Industrial Crops & Products

Spanish Satureja montana L. hydrolate: ecotoxicological study in soil and water non-target organisms

--Manuscript Draft--

Manuscript Number:	INDCRO-D-21-04492R3
Article Type:	VSI:AAIC2021
Section/Category:	Non-food bioactive products
Keywords:	Hydrolate; Satureja montana; ecotoxicity
Corresponding Author:	María Rosa Pino-Otín Universidad San Jorge SPAIN
First Author:	María Rosa Pino-Otín
Order of Authors:	María Rosa Pino-Otín
	Juliana Navarro
	Jonatan Val
	Francisco Roig
	Ana M. Mainar
	Diego Ballestero
Abstract:	Despite the current popularity of herbal and flower hydrolates and the multiple applications found in the food, wood, pharmaceutical, pesticide, perfume, cosmetic, and aromatherapy industries, the effect of hydrolates in the environment is poorly known. This study evaluates the ecotoxicity of the Satureja montana L. hydrolate on water and soil bioindicators and also on the fluvial periphyton mesocosms for a more ecological point of view. The acute toxicity of the fresh water invertebrate Daphnia magna, the bacteria Vibrio fisheri , and the earthworm Eisenia fetida, was quantified as well as the phytotoxic effect on the plant Allium cepa L . Communities of river periphyton were used to study the impact of the hydrolate on the freshwater ecosystems. The taxonomic study of these communities revealed a rich diversity of diatoms. The hydrolate of S. montana showed a high ecotoxicity at very low percentages of hydrolate in all organisms tested and in the periphyton communities. The LC 50 varies from 0.05% to 4.25%, with a clear dose-dependent relationship. The effect of the hydrolate in decreasing order was: A. cepa > D. magna > V. fisheri > periphyton > E. fetida. The strong phytotoxic effect on A. cepa allows exploring possible uses of the hydrolate as a bio-herbicide. The Gas Chromatography Mass Spectrometry (GC-MS) characterisation of the hydrolate reveals 1 alcohol and 6 terpenes. Carvacrol (89.03%) and thymol (6.66%) are the volatile compounds found in the highest proportion, products that have multiple biological properties and known synergistic effects, which could explain the high bioactivity of the hydrolate. Our study suggests that S. montana hydrolate could impact different trophic levels of the river ecosystems and can affect the soil functions affecting earthworms due its powerful bioactivity on a wide range of non-target organisms even in complex communities such as the fluvial periphyton. Therefore, although hydrolates can become a good alternative to synthetic products, the use of these products is not free of environmental risks and their release to the environment should be evaluated.

Spanish *Satureja montana L.* hydrolate: ecotoxicological study in soil and water non-target organisms

Mª Rosa Pino-Otin[1], Juliana Navarro[2], Jonatan Val[3], Francisco Roig[1], Ana M. Mainar[4], Diego Ballestero[1]

(1) Universidad San Jorge. Villanueva de Gállego, 50830 Zaragoza, Spain

(2) CITA, Unidad de Recursos Forestales, Avda. Montañana 930, 50059-Zaragoza, Spain

(3) Colegio Internacional Ánfora. c/ Pirineos, 8, 50410 Cuarte de Huerva, Zaragoza, Spain

(4) I3A, Universidad de Zaragoza, c/ Mariano Esquillor s/n, 50018 Zaragoza, Spain

- Mª Rosa Pino-Otín: rpino@usj.es (corresponding author)
- Juliana Navarro: jnavarroro@cita-aragon.es
- Jonatan Val: jval@colegiointernacionalanfora.com
- Francisco Roig: fjroig@usj.es
- Ana M. Mainar: ammainar@unizar.es
- Diego Ballestero: dballestero@usj.es

Corresponding author:

Mª Rosa Pino-Otín. Universidad San Jorge. Campus Universitario Villanueva de Gállego

Autovía A-23 Zaragoza-Huesca, km. 510 50830 Villanueva de Gállego. Zaragoza (Spain).

T. 0034 976 060 100

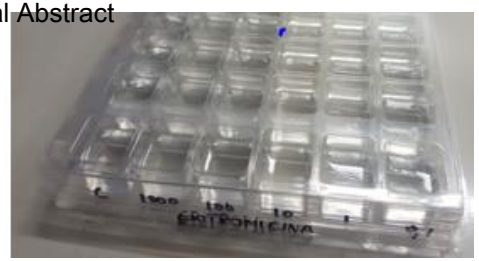
M.0034 676 22 79 90

Fax. (34) 976 077 584

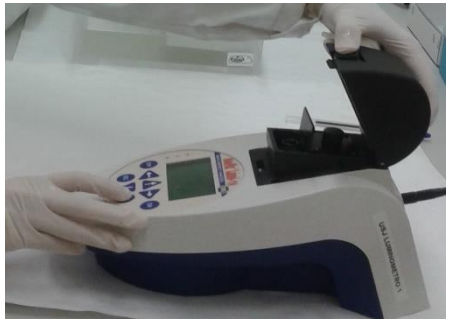
rpino@usj.es

www.usj.es

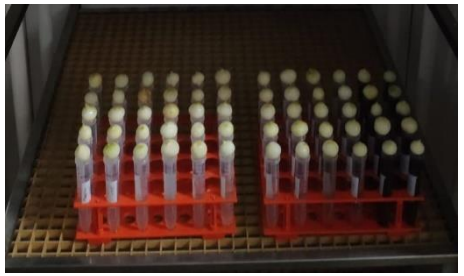
Graphical Abstract



Daphnia magna



Vibrio fisheri



Allium cepa

Eisenia fetida

Individual toxicity

Acute toxicity

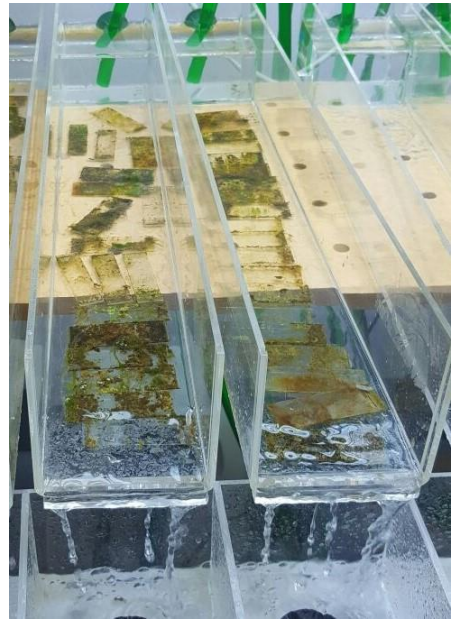
Satureja montana hydrolate

Community effects

Photosynthesis yield

Periphyton communities

River microbial community



HIGHLIGHTS

- *Satureja montana* hydrolate is highly phytotoxic on *Allium cepa* (LC_{50} =0.05%)
- The hydrolate has a high ecotoxicity on *Daphnia magna* and *Vibrio fisheri* (LC_{50}<1%)
- *Eisenia fetida* is the single organism more resistant to the hydrolate (LC_{50}=4.25%)
- Communities of fluvial periphyton are also sensitive to hydrolate (LC_{50}=4.23%)
- Carvacrol (89%) and thymol (7%) are the main volatile compounds of the hydrolate

Spanish *Satureja montana L.* hydrolate: ecotoxicological study in soil and water non-target organisms

Mª Rosa Pino-Otin, Juliana Navarro, Jonatan Val, Francisco Roig, Ana M. Mainar, Diego Ballestero

Abstract

Despite the current popularity of herbal and flower hydrolates and the multiple applications found in the food, wood, pharmaceutical, pesticide, perfume, cosmetic, and aromatherapy industries, the effect of hydrolates in the environment is poorly known. This study evaluates the ecotoxicity of the *Satureja montana L.* hydrolate on water and soil bioindicators and also on the fluvial periphyton mesocosms for a more ecological point of view. The acute toxicity of the fresh water invertebrate *Daphnia magna,* the bacteria *Vibrio fisheri*, and the earthworm *Eisenia fetida,* was quantified as well as the phytotoxic effect on the plant *Allium cepa L*. Communities of river periphyton were used to study the impact of the hydrolate on the freshwater ecosystems. The taxonomic study of these communities revealed a rich diversity of diatoms. The hydrolate of *S. montana* showed a high ecotoxicity at very low percentages of hydrolate in all organisms tested and in the periphyton communities. The LC_{50} varies from 0.05% to 4.25%, with a clear dose-dependent relationship. The effect of the hydrolate in decreasing order was: A.*cepa* > *D. magna* > *V. fisheri* > periphyton > *E. fetida.* The strong phytotoxic effect on *A. cepa* allows exploring possible uses of the hydrolate as a bio-herbicide. The Gas Chromatography Mass Spectrometry (GC-MS) characterisation of the hydrolate reveals 1 alcohol and 6 terpenes. Carvacrol (89.03%) and thymol (6.66%) are the volatile compounds found in the highest proportion, products that have multiple biological properties and known synergistic effects, which could explain the high bioactivity of the hydrolate. Our study suggests that *S. montana* hydrolate could impact different trophic levels of the river ecosystems and can affect the soil functions affecting earthworms due its powerful bioactivity on a wide range of non-target organisms even in complex communities such as the fluvial periphyton. Therefore, although hydrolates can become a good alternative to synthetic products, the use of these products is not free of environmental risks and their release to the environment should be evaluated.

1. Introduction

The *Satureja* L genus. (Lamiaceae Family) has numerous species widely distributed throughout the world, most of them are shrubs or aromatic herbs with interesting biological properties (Saeidnia et al., 2016). *Satureja montana* L., also called winter savory, is a perennial shrub with white or slightly pink flowers (Lawless, 2002), industrially grown and appreciated for its medicinal, bactericidal and fungicidal properties (Lopez-Cobo et al., 2015).

Satureja montana essential oil (EO) has a wide variety of pharmacological properties as antimicrobial, antifungal, antioxidant, antispasmodic, antiviral, and antidiarrheal (Caprioli et al., 2019; Jafari et al., 2018; Tepe and Cilkiz, 2016). This EO also has insecticidal (Tepe, 2015) and nematocidal (Faria et al., 2016) properties. The EO from the same Spanish plant, *S. montana,* recently have shown nematocidal properties as well (Navarro-Rocha et al., 2020).

During distillation of any plant biomass to obtain these EOs, a certain proportion of them becomes dissolved in distillation or condensate water leading to a mixture named hydrolate, containing a variable quantity of EOs and volatile, water-soluble, secondary active metabolites (Labadie et al., 2015; Zheljazkov and Astatkie, 2011). Hydrolates are products of steam distillation and have been considered a simple waste. This consideration has recently changed and many researchers have found interesting biological properties of these aqueous fractions such as antifungal, antibacterial, and antioxidant activities (Tornuk et al., 2011; Prusinowska et al., 2016; Maia et al., 2013; Franzener et al., 2007; Di Vito et al., 2021).

Consumers´ increasingly demand products with good environmental qualities, low toxicity and safe for health so hydrolates appear as a good option to traditional treatments. Additionally, hydrolates are readily available, inexpensive, and easy to produce (Tornuk et al., 2011). Hydrolates can act as repellants, herbicides (Politi et al., 2020), and nematicides (Andres et al., 2018).

Hydrolates are widely used in the cosmetic industry and used as diluted in refreshing drinks (D'Amato et al., 2018). The current research now also points to the use of hydrolates in the food industry to prevent the growth of pathogenic microorganisms. Hydrolates also prevent organoleptic properties of treated products and also possible risks to the health of consumers (Sagdic et al., 2013) such as the appearance of carcinogenic by-products in chlorine-based sanitisers when reacting with organic matter (Gil et al., 2009).

Although not well studied, the hydrolate of *Satureja* genus can act as a fungicide to prevent food spoilage (Boyraz and Ozcan, 2006) and have been proposed as an environmentally safer alternative to prevent saprophytic and pathogenic fungi from damaging agri-food products (Boyraz and Ozcan, 2006). Antimicrobial activity has also been reported for hydrolates of *Satureja* of different species (Sagdic and Ozcan, 2003; Chorianopoulos et al., 2008; Giaouris et al., 2008; Sagdic et al., 2013; Sahan and Tornuk, 2016). On the other hand, natural antioxidants obtained from hydrolates could be a good alternative to synthetic antioxidants employed in the pharmaceutical and food industry, possibly with little effect on organoleptic properties as described in the literature for hydrolates of *Satureja* genus (Cabana et al., 2013).

Recently an Italian *S. montana hydrolate* showed important antimicrobial and antifungal properties in a wide variety of microorganisms (Di Vito et al., 2021). Interestingly, the compounds of the hydrolate were better able to inhibit microbial growth than EO from the same plant because they showed activity at lower concentrations.

