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Toxicity assessment of five emerging pollutants, alone and in binary or ternary mixtures, towards three aquatic organisms

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Abstract :

Despite a growing scientific attention on ecological impact of emerging pollutants (EPs) such as pharmaceuticals, personal care products, and pesticides, knowledge gaps remain regarding mixture toxicity and effects on aquatic organisms. Several EPs were screened in seawater (Normandy, France), and the ecotoxicity of five compounds, chosen on their occurrence in ecosystems and use worldwide, was assessed and were the biocides methylparaben (MP) and triclosan (TCS), a pesticide degradation product (AMPA), and the pharmaceuticals venlafaxine (VEN) and carbamazepine (CBZ). The acute or sub-chronic toxicity, alone or in binary/ternary mixtures of three of them (CBZ, AMPA, and MP), was assessed on one marine and two freshwater organisms: *Crassostrea gigas*, *Pseudokirchneriella subcapitata*, and *Daphnia magna*. TCS and AMPA were, respectively, the most ($EC_{50} < 1 \text{ mg L}^{-1}$) and the least ($EC_{50} > 50 \text{ mg L}^{-1}$) toxic chemicals for the four endpoints (algal growth inhibition, daphnia immobilization, oyster embryotoxicity, and metamorphosis). The anxiolytic VEN ($EC_{50} < 1 \text{ mg L}^{-1}$) was particularly toxic to oyster larvae showing sensitivity difference between freshwater and marine organisms. If all the mixtures appeared to be in the same range of toxicity, the joint-toxic effects mainly led to synergistic or antagonistic interactions compared to single-compound toxicity. The data also highlighted species-dependent differing models of toxicity and underscored the need for an awareness of cocktail effects for better ecological risk assessment.

Keywords : Pharmaceuticals, Personal care products, Pesticides, Mixture and single-compound toxicity, Freshwater species, Marine bivalve

Introduction

Nowadays, over a thousand emerging pollutants (EPs), their metabolites and their transformation products are present in aquatic environments worldwide (NORMAN 2016). However, they are currently not included in routine monitoring programs, and their fate, behaviour and ecotoxicological effects remain little known. Amongst EPs, the classes of Pharmaceuticals and Personal-Care Products (PPCPs) and polar pesticides are predominant in European hydrosystems (NORMAN 2016). They received a growing scientific attention in the last decades in particular because of their intrinsic properties to cause biological effects (e.g., Daughton and Ternes 1999; Boxall et al. 2012; Fabbri and Franzellitti 2016). They are found ubiquitously in all aquatic compartments at concentrations reaching few ng.L^{-1} in coastal marine waters of developed countries (Gaw et al. 2014; Fabbri and Franzellitti 2016) to up to several dozen $\mu\text{g.L}^{-1}$ in surface waters and urban effluents (e.g., Daughton and Ternes 1999; Kolpin et al. 2002; Munaron et al. 2012; Chiffre et al. 2016); exceeding sometimes the safety threshold for predicted environmental concentrations in surface waters of $0.01 \mu\text{g.L}^{-1}$ (EMEA 2006). In addition, because of their physical and chemical properties ($\log K_{ow} > 3$) and/or because of their continual infusion into the aquatic media, some PPCPs and pesticides may persist in environment and bioaccumulate in animal tissues (e.g. Álvarez-Muñoz et al. 2015a,b; Fabbri and Franzellitti 2016). Numerous compounds like antimicrobial disinfectants and paraben preservatives, used for example in cosmetic, pharmaceutical or food industries, rise concern in particular because of their suspected or proven endocrine effects in aquatic organisms (Brausch and Rand 2011); and thus need to be investigated in risk assessments. One can cite the antimicrobial triclosan (a diphenyl ether derivative) which is highly prevalent in aquatic environments throughout the world (Kolpin et al. 2002; Halden and Paull 2005), and suggested as a candidate priority substance (von der Ohe et al. 2012). The continuous release of pharmaceutical residues also becomes an environmental issue because they can

