifuged (10 min, 3000 rpm) and adjusted to an optical density (OD) of 0.15 for the test suspensions. The OD was measured by a spectrophotometer (λ 685 nm). After pre-tests to adjust the concentration range, the fipronil concentrations (0.25, 0.5, 0.75, 1, 1.5 or 2 mg/L) were tested under similar light and shaking conditions as those during growth. The pH of the solutions was checked at the beginning and end of the measurements. Experiments were performed at 20, 25 or 30°C. The algal photosynthetic yield of photosystem II was measured using a mini-PAM fluorometer (Walz, Effeltrich, Germany), as previously described (Pino, et al. 2016). Each concentration was tested in triplicate. EC_{50} values (photosynthetic yield) after 6-h exposures were calculated.

2.4 Periphyton communities assay

2.4.1 Colonisation

Periphyton communities were obtained from the Gallego River (Zaragoza, Spain). Artificial substrates, consisting of a flat, heavy rock equipped with methacrylate racks containing 24 microscope slides, were placed under water (10–15 cm depth) in May 2017. Periphyton reached an average thickness of 0.75 mm. This methodological approach has been developed and tested in a previous study (Navarro, Guasch, and Sabater 2002).

2.4.2. Periphyton characterisation and water analysis

After the colonisation period, periphyton-colonised slides were transported to the laboratory, and one slide was prepared for taxonomic identification. Diatom frustules were obtained by oxidation with hydrogen peroxide and were mounted on permanent slides with Naphrax resin. Cell count and identification were performed using a Leica light microscope at 1000X total magnification (diatoms) or 100, 400 and 1000X total magnification (other microalgae). Results are expressed as the number of individuals per cm^2 of biofilm as well as density (number of individuals per mL). The river water was measured at the start of colonisation, 15 days later and at the end of colonization. Substrates were collected with a sample of river water (Table 1). Water samples were also analysed for chlorophyll *a*, *b* and *c*. The Trophic State and Margalef Indexes were calculated.

2.4.3. Dose and time response curves in flow-through artificial channels

Flow through methacrylate channels (90 cm long and 10 cm wide) connected to separate water reservoirs was used for dose-response experiments with periphyton (Fig. 1). Reservoirs were submerged in a thermostatic bath at 23°C. Aquarium pumps re-circulated the water from the reservoirs through every channel at 0.113 m^3/h. Every reservoir contained 4 L of water. Light was provided by fluorescence lamps (Blau Aquaristic T5HO: 39 W, 10,000°K and 80 µmol photon m^{-2} s^{-1} at the channel surface). Slides colonised by periphyton were placed horizontally on the bottom of the flow-through channels (Fig. 1). The effect of fipronil on the photosynthetic efficiency of the periphyton was evaluated as described by (Val, et al. 2016), using a portable pulse amplitude modulation fluorometer (MINI-PAM, Walz). The yield reflects the efficiency of the photochemical energy conversion process (Consalvey, et al. 2005).

The dose-response experiment was designed using 0.1, 0.25, 0.75, 1 and 2 mg/L of fipronil according to pre-tests. Toxicity was evaluated at these concentrations in two different media to assess the possible effect of chemical composition of river water: the river water or buffer solution (MOPS), adjusted to a pH of 7.5 using KOH. One channel

with river water and another with MOPS, both without fipronil, were used as the negative controls. The photosynthetic efficiency of the periphyton was measured in triplicate after 1 and 13 h. Three slides of periphyton were placed in each channel.

In addition to the dose-response experiment, a time-response curve with regard to the effect of fipronil on periphyton was performed. For this endeavour, one periphyton slide was placed in the flow-through artificial channels with river water and the other with MOPS; both were exposed to 2 mg/L fipronil (the maximum soluble concentration in water). The photosynthetic efficiency was measured every 30 min for 24 h.

2.5 Water and soil microorganisms assays

2.5.1. Water samples

Water samples were collected on 17 October 2017 from the Gallego River and transported to the laboratory according to standard procedures. The physicochemical characteristics of this water are provided in Table 1. In addition, conductivity (2340 µS/cm), dissolved oxygen (11.1 mg/L O_2 and % sat, 128.2 mg/L O_2), pH (8.1) and temperature (22.1ºC) were measured *in situ*. For genetic analysis, microorganisms were extracted from 1 L of the river water that was filtered through a 0.22 µm filter, resuspended in a Falcon tube with 10 mL Milli-Q water, centrifuged at 5,000 *g* and stored at -80°C until sequencing.

2.5.2 Soil samples

The soil was obtained on 30 October 2017 from a crop field free of pesticides or other contaminants (Montañana, Zaragoza, Northeast Spain). Soil was sieved at < 2 mm and stored at 4°C until use. The texture of the soil was: 37.3% sand, 24.7% silt and 38.0% clay; the content of organic matter was 3.8%; and the pH was between 7.8 and 8. Microbes were extracted from 20 g of the soil, to which 100 mL of sterile water was added. In Falcon tubes with 10 ml Milli-Q water, they were sonicated for 1 min and

centrifuged at 1000 *g* for 10 min. The supernatants were collected aseptically. The process was repeated five times. The portion of the resulting supernatant containing the soil microorganisms was stored at -4ºC to be assayed in the Biolog EcoPlate. The remaining liquid was filtered with a 0.2 µm filter with a vacuum kitasato; the filter content was carefully washed with Milli-Q water and centrifuged at 5000 *g* for 10 min. The supernatant was removed with an eyedropper and the pellets were stored at -80°C until sequencing.

2.5.3. Genetic sequencing of river and soil microorganisms

To better interpret the effect of fipronil on the metabolism of microbial communities, it is necessary to know its taxonomic composition and the predominant taxa. For this, the most accurate tool is genetic sequencing.

Genetic sequencing of microorganisms in Gállego River water samples and soil was performed in the Genomics Unit Cantoblanco, Science Park (Madrid, Spain). Bacterial genomic DNA from the samples (previously homogenised in phosphate-buffered saline [PBS]) was extracted from 200 µL aliquots after proteinase K and RNAse digestion using G-spin columns (INTRON Biotechnology, South Korea). Quant-IT PicoGreen reagent (Thermo Fischer, EEUU) was used to determine DNA concentrations. DNA samples were used to amplify the V3-V4 region of the 16S ribosomal RNA (rRNA) gene, as previously described (Caporaso, et al. 2011; Caporaso, et al. 2012; Pino-Otin, et al. 2019).

Polymerase chain reaction (PCR) products included extension tails that allowed sample barcoding. Amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent, EEUU), and the concentration was estimated by real-time PCR (Kapa Biosystems, Hoffmann-La Roche Switzerland). Later, DNA samples were sequenced on an Illumina MiSeq Instrument under a 2 x 300 protocol. Finally, reads were quality filtered according to Illumina standard values, demultiplexed and fastq files were mapped against the GreenGenes database using current applications of Base Space (16S Metagenomics,

Illumina). In the run, 94.4% of the 137,961 total reads for water microorganisms passed quality filtering and 93.5% of the 154,842 total reads for soil microorganisms passed quality filtering.

2.5.4. Microbial metabolism: ability to degrade different carbon sources

The ability of water microbial community to utilise several carbon sources after fipronil exposure was analysed with a Biolog EcoPlate test that contains 31 different carbon sources plus a blank (water) in triplicate. Fipronil dilutions in river water containing water microorganisms (0.1 or 1 mg/L) were prepared in a final volume of 150 µL in Biolog plate wells. Regarding soil microorganisms, soil particles were removed from the supernatant containing the soil microorganisms (section 2.5.2) by low-speed centrifugation at 500 *g* for 2 min before inoculating the Biolog plates. Then, the supernatant was added to each well and the same fipronil dilutions (0.1 or 1 mg/L) were prepared in a final volume of 150 µL. Each concentration was tested in triplicate. All manipulations were performed under sterile conditions in a flow chamber. The plates were incubated in the dark at 20, 25 and 30°C for 7 days under sterile conditions. The final pH for dilutions was between 7.7 and 8.1 and river water was strongly agitated with magnetic stirring before testing to ensure oxygenation.

The Optical Density (OD, λ 590 nm) of each well was measured just after inoculation and once a day using an Anthos 2010 microplate reader and ADAP 2.0 software (Biochrom, Ltd., Cambridge, England), as previously described (Muniz, et al. 2014; Pino-Otin, et al. 2019). The rate of utilisation of the carbon sources was thus assessed as the reduction of tetrazolium violet redox (Pohland and Owen, 2009).

2.6 High performance liquid chromatography (HPLC) analysis

The conditions studied in the ecotoxicity tests (type of medium, temperature and time) were analysed through HPLC, to monitor the behaviour and solubility of fipronil. One-hundred mL solutions were prepared for each studied condition. The same river water

samples used in the ecotoxicity tests for *D. magna, C. reinhardtii*, water microorganisms and periphyton communities (see Table 1) were prepared, as well as MOPS and synthetic freshwater solutions (same than *Daphnia* test). Along with fipronil, ethiprole was added as an internal standard (0.2 mg/L of each). An ultrasonic bath was used for the dissolution of the products. Solutions were prepared in triplicate at the different temperatures tested in the ecotoxicity tests and kept in darkness.

All samples were analysed using an Agilent 1100 HPLC unit coupled to the Bruker MicroTOF-Q high resolution mass spectrometer equipped with a Poroshell 120 column, with Q-TOF hybrid analyser and electrospray (APCI and APPI) systems. As a mobile phase, a ratio of 60/40 (acetonitrile/water) was used. Ethiprole and acetonitrile (HPLC quality) were purchased from Scharlab. Samples were analysed with an isocratic mobile phase with a flow of 1 mL/min. Five µL of each solution was injected into the equipment.

2.7 Statistics and graphical representation

Dose-response curves for *D. magna* mobility and *C. reinhardtii* and periphyton community photosynthetic yield were calculated with a logit logistic regression using XLSTAT (2014.5.03) software to obtain the corresponding EC_{50} values and standard errors (SE). Dose-response models were statistically tested using a chi-square test. Later, *t*-tests were performed to compare parameters (i.e. EC_{50}) of dose-response curves using R statistical software, namely the CompParm function from the drc package.

