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Ecotoxicity of a novel biopesticide from Artemisia absinthium on non-target aquatic organisms

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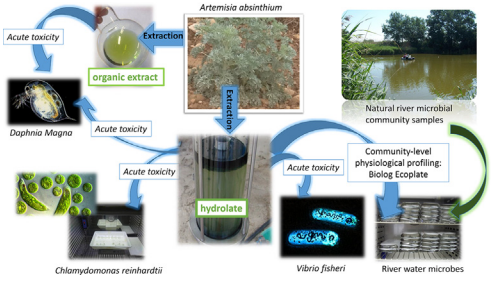
HIGHLIGHTS

- Aquatic ecotoxicity *of a novel biopesticide from Artemisia absinthium is analysed.*
- We studied the ecotoxicity of the hydrolate and the organic extract of this plant.
- The hydrolate cause toxicity on aquatic non-target organisms from 0,2% dilution.
- The organic extract of *Artemisia abshintium* is highly toxic to *Daphnia magna.*
- The hydrolate affects the physiology of a natural river microbial community.

GRAPHICAL ABSTRACT



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ABSTRACT

Biopesticides are increasingly being used to replace synthetic pesticides for pest control. This change raises concern for its environmental impacts, especially on non-target organisms. In this study, the ecotoxicological effects of a potential nematicide from Spanish populations of *Artemisia absinthium* (var. Candial) were evaluated on freshwater and aquatic non-target organisms. The study focused on the aqueous extract (hydrolate), the principal component of which ((−) -(Z) −2,6-dimethylocta-5,7-diene-2,3-diol) is responsible for its nematicidal effect. Until now, the hydrolate has been considered a byproduct of the process used to obtain essential oils, and there are no studies on its ecotoxicity from any plant with biopesticide properties. Our results indicated that *A. absinthium* hydrolate caused acute toxicity for non-target organisms at dilutions as low as 0.2%. The sensitivity of the organisms, from the most to the least sensitive, was: *Daphnia magna* ($LC_{50} = 0{,}236\%$) > *Vibrio fisheri* ($LC_{50} = 1{,}85\%$) > *Chlamydomonas reinhardtii* ($LC_{50} = 16{,}49$). Moreover, the *A. absinthium* organic extract was highly toxic to *D. magna* ($LC_{50} = 0{,}093$ mg/L). *A. absinthium* hydrolate toxicity was also tested on a natural river microbial community. Bacterial growth was not affected; the physiology of the community was only slightly modified, namely through an increased ability to degrade different substrates, mainly carbohydrates.

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This study provides for the first time an exhaustive assessment of the environmental exposure of a plant-derived biopesticide and shows that these products may cause a broad range of toxicity on non-target aquatic organisms.

1. Introduction

Over the past 100 years, the world population has grown exponentially and consequently forced a shift to the use of intensive production systems for global agricultural (Kohler and Triebskorn, 2013). This change caused unprecedented pressure on natural resources (e.g., water) as well as the release of thousands of tons of synthetic pesticides to prevent the adverse effects of pests and improve crop yield (Liu et al., 2015).

The indiscriminate use of chemical pesticides has provoked many well-known and serious problems, including genetic resistance of pest species, product residues, toxicity to non-target organisms and impacts on biodiversity. Moreover, humans have also been impacted; acute and chronic toxicity of farmworkers, applicators and even consumers have been described (Brack et al., 2007; Dinesh et al., 2014; George et al., 2014; Kunz and Kemp, 1994; Lew et al., 2009; Rosner and Markowitz, 2013; Zhang et al., 2009). In addition, continuous application of synthetic pesticides in agriculture over the years has caused pesticide residue accumulation in the environment. Indeed, more than 99.7% of pesticides persist and are accumulated in the environment (Brack et al., 2007; Pimentel, 1995; Shao and Zhang, 2017; Tejada et al., 2017). However, most of their environmental effects remain unclear.

The problems caused by synthetic pesticides and their residues have invigorated research for alternatives. Growing attention has been directed toward environmentally friendly pesticides/insecticides like traditional plant-based agents that would both act against pests and prevent undesired health and environmental impacts (Dinesh et al., 2014). Plants have evolved over 400 million years to defend themselves from parasites and predators through the development of chemical-protection substances with insecticidal, bactericidal, repellent and other activities (Dinesh et al., 2014). These substances are currently being explored as promising alternatives to synthetic pesticides (2011, Benelli et al., 2016, Govindarajan and Benelli, 2016). Plant-based chemicals have shown numerous advantages over conventional pesticides: they are cheaper, safer to farmers during handling and viable for use on small farms (Amoabeng et al., 2014). Due to their natural origin, biopesticides are considered to be safer and less harmful to non-target organisms (Shao and Zhang, 2017; Varma and Dubey, 1999). Unlike conventional pesticides, which are based on a single active ingredient, plant-based formulations have more complex chemistries that should limit development of pest resistance against them (George et al., 2014; Jaya et al., 2014; Miresmailli et al., 2006). In addition, some botanical pesticide active ingredients apparently degrade rapidly when exposed to sunlight, air and moisture and are readily broken down by detoxification enzymes (El-Wakeil, 2013; Varma and Dubey, 1999). For these reasons, many of the plant-based formulations are on the “Generally Recognised as Safe” (GRAS) list fully approved by the U.S. Food and Drug Administration (FDA) and Environment Protection Agency (EPA) (Kedia et al., 2015).

Thus, plant based formulations are considered safer to the environment (Benelli, 2015; Govindarajan et al., 2016; Pavela, 2015) than the common synthetic chemicals. However, as far as we know, the latest generation of biopesticides poses problems for wildlife, perhaps not directly through receptor interaction in non-target species, but perhaps indirectly affecting interactions between species. (Kohler and Triebskorn, 2013). Recent studies suggested that biopesticides can exhibit high but transient biocidal effects (Shao and Zhang, 2017). Nevertheless, only scarce information about the environmental effects of biopesticides is available. **Considering that biopesticides are increasingly replacing synthetic products in pest control, it is necessary to assess their ecotoxicity and especially their non-target effects on the environment, which is largely unknown.**

Artemisia, the largest genus of the family Asteraceae, comprises over 500 species native to Europe, North America and Asia; these species are commonly cultivated in temperate climatic zones (Bora and Sharma, 2011a; He et al., 2009). This genus is a rich source of plant-derived pesticides (Duke et al., 1988). *Artemisia absinthium* L. (wormwood) is a strongly aromatic plant that bears numerous oil-producing glands on leaves, stems and flowering branches (Chiasson et al., 2001; Mihajilov-Krstev et al., 2014). Its essential oil (EO) and other forms of herbal preparations are traditionally used in ethnopharmacology and ethnomedicine (Abad et al., 2012; Bora and Sharma, 2011b). Botanicals are derived from fresh or dried plants, plant parts, or from plants isolated or extracted in water, ethanol or other organic solvents as well as with supercritical CO_2 techniques. The composition of the *A. absinthium* EO and its biological effects have been widely studied (Bailen et al., 2013; Dane et al., 2016; Judzentiene et al., 2012; Tehrani et al., 2012). Absinthium EOs are larvicidal against mosquitoes (Duke et al., 1988; Govindarajan and Benelli, 2016), acaricidal (Chiasson et al., 2001) and repel insects (Croom, 1981; Mihajilov-Krstev et al., 2014) and ticks (Jaenson et al., 2005). In addition, these EOs have fungicidal effects (Umpierrez et al., 2012). *Absinthium* EOs are also antimicrobial (Erel et al., 2012; Juteau et al., 2003; Mihajilov-Krstev et al., 2014) and exhibit antiprotozoal effects against *Leishmania aethiopica* and *L. donovani* (Tariku et al., 2011).

While most of these studies have been conducted with EOs or organic extracts, other products from plants such as hydrolates that result from vapor pressure extraction of EOs have been scarcely studied. These aqueous extracts generally have water-soluble volatile compounds and possibly large amounts of active ingredients such as acids, aldehydes or amines. Until now, this product has been considered a mere byproduct of the process used to obtain EOs. There are a few studies that have focused on the antifungal, antibacterial and antioxidant activities of hydrolates from different plants (Boyraz and Ozcan, 2006; Boyraz and Özcan, 2005; Franzener et al., 2007; Maia et al., 2013; Prusinowska et al., 2016; Tornuk et al., 2011). Hydrolates could be a sustainable source of new biopesticides. Hydrolates of *Lavandula intermedia* (var. super) and *L. luisieri* were strongly nematicidal against *Meloidogyne javanica* (Andres et al., 2017), and the *L. luisieri* hydrolate showed strong phytotoxic activity against both *Lactuca sativa* and *Lolium perenne* (Julio et al., 2016).

Hydrolates could potentially enter the environment either by its use as a biopesticide or as a residue from EO extraction. Due to its solubility in water, it would presumably be more bioavailable than an organic extract. However, there are no studies about plant-derived hydrolate ecotoxicity. Less information is available about

the effects of aqueous compounds or hydrolate byproducts from *A. absinthium*. As far as we know, only the inhibition of *Phytophthora parasitica* and *P. capsici* using aqueous *A. absinthium* extract has been reported (Ali et al., 2015). No data are available about its ecotoxicity.

Spanish populations of *A. absinthium* (var. Candial) have been domesticated for cultivation and are thus chemically stable (Bailen et al., 2013; Gonzalez-Coloma et al., 2012; Julio et al., 2015a; Martin et al., 2011). The hydrodistilled and semi-industrial vapor-pressure EOs from these *A. absinthium* populations showed antifungal (Julio et al., 2015a), nematicidal (Garcia-Rodriguez et al., 2015) and leishmanicidal and trypanocidal activities (Gonzalez-Coloma et al., 2012; Martinez-Diaz et al., 2015). The properties of the hydrolate as a biopesticide have been quite promising. Recently, it was found to have nematicidal activity (Julio et al., 2017b).

