

# Preliminary ecotoxicity assessment of selected flavour and fragrance compounds using Microtox assay

Wojciech Kołodyński<sup>1,\*</sup>, Katarzyna Piekarska<sup>1</sup>, and Daniel Strub<sup>2,3</sup>

<sup>1</sup>Wrocław University of Science and Technology, Faculty of Environmental Engineering, Wyb. Wyspiańskiego 27, 50-370 Wrocław, Poland

<sup>2</sup>Wrocław University of Science and Technology, Faculty of Chemistry, Wyb. Wyspiańskiego 27, 50-370 Wrocław, Poland

<sup>3</sup>Liquid Technologies sp. z o.o., ul. Chełmońskiego 12, 51-630 Wrocław, Poland

**Abstract.** The bioluminescence inhibition bioassay using marine bacteria *Vibrio fischeri* is widely used as a tool to assess the toxicity of chemical compounds, because of the many benefits comprising cost and time effectiveness, rapidness, sensitivity, and ease of operation. In addition, the test is used for various types of organic and inorganic compounds. Due to the insolubility of organic compounds in water, a suitable solubilizer should be applied. The ecotoxicity of the solvent should be negligible in relation to marine bacteria. On account of superior human activities the synthesis of new chemical substances is inextricably linked to the emergence of chemical compounds that are a potential threat on environment. A Microtox system utilizing the 81.9% Basic Test with 14 dilutions was applied to pre-evaluate the ecotoxicity of  $\beta$ -cyclocitral oxime, citronellal oxime and perillaldehyde oxime. Substances solutions with an initial concentration of 0.036% in DMSO were prepared. The preliminary results show that the studied scent compounds are characterized by quite high toxicity. In order to confirm the received ecotoxicity values, it is necessary to carry out additional surveys using another range of concentrations and biotests on organisms at each trophic level.

## 1 Introduction

Over the last years, a constantly growing request for synthesis of new fragrance components has been observed [1]. The olfactory potential depends mainly on the structure of the designed molecules and an osmophore (functional group) [2, 3]. The chemical groups responsible for a pleasant smell include: ester, ketone, aldehyde, ether and hydroxyl groups, whereas the presence of thiocarbonyl, thioformyl, thioether, thiol and amine groups result most often in unobjectionable smell. Fragrances with slight changes in the chemical structure may differ significantly in aroma [4]. A small change in the spatial structure caused by the removal or addition of one atom or chemical group in the molecule may be sufficient to

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\* Corresponding author: [wojciech.kolodynski@pwr.edu.pl](mailto:wojciech.kolodynski@pwr.edu.pl)

increase the intensity of the smell. Additionally the concentration of the substance plays a key role in the perception of the fragrance, because it can affect the quality of the scent [5]. Development of synthetic methods for the preparation of natural compounds was possible by unravelling their structures. Moreover, modern chemistry manners have allowed both the chemical synthesis of compounds existing in nature as components of essential oils or animal products, as well as the discovery of fragrant molecules that do not exist in nature. Fragrance ingredients are divided into natural and synthetic origin compounds. Due to the high cost of scents isolated from natural sources, more often aroma compounds are prepared via synthesis [4]. Upwards of 3000 scent-bearing compounds are applied in perfumery industry [3]. The largest amount of fragrance compounds is present in cosmetics, perfumes and household chemicals [6].

Chemical synthesis of new products is associated with the conduct of many tests to check the quality, density, purity, structure, physical-chemical properties, as well as toxicity and ecotoxicity in order to estimate the potential risk to the environment [7]. Microbiotests based on bioindicators have been available on the market for several years. They allow to determine whether a given substance may pose a threat to the environment. The toxikit microbiotests use test organisms in immobilized or dormant form, which allows them to be applied on demand if required [22]. In addition, they are an alternative to classical analytical methods [23]. This allows to avoid the step of cultivating test organisms in laboratory conditions [22]. Toxicity action of the tested chemicals is manifested by metabolic disturbances resulting from physiological and conservative changes of test organisms [10]. In these studies, the toxicological hazard assessment of the three fragrances was investigated. Test compounds, namely  $\beta$ -cyclocitral oxime, citronellal oxime, perillaldehyde oxime were supplied by the Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Science and Technology. These fragrances can be further used in household chemicals, cosmetics and perfumes or can become a substrate for the synthesis of another odour components [8]. Due to the increasingly frequent withdrawal of fragrances from the market and use, it is necessary to search for new fragrances. The scents or their derivatives may be delivered to the environment, therefore their impact on living organisms must be known, as it was in the case of camphor oxime and citral oxime ethers.