behave as persistent compounds (Daughton and Ternes 1999), and concern has been voiced for the psychotropic drugs for which global consumption is increasing (Beausse 2004). Antiepileptics were among the most predominant therapeutic classes prescribed in several countries at the end of the 90s (Beausse 2004) and nowadays, carbamazepine is increasingly found in aquatic environments (Ternes 1998; Daughton and Ternes 1999; Seiler et al. 1999; Chiffre et al. 2016). This compound is even in the top 5 of the most occurring molecules in the marine environment worldwide (Fabbri and Franzellitti 2016). Between 1980 and 2001 in France, the prescription of antidepressants grew sevenfold, with venlafaxine being one of the most consumed molecules (Olié et al. 2002; Amar and Balsan 2004; ANSM 2012). This compound was found to be the most prevalent antidepressant in the Orne river (France) at the $\text{ng}\cdot\text{L}^{-1}$ range, and is even detected at sea, 17 km downstream the point of release (Minguez et al. 2016). Finally there is an increase in the use of pesticides worldwide, which is seen as a consequence of human population growth and its impact has reached global significance (Köhler and Triebkorn 2013). For example, the glyphosate-based formulations are amongst the most used herbicides in the world (Howe et al. 2004). Glyphosate has often been considered relatively non-toxic for humans and fauna (Giesy et al. 2000; Williams et al. 2000) with an environmentally low impact because of its rapid biodegradation and strong adsorption to soil particles (Agritox 2016; PPDB 2016). However, a degradation product, the aminomethylphosphonic acid (AMPA), was detected much more frequently in US streams and treatment plant effluents than glyphosate itself (present in 67.5% of the samples vs 17.5%, respectively; Kolpin et al. 2006) and AMPA is, even now, the most prevalent pesticide molecule in French stream waters (Botta et al. 2009; Commissariat Général au Développement Durable 2013).

Quantification of emerging pollutants in the different environmental compartments is essential for gaining knowledge on their occurrence and fate. In this study, we first performed

an environmental screening of 21 molecules belonging to three classes of emerging compounds (pharmaceuticals, biocides and pesticides) in coastal seawaters. Based on our screening and on literature (data about occurrence and use worldwide), we tested the ecotoxicity of five molecules that were: (1) the anxiolytic venlafaxine (VEN) and the antiepileptic carbamazepine (CBZ); (2) the preservative methylparaben (MP) and the antimicrobial triclosan (TCS); and (3) the aminomethylphosphonic acid (AMPA). In order to obtain ecologically-relevant data on their potential ecological risks, we studied the toxicity of these five chemicals alone as well as the joint-action toxicity of three of them (CBZ, MP and AMPA) in binary and ternary combinations to mimic environmentally realistic mixtures. We carried out several bioassays using two freshwater and one marine species according to standard guidelines: embryotoxicity and metamorphosis test on the Pacific oyster *Crassostrea gigas*, a worldwide leading aquaculture species, whose the larvae are particularly susceptible to a large range of pollutants (e.g., Mottier et al. 2013; Di Poi et al. 2014); growth inhibition assay on the freshwater green alga *Pseudokirchneriella subcapitata*, which is an important primary producer (Blaise and Vasseur 2005); and immobilization test on the freshwater crustacean *Daphnia magna*, which occupies a key position in the aquatic food web by linking primary and secondary productions (Dodson and Hanazato 1995; Tatarazako and Oda 2007; Miner et al. 2012). With regard to the constitution of the mixture (different families of molecules and different biological targets), we hypothesized that the combined effects will lead to synergistic interactions between the compounds, i.e. the toxic effects caused by the mixture will exceed the sum of effects of the separate constituents, and aimed to bring new insights in the ecotoxicological risk assessment of emerging pollutants.

Material and methods

Analytical quantification in seawater

In October 2014, we conducted an environmental screening of 21 emerging compounds (Table 1) that belonged to three different chemical classes (9 pharmaceutical products, 9 biocides, 2 pesticides and 1 degradation product) in seawater samples collected in the district of Meuvaines (49°20'38"N 0°34'19"W)/Ver-sur-Mer (49°20'45"N 0°31'99"W), an important shellfish harvesting area on the Normandy coastline (Figure 1).

Pesticides (glyphosate, AMPA, glufosinate) were quantified by UPLC and Fluorometric detection (in accordance with NF ISO 21458) using UPLC Acquity with FLR detector (Waters[®], Milford, MA, USA) and a column Acquity BEH C18 -2.1x150 mm, 1.7µm (Waters[®], Milford, MA, USA). The 18 other compounds were extracted from 1 litre of sampled seawater with Solid Phase Extraction (HLB Oasis 200mg; Waters[®], Milford, MA, USA). The extract was then analysed by ultra-performance liquid chromatography (LC) and MS-MS detection using a UPLC Agilent 1290 Infinity with Agilent 6460 Triple Quadrupole (Agilent Technologies[®], Santa Clara, USA) and a Column Acquity BEH C18 -2.1 mm×150 mm, 1.7 µm column (Waters[®], Milford, MA, USA). Two different chromatographic methods were used: for the biocides quantification, the gradient mobile phase was methanol and MilliQ ultrapure water and for pharmaceuticals, the gradient mobile phase was prepared with acetonitrile or MilliQ ultrapure water acidified (0.05 % formic acid). For each compound, an isotopically-labeled surrogate (internal standard) was associated for LC-MS-MS quantification in Multiple Reaction Monitoring Analysis (Supplemental Data, Table S1).