These findings highlight the potential of hydrolates as promising consumer products of commerce in the food, wood, pharmaceutical, pesticide, perfume, cosmetics, and aromatherapy industries. For example, the value of the hydrophilic EO fractions of aromatic oils that escape into the hydrolate was estimated to be worth US$50–100 million in India (Pangarkar, 2008).

Moreover, the exploitation of these by-products is an opportunity to extend their life cycle in accordance with the circular economy guidelines and reduce the environmental impacts of waste generation.

While potential uses are being studied, very little is known about ecotoxicity in non-target organisms once these products reach the environment. Hydrolates could spread in the environment either as a residue from EO extraction or as waste after consumption.

The first studies carried out on the ecotoxicity of hydrolates have shown that they are not at all harmless for the environment. Studies on the ecotoxicity of hydrolates from *Lavandula luisieri* (Rozeira) and *Artemisia absinthium L.* on the aquatic (Pino-Otin et al., 2019a) and edaphic environments (Pino-Otin et al., 2019c; Pino-Otin et al., 2019b) show affection on individual organisms as well as entire communities. Some studies also point to effects on fish: *Cinnamomum zeylanicum* J. Presl hydrolate is toxic on carps (*Cyprinus carpio*) in aquariums (Gulec et al., 2013),

and *Lippia alba* (Mill.) N.E. Brown hydrolate showed sedative (da Silva et al., 2018) and anaesthetic effects (Maia et al., 2019) in tambaqui (*Colossoma macropomum)*.

The aim of this study was to evaluate the ecotoxicity of *S. montana L.* hydrolate on the environment. For this, water indicator organisms, the freshwater invertebrate *Daphnia magna* and the marine bacterium *Vibrio fisheri,* have been used. In addition, river periphyton communities were used to study the effects of this hydrolate on a river, allowing a more ecological perspective of the effects than individual organisms. Finally, two bioindicators: a soil invertebrate (the earthworm *Eisenia fetida*) and a plant (*Allium cepa*) were chosen to evaluate the impact of the hydrolate on the soil environment.

2. Material and methods

2.1 Hydrolate

A Spanish population of *S. montana* were submitted to a four year pre-domestication process to develop a parental line named SAMO-0 (Navarro-Rocha et al., 2020). Field trials located in Ejea de los Caballeros (Aragón, Spain, 42°8′8.73″ N, 1°12′31.50″ W / 346 m a.s.l) allowed obtaining a population of the 40 plants of *S. montana SAMO-0*. From these plants a total of 75 kg of fresh biomass was obtained, on which aerial parts of the plant (leaves and flowers) distillation was carried out in a stainless steel extraction plant with a pressure reducing valve, similar to previous studies (Navarro-Rocha et al., 2020). The hydrolate (aqueous phase) was decanted from the EO in a separatory funnel and then filtered before being used.

2.2 Hydrolate characterisation and computed physicochemical properties

The hydrolate was extracted with hexane (Chizzola et al., 2021)) and the content of volatile organic compounds were analysed by gas chromatography-mass spectrometry (Series 5973, Agilent Technologies) fitted with a HP-5MS column (30 m, 0.25 mm i.d., 0.25 µm film thickness). Helium was used as a carrier gas at 1mL/min. The oven program was held at 60 ºC for 1 min, increased to 246 ºC at 3 ºC/min, and finally increased to 280ºC and held for 1 min. The working parameters used were an injector temperature of 230 ºC split mode (split ratio 20:1) and a transfer line temperature of 240 ºC. The mass spectrometer was performed in the electron impact mode at 70 eV and MS source and MS quad temperatures were 230 ºC and 150 ºC, respectively.

Peak identification was achieved by comparing with standards and/or mass spectra with the National Institute of Standards and Technology MS Search Program 2.0 data and Wiley 275 libraries. Finally, values obtained using an *n*-alkane series (C8–C20) with the same analysis conditions were compared with the retention index (RI) previously reported. External standard was used for the quantification of thymol and carvacrol:

carvacrol (CAS: 499-75-2) with a minimum purity of 98.0% and thymol (CAS: 89-83-8) with a purity of 100.0% (provided by Sigma-Aldrich).

The quantum continuum method COSMO-RS (Klamt et al., 1998; Eckert and Klamt, 2002) was used for predicting the soil adsorption coefficient, log K_{oc}, to assess the mobility in soil of the main compounds of the hydrolate. The pre-optimised three-dimensional chemical structures of carvacrol and thymol were procured from the PubChem database.

A continuum model provided with density functional theory (parametrisation bvp86/dga1) was used to carry out the calculations (parametrisation bvp86/dga1) following the procedures described by (Martinez-Lopez et al., 2018). Water solubility, boiling temperature, octanol-water partition coefficient and vapour pressure were also estimated.

2.3 Daphnia magna assay

These assays were performed following the methodology of OECD (204) and the procedure provided by the Daphtoxkit FTM magna (1996) from Vidrafoc (Spain) (ref. DM121219)

Daphnia eggs incubation lasted 72 h at 20–22 °C with 6000 lx light in a TOXKIT model CH-0120D-AC/DC incubator (provided by ECOTEST, Spain). Two hours before the assay with the hydrolate neonates were feeded with one vial of spirulina.

A pre-test was started to assess the range of concentrations to be tested with the following hydrolate concentrations: 0.1, 1, 10, 50, and 100% v/v. This allowed us to determine that the toxicity ranges were between 0.1% and 10% and to better outline the toxicity curve. The final test was carried out at the following concentrations: 0.01, 0.1, 0.5, 1, and 10%. Concentrations of hydrolate were prepared in synthetic freshwater (ISO 6341 2012) adjusting the pH between 7-7.5 using 0.1 M NaOH. Synthetic freshwater was used as a negative control. After feeding the neonates as described before, 25 organisms were used in the assay. Five replicates were performed for each concentration of hydrolate. Five organisms were placed on each replicate. The assay was carried out in complete

144 darkness for 24 h at 20-22 ºC. After that, the EC_{50} (hydrolate concentration that causes 50% of
145 immobilisation) was calculated considering immobile those daphnids that after 15 s of agitation could
146 not move.

147 **2.4 *Vibrio fisheri* assay**

148 The bioluminescence inhibition assays were performed following the methodology described on the
149 *V. fisheri* acute toxicity test (UNE-EN-ISO 11348-3 2009). The *V. fisheri* (strain NRRL-B-11,177)
150 used in this assay was purchased from Macharey-Nagel (ref. 945 006).
151 Lyophilised *V. fisheri* was rehydrated with the reactivation solution provided by the manufacturer and
152 stored for 5 min at 4 ºC. Then, dilutions of the hydrolate were prepared using a 2% NaCl stock
153 solution (v/v): 0.05, 0.1, 0.25, 0.4, 0.5, 1, 2, and 10%. Solutions NaOH and HCl 0.1M respectively
154 were used to obtain a pH solution between 6-8. The test was repeated in quadruplicate. Four tubes
155 with bacteria and without hydrolate solutions were the negative controls.
156 To start the assay, test tubes at 15 ºC in a water bath were filled with 0.5 mL of the reactivated
157 bacteria. After solution equilibration for 10 min, the basal luminescence was obtained with the first
158 measurements. After initial measurements, 0.5 mL of each hydrolate dilution to be tested was added
159 to the tubes and, after 30 min, the second luminescence inhibition measurements were performed.
160 Those measurements were obtained with a Biofix® Lumi-10 luminometer (Macharey-Nagel) using
161 the acute mode (Biotox B) equipped with an ultra-fast single-photon counter detector that covered
162 the 3.806–660 nm spectral range. The endpoint of the test was the loss in bacterial light production.
163 The EC_{50} (hydrolate concentration that induce a 50% loss of bioluminescence) was expressed as
164 percentage of luminescence inhibition and calculated for each concentration in comparison with the
165 control.

166 **2.5 *Eisenia fetida* assay**

167 These tests were carried out following the methodology of the OECD 207 (1984). Adult *E. fetida*
168 specimens, were acquired from Todoverde company (Spain) with the following characteristic:
169 developed clitella, two months old and weights between 300-600 mg.
170 Before the test, all organisms were subjected to 15 days of acclimatization under controlled
171 conditions: 18-25 ºC, pH=7.5-8.0 and 80-85% humidity in a sphagnum peat substrate (Flower
172 Company, Spain).

The tests were carried out in 1 L polypropylene boxes fitted with a perforated lid to facilitate ventilation and oxygen supply and to avoid leakage od the specimens, as well as excessive water evaporation.

The boxes were filled with five hundred grams (wet weight) of standardised OECD soil substrate consisting of industrial fine sand, kaolin clay (provided by Imerys Ceramics España, S.A.), and sphagnum peat (provided by Verdecora vivarium, Spain) in a 7:2:1 ratio.

The water content was determined by the weight difference between the soil sample and the sample heated for 24 h at 105 ºC. Deionised water was used to obtain a water content of 35%. Soil pH was controlled with a pH-meter and a 1 M KCl solution.

Several test concentrations of hydrolate in distilled water were prepared: 0.1, 1, 10, 50, and 100%. Negative controls were standard soil without hydrolate. The assay was carried out with three replicates for each concentration. Each box contained 10 earthworms, standard soil and the hydrolate.

The experimental conditions of the assay were the following: temperature of 20 ± 2 ºC, under 80–85% relative humidity and 400-800 lux of constant light. The LC_{50} values (Lethal Concentration 50) were determined using log-Probit analysis (Bliss 1934) after 14 days treatment.

2.6 *Allium cepa* assay

Acute toxicity experiments with *A. cepa* were carried out following the methology described by Fiskesjö (1993). Bulbs of *A. cepa* (variety Stuttgarter Riesen 14/21) were provided by Fitoagrícola Company (Spain) and stored in a dry environment at a temperature between 10 and 20 °C in the dark to reduce the risk of fungal growth.

Before the test, young bulbs were peeled avoiding the damage to the root ring. 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. Five replicates were performed for each concentration: 0.001, 0.01, 0.1, and 0.5% v/v and a negative control with water only.

The assay was carried out in the dark at 25 ºC over 72 h and solutions were renewed every 24 h.

2.7 Periphyton communities mesocosm assay

2.7.1 Colonisation

Microscope slides in a methacrylate racks were located in the Gállego River (Zaragoza, Spain) at 15 cm depth on 24 June 2019 in order to obtain periphyton river communities.

When the thickness of the periphyton layer was adequate (around 0.75 mm after 5 weeks) to contain similar algal communities (Navarro et al., 2002), 24 slides were collected. In the laboratory, a sample of each slide was destined to the taxonomic study.

At the same time, to know the physical-chemical characteristics of the river, water sample was also taken in the same place and analysed in the Pyrenean Institute of the Higher Centre for Scientific Research (Support Information 1).

2.7.2 Taxonomic identification

For the taxonomic study of the algae from the slide samples, the Utermöhl technique adapted to inverted microscopy was used (UNE-EN 15204, 2007).

Periphyton samples were oxidated with hydrogen peroxide and a suspension containing clean frustules were obtained mounted and fixed on slides with Naphrax© resin. Then, algae were counted and identified according to UNE-EN 13946, UNE-EN 15204, and UNE-EN 14407.

Cell count and identification were carried out with a Leica light microscope at 1000 magnifications (diatoms) and 100, 400, and 1000 magnifications (other microalgae). Finally, the count allowed obtaining the number of individuals per cm^2 of biofilm.

2.7.3 Flow-through artificial channels assays

Continuous flow methacrylate channels fed by separate water tanks, were used for toxicology experiments. The closed water circuit for each channel is kept in motion (0.113 m^3 / h) thanks to a system of motors. The volume of each channel was 4 L. Water temperature was maintained al 23 ºC by means of a thermostatic bath and measured periodically in the assays.

Before starting the ecotoxicology test, the river slides that had been colonized by river periphyton (mesocosms) were acclimated in one of the channels at 23 ºC.

When the test started, the slides were laid on the bottom of the channels horizontally with the colonized surface facing up and exposed in a geometric series of concentrations: 0.1, 1, 10, 15, and 25 %v/v of hydrolate in buffer solution (MOPS, 0.01 M). HCl or NaOH were used to adjust the pH to 7.5. Negative control was a channel with MOPS only. In order to simulate the sunlight in a specific

spectrum for the cultivation of algae, Blau aquaristic lamps were used (T5HO, 39 w/10.000 °K, 80 µmol photon m^{-2} s^{-1} on the channel surface).

After 2 hours of exposure to the hydrolate, the photosynthetic yield of the mesocosm (efficiency of the photochemical energy conversion process) was evaluated (Consalvey et al., 2005) using a MINI-PAM-II Photosynthesis Yield Analyzer (Walz, Germany). In addition, measurements were made at time 0 to control the possible change in the composition of the mesocosm.

