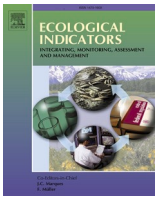
Ecological Indicators 142 (2022) 109173

Contents lists available at ScienceDirect

Ecological Indicators

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



Ecotoxicity of basil (*Ocimum Basilicum*) extract in aquaculture feeds: Is it really eco-safe for the aquatic environment?

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ARTICLE INFO

Keywords:
Acute toxicity
Aquatic ecosystems
Daphnia magna
Paracentrotus lividus
Plant extract
Trophic level

ABSTRACT

Plant extract and essential oils are gaining application in aquaculture, but data about their environmental impact are limited and their potential effects on aquatic organisms are largely unknown. For this study, ecotoxicity tests were performed under standardized conditions on fish feed supplemented with 3 % w/w of a basil supercritical extract (F1-BEO; substance A), F1-BEO extract (substance B), and fish feed without F1-BEO extract (substance C) on three model species of different trophic levels (bacteria, primary producer, primary consumer) considered representative for freshwater (*Aliivibrio fischeri*, *Raphidocelis subcapitata, Daphnia magna*) and marine (*A. fischeri*, *Phaeodactylum tricornutum*, *Paracentrotus lividus*) ecosystems. Ecotoxicological response was largely comparable within the same trophic level (whichever the ecosystem). EC_{50} was not calculable in the concentration range here tested (3.9–500 mg/L) for freshwater and marine microalgae, suggesting that none of the substances were toxic for primary producers. Reduction of *A. fischeri* bioluminescence at the tested concentration (0.5–10 mg/L) was observed only for substance A (EC_{50} 9.53 mg/L and 9 mg/L for freshwater and marine ecosystems, respectively). Notably, in *P. lividus* embryotoxicity was higher for substances A (EC_{50} 1.80 mg/L) and C (EC_{50} 4.6 mg/L) than for substance B (EC_{50} 7.10 mg/L), suggesting a toxic effect due to feed dissolution. In contrast, substance B was more toxic (EC_{50} 0.34 mg/L) in *D. magna* than substances A (EC_{50} 3.98 mg/L) and C (EC_{50} 5.50 mg/L). Based on the Globally Harmonized System of Classification and Labelling of Chemicals, all substances were categorized Acute 2, except for substance A which was categorized Acute 1 for *D. magna*. Overall, the substances were found to be potentially toxic for an aquatic ecosystem, especially for primary consumer. Further study of plant extract and essential oils is needed to better understand their effects and fate on the aquatic environment.

1. Introduction

The ever wider use of antibiotics in human and veterinary medicine has led to an increase in the circulation of antibiotic-resistant bacteria (Amarasiri et al., 2020; Serwecińska, 2020). In veterinary medicine, antibiotics have long been used in therapy and animal production (Sicuro et al., 2020; Palma et al., 2020). The World Health Organization (WHO) has stated that antimicrobial resistance is a global concern for the 21st century (Talebi Bezmin Abadi et al., 2019). In addition to measures to improve surveillance and diagnosis of infection and promote rational antibiotic use (Ben et al., 2019; Bui et al., 2022), strengthened and coordinated actions are needed to achieve successful results.

In this context, plant extracts (PEs) and essential oils (EOs) could play a key role as natural antimicrobials (Yu et al., 2020; El-Tarabily et al., 2021). Such compounds are a liquid, volatile, rarely coloured, and lipid-soluble mixture of terpenes and terpenoid that are biosynthesized by aromatic plants for attractive or defensive purposes (Mohammedi et al., 2020). EOs for example typically have 20–60 constituents, of which two or three are major compounds (20–70 % of total amount) and the other components occur in traces (Ferraz et al., 2022a).

PEs and EOs have traditionally been used for their antimicrobial

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https://doi.org/10.1016/j.ecolind.2022.109173
Received 24 May 2022; Received in revised form 7 July 2022; Accepted 11 July 2022
Available online 16 July 2022

activity in folk medicine (Stefanello et al., 2011). Scientific studies demonstrating this property are relatively recent, however, and in most cases concern pathogens that affect humans (Ahmad et al., 2021). The use of PEs and EOs in veterinary medicine for pet animals and livestock holds interest since they could offer an alternative to synthetic antimicrobials (Ebani and Mancianti, 2020; Sicuro et al., 2020) against infection and improve production quality (i.e., meat, eggs, milk, honey, seafood), without the residues of conventional drugs in food (Evangelista et al., 2021).

Essential oils are volatile liquids obtained by distillation of any part of a plant or mechanical extraction from the epicarp of a citrus fruit at room temperature (van Beek and Joulain, 2018). Hydrolates can be obtained as a by-product of distillation to extract EOs (ISO, 2013a). The International Organization for Standardization (ISO) defines a hydrolate as the distilled water that remains after distillation and is typically rich in water-soluble essential oil components (ISO, 2013a; Bicchi and Joulain, 2018), whereas an extract is "a product obtained by treating a natural raw material with a solvent and then, after filtration, removing the solvent by distillation, unless a non-volatile solvent is used" (ISO, 1997).

Supercritical fluid extraction (SFE) of plant materials with solvents such as carbon dioxide (CO_2) is gaining popularity. SFE allows plant material to be processed at low temperatures, limiting thermal degradation, without the use of toxic solvents (Khajeh et al., 2004). Currently, SFE is used primarily for large-scale decaffeination of coffee and tea and the production of hop extracts. It is attracting growing interest for other industrial applications at various scales of operation (Babova et al., 2016). SFE with CO_2 can extract natural compounds, especially those unstable at high temperature. It is the most widely used method in the food and pharmaceutical industry as the extracts contain no organic residues (Wang et al., 2021). Furthermore, extraction can be carried out at low temperature and moderate pressure (Yang et al., 2020).