Laboratory bioassays

Chemical products

Based first on the concentration levels of the compounds in the harvesting area (Table 1) and/or based on consumption data and occurrence worldwide when necessary, we analyzed the toxicity of five compounds: the pharmaceuticals venlafaxine (VEN; anxiolytic) and carbamazepine (CBZ; anticonvulsant and antiepileptic), the biocides methylparaben (MP; preservative) and triclosan (TCS; antimicrobial), and a glyphosate degradation product, the aminomethylphosphonic acid (AMPA). To mimic environmentally realistic mixtures of emerging pollutants, we selected one compound in each chemical class and were: CBZ, MP and AMPA mixed in binary and ternary combinations.

The molecules were purchased in analytical grade (purity > 98%) from Sigma Aldrich® (St Louis, MO, USA): VEN (CAS No. 99300-78-4), CBZ (CAS No. 298-46-4), MP (CAS No. 99-76-3), TCS (CAS No. 3390-34-5) and AMPA (CAS No. 1066-51-9). For the preparation of the test solutions, the chemicals were dissolved in the appropriate medium; while AMPA and VEN were soluble in water, the carrier solvent dimethyl sulfoxide (DMSO; CAS No. 67-68-5) was used to dissolve MP, TCS, and CBZ in the solutions with a final proportion comprised between 0.005% and 0.1% of the volume at most. In each bioassay, the effect of DMSO was also tested in parallel to controls (i.e. seawater or culture medium with no added chemicals) and no effect was observed.

Test solutions

For the compounds alone, the bioassays were first applied using a broad range of concentrations from 1×10^{-7} for the minimum to $1 \times 10^7 \mu\text{g.L}^{-1}$ at most by serial dilutions with a factor 10. Then the concentration arrays were narrowed with a dilution factor of 2.5 and

lowered when necessary. The range was not narrowed when the EC_{50} values was above 100 $mg.L^{-1}$. Based on the EC_{50} values of the molecules alone, the mixtures were realized by using the lowest toxic compounds of each class of pollutants in order to test for the hypothesis of an enhancement of toxicity (additive or synergistic action) when mixed together. Three binary mixtures and one ternary mixture were tested and the combinations were: Mixture 1 = CBZ + MP + AMPA; Mixture 2 = CBZ + MP; Mixture 3 = CBZ + AMPA; Mixture 4 = AMPA + MP. The toxicity of these mixtures was tested by sequential dilutions ranging from the actual environmental concentrations (i.e. 1 X), by taking the highest concentration for each compound (given in Table 1), to up to 1×10^7 X with a dilution factor of 10. The range was eventually narrowed with a factor of 2.5 or lowered when relevant. Because AMPA was not detected at the time of the sampling (October 2014), the environmental level of 500 ng.L^{-1} was used (average measured after monthly quantification from between February to November 2014; Bouchart and Houssin 2014) to make the concentration range.

Algal growth inhibition tests

Freshwater algal tests were conducted following the NF EN ISO 8692 guideline (2012) using the microalga *Pseudokirchneriella subcapitata* AC152 obtained from Algobank® (Caen, France). We used the ISO 8692 standard rather than the OECD 201 (2006); however, the two methods are quite similar in terms of experimental conditions (see the document ENV/JM/MONO(2008)28 for further details on comparisons). We performed the tests in 96-well cell culture plates instead of glass vessels in order to miniaturize the assays. The algal growth inhibition tests were conducted at $20 \pm 1^\circ\text{C}$ with continuous shaking at 100 rpm and luminosity (white light at 4,000 lux). Each substance, the medium and the algal inoculums were mixed to obtain an initial algal concentration of $10^4 \text{ cells.mL}^{-1}$ in 0.21 mL of bioassay

volume. At least three replicates were used per concentration. Cell density was measured after 72 h of exposure. The results were expressed as average growth rates calculated from cell numbers based on chlorophyll fluorescence measurements (680 nm, TECAN Infinite[®] M200 microplate reader). The % of inhibition of average specific growth relative to controls were calculated for each concentration. All the controls (chemical-free culture medium and DMSO) met the acceptability criteria, i.e. an algal growth factor higher than 32 after 72 h from the initial concentration, and toxicity for the reference chemical potassium dichromate ($K_2Cr_2O_7$) was between 0.24 and 1.03 mg.L⁻¹ for the 24 h-EC₅₀.