The microbial activity of each Biolog EcoPlate microplate was expressed as the average well colour development (AWCD) and determined according Garland and Mills (1991) and a previous study (Pino-Otin, et al. 2019) as follows:

$$AWCD = \sum_{i=0}^{i=12} \left(OD_{t=x_i} - OD_{t=x_0}\right) \qquad [1]$$

where OD_i is the optical density value from each well at any given time, after subtracting $OD_{t = X0}$ from $OD_{t = Xi}$ of that well. The variance relationship between AWCD values of the

three replicates and Student's independent sample *t*-tests were used to assess significance using XLSTAT software (2014.5.03).

For HPLC experiments, peak area data were obtained by integrating the fipronil and ethiprole peaks with the Data Analysis 4.2 (Bruker) programme in order to take advantage of the direct relationship that exists between the integration of the peak area and the concentration of the solution.

3. Results

3.1. Effects of fipronil on *D. magna*

D. magna mortality increased with temperature in both types of media (Fig. 2). Toxicity was slightly higher in river water compared to standard water at 18°C, but it was similar at the other two temperatures. The t tests showed the greatest differences between river water at 18°C versus standard water at 25°C (p=0,054) and river water at 25°C versus standard water at 18°C (p=0.068). In the case of synthetic water, fipronil median lethal concentration (LC_{50}) values (in mg/L) were 0.38 at 18°C, > 0.10 at 23°C, > 0.07 at 25°C. For river water, the LC_{50} values were 0.16 at 18°C, > 0.1 at 23°C, > 0.07 at 25°C; see Fig. 2 for confidence limits.

Notably, fipronil underwent oscillations in its solubility over 24 h depending on the temperature and the type of solvent (Fig. 2). In the case of *Daphnia* standard water, fipronil solubility increased after 24 h, especially at 23 and 25°C. In the case of river water, the opposite occurred: the solubility of fipronil decreased at 24 h, especially at 23 and 25°C. Fipronil solubility was also checked in distilled water; it presented very similar results to those obtained with the standard *Daphnia* water (data not shown).

3.2. Effects of fipronil on *C. reinhardtii*

C. reinhardtii photosynthetic activity was affected by fipronil, but only at 20°C and only in MOPS buffer (Fig. 3). LC50 and LC_{10} values for MOPS at this temperature were 2.44

mg/L (95% confidence interval [CI] 1.97–3.32) and 0.12 mg/L (95% CI 0,07–0,18), respectively, according chi-square test.

No effects could be detected in the other experimental conditions: MOPS at 25ºC and 30°C or any conditions in river water.

Fipronil solubility at 20°C in river water and MOPS at the beginning of the experiment and after 6 h was analysed through HPLC (Fig. 3, down). Under these conditions, fipronil solubility in MOPS buffer showed a slight decrease after 6 h.

3.3. Effects of fipronil on periphyton communities

3.3.1. Periphyton community analysis

Algae from three phyla (Dinophyta, Crypotophyta and Chlorophyta) and the subclass Bacillariophyceae, together with the photosynthetic bacterial phylum Cyanobacteria, were identified (Fig. 4). Bacillariophyceae presented the greatest species diversity (17). The most common species (in decreasing order) were *Achnanthes lanceolata*, *Amphora veneta*, *Cymbella silesiaca* Bleisch, 1864 and *Achnanthidium minutissimum*. Among the green algae chlorophytes, there were up to seven different species, the most abundant being *Scenedesmus* sp. There were small differences in the composition and dominance of species among the 10 studied samples (one from each experimental condition), although most species were present in all samples (Supporting Information 1).

3.3.2 Fipronil dose-response curves

The evolution of the photosynthetic yield of the river periphyton when exposed to different doses of fipronil after 1 or 13 h in river water and MOPS is presented in Fig. 5. In MOPS, there was a clear decrease in photosynthetic yield as the fipronil dose increased, especially after 13 h (Fig. 5a); however, the effect was lower in river water (Fig. 5b).

The LC_{50} and LC_{10} values for MOPS were 0.74 mg/L (95% CI 0.62–0.89) and 0.02 mg/L (95% CI 0.01–0.04), respectively (Fig. 6).

361

362 **3.3.3 Assessing the influence of time in fipronil toxicity**

363 Time clearly increased fipronil toxicity in MOPS (EC_{50T} = 10 hours), but again, there was
364 no toxicity in river water. Fig. 7 shows periphyton photosynthetic yield changes over time
365 after exposure to 2 mg/L of fipronil in MOPS (blue line) and river water (red line) over 24
366 h.

367

368 **3.3.4 Fipronil solubility in river water and MOPS**

369 Fipronil presented higher solubility in MOPS compared to river water at 23ºC, but it
370 decreased progressively up to 24 h. The solubility in river water tends to increase (Fig.
371 5c).

372

373 **3.4. Effects of Fipronil on water and soil microorganisms**

374

375 **3.4.1. Effect of fipronil in microorganisms' metabolism**

376 There are hardly any differences in the ability of microorganisms to degrade the different
377 carbon sources present in the EcoPlate, regardless of the presence of fipronil (Fig. 8,
378 river water and soil microorganisms are shown in the first and second columns,
379 respectively). Differences from control due to temperature are also very small (all p
380 values were between 0,08-0,9).
381 Fipronil solubility was maintained throughout the 96-h assay in both solvents, river water
382 and MOPS (See HPLC graphs, Fig.9), although solubility fluctuated and increased with
383 time in both media (central figure, Fig. 9).

384

385 **3.4.2 Genetic identification of microbial populations**

386 River microorganism sequencing comprised 165,548 total reads, of which 156,405
387 passed filter of quality (94.5%). Each level of organisation was successfully sequenced:
388 > 94% phyla, 65% family, 61.29% genus and 28.3% species. Soil microorganism

389 sequencing involved 209,275 reads, of which 196,682 passed filter of quality (90%). All
390 levels of organisation were optimally sequenced (> 95%) except for genus (sequenced
391 in 41% of microorganisms). The chart in Fig. 10 and Support information 2 show the per
392 cent taxa abundance for the different taxonomic levels for water and soil microorganisms
393 within each taxonomic level.
394
395 **3.4.2.1 Water microorganisms**
396 There were three predominant water bacteria phyla: Proteobacteria (41.19%),
397 Cyanobacteria (32.07%) and Bacteroidetes (10.95%). A small number of bacterial reads
398 (8.97%) could not be identified, indicating the extent of novel sequences in this study.
399 Proteobacteria can be classified in alpha, beta, gamma and delta families based on 16S
400 rRNA. We found the four classes: Gammaproteobacteria (36.99% of Proteobacteria,
401 14.25% of the total taxa), Alphaproteobacteria (28.50% of Proteobacteria; 10.98% of
402 total taxa), Betaproteobacteria (19.91% of Proteobacteria; 7.67% of total taxa) and
403 Deltaproteobacteria (11.25% of Proteobacteria; 4.33% of total taxa).
404 The most abundant order among Gammaproteobacteria was Xanthomonadales (34.32%
405 of Proteobacteria; 4.89 % of total taxa) and the order Rhodobacterales was the
406 predominant one among Alphaproteobacteria (53.05% of the Alphaproteobacteria;
407 5.82% of total taxa).
408 Among Betaproteobacteria, the dominant order was Burkholderiales (69.46% of the
409 Betaproteobacteria; 5.33% of total taxa). Finally, Myxococcales was the predominant
410 order (48.97% of the Deltaproteobacteria; 2.12% of total taxa) among
411 Deltaproteobacteria.
412 Virtually all Cyanobacteria members of our samples were from class
413 Oscillatoriophycideae (91.33% of Cyanobacteria reads; 27.40% of total taxa) and all the
414 bacteria in this class are of the order Chroococcales. Beyond the order, it has been
415 difficult to classify.

We found the class Sphingobacteriia as the most abundant among Bacteroidetes phylum (76.07% of the Bacteroidetes; 7.79% of total reads).

3.4.2.2 Soil microorganisms

In soil samples, Proteobacteria were also the main bacterial phylum (79,91%), followed distantly by Bacteroidetes (10.40%) and Actinobacteria (6.77%).

Gammaproteobacteria (44.63% of Proteobacteria, 34.42% of the total taxa) was also the predominant Proteobacteria class, followed by Betaproteobacteria (24.36% of Proteobacteria; 17.69% of total taxa), Alphaproteobacteria (21.58% of Proteobacteria; 15.67% of total taxa) and Epsilonproteobacteria (8.92% of Proteobacteria; 6.48% of total taxa).

Among the Gammaproteobacteria class, virtually all belonged to the order Pseudomonadales (98.80% of Gammaproteobacteria, 32.03% of the total taxa). All of those microorganisms belonged to the family *Pseudomonadaceae* and the genus *Pseudomonas*.

Betaproteobacteria was almost entirely comprised of the order Burkholderiales (96.45% of Betaproteobacteria, 17.07% of the total taxa). The main family was *Oxalobacteraceae* (80.26% of Burkholderiales, 13.7% of total reads), and among them, *Janthinobacterium* was the predominant genus (91.23% of *Oxalobacteraceae*, 12.50% of total reads)

The main order of Alphaproteobacteria was Sphingomonadales (59.91% of Alphaproteobacteria, 9,39% of total reads), all of them from the family *Sphingomonadaceae*.

The class Sphingobacteriia predominated among Bacteroidetes (82.32% of Bacteroidetes, 7.80% of total reads).

4. Discussion

Daphnia magna has the lower LC_{50} values among the organisms tested in this study, an expected outcome given the nature and mode of action of the fipronil, with an LC_{50} from

0.07 to 0.38 mg/L. The river periphyton presented slightly higher values (EC_{50} of 0.74 mg/l), and the algae *C. reinhardtii* has the higher values of LC_{50} to fipronil (2.44 mg/l). The metabolism of river bacterial communities was not affected. Notably, as observed in the different experiments, fipronil solubility changed over time, at different temperatures and in different environments. Hence, these factors may differentially affect its toxicity depending on the organism on which it acts.

