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Toxicity of emerging antifouling biocides to non-target freshwater organisms from three trophic levels

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Highlights (max 85 characters; 3 to 5 bullet points)

- Tralopyril caused lethal and sublethal effects on algae, daphnids and zebrafish
- Triphenylborane pyridine (TPBP) was lethal to zebrafish and daphnids but not to *algae*
- Tralopyril and TPBP altered general stress-related and compound-specific proteins
- Tralopyril and TPBP identified as posing potential risk to freshwater environments
- Capsaicin did not cause mortality up to 1mg L$^{-1}$, appears to be environment-friendly

Abstract

Antifouling (AF) systems provide the most cost-effective protection against biofouling. Several AF biocides have, however, caused deleterious effects in the environment. Subsequently, new compounds have emerged that claim to be more environment-friendly, but
studies on their toxicity and environmental risk are necessary in order to ensure safety. This work aimed to assess the toxicity of three emerging AF biocides, tralopyril, triphenylborane pyridine (TPBP) and capsaicin, towards non-target freshwater organisms representing three trophic levels: algae (*Chlamydomonas reinhardtii*), crustacean (*Daphnia magna*) and fish (*Danio rerio*). From the three tested biocides, tralopyril had the strongest inhibitory effect on *C. reinhardtii* growth, effective quantum yield and adenosine triphosphate (ATP) content. TPBP caused sub-lethal effects at high concentrations (100 and 250 µg L$^{-1}$), and capsaicin had no significant effects on algae. In the *D. magna* acute immobilisation test, the most toxic compound was TPBP. However, tralopyril has a short half-life and quickly degrades in water. With exposure solution renewals, tralopyril's toxicity was similar to TPBP. Capsaicin did not cause any effects on daphnids. In the zebrafish embryo toxicity test (zFET) the most toxic compound was tralopyril with a 120h - LC$_{50}$ of 5 µg L$^{-1}$. TPBP's 120h - LC$_{50}$ was 447.5 µg L$^{-1}$. Capsaicin did not cause mortality in zebrafish up to 1 mg L$^{-1}$. Sub-lethal effects on the proteome of zebrafish embryos were analysed for tralopyril and TPBP. Both general stress-related and compound-specific protein changes were observed. Five proteins involved in energy metabolism, eye structure and cell differentiation were commonly regulated by both compounds. Tralopyril specifically induced the upregulation of 6 proteins implicated in energy metabolism, cytoskeleton, cell division and mRNA splicing whilst TPBP lead to the upregulation of 3 proteins involved in cytoskeleton, cell growth and protein folding. An ecological risk characterization was performed for a hypothetical freshwater marina. This analysis identified capsaicin as an environment-friendly compound while tralopyril and TPBP seem to pose a risk to freshwater ecosystems. Noneless, more studies on the characterization of the toxicity, behaviour and fate of these AF biocides in the environment are necessary since this information directly affects the outcome of the risk assessment.

Keywords: Antifouling biocides; Ecological risk assessment; Non-target freshwater organisms; Proteomic differential analysis; Multidimensional protein identification technology (MudPIT)

1. Introduction

Biofouling is a natural process defined by the settlement and growth of unwanted organisms on submerged surfaces. This phenomenon may lead to major economic and environmental losses (i.e. through the transport and dissemination of non-indigenous species) and therefore biofouling prevention is essential. Traditional antifouling (AF) systems based on
coatings and paints offer a cost-effective way to prevent biofouling. The use of biocides in these systems promotes additional protection against biofouling. However, in the past, the leaching of such biocides into the aquatic environment has caused deleterious effects on various organisms (Sousa et al., 2014; Thomas et al., 2001). Therefore, it is essential to perform a proper risk assessment of emerging biocides that claim to be more environment-friendly than those currently in use. Furthermore, EU Regulation No 528/2012, also known as Biocidal Product Regulation (BPR), demands a higher level of protection for humans and the environment in order to approve the use of certain substances and products under specific product types. AF products are classified under product type 21 of the BPR (EU, 2012).

In this study we investigated the toxicity of three AF biocides: tralopyril (4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile), TPBP (triphenylborane pyridine) and capsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-6-nonenamide). Among the three, tralopyril is the only one approved for use in Europe under the BPR as AF biocide (EU, 2012). Tralopyril is the main active substance of ECONEA®, marketed as a non-persistent and biodegradable biocide, efficient in controlling fouling by barnacles, hydroids, mussels and polychaetes (Thomas and Brooks, 2010). Its half-life has been estimated to be 6.1 and 8.1 h in both sea and riverine waters (Oliveira et al., 2016b). Tralopyril is thought to act as an uncoupler, affecting ATP production in the mitochondria (European Chemicals Agency, 2014). To the best of our knowledge there are no measured environmental concentration data reported for this biocide. In fact, only a single attempt to determine its occurrence has been made so far, at a seawater harbor in Portugal, but it was not detected (Oliveira et al., 2016b). However, tralopyril was only approved to be used in AF paints, under the BPR, in September of 2014. In an earlier study we have predicted that this compound might be a potential threat to the marine ecosystems as the ratio between the predicted environmental concentration (PEC) in a seawater marina and the predicted no effect concentration (PNEC) using bioassays with early-life stages of marine species was higher than 1 (Oliveira et al., 2014).

TPBP, marketed as Borocide®, is an organoborane compound used as a broad spectrum biocide mainly in Japan (Okamura and Mieno, 2006; Wendt et al., 2013), where it has been the predominant biocide used in AF systems since 1995 (Mochida et al., 2012). The mechanisms behind its toxicity are still unknown, likely due to its limited distribution on the worldwide AF market. TPBP is reported to undergo abiotic degradation through hydrolysis and photolysis in natural seawater (Zhou et al., 2007). The estimated half-lives reported for seawater vary from 6.6 h up to 6 months depending on the degradation pathway, pH and temperature (Zhou et al., 2007). The highest environmental concentration reported for TPBP is 21 pg L⁻¹ in a fishing port.
in Hiroshima Bay, Japan (Mochida et al., 2012). In our previous study, TPBP was found to pose a risk to seawater ecosystems (Oliveira et al., 2014).

Capsaicin is a naturally occurring compound, derived from the chili pepper plant (from the genus Capsicum). It is typically used as an animal repellent (Gervais et al., 2008) due to its capacity to interfere with the organisms ability to attach to surfaces (Angarano et al., 2007; Xu et al., 2005). Environmental concentrations are unknown, but previous studies have suggested a low ecological risk from the use of this substance (Oliveira et al., 2014; Wang et al., 2014).

This study aimed to cover the lack of knowledge on the effects of tralopyril, TPBP and capsaicin in freshwater ecosystems. For this, the toxicity of tralopyril, TPBP and capsaicin was investigated in three non-target freshwater model organisms representing three trophic levels, namely the green algae Chlamydomonas reinhardtii, the crustacean Daphnia magna and the fish Danio rerio (zebrafish). In addition to the conventional mortality tests, sub-lethal effects were also assessed in the algae and zebrafish. Moreover, the effects of tralopyril and TPBP on the zebrafish embryo proteome were investigated aiming to better understand the perturbed pathways and mechanism of action. Lastly, based on our data we performed a risk assessment for the three AF compounds in freshwater marinas.