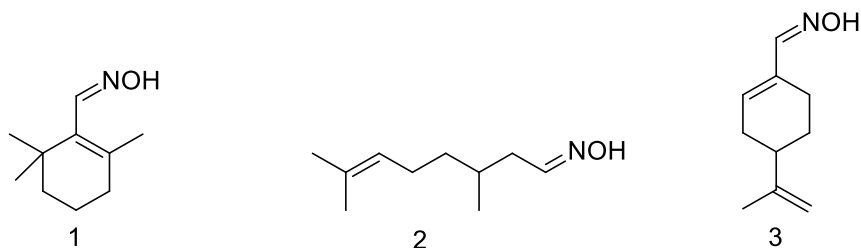
Many substances used in the cosmetic industries have an allergenic effect. In addition, they may accumulate in living organisms [8]. Fragrance components present in cosmetics commonly lead to the allergic response of the human body [9]. For example, polycyclic and nitro musk are one of the substances applied in the cosmetic industry that have a negative impact on people. These compounds and their derivatives are characterized by high toxicity and bioaccumulation [8].

Fast and reliable assessment of the acute ecotoxicity of a new substance can be carried out by employing Microtox test. This biotest is widely applied to monitor toxicity due to many benefits encompassing cost and time effectiveness, rapidness, sensitivity, and ease of operation. Biotest based on the inhibition of luminescence in marine bacteria *Vibrio fischeri* is utilized to all types of matrices, both organic and inorganic compounds. Furthermore, conducting a complete ecotoxicity assessment requires the usage of bioassays based on organisms from each trophic level. In addition, surveys based on marine luminescent bacteria are frequently first of the bioassays utilized in the creation of biotest battery on account of the speed and cost of the procedure for its execution [11, 12].

The Microtox Acute Toxicity Test utilize a clonal strain of bioluminescent bacteria supplied in lyophilized vial format. During the toxicological evaluation of organic substances that do not dissolve in the aquatic environment, solubilization is used. For this purpose, carrier solvents such as DMSO, acetone or ethanol may be necessary to solubilize chemicals. Finding the right solubilizer is the main challenge when assessing the ecotoxicity of organic compounds. However, solvents possess their own ecotoxicity and should be selected so that

they do not affect the results of the test substance. Therefore, an important step during the assessment of the ecotoxicity of chemical substances is to eliminate the toxic effects of the solubilizer. Therefore, an analysis based on a comparison of the ecotoxic effect of the solvent and the solvent with dissolved substance is performed.

In the paper we present preliminary studies on the ecotoxicity of three flavour and fragrance substances, namely  $\beta$ -cyclocitral oxime, citronellal oxime and perillaldehyde oxime. The chemical structures of  $\beta$ -cyclocitral oxime, citronellal oxime and perillaldehyde oxime are presented in Figure 1. The aim of the research was to determine the level of ecotoxicity of the compounds listed above. Microtox system was chosen for ease of operation and high sensitivity.



**Fig 1.**  $\beta$ -cyclocitral oxime (1), citronellal oxime (2), perillaldehyde oxime (3) chemical structures.

## 2 Materials and methods

### 2.1 Microtox toxicity analysis

The Microtox toxicity analysis is based on a specific clonal strain *Vibrio fischeri* marine bacteria, earlier referred as *Photobacterium phosphoreum* [11]. Light production is directly related to the metabolic activity of bioluminescent microorganisms [12]. The test relies on disrupting the emission of light in optimal environmental conditions as a result of toxic substances acting on the marine bacteria *Vibrio fischeri*, which lead to the reduction of light emission. The reaction mechanism is that marine luminous bacteria contain luciferase that catalyses the oxidation of a reduced flavin mononucleotide and aliphatic long-chain aldehyde by oxygen. As a result of this reaction, an unstable complex is formed which in next step emits photons during decomposition with its highest intensity at wavelength 490 nm. Bioluminescence is directly related to respiration through the electron transport chain, therefore the bacteria are used to evaluate the toxicity of the test substances [10, 13]. The Microtox 81,9% Basic Test with 14 dilutions was chosen to provide a wider range of tested concentrations in view of the fact that the ecotoxicity of tested substances was completely unknown. The ecotoxicity of fragrance substances dissolved in DMSO was measured. Possible toxicity carrier solvent DMSO, a substance that facilitates the dissolution of organic compounds in water, has been tested in previous studies to assess the potentially ecotoxicity effects on bioluminescent bacteria [7]. For this reason, the re-evaluation of the ecotoxicity of carrier solvent DMSO is omitted.