4.1 Effect of fipronil on *D. magna*

The short-term acute toxicity values of fipronil for *D. magna* in this study are in the range of those described previously: LC_{50} values from 0.08 to 0.19 mg/L (Hayasaka, et al. 2012b; Iwafune, et al. 2011) USEPA, 2000). Other closely related cladocerans, such as *Daphnia pulex* or *Ceriodaphnia dubia*, presented LC_{50} values in the same range, from 0.0156 mg/L to 0.51 mg/L (Stark and Vargas 2005). Daphnids have a GABA receptor, albeit in a different conformation compared to vertebrates (Jackel, Krenz, and Nagy 1994). Previous studies (Barry 2002) have shown that cholinergic and GABAergic drugs modulate neckteeth development in *D. pulex*. The authors proposed that these chemicals modulate the release of neurohormones; mechanism of action may underscore the effect of fipronil in *D. magna*.

An increase in temperature enhanced fipronil toxicity in both synthetic and river water. A modulating effect of temperature on insecticide toxicity has been described for insects (Musser and Shelton 2005; Srigiriraju, Semtner, and Bloomquist 2010). However, while organophosphates may exhibit enhanced toxicity as the temperature increases, pyrethroids have shown the opposite behaviour (Arthur 1999; Athanassiou, et al. 2008; Vayias, Athanassiou, and Buchelos 2006; Kavallieratos, et al. 2009). As far as we know, only one previous study examined the influence of temperature on fipronil and found that this insecticide presented biphasic behaviour in the heteroptera *Apolygus lucorum*: toxicity decreased from 15 to 20°C and increased from 20 to 35°C (Ma, et al. 2012).

Fipronil is very stable in neutral aqueous pH solution at 18–25ºC (Bobe, et al. 1998; Ramesh and Balasubramanian 1999). In this study, its solubility increased in standard water and decreased in river water over 24 h; these differences were less marked at 18°C compared to the other temperatures. Fipronil solubility decreases considerably at higher salinities (Goff, et al. 2017). This factor may explain its lower solubility in river water, which contains more dissolved solids and salts (Table 1). However, its toxicity in the standard medium and river water at 23 and 25°C was similar. Therefore, solubility – which also depends on the temperature – does not seem to play a key role in explaining the differences in toxicity. Temperature-related changes in toxicity likely rely on mechanisms other than changes on fipronil physicochemical characteristics, such as the metabolic activity of *Daphnia*. At 25°C, *Daphnia* may present an accelerated life cycle so an increase in Daphnia activity and feed consumption would lead to a consequent increase in toxicant intake (Betini, et al.).

4.2 Effect of fipronil on *C. reinhardtii*

This study is the first to assess the short-term acute toxicity of fipronil on *C. reinhardtii*. The EC_{50} values are higher than those from previous studies on different algae. Studies on the marine phytoplankton *Dunaliella tertiolecta* resulted in an EC_{50} of 0.63 mg/L (Overmyer, et al. 2007). The R- and S-fipronil enantiomers showed EC_{50} values of 0.29 mg/L and 1.50 mg/ L for *Scenedesmus obliguus* (Qu, et al. 2014). The United States Environmental Protection Agency (USEPA, 2007) has provided some ecotoxicity values of fipronil for diatoms like *Navicula pelliculosa* and *Skeletonema costatum*: 5-day EC_{50} values of 0.12 mg/L and > 0.14 mg/L, respectively. In addition, the green alga *Selenastrum capricornutum* showed a 5-day EC_{50} 0.14mg/L and the blue-green alga *Anabaena flos-aquae* presented an EC_{50} > 0.17 mg/L. All these studies were based on long-term exposure tests (several days). The use of a very specific endpoint, such as photosynthesis, rather than more integrative measures like growth, together with the acute short-term test approach, could explain the higher EC_{50} obtained in this study.

499 The fipronil mechanism of action on unicellular algae remains unknown. In the
500 biosorption process, organic contaminants are absorbed at the surface of the cell wall
501 (Qu, et al. 2014), a phenomenon that may commence bioaccumulation. Hence, the
502 insecticide may damage the functions or structures of the algal cell. For example, *S.*
503 *capricornutum* exposed to carbofuran and diuron exhibits significantly reduced growth
504 and suffer physiological (chlorophyll *a* content) and morphological (complexity and cell
505 size) changes (Mansano, et al. 2017). In other cases, sublethal effects occur and
506 although cell viability is not affected, pesticide exerted genotoxic effects (Martinez, Di
507 Marzio, and Saenz 2015). However, members of the genus *Chlamydomona* have a
508 complex cell wall that is formed by hydroxyproline-rich glycoprotein and crystalline layers
509 (Voigt 1988) that may act as a protective barrier (Sena, et al. 2010) and thus explain their
510 resistance to this pesticide. Regarding the differences in toxicity depending on the
511 utilised media (river water or MOPS), the affinity of fipronil to organic matter or
512 suspended solids, due to its high logKow values, may reduce its bioavailability for *C.*
513 *reinhardtii* in river water (Table 1). This factor may underlie why we did not detect the
514 effect of fipronil on the algae in this medium in any of the tested conditions. Modulation
515 of freshwater ecotoxicology by the presence and quality of suspended and dissolved
516 materials has been explained for Delgado-Moreno (Delgado-Moreno, et al. 2011) for
517 pyrethroids and for other pesticides in surface water (Hernandez-Antonio and Hansen
518 2010; Knauer, et al. 2017). Lajko et al. (Lajko, et al. 1997) observed a suppression of the
519 photosynthetic activity by respiration when the cells are incubated at temperatures 5–
520 10ºC higher than the cultivation temperature, especially in short-term incubation periods.
521 This photosynthesis suppression could explain why fipronil had no detectable changes
522 on algae photosynthesis in MOPS at 25 or 30°C.
523
524 **4.3 Effect of fipronil on periphyton**
525 This study showed that an insecticide such as fipronil may pose hazards to freshwater
526 primary producers and decomposers. It has been demonstrated that herbicides (Paule,

et al. 2016; Chaumet, et al. 2019; Dorigo, et al. 2010) and their mixtures (Ham, et al. 2014; Morin, et al. 2012; Pesce, et al. 2011) affect the microbial and photosynthetic fractions of the periphyton. However, as far as we know there is no information on the ecotoxicity effect of insecticides on biofilms.

The periphyton is a complex community composed of algae, bacteria, fungi, protozoa and invertebrates that develops on an underwater substratum (Seguin, Druart, and Le Cohu 2001). Biofilms integrate the effects of environmental conditions over extended periods of time, so ecotoxicity results using periphyton provide more environmentally realistic results (Kraufvelin 1998; Sabater, et al. 2007). Periphyton, and particularly attached diatoms, are the dominant primary producers in small rivers (Schagerl and Donabaum 1998) and are very sensitive to qualitative or quantitative environmental changes that have consequences for the whole trophic web (Denoyelles, Kettle, and Sinn 1982). Our results showed that fipronil affects the periphyton photosynthetic yield. These data indicate that at least the photosynthetic organisms among the biofilm community (e.g. diatoms, which are the most abundant and diverse in our samples) are affected by the insecticide.

Fipronil impacted photosynthetic yield at concentrations lower than those required by *Chlamydomonas* species, although the periphyton structure could be expected to show some resistance to the effect of fipronil. The sensitivity of biofilms to toxicants may depend on its thickness (Navarro, Robinson, and Behra 2008), age, composition and velocity of the water flow (Sabater, Navarro, and Guasch 2002). In addition, other mechanisms of resistance, such as an exopolysaccharide (EPS) matrix, provide protection against pollutants like heavy metals (Nocelli, et al. 2016), predation and environmental fluctuations (Headley, et al. 1998; Miao, et al. 2019; Manning, et al. 2018). Although pesticide solubility is a key parameter for absorption of dissolved organics to biofilms, the lipophilic nature of a contaminant seems to also be an important factor (Headley, et al. 1998). In addition, the longer exposure time (13 vs. 1 h) and the flow velocity – which reduces the boundary layer and thus increases the uptake of toxicants

555 in biofilms – may explain this apparently higher sensitivity of periphyton compared to *C.*
556 *reinhardtii.*
557 Similar to *C. reinhardtii*, fipronil was more toxic in MOPS medium compared to river
558 water. The mechanism is likely to be similar and related to the adsorption of insecticide
559 to suspended organic matter in river water. A recent study found that organic matter
560 attenuates the toxic effects of CuO nanoparticles to the microbial community in sediment
561 biofilms (Miao, et al. 2019). On the other hand, the solubility of fipronil in river water and
562 in MOPS fluctuated over time, but it remained higher in MOPS compared to river water
563 at all time points (Fig. 5c).
564
565 **4.4 Effect of fipronil on river and soil microbial community**
566 The metabolism of microbial community, under experimental conditions including
567 different temperatures, was not affected by fipronil, as shown by the AWCD results. This
568 can be explained by the bioavailability of fipronil but also by the taxa composition of the
569 microbial communities.
570 While fipronil solubility oscillated during the experiment, it remained soluble in river water
571 or MOPS (Fig. 9). However, mechanisms of adsorption to organic matter suspended in
572 river samples could make fipronil less bioavailable for river bacteria (Peret, et al. 2010).
573 Despite the physicochemical aspects of fipronil, the main factor for the lack of a pesticide
574 effect is likely due to the microbial community biology. Community responses are
575 extremely complex, and the literature commonly records both positive and negative
576 responses of different bacteria to each pesticide (Ros, et al. 2006; Lo 2010; Itoh, et al.
577 2014; Rouze, et al. 2019). A study focused on the effect of low fipronil concentrations
578 (50 and 5 µM) on *Escherichia coli* resulted in no lethality (Bhatti et al., 2019). Among the
579 microbial community, those species sensitive to fipronil can be replaced during the
580 experiment by survivors (Xia, et al. 2013), a phenomenon that compensates for losses
581 and prevents (or masks) changes to the metabolic pattern of the community as a whole.
582 Likewise, literature showed that the presence of fipronil residues in the soil does not