2. Material and Methods
2.1. Chemicals and exposure solutions

TPBP (99 % purity) was kindly donated by Hokko Chemical Industry Co, Japan. Tralopyril (PESTANAL®, analytical standard, Fluka), capsaicin (C 95 %, from Capsicum sp.), dimethyl sulfoxide (DMSO; 99.7 % purity), tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), iodoacetamide (IAA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Protease Inhibitor Cocktail (50x) were purchased from Sigma–Aldrich (Switzerland). Trypsin (sequencing grade) was obtained from Roche Applied Science (Switzerland) and the dye for the Bradford test from Bio-Rad (Switzerland). Fused silica tubing was purchased from BGB Analytik AG (Switzerland). RP (C18) and SCX resins were obtained from Macherey–Nagel AG (Switzerland). HPLC solvents were from Acros Organics (Belgium) and other conventional chemicals were either from Sigma–Aldrich or Fluka (Switzerland).

Stock solutions were made by dissolving the test compounds - tralopyril, TPBP and capsaicin - in DMSO. Exposure solutions were prepared by dissolving the stock solutions in the media used for the different species. Final DMSO concentrations in the exposure solutions
varied between 0.01 % and 0.03 %.

2.2 Species and endpoints

Three model freshwater species were used to perform various toxicity tests: the green algae *C. reinhardtii*, the crustacean *D. magna* and the teleost fish *D. rerio*. Mortality was assessed for the algae and the fish together with the calculation of the median lethal concentration (LC50) whenever possible. For *D. magna*, immobilization instead of mortality was assessed and the median effective concentrations (EC50) calculated. Sub-lethal effects were also assessed in *C. reinhardtii* and *D. rerio*. In the algae, the photosynthetic activity of PSII and the ATP content at 4 and 24 h were examined. In zebrafish, sub-lethal effects at the morphological level were assessed daily from one up to 5 days post fertilization (dpf). The different EC50 were calculated for each sub-lethal effect whenever possible. For tralopyril and TPBP in zebrafish, additional experiments were performed to assess the effects of different exposure scenarios. Lastly, a global proteomics analysis was performed to study the effects of tralopyril and TPBP on the zebrafish embryo proteome.

2.2.1. *Chlamydomonas reinhardtii*

*C. reinhardtii* exposures were made in Talaquil medium (Le Faucheur et al., 2005). For this, several stock solutions were made and autoclaved: 0.5 M CaCl2·2H2O, 0.15 M MgSO4·7H2O, 0.05 M K2HPO4·3H2O, 1 M NH4Cl and 0.1 MOPS (pH=7.5). Additionally, a filtered 0.24 M NaHCO3 and a 1 M solution of essential trace metals in ethylenediaminetetraacetic acid (EDTA) were used but not autoclaved. Per one L of Talaquil solution, 1 mL of each solution was added except NaHCO3 and MOPS, of which 5 and 100 mL were added, respectively. Algae cells in late exponential phase were harvested and added to the exposure vessels. The initial cell density was 2.5x10^5 in 50 mL test solutions. The algae exposure was made with a moderate PAR intensity of 80 ± 1 μmol m^−2 s^-1 provided by cool-white fluorescent lamps at a temperature of 25 °C. The tested concentrations for each compound were selected based on range finding tests up to a maximum of 1mg L^-1. The endpoints tested were based on the study by Nestler et al (2012) with slight modifications. Cell density (cells mL^-1) measurements were made using an electronic particle counter (Casy, OMNI Life Science GmbH, Switzerland) at time points 0, 4 and 24 h. Growth was defined as the number of cells measured at 24 h subtracted by the number of cells at T0 after a ln(x) transformation of the data, divided by the exposure time (24 h). The validation criterion for the
test performance was the doubling of the cell number in the control after 24 h. All other endpoints were normalized to the number of cells.

The effective quantum yield of PSII ($\Phi_{\text{PSII}}$) was determined using a pulse-amplitude-modulated (PAM) chlorophyll fluorimeter (IMAGING-PAM M-Series, Heinz Walz GmbH, Germany). Aliquots from each treatment were transferred to the PAM cells and the maximum fluorescence measured under actinic light illumination (82 $\mu$mol m$^{-2}$s$^{-1}$), after the application of three saturation pulses, one minute apart from each other. Three measurements of the fluorescence yield of PSII under actinic radiation ($F'$) and the maximum fluorescence yield in the light adapted state ($F_{m'}$) were averaged. The effective quantum yield was calculated based on the formula $\Phi_{\text{PSII}} = (F_{m'} - F')/ F_{m'}$.

ATP levels were assessed using a bioluminescence-based kit (BacTiter-GloTM Microbial Cell Viability Assay; Promega, USA). 30 $\mu$L of cell suspensions from each treatment were transferred in triplicate to an opaque white 96-well plate (Greiner Bio-One) and 15 $\mu$L of the reagent volume added (Nestler et al., 2012). Luminescence was measured on an Infinite M200 plate reader (TECAN, Switzerland). A cell free control was added in all measurements for background luminescence assessment. Final values were obtained by subtracting these background values from the values obtained for each culture condition.

2.2.2. *Daphnia magna*

*D. magna* acute immobilization test was performed according to the Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2004). The animals, obtained from the same healthy stock, were kept in filtered natural water from lake Greifensee, Switzerland, at a constant temperature of 22 ºC and 14/10 h light/dark cycle and fed daily. All tests were done at conditions similar to the described normal maintenance. Young daphnids aged less than 24 h at the start of the test were exposed for 48 h without aeration or food to concentrations of each single biocide selected in a range finding test. Four replicates of five animals each per treatment were exposed in glass vessels filled with 10 mL of exposure solution. After 24 and 48 h, the number of immobilized animals was recorded. Potassium dichromate was used as a positive control. EC$_{50}$ values after 24 and 48 h were calculated for all compounds. Additional experiments with exposure solution exchange were performed for TPBP (renewal every 24 h) and tralopyril (renewal either every 24 h or every 12 h). The solution exchange was carried out by carefully transferring the daphnids to new vessels containing the same amount of freshly spiked medium.
2.2.3. *Danio rerio*

Maintenance, breeding and embryo collection from wildtype zebrafish were performed as described in Groh et al. (2015). The fish embryo exposures were performed in E3 medium (pH 8.6). For this medium a 60 times concentrated stock solution was prepared containing 17.2 g NaCl, 0.76 g KCl, 2.9 g CaCl₂·2H₂O and 4.9 g MgCl₂·6H₂O in 1 L nanopure water. For the actual medium, 16.6 mL of this stock solution and 9 mL of a 0.3 mM NaHCO₃ solution were added to 1 L of nanopure water (ISO, 2007). Fish embryos were used to perform the zebrafish embryo toxicity assay (zFET) with tralopyril, TPBP and capsaicin. Besides, zebrafish were exposed to both tralopyril and TPBP and used for differential proteomics analysis by Multidimensional Protein Identification Technology (MudPIT).