2.8 Statistics and graphical representation

The EC_{50} values and the standard errors (SE) of the dose-response curves were calculated with a logistic regression using the XLSTAT software (2014.5.03). The chi-square test was used to statistically test the dose-models. Regarding periphyton taxonomy, the whole lineage of each of identified taxa was searched in the Algaebase (Guiry and Guiry, 2021) and NCBI taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy). Using these full lineages, a Krona chart (Ondov et al., 2011) was constructed to illustrate the distribution pattern of periphyton phyla in density-scraped surface.

3. Results and Discussion

3.1 Hydrolate composition and calculated physicochemical properties

The hydrolate composition studies focused on the identification of volatile organic compounds following a hypothesis similar to that proposed by Di Vito (Di Vito et al., 2019), which takes into account the effectiveness of some terpenoids even at low concentrations.

The Gas Chromatography Mass Spectrometry analysis reveals that the compounds detected in the hydrolate are 1 alcohol and 6 terpenoids. The hydrolate composition was the following: 89.03% carvacrol (CAS 499-75-2), 6.66% thymol (CAS 89-83-8), 1.34% terpinen-4-ol (CAS 562-74-3), 1.02% 1-octen-3-ol (CAS 3391-86-4), 0.99% borneol (CAS 464-45-9), 0.56% 1,8-cineole (CAS 470-82-6) and 0.41% linalool (CAS 78-70-6). The quantification of the major volatile compounds (carvacrol and thymol) by external standard showed a hydrolate concentration of 24.32 µg/mlof carvacrol and 1.05 µg/ml of thymol. Carvacrol (2-methyl-5-isopropylphenol) and thymol (2-isopropyl-5-methylphenol), both monoterpenoid phenols, are very common in EOs. Although the composition of hydrosols can vary based on plant part, different plant growth stages, geographical

locations, or under changing management practices, these results are in line with what is found in an Italian variety of *S. montana* with the same technique of characterisation (Di Vito et al., 2021).

On the other hand, Table 1 shows the results estimated by COSMO-RS for the soil adsorption coefficient, log K_{oc}. Boiling temperature, water solubility, vapour pressure, and octanol-water partition coefficient were also included together with the values found in the literature. The concordance between both data series is adequate. It should be noted that good correspondence was found for the octanol-water partition coefficients.

The presence of these two monoterpenes in the hydrolate suggests that *S. montana* hydrolate can be considered an interesting bioactive and a very cheap EO distillation product. It can become the main distillation product, rather than a by-product, with the advantage in addition to recovering a waste. In fact, Di Vito (Di Vito et al., 2021) found that hydrolates were more effective at inhibiting microbial growth than the EO from the same plant. In thymol-rich hydrolates, the hydrolate appeared to be more cytocidal or cytostatic than the EO, probably due to the hydrophilic behaviour of the hydrolate, which improves the bioavailability of terpene (Di Vito et al., 2019).

Growing evidence shows powerful pharmacological activities of both carvacrol and thymol (Lombrea et al., 2020). Their bactericidal properties are well known (Walczak et al., 2021; Badawy et al., 2019; Garcia-Salinas et al., 2018), as well as their antifungal (Zhang et al., 2019) and antiparasitic properties (Seo et al., 2012; Giannenas et al., 2003; Kordali et al., 2008). Antitumoral and anti-inflammatory activities among others are also being detected (Silva et al., 2012, Elshafie et al., 2017). However, very little is known about their ecotoxicity effects on non-target aquatic organisms and there is no information on soil ecotoxicity. In this study the ecotoxicity of the hydrolate of *S. montana* has been studied for the first time.

3.2 Ecotoxicity of *Satureja montana* hydrolate

The hydrolate reveals to have a very important bioactivity against a very different series of non-target organisms. The results also show a very clear dose-dependent relationship, and the effects appear with exposures to very small percentages of hydrolate, ranging from 0.05% to 4.3%. From highest to lowest effect considering the LC_{50} values obtained: *A. Cepa* > *D.magna* > *V. fisheri* > periphyton > *E. fetida.*

3.2.1 Effects of *Satureja montana* hydrolate on immobilisation of *Daphnia magna*

286 The effect of the hydrolate on the survival of *D. magna* after a 24 h exposure can be seen in Figure
287 1. The results show a clear dose-response curve. The LC_{50} value was 0.43% (SE interval of 0.66–
288 0.27) and LC_{10} value (Lethal Concentration 10) was 0.07% (SE interval of 0.13–0.02). The chi-square
289 test showed good significance ($P < 0.0001$).
290 The mechanism of action of *S. montana* hydrolate on aquatic invertebrates has not been studied as
291 far as we know. However, the ranges of toxicity in *D. magna* values are similar to those described
292 for other hydrolates, such as *A. absinthium* hydrolate with a LC_{50} value of 0.24% (Pino-Otin et al.,
293 2019a).
294 The high toxicity of this hydrolate on *D. magna* could lie in the mechanism of action of its
295 components, mainly carvacrol and thymol. For example, EOs whose main composition is carvacrol
296 and thymol have been shown to be highly bioactive (Lombrea et al., 2020). In fact, ECHA states for
297 carvacrol the 48h EC_{50} value (Effective Concentration 50) for *D. magna* is 8.74 (7.36–11.79) mg/L
298 and thymol has a LC_{50} value (48 h) of 5.94 (5.45–6.53) mg/L on *D. magna* (Seo et al., 2012).
299 Carvacrol is a monoterpenoid with a low molecular weight of 150.22 g/mol (PubChem release
300 2021.05.07) and low water solubility (see Table 1). It is a weak acid (based on its pKa = 10.38), so
301 it will poorly ioinized. The most predominant forms in water will be the non-ionising ones because
302 are more liposoluble, so they may be more available to cross biological membranes. Moreover,
303 carvacrol has a $logK_{(octanol/water)}$ (see Table 1) which makes it have a medium-high solubility through
304 cell membranes (Zarybnicky et al., 2018). Therefore, carvacrol likely can easily access *Daphnia*
305 organisms through its body. *D.magna* is also a filter feeder that can strain microscopic food particles
306 from the water.
307 Literature focused on the bactericidal activity of carvacrol and thymol and showed that these
308 mononterpenes cause changes in the permeability of the bacterial cell membrane because they are
309 capable of producing functional and structural damage (Sikkema et al., 1995; Lambert et al., 2001)
310 so this behaviour is expected to be similar in the cell membranes of protists. For example, the effect
311 of *Origanum vulgare L.* EO on several parasitic protozoa (Giannenas et al., 2003; Santoro et al.,
312 2007) and other protists (Gaur et al., 2018) may be due to the presence of these phenolic compounds
313 (thymol and carvacrol) that interact with the permeability of the cytoplasmic cell membrane. This EO
314 seemed also to have an antiparasitic effect against intestinal parasitic flatworms (Pensel et al., 2014).

Thymol, although to a lesser extent in the hydrolate, can also play a role in bioactivity since it has similar properties to carvacrol as an isomer. Thymol also has low water solubility and is a weak acid with a very similar pKa (10.59) and logK to carvacrol (see Table 1), so similar effects on cell membranes are expected. According to Lambert (Lambert et al., 2001), thymol ties to membrane proteins and increases the permeability of the bacterial cell membrane.

On the other hand, the combination of both monoterpenes can present a synergistic action (Bouhtit et al., 2021) which would explain these intense effects of the hydrolate not only on *D. magna* but on all of the bioindicators studied. The rest of the volatile components identified, although in a much smaller proportion, can also make a contribution that cannot be ruled out.

3.2.2. Effects of *Satureja montana* hydrolate on *Vibrio fisheri* light production

Figure 2 shows the decrease in bioluminescence of *V. fisheri* when exposed to Satureja hydrolate. The dose-response values showed a very good significance (*P* <0.0001). The LC_{50} was 0.58% (0.62–0.54) and the LC_{10} was 0.10% (0.12–0.09) of the hydrolate dilution.

The survival of the marine bacterium *Vibrio fisheri* is affected at very low concentrations of hydrolate exposure, actually quite minor to those described for other hydrolates, such as *A. absinthium* hydrolate with a LC_{50} value of 1.85% (Pino-Otin et al., 2019a). As far as we know, there are no precedents in the literature for the effect of this hydrolate or its two main components on this bacterium.

However, the bactericidal effects of carvacrol and thymol (or EOs rich in them) on other gram-negative bacteria have been widely described (Chung et al., 2018; Gomez-Sequeda et al., 2020; Porter and Monu 2019; Osaili et al., 2021; Palaniappan and Holley, 2010). It is well documented that carvacrol (Ait-Ouazzou et al., 2013; Sikkema et al., 1995) and thymol (Trombetta et al., 2005) would damage the bacterial cytoplasmic membrane producing its structural disorganisation leading to a failure in cellular permeability. The combination of both monoterpenes showed additive effects, improving the rapidity of the antimicrobial action (Iten et al., 2009; Zhou et al., 2007; Netopilova et al., 2018; Gutierrez-Fernandez et al., 2013), which would help explain the great effect of the hydrolate on *V. fisheri.*

3.2.3 Effects of *Satureja montana* hydrolate on *Eisenia fetida* mortality

The dose-response curve of the earthworm *E. fetida* after 14 days of exposure to the hydrolate of *S. montana* can be seen in Figure 3. The hydrolate causes mortality in *E. fetida* with LC_{50} = 4.25% (SE interval of 6.79–2.51) and a LC_{10} value of 0.34 % (SE interval of 0.69–0.11) of hydrolate ($P < 0.0001$). The effect of the hydrolates on the earthworm *E. fetida* is remarkable, although it is the least affected organism of those analysed in this study. Regarding the effect of other hydrolates reported in the literature on *E. fetida*, it is the one that has the greatest effect on this earthworm, above the hydrolate of *Artemisia absinthium* (Pino-Otin et al., 2019c) and much more than that of *Lavandula luisieri* (Pino-Otin et al., 2019b). To our knowledge, there are no other studies of hydrolate ecotoxicity on *E. fetida* in the literature.

It is surprising that toxicity of several EOs rich in carvacrol (Benelli et al., 2019b), thymol (Benelli et al., 2019c), or both (Pavela et al., 2020; Benelli et al., 2019a) was minimal, leading to no or very low mortality on *E. fetida*. This suggests that these monoterpenes have more bioavailability in the liquid phase of the hydrolate than in the oil.

Carvacrol is a compound not easily biodegradable (ECHA). This fact, together with the values of log Koc and log Kow, indicates that carvacrol is one that potentially meets the M (Mobile) or vM (very Mobile) UBA criteria.

The hydrolate applied to the soil during the test allows *E. fetida* to be exposed to these monoterpenes by ingesting the impregnated soil particles (Suthar et al., 2008). However, the main route of exposure is probably the contact of the hydrosol with the worm's body. The biochemical composition of the earthworm cuticle is well known and it is highly tolerant of water absorption and loss (Wallwork, 1983), so high water permeability through the body wall can occur (Laverack, 1963). Contaminants present in pore water will therefore be available to earthworms through dermal absorption (Vijver et al., 2003). Therefore, the greater risk of ecotoxicity of hydrolates compared with EOs, in terrestrial environments due to their greater bioavailability, is an aspect to take into account.

3.2.4 Effects of *Satureja montana* hydrolate on *Allium cepa* roots elongation

Figure 4 shows the inhibition of the elongation of the roots of *A. cepa* after 72 h of exposure to the hydrolate. The hydrolate of *S. montana* has a high phytotoxicity against *A. cepa*, being the most sensitive organism of those analysed in this study. As can be seen, a strong dose-response effect

371 is produced with an EC_{50} value of 0.05 (SE interval of 0.056–0.046) and an EC_{10} value of 0.013 (SE
372 interval of 0.015–0.011) of the dilution ($P < 0.0001$).
373 Among the hydrolates tested in the literature, *A. abshintium* with an LC_{50} of 3.87% (Pino-Otin et al.,
374 2019c) and *L. luisieri* with a LC_{50} value of 2.2% (Pino-Otin et al., 2019b), the hydrolate of *S. montana*
375 is the one with the greatest effect on *A. cepa* by far (LC_{50} = 0.05%).
376 Although there are no other studies on hydrolates, studies of EOs rich in carvacrol and thymol have
377 reported an important activity on the germination of *A. cepa*. For example, the *Origanum vulgare L.*
378 EO affects the allelopathic and mitotic activity of *A. cepa* on its root elongation **(**Dra**g**oeva et al.,
379 2008, Grondona et al., 2014). Also, phytotoxicity of thymol affects the root length and germination of
380 *A. cepa* (Mattos et al., 2019).
381 Essential oils isolated from other Lamiaceas with carvacrol and thymol as main constituents inhibited
382 the seedling growth and seed germination of *Amaranthus retroflexus L.*, *Chenopodium album L.,*
383 and *Rumex crispus L.* (Kordali et al., 2008). In addition, carvacrol and thymol exhibit genotoxic,
384 cytotoxic and phytotoxic activities in seeds of *Lactuca sativa L.* and *Sorghum bicolor* L. (Alves et al.,
385 2018).
386 Regarding mechanisms of action, carvacrol is clastogenic, causing damage to the DNA and inciting
387 membrane leakage, but it has not had a detectable effect on cell microtubules (Chaimovitsh et al.,
388 2017; Alves et al., 2018). Recently, Araniti (Araniti et al., 2020) proposed that thymol-induced
389 phytotoxicity could be related to a combined oxidative and osmotic stress that resulted in reduced
390 plant development. In addition, thymol presents aneugenic and clastogenic mechanisms of action,
391 promoting damage to the DNA and the mitotic spindle (Alves et al., 2018).
392 Therefore, *S. montana* hydrolate can combine the sets of actions of its two main components, which
393 would explain its high phytotoxicity on *A. cepa*. Although no specific synergistic effects of the
394 combination of carvacrol and thymol have been reported for phytotoxicity or herbicidal effect, there
395 are studies that suggest that both products can act synergistically, increasing the effect of
396 phytotoxicity when combined with other components of EOs (Koiou et al., 2020; Vasilakoglou et al.,
397 2013).

