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Research article

Impact of citronellol on river and soil environments using non-target model organisms and natural populations

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ABSTRACT

Citronellol is an acyclic monoterpenoid with a wide range of pharmacological activities (antibacterial, anti-fungal, anti-lice, repellent, lipolytic, anti-allergic, anti-inflammatory, antispasmodic, antidiabetic, anti-cholesterol, among other) and potential to replace synthetic products. However, the impact of citronellol on the environment remains unknown.

We analysed, for the first time, the environmental impact of citronellol on river and soil environments using non-target model organisms and natural populations. The acute toxicity of citronellol on the aquatic invertebrate *Daphnia magna*, the plant *Allium cepa* L *and the* earthworm *Eisenia fetida* was quantified. The effect of citronellol in a river ecosystem was analysed using river periphyton communities taxonomically characterised and a river microbial community characterised through 16 S rRNA gene sequencing. Finally, a microbial community from natural soil was used to monitor the effect of citronellol on the soil ecosystem.

The results showed that *E. fetida* was most sensitive to citronellol (LC_{50} = 12.34 mg/L), followed by *D. magna* (LC_{50} = 14.11 mg/L). Citronellol affected the photosynthesis of the fluvial periphyton (LC_{50} = 94.10 mg/L) and was phytotoxic for *A. cepa*. Furthermore, citronellol modified the growth and metabolism of both fluvial (LC_{50} = 0.19% v/v) and edaphic (LC_{50} = 5.07% v/v) bacterial populations. The metabolism of the microorganisms in the soil and water exposed to citronellol decreased with respect to the control, especially their ability to metabolise carbohydrates.

Our results show that citronellol has a negative impact on the environment. Although acute effects cannot be expected, it is necessary to quantify the environmental levels as well as the long-term and persistent effects of this monoterpene.

1. Introduction

Medicinal plant essential oils are rich in secondary metabolites that have evolved to become a powerful defence mechanism for plants. Among these metabolites are terpenes, the study of which has revealed several pharmacological effects widely described in the literature, such as anti-inflammatory properties (Lima et al., 2013), antihyperalgesic activity (Santos et al., 2016) or antioxidant properties (Seol et al. 2016). Therefore, terpenes are important alternatives for the development of new drugs with few side effects and reduced costs and time (Jansen and Shenvi 2014).

Citronellol ($C_{10}H_{20}O$) is an acyclic monoterpenoid with a characteristic rose-like odour present in the essential oil of several plants, such as the genus *Cymbopogon* in the Poaceae family, with about 55 species native to the warm, tropical regions of Asia (de Castro et al., 2010) or *Pelargonium*, one of the seven genera belonging to the Geraniaceae family, which includes almost 280 species mainly from South Africa (Szutt et al. 2019), among others.

Citronellol has a wide range of pharmacological activities that make it a promising molecule in many areas of research (Abe et al., 2003; Su et al., 2010; Kobayashi et al., 2016; Menezes et al., 2010; Srinivasan and Muruganathan 2016; Batubara et al., 2015; Santos et al., 2019) and a

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viable alternative to synthetic chemicals. This monoterpene has antibacterial (Kotan et al. 2007) and antifungal (Zore et al., 2011; Shin and Lim 2004; Viollon and Chaumont 1994) properties against species of clinical relevance. Furthermore, citronellol also has repellent (Nerio et al. 2010; Michaelakis et al., 2014), insecticidal (Tabari et al., 2017; Baldin et al., 2015), fungicidal (Juarez et al., 2016) and herbicidal (Lins et al., 2019; Kordali et al. 2007a, 2007b) properties.

Thus, it could be expected that, as a natural product, citronellol, in addition to generating fewer side effects in its pharmacological uses, could also be used as a biopesticide or repellent, presumably with less environmental impact than synthetic chemicals with similar properties.

However, to our knowledge, these eco-friendly properties have not been systematically studied, and the impact of citronellol on the environment remains unknown, although it is foreseeable that the consumption of this product of natural origin will increase worldwide in the future.

Little is currently known concerning the occurrence of citronellol in the environment, but there are already indications that it can reach rivers, since it has been detected in grey wastewater from bathrooms at concentrations of 2.8 μg/L (Eriksson et al., 2003).

Although the effects of citronellol on target organisms have been studied, little is known about its environmental relevance on non-target organisms. Studies have reported inhibitory effects on green algae (Ikawa et al., 1992) and on germination and root elongation of several plants (Batish et al., 2006; De Martino et al., 2010; Singh et al., 2002). However, nothing is known of their effect on indicator organisms of aquatic or terrestrial ecosystems.

In this context, we analysed, for the first time, the environmental impact of citronellol on river and soil environments using different endpoints from aquatic and soil non-target bioindicators. To quantify the ecotoxicity of citronellol in the aquatic environment, acute toxicity on the aquatic invertebrate *Daphnia magna* was monitored. In addition, from a more ecological point of view, the impacts of this monoterpene on a river ecosystem were analysed using river periphyton communities and a river microbial community from the nekton.

The evaluation of the effect of citronellol on the soil environment was carried out using three bioindicators: a plant (*Allium cepa* L.), a soil invertebrate (the earthworm *Eisenia fetida*) and a microbial community from a natural soil.

Combined with the ecotoxicity study, the taxonomic analysis of the river, the soil microbial communities and the periphyton communities provide a complete picture of the effect of this natural product on the environment.

2. Material and methods

2.1. Citronellol

Citronellol (3,7-dimethyloct-6-en-1-ol) was purchased from Sigma-Aldrich (CAS 106-22-9), with a minimum purity of 95.0% and a molecular weight of 156.27 gr/mol.

2.2. Daphnia magna assay

Daphnia magna (water flea) assays were performed following the standard operational procedures of the Daphtoxkit FTM magna (1996) from Vidrafoc (Spain) (ref. DM121219) in accordance with OECD 202 (2004) guidelines. The kit provided *D. magna* ephippia, synthetic freshwater and spirulina microalgae as a source of food for *D. magna* and was stored at 5 °C until use.

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, Spain). The neonates were pre-fed with one vial of the spirulina microalgae 2 h before exposure to citronellol. Several concentrations of citronellol were prepared in synthetic freshwater (ISO 6341 2012) at the following concentrations used in the test: 0.85, 8.5, 85 and 855 mg/L. Synthetic freshwater was used as a negative control. The pH of the solutions was adjusted to 7–7.5 using 0.1 M NaOH. After 2 h of feeding, 25 organisms (24-h-old) were used. Each concentration of citronellol was performed in five replicates per plate of five organisms per well. Daphnids were incubated in complete darkness for 24 h at 20–22 °C. After a 24-h exposure period, 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 LC_{50} (chemical concentration resulting in 50% immobilisation).

2.3. Periphyton community assay

2.3.1. Colonisation

Periphyton river communities were obtained from supports consisting of methacrylate racks with 24 bonded microscope slides that were located in the Gállego River (Zaragoza, Spain) at a depth of 15 cm on June 24, 2019.

After 5 weeks, the algal biofilm reached an average thickness of around 0.75 mm, which ensured that the algal biofilm showed communities of similar biomass and physical dimensions (Navarro et al. 2002). Then, the periphyton-colonised slides were transported to the laboratory, and a sample of each slide was prepared for taxonomic identification.

2.3.2. Water samples

A water sample was collected at the same time and place as the periphyton slides, and the physicochemical parameters of the river were measured (Pyrenean Institute of the Higher Center for Scientific Research; Support information 1).

2.3.3. Taxonomic identification

In the laboratory, we proceeded to identify and count algae from the samples of periphyton according to the Utermöhl technique adapted to inverted microscopy (UNE-EN 15204, 2007). Subsequently, samples were treated to obtain a suspension of clean frustules by oxidation with hydrogen peroxide, mounted on permanent slides with Naphrax © resin, to identify, count and interpret benthic diatoms in the periphyton sample, according to UNE-EN 13946, UNE -EN 15204 and UNE-EN 14407.

Cell counting and identification (to the maximum possible taxonomic level) were performed under a Leica light microscope at a 1000 magnification (diatoms) and at 100, 400 and 1000 magnification (other microalgae). The results were expressed as the number of individuals per $cm^2$of biofilm as well as density, number of individuals per mL.

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

The toxicology experiments were carried out in flow-through methacrylate channels connected to separate water reservoirs. A system of motors connected to different reservoirs fed a closed water circuit through each channel at 0.113 m^3/h. A thermostatic bath maintained the water temperature at 23 °C. Each reservoir had the same volume (4 L).

Prior to the ecotoxicological tests, the slides colonised by periphyton recently transported from the river were placed horizontally in an acclimatisation channel at 23 °C. The slides were then placed horizontally at the bottom of six flow-through artificial channels (mesocosms).

Light was provided by lamps with a light spectrum similar to that of sunlight and specific for the cultivation of algae lamps (Blau Aquaristic, T5HO, 39 w/10,000 K, 80 μmol photon/(m^2 s) on the channel surface), allowing periphyton communities to carry out photosynthesis as under real conditions. Periphyton communities were exposed to a geometric series of the following concentrations: 0.1, 1.0, 10, 50 and 200 mg/L of citronellol in buffer solution (MOPS, 0.01 M), adjusted to a pH of 7.5 using NaOH or HCl. One channel with MOPS without citronellol was used as a negative control. The temperature was measured periodically throughout the experiment to ensure that it remained constant.

