

Comparatory Evaluation of the Ecotoxic Effects of Flavour and Fragrance Aldehydes, Ketones, Oximes, and Oxime Ethers

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Abstract

Due to their toxicity, some aroma chemicals are banned by national, regional, and international regulatory bodies. As a result, the development of novel fragrance compounds is in line with the goals of several industrial sectors, including household chemistry, cosmetics, and fragrance. In this study, we examine the effects of new potential fragrances of the oxime ether class on bacteria (*Alivibrio fischeri*), microalgae (*Raphidocelis subcapitata*), plants (*Spirodela polyrhiza*) and crustaceans (*Daphnia magna*). We assessed a total of 24 substances including commercially relevant flavors and fragrances such as perillartine, buccoxime, stemone or citral oxime, and compared the oxime ethers' and oximes toxicity to that of the parent carbonyl compounds. The toxicity of the oxime ethers ranged from non-acute (class I), low-acute (class II), acute (class III), high-acute (class IV), and very high-acute toxicity (V). In most cases, the toxicity of oxime ethers was similar to or lower than the toxicity of commercially exploited flavours and fragrances. The most sensitive organisms were *A. fischeri* and *S. polyrhiza*, and all of the compounds showed very high acute toxicity toward it.

Introduction

The development of the industry can be destructive to the environment and the most obvious is the fact that all new compounds introduced to the market should be tested in terms of their environmental impact (Walker et al. 2012, Yao et al. 2018). Since the 1980s, it has been widely preferred to carry out ecotoxicological tests for this purpose (Chapman 1986). Microbiotests are widely used to evaluate the biological activity of chemical samples and solid / liquid waste (Persoone et al. 2009). They are based on various types of organisms, which also represent different trophic levels of the aquatic food chain (producers, consumers, and destructors): bacteria, algae, protozoa, crustacea, and rotifers (M. Kaza 2007). The advantages of microbiotests are reproducibility, precision, speed, simplicity, standardisation, and sensitivity (Johnson 2005). There is a constant need to upgrade existing knowledge on the influence and toxicity of chemical compounds on the aquatic environment. In the European Union, REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) regulates the implementation of chemicals, including the need to provide adequate ecotoxicological information (Narula et al. 2006). Therefore, by the end of 2017 the ECHA (European Chemicals Agency) database contained 67 005 registrations from 12 242 companies (European Chemicals Agency 2018).

Terpenes are one of the largest groups of secondary metabolites in plants and consist of isoprene units. Terpenoids are a modified class of terpenes with added functional groups. Terpenes and terpenoids are produced by plants, animals, and marine organisms (Perveen 2018). These compounds are commonly used in the agriculture, pharmaceutical, chemical and food industries as flavours, solvents, fragrances, pharmaceuticals, pesticides and also as chiral intermediates (Rubulotta &Quadrelli 2019, Schwab et al. 2013). Environmental problems can arise when these types of compounds enter sewage effluent as volatile organic compounds (VOCs) (González et al. 2019). Terpenes and their derivatives are transmitted to the environment from cleaning products, air fresheners, cosmetics, perfumes, and food waste (Kim et al. 2015). European regulators prohibit the use of certain fragrance compounds due to their toxicity.

Therefore, the search for new fragrance compounds aligns with the interests of industries such as cosmetics, perfumery, and household chemistry. In this article, we examine the influence of new potential fragrances of the oxime ether class (Table 1) that were developed in our group (Balcerzak et al. 2022a) on bacteria (*Alivibrio fischeri*) (Engelbrecht et al. 1983), microalgae (*R. subcapitata*) (Pavlić et al. 2005), plant (*S. polyrhiza*) (Hoang & Schubert 2017), and crustacean (*D. magna*) (Carvalho 1987). To the best of our knowledge, this is the first report on the ecotoxicity of low-molecular-oxime ethers. We have evaluated 24 compounds in total and compared the toxicity of oxime ethers to parental oximes and carbonyl compounds, including commercially exploited and relevant flavours and fragrances.

Table 1

Olfactory profile of investigated carbonyl compounds, their oximes, and oxime ethers. Commercially exploited chemicals have been marked grey.