Despite the growing interest in EO and PE in livestock, the scientific community has paid far less attention to their potential environmental impact (Ferraz et al., 2022a). One explanation for this lack of research is the general belief that plants, and their components are generally natural and safe. Some plants, however, produce highly toxic metabolites (Zárybnick et al., 2018; Falkowski et al., 2020), necessitating assessment of their potential toxic effects on non-target organisms.

The number of drugs authorized in aquaculture is limited and the spread of antibiotic resistance has significantly reduced current options for treating fish diseases (Santos and Ramos, 2018). Several EOs and PEs from aromatic plants are known to have biological activity (Radünz et al., 2019). Basil (*Ocimum basilicum*), for instance, one of the world's most popular aromatic herbs, has been shown to be an effective antioxidant, antimicrobial, insecticidal, nematocidal, and fungistatic agent in aquaculture (Brum et al., 2018; El-Ekiaby, 2019; Amor et al., 2021; Magara et al., 2022).

Two recent studies assessed the long-term changes in serum blood biochemical parameters and biomarkers of antioxidant stress in rainbow trout (*Oncorhynchus mykiss*) fed with a commercial fish diet supplemented with a basil supercritical extract (F1-BEO) up to 3 % w/w (Magara et al. 2022; Pastorino et al., 2022). Its ecotoxicity profile in aquatic ecosystems has not been assessed to date, however. For the present study, ecotoxicity tests with F1-BEO extract, fish feed supplemented with 3 % w/w F1-BEO and fish feed without F1-BEO extract (comparison condition) were performed on three species of different trophic levels and considered representative for freshwater (*Aliivibrio fischeri*, bacterium; *Raphidocelis subcapitata* primary producer, *Daphnia magna*, primary consumer) and marine ecosystems (*Aliivibrio fischeri*, bacterium; *Phaeodactylum tricornutum*, primary producer; *Paracentrotus lividus*, primary consumer) (Parvez et al., 2006; Baudo et al., 2011). Ecotoxicological bioassays on three species at different levels of biological complexity and ecological niches can yield information about the effects of contaminants on community and ecosystems (Baudo et al., 2011). The use of assay batteries for evaluation of the ecotoxicity of chemical substances was introduced in European legislation by the REACH Regulation, which refers to the OECD protocols for the choice of the most suitable methods and indicators for acute and chronic toxicity tests (Parvez et al., 2006; Oliva et al., 2021).

2. Material and methods

2.1. Chemical profile of basil supercritical fluid extract (F1-BEO)

The basil supercritical fluid extract (F1-BEO) was the same as that used in previous studies (Magara et al., 2022; Pastorino et al., 2022). F1-BEO was extracted by Exenia Group s.r.l. (Pinerolo, Italy) from dried, clean sweet basil leaves (size 0.3 to 0.5 cm; residual humidity 10 %) using a supercritical fluid extractor (SCF-100; Separeco s.r.l, Pinerolo, Italy). Spectrophotometric analysis showed that the F1-BEO contained bioactive compounds, total polyphenol content and total flavan-3-ol content of 32.97 ± 1.63 mmol gallic acid equivalent (GAE) per 100 g of fresh weight and 21.21 ± 1.04 mmol A2-type proanthocyanidin content equivalent (A2-PACE) per 100 g of fresh weight, respectively. Several polyphenolic compounds were identified in the F1-BEO extract by HPLC-ESI-MS/MS (Fig. 1). The F1-BEO also contained several volatile organic compounds, mainly linalool (25.29 %), α-bergamotene (19.34 %) and estragole (18.79 %) (Table 1). The F1-BEO fraction was composed of about 10 % fats: palmitic acid, linoleic acid, and oleic acid accounted for 77 % of the total fatty acid content (GC–MS and GC-FID analysis).

2.2. Ecotoxicity bioassay of substances

Ecotoxicity bioassays were performed on: commercial feed (Alterna Eel, Skretting; ingredients: fish meal, fish oil, wheat red dog, wheat gluten, blood meal from poultry, a soya bean protein concentrate, swine haemoglobin, whey powder; proximate composition: protein 48 %, lipid 11 %, ash 8 %, fibre 1 %) supplemented with 3 % w/w F1-BEO (substance A) which is the feed with the higher basil extract inclusion by weight used by Magara et al. (2022) and Pastorino et al. (2022); the F1-BEO extract (substance B); a commercial feed (Alterna Eel, Skretting) without F1-BEO supplement (substance C).

Substances A and C (3 % w/w F1-BEO and feed without F1-BEO, respectively) were prepared following the protocol proposed by Magara et al. (2002). Briefly, for the preparation of the substance A, the F1-BEO derived from the supercritical fluid extraction of basil was added to the commercial feed flour in the proportions of 3 % w/w (3 g of F1-BEO in 100 g of feed flour). A control diet without F1-BEO (only feed flour Alterna Eel, Skretting) was also made (substance C). Then, the mixture was subsequently mixed to obtain a suitable material for pellet preparation. The pellets were obtained using a 4.0 mm die meat grinder and dried at 30 °C for 48 h. Substances A and C were then powdered with a pestle and stock solutions of 500 mg/L were prepared with 0.5 % dimethyl sulfoxide (DMSO) as solvent (Ferraz et al., 2022b). The solutions were treated by sonication at 40 Hz for 20 min to disaggregate the clusters. Toxicity of the 0.5 % DMSO solution was also tested as negative control to minimize any possible effects of the solvent on the results (OECD, 2019).