2.2.3.1. zFET

The zFET was performed according to the OECD guideline (OECD, 2013) in 24-well plates (Cellstar Greiner Bio-One, Germany) with some modifications (EU, 2012). Briefly, newly fertilized eggs were exposed from ~1 hour post fertilization (hpf) until 120 hpf to either tralopyril, TPBP or capsaicin in E3 medium. For tralopyril and TPBP, additional experiments using different exposure scenarios were performed (Figure 1), namely exposure for 24 h (starting either at ~1 hpf or ~ 96 hpf) and for 48 h (starting at ~ 72 hpf). The embryos were kept at 26 °C and a 14/10 h light/dark cycle. Every 24 h, inspections for lethal and sub-lethal effects were performed under the microscope and 90 % of the solutions were renewed afterwards. Coagulation of fertilized eggs, lack of somite formation, lack of tail detachment from the yolk sac and lack of heartbeat were considered indicators of lethality. The recorded sub-lethal endpoints included hatching time, inflation of the swim bladder, deformations of the tail, presence of heart or yolk sac edema, heart beat rate (the number of beats counted per 30 s), locomotion difficulties and differences in the blood flow (bf) velocity (normal: continuous bf; abnormal: slow or interrupted bf).

![Graph showing zebrafish embryo development and exposure scenarios](image-url)
Figure 1 – *Danio rerio*: scheme of the 4 exposure scenarios tested. F – time when fertilization occurred (0 h); hpf – hours post fertilization.

2.2.3.2. Proteomics analysis

The proteomics analysis was based on procedures published earlier (Groh et al., 2011). Briefly, zebrafish embryos (25 per treatment) were exposed in Petri dishes from ~1 hpf until 120 hpf to three sub-lethal concentrations of tralopyril (1, 2 and 4 μg L⁻¹) or TPBP (75, 100 and 250 μg L⁻¹), plus solvent (0.01 % DMSO) and blank (E3 medium) controls. At the end of the exposure, embryos from each treatment were pooled in an Eppendorf tube, anesthetized with MS222 (tricaine methanesulfonate; 200 mg L⁻¹), quickly washed in ice-cold phosphate-buffered saline and frozen in liquid nitrogen. Samples were kept at -80 ºC until further analysis.

Protein extraction and digestion

For the protein extraction, the sample was placed in the lysis buffer (7 M urea, 2 M thiourea, 1 % CHAPS, 2 % Triton X-100, 100 mM Tris–HCl, 1 x Protease Inhibitor Cocktail, pH 8), homogenized with a pestle (30 strokes) followed by vortex homogenization (3 times 10 s with 3 min breaks on ice) and lastly sonicated on ice with three successive 10 s bursts (30 s pauses). The sample was finally centrifuged for 20 min at 13000 rpm and 4 ºC. The proteins were precipitated from the supernatant using a conventional methanol/chloroform method. The pellet formed after the precipitation was air-dried for 5 min and redissolved in resolubilization buffer (9 M urea, 2 M thiourea, 0.1 M Tris–HCl, pH 8.5). The pellet was wetted with 0.2 M NaOH, to improve the solubility before adding the resolubilization buffer. The protein concentration was measured by the Bradford method. 100 μg of proteins were reduced with TCEP, alkylated (carbamidomethylated) with IAA and digested with trypsin (100:1 ratio) overnight (14 to 16 h) at 37 ºC with shaking. The reaction was quenched by adding formic acid. Samples were then filtered (0.45 μm, Durapore PVDF, Merck Millipore, Switzerland), placed into glass vials and loaded onto the nano-HPLC (Ultimate 3000, Dionex, Switzerland).

Shotgun proteomics

After loading, the peptide mixture was trapped on a commercial C18 column (Dionex, 5 mm, 300 μm ID, 5 μm, 100 A, C18 Acclaim PepMap 100), eluted onto an in-house filled SCX column (3.5 cm, 5 μm, Nucleosil 100-5 SA) followed by an analytical in-house filled C18 column (4.5 cm, 3 μm, Nucleodur C18 Pyramid) linked in series. A 25 μm ID fused silica linked the SCX to the analytical column. Both in-house built columns were made from fused silica tubing (100 μm ID, 375 μm OD). The SCX column was closed with a frit and pressure-filled. The analytical column was pulled to a needle tip using a needle puller (Sutter Instrument
Co., Model P-2000, Science Products AG, Switzerland) before pressure-filling. The SCX column and analytical column were exchanged after three analytical runs. The sample was directly sprayed from the analytical column into the LTQ-Orbitrap XL (Thermo Scientific, Germany). The instrument was tuned on the doubly charged protonated molecular ion of angiotensin (m/z = 648.8), and calibrated using a mixture from Thermo Scientific (CalMix, pos. ProteoMass LTQ/FT Hybrid, Supelco, Switzerland). It was operated at 1.2 kV spray voltage in positive ion mode with the tube lens set to 135 V and the ion transfer capillary temperature to 200 °C. Peptides were sequentially eluted from the SCX resin to the reverse phase resin by a fully automated standard 11-step MudPIT protocol with increasing salt concentrations, followed by an organic gradient (Groh et al., 2011). One full scan FT mass spectrum (300–1,600 m/z, resolution of 60’000) was run in parallel to seven data-dependent MS/MS scans acquired in the linear ion trap with normalized collision energy set to 35 %. Precursor ions for MS/MS analysis were selected scan-dependently with dynamic exclusion set to 60 s and minimal signal intensity of the protonated molecular ion to $10^3$ counts. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific). Three technical replicates of each sample were analyzed.

**LC-MS/MS data analysis**

Scripts developed in-house were used to generate peak lists by converting the raw data files into MASCOT generic format files (mgf). The data generated was searched for peptide hits using the Open Mass Spectrometry Search Algorithm (OMSSA; Geer et al., 2004) against an in-house curated protein database containing the available protein sequences for *Danio rerio* downloaded from NCBI in 2014. Redundant protein entries were partially removed by the software CD-HIT (Li and Godzik, 2006) with the threshold set to 0.95. The database was supplemented with the sequences of common contaminants that could originate from sample processing, such as highly abundant human proteins (e.g. keratins) and trypsin. Equal numbers of randomized sequences produced by the decoy.pl PERL script freely available from MASCOT (Matrix Sciences, Boston, MA, USA) were also added to the database to be used for calculation of false discovery rates (FDR) based on the target-decoy database approach (Käll et al., 2008). During database search carbamidomethyl-cysteine was set as fixed modification. Protein N-terminal acetylation, oxidation of methionine, deamidation of asparagine and glutamine and peptide N-terminal formation of pyroglutamic acid were set as variable modifications. Tryptic specificit was set to a maximum of two missed cleavages. The charge of precursors to be searched was +2 and +3, and minimum precursor charge to start considering multiply charged products was set to +2. Only discrete hits with FDR less than 1 % were
considered for further analysis. Protein quantitation was performed based on a label-free approach that takes into account the spectral counts for observed peptide hits. With this objective the OMSSA files from the two technical replicates were combined and analyzed by G-test (Zhang et al., 2006). Only proteins that had more than twenty counts in at least one of the conditions were considered. For each protein, the normalized proportion of its counts as a function of the protein length (Normalized spectral abundance factors; NSAFs, Zybaïlov et al., 2006) were calculated for all experimental conditions. The protein fold changes were then estimated by dividing this value in the exposure conditions by the corresponding value in the control condition.