The effect of citronellol on the photosynthetic efficiency of the periphyton was evaluated as described before (Val et al., 2016), measuring the photosynthetic yield that reflected the efficiency of the photochemical energy conversion process (Consalvey et al., 2005), using a MINI-PAM-II Photosynthesis Yield Analyzer (Walz, Germany) after 1 and 2 h of exposure and in triplicate.

As a control for each of the measurements, control was taken at time 0, as the composition of each community can vary and affect the toxicity of the compounds.

2.4. Water and soil microorganism assays

2.4.1. Water samples

Water samples were collected from the Gállego River (Zaragoza, Spain) at the same time as the periphyton samples and transported to the laboratory according to standard procedures (ISO 19458:2006, AENORISO 19458:2006, AENOR).

For genetic analysis, microorganisms were extracted from 5 L of the river water that was filtered through a 0.22-μm cellulose nitrate filter (Sartorius), using a vacuum pump kitasate, resuspended in a sterile Falcon tube with 50 mL of phosphate-buffered saline (PBS) and centrifuged 10 min at 5000 g. The supernatant was discarded, and the pellet was stored at −80 °C until sequencing.

For the ecotoxicity assays, 1 L of river water was filtered through a 70-μm nylon sieve (BD Falcon) to remove debris and stored at 4 °C in the dark until use.

2.4.2. Soil samples

Soil samples were obtained on July 5, 2019, from a crop field free of pesticides or other contaminants (CITA, Zaragoza, NE Spain). The soil composition was analysed (CITA Soil and Irrigation Unit, Support information 1).

For the genetic analysis, 100 mL of sterile water was added to 20 g of soil, and after stirring for 30 min under sterile conditions, the sample was left to stand for 1 h. Subsequently, 10 mL of the sample was divided into Falcon tubes that were sonicated for 1 min and centrifuged at 1000 g for 10 min. The supernatant was collected under sterile conditions, and the soil microorganisms were obtained by filtering the supernatant through a 0.22-μm cellulose nitrate filter (Sartorius) with a vacuum Büchner flask. The filter content was carefully washed with sterilised PBS and centrifuged at 5000 *g* for 10 min. Subsequently, it was removed with an eyedropper, and the pellets were stored at −80 °C until sequencing.

Prior to the ecotoxicity assays, 10 g of soil was filtered through a 2-mm sieve (Becton Dickinson, Spain). To the 10 g of pre-sieved soil, 95 mL of sterile water was added, and the sample was left stirring in an Erlenmeyer flask for 30 min and standing for 1 h. Subsequently, 10 mL of the top of the Erlenmeyer flask was placed in Falcon tubes and centrifuged for 10 min at 1000 g, collecting the supernatant under sterile conditions. This cycle was repeated five times. The total supernatant obtained was filtered to remove through a 70-μm nylon sieve (Becton Dickinson, Spain) to remove suspended soil debris, obtaining a sufficient sample amount for inoculation in Biolog plates.

2.4.3. Community-level physiological profiling (CLPP) of water and soil microbes

We used the Biolog EcoPlate test (Tiselab S.L., Spain) to assess the effects of citronellol on the metabolism of microbial communities in water and soil and, specifically, changes in the use of 31 different carbon sources, as described before (Pino-Otin et al. 2017, 2019a).

For the ecotoxicity test, the following concentrations of citronellol were prepared: 0.5, 12.5, 25, 37.5 and 50%, in a final volume of 150 μL in the wells of a Biolog plate with prefiltered river water (see 2.4.1) or the supernatant obtained from the soil sample (see 2.4.2) to assess the toxicity of citronellol on microbial communities in water and soil, respectively. The final pH of the solutions ranged between 6 and 7. Each concentration was tested in triplicate, and all manipulations were performed under sterile conditions in a flow chamber. The plates were incubated in the dark at 25 °C for 6 days under sterile conditions.

The optical density (OD, wavelength 590 nm) of each well was measured immediately after inoculation and once a day using a Synergy H1 Microplate reader (BIO-TEK. EEUU) and the Gen5™ data analysis software. The carbon use rate was thus assessed as the reduction of tetrazolium violet redox (Pohland and Owen, 2009).

2.4.4. Genetic sequencing of river and soil microorganisms

To better interpret the effect of this monoterpene on the metabolism of microbial communities, its taxonomic composition and the predominant taxa were studied through genetic sequencing.

Genetic sequencing of water and soil microorganisms was performed in the Genomics Unit Cantoblanco, Science Park (Madrid, Spain). Bacterial genomic DNA of samples (previously homogenised in 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) was used to determine the DNA concentration. The DNA samples were used to amplify the V3–V4 region of the 16 S ribosomal RNA (rRNA) gene, as previously described (Caporaso et al. 2011, 2012; Pino-Otin et al., 2019a).

We used the Bioanalyzer 2100 (Agilent, EEUU) to analyse individual amplicon libraries, and the concentration was estimated by real-time PCR (Kapa Biosystems). The Illumina MiSeq Instrument served to sequence DNA samples under a 2 × 300-protocol. 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 (16 S Metagenomics, Illumina).

In the run, 100% of a total of 85,525 reads passed the quality filtering for water microorganisms and 100% of a total of 145,498 reads for soil microorganisms.

2.5. Eisenia fetida assays

Adult individuals of *Eisenia fetida* were acquired from the composters of Todo Verde (Spain). Before testing, earthworms were acclimatised over 15 days in a sphagnum peat-conditioned substrate from the Spanish Flowers Company (Spain) and kept at stable conditions: 18–25 °C, pH 7.5–8 and 80–85% humidity.

For the ecotoxicity test, adult earthworms in the same vital stage were selected: above 60 days of age, with clitellum, with similar size and weights between 300 and 600 mg. The toxicity tests were carried out following the indications of the OECD 207 (1984) methodology, as described previously (Pino et al., 2015).

The artificial soil, according to the OECD 207 standard, was prepared with quartzitic sand (Imerys Ceramics España, S.A., Spain), kaolinic clay (Imerys Ceramics España, S.A., Spain) and commercial black peat (Verdecora vivarium, Spain) at a ratio of 7:2:1. The moisture of the substrate was adjusted with deionised water in an amount equivalent to 35–45% of the dry soil weight. Polypropylene containers with a capacity of 1 L and a perforated lid to allow breathing, reducing moisture loss, were filled with 600 mg of this artificial soil.

Ten earthworms were placed in each container, and 120 mL with the following concentrations of citronellol were added: 0.1, 1.0, 10, 100 and 200 mg/L. Negative controls were prepared using the same procedure without citronellol. Each concentration was tested in triplicate.

The containers were kept under controlled environmental conditions at 20 ± 2 °C, 80–85% relative humidity and 400–800 lx of light. Earthworm mortality was measured after 14 days of treatment.

2.6. Allium cepa assay

Bulbs of *A. cepa* (var. Stuttgarter Riesen, 14/21 gauge) were acquired from the Fitoagrícola Company (Spain) and stored in a humidity-free environment at a temperature between 10 and 20 °C in the dark to

prevent the growth of fungi. Young bulbs were peeled before the test, avoiding damage to the root ring.

Acute toxicity experiments with *A. cepa* were conducted according to Fiskesjö (1993). The bulbs were placed in 15-mL tubes using mineral water (VERI, Aguas de San Martín de Veri S.A., Spain) as the growth medium because of its adequate content of Ca + Mg (https://www.veri.es/es/el-producto). Ecotoxicological tests were performed using 12 replicates for each concentration: 0.03, 0.3, 3.0, 30 and 300 mg/L. The negative control only contained water. The bulbs were cultivated in a dark chamber at 25 °C for 72 h, and the test solutions were renewed every 24 h.

2.7. Statistics and graphical representation

Dose-response curves for *D. magna* mobility, *E. fetida* survival, *A. cepa* root elongation and periphyton photosynthetic yield were calculated with a logit logistic regression using the XLSTAT (2014.5.03) software to obtain the corresponding LC_{50} values and standard errors (SE). Dose-response models were statistically tested using a chi-squared test. The variance relationship between average well color development (AWCD) values of the three replicates and Student's t-test on two independent samples to assess significance were calculated using the XLSTAT software (2014.5.03).

The microbial activity of each Biolog EcoPlate microplate was expressed as the AWCD and determined according to Garland and Mills (1991), as described in previous studies (Pino-Otin et al., 2019a):

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

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.

3. Results

3.1. Effects of citronellol on D. magna

The dose-response curve for *D. magna* after a 24 h of exposure to citronellol is shown in Fig. 1 (a). All toxicity values analysed with the chi-squared test were highly significant ($P < 0.0001$). The LC_{50} value was 14.11 mg/L (s.e. interval of 7.52–26.70) and the LC_{10} was 1.59 mg/L (s.e. interval of 0.41–3.40).

3.2. Effects of citronellol on river periphyton

The concentration-response curve of the photosynthetic yield of river periphyton after 2 h of exposure to citronellol can be seen in Fig. 1 (b). As before, the chi-squared test of toxicity values was highly significant ($p < 0.0001$).

The LC_{50} value was 94.10 mg/L (s.e. interval of 85.38–103.91), and the LC_{10} was 32.17 mg/L (s.e. interval of 26.99–37.17).

3.3. Taxonomic study of the periphyton

The taxonomic identification of the periphyton showed a remarkable diversity, including green and red algae, diatoms and Cyanobacteria (see Fig. 2 for the composition and abundance of the main phyla and Support Information 2 to access the complete taxonomic list).