398 **3.3 Mesocosms experiments on river periphyton**

In addition to studying the effects of hydrolate toxicity on individual indicator organisms, more complex models are needed that incorporate complete natural communities of organisms that are representative of the ecosystem to be studied, in this case the river. These complex models allow the study of effects with greater environmental realism.

The periphyton is an underwater substratum composed by a complex community of both autotrophic and heterotrophic organisms. Fungi, bacteria, protozoa, algae and invertebrates develop a community that functions as an autonomous ecosystem (Seguin et al., 2005; Cohu, 2001).

3.3.1 Taxonomic study of periphyton

The taxonomic identification of the periphyton shows a rich diversity of diatoms that make up almost the entire sample (99% indiv/cm^2). Diatoms are very frequent in small rivers (Schagerl and Donabaum, 1998). Periphytic diatomic populations have been used as indicators of water system quality (Lecointe et al. 1993; Vinebrooke and Graham, 1997). They recorded changes over time by being exposed to water conditions, so they are excellent indicators of ecotoxicity (Sabater et al., 2007). The periphyton of our samples also contains some Chlorophyceae and Ulvophyceae (0.01% and 0.34% indiv/cm^2 respectively). Furthermore, a small fraction of cyanobacteria (0.64% indiv/cm^2) has been identified (Figure 5).

Among the diatoms—all belonging to the Bacillariophyceae class—the most abundant genus is *Achnanthidium (Cocconeidales order)*, which has a cosmopolitan distribution and represents more than 50% of the total diatoms in our samples. *A. pyrenaicum* (Hustedt) Kobayasi, *A. minutissimum* (Kützing) Czarnecki, *A. eutrophilum* (Lange-Bertalot) Lange-Bertalot (33, 20 and 4% of the total Bacillariophyceae respectively) are the species identified. Bacillariophyta are very abundant in aquatic ecosystems (Isakova and Veisberg, 2019).

The second order of diatoms in abundance was *Nitzschia* (Bacillariales order). Nitzschia is a common pennate diatom whicμh includes several species of diatoms with similar morphology and found mostly in colder water. The genus found were: *N. inconspicua* Grunow (8% of the total Bacillariophyceae), *N. microcephala* Grunow in Cleve & Möller (5%), *N. amphibia* Grunow (2%), and *N. fonticola* Grunow in Cleve & Möller (1%).

Other diatoms found in a proportion greater than 1% were: *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot (6%); *Staurosira venter* (Ehrenberg) Cleve & Möller (4%); and *Gomphonema saprophilum* (Lange-Bertalot & Reichardt) Abarca Jahn Zimmermann & Enke (2%).

3.3.2. Effects of Satureja montana hydrolatel on river periphyton

The individual organisms of these communities probably have a certain degree of protection as part of the community, and together with *E. fetida*, fluvial periphyton is the least affected by *S. montana* hydrolate among the organisms tested in this study; however, LC_{50} values are also low.

The changes in the photosynthetic yield of river periphyton after 2 h of exposure to hydrolate can be seen in Figure 6. A very clear dose-effect response can be appreciated.

The LC_{50} value was 4.23% (SE interval of 4.83–3.66) and the LC_{10} value was 0.50% (SE interval of 0.38–0.65) ($P < 0.0001$).

To the best of our knowledge, this is the first time that hydrolate ecotoxicity has been studied in a periphyton community. Some studies focus on the effect of EOs, most of them with carvacrol or thymol as main constituent, on algae or cyanobacteria from biofilms from wall surfaces can be found. These studies showed that these compounds inhibited the photosynthetic activities of the cyanobacteria or algae (Bruno et al., 2019; Genova et al., 2020; Candela et al., 2019).

The effect of thymol or carvacrol on river periphyton is unknow, but species of algae isolated from biofilms formed on deteriorated painted surfaces exposed to different concentrations of thymol undergo total inhibition of algae growth (de Saravia et al., 2018). Among these algae, Chroococcales (Cyanophyta) are predominant, which were also present in our samples. However, in those cases in which wall surfaces biofilms taxonomy has been analysed, the composition of taxa is very different from that of river biofilms. We have not been able to find any study on the effect of hydrolates, carvacrol, or thymol on diatoms, which are the most abundant photosynthesising organisms in our sample.

Unicellular green alga, *Pseudokirchneriella subcapitata,* was exposed to p-thymol, and an EC_{50} = 7.4 mg/L was found (Tamura et al., 2013), but the behavior of the individual organism probably differs from what it would have as part of the periphyton, so it is not possible to compare.

In order to have some reference value regarding the effect of natural products on the periphyton, citronellol affects photosynthesis of the fluvial periphyton at a LC_{50} value of 94.10 mg/L, therefore, being less toxic than *S. montana* hydrolate (Pino-Otin et al., 2021).

Finally, the mode of action of *S. montana* hydrolate on periphyton must be sought in the interaction of carvacrol and thymol with the cell membrane, causing alteration of the lipid bilayer (Ben Arfa et al., 2006) and its possible synergistic effect.

3.3 Environmental relevance

Hydrolates can become a good alternative to synthetic products with a wide range of uses (D'Amato et al., 2018). However, the powerful bioactivity of *S. montana* hydrolate on a wide range of non-target organisms, as well as other hydrolates studied, points out that the use of these products is not free of environmental risks and their release to the environment should be evaluated.

Our results suggest that the hydrolate of *S. montana* could affect the delicate network of trophic interactions in river ecosystems of both producers (periphyton) and consumers (*D. magna*) with important functions in freshwater food webs. Changes in herbivore-producer interactions (Relyea and Hoverman, 2006) or in interspecific competition due to differential sensitivity of competitors to hydrolate constituents (Relyea, 2006; Fairchild and Eidt, 1993) would be expected.

On the other hand, *S. montana* hydrolate can affect the soil functions affecting earthworms. Earthworms are connected to the chemical and physical processes of the terrestrial environment, being at the base of the trophic chains. *Eisenia fetida* is involved in nutrient cycling ,moisture content, soil aeration, and overall soil structure, so their alteration can lead to a physical and chemical changes in the dynamics of the terrestrial environment (Cortez and Bouche, 1992). Furthermore, our results show the hydrolate of *S. montana* as a potent phytotoxic agent, which is accumulated in soils and can also affect the yield of plantations. This phytotoxicity, however, opens up interesting possibilities for the use of this hydrolate as a bio-herbicide.

We believe that our results should be taken into consideration for future applications of hydrolates as alternatives to products of non-natural origin in a safe way for the environment. Future research should focus on determining the biodegradability and persistence of hydrolates in the environment, predictably inferior to synthetic products, although so far nothing is known with certainty in this

regard. Hydrolates seem to have a lower capacity to generate resistances ((Lewis and Ausubel, 2006) which opens up important expectations for new safe applications.

Hydrolates seem to have a lower capacity to generate resistances (Lewis and Ausubel, 2006) which offers new possibilities for safe applications.

4. Conclusions

The properties of *S. montana* hydrolate are beginning to be described; however, this is the first study to analyse the ecotoxicity of this hydrolate.

The characterization of the hydrolate of *S. montana* showed that carvacrol and thymol were the main volatile components whose bioactivity and pharmacological properties have been widely described both individually and in synergy.

The *S. montana* hydrolate has a high ecotoxicity with very low percentages against a very different series of non-target soil and water organisms with a clear dose-dependent relationship. Among aquatic organisms, *D. magna* and *V. fisheri* are especially sensitive with LC_{50} < 1% of hydrolate. The fluvial periphyton, complex communities with a higher degree of resistance to disturbances, are also sensitive to hydrolate. In soil organisms, although *E. fetida* is somewhat more resistant, the strong phytotoxic effect on *A. cepa* is worth noting, which makes it possible to explore its properties as a bio-herbicide.

This study indicates that although hydrolates can become a very interesting option to replace synthetics in many applications, they are not harmless for the environment and their release should be controlled.

Acknowledgements

The authors thank the financial support of Gobierno de Aragón: Departamento de Ciencia, Universidad y Sociedad del Conocimiento (Group E39_20R) and Cátedra NOVALTIA.

The authors would like to thank Dr. María Ángeles Sanz García for her collaboration in the characterization of the hydrolate and in the interpretation of the results.

REFERENCES

Ait-Ouazzou, A., Espina, L., Gelaw, T.K., de Lamo-Castellvi, S., Pagan, R., Garcia-Gonzalo, D., 2013. New insights in mechanisms of bacterial inactivation by carvacrol. *J. Appl. Microbiol.* 114, 173-185. http://dx.doi.org/10.1111/jam.12028.

Alves, T.D., Pinheiro, P.F., Praca-Fontes, M.M., Andrade-Vieira, L.F., Correa, K.B., da Cruz, F.A., Lacerda, V., Ferreira, A., Soares, T.C.B., 2018. Toxicity of thymol, carvacrol and their respective phenoxyacetic acids in *Lactuca sativa* and *Sorghum bicolor*. *Ind. Crop and Prod.* 114, 59-67. http://dx.doi.org/10.1016/j.indcrop.2018.01.071.

Andres, M.F., Gonzalez-Coloma, A., Munoz, R., De la Pena, F., Julio, L.F., Burillo, J., 2018. Nematicidal potential of hydrolates from the semi industrial vapor-pressure extraction of Spanish aromatic plants. *Environ. Sci. and Pollut* Research 25, 29834-29840. http://dx.doi.org/10.1007/s11356-017-9429-z.

Badawy, M.E.I., Marei, G.I.K., Rabea, E.I., Taktak, N.E.M., 2019. Antimicrobial and antioxidant activities of hydrocarbon and oxygenated monoterpenes against some foodborne pathogens through in vitro and in silico studies. *Pestic. Biochem. Phys.* 158, 185-200. http://dx.doi.org/10.1016/j.pestbp.2019.05.008.

Benelli, G., Pavela, R., Maggi, F., Wandjou, J.G.N., Fofie, N., Kone-Bamba, D., Sagratini, G., Vittori, S., Caprioli, G., 2019a. Insecticidal activity of the essential oil and polar extracts from *Ocimum gratissimum* grown in Ivory Coast: Efficacy on insect pests and vectors and impact on non-target species. *Ind. Crop. Prod.* 132, 377-385. http://dx.doi.org/10.1016/j.indcrop.2019.02.047.

Benelli, G., Pavela, R., Petrelli, R., Cappellacci, L., Bartolucci, F., Canale, A., Maggi, F., 2019b. Origanum syriacum subsp. syriacum: From an ingredient of Lebanese 'manoushe' to a source of effective and eco-friendly botanical insecticides. *Ind. Crop. Prod.* 134, 26-32. http://dx.doi.org/10.1016/j.indcrop.2019.03.055.

534 Benelli, G., Pavela, R., Zorzetto, C., Sanchez-Mateo, C.C., Santini, G., Canale, A., Maggi, F., 2019c.
535 Insecticidal activity of the essential oil from *Schizogyne sericea* (Asteraceae) on four insect pests
536 and two non-target species. *Entomol. General.* 39, 9-18.
537 http://dx.doi.org/10.1016/j.indcrop.2019.03.055.

538 Bouhtit, F., Najar, M., Agha, D.M., Melki, R., Najimi, M., Sadki, K., Boukhatem, N., Bron, D.,
539 Meuleman, N., Hamal, A., Lagneaux, L., Lewalle, P., Merimi, M., 2021. New anti-leukemic effect of
540 carvacrol and thymol combination through synergistic induction of different cell death pathways.
541 *Molecules* 26. http://dx.doi.org/10.3390/Molecules26020410.

542 Boyraz, N., Ozcan, M., 2006. Inhibition of phytopathogenic fungi by essential oil, hydrosol, ground
543 material and extract of summer savory (*Satureja hortensis* L.) growing wild in Turkey. *Int. J. Food.*
544 *Microbiol.* 107, 238-242. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.10.002.