Daphnia magna acute immobilization tests

Immobilization tests were conducted following the guideline NF EN ISO 6341 (1996) using the water flea *Daphnia magna* Straus (Cladocera, Crustacea) obtained from the Institut National de l'Environnement Industriel et des Risques (INERIS, Verneuil-en Halatte, France) and from the Labéo Franck Duncombe (Saint-Contest, France). We also used the ISO 6341 standard rather than the OECD 202 (2004) but the two methods are similar (see the document ENV/JM/MONO(2008)28 for further details on comparisons). Tests were ran using neonates less than 24 h-old obtained from continuous parthenogenetic reproduction of adult females. Static 48h-toxicity tests were performed in 10 mL-culture volume at a temperature of 20 ± 1°C, without food addition and in total darkness. At least three replicates were used per concentration with 5 neonates per replicate. Immobility (i.e., no movement following shaking of the test pill bottles) was the endpoint for effect calculation observed at 48h. All the controls (chemical-free culture medium and DMSO) met the acceptability criteria (no more than 10 % mortality) and the 24 h-EC₅₀ for the reference chemical potassium dichromate ($K_2Cr_2O_7$) ranged between 0.6 and 2.1 mg.L⁻¹.

Oyster embryo-larval development and metamorphosis tests

The methods are fully described in Di Poi et al. (2014).

Embryotoxicity. Acute tests were conducted following the standardized AFNOR XP-T-90-382 procedure (2009) and conditioned oyster genitors were provided by the Guernsey Sea Farm hatchery Ltd[®] (Guernsey, UK). Spawning was induced by thermic stimulation (succession of 30-min cold and warm bathes of 16 and 28°C, respectively) in filtered seawater, and the eggs were fertilized with sperm at a 1:6 (egg:sperm) ratio. Twenty minutes post-fertilization, embryos were distributed into glass pillboxes at a density of 60,000 embryos.L⁻¹, and exposed in a final volume of 25 mL of filtered and sterilized natural seawater (0.22 µm; Steritop[®] Millepore) for 36 h at 22 ± 1°C in dark. Observations were conducted on fixed organisms (8% formalin solution) with an inverted binocular microscope (Leica[®] DM IRB), and the net percentage of normal development (NPN), i.e. number of normal D-shaped larvae vs. number of embryos and abnormal larvae adjusted to the controls, was calculated. Each concentration was tested at least in triplicate. All bioassays met the acceptability criteria with EC₅₀ of Cu²⁺ between 6-16 µg.L⁻¹ and mean rates of normal development for the different control groups reaching 78.5 ± 1.6 % for chemical-free seawater controls, 75.8 ± 2.1 %, 79.7 ± 3.3 %, 79.3 ± 2.1 % for 0.005%, 0.01% and 0.1% DMSO controls, respectively.

Metamorphosis. Twenty-one day-old pediveliger larvae were provided by the SATMAR hatchery[®] (Société ATLantique of MARiculture, Gatteville-le-Phare, France). Larvae were exposed in 12-well culture plates with a density of 50-80 individuals per well in a final volume of 1.5 mL of filtered and sterilized seawater (0.22 µm; Steritop[®] Millepore) for 24 h at 22 ± 1°C in dark. Epinephrine at 10⁻⁴ M (CAS No. 51-43-4; Sigma Aldrich[®], Saint-Louis, MO, USA) was added to promote larval settlement and metamorphosis (Coon and Bonar, 1987). Observations were conducted on fixed organisms (8% formalin solution) with an

inverted binocular microscope (Leica[®] DM IRB). The net percentage of metamorphosis rate (NPM) was assessed by counting the number of metamorphosed larvae vs. total number of dead larvae and non-metamorphosed larvae adjusted to the controls. Each concentration was tested at least in triplicate. Mean rates of metamorphosis for the different control groups reached 80.4 ± 1.8 % for chemical-free seawater controls, 77.7 ± 2.0 %, 83.1 ± 2.8 %, 73.0 ± 1.7 % for 0.005%, 0.01% and 0.1% DMSO controls, respectively.

Data treatment and statistics

Dose-response data analysis

For each endpoint, non-linear regression using the hill equation was used to calculate Effective Concentrations (EC₁₀, EC₂₀, EC₅₀) based on nominal concentrations. These regressions were obtained using the Excel[™] macro REGTOX (Vindimian 2012). Estimates of EC₁₀ and EC₂₀ were taken as equivalent to No Observed Effect Concentration (NOEC), and Lowest Observed Effect Concentration (LOEC), respectively as suggested in OECD guidelines (OECD 201 2006). For each bioassay, toxicity between the four mixtures was compared using a non-parametric Kruskal-Wallis test based on raw data (Supplemental Data, Table S2) following normality tests of Shapiro-Wilk. $p < 0.05$ was chosen as the significance level.

Toxicity of mixtures

Joint-action toxicity data were analyzed based on the Synergistic Ratio (SR) model after Hewlett and Plackett (1959):

$$\text{SR index} = \frac{\text{EC50 of Chemical acting alone}}{\text{EC50 of Chemical + additive (mixture)}}$$

where EC_{50} of the mixture obtained in X were recalculated by taking into account the actual concentrations of each compound used in the mixture in $mg.L^{-1}$; and $SR = 1$ describes that the joint action is additive; $SR < 1$ describes antagonism; $SR > 1$ describes synergism.