The two classes of green algae were Chloropyceae and Ulvophyceae. The first with four genera and the second with three, with *Oedogonium* sp. and *Gongrosira*, respectively, being the most abundant ones. We also found red algae, the Rhodophyta division, only containing the genus *Bangia.* In the subclass of the diatoms Bacillariophyceae, we found the greatest diversity of species, up to 32, among which three species predominated in decreasing order: *Achnanthidium pyrenaicum* (Hustedt) Kobayasi, *Achnanthidium minutissimum* (Kützing) Czarnecki and *Nitzschia inconspicua Grunow*.

Finally, six genera of Cyanobacteria, a large and diverse phylum in the kingdom *Bacteria*, were also identified, with the genus *Pleurocapsa* being the most abundant one.

3.4. Effects of citronellol on aquatic microorganisms

3.4.1. Effect of citronellol on microbial metabolism

Fig. 3 (a) shows the AWCD values obtained from the 6-day incubation of the microorganisms obtained from river water exposed to several concentrations of citronellol in the Biolog Ecoplate. Citronellol had a remarkable toxicity on river microorganisms with respect to the control (black line), even at minor concentrations (0.5 and 12.5%; $p > 0.05$), but especially at higher concentrations (25–37.5%; $p < 0.01$), showing a clear dose-response behaviour. The greatest effect was detected at the 50% concentration ($p = 0.002$).

The AWCD data at 48 h of exposure were calculated as a dose-response curve that allowed for the calculation of an EC_{50} value of 0.198% (s.e. interval of 0.061–0.424) and an average EC_{10} value of 0.004% (s.e. interval of 0.000–0.016) These toxicity values were highly significant (Chi-square test, $P < 0.0001$).

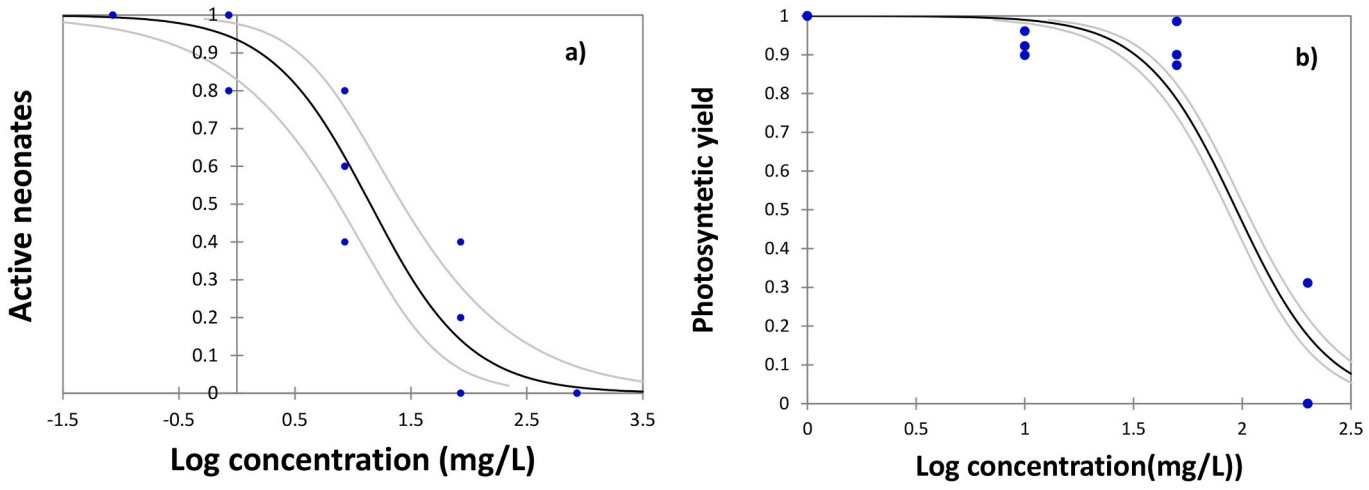

Fig. 1. Dose-response curve of *D. magna (a) and* photosynthetic yield of river periphyton (b) after a 24 h and 2 h of exposure to citronellol respectively. For *D. magna*, curve is the average value of five replicates. Photosynthetic values of periphyton are expressed as the percentage of the control and each dose was measured in triplicate. Pale grey lines indicate the confidence limits (95%).

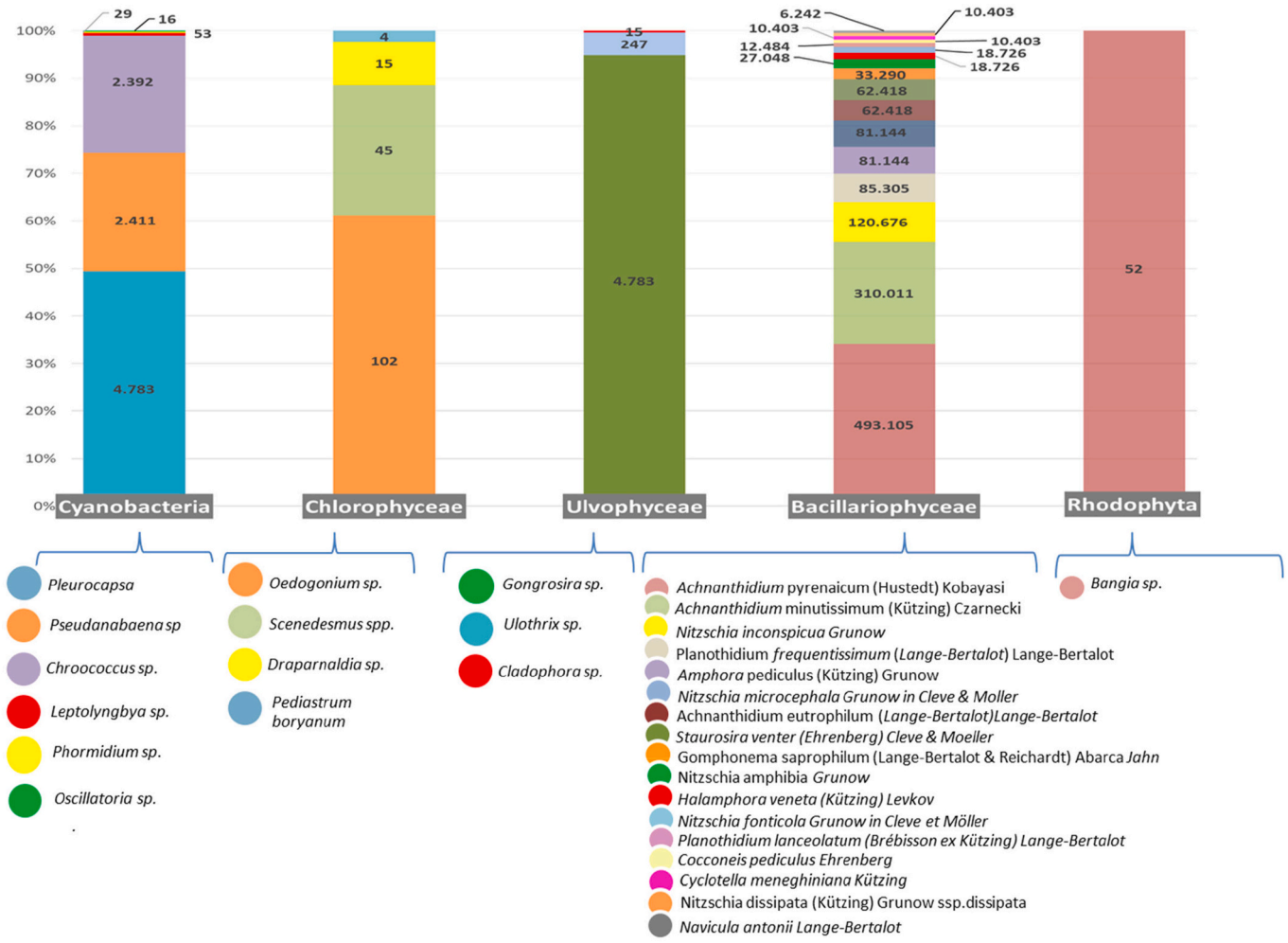

Fig. 2. Abundance (individuals/ml) of taxa within each phylo found in the river periphyton communities (biofilms) obtained from the Gallego River (Zaragoza, Spain) on June 2019.1 individual = 1 organism (colony, filament, thallus, frustum or cell).

These AWCD values provide the mean values of the plate containing the 31 different carbon sources in triplicate. Subsequently, we also investigated the differences in sensitivity for each group of metabolites. For this, the 31 carbon sources were grouped within five functional classes (carbohydrates, polymers, carboxylic and ketonic acids, amino acids and amines/amides) that show aggregated responses in Biolog assays (Weber and Legge 2009; Zak et al., 1994; Lehman et al., 1995). In Fig. 4 (a), the variation of AWCD for each group of metabolites is shown (subtracting values from the negative control) for two concentrations of citronellol: the lowest, 0.5%, and the highest, 50%, to perceive the changes associated with the dose. As seen in Fig. 4, after 48 h of exposure, the metabolism of all functional classes decreased with respect to the control. In addition, with increasing citronellol doses, the metabolism of amino acids and amines/amides decreased. The ability to metabolise carbohydrates decreased most significantly. Carboxylic and ketonic acids underwent little change with increasing doses. The ability to metabolise polymers decreased after 48 h.