545 Bruno, L., Rugnini, L., Spizzichino, V., Caneve, L., Canini, A., Ellwood, N.T.W., 2019.
546 Biodeterioration of Roman hypogea: the case study of the Catacombs of SS. Marcellino and Pietro
547 (Rome, Italy). *Ann Microbiol* 69, 1023-1032. http://dx.doi.org/10.1007/s13213-019-01460-z.

548 Cabana, R., Silva, L.R., Valentao, P., Viturro, C.I., Andrade, P.B., 2013. Effect of different extraction
549 methodologies on the recovery of bioactive metabolites from *Satureja parvifolia* (Phil.) Epling
550 (Lamiaceae). *Ind Crop. Prod.* 48, 49-56. http://dx.doi.org/10.1016/j.indcrop.2013.04.003.

551 Candela, R.G., Maggi, F., Lazzara, G., Rosselli, S., Bruno, M., 2019. The essential oil of T*hymbra*
552 *capitata* and its application as a biocide on stone and derived surfaces. *Plants-Basel* 8.
553 http://dx.doi.org/10.3390/plants8090300.

554 Caprioli, G., Lupidi, G., Maggi, F., 2019. Comparison of chemical composition and antioxidant
555 activities of two Winter savory subspecies (*Satureja montana* subsp. variegata and *Satureja*
556 *montana* subsp. montana) cultivated in Northern Italy. *Nat. Prod Res.* 33, 3143-3147.
557 http://dx.doi.org/10.1080/14786419.2018.1516661.

558 Chaimovitsh, D., Shachter, A., Abu-Abied, M., Rubin, B., Sadot, E., Dudai, N., 2017. Herbicidal
559 activity of monoterpenes is associated with disruption of microtubule functionality and membrane
560 Integrity. *Weed. Sc.* 65, 19-30. http://dx.doi.org/10.1614/ws-d-16-00044.1.

561 Chung, D., Cho, T.J., Rhee, M.S., 2018. Citrus fruit extracts with carvacrol and thymol eliminated 7-
562 log acid-adapted *Escherichia coli* 0157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes*:
563 A potential of effective natural antibacterial agents. *Food. Res. Int.* 107, 578-588.
564 http://dx.doi.org/10.1016/j.foodres.2018.03.011.

565 Consalvey, M., Perkins, R.G., Paterson, D.M., Underwood, G.J.C., 2005. Pam fluorescence: A
566 beginners guide for benthic diatomists. *Diatom. Res* 20, 1-22.

567 Cortez, J., Bouche, M.B., 1992. Do earthworms eat living roots. *Soil. Biol. Biochem.* 24, 913-915.

568 Chizzola, R. Billiani, F. Singer, S. Novak, J. 2021.Diversity of Essential Oils and the Respective
569 Hydrolates Obtained from Three Pinus cembra Populations in the Austrian Alps. *Appl. Sci.* 11, 5686.
570 https://doi.org/10.3390/app11125686

571 D'Amato, S., Serio, A., Lopez, C.C., Paparella, A., 2018. Hydrosols: Biological activity and potential
572 as antimicrobials for food applications. *Food Control* 86, 126-137.
573 http://dx.doi.org/10.1016/j.foodcont.2017.10.030.

574 da Silva, H.N.P., Sousa, E.M.D., Maia, J.L.D., Pinheiro, M.T.L., Lameirao, S., Mourao, R.H.V., Maia,
575 J.G.S., Baldisserotto, B., da Silva, L.V.F., 2018. *Lippia alba* (Verbenaceae) hydrolate as sedative of
576 tambaqui (*Colossoma macropomum*) juveniles in simulated transport conditions. *Aquac. Res.* 49,
577 128-134. http://dx.doi.org/10.1111/are.13441.

578 de Saravia, S.G.G., Rastelli, S.E., Blustein, G., Viera, M.R., 2018. Natural compounds as potential
579 algaecides for waterborne paints. *Coat. Technol. Res.* 15, 1191-1200.
580 http://dx.doi.org/10.1007/s11998-018-0099-7.

581 Di Vito, M., Bellardi, M.G., Mondello, F., Modesto, M., Michelozzi, M., Bugli, F., Sanguinetti, M.,
582 Sclocchi, M.C., Sebastiani, M.L., Biffi, S., Barbanti, L., Mattarelli, P., 2019. *Monarda citriodora*
583 hydrolate vs essential oil comparison in several anti-microbial applications. *Ind. Crop. Prod.* 128,
584 206-212. http://dx.doi.org/10.1016/j.indcrop.2018.11.007.

585 Di Vito, M., Smolka, A., Proto, M.R., Barbanti, L., Gelmini, F., Napoli, E., Bellardi, M.G., Mattarelli,
586 P., Beretta, G., Sanguinetti, M., Bugli, F., 2021. Is the antimicrobial activity of hydrolates lower than
587 that of essential oils? *Antibiotics-Basel* 10. http://dx.doi.org/10.3390/antibiotics10010088.

588 Dragoeva, A.P., Nanova, Z.D., Kalcheva, V.K., 2008. Allelopathic activity of micropropagated
589 *Origanum vulgare* ssp hirtum and its effect on mitotic activity. *Allelopathy J.* 22, 131-142.

590 Eckert, F., Klamt, A., 2002. Fast solvent screening via quantum chemistry: COSMO-RS approach.
591 *Aiche J.* 48, 369-385. http://dx.doi.org/10.1002/aic.690480220.

592 Elshafie, H.S., Armentano, M.F., Carmosino, M., Bufo, S.A., De Feo, V., Camele, I., 2017. Cytotoxic
593 activity of *Origanum vulgare* L. on hepatocellular carcinoma cell liine HepG2 and evaluation of its
594 biological activity. *Molecules* 22. http://dx.doi.org/10.3390/Molecules22091435.

595 Fairchild, W.L., Eidt, D.C., 1993. Perturbationfo the aquatic invertebrate community of acidic bog
596 ponds by the insecticide Fenitrothion. *Arch. Environ. Con. Tox.* 25, 170-183.

597 Faria, J.M.S., Rodrigues, A.M., Sena, I., Moiteiro, C., Bennett, R.N., Mota, M., Figueiredo, A.C.,
598 2016. Bioactivity of *Ruta graveolens* and *Satureja montana* essential oils on *Solanum tuberosum*
599 hairy roots and *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures. *J. Agr. Food.*
600 *Chem.* 64, 7452-7458.

601 Fiskesjö, G. The allium test in wastewater monitoring. 1993. Envir. Tox. and Water Quality 8, 291-
602 298.

603 Franzener, G., Martinez-Franzener, A.D., Stangarlin, J.R., Czepak, M.P., Freitas Schwan-Estrada,
604 K.R., Silva Cruz, M.E., 2007. Antibacterial, antifungal and phytoalexins induction activities of
605 hydrolates of medicinal plants. *Semin-Cienc Agrar.* 28, 29-38.

606 Garcia-Salinas, S., Elizondo-Castillo, H., Arruebo, M., Mendoza, G., Irusta, S., 2018. Evaluation of
607 the antimicrobial activity and cytotoxicity of different components of natural origin present in essential
608 oils. *Molecules* 23. http://dx.doi.org/10.3390/Molecules23061399.

609 Gaur, S., Kuhlenschmidt, T.B., Kuhlenschmidt, M.S., Andrade, J.E., 2018. Effect of oregano
610 essential oil and carvacrol on *Cryptosporidium parvum* infectivity in HCT-8 Cells. *Parasitol. Int.* 67,
611 170-175. http://dx.doi.org/10.1016/j.parint.2017.11.001.

612 Genova, C., Fuentes, E., Sanmartin, P., Favero, G., Prieto, B., 2020. Phytochemical compounds as
613 cleaning agents on granite colonized by phototrophic subaerial biofilms. *Coatings* 10.
614 http://dx.doi.org/10.3390/COATINGS10030295.

615 Giannenas, I., Florou-Paneri, P., Papazahariadou, M., Christaki, E., Botsoglou, N.A., Spais, A.B.,
616 2003. Effect of dietary supplementation with oregano essential oil on performance of broilers after
617 experimental infection with *Eimeria tenella*. *Archi. Anim. Nutr.* 57, 99-106.
618 http://dx.doi.org/10.1080/0003942031000107299.

619 Gil, M.I., Selma, M.V., Lopez-Galvez, F., Allende, A., 2009. Fresh-cut product sanitation and wash
620 water disinfection: Problems and solutions. *Int. J. of Food. Microbiol.* 134, 37-45.
621 http://dx.doi.org/10.1016/j.ijfoodmicro.2009.05.021.

622 Gomez-Sequeda, N., Caceres, M., Stashenko, E.E., Hidalgo, W., Ortiz, C., 2020. Antimicrobial and
623 antibiofilm activities of essential oils against *Escherichia coli* O157:H7 and Methicillin-resistant
624 *Staphylococcus aureus* (MRSA). *Antibiotics-Basel* 9. http://dx.doi.org/10.3390/antibiotics9110730.

625 Grondona, E., Gatti, G., Lopez, A.G., Sanchez, L.R., Rivero, V., Pessah, O., Zunino, M.P., Ponce,
626 A.A., 2014. Bio-efficacy of the essential oil of oregano (*Origanum vulgare* Lamiaceae. Ssp Hirtum).
627 *Plant. Food. Hum. Nutr.* 69, 351-357. http://dx.doi.org/10.1007/s11130-014-0441-x.

628 Guiry, M.D., Guiry, G.M., 2021. AlgaeBase. World-wide electronic publication, National University of
629 Ireland, Galway.

630 Gulec, A.K., Altinterim, B., Aksu, O., 2013. Determination of lethal concentration (LC50) values of
631 *Cinnamomum zeylanicum* hydrosol on carp fish. *Iran J. of Fish. Sci.* 12, 34-44.

632 Gutierrez-Fernandez, J., Garcia-Armesto, M.R., Alvarez-Alonso, R., del Valle, R., de Arriaga, D.,
633 Rua, J., 2013. Antimicrobial activity of binary combinations of natural and synthetic phenolic
634 antioxidants against *Enterococcus faecalis*. *J. Dairy Sc.* 96, 4912-4920.
635 http://dx.doi.org/10.3168/jds.2013-6643.

636 Isakova, N.A., Veisberg, E.I., 2019. Factors determining the organization of periphyton community
637 bacillariophyta. *Inland Water Biol.* 12, 45-52. http://dx.doi.org/10.1134/s1995082919050092.

638 Iten, F., Saller, R., Abel, G., Reichling, J., 2009. Additive antimicrobial effects of the active
639 components of the essential oil of *Thymus vulgaris* - chemotype carvacrol. *Planta Med.* 75, 1231-
640 1236. http://dx.doi.org/10.1055/s-0029-1185541.

641 Jafari, F., Farmani, F., Zomorodian, K., Moein, M., Faridi, P., Zarshenas, M.M., 2018. A study on
642 essential oil chemical compositions, antioxidant, and antimicrobial activities of native and endemic
643 satureja species growing in Iran. *Pharm. Chem. J.* 52, 63-68. http://dx.doi.org/10.1007/s11094-018-
644 1766-9.

645 Klamt, A., Jonas, V., Burger, T., Lohrenz, J.C.W., 1998. Refinement and parametrization of COSMO-
646 RS. *J. Phys. Chem A* 102, 5074-5085. http://dx.doi.org/10.1021/jp980017s.

647 Klamt, A., Eckert, f., 2000. COSMO-RS: a novel and efficient method for the a priori prediction of
648 thermophysical data of liquids. *Fluid Phase Equilibria* 172, 43-72.

649 Klamt, A., F. Eckert f., Diedenhofen, m., 2002a. Prediction of soil sorption coefficients with a
650 conductor-like screening model for real solvents. *Environ. Toxicol. and Chemistry* 21, 2562-2566.

651 Klamt, A., F. Eckert, f., Hornig, m., Beck M. E., Burger, t., 2002b. Prediction of aqueous solubility of
652 drugs and pesticides with COSMO-RS. J. of Comput. *Chemistry* 23, 275-281.

653 Koiou, K., Vasilakoglou, I., Dhima, K., 2020. Herbicidal potential of lavender (*Lavandula angustifolia*
654 Mill.) essential oil components on bristly foxtail (*Setaria verticillata* (L.) P. Beauv.): comparison with
655 carvacrol, carvone, thymol and eugenol. *Arch. Biol. Sci.* 72, 223-231.
656 http://dx.doi.org/10.2298/abs200106016k.

657 Kordali, S., Cakir, A., Ozer, H., Cakmakci, R., Kesdek, M., Mete, E., 2008. Antifungal, phytotoxic and
658 insecticidal properties of essential oil isolated from Turkish Origanum acutidens and its three
659 components, carvacrol, thymol and p-cymene. *Bioresource Technol.* 99, 8788-8795.
660 http://dx.doi.org/10.1016/j.biortech.2008.04.048.