Compound		
-	Buccoxime (fresh, fruity, natural, buchu, clean)	-
-	Stemone (galbanum, fig, leafy, green, weedy, cortex)	-
Propiophenone (hawthorn, lilac, floral, aromatic, cherry, herbal)	Propiophenone oxime (floral, gerbera, and geranium)	Propiophenone oxime <i>O</i> -ethyl ether (Fruity (red gooseberry) with citrusy (lime) nuances
Dihydrocinnamaldehyde (green, aldehydic, floral, melon)	Dihydrocinnamaldehyde oxime (delicate, cinnamon-like)	Dihydrocinnamaldehyde oxime <i>O</i> -methyl ether (spicy, cinnamon-like)
<i>o</i> -Tolualdehyde (cherry)	<i>o</i> -tolualdehyde oxime (chemical, phenolic)	<i>o</i> -tolualdehyde oxime <i>O</i> -methyl ether (cresol-like, floral, narcissus, ylang and tuberose notes)
<i>m</i> -Tolualdehyde (sweet, fruity, cherry, almond, bitter, almond, phenolic)	<i>m</i> -tolualdehyde oxime (chemical, phenolic)	<i>m</i> -tolualdehyde oxime <i>O</i> -methyl ether (fruity, grapefruit, camphor-like with cherry tree notes)
<i>p</i> -Tolualdehyde (fruity, cherry, phenolic)	<i>p</i> -tolualdehyde oxime (oriental, tonka bean, and heliotropine notes)	<i>p</i> -tolualdehyde oxime <i>O</i> -methyl ether (fresh, herbal, peach with a watery note)
Citral (lemon)	Citral oxime (diffusive musty odour, suggestive of sea weeds)	-
-	Cyclocitral oxime (intense, earthy with patchouli, moss, and leather notes)	Cyclocitral oxime <i>O</i> -methyl ether (tropical, cyclocitral-like)
Perillaldehyde (fresh, green, oily, grassy)	Perillaldehyde oxime (perillartine) (weak, similar to a grated carrot root)	Perillaldehyde oxime <i>O</i> -methyl ether (sea-floral/floral with the sweetness of heliotrope)

Materials and Methods

Reagents

Buccoxime CAS# 75147-23-8 purity: >99%, stemone CAS# 22457-23-4 purity: >99%, citral CAS# 5392-40-5, purity: 96%, dihydrocinnamaldehyde CAS# 104-53-0, purity: 95%, perillaldehyde CAS# 2111-75-3, purity 92%, propiophenone CAS# 93-55-0, purity 99%, *m*-tolualdehyde CAS# 620-23-5, purity 97%, *o*-tolualdehyde CAS# 529-20-4, purity 97%, and *p*-tolualdehyde CAS# 104-87-0, purity: 97%, were purchased from Sigma-Aldrich, TCI or Perfumers World. The remaining oximes (propiophenone oxime CAS# 2157-50-8, purity > 99%; citral oxime CAS# 13372-77-5, purity 99%; cyclocitral oxime CAS# 73661-48-0, purity 99%; dihydrocinnamaldehyde oxime CAS# 1197-50-8, purity 98%; perillaldehyde oxime CAS# 30950-27-7, purity 90%; *m*-tolualdehyde oxime CAS# 41977-54-2, purity > 99%; *o*-tolualdehyde oxime CAS# 24652-62-8, purity 97%; *p*-tolualdehyde oxime CAS# 3235-02-7, purity > 99%), and all oxime ethers (cyclocitral oxime *O*-methyl ether CAS# 169256-92-2, purity 96%; dihydrocinnamaldehyde oxime *O*-methyl ether CAS# 69163-87-7, purity 94%; perillaldehyde oxime *O*-methyl ether (lack of CAS#), purity 98%; propiophenone oxime *O*-ethyl ether (lack of CAS#), purity 95%; *m*-tolualdehyde oxime *O*-methyl ether CAS# 95082-15-8, purity 99%; *o*-tolualdehyde oxime *O*-methyl ether CAS# 646051-32-3, purity 97%; *p*-tolualdehyde oxime *O*-methyl ether CAS# 70286-36-1, purity 99%) were described in our previous reports (Balcerzak et al. 2019, Balcerzak et al. 2022b). Oximes were prepared in basic conditions from corresponding carbonyl compounds in the reaction with hydroxylamine. After purification oximes were *O*-alkylated with alkyl halides and purified by vacuum distillation. Chromatographic and spectral data are available for both oximes (Balcerzak et al. 2019) and oxime ethers (Balcerzak et al. 2022b).

Ecotoxicological tests:

All microbiotests were purchased from Microbiotests Inc., except Microtox reagents, which were purchased from Modern Water. The procedures were carried out according to the norms (Spirodela duckweed toolkit ISO norm 20079, Algaltoxit F ISO norm 8692 and OECD Guideline 201, Daphtoxkit F magna ISO norm 6341 and OECD Guideline 202, Microtox ISO norm 11348-3). We modified the introduction of compounds in cultures tested because of their low solubility in water. Initial concentrations and appropriate dilutions of compounds were prepared in DMSO. The DMSO concentration in the assays was not greater than 0.5% for *Daphnia magna* and *R. subcapitata* and not greater than 0.25% for *Spirodela polyrhiza*. This level of DMSO did not affect culture development. The toxic effect was transformed into toxic units (TU) with the formula: $TU = [1/EC_{50}] \cdot 100$ and classified with the hazard classification system described by G. Persoone and co-workers (Persoone et al. 2003).

Daphtoxkit F™

The same standard freshwater was prepared for hatching and medium. Three days before the appropriate tests, rinsed ehippia were transferred to a Petri dish in 50 ml of preaerated standard freshwater and incubated at 22 ° C under continuous illumination of a minimum of 6000 lux. The neonates were pre-fed with spirulina powder 2 h before the toxicity test. The test plate control wells were

filled with 10 mL of standard freshwater. The other wells were filled with 9.95 mL of standard freshwater and 0.05 mL of appropriate tested concentration and 5 neonates were transferred to each well as described by the producer. The number of dead and immobilized neonates was determined after 24 and 48 h of incubation in darkness at 20°C.