3.4.2. *Genetic identification of microbial populations*

Kingdom, phylum, class and order were optimally sequenced (above 95% of taxa) and less in the case of family, genus and species (59.97, 53.80 and 18.67%, respectively). The charts in Fig. 6 show the percentages of taxa abundance for the different taxonomic levels of aquatic microorganisms within each taxonomic level. From inside to outside the circle: phylum, class, order, family, genus and species.

Water samples showed a high diversity of bacterial taxa; the dominant phylum was Cyanobacteria (44.20% of bacterial reads), followed by Proteobacteria (34.49%) and Bacteroidetes (11.24%); 10.08% of bacterial reads could not be identified, and therefore, there were novel sequences in the samples. All of them are common in freshwater, although in our case, Cyanobacteria dominated Proteobacteria and Bacteroidetes, which are usually the predominant groups, in this order (Madigan et al., 2015).

The phylum Cyanobacteria is a large and heterogeneous group from the morphological and ecological point of view of oxygenic phototrophic bacteria (Madigan et al., 2015), present in rivers (Sun et al., 2017; Genuario et al. 2017; Oikawa et al., 2015). Most Cyanobacteria in our samples belonged to the class Oscillatorioriophycideae (74.72% of cyanobacterial reads; 32.53% of total taxa), and almost all bacteria belonged to the order Chroococcales (99.95% of the class Oscillatorioriophycideae; 32.52% of total reads) characterised by coccoid cells often in a mucilaginous envelope (Casamatta and Hasler 2016). Chroococcales have been identified as the dominant cyanobacterial group in freshwater biotopes (McGregor et al. 2007). Among Cyanobacteria, Nostocophycideae was the second class found in our samples, albeit in a much smaller proportion (18.99% of cyanobacterial reads; 8.27% of total taxa), frequently filamentous.

Proteobacteria is an abundant phylum of Gram-negative bacteria, constituting approximately 40% of the freshwater bacterial communities (Battistuzzi and Hedges 2009). They are usually fast-growing species that respond quickly to pulses of organic nutrients (Madigan et al., 2015). Beta and alpha proteobacteria are usually the most

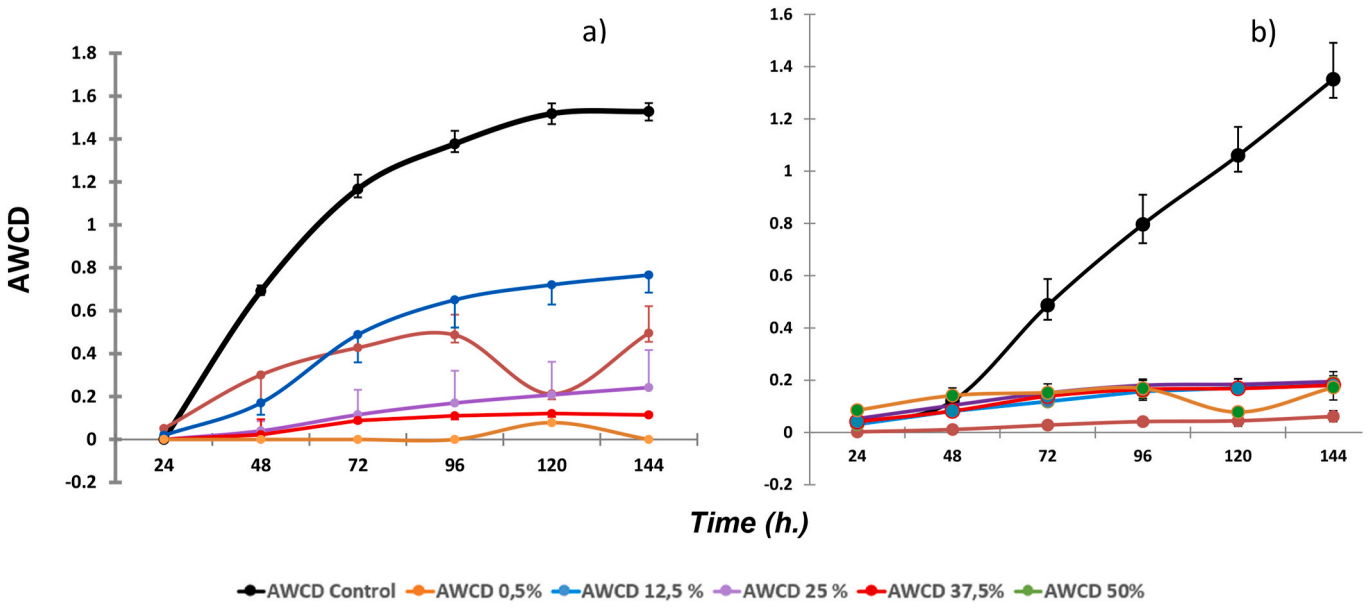

Fig. 3. Average well color development (AWCD) of metabolized substrates in Biolog EcoPlates based on 144 h incubation of river microorganisms (a) and soil microorganisms (b) exposed to citronellol. Concentrations of citronellol can be seen at the bottom of the figure. Values can be compared to a reference control value (microorganisms that have not been treated with citronellol, 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 colour in this figure legend, the reader is referred to the Web version of this article.)

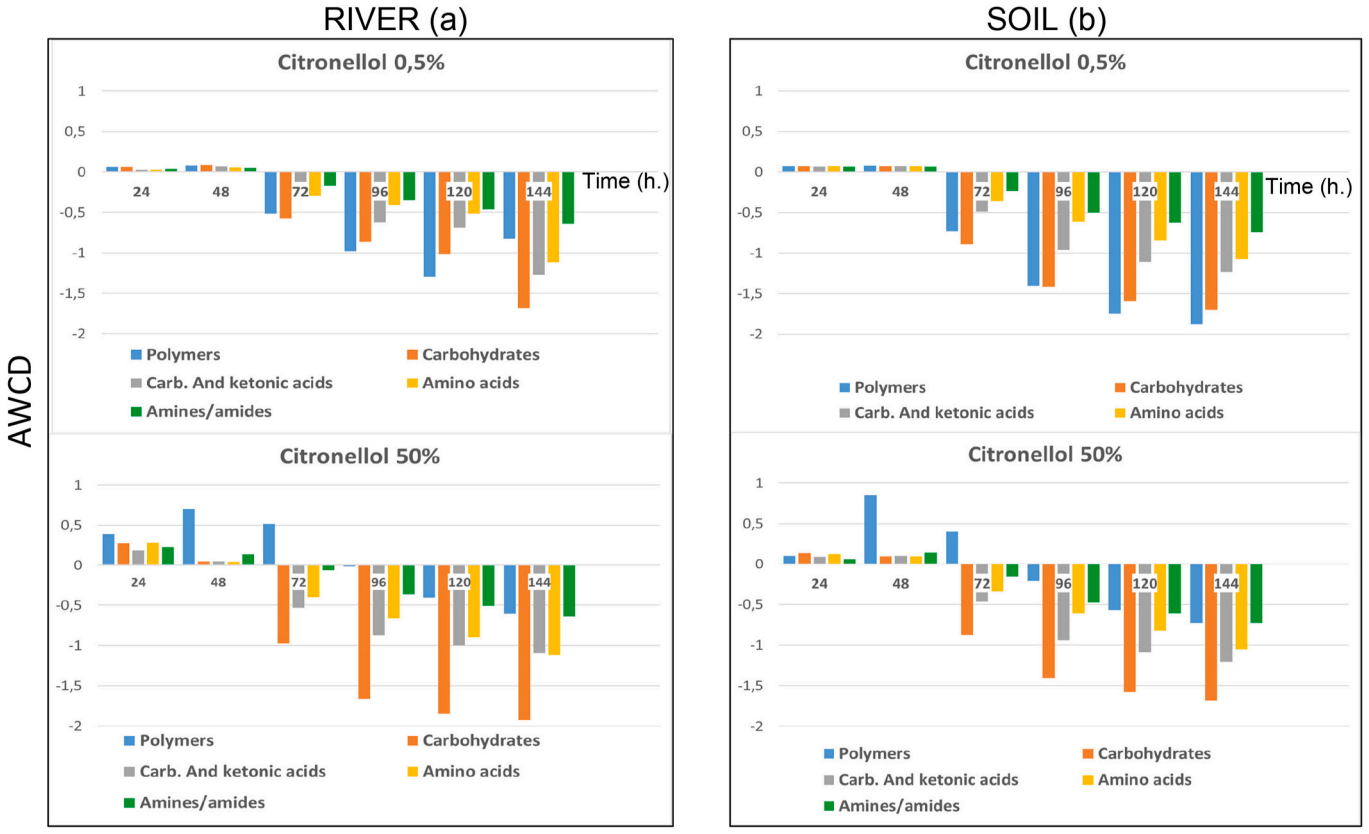

Fig. 4. Bars represent the variation of AWCD for each group of metabolites of control, of water bacteria communities (a) and soil bacteria (b) treated with citronellol during 144 h. Values were obtained by subtracting those from the negative control.

abundant classes of Proteobacteria in freshwater habitats (Madigan et al., 2015), followed by Gammaproteobacteria, as found in our samples: Betaproteobacteria (44.74% of Proteobacteria; 15.20% of total taxa), Alphaproteobacteria (27.67% of Proteobacteria; 9.40% of total taxa). We also found other Proteobacteria classes usually less abundant in freshwater, such as Gammaproteobacteria (19.07% of Proteobacteria,

6.48% of the total taxa).