583 adversely affect abundance and activity of two rhizosphere bacterial strains,
584 *Staphylococcus arlettae* and *Bacillus thuringiensis* (At, Karthikeyan, and Thanga 2019).
585 Maute (Maute, et al. 2015) used Biolog plates to study the effect of the fipronil on
586 microorganisms in Australian grassland and found that pesticide treatment seems to not
587 affect the functional diversity of the bacterial community of this arid zone – although
588 microorganisms present in the samples were not identified.
589 Bacteria can use fipronil as a source of carbon and biodegrade it, as it has been
590 described to *S. arlettae* and *B. thuringiensis* (At, Karthikeyan, and Thanga 2019),
591 *Acinetobacter calcoaceticus* and *Acinetobacter oleivorans* (Uniyal, et al. 2016b),
592 *Stenotrophomonas acidaminiphila* (Uniyal, et al. 2016a) and *Paracoccus* sp. (Kumar,
593 Singh, and Gupta 2012). Even *E.coli* (Bhatti, et al. 2019) may use fipronil as carbon
594 source. Moreover, previous studies have shown that predominant bacterial taxa in our
595 samples are specialists for degradation of high molecular weight compounds, including
596 pesticides. For example, Gammaproteobacteria were the most abundant in our river
597 water samples. This class include important degraders of organic compounds and
598 pesticides in water (Holmsgaard, et al. 2017), soil (Paul, et al. 2006; Newman, et al.
599 2016) and sediments (Fang, et al. 2014). In freshwater bacterial communities where
600 Alphaproteobacteria predominant, there was no significant effect on bacterial community
601 structure or composition after exposure to the fungicide tebuconazole (Pascault, et al.
602 2014). Burkholderiales, the main order found in both the river and soil samples among
603 Betaproteobacteria, can degrade a vast array of aromatic compounds (Thoetkiattikul, et
604 al. 2017), including insecticides such as chlorpyrifos in water (Ferrario, et al. 2017) and
605 soil (Kim and Ahn 2009), as well as dimethoate, fenitrothion or malathion in soil (Kim and
606 Ahn 2009). Curiously, insecticide properties of Burkholderiales itself have been reported
607 (Ruiu 2015). The class Sphingobacteriia that dominated among Bacteroidetes in our soil
608 samples contains bacteria that are also very active in the degradation of organic
609 compounds (Bauer, et al. 2006; Bissett, Bowman, and Burke 2008).
610 Sphingobacteriia members are also able to degrade benzene compounds (Li, et al.

611 2012) and seem to be able to degrade the insecticide bifenthrin and absorb it as nutrition
612 (Zhang, et al. 2017).
613 Cyanobacteria, an important presence in our river samples, can metabolise methyl
614 parathion from culture medium (Fioravante, et al. 2010). However, when tests are
615 performed using individual organisms, the sensitivity of Cyanobacteria to pesticides is
616 inconclusive (Ma, et al. 2006; Stratton and Corke 1982; Sabater and Carrasco 2001a;
617 Sabater and Carrasco 2001b). At the community level, the effect of phytosanitary
618 products has been extensively studied in rice field, where insecticides seems to promote
619 the growth and nitrogen-fixing activity of rice field native cyanobacteria (reviewed by
620 (Kaushik, et al. 2019). Finally, *Pseudomonas* spp. were also very abundant among our
621 soil microorganisms. These bacteria maintain their production of plant growth promoting
622 substances in the rhizosphere, after fipronil exposition (Ahemad and Khan 2011b). In
623 addition these bacteria can degrade organophosphorus pesticide (Jariyal, et al. 2018)
624 and are tolerant to the insecticide dimethoate and the pesticide pentachlorophenol
625 (Hassen, et al. 2018)
626
627 **4.5 Environmental relevance**
628 Our results suggest that fipronil could affect different trophic levels of the river
629 ecosystems and their complex network of communities of periphyton, algae, fungi,
630 protozoa and invertebrates, many of them on the bottom or with important functions in
631 freshwater food webs. For example, changes in herbivore-producer interactions were
632 expected if fipronil affects primary producers (algae, periphyton) that would lead to a
633 reduction in herbivorous invertebrate densities and a top-down impact on trophic
634 cascades (Relyea and Hoverman 2006). Interestingly, sometimes the result of the
635 interaction can be different. For example, after the application of the insecticide
636 deltamethrin, a phytoplankton bloom occurred due to the nutrients released by the
637 decomposing herbivorous arthropods that were killed by the insecticide (Knapp, et al.
638 2005). Changes in interspecific competition due to differential sensitivity of competitors

to pesticides are also possible. Species that are more sensitive to the pesticide are eliminated and those that are more tolerant come to dominate, changing invertebrate community (Relyea 2006; Fairchild and Eidt 1993). For example, when moderate concentrations of insecticides are administered to communities containing both cladocerans (such as *D. magna*) and copepods, a dramatic decrease in the more sensitive cladocera species and a substantial increase in the abundance of copepods can be observed (vandenBrink, et al. 1996; Relyea 2006).

Fipronil is present in freshwaters in the range of ng/L (Michel, et al. 2016) up to 2 µg/L (Ensminger, et al. 2013), concentrations lower than those capable of producing acute ecotoxicity as shown by our results.

However, Its bioaccumulation in different aquatic organisms has been reported (Qu, et al. 2016; Dang, et al. 2016; Lopez-Pacheco, et al. 2019). In some cases, concentrations in animal tissues are similar to those found in river water. For example, in eel liver, fipronil has been found at a concentration of 19.91 ng/g (Michel, et al. 2016). These findings suggest that, although the initial exposure of aquatic and terrestrial organisms to this insecticide does not reach ecotoxicity values, repeated and long-term exposure can lead to its bioaccumulation both in its original form and in its by-products. Hence, the chronic effects of fipronil toxicity cannot be ignored.

5 Conclusions

This study provided a comprehensive overview of the effect of the insecticide fipronil in a freshwater environment, considering its behaviour in different media and temperatures, and studying the effects not only on standard organisms but also on communities, including soil microorganisms. Fipronil affected the survival of *D. magna*, the photosynthetic activity of *C. reinhardtii* and the freshwater periphyton. However, soil and water bacterial communities identified through 16S rRNA gene sequencing were minimally affected by fipronil, probably due to the presence of specialist bacteria that can degrade pesticides. River water, in the case of *C. reinhardtii* and the freshwater

periphyton, seems to interfere with fipronil toxicity, but this effect was not detected in the case of *D. magna*. Temperature increases the toxicity of the insecticide in the case of *D. magna*; this aspect must be considered in the context of expected global warming. Despite the fact that fipronil is present in freshwaters and soils in concentration ranges lower than the detected ecotoxicity values, these results highlight that toxic effects in the environment cannot be excluded given the prolonged presence of this insecticide in the environment and its bioaccumulation capacity.

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Assay	*D. magna*	*C. reinhardtii*	Bacterial communities of river	Periphyton communities		
				Biofilm substrate placement	15 days from the biofilm substrate placement	Biofilm substrate collection
Date	13/07/2016	29-09-17	17-10-17	16-05-17	20-06-17	11-07-17
Conductivity (µs/cm)	2423,8	2348	2853	2368	2187	2363
pH	7,8	7,7	7,92	8,17	8,14	8,46
Total Suspended Solids (mg/L)	22	2,2	4,2	4,3	3,5	12,1
Total Dissolved Solids (mg/L)	563	1716	1360	1640	1500	1560
Fluorides (mg/L)		0,088	0,094	0,065	0,05	0,051
Chlorides (mg/L)	58,1	414,625	507,599	187,897	190,952	190,209
Nitrites (mg/L)	0,49	0	0	0	0	0
Bromides (mg/L)		0,392	0,567	0,253	0,214	0,264
Nitrates (mg/L)		14,827	13,563	6,668	5,936	5,285
Phosphates (mg/L)	0,28	0	0	0	0	0
Sulphates (mg/L)	157,3	330,936	383,113	162,035	163,198	168,133
Total alkalinity (mg/L)		262,1	223,26	276,24	215,18	236,68
Sodium (mg/L)		255,309	332,718	196,548	191,93	164,028
Ammonium (mg/L)	> 0,10	1,797	0	0,033	0,038	0
Potassium (mg/L)		3,103	3,623	2,098	2,135	2,49
Calcium (mg/L)		178,587	198,203	130,42	128,442	115,446
Magnesium (mg/L)		29,776	31,683	33,391	19,758	7,473
Total organic carbon (mg/L)	5,5	1,3	2,2	1,42	1,6	1,11
Total Nitrogen (mg/L)				4,54	3,93	3,72
Chlorophyll a (µg/L)				2,01	2,47	18,54
Chlorophyll b (µg/L)				0,76	1,22	6,53
Chlorophyll c (µg/L)				96,80	78,78	201,20
TSI (Chlorophyll a)				37,00	39,00	59,00
Margalef Index				1,36	1,84	2,00
Moss index				1,27	1,62	1,77

Table 1. Physical-chemical, biological and ecological parameters of the different river water samples from the Gállego River (Ebro river tributary, Villanueva de Gállego, Zaragoza, Spain), used in the assays.