2.3 Statistics

LC₅₀ and EC₅₀ estimates were obtained by nonlinear fitting to a four parameter logistic equation through the least squares statistical method using Prism V. 5.0.C (Graph Pad, CA, USA). When it was not possible to fit a dose-response curve to the data due to the lack of response, the significance of the differences between the observed effects at each tested concentrations was evaluated using a one-way ANOVA followed by a Dunnett’s Multiple Comparison post hoc test. Homogeneity of variances was tested using the Brown-Forsythe test.

3. Results

All experiments met the requirements for control survival and normal development of ≥ 90 %. No significant differences in response were observed between freshwater and DMSO for all of the endpoints tested across the three species exposed to tralopyril, TPBP and capsaicin, with the exception of the differential proteomics analysis. Therefore, since DMSO was used in all solutions to dissolve the biocides, treatments were compared to the respective DMSO control. Similar approaches, where treatments are compared with solvent control have previously been reported in the literature (Kamel et al., 2012; Lacaze et al., 2015; Oliveira et al., 2016a). The percentage of DMSO used in the solvent controls was 0.01 % with the single exception of the *D. magna* capsaicin series where a higher solvent exposure percentage of 0.03 % was used. In this latter case there were no significant differences between both controls either.

Nominal concentrations were used throughout this study for TPBP and capsaicin. For tralopyril we measured the concentrations in freshly prepared E3 medium solutions, used for
the zebrafish bioassays. Results showed that, at the start of the exposures, nominal concentrations were very similar to the measured ones (Table S1 in supplementary material).

3.1 – Toxicity to the green algae *C. reinhardtii*

The results of toxicity tests with *C. reinhardtii* are shown in Figure 2. Among the three tested biocides, tralopyril was the most toxic to *C. reinhardtii* and caused lethal and sub-lethal effects on both the effective quantum yield of PSII (ΦₚₛⅡ) and ATP levels in a dose-dependent manner. The calculated LC₅₀₂₄h and the confidence interval (shown in brackets) were 71 (66.8 - 75.6) μg L⁻¹. After 4 h of exposure the calculated EC₅₀₄h values were 70.4 (66.60 – 74, 50) μg L⁻¹ and 60.48 (52.79 – 69.30) μg L⁻¹ for ΦₚₛⅡ and ATP levels, respectively. After 24 h an increase of the EC₅₀₂₄h for both ATP and ΦₚₛⅡ was observed (especially at 75 and 100 μg L⁻¹ treatments where we suspect that some algae cells had recovered from the exposure and were able to continue growing) whilst for concentrations higher than 100 μg L⁻¹ there was nearly 100 % cell mortality.

The growth inhibition caused by TPBP was negligible. Significant differences on the ΦₚₛⅡ were observed only at the maximum concentration tested. ATP levels were higher in exposed algae. However, statistically significant effects were detected in the two highest concentrations after 4 h and at 250 μg L⁻¹ after 24h.

Capsaicin did not cause significant alterations in growth, ΦₚₛⅡ or ATP content in *C. reinhardtii*. 
Figure 2 – *Chlamydomonas reinhardtii*: effects of tralopyril (1st row), TPBP (2nd row) and capsaicin (3rd row) on the algae growth after 24 h (1st column), effective quantum yield of PSII ($\Phi_{\text{PSII}}$) (2nd column) and ATP levels (3rd column). The three levels of significance are indicated by a ($p < 0.001$), b ($p < 0.01$) and c ($p < 0.05$).

### 3.2 – Toxicity to the crustacean *D. magna*

Following the OECD guidelines, a continuous exposure of 48 h was performed with young daphnids aged less than 24 h. Two EC$_{50}$ were calculated: after 24 and 48 h. Results from the *Daphnia* acute immobilization test using tralopyril and TPBP are summarized in Figure 3. Our results show that capsaicin didn’t exert toxic effects up to 1 mg L$^{-1}$ (for this reason the graph for capsaicin is not shown in Figure 3) and that TPBP was more toxic than tralopyril (EC$_{50}$,TPBP$_{48h}$ = 8.8 (8.0 – 9.7) µg L$^{-1}$ and EC$_{50}$,tralopyril$_{48h}$ = 25.2 (23.1 – 27.4) µg L$^{-1}$). Due to the similarity of the EC$_{50}$ obtained for tralopyril at 24 h and 48 h, and knowing that it degrades rapidly in a few hours (Kempen, 2011; Oliveira et al., 2016b), additional experiments with exposure solution renewal were performed. When the renewal was performed after 24 h, the EC$_{50}_{48h}$ for tralopyril decreased substantially. With renewals performed every 12 h there was a further decrease of both EC$_{50}_{24h}$ and EC$_{50}_{48h}$, indicating that the actual concentration of the compound increased and hence the effect concentration strongly depends on tralopyril degradation and frequency of the media renewal.
On the other hand, both EC₅₀ values calculated for TPBP (with one media renewal after 24 h) were very similar to those from the continuous exposure, suggesting that TPBP's EC₅₀ are independent from the media renewal and thus TPBP does not degrade in the 48 h of the test duration. Therefore, we did not test more frequent medium renewal protocols for this compound.

![Graph showing EC₅₀ values for tralopyril and TPBP](image)

Figure 3 – *Daphnia magna* immobilization test: median effective concentrations (EC₅₀) plus the 95% confidence intervals calculated after 24 and 48 h of exposure to tralopyril and TPBP with and without exposure solution renewal.

### 3.3 – Toxicity to the zebrafish *D. rerio*

Results obtained for the three AF biocides are summarized in Table 1. Following continuous exposure (from ~1 hpf until 120 hpf), tralopyril was the most toxic biocide. The main sub-lethal effects of tralopyril were the inhibition of the swim bladder inflation and deformation of body axis (lordosis) at higher concentrations (6 and 8 µg L⁻¹). TPBP was less toxic overall, but more sub-lethal effects were observed: it inhibited the inflation of the swim bladder, caused heart edema, reduction of the blood flow, locomotion difficulties and changes in the heart beat rate. The latter increased significantly at 50 µg L⁻¹ (p<0.01) and started to decrease at 100 µg L⁻¹, with a statistical significance achieved at 250 µg L⁻¹. In addition, high TPBP concentrations seemed to induce light sensitivity. This effect was observed in embryos with decreased heart rate and poor blood flow (250 and 375 µg L⁻¹). Despite their extreme locomotion difficulties, these embryos tried to swim away from light. This observation requires further studies for confirmation. Capsaicin did not cause mortality up to 1 mg L⁻¹. Interestingly, statistically significant increase of the heart rate was observed at all tested concentrations of capsaicin.
Due to its low toxicity, capsaicin was not further assessed, while the effects of tralopyril and TPBP were additionally evaluated with other exposure scenarios (Table 1 and Figure 4). These experiments were performed in order to understand whether a shorter exposure at different stages of embryo development would lead to the same extent of toxic effects. For this, two 24 h exposures (during the first 24h and from 96 to 120 hpf) and one 48 h exposure (from 72 to 120 hpf) were assessed (Figure 1).