Results

Occurrence of emerging compounds in seawater

The concentrations of the 21 compounds screened in coastal waters are reported in Table 1. For pharmaceutical residues, the levels of contamination were equivalent between the two sampling sites. Amongst the 9 molecules screened, 5 pharmaceuticals were quantified at concentrations ranging from 0.81 to 1.74 $ng.L^{-1}$; the highest concentration molecule was oxazepam (1.61 $ng.L^{-1}$ in average). The concentrations of biocides were higher at Meuvaines than at Ver-sur-Mer for 5 biocidal compounds; and levels of methylparaben and triclosan were the most elevated at both sites (averages of 11.12 $ng.L^{-1}$ and 9.13 $ng.L^{-1}$, respectively). Concentrations of the two pesticides (glyphosate, glufosinate) and of AMPA were under the limit of quantification (0.05 $ng.L^{-1}$) at both sites at the time of the sampling (October 2014).

Toxicity of single molecules

The toxicity was evaluated for the five molecules based on nominal concentrations (Table 2). According to the ecotoxicological parameters (EC_x), triclosan and AMPA were, respectively, the most and the least toxic compounds for all species. The EC_x values for each compound were in the same range for the two freshwater species. They were as low as 0.03 $mg.L^{-1}$ in *P. subcapitata* and 0.23 $mg.L^{-1}$ *D. magna* for triclosan, to higher than 100 $mg.L^{-1}$ for carbamazepine and AMPA. The EC_{50} values of venlafaxine and methyl-paraben were, respectively, 47.58 $mg.L^{-1}$ and 35.25 $mg.L^{-1}$ in green algae and 141.28 $mg.L^{-1}$ and 41.23 $mg.L^{-1}$

¹ in the daphnia. In the *C. gigas* embryotoxicity test, the EC₅₀ values ranged from 0.04 mg.L⁻¹ for triclosan up to 50.78 mg.L⁻¹ for AMPA. In the metamorphosis test, triclosan and venlafaxine were the most toxic compounds (EC₅₀ = 0.34 and 0.31 mg.L⁻¹, respectively). Except for triclosan which appeared highly toxic whatever the organism tested, the early life stages of *C. gigas* were more sensitive than freshwater species (EC_xs in *C. gigas* < EC_xs in *D. magna* and *P. subcapitata*).

Joint-action toxicity of mixtures

The toxicity of mixtures varied from 1,793 X in *P. subcapitata* to more than 900,000 X in *C. gigas* and *D. magna* (Table 3). Based on EC₅₀s and for the four bioassays, the mixtures can be ranked as: mixture 2 (MP + CBZ) < mixture 3 (AMPA + CBZ) < mixture 1 (AMPA + MP + CBZ) < mixture 4 (AMPA + MP). However, there was no statistical difference in their range of toxicity for each bioassay (Kruskal-Wallis tests, $p > 0.05$); the ternary mixture did not appear as the most toxic cocktail.

On the basis of EC₅₀s (recalculated in mg.L⁻¹ according to the environmental values), all the mixtures were found to be more toxic to the three species than the chemicals applied alone. There was a high variability in the SR ratios ranging from 0.09 to 144 (Table 4). In the green algae, most SR ratios calculated with reference to CBZ, AMPA and MP agreed with the model of synergism action (SR > 1); by contrast a model of additive action was suggested in the case of MP in the ternary mixture (SR = 1). The synergistic ratios derived from the AMPA + MP mixture, and from the MP + CBZ mixture with reference to CBZ (27 < SRs < 144) were significantly higher than the other SR values (SR < 10) indicating higher potency. In the daphnia, the joint-action toxicity of the ternary mixture and the AMPA + CBZ mixture agreed with antagonistic models, whatever the chemical of reference (SR < 1); whereas the SR for

the mixture 2 (MP + CBZ) and for the mixture 4 with reference to AMPA were consistent with a synergistic model ($SR > 1$). In the *C. gigas* metamorphosis bioassay, most mixtures fitted with an antagonistic model ($SR < 1$); whereas in the embryotoxicity bioassay, both concepts of synergism and antagonism were observed according to the mixtures. The SR model revealed that the interactions were conformed to the model of synergism for all the mixtures with reference to AMPA, and for mixtures 2 and 4 ($SR > 1$), but the mixtures with reference of CBZ (mixtures 1 and 3) and MP (mixture 1) were rather antagonistic.