661 Labadie, C., Ginies, C., Guinebretiere, M.H., Renard, C., Cerutti, C., Carlin, F., 2015. Hydrosols of
662 orange blossom (*Citrus aurantium*), and rose flower (*Rosa damascena and Rosa centifolia*) support
663 the growth of a heterogeneous spoilage microbiota. *Food Res. Int.* 76, 576-586.
664 http://dx.doi.org/10.1016/j.foodres.2015.07.014.

665 Lambert, R.J.W., Skandamis, P.N., Coote, P.J., Nychas, G.J.E., 2001. A study of the minimum
666 inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl*
667 *Microbiol.* 91, 453-462. http://dx.doi.org/10.1046/j.1365-2672.2001.01428.x.

668 Laverack, M.S., 1963. The Physiology of Earthworms, Pergamon Press, Oxford.

669 Lawless J., 2002. The Illustrated Encyclopedia of Essential Oils. Thorsons (London).

670 Lecointe, C., Coste, M., Prygiel, J., 1993. Omnidia-software for taxonomy, calculation of diatom
671 indexes and inventories management. *Hydrobiologia* 269, 509-513.
672 http://dx.doi.org/10.1007/bf00028048.

673 Lewis, K., Ausubel, F.M., 2006. Prospects for plant-derived antibacterials. *Nat. Biotechnol.* 24, 1504-
674 1507. http://dx.doi.org/10.1038/nbt1206-1504.

675 Lombrea, A., Antal, D., Ardelean, F., Avram, S., Pavel, I.Z., Vlaia, L., Mut, A.M., Diaconeasa, Z.,
676 Dehelean, C.A., Soica, C., Danciu, C., 2020. A Recent insight regarding the phytochemistry and
677 bioactivity of *Origanum vulgare* L. Essential Oil. *Int. J. Mol. Sci.* 21.
678 http://dx.doi.org/10.3390/ijms21249653.

679 Lopez-Cobo, A., Gomez-Caravaca, A.M., Svarc-Gajic, J., Segura-Carretero, A., Fernandez-
680 Gutierrez, A., 2015. Determination of phenolic compounds and antioxidant activity of a
681 Mediterranean plant: The case of *Satureja montana* subsp kitaibelii. *J. Funct. Food* 18, 1167-1178.
682 http://dx.doi.org/10.1016/j.jff.2014.10.023.

683 Maia, A.J., Schwan-Estrada, K.R.F., Faria, C., Jardinetti, V.D., Botelho, R.V., 2013. Bud break of
684 benitaka grapevines treated with *Gallesia integrifolia* hydrolate. *Rev. Bras. Frutic.* 35, 685-694.
685 http://dx.doi.org/10.1016/j.aquaculture.2018.11.058.

686 Maia, J.L.D., Sousa, E.M.D., da Silva, H.N.P., Pinheiro, M.T.L., Mourao, R.H.V., Maia, J.G.S.,
687 Lameirao, S., Baldisserotto, B., da Silva, L.V.F., 2019. Hydrolate toxicity of *Lippia alba* (Mill.) N. E.
688 Brown (Verbenaceae) in juvenile tambaqui (*Colossoma macropomum*) and its potential anaesthetic
689 properties. *Aquaculture* 503, 367-372. http://dx.doi.org/10.1016/j.aquaculture.2018.11.058.

690 Martinez-Lopez, J.F., Pardo, J.I., Urieta, J.S., Mainar, A.M., 2018. Excess molar enthalpies of R-
691 fenchone + butan-1-ol or + pentan-1-ol. Modeling with COSMO-RS and UNIFAC. *J. Chem.*
692 *Thermodyn.* 120, 13-20. http://dx.doi.org/10.1016/j.jct.2018.01.005.

693 Mattos, B.D., da Silva, L.R., de Souza, I.R., Magalhaese, W.L.E., Leme, D.M., 2019. Slow delivery
694 of biocide from nanostructured, microscaled, particles reduces its phytoxicity: A model investigation.
695 *J. Hazard. Mater.* 367, 513-519. http://dx.doi.org/10.1016/j.jhazmat.2018.12.117.

696 Navarro-Rocha, J., Andres, M.F., Diaz, C.E., Burillo, J., Gonzalez-Coloma, A., 2020. Composition
697 and biocidal properties of essential oil from pre-domesticated Spanish *Satureja Montana*. *Ind. Crop.*
698 *Prod.* 145. http://dx.doi.org/10.1016/j.indcrop.2019.111958.

699 Navarro, E., Guasch, H., Sabater, S., 2002. Use of microbenthic algal communities in
700 ecotoxicological tests for the assessment of water quality: the Ter river case study. *J. Appl. Phycol.*
701 14, 41-48. http://dx.doi.org/10.1023/a:1015242301451.

702 Netopilova, M., Houdkova, M., Rondevaldova, J., Kmet, V., Kokoska, L., 2018. Evaluation of in vitro
703 growth-inhibitory effect of carvacrol and thymol combination against *Staphylococcus aureus* in liquid
704 and vapour phase using new broth volatilization chequerboard method. *Fitoterapia* 129, 185-190.
705 http://dx.doi.org/10.1016/j.fitote.2018.07.002.

706 Ondov, B.D., Bergman, N.H., Phillippy, A.M., 2011. Interactive metagenomic visualization in a Web
707 browser. *Bmc Bioinformatics* 12. http://dx.doi.org/10.1186/1471-2105-12-385.

708 Osaili, T.M., Hasan, F., Dhanasekaran, D.K., Obaid, R.S., Al-Nabulsi, A.A., Ayyash, M., Karam, L.,
709 Savvaidis, I.N., Holley, R., 2021. Effect of active essential oils added to chicken tawook on the
710 behaviour of *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* 0157:H7 during storage.
711 *Int. J. Food. Microbiol.* 337. http://dx.doi.org/10.1016/j.ijfoodmicro.2020.108947.

712 Palaniappan, K., Holley, R.A., 2010. Use of natural antimicrobials to increase antibiotic susceptibility
713 of drug resistant bacteria. *Int. J. Food. Microbiol.* 140, 164-168.
714 http://dx.doi.org/10.1016/j.ijfoodmicro.2010.04.001.

715 Pangarkar, V.G., 2008. Microdistillation, thermodistillation and molecular distillation techniques.In;
716 Handa, S. S., Khanuja, S.P.S., Longo, G. and Rakesh, D.D. Eds., Extraction Technologies for
717 Medicinal and Aromatic Plants. United Nations Industrial Development Organization and
718 International Center for Science and High Technology, Trieste, Italy, 129-143.

719 Pavela, R., Morshedloo, M.R., Mumivand, H., Khorsand, G.J., Karami, A., Maggi, F., Desneux, N.,
720 Benelli, G., 2020. Phenolic monoterpene-rich essential oils from Apiaceae and Lamiaceae species:
721 insecticidal activity and safety evaluation on non-target earthworms. *Entomologia Generalis* 40, 421-
722 435. http://dx.doi.org/10.1127/entomologia/2020/1131.

723 Pensel, P.E., Maggiore M.A., Gende, L.B., Eguaras M.J., Denegri, M.G., Elissondo M.C., 2014.
724 Efficacy of Essential Oils of *Thymus vulgaris* and *Origanum vulgare* on *Echinococcus granulosus*.
725 *Interdiscip. Perspect. Infect. Dis.* 693289. http:// dx.doi.org /10.1155/2014/693289.

726 Pino-Otin, M.R., Ballestero, D., Navarro, E., Gonzalez-Coloma, A., Val, J., Mainar, A.M., 2019a.
727 Ecotoxicity of a novel biopesticide from *Artemisia absinthium* on non-target aquatic organisms.
728 *Chemosphere* 216, 131-146. http://dx.doi.org/10.1016/j.chemosphere.2018.09.071.

729 Pino-Otin, M.R., Langa, E., Val, J., Mainar, A.M., Ballestero, D., 2021. Impact of citronellol on river
730 and soil environments using non-target model organisms and natural populations. *J. Environ.*
731 *Manage.* 287. http://dx.doi.org/10.1016/j.jenvman.2021.112303.

732 Pino-Otin, M.R., Val, J., Ballestero, D., Navarro, E., Sanchez, E., Gonzalez-Coloma, A., Mainar,
733 A.M., 2019b. Ecotoxicity of a new biopesticide produced by *Lavandula luisieri* on non-target soil
734 organisms from different trophic levels. *Sci. Total Environ.* 671, 83-93.
735 http://dx.doi.org/10.1016/j.scitotenv.2019.03.293.

736 Pino-Otin, M.R., Val, J., Ballestero, D., Navarro, E., Sanchez, E., Mainar, A.M., 2019c. Impact of
737 *Artemisia absinthium* hydrolate extracts with nematicidal activity on non-target soil organisms of
738 different trophic levels. *Ecotox. Environ. Safe.* 180, 565-574.
739 http://dx.doi.org/10.1016/j.ecoenv.2019.05.055.

740 Politi, M., Menghini, L., Conti, B., Bedini, S., Farina, P., Cioni, P.L., Braca, A., De Leo, M., 2020.
741 Reconsidering hydrosols as main products of aromatic plants manufactory: The Lavandin
742 (Lavandula x intermedia) case study in Tuscany. *Molecules* 25.
743 http://dx.doi.org/10.3390/Molecules25092225.

744 Porter, J.A., Monu, E.A., 2019. Evaluating the antimicrobial efficacy of white mustard essential oil
745 alone and in combination with thymol and carvacrol against Salmonella. *J Food Protect.* 82, 2038-
746 2043. http://dx.doi.org/10.4315/0362-028x.jfp-19-029.

747 Prusinowska, R., Smigielski, K., Stobiecka, A., Kunicka-Styczynska, A., 2016. Hydrolates from
748 lavender (Lavandula angustifolia) - their chemical composition as well as aromatic, antimicrobial and
749 antioxidant properties. *Nat. Prod. Res.* 30, 386-393.
750 http://dx.doi.org/10.1080/14786419.2015.1016939.

751 Relyea, R., Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in
752 freshwater systems. *Ecol. Lett.* 9, 1157-1171. http://dx.doi.org/10.1111/j.1461-0248.2006.00966.x.

753 Relyea, R.A., 2006. The impact of insecticides and herbicides on the biodiversity and productivity of
754 aquatic communities - Response. *Ecol. Appl.* 16, 2027-2034. http://dx.doi.org/10.1890/1051-
755 0761(2006)016[2027:tioiah]2.0.co;2.

756 Sabater, S., Guasch, H., Ricart, M., Romani, A., Vidal, G., Klunder, C., Schmitt-Jansen, M., 2007.
757 Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Anal.*
758 *Bioanal. Chem.* 387, 1425-1434. http://dx.doi.org/10.1007/s00216-006-1051-8.

759 Saeidnia, S., Gohari, A.R., Manayi, A., Kourepaz-Mahmoodabadi, M., 2016. Satureja:
760 Ethnomedicine, Phytochemical Diversity and Pharmacological Activities. Springer Briefs in
761 *Pharmacol. and Toxicol.* Springer Cham Heidelberg New York Dordrecht London.
762 http://dx.doi.org/10.1007/978-3-319-25026-7_1

763 Sagdic, O., Ozturk, I., Tornuk, F., 2013. Inactivation of non-toxigenic and toxigenic *Escherichia coli*
764 O157:H7 inoculated on minimally processed tomatoes and cucumbers: Utilization of hydrosols of
765 Lamiaceae spices as natural food sanitizers. *Food Control* 30, 7-14.
766 http://dx.doi.org/10.1016/j.foodcont.2012.07.010.

767 Santoro, G.F., Cardoso, M.D.G., Guimaraes, L.G.L., Salgado, A., Menna-Barreto, R.F.S., Soares,
768 M.J., 2007. Effect of oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) essential oils
769 on *Trypanosoma cruzi* (Protozoa : Kinetoplastida) growth and ultrastructure. *Parasitol. Res.* 100,
770 783-790. http://dx.doi.org/10.1007/s00436-006-0326-5.

771 Schagerl, M., Donabaum, K., 1998. Epilithic algal communities on natural and artificial substrata in
772 the River Danube near Vienna (Austria). *Archiv Fur Hydrobiologie*, 153-165.

773 Seguin, F., Druart, J.C., Le Cohu, R., 2001. Effects of atrazine and nicosulfuron on periphytic diatom
774 communities in freshwater outdoor lentic mesocosms. *Ann. Limnol-Int. J. Lim.* 37, 3-8.
775 http://dx.doi.org/10.1051/limn/2001004.