Algatoxkit F™

Standard freshwater was prepared for medium. The algae were deimmobilised and optical density measurements of the algal suspensions were carried out using a Jenway 6300 spectrophotometer. The concentrated algal inoculum was prepared. The control long cells were filled with 24.75 mL of standard freshwater. The remaining long cells were filled with 24.62 mL of standard freshwater and 0.125 mL of the appropriate concentration. To all long cells 0.25 mL of concentrated algal inoculum was added. Each concentration and the control were assayed in three replicates. The measurement of optical density was carried out after 24, 48, and 72 h of incubation with a constant uniform illumination supplied by cool white fluorescent lamps with sideways illumination of 10,000 lux. The calculation of the inhibition of algal growth in each concentration of toxicants was made using software for the treatment of Algatoxkit data supplied by the manufacturer (Microbiotests Inc.).

Spirodela Duckweed toxkit

The Steinberg medium was prepared for growth and test medium. Three days before appropriate tests, turions were placed in a Petri dish filled with 30 mL of Steinberg medium and incubated at 25°C with continuous top illumination (at least 6 000 lux) on the surface. The multiwell plate was filled with Steinberg medium: 1 mL for control and 0.997 mL for the appropriate concentration tested. 2.5 µL of the test solutions were added to the test wells. The germinated turions were transferred to all rows of a multiwell plate (1 germinated turion in each row). The photo of the multiwell plate was taken after setting up the experiment and after 3 days of incubation at 25°C, with a continuous illumination of 6000 lux (at the top of the multiwell). Appropriate measurements (Image J software) and calculations (excel sheet available from MicroBioTest Inc.) of the area of the fronds were made.

Microtox

Microtox analysis is based on a specific clonal strain of *Alivibrio fischeri* (formerly *Vibrio fischeri*). These bacteria under optimal environmental conditions emit light. When a substance negatively affects *A. fischeri*, the light emission is reduced. Microtox acute toxicity test was carried out using the Model 500 Microtox Analyzer and 81.9% Basic Test protocol (Rosen et al. 2008) with 14 dilutions. The tested compounds were dissolved in dimethyl sulfoxide (DMSO). Due to the lack of EC₅₀ data, 0.036% of the fragrances in DMSO were prepared and tested. The concentrations of the samples used in the calculations took into account the initial dissolution of a sample in DSMO. Therefore, the initial concentration was $81.9/100 \cdot 0.036 = 0.0295\%$. The luminescence intensity was measured after 0, 5, and

15 minutes of exposure time. For the calculations of percent inhibition of light emission, the method was applied as described by Gaudet (I.D. 1994)

Results and Discussion

The toxicity of investigated ketones, aldehydes, oximes, and oxime ethers on *Daphnia magna*, *R. subcapitata*, *Spirodela polyrhiza*, and *A. fischeri* is described below. The test results indicate that the compounds being tested range from non acute toxicity to very high acute toxicity, depending on the organism tested. For example, the most toxic compound for *R. subcapitata* was found to be cyclocitral oxime (Figs. 1 and 2, Table S1) with an EC₅₀ of 0.034 mg/L, followed by citral (EC₅₀ = 0.13 mg/L), which both fall into the V class of toxicity. Figure 2 represents growth inhibition in % vs. the logarithm of the concentration in mg/L. The more the curve is shifted to the left, the higher the toxicity. The slope of the curve is also important; for example, the oxime *o*-tolualdehyde curve has the lowest slope, and the tested range of the concentration logarithm does not reach the 50% inhibition value in the tested concentration, which means that this compound is the least toxic. Other compounds such as *o*-tolualdehyde oxime (EC₅₀ = 1.04 mg/L) and *p*-tolualdehyde (EC₅₀ = 1.27 mg/L) are classified as high acute hazards and fall into the IV class of toxicity. Most of the other compounds, with the exception of the *p*-tolualdehyde oxime *O*-methyl ether (EC₅₀ = 92.35 mg/L) and dihydrocinamaldehyde (EC₅₀ = 75.79 mg/L) which fall into the III class of toxicity, are classified as slight acute hazards or non/low-toxic and fall into the I or II class of toxicity. It should be noted that no EC₅₀ values were given for the cyclocitral oxime *O*-methyl ether and *p*-tolualdehyde oxime as they significantly exceeded the test range. (Table S1). Toxicity values have been previously reported for only 2 compounds, commercially available oximes stemone and buccoxime. The difference from our results might come from the variability in the experimental procedure and in the strain used.

Evaluation of ecotoxic properties on *D. magna* (Table S2, Fig. 3) revealed that all compounds show only two levels of toxicity: high acute toxicity (IV class) and acute toxicity (III class). The least toxic among the compounds tested was propiophenone with an EC₅₀ of 80.14 mg/L. Other compounds that belong to this type of toxicity included buccoxime, citral, citral oxime, cyclocitral oxime, dihydrocinnamaldehyde, perillaldehyde, perillaldehyde oxime, perillaldehyde oxime *O*-methyl ether, and stemone. The highest toxicity was found for cyclocitral oxime with an EC₅₀ of 2.56 mg/L after 48 hours, which shows high acute toxicity, together with the remaining compounds. In almost all cases oxime ethers were comparable or less toxic than parental oximes or commercially available carbonyl compounds. In the series of two isomers (*m*- and *p*-) of tolualdehyde a clear pattern can be noticed where oximation and subsequent alkylation led to the significant decrease of toxicity.