Discussion

Occurrence of emerging pollutants in French northwest coastal waters

From the pharmaceutical residues, psychotropic drugs (carbamazepine, oxazepam, venlafaxine) were the most represented therapeutic class in Normandy coastal waters. This was in agreement with data from Fabbri and Franzellitti (2016) showing the high prevalence of psychotropic drugs, such as antiepileptics, in seawater worldwide. Minguez et al. (2016) also found carbamazepine in relatively high concentrations in the summers 2011 and 2012 further east along the Normandy coastline (i.e. $> 20 \text{ ng.L}^{-1}$ in the Orne river, 2.3 km downstream the point of release of the main wastewater treatment plant of the city of Caen; 1.9 ng.L^{-1} in a marine estuary at 17 km downstream the same treatment plant), which highlighted the potential influence of urban discharges and tourism on pharmaceutical concentrations. Carbamazepine concentrations can also reach 132 ng.L^{-1} and 228 ng.L^{-1} in the Seine and Loire estuaries, respectively (AESN-AQUASCOP 2008). We also quantified the antidepressant venlafaxine in seawater, at a non-negligible level (1.13 ng.L^{-1} in average). Compared to nine other antidepressants (e.g. sertraline, paroxetine, duloxetine, clomipramine, etc.), venlafaxine was found to be also the most prevalent antidepressant in the Orne river

(reaching 4.8 ng.L^{-1}), and was still detected at sea at a concentration of 1.3 ng.L^{-1} (Minguez et al. 2016). Interestingly, the concentrations of biocides, and of methylparaben and triclosan particularly, were higher at Meuvaines than at Ver-sur-Mer. Meuvaines is a small rural village (population of 156 inhabitants) where agriculture represents 40.5% of the local activity (e.g. cereal crop, sugar beet cultures and cattle farming; INSEE 2011) and where biocides may be widely used in breeding and raising livestock. In addition, any pesticide was detected at the time of the environmental monitoring (punctual sampling in October 2014). Polar pesticides are not regularly monitored in the marine environment, in part, because of the analytical challenge due to the compounds' chemical properties (i.e., amphoteric characteristics). In addition, marine waters are usually complex matrices and contaminants are present at low concentrations which require accurate methods of quantification. It also highlighted the need to use more integrative sampling methods using passive sampling device (e.g. POCIS) that allow *in situ* collection of a time-integrated average of hydrophilic organic compounds. However, AMPA was the herbicidal product the most frequently detected in seawater at Meuvaines (Bouchart and Houssin 2015). AMPA concentrations averaged $0.5 \text{ }\mu\text{g.L}^{-1}$ in seawater at Meuvaines over a period of 10 months the same year (based on a monthly monitoring from February to November 2014) with a peak of $1.8 \text{ }\mu\text{g.L}^{-1}$ in April (Bouchart and Houssin 2015). The difference of levels between April (maximum) and October (absence; our study) also suggested that the use of pesticides and thus, the pesticide runoff, varied across the year. The highest presence of AMPA in coastal waters in spring is likely to correlate with a period of high agricultural activities in spring and to its leaching to coastal waters. On the other hand, glufosinate was detected once in February 2014 at a concentration of 100 ng.L^{-1} (Bouchart and Houssin 2015).

Evaluation of single toxicity

The classification of the Commission of the European Communities (1996) considered compounds as very toxic for aquatic organisms when $EC_{50} < 1 \text{ mg.L}^{-1}$, toxic when $1 \text{ mg.L}^{-1} < EC_{50} < 10 \text{ mg.L}^{-1}$, harmful when $10 \text{ mg.L}^{-1} < EC_{50} < 100 \text{ mg.L}^{-1}$, and harmless when $EC_{50} > 100 \text{ mg.L}^{-1}$. Based on nominal concentrations, the biocide triclosan was very toxic to all the species studied since the acute toxicity data were harvested from concentrations below 1 mg.L^{-1} . In the same class, the preservative methylparaben showed either toxic or a harmful proclivity for the oyster metamorphosis test and for the three other bioassays, respectively. The toxicity of AMPA and carbamazepine varied from harmful to harmless according to the species studied. Finally, the toxicity of the anxiolytic venlafaxine varied strongly between very toxic for oyster larvae to harmful for algae and daphnia. In addition, these results showed that early-life stages of *C. gigas* were generally more sensitive than freshwater species (24-36 h- EC_{50} in *C. gigas* < 48-72 h- EC_{50} in *P. subcapitata* and *D. magna*) which supported previous data regarding the toxicity of antidepressants such as fluoxetine, sertraline and clomipramine (Minguez et al. 2014a). In this study, the authors reported a sensitivity gradient between seven aquatic species (4 marine species including *C. gigas*, and 3 freshwater species such as *P. subcapitata* and *D. magna*) and showed that the most sensitive tests were those performed with the bivalve *C. gigas* and the marine microalgae *Skeletonema marinoi*. The impacts of pollutants on the Pacific oyster are particularly of concern. Indeed, *Crassostrea gigas* is a filter-feeding mollusk which dominates the marine bivalve production worldwide. For example in the year 2011-2012, *C. gigas* dominates the production of oysters in France representing 98.9% of the total production (Comité National de la Conchyliculture 2016). Thus a toxic effect at the larval level may induce potentially important population and economic consequences. It therefore suggested that the embryo-larval development and

metamorphosis in *C. gigas* larvae are sensitive endpoints for ecotoxicological assessment, and are important to consider in addition to regulatory model organisms.