Exposure concentrations were determined by pilot testing to define the correct range of dilutions and based on relevant literature (Ferraz et al., 2022b): 0.5–10 mg/L (*A. fisheri*), 3.9–500 mg/L (*R. subcapitata*), 0.01–100 mg/L (*D. magna*), 3.9–500 mg/L (*P. tricornutum*), 0.5–10 mg/L (*P. lividus*).

2.3. Toxicity bioassay: Freshwater organisms

Tests were performed on three species of different trophic levels and considered representative for freshwater ecosystems: *Aliivibrio fischeri* (Gram-negative bacteria; ISO, 2019); *Raphidocelis subcapitata* (algae; ISO, 2012), *Daphnia magna* (Cladocera; ISO, 2013b).

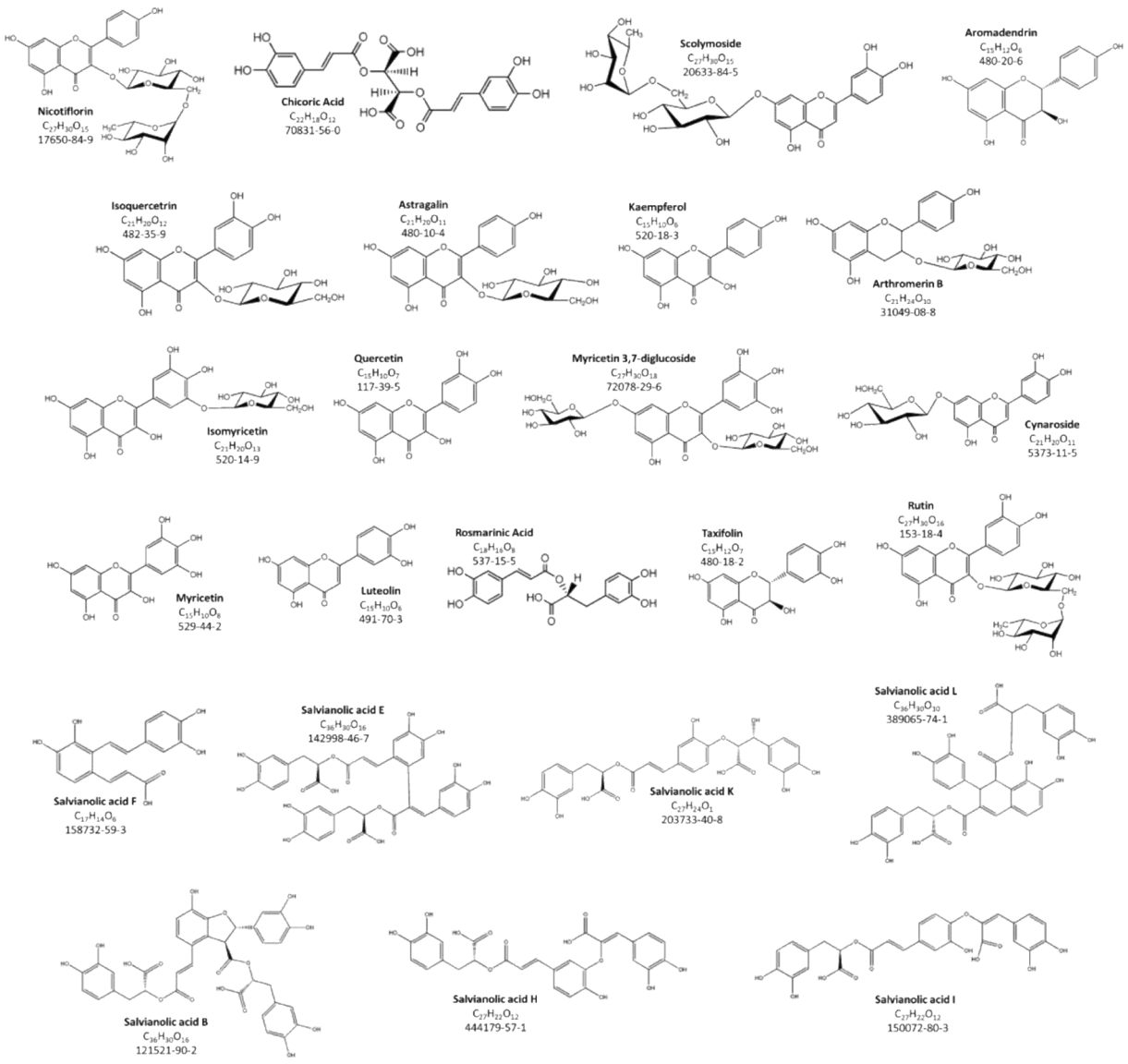

Fig. 1. Polyphenols in F1-BEO identified via HPLC-ESI-MS/MS (Magara et al., 2022).

Table 1
Formulae, identification, and chemical abstracts service identification number (CAS ID) of volatile organic compounds with quantification (in percentage; %) in the F1-BEO extract.