Exposure to tralopyril during 24 h or more always caused some mortality, increasing with longer exposure times. A continuous exposure from ~1 until 120 hpf showed the highest toxicity ($LC_{50}$= 5.02 μg L$^{-1}$), followed by a 48 h exposure from 72 until 120 hpf ($LC_{50}$= 6.38 μg L$^{-1}$), and finally by the 24 h exposure ($LC_{50}$ of 7.27 and 13.3 μg L$^{-1}$ for exposures starting at 96 and ~1 hpf, respectively). The moment when the exposure occurs also has an effect on the $LC_{50}$: in embryos exposed during the first 24 h the compound showed less toxicity ($LC_{50}$= 13.3 μg L$^{-1}$) compared to those exposed at 96 hpf ($LC_{50}$= 7.27 μg L$^{-1}$), when the embryos are already hatched. This result suggests that the chorion may have a protective effect against this compound. In fact, when hatched embryos are exposed (72 hpf and later), there are no significant differences between the $LC_{50}$ calculated for 24 h or 48 h exposure durations (confidence intervals overlap; Figure 4A).

For TPBP, the continuous exposure was also the most toxic ($LC_{50}$= 447.5 μg L$^{-1}$), followed by the 48 h exposure starting at 72 hpf ($LC_{50}$= 855.8 μg L$^{-1}$) (Figure 4B). Effects on the inflation of the swim bladder, heart edema formation, blood flow and heart beat rate were observed following both of these exposure regimes. In turn, a shorter exposure of 24 h, regardless of the starting time, did not cause appreciable mortality or sub-lethal effects (except for the effects on the heart beat rate). Therefore, it was not possible to calculate $LC_{50}$ and $EC_{50}$ for these exposure scenarios. The exact effects on the heart rate depended on the exposure scenario as indicated in Table 1.

Table 1 – *Danio rerio*: Lethal and sub-lethal effects of tralopyril, TPBP and capsaicin at 120 hour post fertilization (hpf) after continuous exposure from 1 until 120 hpf. The median lethal concentration ($LC_{50}$) and the median effective concentration ($EC_{50}$) for the swim bladder inflation (sbi), heart edema (he), blood flow (bf) plus the confidence interval in brackets are presented in μg L$^{-1}$. The heart beat rate (hbr) is presented as an increase (↑) or decrease (↓) plus the lowest concentration at which the effect was statistically different from the solvent control. The same endpoints were measured for tralopyril and TPBP at other exposure times: a 48 h exposure starting at 72 hpf and two 24 h exposures, starting either at 1 or 96 hpf; n.s. – not statistically different; n.o. – not observed.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Exposure duration</th>
<th>Lethal effects μg L⁻¹</th>
<th>Sub-lethal effects μg L⁻¹</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>LC₅₀</td>
<td>EC₅₀_sbi</td>
</tr>
<tr>
<td>Tralopyril</td>
<td>1 until 120 hpf</td>
<td>5.02</td>
<td>(4.86 - 5.19)</td>
</tr>
<tr>
<td></td>
<td>72 until 120 hpf</td>
<td>6.38</td>
<td>(5.88 - 6.93)</td>
</tr>
<tr>
<td></td>
<td>96 until 120 hpf</td>
<td>7.27</td>
<td>(6.32 - 8.36)</td>
</tr>
<tr>
<td></td>
<td>1 hpf until 25 hpf</td>
<td>13.3</td>
<td>(10.66 - 16.59)</td>
</tr>
<tr>
<td>TPBP</td>
<td>1 until 120 hpf</td>
<td>447.5</td>
<td>(434.1 to 461.3)</td>
</tr>
<tr>
<td></td>
<td>72 until 120 hpf</td>
<td>855.8</td>
<td>(820.3 - 892.9)</td>
</tr>
<tr>
<td></td>
<td>96 until 120 hpf</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
<tr>
<td></td>
<td>1 hpf until 25 hpf</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
<tr>
<td>capsaicin</td>
<td>1 until 120 hpf</td>
<td>n.o.</td>
<td>n.o.</td>
</tr>
</tbody>
</table>

Figure 4 – Danio rerio: Survival of the embryos at 120 hpf (full line) and respective confidence intervals (intermittent line) following exposure to tralopyril (A) and TPBP (B), with different exposure scenarios as indicated by the legend: continuous - exposed from 1 to 120 hpf; 48 h - exposed from 72 to 120 hpf; 24 h - either during the first 24 hpf or from 96 to 120 hpf.
3.4. Toxic ratio (TR) as an indication of the mode of action

Important parameters of a certain substance (i.e LC$_{50}$, log K$_{OW}$, etc) may be predicted through their physico-chemical characteristics. The TR is defined as the ratio between an LC$_{50}$ estimated from a QSAR (quantitative structure–activity relationship) model for baseline toxicity and the experimental LC$_{50}$ value for a certain species. In this study we used a windows-based suite of programs developed by the U.S. Environmental Protection Agency (EPA’s Office of Pollution Prevention Toxics) and Syracuse Research Corporation (SRC) named EPISUITE™ to estimate physico-chemical properties and environmental fate of chemicals. Input values were the compound name, CAS number, formula and molecular weight. The resulting zebrafish embryo LC$_{50}$ for tralopyril and TPBP were 1425 μg L$^{-1}$ and 84 μg L$^{-1}$, respectively. No simulations were done for capsaicin since no experimental LC$_{50}$ for zebrafish embryos could be achieved in this study.

A TR of 10 separates “specifically toxic chemicals” from “baseline toxicants” (Maeder et al., 2004). Tralopyril achieved a score of 285 indicating that this chemical has a specific mode of toxic action. This was confirmed by the Assessment Report on tralopyril evaluation for inclusion on the BPR (European Chemicals Agency, 2014), which identified tralopyril as an uncoupler of the mitochondrial oxidative phosphorylation. TPBP achieved a score of 0.9 meaning that its toxicity is most probably based on general narcosis.

3.5 Effects of tralopyril and TPBP on the zebrafish proteome

Eight different samples were analyzed to investigate changes in the proteome of zebrafish embryos exposed to tralopyril and TPBP: E3 medium control (CTRL); 0.01 % solvent control (DMSO); three sub-lethal tralopyril concentrations: 1 μg L$^{-1}$ (TP1), 2 μg L$^{-1}$ (TP2) and 4 μg L$^{-1}$ (TP4); and three sub-lethal TPBP concentrations: 75 μg L$^{-1}$ (TPBP75), 100 μg L$^{-1}$ (TPBP100) and 250 μg L$^{-1}$ (TPBP250).