## 2.2 Calculations

Luminescence intensity was measured by means of a Microtox™ Model 500 Analyser after 0-, 5-, 15-minute exposure time. For the calculations of percent inhibition of light emission, the following equations were applied:

$$R_t = \frac{I_{Ct}}{I_{C0}} \quad (1)$$

$$\Gamma_t = \frac{R_t \cdot I_{S0}}{I_{St}} - 1 \quad (2)$$

$$\%inhibition = \frac{\Gamma_t}{1+\Gamma_t} \cdot 100 \quad (3)$$

where  $R_t$  is a correction for any inhibition induced by a negative control sample (blank cuvette) and  $I_{C0}$ ,  $I_{Ct}$  are absolute light intensities produced by negative control at time  $t$  and at initial time 0, respectively.  $I_{S0}$  and  $I_{St}$  are light intensities produced by a sample at time  $t$  and at initial time 0, respectively.  $\Gamma_t$  is the ratio of light lost at time  $t$  to light remaining at time  $t$ , which can be converted to %inhibition. The final expression of the toxic response of the samples is presented as  $EC_{50}$  which is the Effective Concentration of the samples reducing light emission by 50% and estimated from linear regression of the log of each concentration level of the contaminant versus percent inhibition (%inhibition), as described in [7, 14].

## 2.3 Samples preparation

Samples of  $\beta$ -cyclocitral oxime, citronellal oxime and perillaldehyde oxime were supplied by the Department of Bioorganic Chemistry, Faculty of Chemistry, Wrocław University of Science and Technology. On account of insolubility in water of each of substances, their solubility in DMSO was conducted. It was found that each of the substances easily dissolved in DMSO. According to the article [7], a ecotoxicity comparison of two solubilizers was carried out using Microtox 81.9% Basic Test, namely acetone and DMSO. Due to the lower toxicity of DMSO than acetone to marine bacteria *Vibrio fischeri*, DMSO was chosen as the carrier solvent. Due to the lack of  $EC_{50}$  data, solutions 0.036% of fragrances in DMSO were prepared and tested.

The concentrations of the samples used in the calculations took into account the initial dissolving of the sample in DMSO. Therefore, the initial concentration was:

$$\frac{81,9}{100} \cdot 0,036 = 0,0295\% \quad (4)$$

## 3 Results and discussion

In the first stage of the studies, a screening test was carried out to check whether the test substances inhibit the bioluminescence of marine bacteria *Vibrio fischeri*. All of the tested fragrances showed a toxic effect on bioluminescent bacteria. In the second stage of the study, a series of 14 dilutions was made to find the concentration of the substance causing 50% inhibition of light produced by the bacteria. At the moment of dissolving the fragrances in DMSO and subsequently in water, cloudy milky solutions were formed during the test. At the time the test fragrance was added to the cuvette containing the high concentration of NaCl (derived from 2% in Diluent and 22% in Osmotic Adjusting Solution), no precipitate formed in the solution, therefore a test was carried out according to the standard Microtox protocol (81.9% Basic Test).

The analysis results of acute ecotoxicity measurements are shown in Table 1 as concentration  $EC_{50}$  and ratio of  $1/EC_{50}$ .

**Table 1.**  $EC_{50}$  parameter values of the test substances dissolved in DMSO after 5 and 15 minutes of exhibition to bioluminescent marine bacteria.