The composition of this plant's EOs is well characterised (Bailen et al., 2013; Garcia-Rodriguez et al., 2015; Gonzalez-Coloma et al., 2012; Julio et al., 2015a), and the composition of the hydrolate of has been recently described.

The aim of this study was to characterise the aquatic ecotoxicity of hydrolates and organic extracts from *A. absinthium* L. on an aquatic invertebrate (*Daphnia magna*), a marine bacterium (*Vibrio fisheri*) and a unicellular freshwater alga (*Chlamydomonas reinhardtii*). In addition, the effect of this hydrolate on a river microbial community was analysed in order to evaluate its potential impact on a fluvial ecosystem.

2. Material and methods

2.1. Plant material

A domesticated population of *A. absinthium* (var. Candial) was used. The seedlings were planted in an experimental field at Ejea de los Caballeros (Zaragoza, Spain) in 2008 and harvested in August 2016 (Julio et al., 2015a). Hydrolate was obtained from EO extraction by vapor-pressure steam distillation (Julio et al., 2017b), and the organic extract was sequentially extracted with hexane and ethanol, 1:10 (w/w) dry plant: organic solvent, by maceration at room temperature (48 h).

2.2. Extraction and fractionation

Semi-industrial vapor pressure extraction was carried out in a 3000 L stainless steel industrial distillation facility (http://www.cita-aragon.es). The plant material was steam distilled for 1 h to provide the EO and hydrolate. Chemical characterisation was carried out using chromatographic techniques, mass spectrometry and nuclear magnetic resonance (NMR 1D and 2D) and led to the isolation of monoterpene diols, tetrahydrofuranoid isomer derivatives, tetrahydropyranoid isomer derivatives and tetraols (Julio et al., 2018, submitted). The major active component of the hydrolate was characterised as (−)-(Z)-2,6-dimethylocta-5,7-diene-2,3-diol (Julio et al., 2017b).

2.3. D. magna assay

D. magna (water flea) assays were performed according to the Standard Operational Procedures of the Daphtoxkit FTM magna (1996), which is in accordance with OECD 202 (2004) guidelines. The kit, which included *D. magna* ephippia, syntenthic freshwater and food (spirulina microalgae) was purchased from Vidrafoc (ref. DM030316) and stored at 4 °C.

The eggs were incubated for 72 h at 20–22 °C with 6000 lx light in a TOXKIT model CH-0120D-AC/DC incubator (supplied by ECOTEST). The neonates were pre-fed with one vial of the spirulina microalgae 2 h before exposure to *A. absinthium* hydrolate and EO. Hydrolate was diluted in synthetic freshwater (ISO 6341 2012) at the following dilutions: 10%, 1%, 0,1% and 0,01% (v/v). Synthetic freshwater was used as the negative control. *A. absinthium* organic extract (ethanol and hexane extraction), due its insolubility in water, was diluted in dimethyl sulphoxide (DMSO; 0.5% in water) at the following dilutions: 62%, 6,2%, 0,62% and 0,062%. Previous studies ensured that these DMSO concentrations were innocuous to *D. magna* (at least up to 5% of DMSO). Additionally, positive controls with K_2Cr_2O7 (0.6–2.1 mg/L) were also tested (OECD 202 1984). The pH of the solutions was adjusted to be between 7 and 7.5 using 0.1 M NaOH or 0.1 M HCl solutions. After 2 h of feeding, a total of 25 organisms (24 h old) were used. The organisms were divided into five groups of five organisms per group. Each concentration of hydrolate and EO was performed in five replicates per plate. All concentrations with the daphnids were incubated in complete darkness for 24 h at 20–22 °C. After a 24 h exposure period, immobilised individuals were checked and counted. The daphnids that were unable to swim for 15 s after gentle agitation of the test-vial were considered immobile. The results were calculated as EC_{50} (chemical concentration resulting in 50% in mobilisation).

2.4. V. fisheri assay

These experiments were carried out in accordance with the test conditions and the operating protocol of the *V. fisheri* acute toxicity test (UNE-EN-ISO 11348-3 2009). The lyophilised *V. fisheri* (strain NRRL-B-11,177) that was used for the bioluminescence inhibition assays was purchased from Macharey-Nagel (ref. 945 006). Prior to testing, bacteria were rehydrated using the corresponding reactivation solution provided by the manufacturer. The reactivated bacteria were stored for 5 min at 4 °C and several dilutions of the *A. absinthium* hydrolate were prepared in a 2% NaCl stock solution (v/v): 25%, 10%, 5%, 1% and 0.5%. The pH of the solution was adjusted to 6–8 using either 0.1 M NaOH or 0.1 M HCl solutions. Next, 0,5 mL of the reactivated bacterial suspension was transferred to test tubes tempered at 15 °C in a water bath cuvette. The solution was equilibrated for 10 min and the first measurements were taken to obtain the basal luminescence; subsequently, 0,5 mL of each dilution to be tested was added to the tubes. Luminescence inhibition was measured after 30 min. The test was repeated in triplicate. Three test tubes with bacteria and without hydrolate served as the negative control. Luminescence measurements were obtained with a Biofix® Lumi-10 luminometer (Macharey-Nagel) using the acute mode (Biotox B) equipped with an ultra-fast single-photon counter detector that covered the 3806–660 nm spectral range. The toxic effect values reflect the ratio of the decrease in bacterial light production to the remaining light. The percentage of luminescence inhibition was calculated for each concentration relative to the control and expressed as the EC_{50} (chemical concentration that resulted in 50% bioluminescence reduction).

2.5. C. reinhardtii assay

Unicellular green algae (*C. reinhardtii*; CC125) in the exponential growth phase were maintained under controlled conditions (25 °C, 90 rpm and 130 μE PAR $m^{-2} s^{-1}$) in standard growth medium (*Talaqui*), prepared as previously described (Szivak et al., 2009), except that $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ were used instead of the corresponding sulphates and the pH was adjusted to 7.5. The following dilutions of *A. absinthium* hydrolates were prepared in 25 mL Erlenmeyer flasks immediately before each experiment (v/v): 1OO %, 50%, 10%, 4%, 2% and 1%. Alga without hydrolate were the negative control.

Seventy-two-h-old algae were centrifuged (10 min, 3000 rpm), and the concentrate was adjusted to an optical density (OD) of 0.15. OD was measured by a spectrophotometer (wavelength 685 nm). Similar to Pino et al. (Pino et al., 2016), the alga were inoculated into the flasks 1 min apart and were exposed to the same light and shaking conditions as those during growth. Room temperature was kept between 23 and 26 °C. The pH of the solutions was 7.52 at the beginning of the test. The algal photosynthetic yield, in light, of photosystem II was measured using a Mini-PAM fluorometer (Walz, Effeltrich, Germany). The yield reflects the efficiency of the photochemical energy conversion process. The settings used were as follows: measuring light, level 10; saturation pulse, level 8; length, 0.8 s; gain, 2. The samples used to measure fluorescence were collected after a 1 h exposure. Each sample consisted of 3 mL that was introduced in an optical glass cuvette (Starna Scientific Ltd.; 3,5 mL, 10 mm). Alga were kept in suspension by magnetic shaking. After a 30 s acclimatisation to the new light conditions ($\approx$65–70 μE PAR m^{-2} s^{-1}), consecutive Y(II) measurements were recorded every 10 s. Three replicates (flasks) were prepared for each concentration. Three Y(II) measurements were taken from each replicate. Dose-response curves were generated and the EC_{50} values were calculated after a 1 h exposure.

2.6. Community-level physiological profiling (CLPP) of water microbes with the Biolog EcoPlate

2.6.1. Water samples

Water samples were collected on October 17, 2017 from the Gallego River (Villanueva de Gállego, Zaragoza, Spain). The samples were transported to the laboratory in less than 15 min and stored at 4 °C until use. The physicochemical characteristics of this water are provided in Table 1. The conductivity, pH, dissolved oxygen and water temperature were measured *in situ* and in the laboratory. The other parameters were assessed within 24 h of sampling and transportation to the laboratory according to standard procedures. For genetic analysis, microorganisms were extracted from 1 L of the river water that was filtered through 22 μm filter, resuspended in a Falcon tube with 10 mL Milli-Q water, centrifuged at 5000 g and stored at −80 °C until sequencing.

Table 1
Physicochemical characteristics of water samples collected on October 17, 2017 from the Gállego river (Villanueva de Gállego, Zaragoza, Spain).

Parameter	value	
Conductivity	2853	μs/cm
pH	7,92	
Total Suspended Solids	4,2	mg/L
MO	2,2	mg/L
Total Dissolved Solids	1360	mg/L
Fluorides	0,094	mg/L
Chlorides	507,6	mg/L
Nitrites	0	mg/L
Bromides	0,567	mg/L
Nitrates	13,563	mg/L
Phosphates	0	mg/L
Sulphates	383,11	mg/L
Total alkalinity	223,26	mg/L
Sodium	332,72	mg/L
Ammonium	0	mg/L
Potassium	3623	mg/L
Calcium	198,2	mg/L
Magnesium	31,683	mg/L
Parameter *in situ*	value	
Conductivity	2340	μs/cm
pH	8,1	
Dissolved oxygen	11,1	mg/L
Dissolved oxygen	128.2	% sat.
Water temperature	22,1	°C

2.6.2. Genetic sequencing of river microorganisms

Genetic sequencing of microorganisms in Gállego River water samples was performed in the Genomics Unit Cantoblanco, Science Park (Madrid, Spain). For bacterial analysis, samples were homogenised in PBS, and bacterial genomic DNA was extracted from 200 μL aliquots after proteinase K and RNAse digestion using G-spin columns (INTRON Biotechnology). DNA concentration was determined using Quant-IT PicoGreen reagent (Thermo Fischer), and DNA samples (approximately 3 ng) were used to amplify the V3-V4 region of the 16S ribosomal RNA (rRNA) gene as previously described (Caporaso et al., 2012; Caporaso et al., 2011); these studies showed that this technique of amplicon sequencing reasonably reflects the populations of an ecosystem.