About 2000 unique protein sequences could be detected per sample (Table S2 in supplementary material). The G-test revealed only a few proteins to be significantly regulated by the assessed exposures. Figures 5 and 6 show proteins that fulfill the criteria of G-test significance (p<0.01) and have more than 20 counts in at least one of the conditions.
### Figure 5 – *Danio rerio*: Protein regulation (compared to 0.01 % DMSO) in zebrafish embryos exposed to three concentrations of tralopyril: 1 (TP1 vs. DMSO), 2 (TP2 vs. DMSO) and 4 (TP4 vs. DMSO) µg L⁻¹. Protein regulation in control conditions was also analyzed (CTRL vs. DMSO). The conditions marked with * are statistically significant (G-test, p<0.01).

<table>
<thead>
<tr>
<th>ID</th>
<th>Protein Name</th>
<th>CTRL vs. DMSO</th>
<th>TP1 vs. DMSO</th>
<th>TP2 vs. DMSO</th>
<th>TP4 vs. DMSO</th>
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</thead>
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<td>NP_001116082</td>
<td>vitellogenin 6</td>
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<td><strong>mRNA Splicing</strong></td>
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<tr>
<td>NP_956054</td>
<td>small nuclear ribonucleoprotein D3 polypeptide, like</td>
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### Figure 6 – *Danio rerio*: Protein regulation (compared to 0.01 % DMSO) in zebrafish embryos exposed to three different concentrations of TPBP: 75 (TPBP75 vs. DMSO), 100 (TPBP100 vs. DMSO) and 250 (TPBP250 vs. DMSO) µg L⁻¹. Protein regulation in control conditions was also analyzed (CTRL vs. DMSO). The conditions marked with * are statistically significant (G-test, p<0.01).

<table>
<thead>
<tr>
<th>ID</th>
<th>Protein Name</th>
<th>CTRL vs. DMSO</th>
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<th>TPBP 100 vs. DMSO</th>
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<td>non-histone chromosomal protein HMG-17</td>
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<tr>
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<td>peptidyl-prolyl cis-trans isomerase A</td>
<td>*</td>
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Vitellogenin 6 was the only protein significantly upregulated (p<0.01) by 0.01 % DMSO compared to blank control. Tralopyril exposure significantly modulated the expression of 11 proteins (Figure 5), while TPBP significantly modulated the expression of 8 proteins.
(Figure 6). For both compounds, protein upregulation without a clear dose dependency was the most common response observed.

4. Discussion

Despite the continuous efforts to develop more environment-friendly AF chemicals, it is difficult to avoid any consequences for non-target organisms. One must bear in mind that AF systems need to fulfil their biocidal function in protecting submerged structures. However, it is important to make sure that pollution cases, like that caused by tributyltin in the past, do not reoccur (see (Antizar-Ladislao, 2008; Sousa et al., 2014) for review). Our study aimed to assess the toxicity of the latest generation AF biocides tralopyril, TPBP and capsaicin in the three freshwater (non-target) model species, and to increase our understanding of their mode of action and ecological risk.

Among the three tested biocides, capsaicin was consistently the least toxic to all three species. Tralopyril was the most toxic to algae and zebrafish. In the D. magna acute immobilization test, tralopyril was more toxic than TPBP after 24 h and presented similar toxicities after 48h, but only if concentrations were maintained by means of exposure solution renewal. If no renewal of exposure solutions were made, TPBP exhibited a greater toxicity than tralopyril after 48 h for this crustacean, due to the quick degradation of tralopyril.

Both biocides also seem to have a different mode of action: specific for tralopyril while TPBP appears to be a baseline toxicant (section 3.4). Despite this difference, both compounds caused common morphological effects, e.g. inhibition of the zebrafish swim bladder inflation. This air-filled bladder helps the animals to achieve neutral buoyancy and reduce the energetic cost of swimming (Jönsson et al., 2012). The non-inflation of the swim bladder is usually not immediately lethal to the organisms but greatly diminishes the probability of their long-term survival (Villeneuve et al., 2014; Di Paolo et al., 2015).

Generally, our proteomic data did not show a clear dose-dependent response, which matches observations in the literature (Riva et al., 2011; Shen et al., 2007). The differential proteomic analysis revealed that five proteins were regulated by both tralopyril and TPBP: vitellogenin 6, crystallin β A1b, aspartate aminotransferase 2a, pyruvate kinase isozymes M1/M2 isoforms X1 and non-histone chromosomal protein HMG-17. The joint regulation by compounds that have different modes of action may indicate that these proteins reflect a general stress response rather than a specific effect of the substances analyzed. Vitellogenin 6 was also regulated by DMSO exposure alone. In fact, vitellogenins, used as energy source for
development, are highly abundant in embryos of oviparous vertebrates (Tyler and Sumpter, 1996). Crystallins are major constituents of the eye lens but have other functions as well, such as an ability to inhibit apoptosis and enhance the resistance of cells to stress (Andley, 2007). Both protein families have been frequently reported by differential proteomics studies as responding to various stressors (Groh and Suter, 2015).

Aspartate aminotransferase 2a (AST) and pyruvate kinase isozymes M1/M2 isoforms X1, both upregulated by tralopyril and TPBP, are related to energy metabolism and play essential roles in survival, stress adaptation and tolerance. The upregulation of pyruvate kinase may represent a strategy to maintain the energy levels and cope with toxic stress (Sokolova, 2013). On the other hand, upregulation of AST has been linked to tissue damage (Begum, 2005; Zhang et al., 2014). In several studies, the upregulation of AST (usually together with alanine aminotransferase) has been used as a marker of environmental pollution and fish diseases (Chen et al., 2013; Firat et al., 2011; Loteste et al., 2013). In our study, alanine aminotransferase protein was not detected.

The upregulation of non-histone chromosomal HMG-17 has been found to correlate with processes of cell differentiation rather than proliferation. It unfolds the higher order chromatin structure, influencing the accessibility to DNA and thus enhancing transcription and replication potential of a chromatin template (Herrera et al., 1999; Pogna et al., 2010). Thus, the upregulation of HMG-17 protein may signify that DNA is more vulnerable to the chemical exposure, possibly affecting the normal development of zebrafish embryos.

4.1 Tralopyril-specific effects of exposure

Among the three tested biocides, tralopyril showed the highest toxicity despite its short half-life in water, with D. magna and D. rerio being equally sensitive and much more than C. reinhardtii. Results obtained for the acute toxicity test with Daphnia magna confirmed its fast degradation time since renewing the media caused a decrease of the LC50. Tralopyril's half-life in natural freshwater has been determined to be 8.1 h (Oliveira et al., 2016b). Therefore, an exposure to a constant tralopyril concentration, using for instance passive dosing (Vergauwen et al., 2015), or calculating effect concentrations based on the actual dose seen by the organism, would probably result in a lower LC50 value than the one reported in our study. This short tralopyril half-life is also reflected in the C. reinhardtii bioassay. A 4 h exposure to this biocide caused significant sub-lethal effects on both the ATP content and on the effective quantum yield of PSII (ΦPSII) whilst from the first (4 h) to the second time-point (24 h) a recovery was
observed at concentrations close to the LC$\text{50}$ (71 $\mu$g L$^{-1}$). Although other authors have reported a recovery after exposure to toxic compounds connecting it to the response of the cellular detoxification mechanisms (Nestler et al., 2012; Pillai et al., 2014), we consider that our observations might have been mostly driven by the quick tralopyril degradation. Thus the observed results are thought to come from the recovery of some algal cells that were not permanently impaired after a 4 h exposure. In this way, those cells were able to recover (and possibly to proliferate) leading to an increase in both ATP content and the $\Phi$$_{\text{PSII}}$.