In the case of the effect of flavour and fragrance carbonyl compounds, oximes and oxime ethers on the development of *S. polyrhiza*, the EC₅₀ of all compounds were in the range of $5 \cdot 10^{-5}$ -0.15 mg/L (Fig. 4, Table S3), indicating very high acute toxicity (class V). Perillaldehyde oxime *O*-methyl ether (EC₅₀ = $5 \cdot 10^{-5}$

⁵ mg/L) was the most toxic to *S. polyrhiza* and dihydrocinamaldehyde was the least toxic with EC₅₀ 0.15 mg/L after 72 h.

Microtox

Figure 5 shows the toxicity effect EC₅₀(15) of the tested chemical compounds in relation to the luminescent bacteria *Alivibrio fischeri*. Detailed information on the tested parameters, *i.e.* EC₅₀ values, toxicity units (TU) and estimating equation and 95% confidence range, are included in Table S4 in Supporting Information.

Alivibrio fischeri are naturally luminescent Gram-negative marine bacteria, also known as *Photobacterium phosphorum* NRRL-B-11177 and *Vibrio fischeri*. *A. fischeri* reacts quickly to bioavailable toxicants by decreasing its natural bioluminescence in seconds to minutes, depending on the chemical structure of the toxicant and its concentration (Kurve et al. 2017). Biotests using *Alivibrio fischeri* bacteria are a very useful tool used to assess the degree of toxicity of a given compound (Kaiser & Devillers 1994, Kurvet et al. 2011, Parvez et al. 2006, Suppi et al. 2015). Under optimal conditions, these bacteria are able to use about 10% of the energy that comes from metabolism for glowing. In a polluted environment or in the presence of compounds that have an unfavorable effect on bacterial cells, their metabolic processes are disturbed and the luminescence disappears. Therefore, the reduction in light output reflects the inhibition of bacterial metabolic activity and is proportional to the toxicity of the chemical or test sample (Bulich 1982, Woodland Hastings et al. 1987).

EC₅₀(15) values were obtained from the line of best fit through the four data points on a plot (usually log-log) of test solution concentration vs. Gamma light loss factor. Gamma tells us the ratio of the amount of light lost to the amount of light remaining and can be converted into %effect. The Gamma value cannot be negative. This would prove that the addition of a toxicant stimulates the bacteria to glow. Therefore, negative values were not taken into account for further calculations. The EC₅₀(15) value, corresponding to the halving of light intensity after 15 minutes, is the (anti-logarithm) of the intercept where Gamma = 1, *i.e.* where log Gamma = 0 (Gellert 2000). The lower the EC₅₀ value, the more toxic the sample is. In order to be able to present these values in a clearer way, toxic equivalents were used, where the higher the value of the TU factor, the more toxic the substance is (Ashworth & Symonds 2013, ISO Technical Committee 2018, Pogorzelec & Piekarska 2017, SDI 1992).

All chemical compounds tested by us should be classified as toxic class V (acute toxicity) based on the EC₅₀ parameter (Table S4) and calculated values of the TU factor after 15 minutes of contact of luminescent bacteria with the tested substance. The highest value of this coefficient (347,056) was obtained for *m*-tolualdehyde oxime *O*-methyl ether, and the lowest value of 6,480 for propiophenone oxime *O*-ethyl ether.

Reducing the toxicity in relation to the starting compounds of the synthesis was observed only for cyclocitral oxime *O*-methyl ether and propiophenone oxime *O*-ethyl ether. Such high values of toxicity units obtained in this test compared to other tests could be due to the shortest contact time of the test organisms with volatile chemicals.

Conclusion

The results showed that the evaluated flavours and fragrances belong to five different classes of toxicity. However, the toxicity in most of the cases studied is similar or lower than that of commercially used compounds such as all aldehydes and ketones (citral, cyclocitral, dihydrocinnamaldehyde, propiophenone, tolualdehydes), and oximes stemone, buccoxime, and citral oxime. The most sensitive organisms to all compounds were *A. fischeri* and *S. polyrhiza* (all compounds showed very high acute toxicity), and the most resistant organism to the tested compounds was *R. subcapitata*, where most compounds showed low acute toxicity. This article provides the first ecotoxicological data on volatile oxime ethers. The results show that their potential application poses a similar or lower risk to the aquatic environment compared to the commercially exploited volatile aldehydes, ketones, and oximes.

Declarations

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CRediT authorship contribution statement

Conceptualisation: DJS; methodology: DJS, LB, AT, KP; investigation: DJS, LB, AT; analysis of the data: DJS, LB, AT, KP; writing & review: DJS, LB, KP; Project administration: DJS. All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors are interested in the commercialisation of oxime ethers. This fact did not influence any of the results of this study. All opinions and discussion points are based on the scientific data generated within this and existing studies.