All Betaproteobacteria belong to the order Burkholderiales (90.55% of the Betaproteobacteria; 13.76% of total taxa), which is abundant in other rivers (Xia et al., 2013; Beale et al., 2017; Thoetkiattikul et al., 2017; Staley et al., 2015). These bacterial taxa have a great metabolic variety, with chemoorganotrophs being strictly aerobic, facultative or strictly anaerobic or nitrogen-fixing organisms (Madigan et al., 2015).

Alphaproteobacteria are more competitive under limited nutrient availability and therefore grow well in oligotrophic waters (Madigan et al., 2015). Among Alphaproteobacteria, the order Rhodobacterales predominated in our samples (56.16% of the Alphaproteobacteria; 5.28% of total taxa); it was also predominant in other rivers of Spain (Aguirre et al., 2017) and seems to be important in ecosystem functioning and nutrient cycling (Thiele et al., 2017).

Among the class Gammaproteobacteria, the most abundant order in our samples was Alteromonadales (47,98% of Proteobacteria; 3,11% of total taxa), straight or curved rods cells with a single flagellum. Alteromonadales have also been found in the genetic identification of the river bacterial community of other rives (Xia et al., 2013; Narciso--da-Rocha and Manaia 2016).

The phylum Bacteroidetes, all Gram-negative and anaerobes, has a great metabolic diversity and is probably important in the degradation of various polymers and humic materials (Madigan et al., 2015). Other studies have found this phylum dominating bacterial communities in rivers (Xie et al., 2016). The classes Flavobacteria (48.64% of the Bacteroidetes; 5.39% of total reads) and Sphingobacteria (46.72% of the Bacteroidetes; 5.17% of total reads) were the most abundant Bacteroidetes among our samples.

Flavobacteria are also abundant in typical urban surface waters and in fast-flowing, river-dominated estuaries, along with members of the Sphingobacteria class (Jin et al., 2018; Smith et al., 2019; Narciso--da-Rocha and Manaia 2016).

3.5. Effects of citronellol on Eisenia fetida

The dose-response curve of the earthworm *E. fetida* after 14 days of exposure to citronellol can be seen in Fig. 5 (a). Citronellol affected *E. fetida* survival with an $LC_{50} = 12.34$ mg/L (s.e. interval of 8.54–18.42) and an LC_{10} value of 3.8 mg/L (s.e. interval of 1.32–5.06) of citronellol. Significance was evaluated with the chi-square test, and all values were highly significant ($P < 0.0001$).

3.6. Effects of citronellol on Allium cepa

The inhibition of root growth in *A. cepa* after 72 h of exposure to citronellol can be seen in Fig. 5 (b), with an EC_{50} value of 141.16 mg/L (s.e. interval of 80.08–291.38) and an EC_{10} value of 1.32 mg/L (s.e. interval of 0.48–2.69) of the concentration. All values were highly significant ($P < 0.0001$). These results indicate a dose-effect of citronellol on the phytotoxicity of *A. cepa*, starting at low concentrations.

3.7. Effects of citronellol on soil microorganisms

3.7.1. Effect of citronellol on microbial metabolism

Fig. 3 (b) represents the AWCD values obtained from the 6-day incubation in the Biolog Ecoplate of the microorganisms obtained from soil exposed to citronellol. Based on the results, citronellol could reduce microbial growth at the lowest concentration (all concentrations, $p < 0.05$).

The AWCD data could be calculated at 48 h of exposure as a dose-response curve, allowing the calculation of an average EC_{50} value of 5.076% (s.e. interval of 3.609–6.667) and an average EC_{10} value of 0.753% (s.e. interval of 0.376–1.238). These toxicity values were highly significant (Chi-square test, $p < 0.0001$).

Fig. 4 (b) shows the variation of AWCD for each group of metabolites for the lowest and the highest concentrations of citronellol. Community-level physiological profiling of soil microbes with the Biolog EcoPlate after exposition to citronellol was similar compared to that of river microorganisms. After 48 h of exposure, the metabolism of all functional classes decreases with respect to the control.

At the lower concentration (0.5%), mainly the metabolism of the polymers decreased, whereas at the higher concentration, carbohydrates were mostly affected.

3.7.2. Genetic identification of soil microbial populations

The chart in Fig. 7 shows that, although 14.35% were unknown taxa, Kingdom, phylum, class, order and family were optimally sequenced (above 80% of taxa), and only genus and species were identified below this ratio (76.79 and 37.17%, respectively).

Our soil samples showed a bacterial diversity typical of edaphic ecosystems. The predominant phylum identified was Actinobacteria (54.89% of bacterial reads), followed by Proteobacteria (17.44%) and Firmicutes (13.32%), all of which are frequent phyla in the soil, albeit at different proportions. Generally, Proteobacteria is the dominant phylum in non-contaminated soils (Janssen 2006; Spain et al. 2009) and represents more than one-half of taxa (Madigan et al., 2015). Comparative

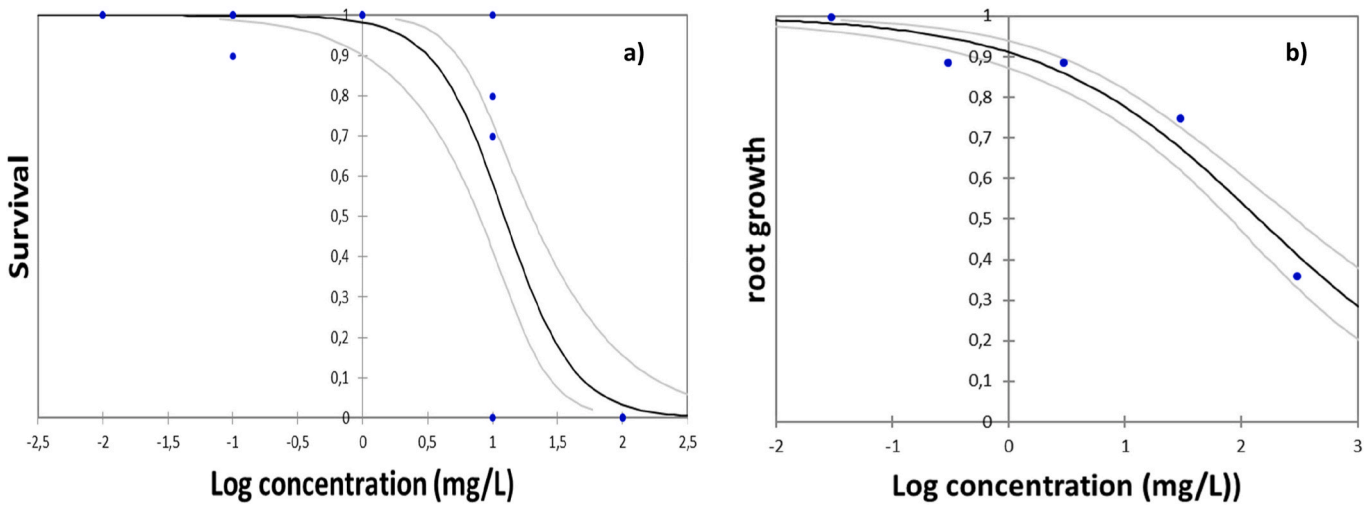

Fig. 5. Curves show the dose-response of *Eisenia fetida* (a) and *Allium cepa (b) after exposure to citronellol during 14 days and 72 h respectively. Curves are the average value of three replicates (a) and twelve replicates (b). Grey lines are the confidence limits (95%).*