Polymerase chain reaction (PCR) products (approximately 450 base pairs) included extension tails that allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle-number PCR. As described before (Aranda et al., 2018), Individual amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent), and a pool of samples was prepared in equimolar amounts. The pool was further cleaned, quantified and the exact concentration was estimated by real-time PCR (Kapa Biosystems). Finally, DNA samples were sequenced on an Illumina MiSeq Instrument under a 2 × 300 protocol. Following sequencing, 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, 91% of reads were positively filtered by the system as acceptable, and >79,5% of the bases had a Q value greater than or equal to Q30.

2.6.3. Sample exposure to hydrolate and the Biolog EcoPlate assay

The ability of water microbial communities to utilise a variety of carbon sources after hydrolate exposure was assessed using a community-level substrate utilisation test based on direct incubation of environmental samples in a Biolog EcoPlate. Dilutions of *A. absinthium* hydrolate in river water (100%, 50%, 25% and 1%) were prepared in a final volume of 150 μL in the wells of a Biolog plate. 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 25 °C for 7 days under sterile conditions. The final pH for hydrolate dilutions was between 7.71 and 8.06.

The OD (wavelength 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 Science Park, Cambridge, England). This method differentiates microbial communities and identifies which substrates are most utilised by these communities (Yu et al., 2012). It is also useful for comparing toxicant effects (Muniz et al., 2014; Pino-Otin et al., 2017; Tiquia, 2010). Every plate had 96 wells; there were 31 different carbon sources plus a blank (water) in triplicate. The rate of utilisation of the carbon sources was thus assessed as the reduction of tetrazolium violet redox (Pohland and Owen, 2009).

2.7. Statistics and graphical representation

2.7.1. D. magna, V. fisheri and C. reinhardtii assays

Dose-response curves for *D. magna* mobility, *V. fisheri* bioluminescence and algal photosynthesis 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-squared test on the logarithm of the likelihood ratio that evaluates if the variables brought significant information by comparing the model as it was defined with a simpler model with only one constant.

2.7.2. Biolog EcoPlate™ assay and physiological diversity of bacterial communities

For statistical analysis, the $OD_{t=0}$ for each well was subtracted from $OD_{t=x}$. Since Biolog EcoPlates contained three replicates of 31 carbon sources (in addition to three negative control wells without a carbon source), the averages and standard deviations that corresponded to each carbon source were also calculated. The microbial activity of each microplate was expressed as the average well color development (AWCD) and was determined as previously described by Garland and Mills, (1991) as follows:

$$AWCD = \sum_{i=0}^{i=12} (OD_{t=x_i} - OD_{t=x_0}) \quad (1)$$

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 ODs at the plateau of the AWCD were also used to calculate the physiological diversity of bacterial communities using Paleontological Statistics Software (PAST 3.0; (Fang et al., 2009; Pino-Otin et al., 2017; Tortella et al., 2013) as follows:

- Shannon-Weaver index (as a measure of richness):

$$H = \sum_{i=1}^{i=12} p_i \log_2 p_i$$

where p_i is the ratio of absorbance of a particular well to the sum of absorbances of all microplate wells. The Shannon-Weaver index was calculated by considering the absorbance values in each well as being equivalent to species abundance.

The variance relationship between the AWCD values of the three replicates and Student's t-test on two independent samples to assess significance were calculated using XLSTAT software (2014.5.03).

3. Results

3.1. Effects of A. absinthium hydrolate on D. magna

The dose-response curve for *D. magna* after a 24 h exposure to *A. absinthium* hydrolate and organic extract are shown in Fig. 1a and b, respectively. All toxicity values analysed with the chi-squared test were highly significant ($P < 0.0001$). The hydrolate was acutely toxic to *D. magna*, with a LC_{10} value of 0.013% (0.002–0.036) and a LC_{50} value of 0.236% (0.111–0.406) hydrolate dilution. In addition, the organic extract sequentially extracted with ethanol showed a LC_{10} value of 0.026 mg/L (0.011–0.041) and a LC_{50} of 0.093 mg/L (0.067–0.124). The organic extract extracted with hexane showed a very similar dose-response curve (not shown) with 0.034 mg/L (0.015–0.052) and 0.103 (0.074–0.135) LC_{10} and LC_{50}, respectively.

3.2. Effects on V. fisheri

Fig. 2 shows the dose-response curve of *A. absinthium* hydrolate on *V. fisheri*, measured as the percentage decrease in bioluminescence. All toxicity values analysed with the chi-squared test were highly significant ($P < 0.0001$). The LC_{10} was 18.52% (14.83–24.09) and the LC_{50} was 1.85% (1.61–2.11) of the hydrolate dilution.

3.3. Effects on the photosynthetic yield of C. reinhardtii

The dose-response curves for *C. reinhardtii* after a 1 h exposure to *A. absinthium* hydrolate are shown in Fig. 3. All toxicity values analysed with the chi-squared test were highly significant ($P < 0.0001$). The hydrolate displayed a clear dose-dependent effect on *C. reinhardtii* photosynthesis. LC_{10} was 2.33% (1.92–2.76) and LC_{50} was 16.49% (14.69–18,59) of the hydrolate dilution.

3.4. Effects on physiology and diversity of water microbes

3.4.1. Genetic analysis of microbial populations

Fig. 4 shows the relative abundance of the eight most common taxons at each classification level. Complete taxonomic classification with all taxa can be found in Supporting Information Fig. SI1. Sequencing results were from 205,746 total reads; 193,902 reads passed quality filtering (94.2%).

A high percentage of taxa within higher levels of organisation (phylum, class, order and family) were succesfully sequenced (greater than 90%). Lower percentages were sequenced at the genus (82.79%) and species (26.36%) levels (Supporting Information, Fig. SI2). Therefore, higher levels of organisation were considered in the discussion (see Fig. 4).

The sunburst charts (Fig. 5) show the relative abundance of the taxons within each taxonomic level; organisation from inside to outside the circle: phylum, class, order, family, genus and species. In circles b and c, the inner circle begins in the class taxonomic level. The "Other" category is the sum of all classifications with less than 3.5% abundance. There were three predominant phylums: Proteobacteria (64.66%) followed by Bacteroidetes (17.28%) and Firmicutes (11.73%).

Proteobacteria are the most abundant creatures in the oceans and other aquatic environments and constitute approximately 40% of the freshwater bacterial biomass (Battistuzzi and Hedges, 2009). Proteobacteria can be classified into three main families based on 16S rRNA: alpha, beta and gamma. All three are represented in the analysed sample, but nearly half of the taxa identified in this phylum are Alphaproteobacteria (45.66% of Proteobacteria; 27% of total taxa), followed by Betaproteobacteria (30% of Proteobacteria; 17.80% of total taxa) and finally Gammaproteobacteria (17.29% of Proteobacteria, 10.26% of the total taxa).

The Alphaproteobacteria, with almost 1000 described species, include most of the oligotrophic bacteria capable of living in an environment that offers very low nutrient levels. Of these, the Sphingomonadaceae predominated in the sample (88.67% of the Alphaproteobacteria; 23.88% of total taxa). The predominant genus was *Novosphingobium* (91.41% of Sphingomonadaceae; 21.83% of total taxa). *Novosphingobium* species have been isolated from a wide range of ecological habitats, including aquatic environments (D'Argenio et al., 2011).

Betaproteobacteria, with almost 500 described species, present a wide variety of metabolic and ecological properties (Madigan et al., 2015). Betaproteobacteria frequently appears among the most abundant members of freshwater bacterioplankton (Lindstrom et al., 2005). Almost all the identified Betaproteobacteria were from order Burkholderiales (98.42% of Betaproteobacteria; 17.52% of total taxa), and of these, almost all were from family Comamonadaceae (95.05% of Burkholderiales and 16.66% of the total). Of this family, the *Limnohabitans* genus, an important group of freshwater bacterioplankton (Kasalicky et al., 2013), predominated (57.3% of the Comamonadaceae and 9.55% of the total taxa).

Gammaproteobacteria include a wide variety of physiological types; while they accounted for only 10.26% of the total taxa, we found a great variety of taxa, among which the *Pseudomonas*, (2.21% of total taxa), *Acinetobacter* (0.89% of total taxa) and *Mannhelmia*