Tralopyril was also found to be very toxic to zebrafish embryos. Sub-lethal effects were observed even if the exposure to the compound only lasted 24 h, especially if the embryos were already hatched. Besides, during continuous exposure, tralopyril was found to be teratogenic, causing lordosis at the higher concentrations tested.

Sub-lethal concentrations of tralopyril led to the upregulation of proteins involved in energy metabolism, cytoskeleton and eye structure formation, cell division and differentiation, and mRNA splicing. From the six proteins specifically regulated by tralopyril exposure, two belong to the group of cytoskeletal proteins. As stated above, the increased expression of the highly abundant protein myosin is thought to be related to general stress. The other protein (LOC563946) yet lacks biochemical annotation.

Tralopyril is thought to uncouple the mitochondrial oxidative phosphorylation by dissipating the proton gradient necessary for ATP formation (European Chemicals Agency, 2014; Oliveira et al., 2016a). Not surprisingly, tralopyril had an effect on the regulation of the cytochrome b-c$_1$ complex subunit 8 protein. This complex catalyzes the transfer of an electron to a higher-potential acceptor protein. This electron transfer is coupled to generate the proton gradient that drives ATP synthesis (Crofts, 2004; Wallace and Starkov, 2000). Surprisingly, however, no oxidative stress proteins were found to be significantly regulated by tralopyril exposure.

Alcohol dehydrogenase – class 3 (ADH3) was also upregulated by tralopyril. This enzyme is found in diverse tissues from different species (Coelho et al., 2012; Park and Kwak, 2009), including zebrafish embryos (Dasmahapatra et al., 2001; Reimers et al., 2004) and is well conserved throughout evolution. ADH3 catalyzes the reversible oxidation of a wide variety of xenobiotic and endogenous alcohols to their corresponding aldehydes (Dasmahapatra et al., 2001; Duester et al., 1999). Its role in scavenging formaldehyde (an intermediate in many metabolic processes) suggests a protective function for the entire enzyme system (Iborra et al., 1992; Jin et al., 2011). Although it is still not known what happens to tralopyril after entering
the zebrafish embryo, our results suggest that ADH3 is associated with the tralopyril-induced biotransformation pathways.

The nuclear migration protein NudC has been reported to play an essential role in mitosis (Kosodo, 2012) being also involved in protein folding and biosynthesis, signal transduction and nucleic acid binding (Zhu et al., 2010), as well as in the regulation of the inflammatory response (Riera and Lazo, 2009). Tralopyril exposure induced the upregulation of this protein at all concentrations, however, significantly only at 1 μg L⁻¹. The same was observed for the small nuclear ribonucleoprotein (snRNP) D3 polypeptide, which is involved in mRNA splicing. Perturbation of snRNPs results in splicing aberrations that can cause or contribute to the development of cancers and genetic diseases (Clelland et al., 2009; Eggert et al., 2006; Lee et al., 2014). Although it would be premature to draw any definite conclusions on the contribution of tralopyril to changes in splicing reactions based solely on the upregulation of this protein, this finding may direct further research on the molecular mechanisms underlying tralopyril's toxicity.

4.2. TPBP-specific effects of exposure

TPBP was toxic to all three tested species with the crustacean D. magna being by far the most sensitive. In the algae, a significant decrease in both ΦPSII and the ATP levels was noted, probably due to baseline toxicity. However, these effects were not strong enough to cause lethality. Compared to tralopyril, TPBP was not as lethal to zebrafish but caused more sub-lethal effects such as heart edema and changes in the heart beat rate, alteration of locomotion and decreased blood flow.

The effects of sub-lethal TPBP concentrations on the zebrafish embryo proteome comprised the regulation of proteins involved in energy metabolism, cytoskeleton and eye structure formation, protein folding, and cell growth and differentiation. As stated before, the proteins commonly regulated by DMSO, tralopyril and TPBP - crystallin and vitellogenin – are thought to reflect a general stress response (Groh and Suter, 2015).

Three proteins were specifically regulated by TPBP exposure: nebulin isoform X3, enhancer of rudimentary homolog and peptidyl-prolyl cis-trans isomerase A. Nebulin is a major filamentous protein constituent of the striated muscle, playing a critical role in the maintenance of the sarcomeric structure (McElhinny et al., 2005; Witt et al., 2006). Changes in the expression of the nebulin gene in zebrafish are involved in nemaline myopathy with the
embryos exhibiting defects in contractile properties (Gibbs et al., 2013; van der Meer, 2005). Although still controversial, nebulin is also thought to be involved in thin filament length regulation in the cardiac muscle where it has been detected in some fish species (e.g. agnathans; Fock and Hinssen, 2002) and mice (Kazmierski et al., 2003). Therefore, nebulin upregulation in TPBP exposed embryos could be related to the locomotion difficulty detected in the zFET at 250 and 375 μg L⁻¹. On the other hand, nebulin regulation may also be linked to the heart defects caused by TPBP, such as heart edema and heart rate changes.

The enzyme peptidyl-prolyl cis-trans isomerase (PPIA) belongs to the cyclophilins family. This protein is involved in protein folding, trafficking, and cell signaling (Zhang et al., 2015). There are also indications of PPIA involvement in several chronic pathologies including disorders of the nervous system, cardiovascular and cardiac diseases (Nigro et al., 2011; Satoh et al., 2011; Seizer et al., 2011).

Lastly, a strong upregulation of the enhancer of rudimentary homolog (ERH) was observed in response to TPBP exposure. This protein, small but highly conserved among eukaryotes, has been linked to transcriptional regulation and cell cycle processes, and is critically required for genomic stability but also in cancer cell survival (Fujimura et al., 2012; Weng et al., 2015).

4.3. Capsaicin-specific effects of exposure

Capsaicin did not cause lethal effects in any of the tested species up to the highest concentration tested (1 mg L⁻¹). In the literature, LC₅₀ of 5.98 mg L⁻¹ for the fish Danio rerio acute toxicity test and 114 mg L⁻¹ for the freshwater algae Selenastrum capricornutum growth inhibition test have been reported (Wang et al., 2014). These values are considerably higher than those we tested.

In the current study, no significant sub-lethal effects were observed for the algae and the crustacean. The only sub-lethal effect observed was a significant increase in the zebrafish heartbeat rate. Interestingly, while a decrease in the heart rate is most commonly reported for chemical exposure, capsaicin caused an increase in the zebrafish heart rate at all concentrations tested. An increase in the heart rate has also been observed in zebrafish embryos exposed to a brominated flame retardant which, at higher tested concentrations, upregulated several genes involved in the cardiac function (Wu et al., 2013). In the case of capsaicin, the implications of
an increased heart rate on the embryos are not known. Hence, it would be interesting to explore whether it has any effects on cardiac function, growth or even survival in the long term in fish and other vertebrates.