Data availability

Data will be made available upon reasonable request.

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Authors agreed with the content of this work and gave explicit consent to submit it.

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Figures

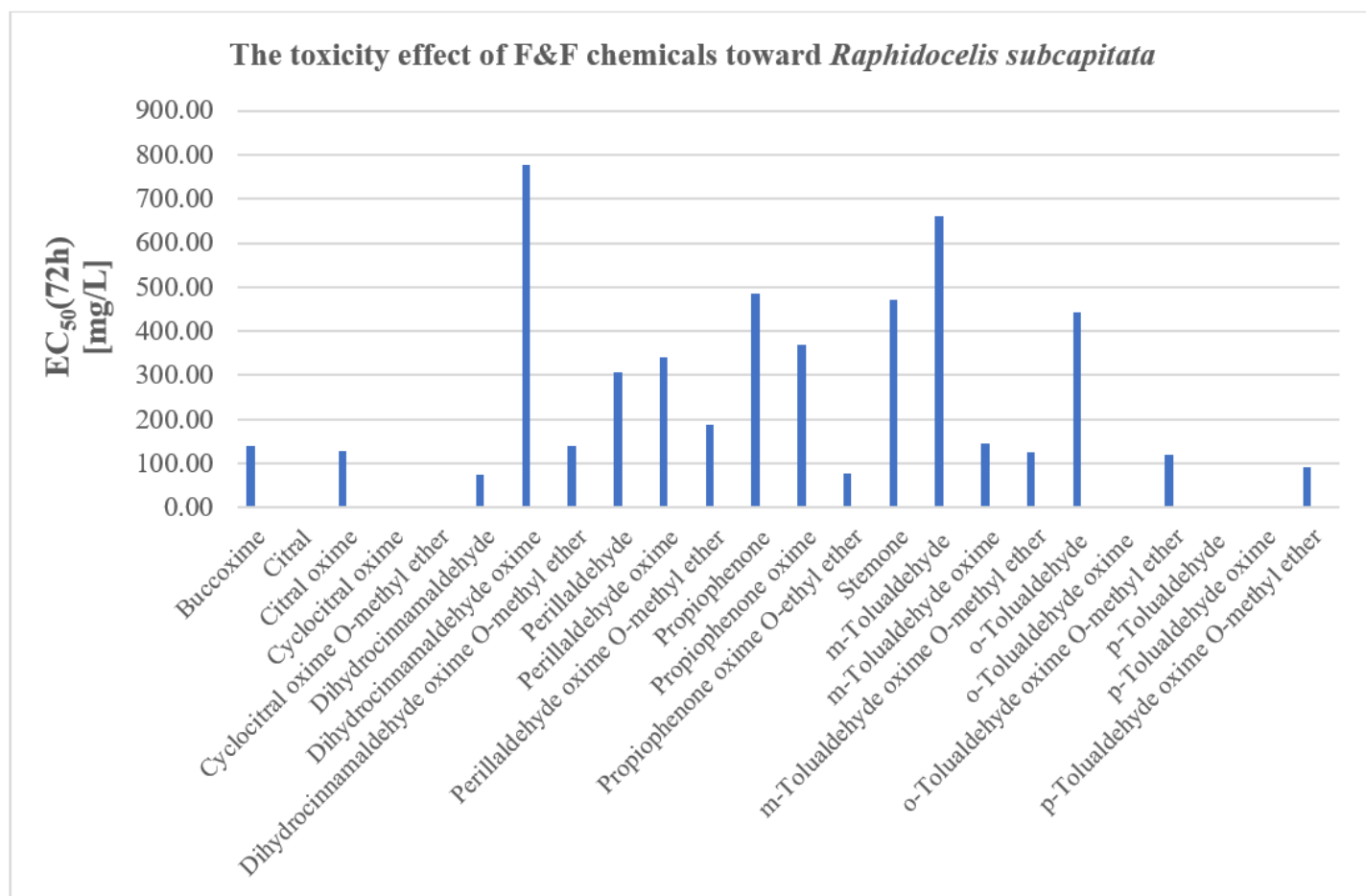


Figure 1

EC₅₀ values of target compounds for *Raphidocelis subcapitata*

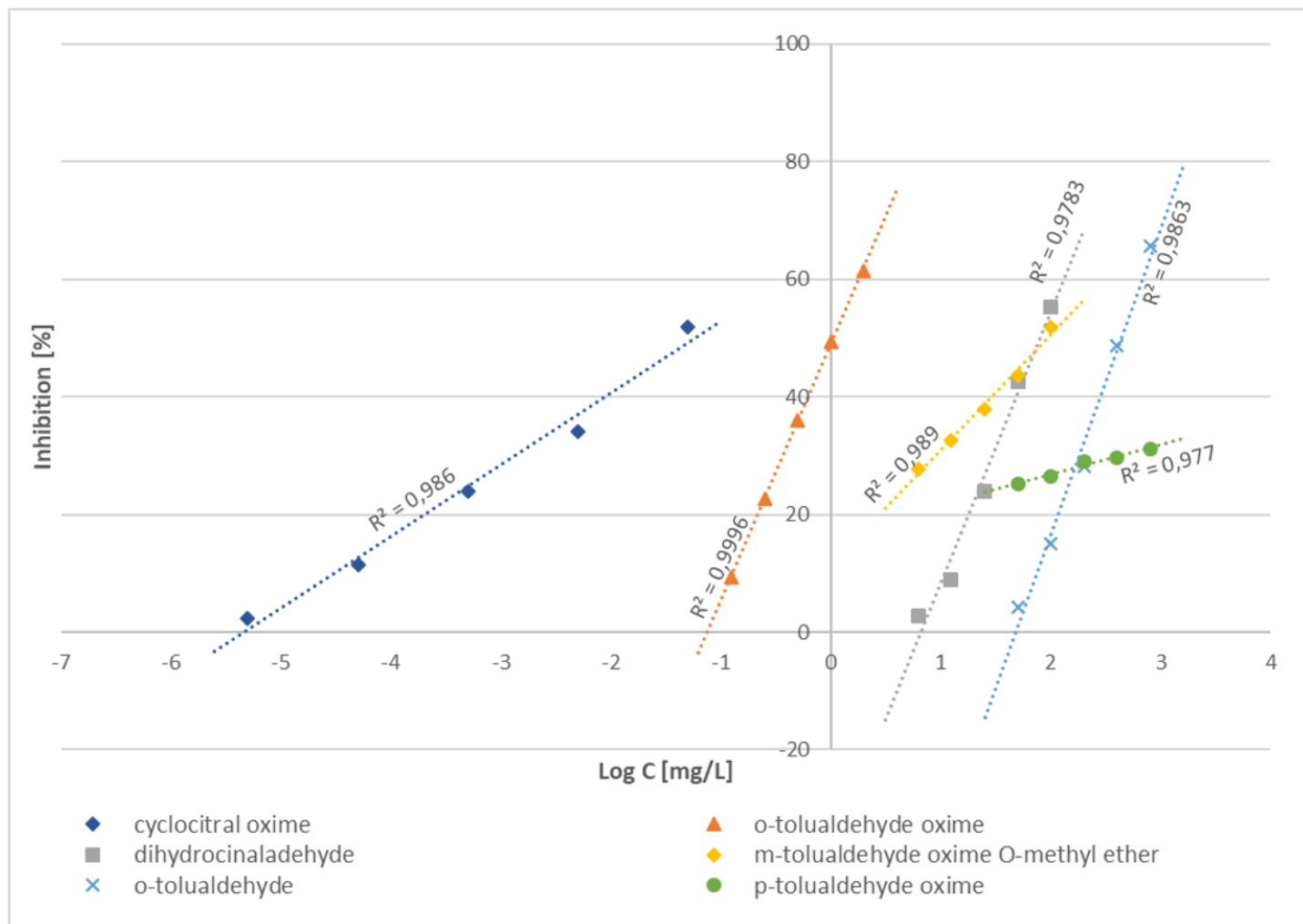


Figure 2

Percentage inhibition of the growth of *R. subcapitata* for selected compounds.