Because of the absence of measured concentrations in the testing vessels during the exposures, we used the nominal concentrations instead of analytically measured ones to determine toxicity values which is a method regularly used in literature (e.g., Orvos et al. 2002; Kim et al. 2007; Terasaki et al. 2009; Bazin et al. 2010). In addition, as we did not know the persistency of the compounds, three bioassays were performed in total darkness and/or with glass vessels to avoid photolysis and sorption as much as possible. Overall, there is little information on the degradation (both biotic and abiotic) of these chemicals in such experimental devices. However, Orvos et al. (2002) reported a high level of recovery of triclosan during the 72 h-toxicity test with the algae *Scenedesmus subspicatus* under a constant light regime. On the other hand, the study of Aranami and Readman (2007) showed that triclosan in 2-L glass bottles was sensitive to irradiation in seawater (about 40% of disappearance after 72h-irradiation) whereas degradation was absent in darkness for 12 days. Interestingly, parabens did not appear so persistent in static systems (Yamamoto et al. 2011) whereas Terasaki et al. (2009) did not show a significant degradation of methylparaben in their 48h-*D. magna* bioassays with a photoperiod of 16-8 light-dark cycle and the latest found a similar EC₅₀ value (average nominal value of 62 mg.L⁻¹). Mottier et al. (2013) also showed a good recovery of AMPA both in glass pillboxes and in culture plates in the 36 h-oyster embryotoxicity and 24 h-metamorphosis bioassays. In addition, it can be said that carbamazepine seems to be relatively persistent, and especially towards direct photolysis (Challis et al. 2014).

Acute effects of pharmaceuticals

The literature review demonstrates a lack of ecotoxicological data for pharmaceuticals, and particularly for psychotropic drugs. Using immobilization as endpoint and based on nominal concentrations, the 48 h-EC₅₀ of carbamazepine in *D. magna* exceeded 100 mg.L⁻¹ which was corroborated by three recent studies (Cleuvers 2003; Kim et al. 2007; Minguez et al. 2016). On the other hand, an other study showed EC₅₀ values reaching approximately 13.8 mg.L⁻¹ after 48 h of exposure (Ferrari et al. 2003). In the algae *P. subcapitata*, toxicity data about growth inhibition were above 100 mg.L⁻¹ after 72 h of exposure, which was supported by the study of Minguez et al. (2016) reporting 72 h-EC₅₀ > 100 mg.L⁻¹. Ferrari et al. (2003) also showed 96 h-NOEC and LOEC higher than 100 mg.L⁻¹ as well. As a comparison, the growth of another algae species, *D. subspicatus*, was inhibited from 74 mg.L⁻¹ after 72 h-exposure to carbamazepine (Cleuvers 2003). The acute toxicity of venlafaxine reached 141.28 mg.L⁻¹ in *D. magna* and 47.58 mg.L⁻¹ in *P. subcapitata* after 48 h and 72 h of exposure, respectively (Minguez et al. 2014b, 2016).

There is no data available for carbamazepine and venlafaxine toxicity in marine bivalves, but using the same larval bioassays in *C. gigas*, a previous study showed that acute toxicity of antidepressants (fluoxetine, sertraline, etc.) was ranged between 0.067 to up to 1 mg.L⁻¹ (Di Poi et al. 2014). In *Mytilus galloprovincialis* embryotoxicity test, the EC₅₀-value was 0.21 µg.L⁻¹ for sertraline (Estévez-Calvar et al. 2016). Aguirre-Martínez et al. (2015) also showed that acute toxicity of the pharmaceuticals ibuprofen, carbamazepine, novobiocin and caffeine, was in the order of mg.L⁻¹ for bacteria bioluminescence, microalgae growth inhibition and urchin fertilization. However, teratogenicity was observed in *Paracentrotus lividus* after exposure to environmental concentrations of drugs at 10 ng.L⁻¹; at this concentration, carbamazepine and ibuprofen were found to reduce significantly the embryo-larval

development. Taken together, these data showed that pharmaceuticals, and particularly antidepressant drugs (e.g., venlafaxine), can be very toxic to marine invertebrates. In addition, some compounds showing no ecological risk (Minguez et al. 2016) and displaying a moderate hydrophobicity (e.g., carbamazepine with a $\log K_{ow} > 2.45$), can potentially bioaccumulate (Ferrari et al. 2004). Such pharmaceuticals are likely to exert long-term effects which cannot be detected after short-term exposures.