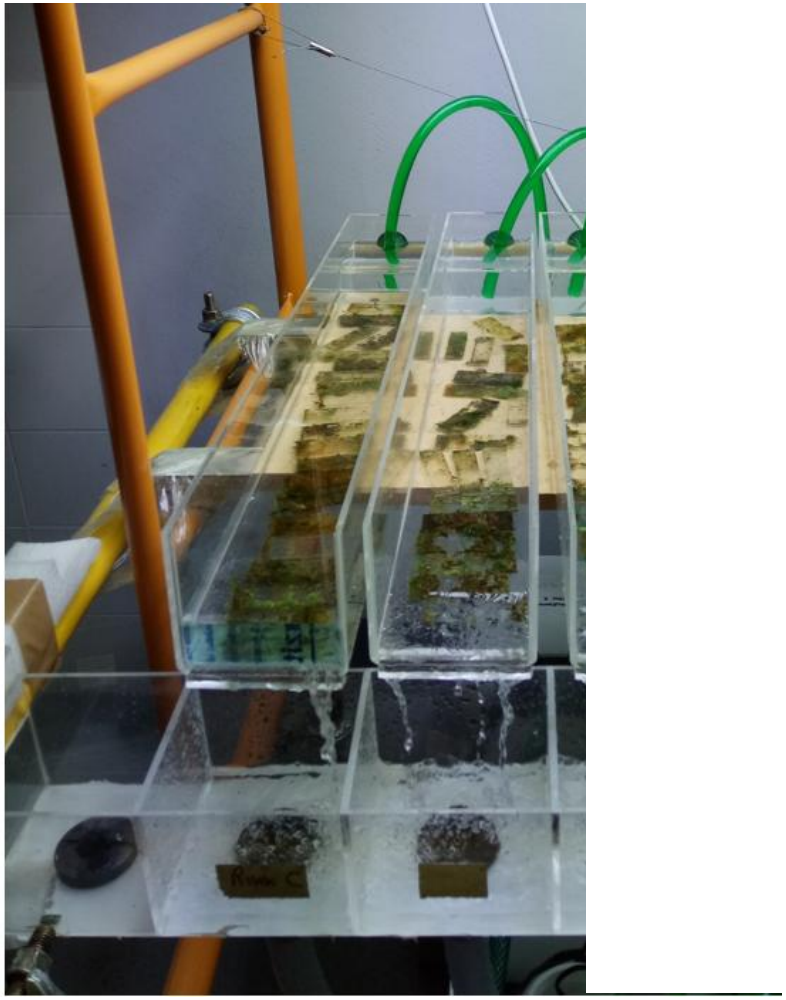

Fig 1. Flow-through artificial channels (mesocosm) where effects of fipronil on river Periphyton communities were analyzed. Periphyton-colonized slides collected from the Gállego River (Villanueva de Gállego, Zaragoza, Spain) can be seen inside the channels.

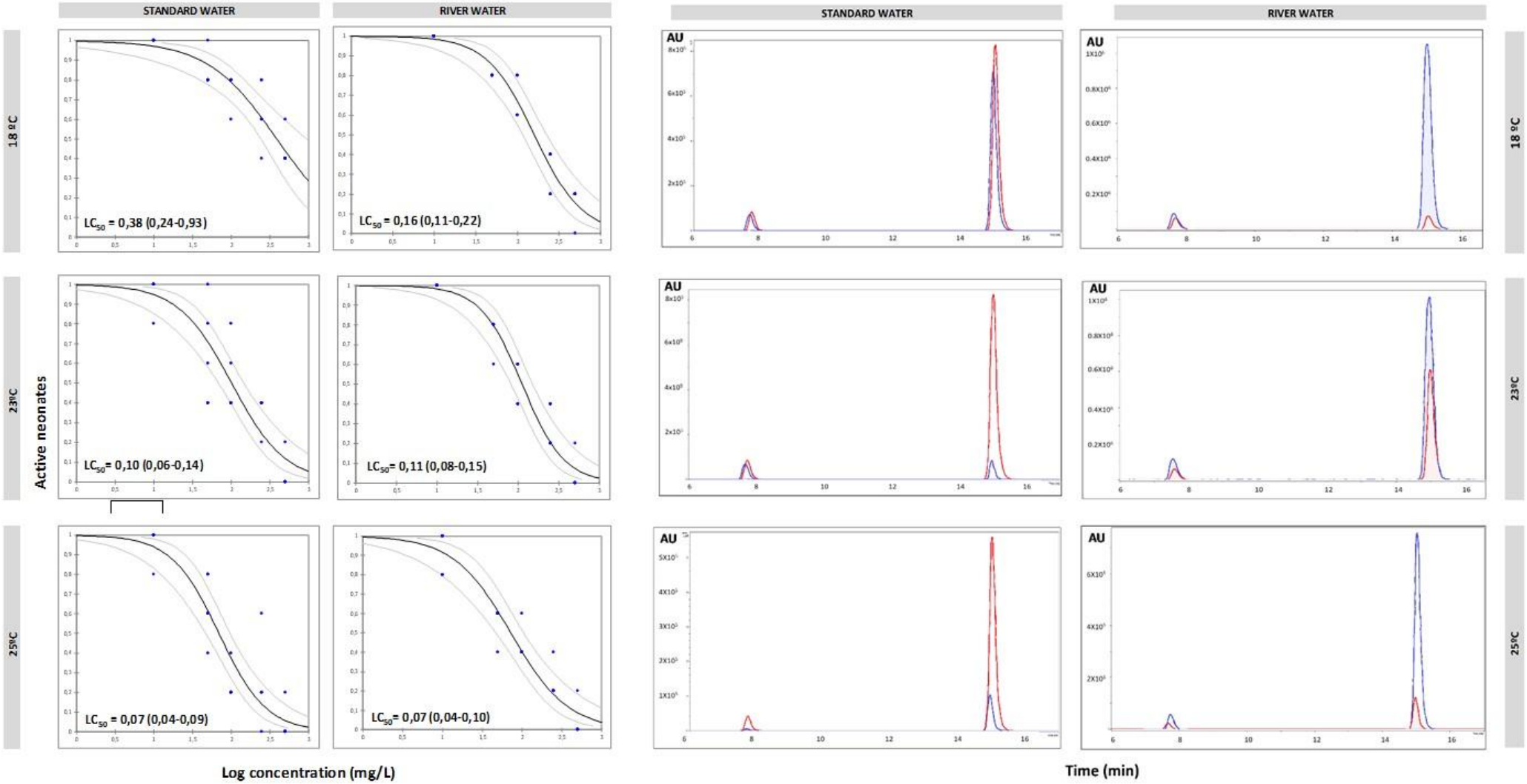

Fig. 2. Dose-response curve of fipronil after a 24 h exposure to *D. magna* (first two columns). The tests were carried out at three temperatures (18º, 23º and 25ºC) and in two media (Daphnia standard water and river water obtained from Ebro river tributary). Pale grey lines indicate the confidence limits (95%). Curve is the average value of five replicates. On the right, HPLC diagrams of detection of fipronil corresponds to the different conditions tested in the ecotoxicity assays. The blue peaks correspond to the measurement made at the beginning of the test, and the red ones at the end, after 24 hours. The peak of fipronil is 14.5 minutes and ethiprole was used as internal standard (peak at 7.5 minutes). AU=absortion units.

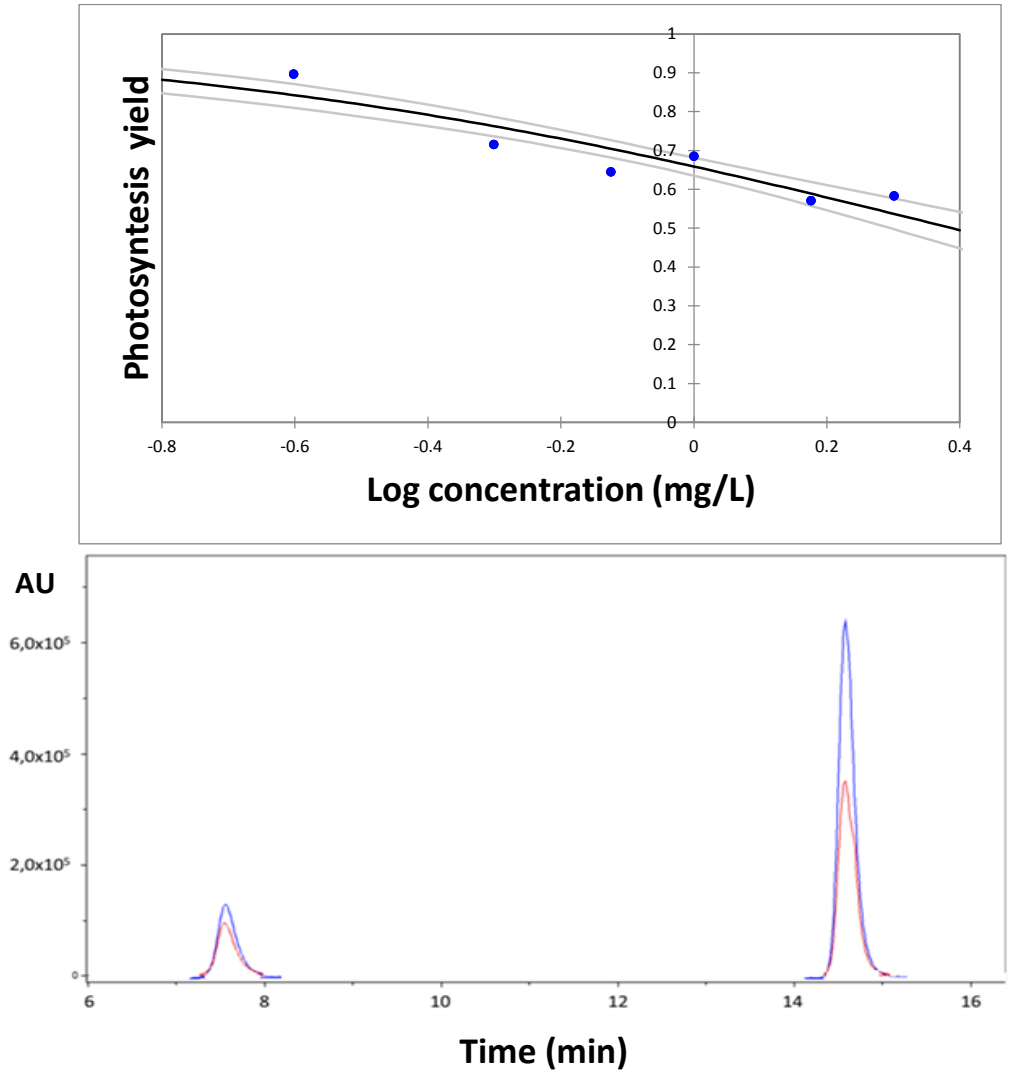

Fig. 3. Dose−response curves of photosynthetic yield of *C. reinhardtii* (up) after 6 h of exposure to fipronil at 20^{0}C, as function of logarithm of the concentration. Photosynthetic values are expressed as the percentage of the control. Pale grey lines indicate the confidence limits (95%). Each dose was tested in triplicate. HPLC diagram of detection of fipronil in MOPS at 0 (blue peak) and 6 hours (red peak) under the same conditions as the ecotoxicity assays, can be seen down. The peak of fipronil is 14.5 minutes and ethiprole was used as internal standard (peak at 7.5 minutes). Measurements were done in triplicate. AU=absortion unit.