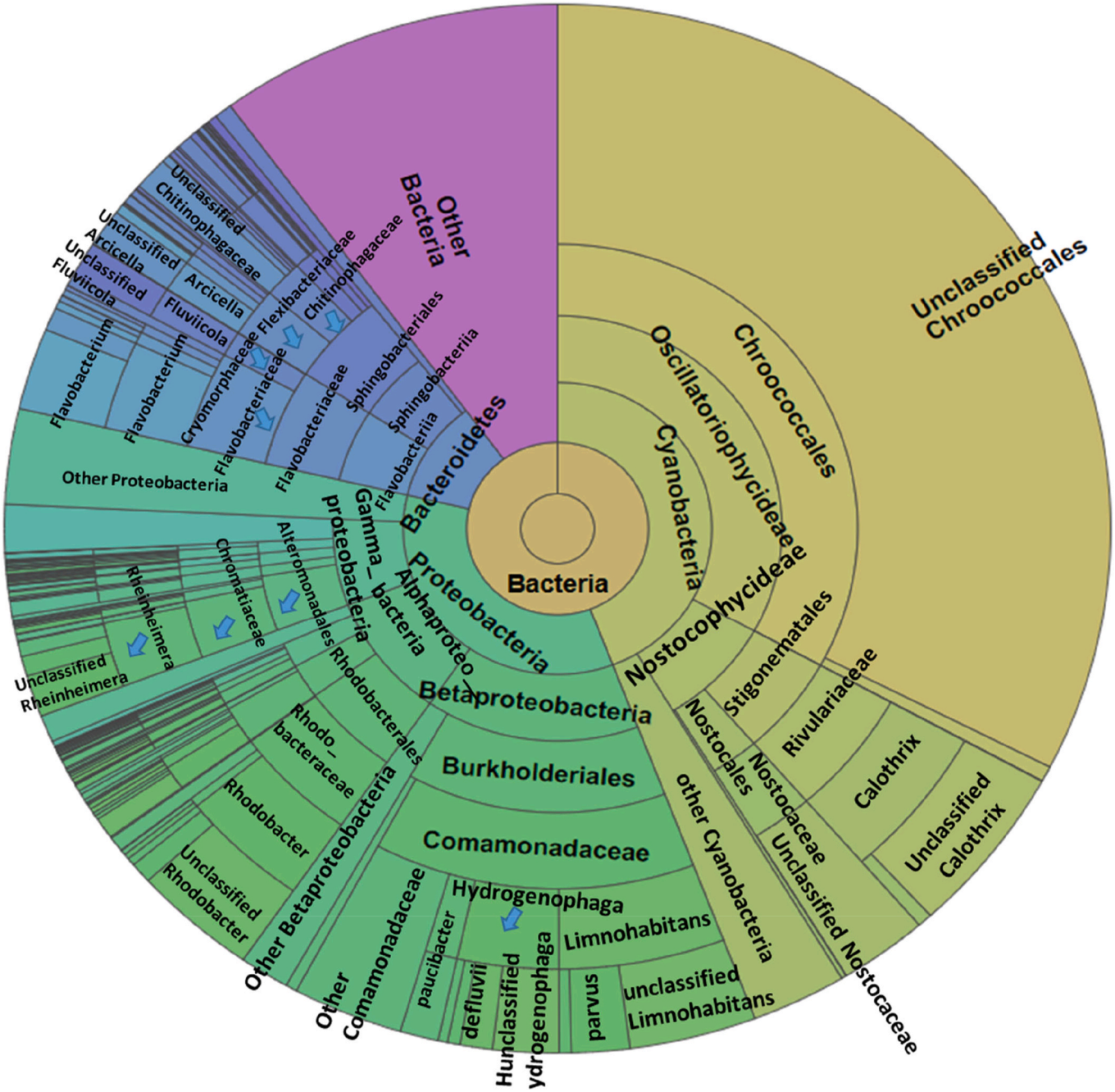

Fig. 6. Relative abundance of the river microbial main taxa within each taxonomic level. From inside the circle to outside: phylum, class, order, family, genus and specie.

phylogenetic analysis of the DNA sequences of cloned 16 S rRNA genes showed that members of the main phylogenetic groups are ubiquitous to almost all soil types: class α-proteobacteria and phyla Actinobacteria are well represented, and together, Acidobacteria and Verrucomicrobia were represented in >75% of 16 S rRNA gene clone library studies of soil bacterial communities. Other classes of the phylum Firmicutes have been detected in 25–75% of respective studies (Zhang and Xu 2008; Hugenholtz et al. 1998).

Actinobacteria, predominant in our samples, are an important phylum of common Gram-positive bacteria in soil and plant matter. They mainly contain aerobic, bacillary or filamentous bacteria (Parte et al., 2012). Most of the species are grouped in the class Actinobacteria, which, in our samples, was also the most abundant one and represented 71.6% of Actinobacteria readings (38.63% of the total taxa) and readings in the order Actinomycetales (accounting for 99.36% of the family Actinobacteria and 38.38% of the total taxa). Actinomycetales have been found in different soil communities, including rhizosphere and root endosphere (Blain et al. 2017), arid and oligotrophic environments (Marcos et al. 2019; Arocha-Garza et al., 2017), cultivated soils, (Zhang et al., 2018), industrially polluted soils (Devpura et al., 2017), and even Antarctic soils (Tytgat et al., 2016). Nocardioidaceae was the most abundant family among the order Actinomycetales (22.56% of Actinomycetales reads; 8.66% of the total taxa).

The class Thermoleophilia had the second highest abundance in the phylum Actinobacteria (20.48% of Actinobacteria and 11.02% of the total taxa), all of them belonging to the order Solirubrobacteales. These bacteria have been identified in different types of soils (Seki et al., 2015), including arid soils (Marcos et al. 2019), contaminated pig

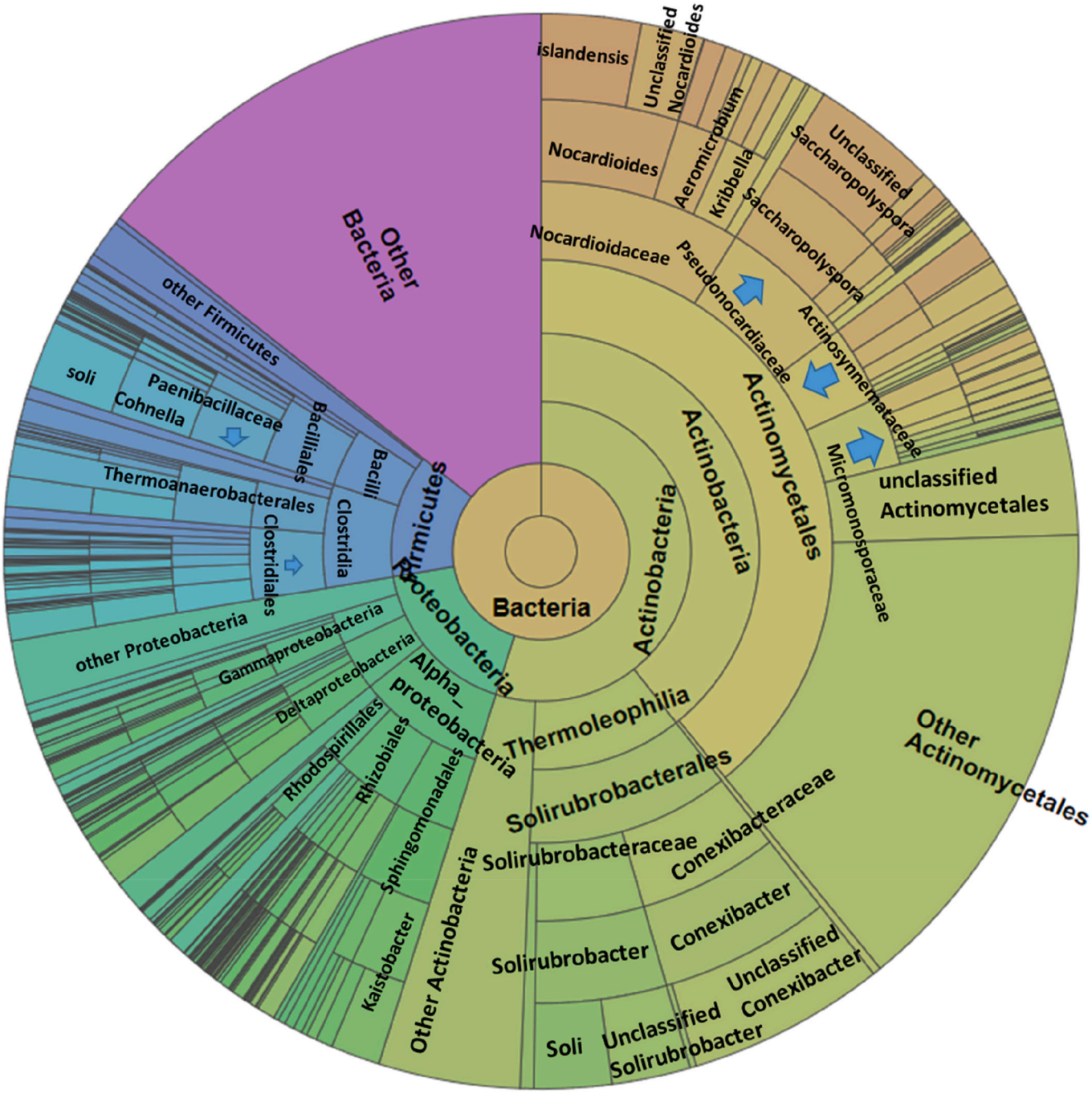

Fig. 7. Relative abundance of the soil microbial main taxa within each taxonomic level. From inside the circle to outside: phylum, class, order, family, genus, and species.

farm-impacted soil (Ouyang et al., 2019), cultivated soils (Shange et al., 2012) and the rhizosphere (Yuan et al. 2015).

The main phylum of Proteobacteria identified was Alphaproteobacteria (53.36% of Proteobacteria reads; 9.30% of the total taxa), followed by Deltaproteobacteria (20.96% of Proteobacteria reads; 3.59% of the total taxa) and Gammaproteobacteria Alphaproteobacteria (13.57% of Proteobacteria reads; 2.32% of the total taxa). At the family, genus and Species level, we found a high diversity of Proteobacteria (Fig. 7).

The phylum Firmicutes contains endospore-forming bacteria, lactic acid bacteria and Gram-positive cocci, abundant in several soils (Ramos et al., 2019), rhizosphere soil (Cordero et al. 2020), wetlands soils (Lv et al., 2014), agricultural soils (Wang et al., 2020) and in soils in Spain similar to that studied here (Pino-Otin et al., 2019b). The class Clostridia was the most abundant one in our samples (57.41% of Firmicutes reads; 7.50% of the total taxa), followed by the class Bacilli (32.61% of Firmicutes reads; 4.26% of the total taxa).

4. Discussion

Our results show that citronellol presents toxicity to non-target organisms and communities in both aquatic and edaphic environments. It can cause mortality in the aquatic invertebrate *D. magna* and affects photosynthesis of the fluvial periphyton. The earthworm *E. fetida* was most sensitive to citronellol, and this monoterpene could also inhibit root growth in *Allium cepa*. The effects followed the order *E. fetida* > *D. magna* > fluvial periphyton > *A. cepa*.