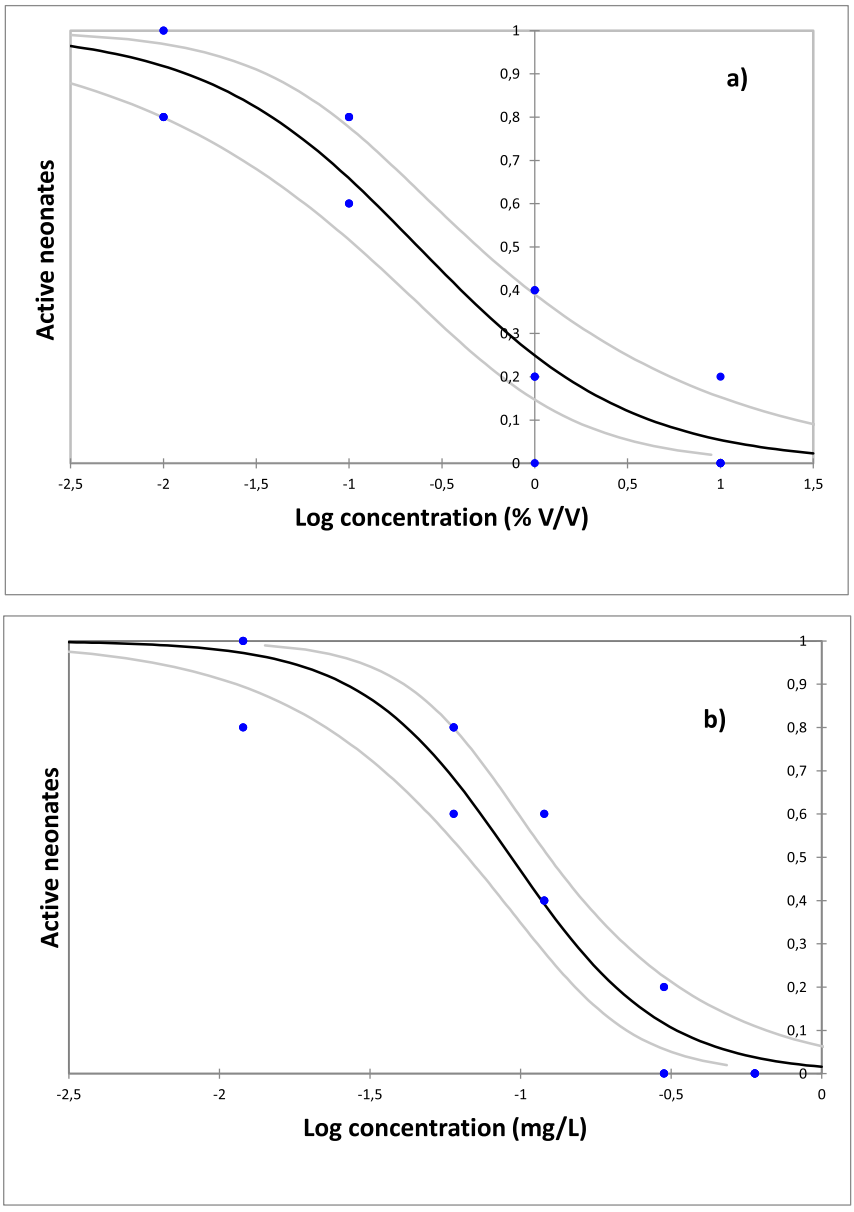


Fig. 1. Dose-response curve of *A. absinthium* L hydrolate **(a)** and vegetal extract (ethanol) **(b)** after a 24 h exposure to *D. magna*. Pale gray lines indicate the confidence limits (95%). Curve is the average value of five replicates.

(2.25% of total) genera predominated.

3.4.2. Biolog plate AWCD

Fig. 6 shows the Biolog plate AWCD during the 7-day incubation; each point represents the average of three replicates. Exposure of bacterial communities to different hydrolate concentrations showed a trend similar to control but with slightly lower values. In the case of the highest concentration (100%), the AWCD decrease was significant (Student's t-test, $p \leq 0.5$). However, 50% hydrolate concentration promoted a small stimulatory effect (i.e., an increase in AWCD from the second to the fifth day).

The dose-response curves for microorganisms exposed to *A. absinthium* hydrolate (measured as AWCD % decrease) are shown in Fig. 7. We found a LC_{10} of 1.64% (0.50–3.29) and a LC_{50} of 74.85% (47.69–145.97) hydrolate dilution. All toxicity values analysed with the chi-squared test were highly significant ($P < 0.0001$).

3.4.3. Physiological diversity of bacterial communities

Maximum ODs values for each of the 31 carbon sources were used to calculate the physiological diversity of bacterial communities (Muniz et al., 2014). River microorganisms exposed to *A. absinthium* hydrolate showed a heterogeneous response in their physiological diversity with some significant changes (Fig. 8). The 50% and 25% hydrolate dilutions provoked significant differences in diversity compared to the control (Student's t-test, $p \leq 0.5$), while the 100% ($p = 0.930$) and 1% ($p = 0.569$) doses were not different from control. Overall, all dilutions (although less markedly in the case of the 1% dilution) increased diversity with respect to the control. Undiluted hydrolate caused a marked decrease in the Shannon-Weaver index after five days. This effect was similar to what was observed for AWCD values (Fig. 6).

3.4.4. Physiological diversity of substrate utilisation

The impact of four *A. absinthium* hydrolate concentrations

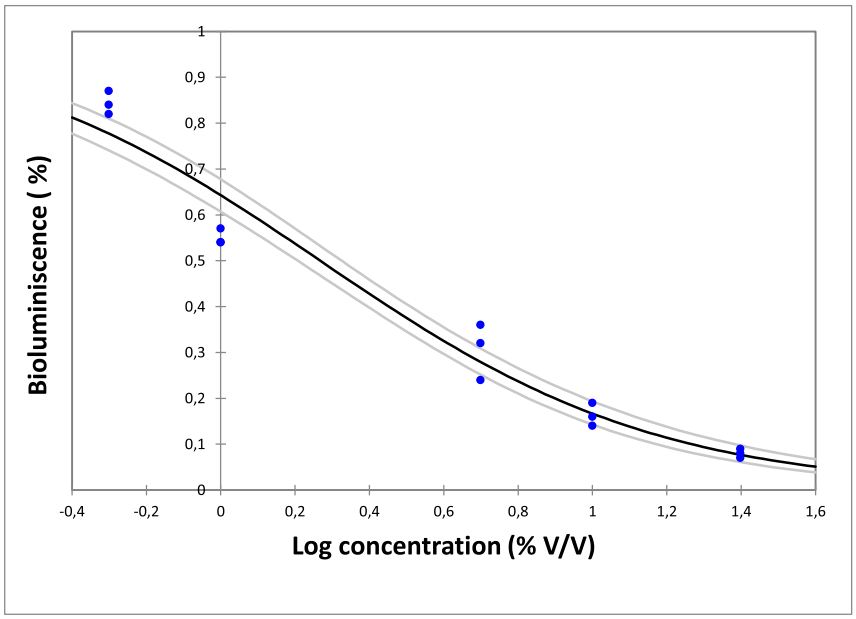


Fig. 2. Concentration-response curves of bioluminiscence loss of *V. fisheri* after 30 min of exposure to *A. absinthium* L hydrolate as a function of logarithm of the concentration. Bioluminiscence values are expressed as the percentage of the control. Pale gray lines indicate the confidence limits (95%). Each concentration was asssayed in triplicate. The points are the values of each triplicate.

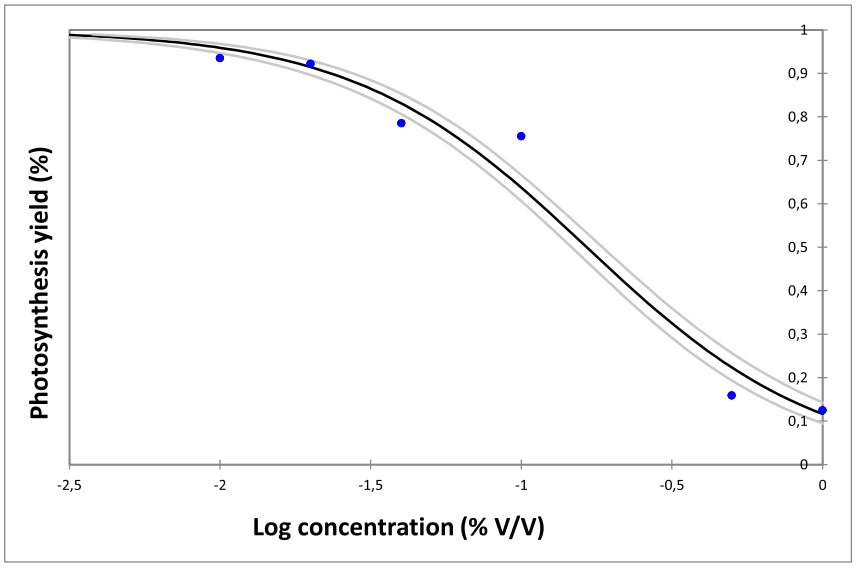


Fig. 3. Concentration-response curves of photosynthetic yield of *C. reinhardtii* after 1 h of exposure to *A. absinthium* L hydrolate as a function of logarithm of the concentration. Photosynthetic values are expressed as the percentage of the control. Pale gray lines indicate the confidence limits (95%). Each point is the average value of three replicates.

during 168 h was assessed on physiological diversity measured as the ability to degrade different carbon sources. The Shannon-Weaver diversity index (H; Fig. 9) was used as a diversity indicator. A higher H value corresponded to the ability to degrade a greater diversity of carbon sources. The 31 carbon sources were grouped within five functional classes (carbohydrates, polymers, carboxylic and ketonic acids, amino acids and amines/amides) according to previous studies (Lehman et al., 1995; Pino-Otin et al., 2017; Weber and Legge, 2009; Zak et al., 1994).

A generalised increase in the capacity to metabolise all substrate groups was observed except for the undiluted extract. On the fifth day, the Shannon-Weaver index decreased for almost all metabolite groups for the undiluted extract. This decrease was in accordance with the AWCD decrease (see section 3.4.2). However, during the initial days, there was a significant increase in the use of polymers, carbohydrates and carboxylic and ketonic acids. Exposure to the 50% hydrolate dilution resulted in a significant increase in the use of polymers, amino acids and amines/amides; this trend was maintained during the entire experiment. At the 25% dilution, the Shannon-Weaver index increased compared to control in the case of polymers ($p = 0.08$), carbohydrates ($p = 0.017$), carboxylic and ketonic acids ($p = 0.189$) and amines/amides ($p = 0.104$); this trend was maintained during the entire experiment. When we applied the 1% hydrolate dilution, we observed statistically significant changes in the use of polymers (slight decrease) and carbohydrates (slight increase).