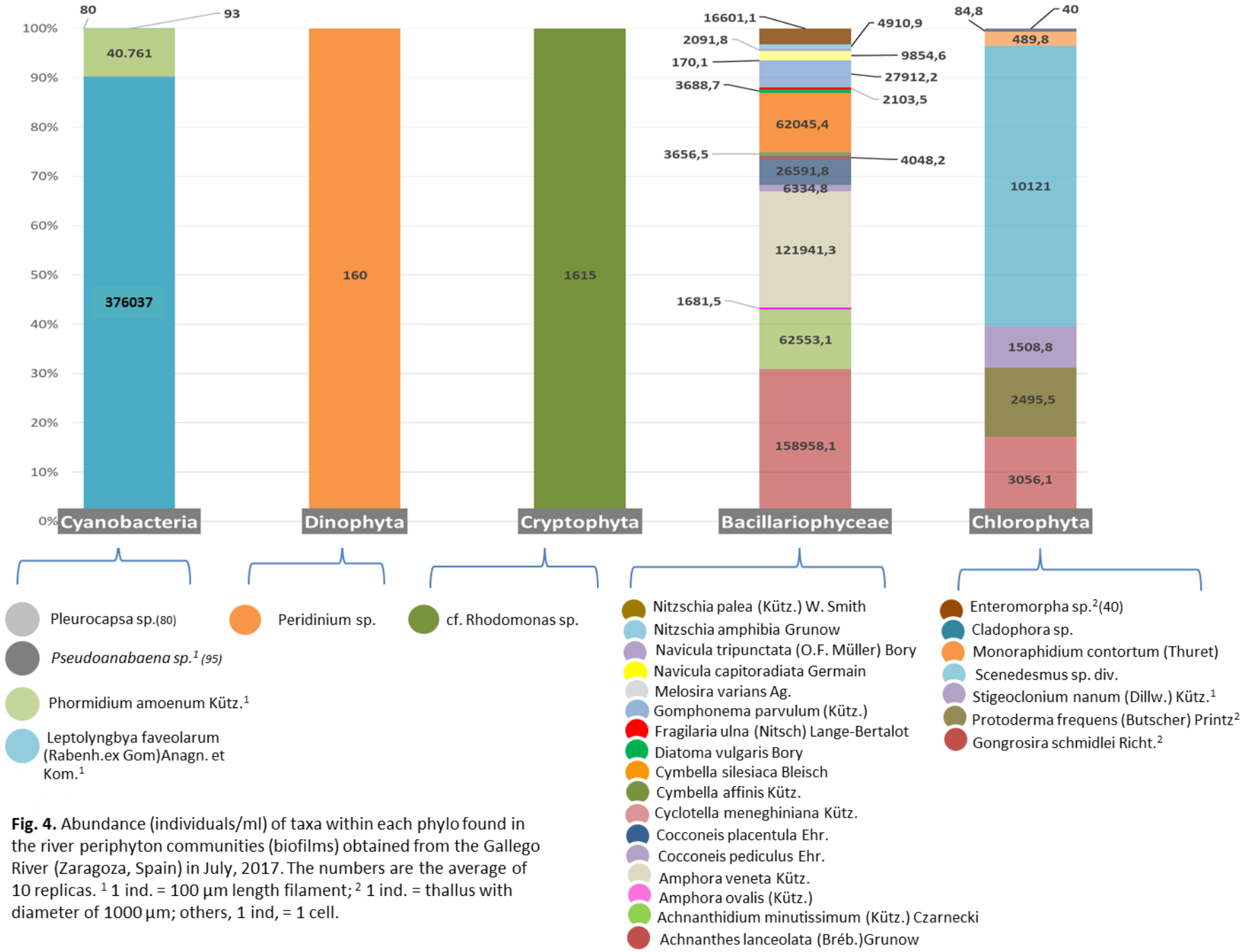

Fig. 4. Abundance (individuals/ml) of taxa within each phylo found in the river periphyton communities (biofilms) obtained from the Gallego River (Zaragoza, Spain) in July, 2017. The numbers are the average of 10 replicas. [1] 1 ind. = 100 µm length filament; [2] 1 ind. = thallus with diameter of 1000 µm; others, 1 ind, = 1 cell.

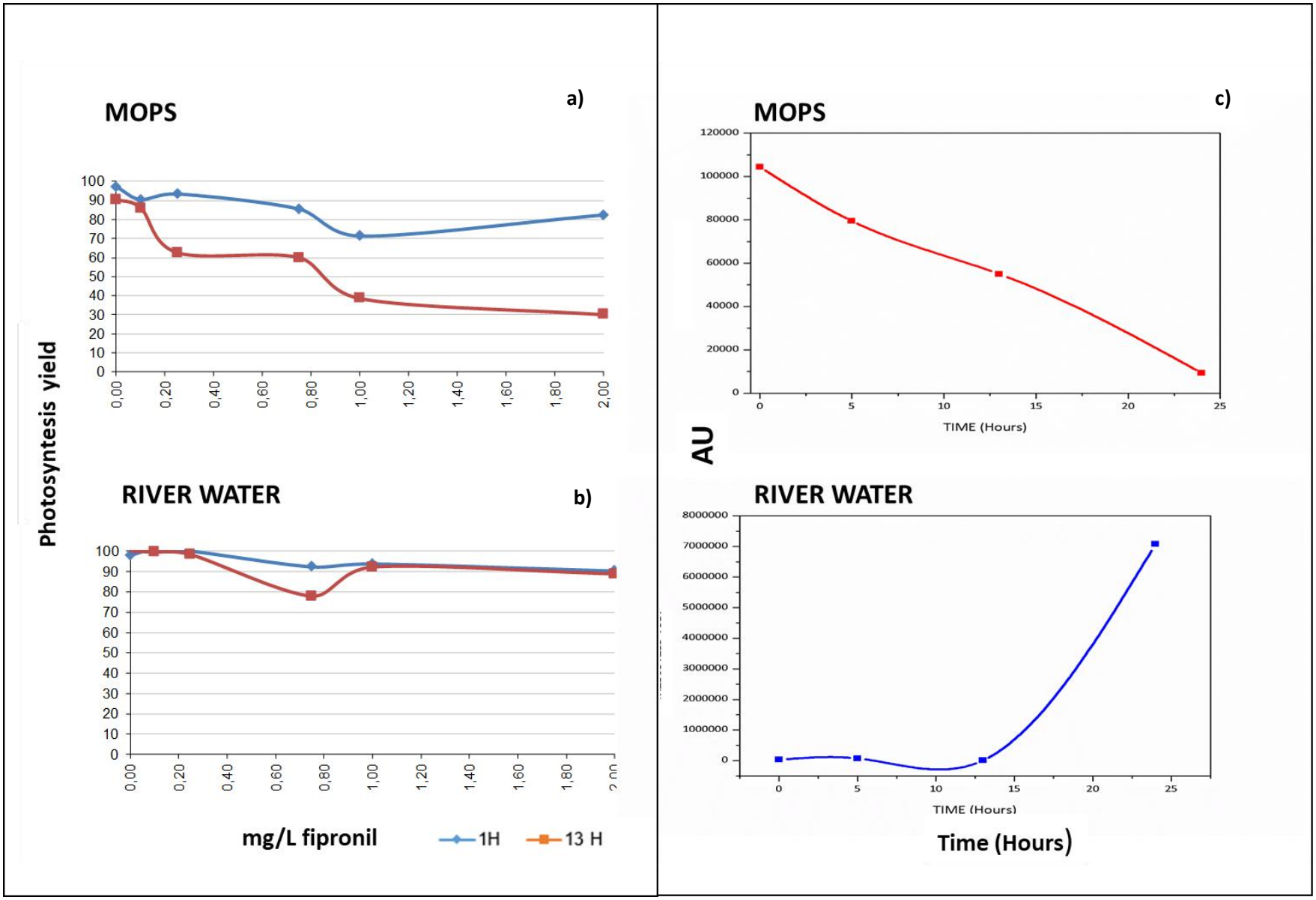

Fig. 5. Dose−response curves of photosynthetic yield of river periphyton after 1 and 13 h of exposure to Fipronil at 23ºC as function of logarithm of the concentration in MOPS (a) and water river (b). Fipronil solubility in river water and MOPS in the experimental conditions of periphyton communities assay can be seen in graph c) where integrated peak area of HPLC analysis are represented. AU=absortion units.