Sample	$EC_{50}$ [%]		$1/EC_{50}$	
	t = 5 min	t = 15 min	t = 5 min	t = 15 min
perillaldehyde oxime	0.000653	0.000684	1531.394	1461.988
$\beta$ -cyclocitral oxime	0.000279	0.000301	3584.229	3322.259
citronellal oxime	0.000231	0.000222	4329.004	4504.505

The results of ecotoxicity measurements of studies scent compounds are quite high. Comparing them with the toxicity values of the pure solvent, the toxicity of the carrier solvent can be considered negligible. Referring to [7], the  $1/EC_{50}$  value after the 5 and 15 minute exposure time is 0.259 and 0,380 for solutions 5%, respectively. This suggests that DMSO is a good solubilizer for further studies of organic compounds as mentioned in the Microtox Acute Toxicity Test, and due to its solubility range, low vapor pressure and low freezing point [11]. However, the results obtained need confirmation with a different range of concentrations using the Microtox test, as well as other ecotoxicity bioassays that use living organisms from different trophic levels. In the interim the conducted research can be considered as an initial assessment of the toxicity of selected investigated scent compounds.

Perillaldehyde oxime, also called perillartine, is a compound 2000 times sweeter than sucrose [15]. Perillaldehyde found in *Pertilla frutescens* (Lamiaceae) is precursor in synthesis perillartine, which is an oxime derivative [16]. May cause skin irritation, serious eye damage or respiratory irritation [17, 18]. Due to the lack of literature reports on perillartine, its ecotoxicity cannot be compared with other studies. However, the perillaldehyde substrate used for the synthesis of perillartine has been tested for genotoxicity. According to the authors of the study, perillaldehyde has no genotoxic potential [24].  $\beta$ -cyclocitral is used as a flavoring ingredient.  $\beta$ -cyclocitral is produced by *Microcystis* species. So far, it has not been demonstrated whether  $\beta$ -cyclocitral may have a negative impact on the environment [19]. In literature, the amount of available data about  $\beta$ -cyclocitral oxime is modest, it is a compound that has not been fully understood yet. Citronellal oxime is a toxic chemical substance to aquatic organisms with long lasting effects, it can also cause eye irritation [20]. Citronellal mutagenicity was tested using *Salmonella* strains. Citronellal was found to be not a mutagenic compound. In addition, it was proven that citronellal inhibited the embryonic development of mosquito *Aedes aegypti* eggs responsible for causing the yellow fever [25]. Citronellal oxime proved to be the most toxic of the substances tested. As the only one of the tested compounds, it showed an increase in ecotoxicity during a longer time of exposure to bioluminescent bacteria *Vibrio fischeri*. Marine bacteria were found to be less sensitive to perillaldehyde oxime and  $\beta$ -cyclocitral oxime. Effective concentrations that inhibits 50% of the light produced by the bacterial culture is much higher than concentration citronellal oxime. For all substances tested no data on their ecotoxicity effects were established before. Only carbonyl substrates were assessed for their toxicity on humans [17–20].

In order to evaluate the potential impact of the studied fragrances on the environment, other ecotoxicity biotests using organisms from different trophic levels should be used. The main problem of tested chemical compounds is their insolubility in water. Most of the ecotoxicological biotest uses aquatic organisms such as *Thamnocephalus platyurus*, *Daphnia*

*magna*, *Chlorella vulgaris* and so on [21]. The lipophilic components must be solubilized in organic solvents. A suitable carrier solvent should have low or no toxicity [11].

## 4 Conclusions

Biological tools, such as bioindicators, are applied to assess the toxic effects of newly formed chemicals. The synthesis of new fragrances and their introduction on the market involves the assessment of ecotoxicity in order to eliminate the potentially negative impact on the environment. The assessment of acute toxicity utilizing the Microtox system revealed quite greatly high EC<sub>50</sub> values of  $\beta$ -cyclocitral oxime, citronellal oxime and perillaldehyde oxime to relation marine bioluminescent bacteria *Vibrio fischeri*. The most ecotoxicity of all three substances tested was citronellal oxime, while the lowest acute toxicity was demonstrated by the perillaldehyde oxime, although the results necessitate confirmation. Other available ecotoxicity bioassays involving organisms at each trophic level are necessary to confirm the obtained results. Moreover, the insolubility properties of the studied fragrance compounds is a challenge in selecting the right conditions when performing the ecotoxicity assessment on aquatic organisms.

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