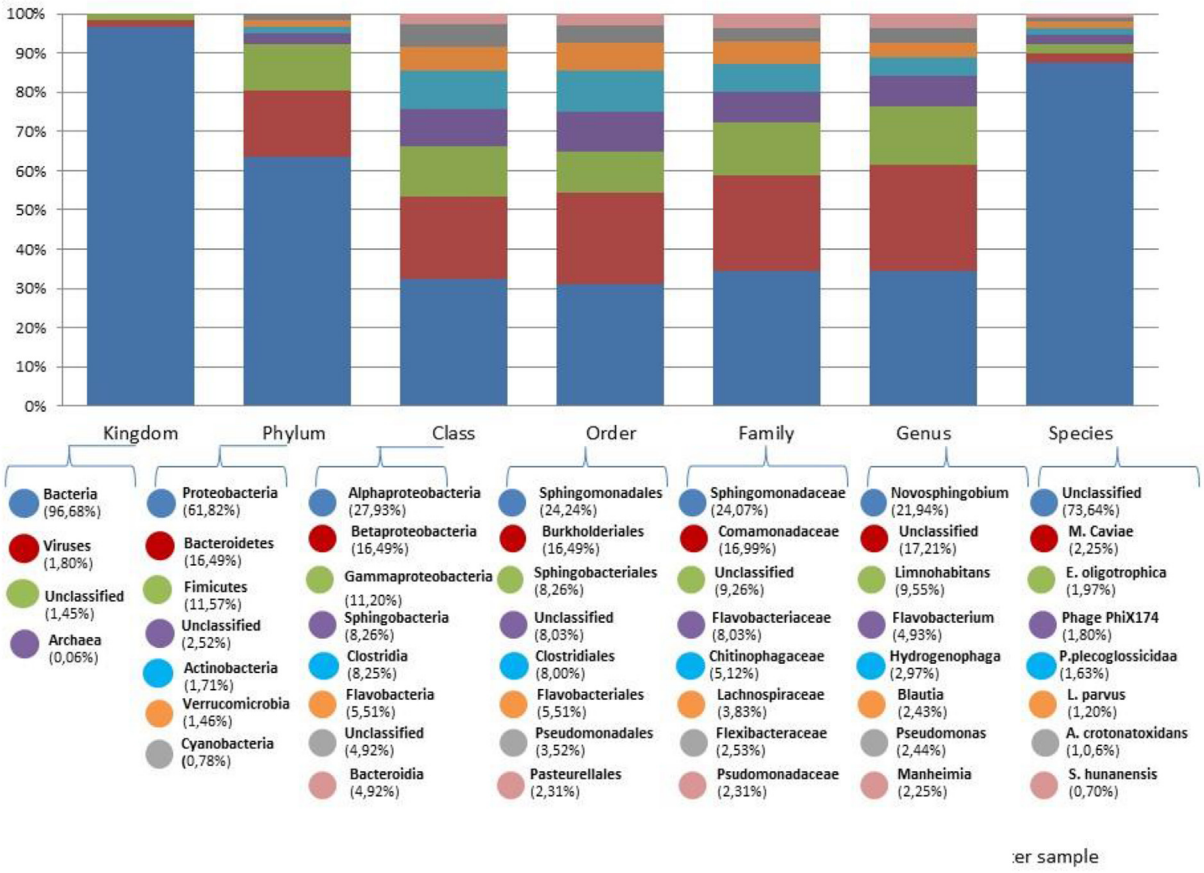


Fig. 4. Relative abundance of the highest 8 taxonomic classifications at each level of microorganisms from river water sample collected on October 17, 2017 from the Gallego river (Villanueva de Gállego, Zaragoza, (Spain).

4. Discussion

We present here for the first time a comprehensive study of the ecotoxicity of a nematicidal byproduct (hydrolate) of a domesticated population of Spanish *A. absinthium* (var. Candial) on three non-target aquatic ecotoxicity indicator organisms and also on the overall aquatic microbial community of a river. This study presents evidence that *A. absinthium* hydrolate can produce an important impact on different non-target aquatic organisms. The tested organisms, presented a wide range of sensitivity to hydrolate dilutions: *D. magna* ($LC_{50} = 0.236\%$); *V. fisheri* ($LC_{50} = 1.85\%$); *C. reinhardtii* ($LC_{50} = 16.49\%$); river microorganisms ($LC_{50} = 74.85\%$). In addition, the sequential ethanol-organic *A. absinthium* extract showed the lowest LC_{50} value on *D. magna* ($LC_{50} = 0.093$). To our knowledge, these ecotoxicological endpoints are the firsts measured for a hydrolate from any plant with biopesticide properties. Even though *A. absinthium* hydrolate caused acute toxicity in previous pure culture *in vitro* studies, we observed that this substance had very little effect on the growth of natural freshwater microbial community populations. Exposure to *A. absinthium* hydrolate resulted in an increased capacity to metabolise all carbon sources. Only the undiluted hydrolate decreased metabolite degradation (and only from the fifth day forward). In all cases the undiluted extract caused the most intense changes. The lack of clear dose-dependency on the effects may be the result of variable sensitivities from the different species found in the community and their broad metabolic spectrum.

4.1. Ecotoxicity effects on single non-target organisms: D. magna, V. fisheri and C. reinhardtii

LC_{50} values in the range of 0.2–16.5% (v/v) were observed for *A. absinthium* hydrolate on *D. magna, V. fisheri* and *C. reinhardtii*. However, it is necessary to know the composition of this hydrolate to better assess its effect. The chemical characterisation of the hydrolate used in this study was recently reported by Julio et al. (2018; submitted) and led to the isolation of a major active component, the (−)-(Z)-2,6-dimethylocta-5,7-diene-2,3-diol (Julio et al., 2017b) and other compounds related to (−)-*cis*-epoxyocimene (Julio et al., 2017a; Moore et al., 1999), the main component of the EO (Julio et al., 2015b).

The monoterpene (Z)-diol is a stereoisomer of 2,6-dimethylocta-5,7-diene-2,3-diol (Agrebi et al., 2012), and it is the most abundant compound in the hydrolate residue. Given its abundance (31,2%; Julio et al., 2018; submitted) and its nematicidal effect (Julio et al., 2017b), this compound might be considered the chemical biomarker of this extract and its potential formulations (Julio et al., 2018; submitted). In addition, this monoterpene diol exhibited a moderate antifeedant effect on the insect *Spodoptera littoralis* (Bailen et al., 2013). Moreover, the nematicidal activity (against *Caenorhabditis elegans*) of a series of monoterpenoids, including some acyclic alcohols like nerolidol, geraniol, citronellol and farnesol, has been reported (Abdel-Rahman et al., 2012). These studies suggest that this monoterpene diol could be the responsible for the high toxicity of *A. absinthium* hydrolate on *D. magna.*

Taken into account that physics and chemical properties for this

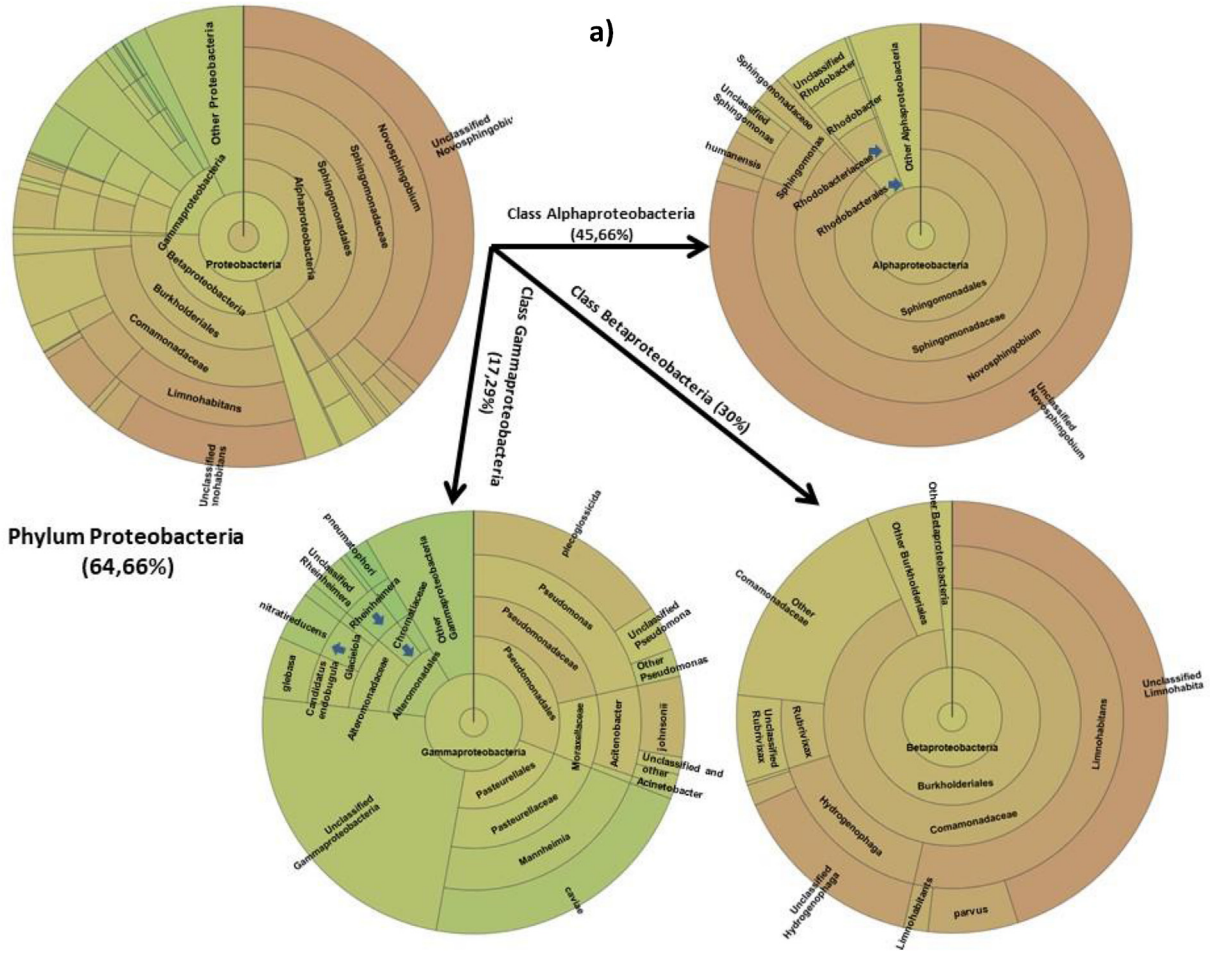


Fig. 5. Relative abundance of the main taxons within each taxonomic level. From inside the circle to outside: phylum, class, order, family, genus and species. In circles b and c the inner circle begins in the class taxonomic level. The "Other" category is the sum of all classifications with less than 3.5% abundance.

monoterpene diol are not been reported, the quantum continuum method COSMO-RS (Eckert and Klamt, 2002; Klamt et al., 1998) has been chosen for predicting water solubility, pka and log P of the monoterpene diol. Calculations were performed using a continuum model with DFT in a two-step procedure as described before (Martinez-Lopez et al., 2018). The water solubility of the monoterpene diol was 715 mg/L, pka = 20.5 and log P = 3.1. Therefore, this compound probably can easily access *Daphnia* organisms through its feeding activity and ingestion.