To the best of our knowledge, no further reports on the sub-lethal effects of capsaicin in freshwater animals exist, except its effects on the attachment of the adult freshwater mussel *Dreissena polymorpha* (Angarano et al., 2007; Cope et al., 1997). Although the mode of action of this compound is still to be elucidated, it seems that its major advantage is the AF effect without a strikingly lethal response.

4.4. Hypothetical Risk Assessment of tralopyril, TPBP and capsaicin

The ecological risk assessment of an AF biocide comprises four main steps: i) hazard identification, ii) dose (concentration) - response (effect) assessment, iii) exposure assessment and iv) risk characterization. These steps represent a systematic process for identifying adverse consequences and their associated probabilities arising from AF system utilization. Organism exposure to AF biocides occurs mostly during their leaching from the AF paints into the water but it may also occur in the dockyards during the spraying and/or painting as well as during the waste disposal after cleaning and scratching of the old coatings (Senda, 2009). The experimental work presented in this study contributes to the establishment of the predicted no effect concentration (PNEC). For the PNECs intended to be used for risk assessment, the utilization of safety factors is highly encouraged (step ii). Due to the scarce or non-existing information about environmental concentrations of these emerging substances, the marine antifoulant model for predicting environmental concentrations (MAMPEC, Version 3.0; Deltares, Netherlands) was used to calculate theoretical predicted environmental concentrations (PEC) occurring in a freshwater marina (step iii). Lastly, the risk characterization was calculated using the PEC/PNEC ratio (step iv). If this ratio is > 1 then the compound may probably threaten wildlife and the ecosystems (European Commission, 2003).

The use of safety factors is not a trivial issue and depends on the type of available toxicity data. It is highly recommended as a precaution to ensure that harmful substances are identified (European Commission, 2003). Although the guidelines for risk assessment support a more protective approach, we consider that a safety factor of 50 applied to the lowest no observed effect concentration (NOEC) of the most sensitive species is sufficient in this study. Besides the *D. magna* acute immobilization test that exclusively reported acute toxicity, other
more comprehensive tests using the algae *C. reinhardtii* and the zebrafish *D. rerio* were performed. The algal growth inhibition test is, in principle, a multigenerational test (European Chemicals Agency, 2015) since it is performed in the exponential phase when the algae are about to reproduce. Furthermore, the zFET arguably covers the most sensitive phase of development, from fertilization until 120 hpf. Thus, we consider that these tests are sensitive enough to support our decision to use an assessment factor of 50, as required by the Biocidal Products Regulation, when dealing with chronic values covering two trophic levels (European Chemicals Agency, 2015). To derive the PNEC, for tralopyril we used the no observed effect concentration (NOEC) assessed for zebrafish embryo mortality after continuous exposure from ~ 1 to 120 hpf (Table 1). For TPBP, the NOEC from the *D. magna* acute immobilization test was used, since this species was the most sensitive to TPBP. For capsaicin, as no toxicity could be observed in this study, we used the LC$_{50}$ reported for *D. rerio* by Wang and colleagues (Wang et al., 2014).

MAMPEC was used to estimate the predicted environmental concentrations (PEC) in a Swiss freshwater marina. *Environment, Compound and Emission* are the three descriptors used to achieve such theoretical estimations. The environment descriptor comprises the information relative to the characteristics of the marina (length, width and depth) along with water, hydrodynamics and sediment features. The compound descriptor comprises the physico-chemical properties of the compound such as molecular mass, log $K_{ow}$, solubility, etc. Lastly, the emission scenarios descriptor comprises the number of ships along with their underwater surface area, leaching rate and theoretical percentage of biocide used in the paint. Although MAMPEC is mostly used to predict marine concentrations, the emission descriptors were based on the same approach as established by the OECD-EU working group for the risk assessment of antifoulants with an adaptation for freshwater marinas as described in the OECD series on Emission Scenario Documents – N° 13: Emission Scenario Document on Antifouling Products (OECD 2005). Nevertheless, this document points out the site specificity of this model – a Swiss marina - stating that a risk assessment based on such specific conditions is inadvisable. However, since no harmonized scenarios exist to predict freshwater environmental concentrations, we used this approach as enlightening information on the possible risks of such compounds in freshwater marinas. The risk characterization based on PEC/PNEC is summarized in Table 2. It has to be noted that the effort to assess the risk of new contaminants is inevitably subjected to a certain degree of uncertainty due to the use of safety factors and the undetermined environmental concentrations substituted by the modeled concentrations.
Table 2 – Hypothetical Ecological Risk Assessment based on the maximum and minimum MAMPEC-predicted environmental concentrations (in μg L\(^{-1}\)) in a freshwater marina (PEC\(_{\text{MAX}}\) and PEC\(_{\text{MIN}}\)) divided by the PNEC, to which an assessment factor of 50 was applied.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Endpoint used for PNEC estimation (μg L(^{-1}))</th>
<th>PEC Swiss marina Max (μg L(^{-1}))</th>
<th>PEC Swiss marina Min (μg L(^{-1}))</th>
<th>Risk Assessment PECmax/PNEC</th>
<th>Risk Assessment PECmin/PNEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>tralopyril</td>
<td>NOEC ((D. \text{rerio}): 4)</td>
<td>1.44</td>
<td>3.17E-01</td>
<td>18.00</td>
<td>3.96</td>
</tr>
<tr>
<td>TPBP</td>
<td>NOEC ((D. \text{magna}): 8)</td>
<td>1.26</td>
<td>2.65E-01</td>
<td>7.88</td>
<td>1.66</td>
</tr>
<tr>
<td>capsaicin</td>
<td>5980* ((D. \text{rerio}))</td>
<td>1.98</td>
<td>5.12E-01</td>
<td>1.7E-2</td>
<td>4.3E-3</td>
</tr>
</tbody>
</table>

(*) Wang et al., 2014

The hypothetical ecological risk assessment performed in this study identified capsaicin as an environment friendly compound. However, its effects on the zebrafish heart beat rate may require further investigation with regard to the consequences in chronic exposure scenarios. On the other hand, tralopyril and TPBP were predicted to pose a risk to freshwater ecosystems even when the lowest PEC is used. Noneless, from the industry’s point of view, capsaicin may have reduced efficiency as an AF biocide whereas tralopyril and TPBP may present greater AF potential. Hence, more studies should be undertaken to better characterise these AF biocides: for instance, in the case of tralopyril, understanding its degradation pathways together with its potential to accumulate in the sediments would be crucial to acknowledge its persistence and bioavailability in the ecosystem. In turn, this could imply a lower PEC, that could reverse the outcome of this result making tralopyril a friendlier and effective biocide alternative. On the other hand, further chronic toxicological studies could also be important to lower the assessment factor used in order to better estimate the PNEC.

Even though the toxicological research history of these biocides is rather short, the resulting risk estimated in this study is in agreement with our previous results for the marine environment (Oliveira et al., 2014). Thus, these preliminary results should alert the users and stakeholders of possible risks associated with the utilization of TPBP and tralopyril particularly because the latter is already accepted as antifoulant under the Biocidal Product Regulation.

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