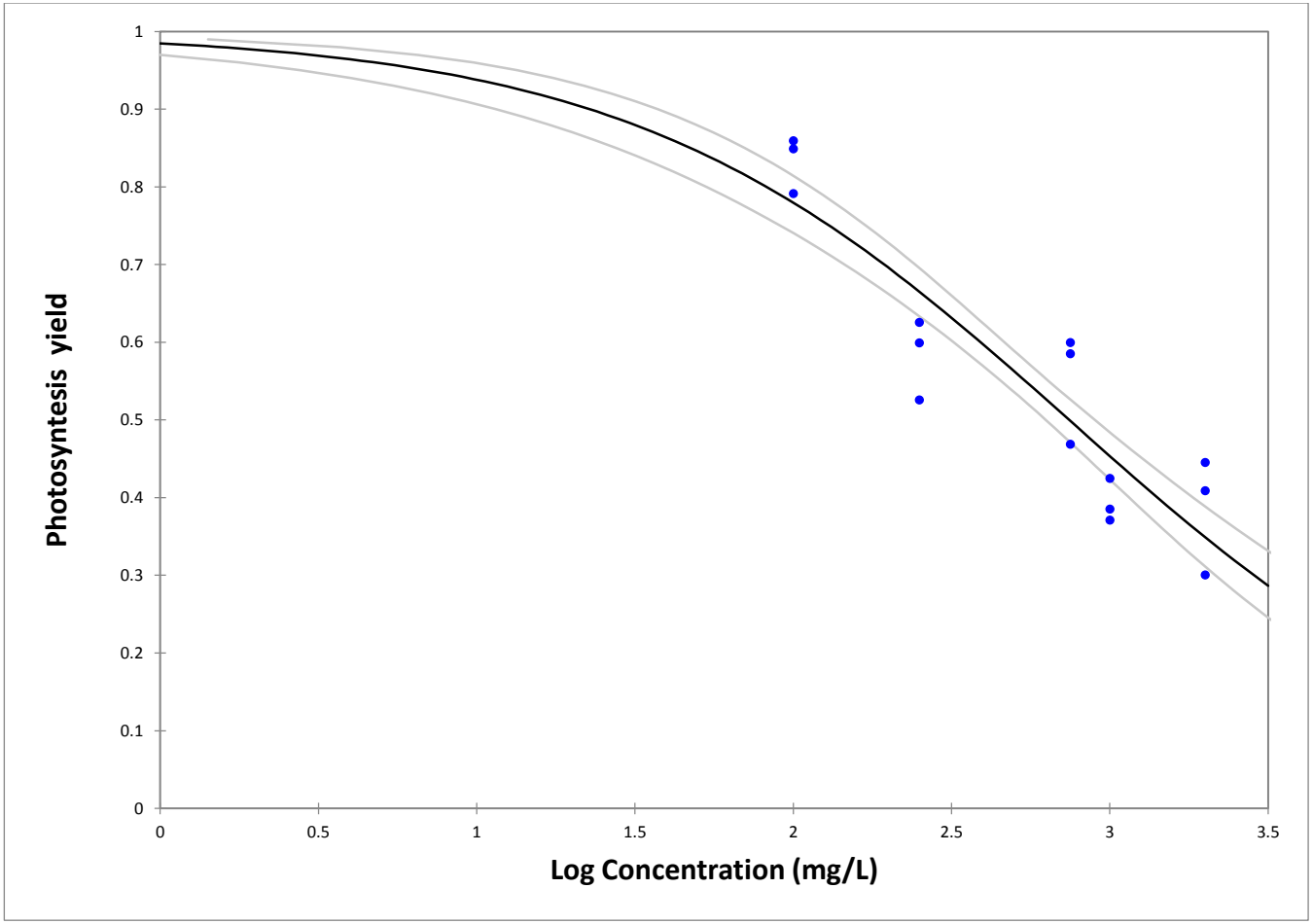

Fig. 6. Dose-response curve of river periphyton after 13 hours of exposition to fipronil in MOPS. Photosynthetic values are expressed as the percentage of the control. Pale grey lines indicate the confidence limits (95%). Each dose was measured in triplicate.

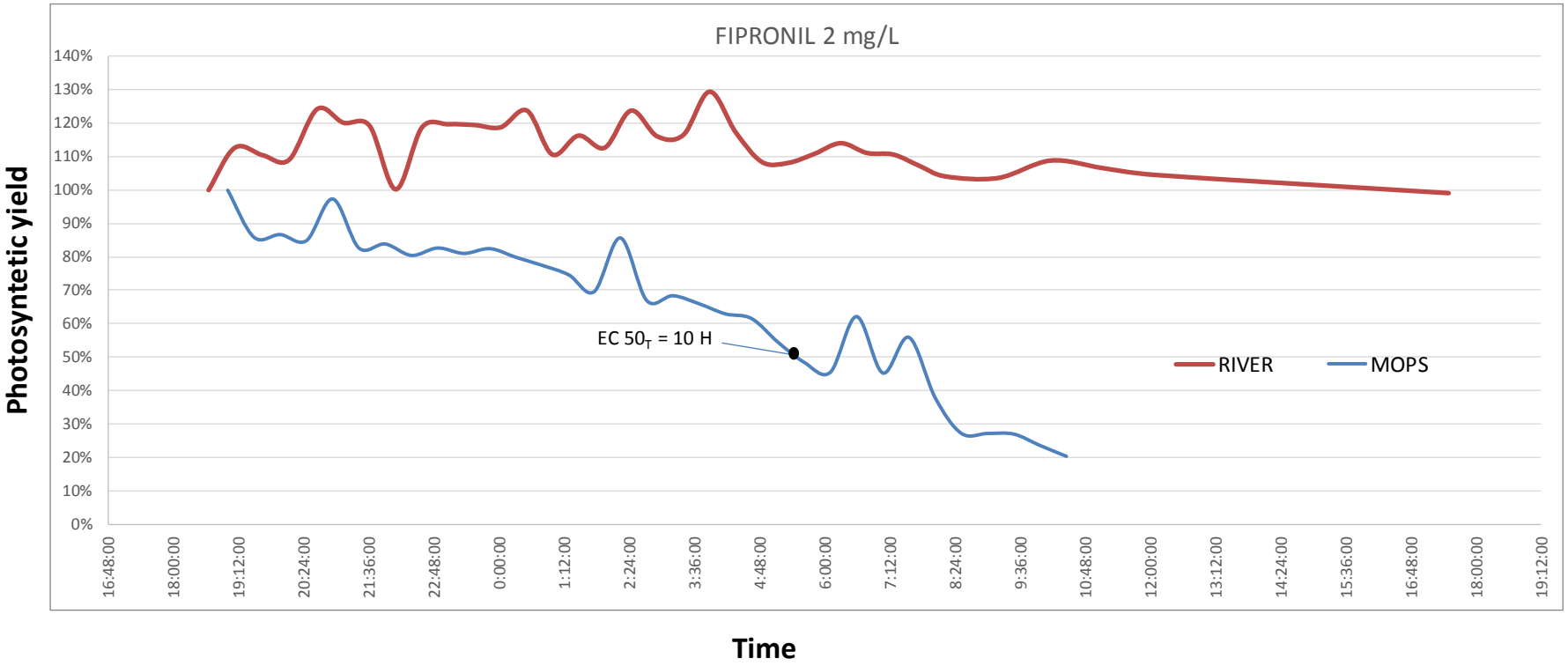

Fig. 7. Response curves of photosynthetic yield of river periphyton after 24 hours to exposure to 2 mg/L of fipronil in river water (red lines) and MOPS (blue lines) al 23^0C. Photosynthetic values are expressed as the percentage of the control.

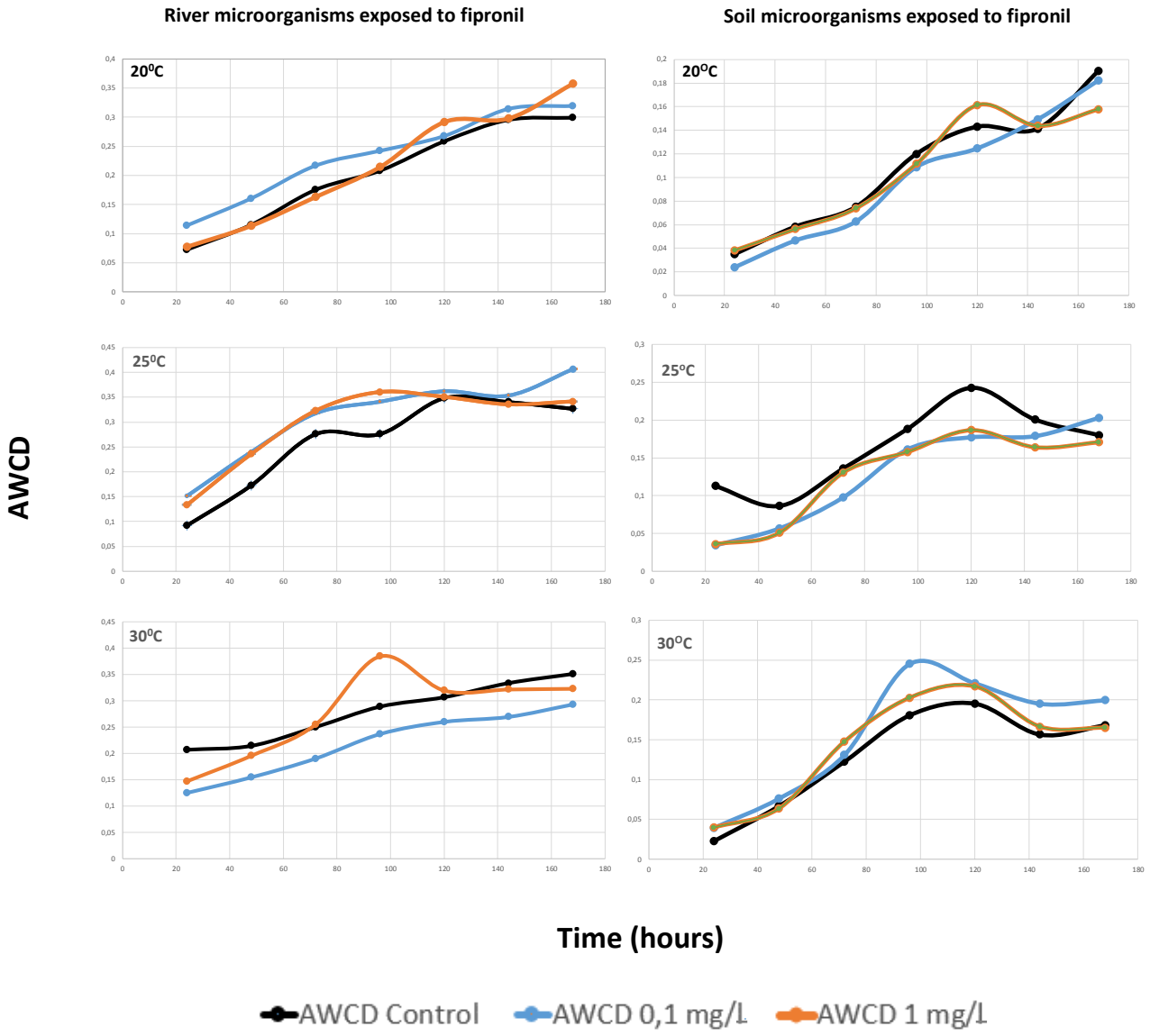

Fig. 8. Average well color development (AWCD) of metabolized substrates in Biolog EcoPlates based on 168-h incubation of river microorganisms (first column) and soil microorganisms exposed to fipronil. Concentrations of the insecticide can be seen at the bottom of the figure. Values can be compared to a reference control value (microorganisms of soil and water that have not been treated with fipronil, only mineral water). Each point is the average value of three replicates.