The organic extract with ethanol or hexane exhibited very similar dose-response curves and LC_{50} values. This similarity may rely on the fact that (Z)-2,6-dimethyl-5,7-octadien-2,3-diol has been isolated from *A. absinthium* EOs (Bailen et al., 2013), and it is expected to also be found in this organic extract.

The mechanism of action of (Z)-2,6-dimethyl-5,7-octadien-2,3-diol, however, is unknown. Terpenoids can cause cytotoxic effects depending on their ability to damage cell membranes (Bakkali et al., 2008). This monoterpene is a member of a class of compounds known as fatty alcohols; it has a low molecular weight of 170.25 g/mol (Wishart et al., 2018), and based on its pKa (20.5), it is weakly acidic and will ionise with difficulty. Thus in water, the non-ionising forms that are more liposoluble will predominate and be more available to cross biological membranes. The basis of the terpenoids mechanism of action have been related to sodium channel activity that increases the permeability of sodium ions in excitable membranes (Holstege et al., 2000; Yakehiro et al., 2000). Generally, EO and terpenoid toxicity in insects is related to neurotoxic effects and growth regulation action (Isman, 2000; Tong and Coats, 2010).

The lethal effects, and the clear dose-response relationships found, of the hydrolate on the marine bacterium *V. fisheri* and in the unicellular algae *C. reindhardtii* might be related to altered cell membrane integrity of compounds similar to (Z)-2,6-dimethylocta-5,7-diene-2,3-diol. This family of compounds may insert into the lipid bilayer to increase its fluidity and consequently cause cytoplasmic leakage and cell death (Camargos et al., 2014). The disruption of cell membrane integrity by interfering with ergosterol biosynthesis and PM-ATPase inhibition has been suggested as toxicity mechanism of geraniol (3,7-dimethylocta-2,6-dien-1-ol) in *Candida* (Sharma et al., 2016). Other acyclic sesquiterpenes (*trans*-nerolidol, *cis*-nerolidol and farnesol) were inserted into the mitochondrial membrane and provoked significant cytochrome P450 inhibition (Spicakova et al., 2017). On the other hand, its antiparasitic effect appears related to interference with isoprenic chain elongation (de Macedo et al., 2002). Antimicrobial activity has also been described for other similar acyclic fatty alcohols such as farnesol and nerodinol (Van Zyl et al., 2010). One of the major modes of antibacterial activity against *Staphylococcus aureus* for these terpenes alcohols has been suggested to be bacterial cell damage and K^+ ion leakage (Inoue et al., 2004). Interestingly, many of these

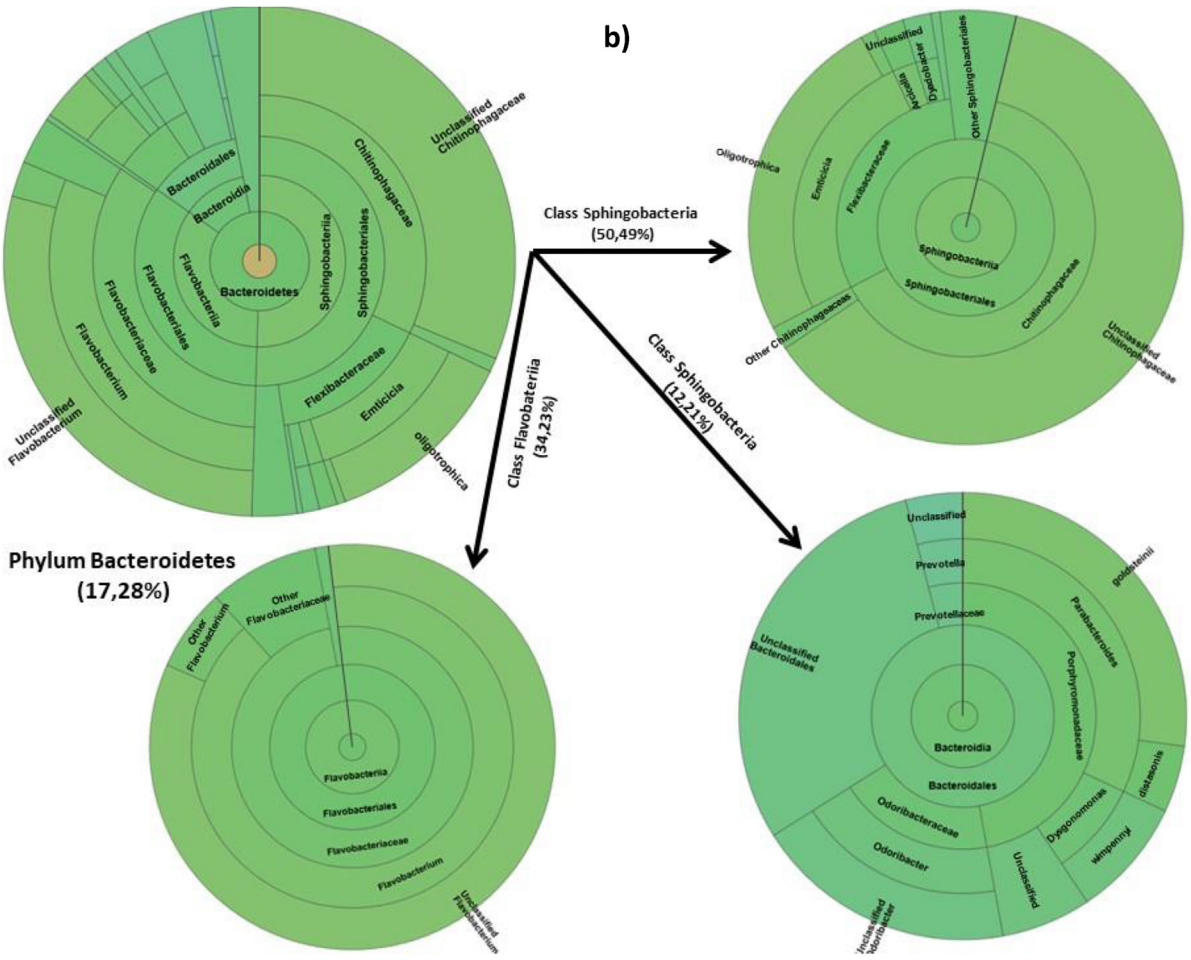


Fig. 5. (*continued*).

studies were performed on gram-pathogenic bacteria, e.g., *S. aureus*, *Escherichia coli* and *Salmonella enterica* (Inoue et al., 2004; Simoes et al., 2008; Tao et al., 2013). These studies suggest that these compounds may cross the complex cell wall, possibly also that of the family Vibrionaceae.