Formula	Compound	CAS ID	Percentage (%)
$C_{10}H_{18}O$	1,8-Cineole	470-82-6	9.33 ± 0.45
$C_{10}H_{18}O$	Linalool	78-70-6	25.29 ± 0.81
$C_{10}H_{12}O$	Estragol	140-67-0	18.79 ± 0.78
$C_{10}H_{12}O_2$	Eugenol	97-53-0	4.49 ± 0.12
$C_{10}H_{10}O_2$	Methylcinnamylate	103-26-4	8.71 ± 0.15
$C_{11}H_{14}O_2$	Methyleugenol	93-15-2	6.58 ± 0.08
$C_{15}H_{24}$	b-Caryophyllene	87-44-5	7.47 ± 0.29
$C_{15}H_{24}$	α-Bergamotene	17699-05-7	19.34 ± 1.09

Briefly, the endpoint for *Aliivibrio fischeri* was inhibition of bacteria when exposed to the sample. Bioluminescence was measured using a luminometer set at 430 nm. The test was performed in triplicate at 15 ± 1 °C for 30 min. The initial bacterial concentration was 10^6 cells, and the maximum testable concentration of the sample was 90 %.

Growth inhibition was the endpoint for the freshwater algae *Raphidocelis subcapitata*. The alga, which was in the exponential growth phase, was exposed to the samples and placed under continuous light for 72 h to stimulate rapid growth. The samples were shaken every 24 h and measured using a spectrophotometer at a wavelength of 670 nm. The test was performed in triplicate at 20 ± 2 °C. The initial concentration of the samples was 10^4 cells, and the sample concentration was 100 %. Nutrients (four stock solutions) were added to each sample according to UNI EN ISO 8692:2012 (ISO, 2012). The test was considered valid if the algal concentration in the negative controls was 16 times the initial concentration after 72 h and if the EC_{50} of the positive controls was 1.19 ± 0.27 mg/L.

Five juvenile *Daphnia magna* aged less than 24 h were exposed to different concentrations of the substances at 20 ± 2 °C and a photoperiod of 16 h of light and 8 h of dark for 48 h. At the end of the test, the number of immobilized specimens was counted. Four replicates were performed for each sample. The criteria in the UNI EN ISO 6341:2013 guideline (ISO, 2013b) were followed to meet the test validity criteria. All tests were performed under standardized conditions using negative and positive controls for each batch of analyses (Table 2); results were within the range of acceptability reported by the specific testing method (Table 2).

Table 2
Positive and negative controls performed on the species of different trophic levels and considered representative of freshwater and marine ecosystems. Negative controls are not available for *Aliivibrio fischeri.* CV denotes coefficient of variation.

Species	Negative control	Value	Positive control	Value
Aliivibrio fischeri	Artificial seawater	–	3,5-dichlorophenol (3.4 mg/L)	20–80 % of inhibition
Raphidocelis subcapitata	culture medium	CV% (μ): max 5 %	$K_2Cr_2O_7$	EC_{50}: 1.19 ± 0.27 mg/L
Daphnia magna	standard ISO freshwater	percentage of immobility: max 10 %	$K_2Cr_2O_7$	EC_{50}: 0.6–2.1 mg/L (24 h)
Phaeodactylum tricornutum	culture medium	CV% (μ): max 5 %	$K_2Cr_2O_7$	EC_{50}: 20.1 ± 5.3 mg/L
Paracentrotus lividus	Artificial seawater	–	$Cu(NO_3)_2$	22.60–68.34 μg/L Cu^{2+}.

2.4. Toxicity bioassay: Marine organisms

The tests were performed on three species of different trophic levels and considered representative for marine ecosystems: *Aliivibrio fischeri* (ISO, 2019), *Phaeodactylum tricornutum* (ISO, 2017), *Paracentrotus lividus* (ISPRA, 2017). Growth inhibition was the endpoint for *P. tricornutum* (ISO, 2017). Algae in the exponential growth phase were placed under continuous light for 72 h to stimulate rapid growth. The samples were shaken and measured with a spectrophotometer at a wavelength of 670 nm every 24 h. The tests were carried out in triplicate at 20 ± 2 °C. The initial concentration of the samples was 10^4 cells. Except for the negative controls (the algal culture medium was already nutrient rich), specific nutrients (S1 + S2 + S3; Piccardo et al., 2021) were added to each sample in accordance with ISO (2017). Positive controls (n = 3) with potassium dichromate were set up. The test was considered valid if the algal concentration in the negative controls after 72 h was 16 times the initial concentration and the EC_{50} of the positive controls was 20.1 ± 5.3 mg/L.

The larval development of the sea urchin *P. lividus* after 72 h of exposure was the endpoint of this study (Piccardo et al., 2021). Eggs were fertilized with sperm, and the fertilized eggs were exposed to the solutions for 20 min. Each sample was added with 2–3 drops of Lugol's fixative to stop cell division at 72 h, and the results were examined under microscopy. Fertilized eggs reached the pluteus larval stage at 72 h of normal development: 100 *plutei* were counted per replicate to determine the percentage of abnormal larvae. Larvae were considered abnormal if their development was halted, arms were absent or of different length, there were extra arms or cross-lateral rods, body width was asymmetrical or displayed other abnormalities according to the literature (ISPRA, 2017). Tests were performed in triplicate. Artificial sea water was used as a negative control and copper (II) nitrate as a positive control (Table 2). Tests were considered valid when the negative control had more than 80 % normally developed larvae and the EC_{50} of the positive control was between 22.60 and 68.34 g/L Cu^{2+} (Table 2).