Furthermore, citronellol strongly modified the growth and metabolism of both riverine and edaphic bacterial populations.

In this sense, citronellol could affect the complex network of interactions in river and soil ecosystems by damaging organisms on the bottom or with important functions in freshwater and soil food webs. It also affects the primary producers of the river environment (periphyton), potentially leading to a reduction in herbivore invertebrate densities and a top-down impact on trophic cascades (Relyea and Hoverman 2006) by changing herbivore-producer interactions.

Changes in the invertebrate community can also occur when *D. magna* is affected and when other invertebrates are less sensitive to citronellol. Changes in interspecific competition have been observed when moderate concentrations of insecticides were administered to communities containing both cladocerans (such as *D. magna*) and copepods. A dramatic decline has been observed in the more sensitive *Cladocera* species and a substantial increase in the abundance of copepods (vandenBrink et al., 1996; Relyea 2006).

Soil invertebrates and soil microbes facilitate proper ecosystem functioning (Schon and Dominati 2020; Hallam and Hodson 2020; Bender, Wagg and van der Heijden 2016).

Earthworms, such as *E. fetida*, are at the base of the terrestrial food web and are important invertebrates in soil ecosystems. They are closely linked to the physical and chemical dynamics of terrestrial environments because of their impacts on nutrient cycling, soil aeration, moisture content, nutrient cycling and overall soil structure (Sofo et al. 2020; Hallam and Hodson 2020; Schon and Dominati 2020). Exposure to citronellol may undermine earthworm ability to deliver ecosystem services. Earthworms constitute the dominant biomass of soil invertebrates (Curry and Schmidt 2007; Lee, 1985; Zhang et al., 1995); reducing earthworm populations and altering microbial metabolism may negatively impact soil ecosystems (Pochron et al., 2021) and crops, based on our results for *A. Cepa*. Although this plant appeared to be highly resistant, effects on root growth were clearly detectable, which would add to the role of an effective herbicide described for other plant species (Lins et al., 2019).

4.1. Effect of citronellol on D. magna

Citronellol is poorly soluble in water, with 307 mg/L at 25 °C and 300 mg/L at 20 °C (https://echa.europa.eu/), which generally impedes its bioavailability. However, since *Daphnia* is a filter organism (it feeds by straining suspended matter and food particles from water), it is quite possible that the effects of citronellol are mainly due to its intake through its food activity.

Although some studies indicate different degrees of toxicity of monoterpenes on terrestrial invertebrates (Benelli et al., 2018a; Reid et al. 2017), to the best of our knowledge, neither the effects of monoterpenes on aquatic invertebrates nor their mechanisms of action have been studied. Probably, like other lipophilic compounds, it can partition into membranes (Sikkema et al. 1994; Bakkali et al., 2008; Camargos et al., 2014) and cause a non-specific reversible disturbance of the functionality and selective permeability, with severe consequences for cellular homeostasis. Terpenoids also affect sodium channel activity, increasing the permeability of sodium ions in excitable membranes (Holstege et al., 2000; Yakehiro et al., 2000).

4.2. Effect of citronellol on E. fetida

Exposure of *E. fetida* to citronellol at relatively low concentrations (LC_{10} = 3.8 mg/L) causes earthworm mortality, reflecting earthworm sensitivity to this product and the high bioavailability of citronellol applied to soil. Exposure can occur through ingestion of particles impregnated with the active product (Suthar et al. 2008; Vijver et al., 2003) and percutaneously. The earthworm cuticle is extremely tolerant of water absorption and loss, potentiating an intense exchange of water through the body wall (Saxena et al. 2014; Wallwork 1983; Laverack 1963).

In addition, the low molecular weight of citronellol (437.1 g/mol) and its hydrophobicity (log Kow = 3.91) could facilitate its permeability in biological membranes, causing damage to cell membranes and producing cytotoxicity (Bakkali et al., 2008), which could explain the intense effect of citronellol on this earthworm species.

As far as we know, there are no toxicity studies for acyclic monoterpenes on *E. fetida*, although plant essential oils (EO) with a high content of monoterpenes (no citronellol) have been studied and seem to show no toxicity on *E. fetida* (Benelli et al. 2018b, 2019), possibly because the concentration of each compound in the EO is lower than when isolated components are tested.

Studies with a series of acyclic monoterpenoids, including citronellol, using other soil model organisms, such as the nematode *Caenorhabditis elegans*, showed that citronellol was one of the thee most efficient compounds with nematocidal effect (Abdel-Rahman et al. 2013).

Several monoterpenes have shown toxicity on other soil invertebrates, such as geraniol against the root-knot nematode *Meloidogyne javanica* (Nasiou and Giannakou 2018) or alpha-pinene monoterpene (Jensen et al., 2020) against *Folsomia Candida*.

4.3. Phytotoxicity of citronellol on A. cepa

Although the effects of pure citronellol have not been tested on *A. cepa*, *Solanum nigrum* extracts containing citronellol showed no negative impact on the germination of onion seeds (Afolayan and Bvenura 2018). However, the concentration of citronellol found in this extract was small (11.98%), and plant extracts usually provide less conclusive results because they are composed of a wide variety of products with potential interactions. The same *S. nigrum* extracts containing citronellol had no effect on the germination of cabbage seeds, although root extract concentrations may hinder germination in tomato seeds (Afolayan and Bvenura 2018).

However, pure monoterpene has a clear phytotoxic activity both regarding root elongation and seed germination. For example, citronellol inhibited the germination and root elongation of *Cassia occidentalis* (broad-leaved) and *Echinochloa crusgalli* (grass). Among a range of root inhibitors, citronellol was the more potent one (Batish et al., 2006).

Furthermore, citronellol can inhibit seed germination in *Raphanus sativus* L. (radish) and *Lepidium sativum* L. (garden cress) (De Martino et al., 2010), reduces the germination of *C. occidentalis* seeds (Singh et al., 2002) and completely inhibiting seed germination and seedling growth of *Amaranthus retroflexus*, *Chenopodium album* and *Rumex crispus* (Kordali et al. 2007a, 2007b). Citronellol also acted as an efficient herbicide when spread on *A. thaliana* leaves (Lins et al., 2019).

The EC_{50} values obtained in this work were in the range of the studies cited: in the case of De Martino (2010), they were practically the same for *Raphanus sativus* L. and *Lepidium sativum* L (around 150 mg/L), but were slightly lower when compared to Singh (2002) for *C. occidentalis* seeds (around 50 mg/L). Batish (2006) obtained EC_{50} values for *C. occidentalis* seeds and *Echinochloa crus-galli* in a wide range from 1670 to 90 mg/L.

The allelopathic and phytotoxicity action mechanisms of monoterpenes have been studied previously. Individual monoterpene molecules, small and amphiphile, could cross the mesh of the cell wall and directly interact with the plant plasma membrane, triggering electrolyte leakage due to alteration of the integrity of the cell membrane (Lins et al., 2019). Toxicity can be produced by alterations in cellular metabolism such as photosynthesis and/or reactive oxygen species (ROS) production (Kaur et al., 2011). However, the disruption of microtubule functionality, described for other monoterpenes, seems to lack for citronellol (Chaimovitsh et al., 2017).

The specific mechanism of action of root growth inhibition by citronellol seems to be the induction of the generation of ROS, resulting

in lipid peroxidation and membrane damage (Kaur et al., 2011).

4.4. Effects of citronellol on photosynthesis of fluvial periphyton

As far as we know, the effects of this monoterpene on a complex aquatic community, such as the periphyton, have never been tested. The periphyton integrates populations of algae, bacteria, fungi, protozoa and invertebrates developing on an underwater substratum (Seguin et al. 2001). This community functions as an autonomous ecosystem, with both autotrophic and heterotrophic metabolism. Although the organisms of these communities would be expected to have a certain degree of protection, the periphyton is highly sensitive to qualitative or quantitative water changes (Denoyelles et al. 1982) and they also represent a temporary record of these changes by being exposed to water conditions for long periods of time. All these factors make them excellent indicators of ecotoxicity, presenting a more complete picture of the environmental conditions than individual organisms (Sabater et al., 2007). However, in the literature, we only found studies on the effects of citronellol on individual organisms, such as the algae *Chlorella* (Ikawa et al. 1992). The concentrations tested by Ikawa were low (20 μL on paper discs) with respect to the ranges of our study, but the differences in the trials make it difficult to compare such studies; although in our periphyton samples, there was a good representation of chlorophytes, their individual behaviour is surely different from what we might expect.

Acyclic terpene alcohols have been described as antiblue-green algal agents, with farnesol being particularly active against *Synechococcus* (Juettner 1983).

There is also evidence that monoterpenes have effects on Cyanobacteria, another group present in our periphyton samples. For example, the phenolic monoterpene carvacrol from the essential oil of *Thymbra capitata* can inhibit microorganisms, such as Cyanobacteria and green alga, colonising outdoor stone surfaces (Candela et al., 2019).

We found no studies on the effect of monoterpenes in relation to the other groups present in our samples, namely diatoms or *Rhodophyta*.

In relation to the mechanism of action of citronellol in the green algae *Chlorella,* Ikawa suggested other possible processes beyond a primary disruption of membrane function, such as respiration, photosynthesis and protein synthesis affectation, and related this activity not only to the lipophilic tail of citronellol but also its hydrophilic head, which could improve its activity (Ikawa et al. 1992).