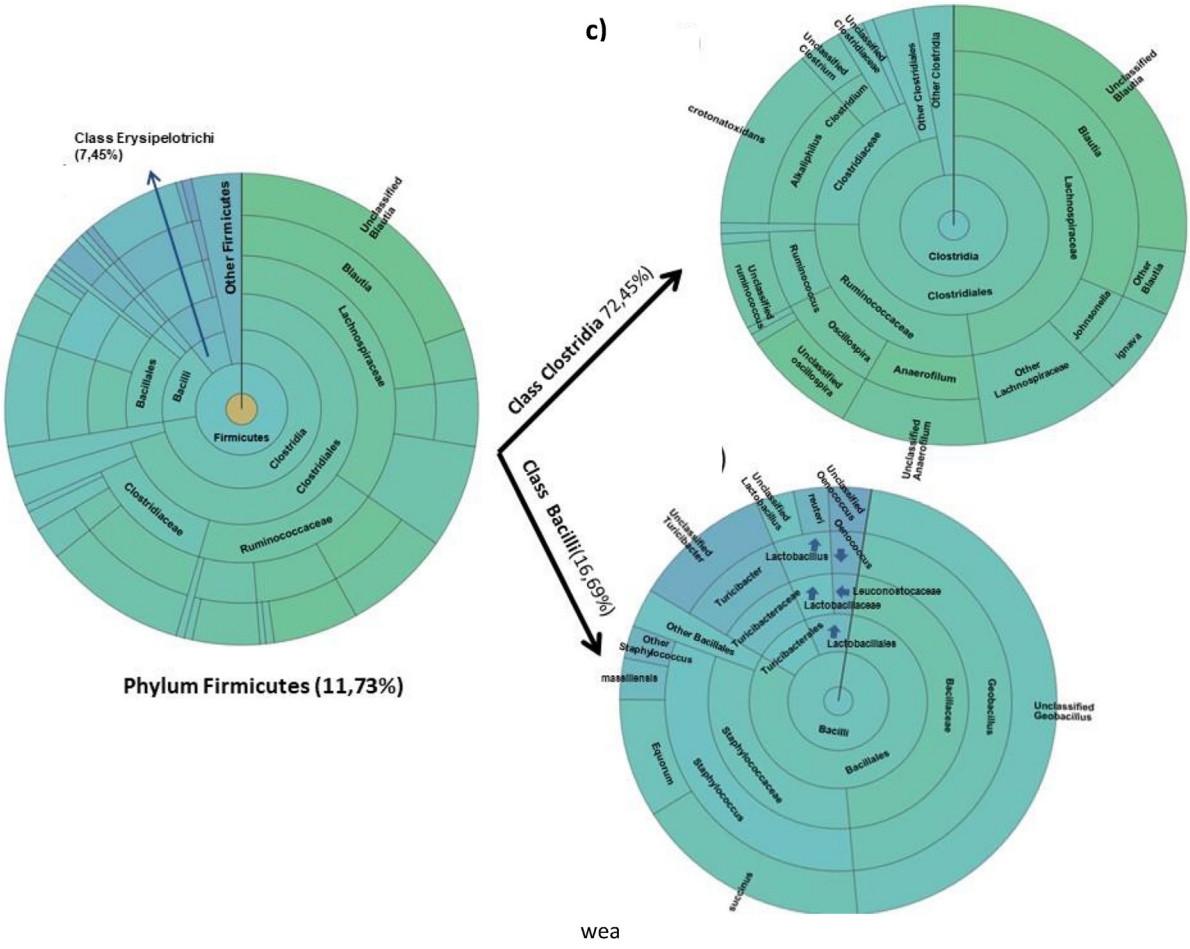
The toxicity of 2,6-dimethylocta-5,7-diene-2,3-diol to *C. reinhardtii* may also relate to its activity on membrane disruption. However, the higher EC_{50} found (i.e. this alga was less sensitive than *V. fisheri* and *D. magna*) may be due to the complex cell wall that is formed by hydroxproline-rich glycoproteins and crystalline layers (Voigt, 1988). As has been already demonstrated for ionic liquids, this complex cell wall may act as a barrier that protects the algae (Sena et al., 2010).

4.2. Effect on microbial freshwater communities

The natural freshwater microbial communities were more resistant to 2,6-dimethylocta-5,7-diene-2,3-diol than *V. fisheri*. This result can be explained because of the physicochemical behaviour of the compound in the river water, and thus its altered bioavailability, as well as different sensitivity of the species present in the natural microbial community. On the other hand, adsorption to organic matter (2.2 mg/L) or suspended solids (4.2 mg/L) that would also make it less bioavailable for river bacteria cannot be excluded. Potential interactions with other high concentration anions in the analysed river water sample, including sulphates, chlorides and nitrates (Table 1), may also be involved. However, monoterpenodiol may be bioavailable, to some degree, for bacteria as shown by changes in the metabolic biodiversity compared to control. Based on the rationale of the "Pollution Induced Community Tolerance Concept" (Blanck et al., 1988), most sensitive bacteria (and probably most abundant ones) would die, while other less abundant but more tolerant species would have replaced them. In the case of a population of bacteria of the same taxon, such as the *D. magna* test, this substitution is not possible, and the tests are also much shorter in time than the biology tests that last 7 days. The metabolic diversity results, which showed an increase, supports this explanation; new niches and opportunities were generated upon exposure to the hydrolate and thus increased the diversity. The increase in diversity in substrate utilisation can also be explained for the same reasons (i.e. appearance of new metabolic pathways).

The genetic study of river microorganisms showed that the predominant phylum was Proteobacteria (64.66% of the microorganisms identified). All Proteobacteria are gram negative, but this phylum has an exceptional variety of organisms with regards to energy generation mechanisms: phototrophs, chemoorganotrophs and chemolithotrophs (Madigan et al., 2015). High initial metabolic diversity in the community would allow it to be more tolerant as a whole to the toxic effects of the hydrolate. It has been described that in mutualistic networks that prevail in microbial communities, compositional diversity and connectivity lead to higher resilience to stressors (Thebault and Fontaine, 2010).

Among Proteobacteria, Alphaproteobacteria were the most



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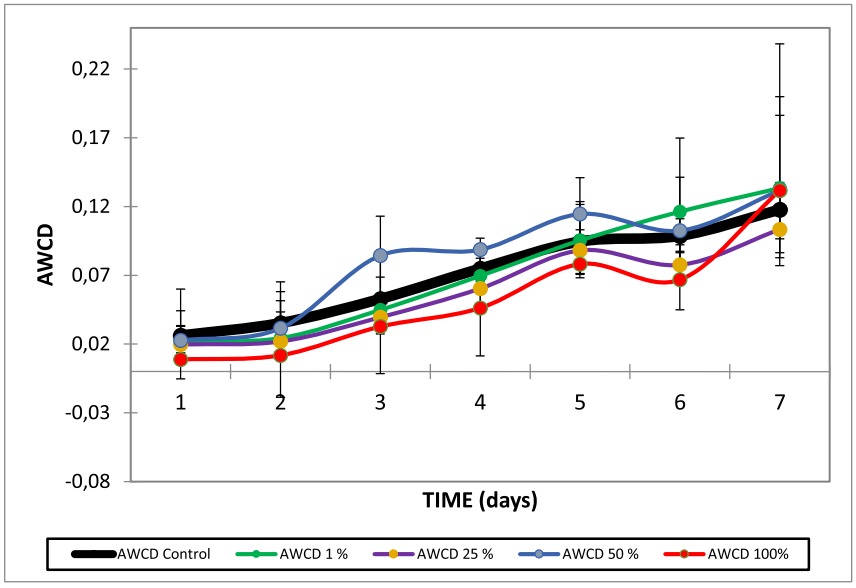

Fig. 6. Average well color development (AWCD) of metabolized substrates in Biolog EcoPlates based on 168-h incubation of river microorganisms exposed to *A. absinthium* L hydrolate Concentrations of hydrolate assayed were: 1-25-50 and 100%. Values can be compared to a reference control value (microorganisms of river water that have not been treated with hydrolate, only mineral water). Each point is the average value of three replicates. Error bars represent the standard deviation of mean of three replicates (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

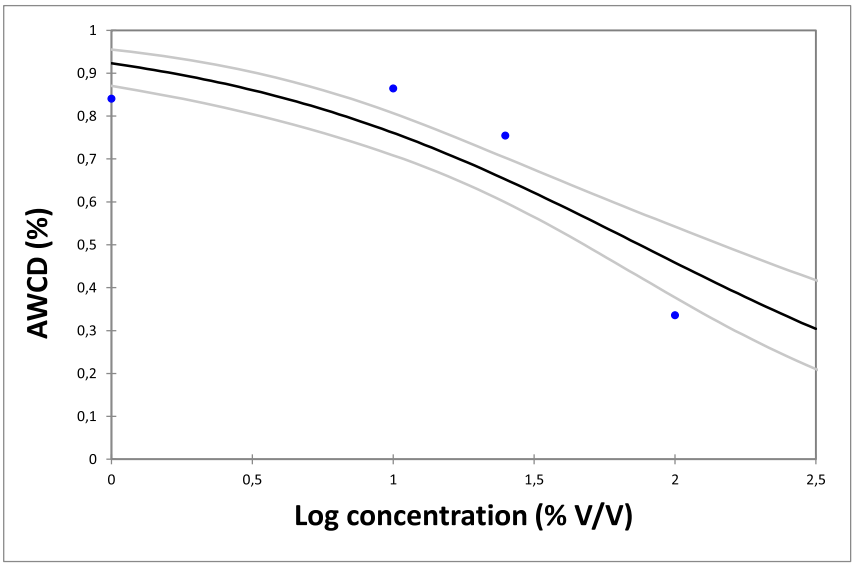

Fig. 7. Concentration-response curves of average well color development (AWCD) of metabolized substrates in BiologEcoPlates after 24 h of exposure of river microorganisms to *A. absinthium* L hydrolate as a function of logarithm of the concentration. AWCD values are expressed as the percentage of the control. Pale gray lines indicate the confidence limits (95%). Each point is the average value of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

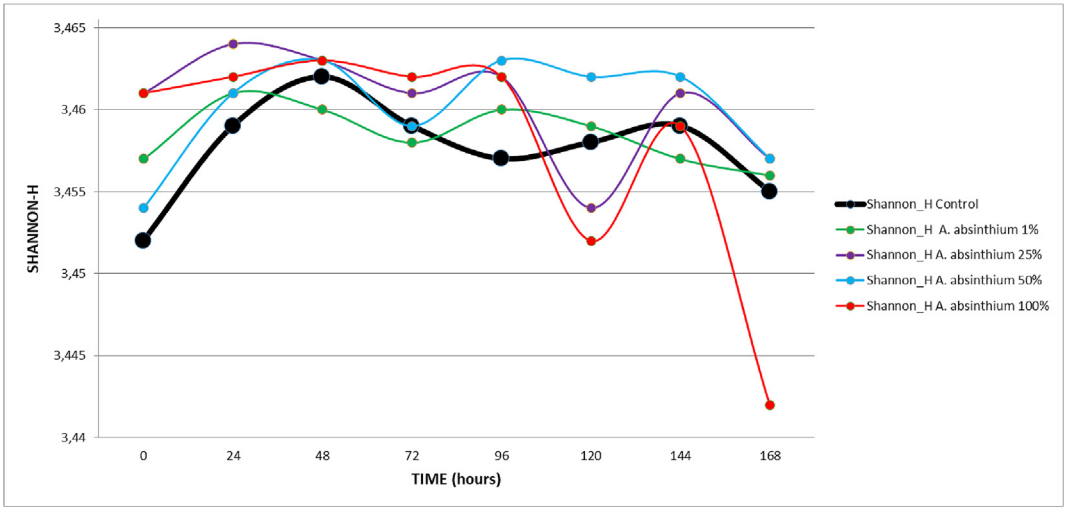

Fig. 8. Diversity indices (Shannon–Weaver index, H) of river microbial communities exposed to *A. absinthium* L hydrolate. 1% and 25% concentrations of hydrolate showed a statistical differences ($p < 0.5$) compared to the control.