2.5. Statistical analysis and toxicological categorization

Concentrations that induced the endpoint in 50 % (EC_{50}), 20 % (EC_{20}), and 10 % (EC_{10}) of the exposed samples were calculated by statistical interpolation from experimental data using the US EPA Toxicity Relationship Analysis Program (TRAP version 1.30), with a Gaussian distribution and logarithmic transformation of exposure variables sized for ecotoxicological tests. Principal component analysis (PCA) was performed to check for trends in ecotoxicological response (EC_{50}, EC_{20}, percentage of effects, maximum concentration, percentage of effects at minimum concentration) between the freshwater (*Aliivibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia magna*) and the marine (*Aliivibrio fischeri*, *Phaeodactylum tricornutum*, *Paracentrotus lividus*) model organisms of different trophic roles (bacteria, primary producers, primary consumers) after exposure to the three substances (A, B, C). The PCA results were plotted using open-source data analysis software RStudio® (RStudio, Inc.).

Solution toxicity was classified according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) adopted by the United Nations (UN, 2019). The system consists of three short-term (acute) categories based on acute toxicity data (mean EC_{50}): Acute 3 in the range of 10–100 mg/L; Acute 2 in the range of 1–10 mg/L; Acute 1 when $EC_{50} \leq 1$ mg/L (UN, 2019).

3. Results

3.1. Freshwater

Ecotoxicological response to the substances is reported in Table 3. Effects at the maximum concentration were observed only for *Daphnia magna* in all substances tested, with higher effects (100 %) noted for substance B (F1-BEO extract), whereas effects at the minimum concentration were observed for substance A (feed with 3 % F1-BEO). EC_{50} was calculated only in *A. fisheri* (9.53 mg/L) exposed to substance B and in *D. magna* in all substances tested, with EC_{50} values ranged from 0.34 mg/L (substance B) to 5.50 mg/L (substance C; feed without F1-BEO). Finally, EC_{20} was calculated only for substance B in *A. fisheri* (5.40 mg/L) and *D. magna* (0.05 mg/L). EC_{10} was never calculable at the concentrations in any of the freshwater organisms.

3.2. Marine organisms

Ecotoxicological response to the substances is reported in Table 4. Effects at the maximum concentration were observed only in *P. lividus* for all substances tested, with higher effects (100 %) noted for substance A, followed by substances B (95.7 %) and C (90 %). Effects at the minimum concentrations were observed for all substances in *P. lividus* (12 %, 18.3 %, and 17 % effect for substances B, C, and A, respectively). EC_{50} was calculated only in *A. fisheri* (9 mg/L) exposed to substance B and in *P. lividus* for all tested substances, with EC_{50} from 1.8 mg/L (substance A) to 7.1 mg/L (substance B). Finally, EC_{20} was calculated only in *P. lividus* for substance A (1.3 mg/L), substance B (5.3 mg/L), and substance C (1.5 mg/L). EC_{10} was never calculable at the tested concentrations in any of the marine organisms.

3.3. Principal component analysis

Eigenvalues revealed that the first two principal components (PC1 and PC2) accounted for a significant portion of total variance (94.6 %), while the two other components (PC3 and PC4) accounted for a much smaller portion of variance (5.4 %). Interpretation of the principal components (PCs) was evaluated using eigenvalues (only PCs with eigenvalues greater than one were retained). The biplot of loadings (variables) and scores (observations) shows which trophic roles are closest to them (Fig. 2). The scores of each trophic role are distinguished by a different symbol and colour (largest symbol denotes average value). Almost all variables moved to PC1 because they were more related to it. The bacteria (*A. fisheri*) and the primary producers (*R. subcapitata* and *P. tricornutum*) are located in the right part of the plot in relation to higher EC_{20} and EC_{50} (fewer sensitive species), whereas primary consumers (*D. magna* and *P. lividus*) are located in the upper left quadrant in relation to higher percentage effects due to exposure to tested substances (more sensitive species).

3.4. Toxicological categorization

Table 5 presents the toxicity category according to the Globally

Table 3
Ecotoxicological response to substances (A - feed with 3% F1-BEO; B - F1-BEO extract; C - feed without F1-BEO) in freshwater organisms. LCL and UCL are 95% confidence lower-level concentration and upper-level concentration intervals associated with EC_{20} and EC_{50}, respectively. NC denotes not calculable; SD standard deviation; %E (max conc) percentage of effect at the maximum concentration; %E (min conc) percentage of effect at the minimum concentration. Data are expressed as mg of substance per litre of solution (mg/L).

Species	Sample	% E (max conc)	SD	% E (min conc	SD	EC_{50}	95 % LCL	95 % UCL	EC_{20}	95 % LCL	95 % UCL
A. fischeri	A	−0.5	0.0	−10.5	0.1	NC	NC	NC	NC	NC	NC
(10–0.5 mg/L)	B	50.5	0.7	−21.0	0.2	9.53	8.41	10.80	5.40	4.55	6.41
	C	−7.4	0.7	−27.8	0.4	NC	NC	NC	NC	NC	NC
R. subcapitata (500–3.9 mg/L)	A	−11.2	0.3	−9.4	1.2	NC	NC	NC	NC	NC	NC
	B	−17.8	6.7	−10.2	0.8	NC	NC	NC	NC	NC	NC
	C	−10.8	2.1	−10.3	0.8	NC	NC	NC	NC	NC	NC
D. magna	A	80.0	0.0	50.0	34.6	3.98	NC	NC	NC	NC	NC
(100–0.01 mg/L)	B	100.0	0.0	35.0	10.0	0.34	0.12	1.01	0.05	0.01	0.37
	C	65.0	10.0	10.0	11.5	5.50	NC	NC	NC	NC	NC

Table 4
Ecotoxicological response to substances (A - feed with 3% F1-BEO; B - F1-BEO extract; C - feed without F1-BEO) in marine organisms. LCL and UCL were 95% confidence lower-level concentration and upper-level concentration intervals associated with EC_{20} and EC_{50}, respectively. NC denotes not calculable; SD standard deviation; %E (max conc) percentage of effect at the maximum concentration; %E (min conc) percentage of effect at the minimum concentration. Data are expressed as mg of substance per litre of solution (mg/L).