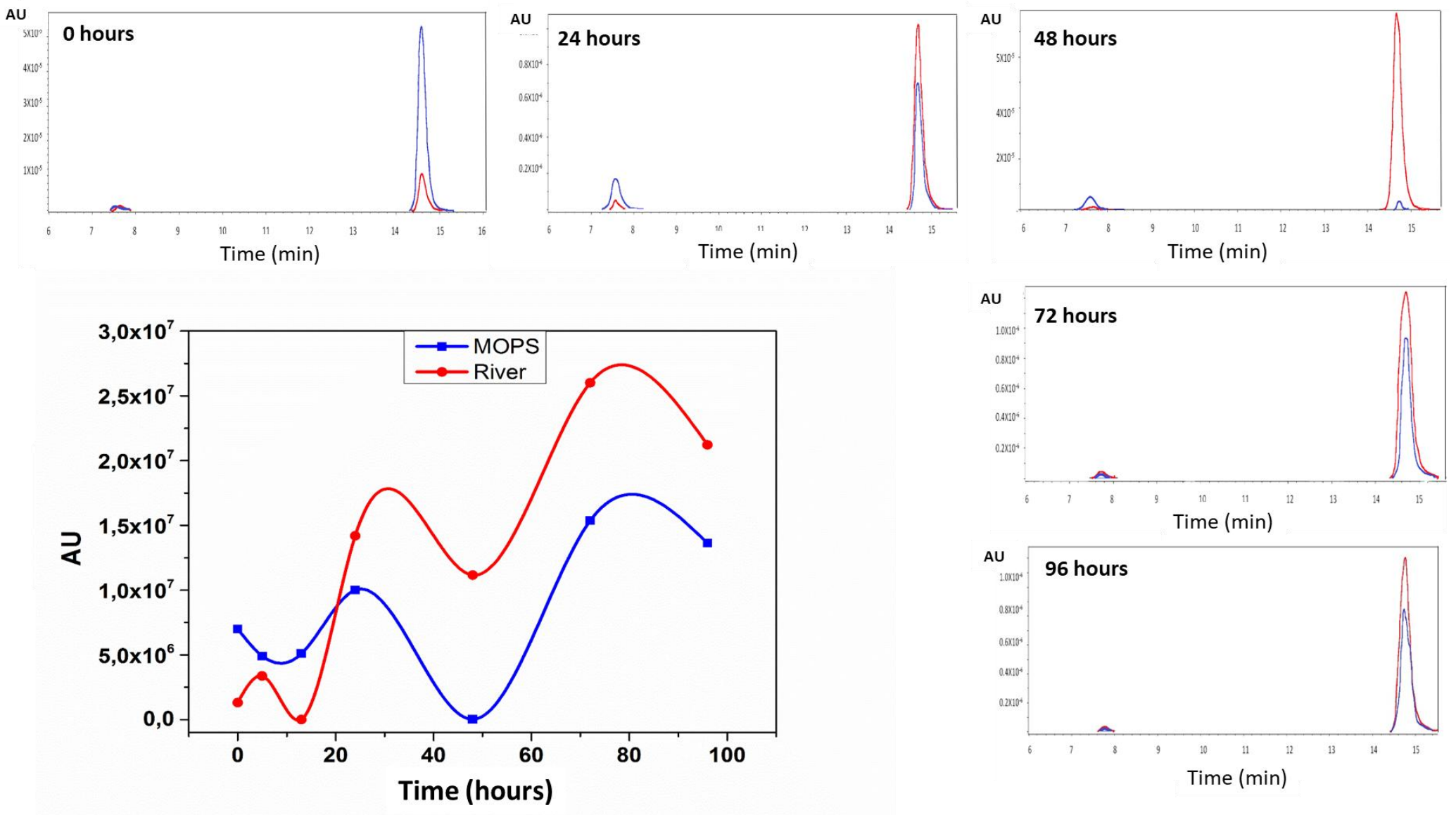

Figure 9. HPLC diagrams of detection of fipronil throughout the 96 hours in the conditions tested in Biolog EcoPlates exposition of microorganisms populations to fipronil in MOPS (blue lines) and river water (red lines). Figures show the bahavior of fipronil solubility at 20^{o}C . HPLC diagrams represents Absortions Units in different times. Ethiprole was used as internal standard (peak at 7.5 minutes). For better monitoring of changes in fipronil solubility, the central figure represents the integrated peak area of HPLC analysis at the same temperature.

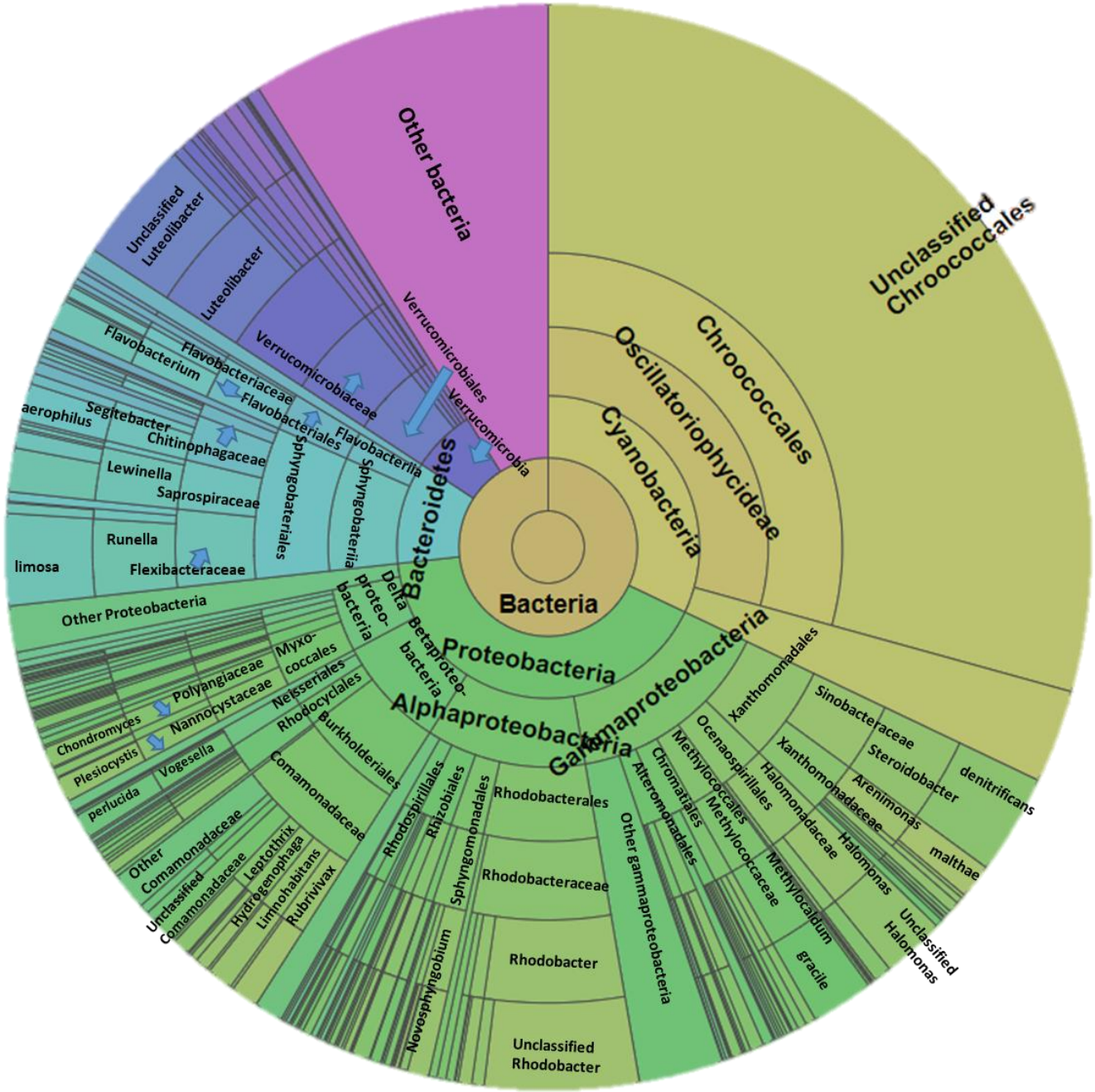

Fig. 10. Relative abundance of the river microbial main taxa within each taxonomic level. From inside the circle to outside: phylum, class, order, family, genus and species.

Credit Author Statement

Mª Rosa Pino Otín: Conceptualization, Formal analysis; Funding acquisition; Investigation, Project administration, Resources, Supervision; Validation, Writing - original draft

Diego Ballestero: Data curation, Investigation, Methodology

Enrique Navarro: Resources, Data curation, review & editing

Ana M. Mainar: Funding acquisition, resources, review & editing

Jonatan Val: Data curation, Investigation, review & editing