4.5. Effect of citronellol on soil and water microorganisms

Several studies have shown that oxygenated monoterpenes and essential oils rich in these components (including citronellol) can be used as antimicrobial agents with clinical or agricultural and dietary interest (Kotan et al. 2007). However, we found no studies on the effects on non-target organisms.

Many plant extracts containing citronellol (especially EO) have antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria of clinical and commercial interest. This is the case for *Rosa damascene* (Ulusoy et al. 2009; Batool et al., 2018; Akram et al., 2020) or the genus *Pelargonium* (Boukhatem et al. 2013; Ouedrhiri et al., 2018). *Beta vulgaris* root methanolic extract demonstrated an inhibitory effect against the pathogenic microorganisms *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* (El-Mesallamy et al., 2020). In another study, EOs from *Citrus aurantifolia* showed antibacterial activity against *Streptococcus mutans* (Lemes et al., 2018). *Vepris macrophylla* EO strongly inhibited the multi-resistant strains *A. baumannii*, *K. pneumoniae* and Staphylococcus *aureus* (Rosato et al., 2018). A novel essential oil obtained from *Eremothecium ashbyii*, also containing citronellol, exhibited antibacterial activities against food spoilage microbes such as *S. aureus, Bacillus subtilis, Bacillus cereus, Listeria monocytogenes*, *Escherichia coli, Salmonella typhimurium, Zygosaccharomyces bailii, Saccharomyces cerevisiae and Candida utilis* (He et al., 2019).

The antibacterial activity of isolated monoterpene has also been studied, clearly showing that citronellol had a broader antibacterial spectrum in both Gram-positive and Gram-negative strains (Kotan et al. 2007). Citronellol showed a rapid bactericidal effect against *E. coli* and antibacterial activity against *Bacillus subtilis*, *Salmonella Typhimurium* and *Staphylococo aureus*, with minimum inhibitory concentrations of 5–370 mg/L (Guimaraes et al., 2019; Lopez-Romero et al., 2015). The antifungal effects of citronellol have been predicted in *in-silico* studies (Silva et al., 2019) and demonstrated in experimental assays (Kordali et al. 2007a, 2007b; Silva et al., 2020).

Therefore, the bactericidal effect of citronellol against microorganisms of clinical or industrial interest is well reflected in the literature. However, no studies on the safety and toxicity of citronellol in non-target microbes could be found in any of the microorganisms that genetic analysis has revealed abundantly in our water or soil samples. There is only evidence of antibacterial activities of citronellol against a species of Burkholderiales *(*Kotan et al. 2007*)* or of other monoterpenes, such as the phenolic monoterpene carvacrol, which shows inhibitory effects on cyanobacteria (Candela et al., 2019). In this sense, our study opens up a new field in this regard.

Based on our results, most natural bacterial communities of soil and water are sensitive to citronellol. However, microbial transformation of monoterpenes has been reported, and it is possible that besides these catabolic reactions, transformations may occur as part of detoxification processes (Marmulla and Harder 2014). Specifically, *Pseudomonas* species can degrade citronellol (Cantwell et al., 1978; Forster-Fromme and Jendrossek 2010).

Regarding the taxa found in our samples, there is evidence that some bacteria belonging to Betaproteobacteria (Foss and Harder 1998) and, specifically, to the order Burkholderiales (Foss et al. 1998) can metabolise oxygenated monocyclic monoterpenes, using them as a carbon source. There is also evidence for the ability to metabolise terpenoids from other bacterial groups found in our samples, such as other Proteobacteria, Actinobacteria and Firmicutes (Lyu et al., 2013; Noike et al., 2012). However, this activity does not seem to impede the effect of citronellol in all bacterial communities of our samples, in which a decrease in the metabolic capacity of practically all functional groups was detected.

The suggested mechanism of action of monoterpenes, including citronellol, at the cellular level in microorganisms is also an action on the cell surface, causing changes in the fluidity of the membrane by inducing perturbation of the membrane potential and/or permeability (Lopez-Romero et al., 2015; Lins et al., 2019). These effects can notably induce cell lysis or death by apoptosis or necrosis. The disruption of cell membrane integrity by interfering with ergosterol biosynthesis has been suggested as the toxicity mechanism of citronellol in *Candida* (Sharma et al., 2020). However, citronellol must first penetrate the bacterial wall of Gram-positive and Gram-negative bacteria. Although it presents differences with respect to the bacterial wall, the *Candida* cell wall weakens due to exposure to citronella. The antimicrobial activity of citronellol could be related to the presence of hydroxyl groups (alcohol compounds), whereas hydrocarbons resulted in a lower activity (Guimaraes et al., 2019).

4.6. Environmental relevance

Citronellol is one of the six most frequently used fragrance compounds (Buckley 2007; Belsito et al., 2008) and present in all of the investigated aerosol spray deodorants on the European market in a study from 1998 and in 47% of domestic and occupational products in an investigation from 2001 (Rastogi et al. 1998, 2001). Numerous natural and artificial flavourings in alcoholic and non-alcoholic beverages (1–4 ppm), hard and soft candies (2–18 ppm), chewing gum (8–9 ppm), ice creams (1–40 ppm), gelatine puddings (2–6 ppm) and baked goods (6–20 ppm) contain various amounts of citronellol. The annual consumption of citronellol by perfumers in the U.S., Japan and some European countries was estimated in hundreds (Bedoukian 1985) to

thousands of tons (Pisano 1986). In addition, citronellol has other promising properties, and its use will probably be extended in the near future, for example replacing synthetic products such as antibiotics (Kotan et al. 2007) or being combined with them (Cheesman 2017), thereby reducing the doses of synthetic products as a palliative effect to mitigate resistance (Lewis and Ausubel 2006; Inui et al., 2007). In this sense, its presence in the environment will foreseeably increase.

Here, we show that natural products come with their own set of environmental impacts. Therefore, it is relevant to know their possible effects on non-target organisms. The United States Environmental Protection Agency (USEPA) has stated that for citronellol, "no adverse effects to non-target organisms or the environment are expected as. This should be reviewed due the effects on a key non-target organism in water and soil, detected in this study.

The toxicity of citronellol in aquatic organisms such as *D. magna* or in river periphyton is significantly lower than that found for synthetic insecticides such as fipronil in the same tests (Pino et al., 2020). However, this is not always the case. For example, citronellol showed lower LC_{50} values for *E. fetida* than those found for 18 drugs evaluated with the same test (Pino et al., 2015).

It is important to gain information about the occurrence of citronellol in the environment. In screening analyses, citronellol was detected in all waste and river water samples from the Danube (Milic et al., 2014) and in municipal wastewater from Göteborg, Sweden (Paxeus and Schroder 1996). The few studies that quantified the appearance of citronellol in the environment indicate ranges (Eriksson et al., 2003), lower than those we obtained for all the non-target organisms studies; therefore, acute ecotoxicity effects of this terpene in the environment are currently not expected. However, more studies are needed to confirm these levels of presence of this monoterpene in the environment.

Finally, environmental effects of citronellol cannot be ruled out. A greater use of these products can increase their release into water and soil through irrigation or the application of municipal biosolids to soil, similar to other emerging pollutants (Tijani et al., 2016). Once in the environment, citronellol could remain and accumulate after repeated applications of biosolids or via irrigation. Therefore, although these concentrations are not immediately toxic, the cumulative, chronic and long-term effects should be studied.

Little is known about the behaviour and pathways of this product in the environment. The oxidation pathways of citronellol exposed to air have been studied for the formation of oxidation products (Rudback et al., 2014) and its biodegradation by some bacteria, particularly *Pseudomonas* (Forster-Fromme and Jendrossek 2010), but under conditions that do not simulate environmental exposure.

Therefore, it is necessary to know the dynamics that citronellol can follow in soils or rivers to predict whether its environmental impact may be relevant with increasing use. Furthermore, information on the ecotoxicity of this substance, provided by The European Agency for Chemical Substances and Mixtures (ECHA), is limited. This study therefore fills an important knowledge gap.

5. Conclusions

The acute toxicity of citronellol was evaluated for the first time on aquatic invertebrates, such as *D. magna*, the plant *A. cepa* L and the earthworm *E. fetida*, together with the influence on river periphyton communities and river and soil bacterial populations, all of them taxonomically characterised.

Eisenia fetida was the model organism most sensitive to citronellol, followed by *D. magna.* The fluvial periphyton was also affected, and citronellol was phytotoxic for *A. cepa*. Moreover, the growth and metabolism of both fluvial and edaphic bacterial populations were strongly modified by citronellol, provoking a decrease in carbohydrate metabolism after 48 h of exposure.

Therefore, if this monoterpene is considered to be used at an industrial level or its use is increased as an alternative to synthetic products, these results should be taken into account to better understand its toxicity on non-target organisms.

Credit author statement

Ma Rosa Pino Otín: Conceptualization, Formal analysis; Funding acquisition; Investigation, Project administration, Resources, Supervision; Validation, Writing – original draft. Elisa Langa: Data curation, Investigation, Methodology, review & editing. Jonatan Val: Data curation, Investigation. Ana M. Mainar: Funding acquisition, Resources, review & editing. Diego Ballestero: Conceptualization, Investigation, Supervision; Validation, review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.112303.

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