Species	Sample	%E (max conc)	SD	% E (min conc)	SD	EC_{50}	95 % LCL	95 % UCL	EC_{20}	95 % LCL	95 % UCL
A. fischeri	A	−6.5	0.1	−13.5	0.8	NC	NC	NC	NC	NC	NC
(10–0.5 mg/L)	B	53.6	0.7	−16.0	1.5	9.0	8.0	10.2	NC	NC	NC
	C	−5.4	0.7	−20.6	1.8	NC	NC	NC	NC	NC	NC
P. tricornutum	A	−12.1	0.6	−13.3	0.3	NC	NC	NC	NC	NC	NC
(500–3.9 mg/L)	B	−13.5	0.6	−13.0	0.6	NC	NC	NC	NC	NC	NC
	C	−12.9	0.2	−12.9	0.2	NC	NC	NC	NC	NC	NC
P. lividus	A	100.0	0.0	18.3	0.6	1.8	1.6	1.9	1.3	1.0	1.5
(10–0.5 mg/L)	B	95.7	2.5	12.0	2.6	7.1	6.8	7.5	5.3	4.8	5.8
	C	90.0	1.0	17.0	5.2	4.6	2.8	4.5	1.5	0.0	4.5

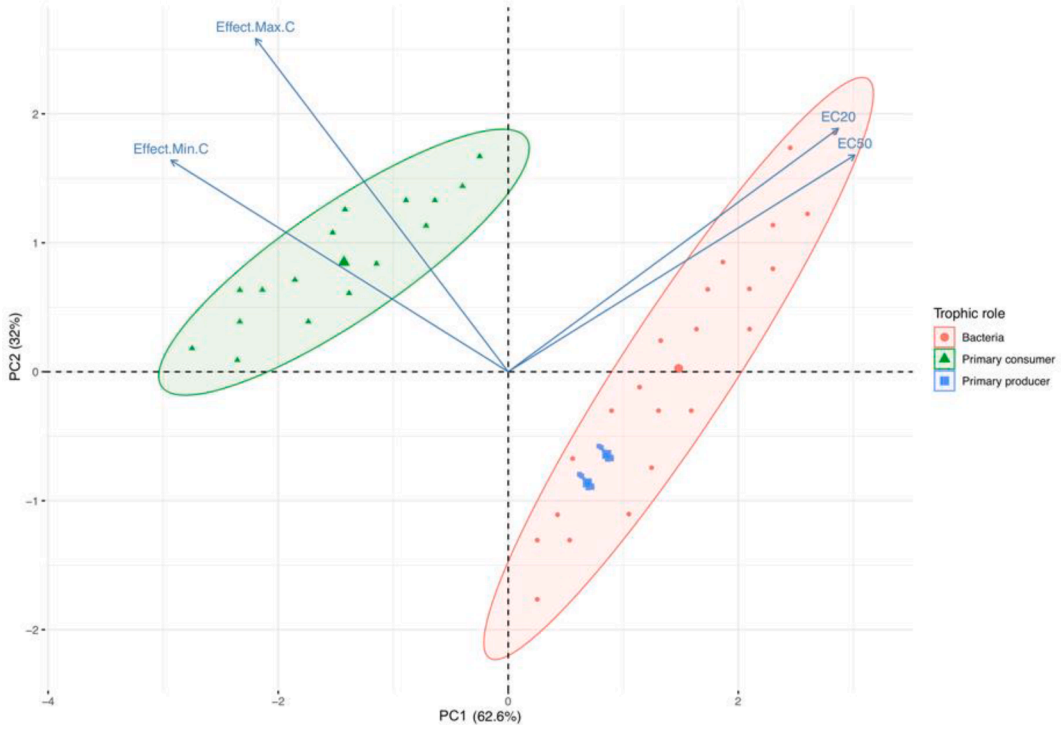

Fig. 2. Biplot of loadings (variables) and scores (observations) in the principal component analysis. The scores of each trophic role are denoted by a symbol (largest symbol denotes average value); each trophic role has a different colour (red for bacteria, blue for primary producer, green for primary consumer).

Table 5
Substances (A - feed with 3 % F1-BEO; B - F1-BEO extract; C - feed without F1-BEO) tested in freshwater and marine model organisms and their toxicological categorization (EC_{50}) according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Acute 3: 10–100 mg/L; Acute 2: 1–10 mg/L; Acute 1: $EC_{50} \leq 1$ mg/L.