776 Seo, S.M., Park, H.M., Park, I.K., 2012. Larvicidal Activity of Ajowan (Trachyspermum ammi) and
777 Peru Balsam (*Myroxylon pereira*) Oils and Blends of Their Constituents against Mosquito, *Aedes*
778 *aegypti*, Acute Toxicity on Water Flea, *Daphnia magna*, and Aqueous Residue. *J. Agr. Food. Chem.*
779 60, 5909-5914. http://dx.doi.org/10.1021/jf301296d.

780 Sikkema, J., Debont, J.A.M., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons.
781 *Microbiol. Rev.* 59, 201-222. http://dx.doi.org/10.1128/mmbr.59.2.201-222.1995.

782 Silva, F.V., Guimaraes, A.G., Silva, E.R.S., Sousa-Neto, B.P., Machado, F.D.F., Quintans, L.J.,
783 Arcanjo, D.D.R., Oliveira, F.A., Oliveira, R.C.M., 2012. Anti-Inflammatory and anti-ulcer activities of
784 carvacrol, a monoterpene present in the essential oil of oregano. *J. Med. Food* 15, 984-991.
785 http://dx.doi.org/10.1089/jmf.2012.0102.

786 Suthar, S., Singh, S., Dhawan, S., 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb
787 and Cd) in soils: Is metal bioaccumulation affected by their ecological category? *Ecol. Eng.* 32, 99-
788 107. http://dx.doi.org/10.1016/j.ecoleng.2007.10.003.

789 Tamura, I., Kagota, K., Yasuda, Y., Yoneda, S., Morita, J., Nakada, N., Kameda, Y., Kimura, K.,
790 Tatarazako, N., Yamamoto, H., 2013. Ecotoxicity and screening level ecotoxicological risk
791 assessment of five antimicrobial agents: triclosan, triclocarban, resorcinol, phenoxyethanol and p-
792 thymol. J. of Applied Toxicol. 33, 1222-1229.

793 Tepe, B., 2015. Inhibitory Effect of Satureja on Certain Types of Organisms. *Rec. Nat. Prod.* 9, 1-
794 18. http://dx.doi.org/10.1002/jat.2771.

795 Tepe, B., Cilkiz, M., 2016. A pharmacological and phytochemical overview on Satureja. *Pharm. Biol.*
796 54, 375-412. http://dx.doi.org/10.3109/13880209.2015.1043560.

797 Tornuk, F., Cankurt, H., Ozturk, I., Sagdic, O., Bayram, O., Yetim, H., 2011. Efficacy of various plant
798 hydrosols as natural food sanitizers in reducing *Escherichia coli* O157:H7 and *Salmonella*
799 *Typhimurium* on fresh cut carrots and apples. *Int. J. Food Microbiol.* 148, 30-35.
800 http://dx.doi.org/10.1016/j.ijfoodmicro.2011.04.022.

801 Trombetta, D., Castelli, F., Sarpietro, M.G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti,
802 G., Bisignano, G., 2005. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob.*
803 *Agents. Ch.* 49, 2474-2478. http://dx.doi.org/10.1128/aac.49.6.2474-2478.2005.

804 Vasilakoglou, I., Dhima, K., Paschalidis, K., Ritzoulis, C., 2013. Herbicidal potential on *Lolium*
805 *rigidum* of nineteen major essential oil components and their synergy. *J. Essent. Oil Res.* 25, 1-10.
806 http://dx.doi.org/10.1080/10412905.2012.751054.

807 Vijver, M.G., Vink, J.P.M., Miermans, C.J.H., van Gestel, C.A.M., 2003. Oral sealing using glue: a
808 new method to distinguish between intestinal and dermal uptake of metals in earthworms. *Soil Biol.*
809 *Biochem.* 35, 125-132. http://dx.doi.org/10.1016/s0038-0717(02)00245-6.

810 Vinebrooke, R.D., Graham, M.D., 1997. Periphyton assemblages as indicators of recovery in
811 acidified Canadian Shield lakes. *Can. J. Fish. Aquat. Sci*. 54, 1557-1568.
812 http://dx.doi.org/10.1139/cjfas-54-7-1557.

813 Walczak, M., Michalska-Sionkowska, M., Olkiewicz, D., Tarnawska, P., Warzynska, O., 2021.
814 Potential of carvacrol and thymol in reducing biofilm formation on technical surfaces. *Molecules* 26.
815 http://dx.doi.org/10.3390/Molecules26092723.

816 Wallwork, J. A., 1983. Annelids: The First Coelomates., Edward Arnold, London

817 Zarybnicky, T., Bousova, I., Ambroz, M., Skalova, L., 2018. Hepatotoxicity of monoterpenes and
818 sesquiterpenes. *Arch. Toxicol.* 92, 1-13. http://dx.doi.org/10.1007/s00204-017-2062-2.

819 Zhang, J.H., Ma, S., Du, S.L., Chen, S.Y., Sun, H.L., 2019. Antifungal activity of thymol and carvacrol
820 against postharvest pathogens *Botrytis cinerea*. *J. Food Sci. Tech. Mys*

821 . 56, 2611-2620. http://dx.doi.org/10.1007/s13197-019-03747-0.

822 Zheljazkov, V.D., Astatkie, T., 2011. Effect of residual distillation water of 15 plants and three plant
823 hormones on Scotch spearmint (Mentha x gracilis Sole). *Ind. Crop. Prod.* 33, 704-709.
824 http://dx.doi.org/10.1016/j.indcrop.2011.01.011.

825 Zhou, F., Ji, B.P., Zhang, H., Jiang, H., Yang, Z.W., Li, J.J., Li, J.H., Yan, W.J., 2007. The
826 antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the
827 foodborne pathogen *Salmonella typhimurium*. *J. of Food Safety* 27, 124-133.
828 http://dx.doi.org/10.1111/j.1745-4565.2007.00064.x.

829

Table 1. COSMO-RS estimations for the soil adsorption coefficient, log K_{oc}. Boiling temperature, water solubility, vapour pressure, and octanol-water partition coefficient with the values found in the literature

No	Compound	boiling_T[C]	VapPress[Pa]	w_solub[mg/l][1]	LogKow[2]	LogKoc[3]
1	Carvacrol	240	10.9	576	3.25	2.6
		(237-238) ºC[c]	3.9 Pa at 25ºC[d]	333 mg/L at 20ºC[a]	3.33 at 40 ºC[b]	
2	Thymol	239	12.1	172	3.27	2.6
		(231.8 - 233.5) °C[g]	2.2 Pa at 25ºC[h]	(800-980) mg/L at 20 -25°C[e]	3.13-3.3 at 25 ºC[f]	

T, temperature; VapPress, Vapor pressure; w. solub, water solubility; LogKow, octanol-water partition coefficient; LogKoc, organic carbon-water partition coefficient.

1.- (Klamt et al. 2002b)
2.- (Klamt and Eckert 2000)
3.-(Klamt, Eckert and Diedenhofen 2002a)

Experimental values:
a https://echa.europa.eu/registration-dossier/-/registered-dossier/23562/4/9/?documentUUID=fff5e95a-ac48-44a9-99a7-aa608aca8576
b https://echa.europa.eu/registration-dossier/-/registered-dossier/23562/4/8/?documentUUID=6f1b80ed-e411-4ac6-8169-33852cf9559c
c https://echa.europa.eu/registration-dossier/-/registered-dossier/23562/4/4/?documentUUID=fd1bc27e-c99e-4572-a676-78d9bdadb03d
d https://echa.europa.eu/registration-dossier/-/registered-dossier/23562/4/7/?documentUUID=cba14a0c-14e0-429d-ac0f-6d4334557d25
e https://echa.europa.eu/es/registration-dossier/-/registered-dossier/11030/4/9/?documentUUID=13721e58-77b6-4d81-9d00-cc0eb52f1329
f https://echa.europa.eu/es/registration-dossier/-/registered-dossier/11030/4/8/?documentUUID=42d8d808-a220-48c9-8a09-392f31c9f9b8
g https://echa.europa.eu/es/registration-dossier/-/registered-dossier/11030/4/4/?documentUUID=f16e19d3-b5fd-4af1-8809-38c739826c27
h https://echa.europa.eu/es/registration-dossier/-/registered-dossier/11030/4/7/?documentUUID=af7cadf2-e500-4179-b1f1-8636231a1403

Support Information. Physical-chemical parameters of the river water sample collected from the Gállego River a Ebro river tributary (Montañana, Zaragoza, Spain) on June 24, 2019 for periphyton assays.

River water parameters	value
Conductivity (µs/cm)	2317
pH	7.76
Total Suspended Solids (mg/L)	5.1
Organic matter	3.0
Total Dissolved Solids (mg/L)	1586.4
Carbonates (mg/L)	0.0
Bicarbonates (mg/L)	249.4
Fluorides (mg/L)	0.088
Chlorides (mg/L)	475.195
Nitrites (mg/L)	0
Bromides (mg/L)	0.493
Nitrates (mg/L)	14.479
Phosphates (mg/L)	0
Sulphates (mg/L)	358.835
Total alkalinity (mg/L)	249.4
Total organic carbon (mg/L)	2.96
Total Nitrogen (mg/L)	3.77

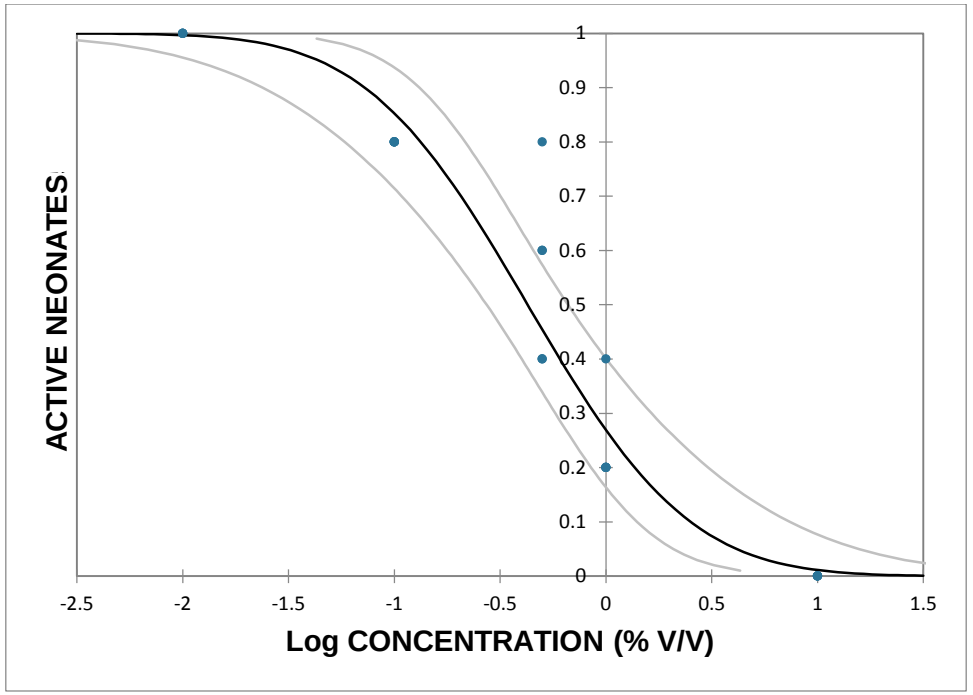


Figure 1. Dose-response curve of *D. magna* after a 24 h of exposure to *S. montana* hydrolate. *C*urve is the average value of five replicates. Pale grey lines indicate the confidence limits (95%).