abundant (27% of total taxa). These bacteria are characterised by slow growth, low rates of metabolism and generally low population density. They are strict or facultative aerobes and can live in environments with low nutrient concentrations. The Sphingomonadaceae family predominated in the sample (23.88% of total taxa), and this taxon includes chemoorganotrophs, strict aerobes and facultative aerobes that can metabolise a broad spectrum of complex polysaccharides (Madigan et al., 2015). The genus *Novosphingobium* (91.41% of Sphingomonadaceae and 21.83% of total taxa) show high metabolic versatility (Kumar et al., 2017). This fact could explain the most significant increase in carbohydrate metabolism observed at virtually all hydrolate concentrations (see Fig. 9, red bars) of the groups not adversely affected by the hydrolate. In addition, *Novosphingobium* species contain genes that encode components involved in alanine, aspartate and glutamate metabolism that could explain the increase in amino acid metabolism at the 50% hydrolate concentration. *Novosphingobium* strains can also reduce nitrate (Takeuchi et al., 2001), metabolise different types of environmental sulphur compounds (Kumar et al., 2017) and were found to harbor a wide variety of mono- and dioxygenases that mediate the metabolism of several aromatic compounds, (Kumar et al., 2017).

Betaproteobacteria (30% of protobacteria, 17.80% of total taxa) also display a wide variety of metabolic and ecological properties. Almost all of the identified Betaprotebacteria were from the order Burkholderiales, which has an extraordinary nutritional spectrum, including the ability to use up to 100 different organic substrates: fatty acids, sugars, polyalcohols, aromatics, amino acids and others Tortora et al., 2007. In the sample, the genus *Limnohabitans* (freshwater bacterioplankton) predominated. Previous studies found that monosaccharides (glucose and fructose) and some amino acids (L-alanine, L-cysteine, glutamine and glutamate) were the most widely utilised substrates (Kasalicky et al., 2013) by members of this genus. This fact would correspond to the increased metabolism of carbohydrates and amino acids in bacteria that are

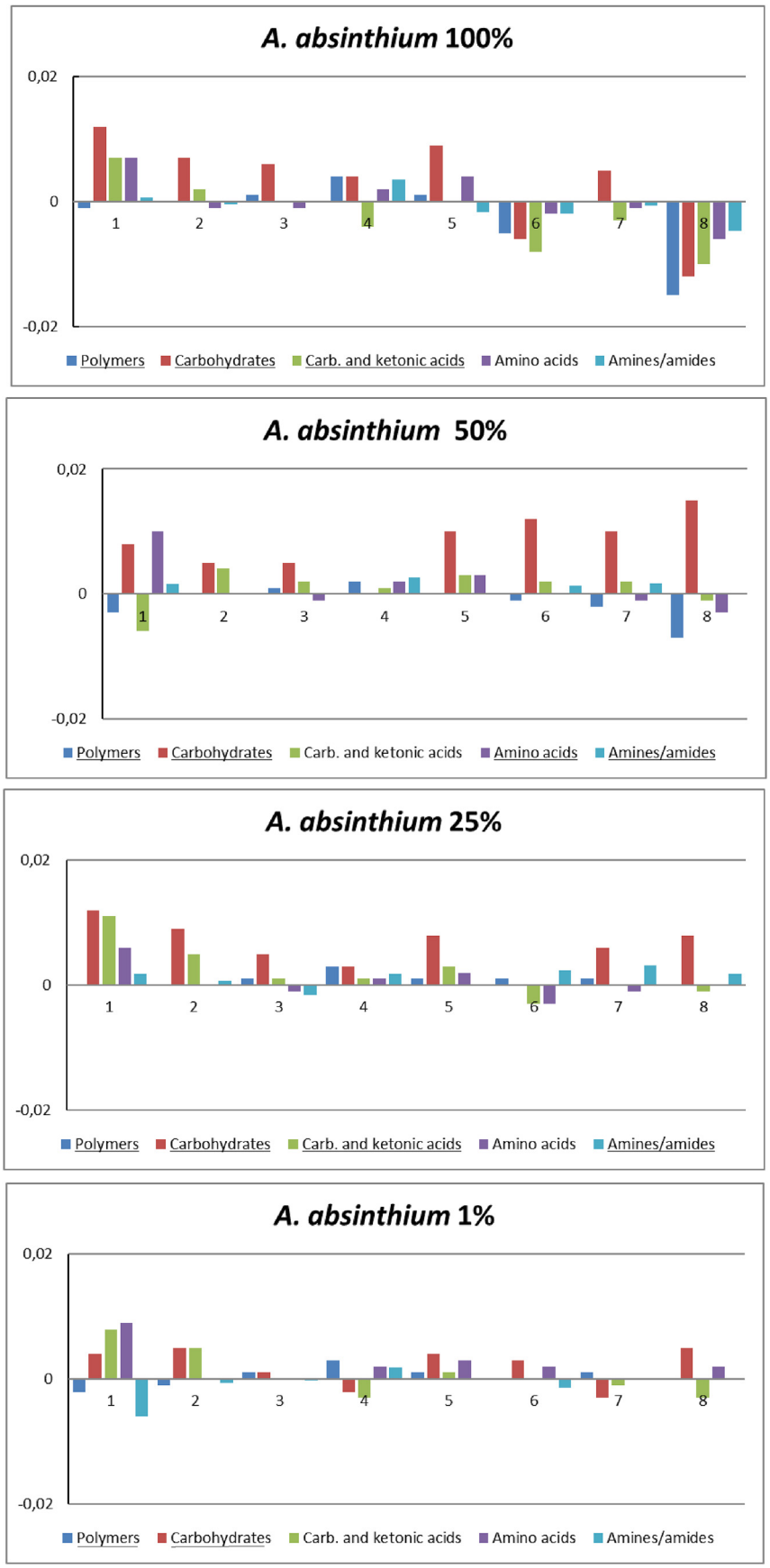

Fig. 9. Shannon index variation of river microorganisms treated with *A. absinthium* hydrolates during 168 h. The bars represent the difference in value of the Shannon index of each concentration compared to the control (x-axis values). Data was measured 8 times at intervals of 23 h (numers in the x axis). Metabolic groups that appear underlined indicates that differences regarding the control are statistically significant ($p < 0.5$).

not affected by the hydrolate. Moreover, some strains of *Limnohabitans* can metabolise butyric, glyceric, pyruvic, fumaric and malic acids.

We found a low proportion of Gammaproteobacteria in the sample (only 10.26% of the total taxa), but the identified species have traits that further explain our findings: they can utilise a great diversity of organic compounds as carbon sources and energy for growth. For example, *Pseudomonas* synthesise an unusually large number of enzymes, can metabolise a wide variety of substrates and can also grow in the presence of minimal amounts of carbon sources (Tortora et al., 2007). In addition, *Pseudomonas* are particularly resistant to potentially toxic chemicals, probably due to efficient control of substance entry through its cell wall and active ejection pump systems in its membranes (Tortora et al., 2007).

A final point to consider is that the high metabolic diversity found in this natural community may lead to the degradation of different components of the hydrolate, including the monoterpene diol, and thus explain the relatively mild observed toxic effects compared to *V. fisheri* pure cultures. For example, different *Pseudomonas* strains, a component of the natural sampled community, can degrade monoterpenes (Marmulla and Harder, 2014). It is even possible that by-products are generated that may be more toxic or at least have different mechanism(s) of action. This fact would explain the remarkable decrease in the metabolic biodiversity of all the metabolites from the fifth day (especially when the undiluted hydrolate was applied), which does not affect the AWCD values that were maintained or even increased. This result could be due to the selective loss of species and the growth of opportunists. In any case, more studies are needed to understand the mechanism(s) of action of *A. absinthium* hydrolate, and specifically its biocidal component, 2,6-dimethylocta-5,7-diene-2,3-diol.

5. Conclusions

This study provides for a first time a comprehensive assessment of the ecotoxicity of a potential plant-based biopesticide on freshwater aquatic non-target organisms. Likewise, it is the first time that attention is focused on the ecotoxicity of a hydrolate. Biopesticides are considered to be safer or to show low non-target toxicity compared to synthetic pesticides. However, this study presents evidence that *A. absinthium* hydrolate, which has nematicidal properties, caused lethal effects on non-target organisms at low concentrations: the cladocera *D. magna*, the bacterium *V. fisheri* and the unicellular alga *C. reinhardtii*. In addition, the organic extract of *A. absinthium* produced very similar results to the hydrolate on *D. magna*. The ecotoxic effects were probably due to its principal component, 2,6-dimethylocta-5,7-diene-2,3-diol. However, when a natural community of river bacteria composed mainly of Proteobacteria with broad-spectrum, ubiquitous metabolic groups that are capable of growing at low concentrations of nutrients was exposed to *A. absinthium* hydrolate, the toxic effects were attenuated. Bacterial growth was not greatly affected and the metabolic diversity of microorganisms underwent small changes, especially an increase in the metabolism of different substrates, mainly carbohydrates. More studies are thus required to better understand the mechanism(s) of action of biopesticides as well as a more complete risk assessment in natural communities of non-target organisms in order to ensure safer incorporation of biopesticides into agricultural practices.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.09.071.

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