Environment	Species	Substance	EC_{50} (mg/L)	GHS classification
Freshwater	*A. fischeri*	B	9.53	Acute 2
	D. magna	A	3.98	Acute 2
		B	0.34	Acute 1
		C	5.50	Acute 2
Marine	*A. fischeri*	B	9.0	Acute 2
	P. lividus	A	1.8	Acute 2
		B	7.1	Acute 2
		C	4.6	Acute 2

Harmonized System of Classification and Labelling of Chemicals (GHS). All substances resulted Acute 2 in the freshwater and the marine ecosystem for bacteria (*A. fischeri*) and primary consumers (*D. magna* and *P. lividus*), except for substance B (F1-BEO extract) categorized as Acute 1 for *D. magna*. The GHS classification is not reported for algae (*R. subcapitata* and *P. tricornutum*) since the EC_{50} was not calculated in the concentration range tested (3.9–500 mg/L). Thus, it can be concluded that none of the three substances were toxic for primary producers.

4. Discussion

The available data on the toxicity of EOs and other PEs in model aquatic organisms are very scarce (Ferraz et al. 2022a). For this study, we report the ecotoxicological response of freshwater and marine model organisms exposed to F1-BEO basil extract and fish feed supplemented with 3 % w/w of F1-BEO. The ecotoxicity of fish feed without F1-BEO is reported for comparison. Despite the difference in ecosystem (freshwater or marine), the ecotoxicological response was generally comparable for the same trophic level, with higher sensitivity noted in primary consumers.

Aliivibrio fischeri lives as a free-living (planktonic) organism or a mutualistic symbiont that colonizes the light-producing organ (photophore) of squids and fish, imparting luminescence for camouflage or prey attraction by the host (Kaeding et al., 2007). Through the electron transport chain, bioluminescence is directly linked to respiration and thus reflects cellular metabolic status as a determinant of xenobiotic-mediated toxicity (Girotti et al., 2008; Abbas et al., 2018). Exposure to toxic substances reduces the production of luminescence: bacterial metabolism is inhibited by the decrease in light emittance that corresponds to the toxicity level of the substance (Abbas et al., 2018). In the present study, a reduction in *A. fischeri* bioluminescence at the tested concentrations (0.5–10 mg/L) was observed only for the F1-BEO extract (mean EC_{50} 9.53 mg/L and 9 mg/L for freshwater and marine ecosystems, respectively). Shukla et al. (2020) investigated the effects of linalool and eugenol (major components of the F1-BEO extract) and found that both compounds could reduce the bioluminescence of *A. fischeri* even at very low concentrations. In contrast, no inhibition of bioluminescence was observed for the other two substances (A and C) tested here.

Microalgae inhabit the world's oceans and seas where they occupy a key trophic level in aquatic ecosystems as primary producers at the base of the marine food chain (Mucha et al., 2003). Because phytoplankton are at the bottom of the aquatic food chain, they are vital to the entire ecosystem; however, very few studies to date have examined the toxic effects of PEs and EOs (papaveraceae, pinaceae, fabaceae, malvaceae, cupressaceae) on microalgae (mainly *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Chlamydomonas reinhardtii*) (Jančula et al., 2007; Duringer et al., 2010; Oliveira et al., 2016; Pino-Otín et al., 2019; Ferraz et al., 2022b). In the present study, the EC_{50} for freshwater and marine microalgae was not calculated at the tested concentrations, suggesting no acute toxic effects on microalgae growth. For the F1-BEO extract (substance B), we may note that linalool was highest in composition percentage, followed by α-bergamotene and estragol. In a previous study, linalool toxicity was tested in *Scendedesmus subspicatus* (growth inhibition test; 96-h period), with an EC_{50} of 141.4 mg/L (Api et al., 2015), confirming the low toxicity of the compound on microalgae.

Unfortunately, the literature offers no data on basil extract toxicity for comparison. With regard to other PEs and EOs, however, Duringer et al. (2010) assessed the ecotoxicity of steam-extracted oils derived from western juniper foliage (*Juniperus occidentalis*) and Port Orford cedar heartwood (*Chamaecyparis lawsoniana*) on *R. subcapitata*; the EC_{50} for *J. occidentalis* EO was 1.7 mg/L at 96 h and was considered moderately toxic to *R. subcapitata*. After exposure to EO from *C. lawsoniana*, the EC_{50} for algal cell growth was reported to be higher than 5 mg/L, leading to the conclusion that the release of *C. lawsoniana* EO into the aquatic environment had no expected acute toxic effects on microalgae. The aqueous extracts from the roots of five papaveraceae plants were tested for their effects on *R. subcapitata* (Jancula et al., 2007); the extracts from *Dicranostigma lactucoides* and *Sanguinaria canadensis* were found to be the most toxic to microalgae after 96 h of exposure (EC_{50} of 21.27 and 23.90 mg/L, respectively) (Jancula et al., 2007).

As regards the toxicity of substances A and C on microalgae, fish feed is a known primary source of waste with the greatest environmental impact in aquaculture. The quantity and quality of waste excreted by fish are determined by dietary intake, digestion, and metabolism (Bureau and Hua, 2010). There is also a link between feed quality, feeding strategy, and waste production (Schneider et al., 2005). Aquacultural waste can be divided into solid and dissolved waste; unused and/or spilled feed by the fish, as well as excreted faeces are the main sources of solid waste, while dissolved waste is nutrient (mainly phosphorus and nitrogen) disintegration/suspension from the solid waste fraction. The increase in organic loading from fish feed into waters may influence the structure, composition, dominance, and biomass of phytoplankton communities (San Diego-Mcglone et al., 2008).