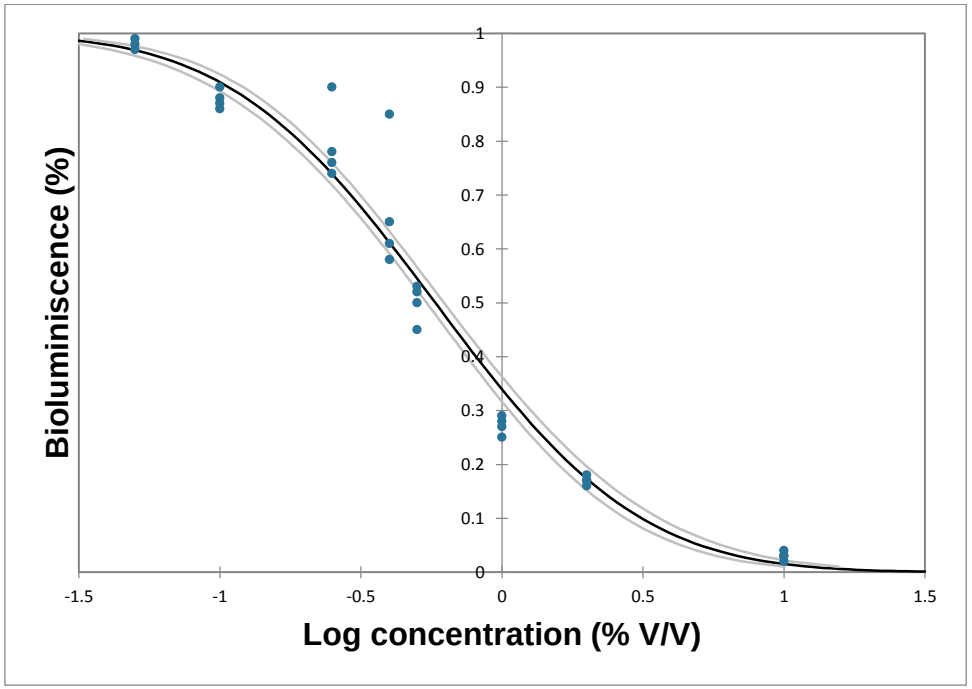


Figure 2. Concentration-response curves of bioluminiscence loss of *V. fisheri* after 30 minutes of exposure to *S. montana* hydrolate as a function of logarithm of the concentration. Bioluminiscence values are expressed as the percentage of the control. Pale gray lines indicate the confidence limits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

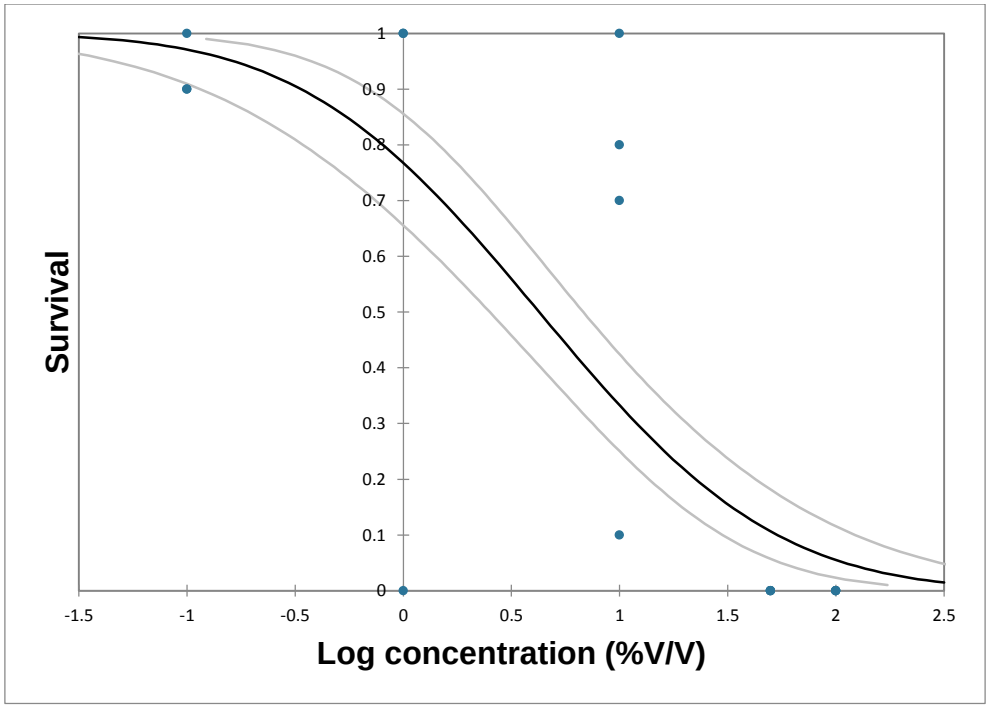


Figure 3. Curve show the dose-response of *Eisenia fetida after exposure to S. montana hydrolate* during *14 days. Curves are the average value of three. Grey lines are the confidence limits (95%).*

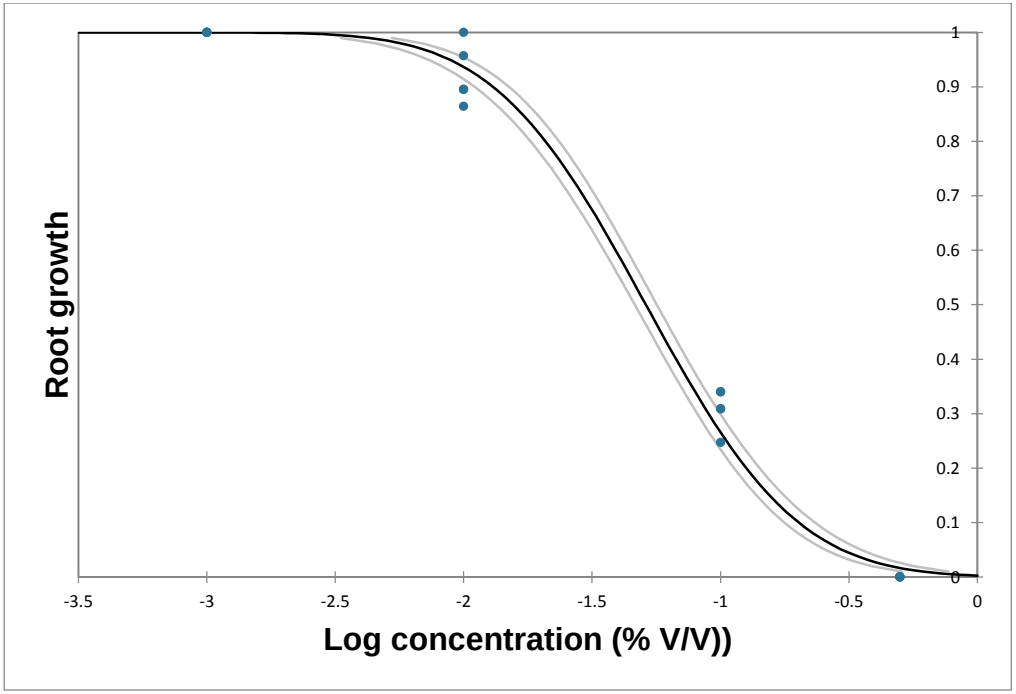


Figure 4. Curve show the dose-response of *Allium cepa after exposure to S. montana hydrolate during 72 hours. Curves are the average value of five replicates. Grey lines are the confidence limits (95%).*

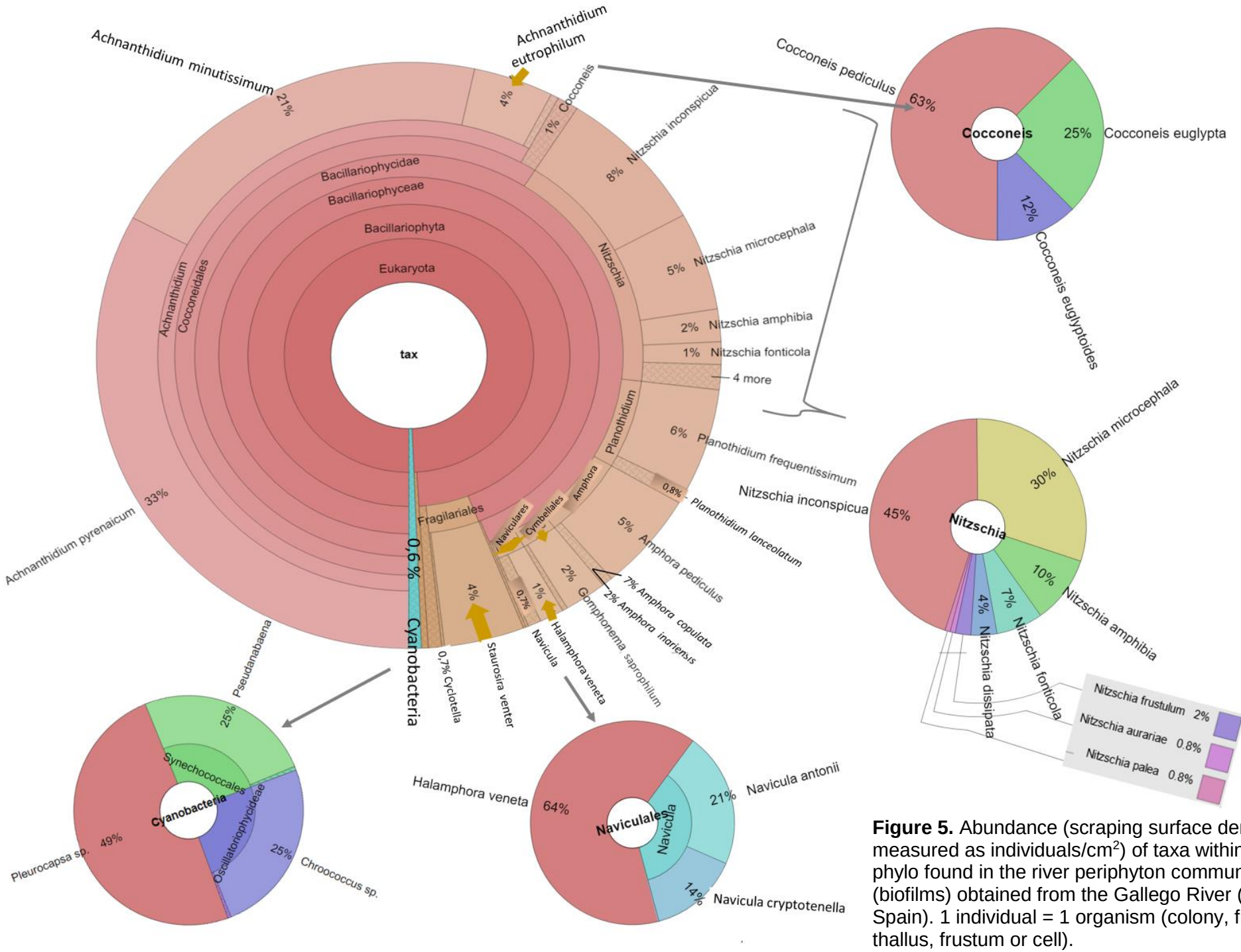


Figure 5. Abundance (scraping surface density measured as individuals/cm^2) of taxa within each phylo found in the river periphyton communities (biofilms) obtained from the Gallego River (Zaragoza, Spain). 1 individual = 1 organism (colony, filament, thallus, frustum or cell).

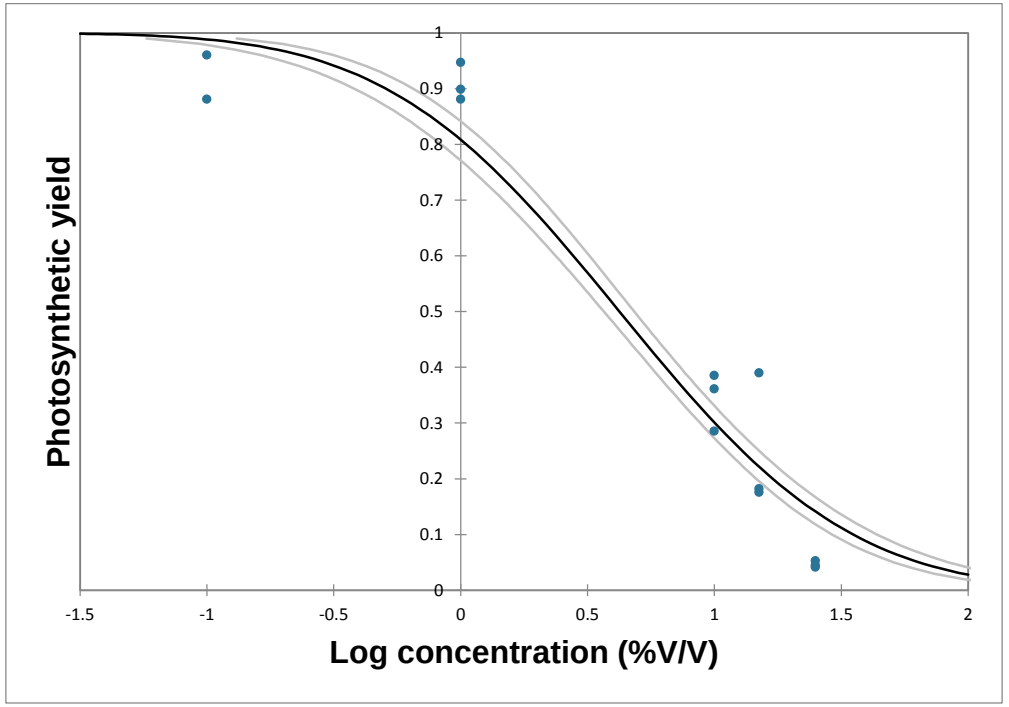


Figure 6. Concentration−response curve of photosynthetic yield of river periphyton after 2 h of exposure to *S. montana hydrolate* at 23ºC as function of logarithm of the concentration. Photosynthetic values are expressed as the percentage of the control. Pale grey lines indicate the confidence limits (95%). Each dose was measured in triplicate.

Credit Author Statement

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

Juliana Navarro: Data curation, Investigation, Supervision

Jonatan Val: Data curation, Investigation, review & editing

Francisco Roig: Data curation, Formal analysis

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

Diego Ballestero: Data curation, Investigation, Methodology, Supervision.