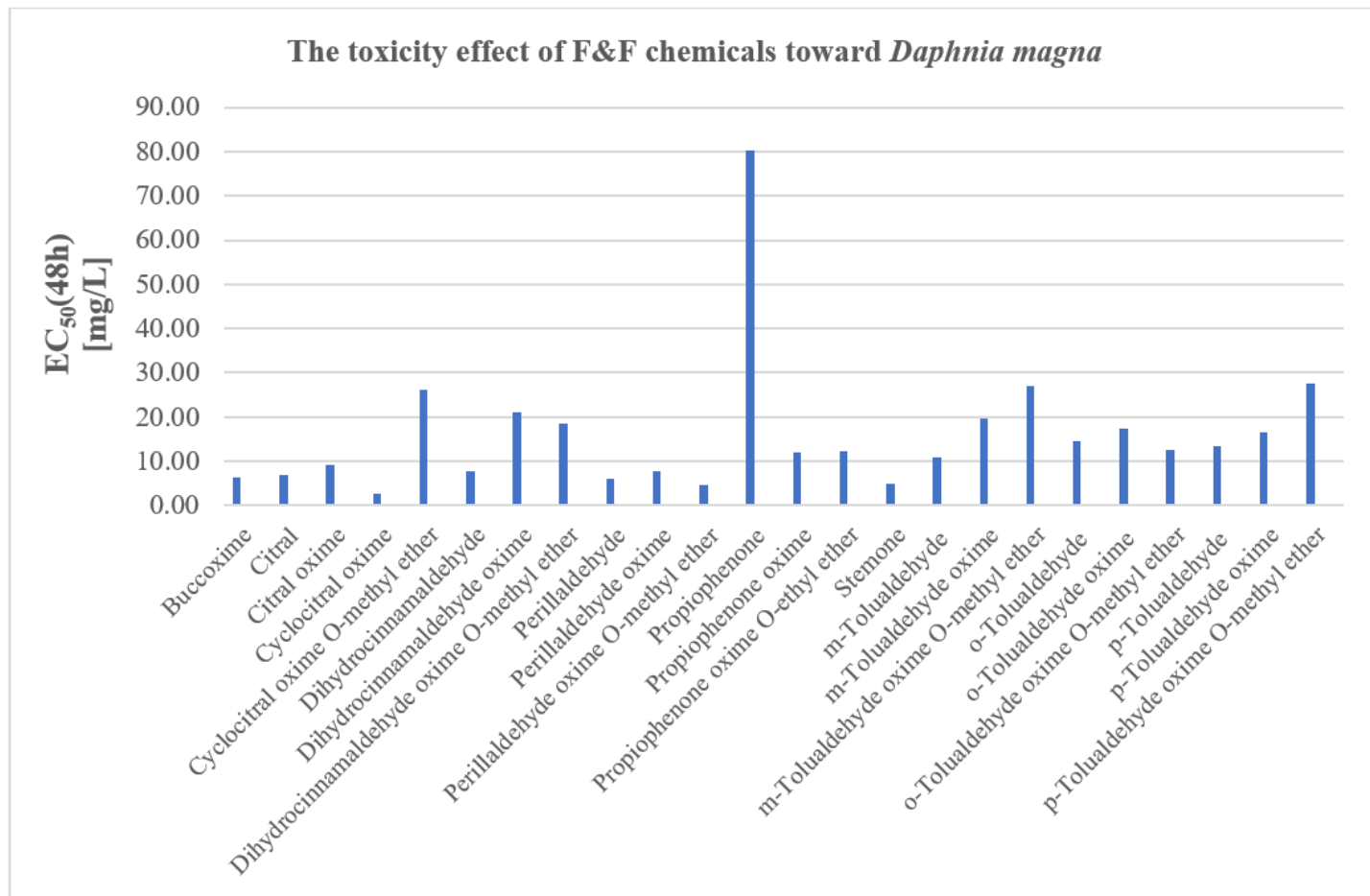


Figure 3

EC₅₀ values, confidence limits, toxic units (TU), and concentration range of target compounds (*Daphnia magna*).