In the present study, dissolution of fish feed in the algal media enhanced the growth of the microalgal species tested with no toxic effects. Differently, the primary consumers in the freshwater and the marine ecosystem displayed acute toxicity in response to all three substances. *Daphnia magna* exposed to F1-BEO (substance B) showed the lowest EC_{50} (0.34 mg/L) compared to the EC_{50} in response to exposure to feed supplemented with F1-BEO (3.98 mg/L) and feed without basil extract (5.50 mg/L). Such findings are corroborated by the chemical composition of the F1-BEO extract as previously stated. A *D. magna* immobilization test (48 h) performed using linalool reported an EC_{50} of 20 mg/L. Eugenol (4.49 % in F1-BEO) was found to be highly toxic at low concentrations for *D. magna* (EC_{50} 0.70 mg/L) (Gueretz et al., 2017). Estragol (18.79 % in F1-BEO) is reported to be toxic for houseflies (Palacios et al., 2009), fruit flies (Cheng et al., 2009), and house dust mites (Lee, 2004), but no data on aquatic organisms are available. Although the toxic effects of PEs and EOs on crustaceans such as *Daphnia magna*, *Daphnia pulex*, *Scapholeberis kingi*, and *Artemia salina* have been studied (Andreu et al., 2018; Seremet et al., 2018; Ishimota et al., 2019; Pavela et al., 2020), only one study (Ferraz et al., 2022b) reported toxicity data for basil extract. *O. basilicum* hydrolate (Ferraz et al. 2022b), composed mainly of 52.5 % eugenol and 38.3 % linalool, showed no acute toxic effects on *D. magna* up to very high concentrations (8000 mg/L). The study did not mention whether the solution was sonicated and had solubilization issues, however.

Daphnia magna was sensitive to fish feed dissolution, with higher toxicity in response to fish feed supplemented with F1-BEO compared to the control fish feed, most likely due to the synergic effect of feed powder and basil extract. The reason for the toxicity was probably due to the solid waste (also known as particulate organic matter) that causes oxygen depletion and ammonia toxicity when it decomposes.

Furthermore, suspended solids (feed powder) floating in the water column can cause gill irritation to *D. magna*, also filling the intestinal tract (Capper, 2006).

Likewise, *Paracentrotus lividus* showed acute toxicity to all substances tested here: a higher percentage of abnormal larvae compared to controls and greater sensitivity to feed supplemented with F1-BEO, followed by feed without basil extract. *Paracentrotus lividus* is a key marine species with larval and adult populations inhabiting planktonic and benthic marine ecosystems, respectively. *P. lividus* has been demonstrated to be highly sensitive to various compounds, and it is widely used to assess the toxicological effects and the environmental impact of a variety of pollutants. The embryos are a highly duplicative cell system; exposure to chemicals has an adverse effect on delicate embryo development (Piccardo et al., 2021; Gharred et al., 2022). As reported for *D. magna*, the dissolution of fish feed probably caused the release of particulate organic matter that altered the water's physiochemical characteristics, making it toxic to *P. lividus* embryos. Changes in the physicochemical parameters of seawater (e.g., total organic content, nitrate, turbidity) could be indicators of eutrophication and have a negative impact on sea urchin embryo-larvae development and animal growth (Ternengo et al., 2018). Zúñiga et al. (1995) found that exposure to organic-waste discharges caused embryotoxicity in the sea urchin *Arbacia spatuligira*.

Finally, no data are available for comparison of acute toxicity of F1-BEO to *P. lividus* embryos. Novaes Simões et al. (2017) evaluated the use of *Lippia alba* EO (composed mainly of linalool [48.69 %] and eucalyptol [10.51 %]) as a sedative in the sea urchin *Echinometra lucunter* and found that a concentration of 150 ppm is sufficient to induce anaesthesia in adult specimens, with possible adverse effects on sea urchin embryos.

5. Conclusions

For this study, the effects of a basil extract (F1-BEO) as-is and supplemented in a commercial fish feed (based on the effectiveness observed in fish [Magara et al., 2022; Pastorino et al., 2022]) were assessed using model organisms for marine and freshwater ecosystems. Our findings suggest that the ecotoxicological responses were comparable in the freshwater and the marine ecosystem for organisms at the same trophic level. The substances appear to be safe for microalgae, whereas they caused toxic effects on primary consumers (*D. magna* and *P. lividus*), particularly the F1-BEO as-is in *D. magna*. The widespread belief that plant-based products are green and safer alternatives to their chemical counterparts lacks empirical data to support this claim. Hence there is an urgent need to assess the safety of other PEs and EOs to better understand their effects on ecosystems.

CRediT authorship contribution statement

Paolo Pastorino: Investigation, Conceptualization, Data curation, Methodology, Writing – original draft. **Marino Prearo:** Investigation, Conceptualization, Methodology, Writing – review & editing. **Serena Anselmi:** Investigation, Methodology, Writing – review & editing. **Andrea Broccoli:** Investigation, Methodology, Writing – review & editing. **Francesca Provenza:** Investigation, Methodology, Writing – review & editing. **Damià Barcelò:** Investigation, Conceptualization, Methodology, Writing – review & editing. **Monia Renzi:** Investigation, Conceptualization, Methodology, Data curation, Supervision, Writing – 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.

Data availability

Data will be made available on request.

Acknowledgments

We would like to thank Alfonso Botto (Exenia Group s.r.l., Pinerolo, Turin, Italy), Cinzia Margherita Bertea, Giuseppe Mannino and Marco Micera (Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy) for providing the chemical profile of the F1-BEO extract.

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