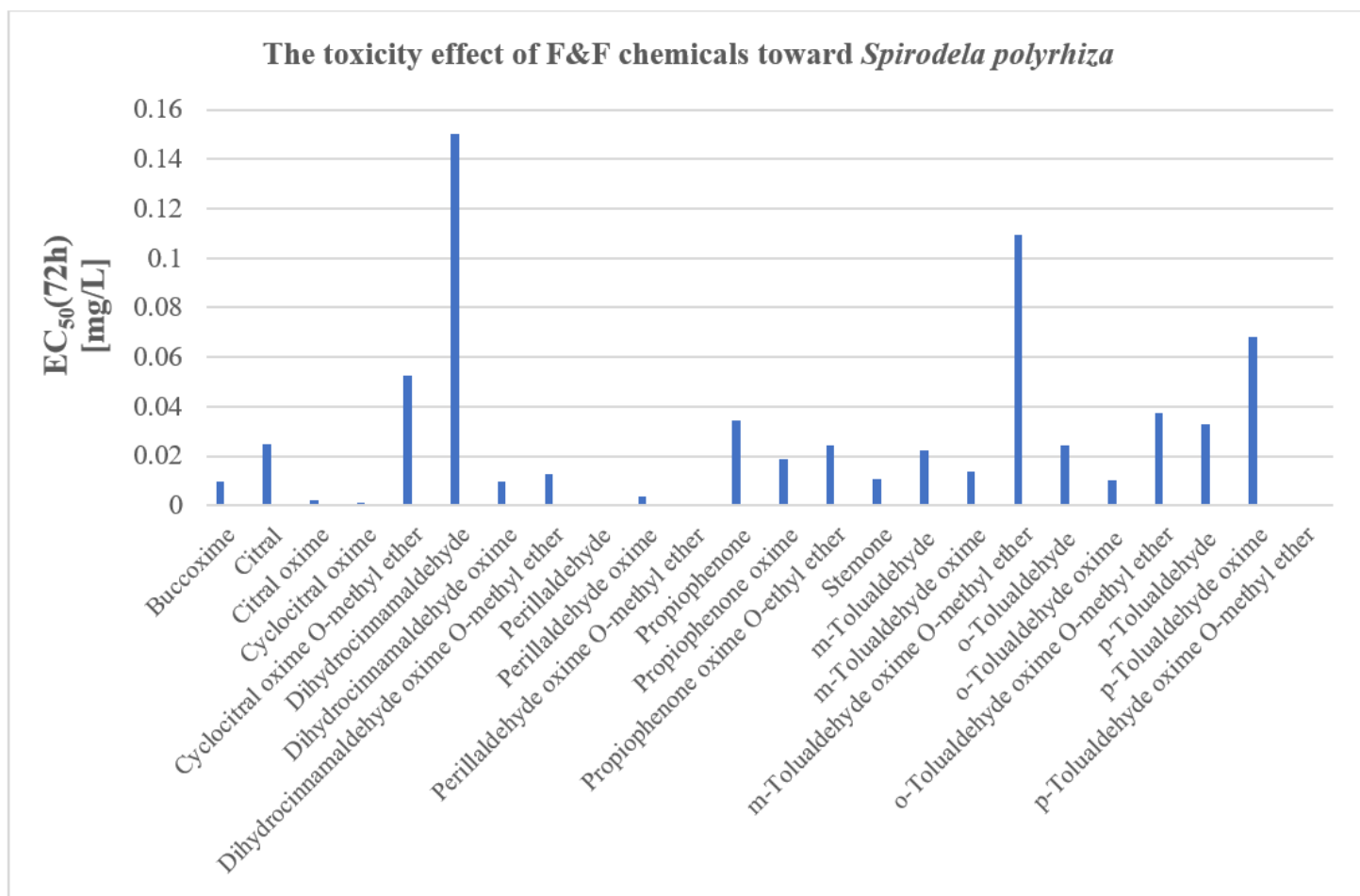


Figure 4

EC₅₀ values of target compounds for *Spirodela polyrhiza*.

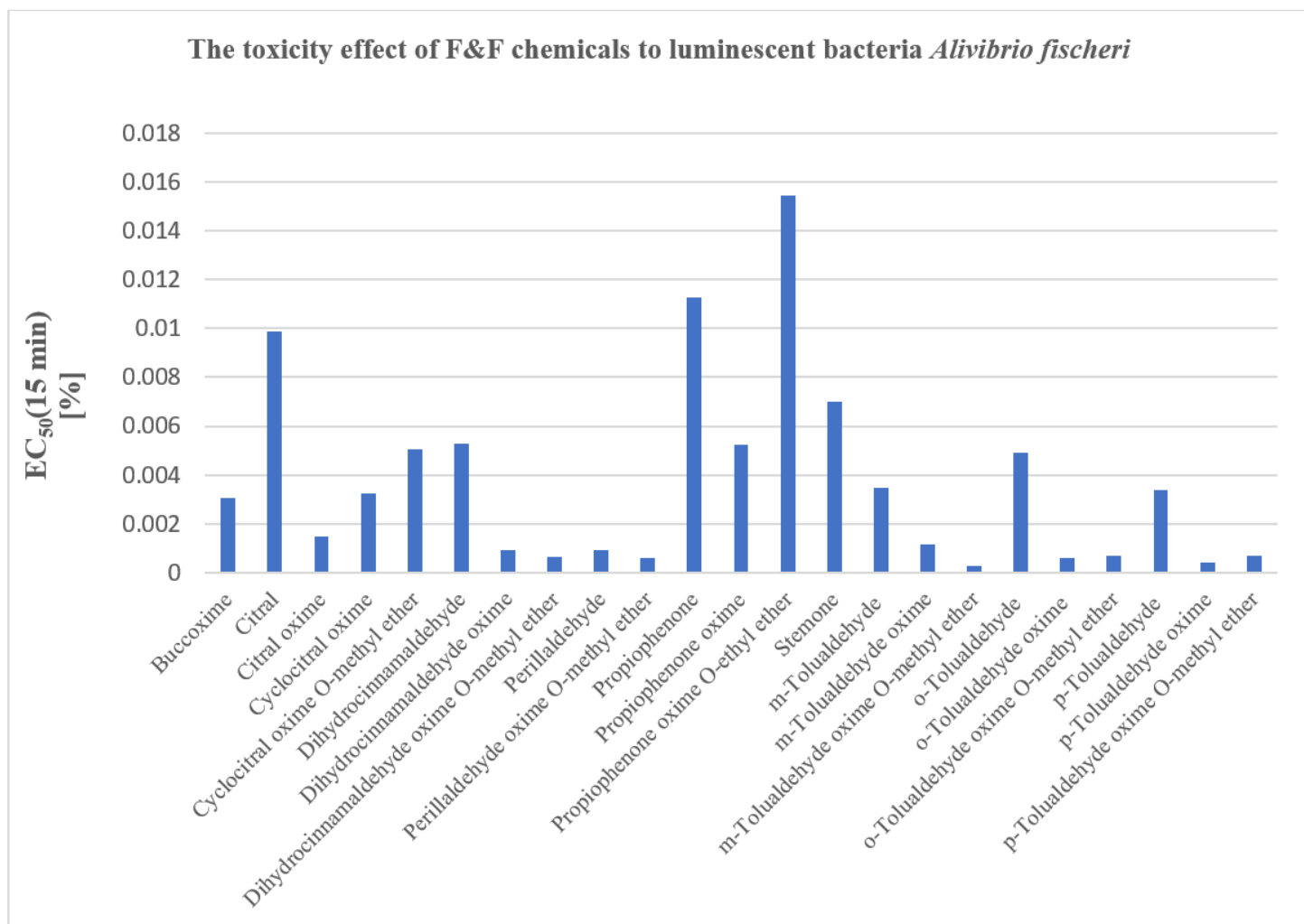


Figure 5

Toxicity effects of flavour and fragrance compounds for *A. fischeri*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryecotoxv2.docx](#)