Acute effects of pesticides

D. magna and *P. subcapitata* are two biological models that have classically been used to guide European legislation on contaminants including pesticides (Agritox 2016). In our study, AMPA did not appear highly toxic to the green alga and daphnia (EC_{50} -values $> 100 \text{ mg.L}^{-1}$). Compared to glyphosate, limited EC_{50} data are available for AMPA, but available values were higher than 100 mg.L^{-1} (e.g. EC_{50} -value = 690 mg.L^{-1} for the immobilization test in daphnia; Agritox 2016; PPDB 2016) which supported our results. On the other hand, EC_{50} -values were lower for oyster early-life stages and were comprised between 50 and 80 mg.L^{-1} (nominal values). In the same laboratory conditions, Mottier et al. (2013) reported similar nominal EC_{50} values, which were 40.62 mg.L^{-1} and higher than 100 mg.L^{-1} for the embryotoxicity and the metamorphosis tests, respectively. As a comparison, Akcha et al. (2012) showed that glyphosate, whose degradation product is AMPA, had no effect on oyster development at low tested concentrations (0.5 to $5.0 \text{ } \mu\text{g.L}^{-1}$). Mottier et al. (2013) reported later that the acute toxicity of glyphosate ranged between 28 mg.L^{-1} (embryo-larval development) to higher than 100 mg.L^{-1} (metamorphosis).

Acute effects of other biocides

The biocides methylparaben and triclosan appeared as the most toxic chemicals to all organisms studied ($1 \text{ mg.L}^{-1} < \text{EC}_{50\text{s}} < 100 \text{ mg.L}^{-1}$ for methylparaben; $< 1 \text{ mg.L}^{-1}$ for triclosan). In the literature, acute toxicity of triclosan has been examined in aquatic invertebrates, fish, amphibians, algae and plants (Brausch and Rand 2011), and the data showed that this chemical was more toxic to similarly studied trophic groups in comparison to other disinfectants (triclocarban, biphenylol). In addition, the same authors reported that for all disinfectants studied, invertebrates are only slightly more sensitive than fish for acute time periods.

The ecotoxicological values that stem from our study were corroborated with literature in *D. magna*. When nominal concentrations were used, the 48 h- $\text{EC}_{50\text{s}}$ reached 0.397 mg.L^{-1} for triclosan (Orvos et al. 2002), and ranged from 54 mg.L^{-1} (Bazin et al. 2010) to 62 mg.L^{-1} (Terasaki et al. 2009) for methylparaben. Yamamoto et al. (2011) used analytically measured concentrations to determine toxicity values and reported a 48 h-effective concentration of 34 mg.L^{-1} for methylparaben which was the least toxic paraben amongst six other forms (e.g. n-butylparaben, n-propylparaben, benzylparaben).

In *P. subcapitata*, 72 h-growth $\text{EC}_{50\text{s}}$ were of 0.025 mg.L^{-1} and 35.25 mg.L^{-1} for triclosan and methylparaben, respectively, which were close to published data reaching 0.53 mg.L^{-1} (nominal value) for triclosan in Yang et al. (2008) and 80 mg.L^{-1} (measured value) for methylparaben in Yamamoto et al. (2011). Moreover, our data coupled with the literature confirmed that triclosan was the most toxic biocidal compound. Our results showed that green algae appeared more sensitive to triclosan than daphnia. High triclosan sensitivity in algae is likely due to the antibacterial characteristics of the compounds which disrupt lipid synthesis (McMurry et al. 1998), destabilize membrane (Lyrgen et al. 2003; Franz et al. 2008) or uncouple oxidative phosphorylation (Newton et al. 2005) and that are similar between algae

and bacteria (Coogan et al. 2007). Furthermore, it can be seen that even if measured concentrations were lacking in our study, methylparaben is classified similarly (i.e. as harmful) based on both nominal and analytical EC_{50} -values in freshwater species.

For the first time, *C. gigas* embryotoxicity and metamorphosis tests have been used in order to assess the toxicity of biocides and showed that oyster larvae were particularly sensitive to triclosan. As a comparison in the marine mussel *Perna perna*, the mean inhibition concentrations of triclosan were 0.490 mg.L^{-1} (1 h- IC_{50}) for fertilization, and 0.135 mg.L^{-1} for the embryo-larval development (48 h- IC_{50}) (Cortez et al. 2012). Based on limited environmental concentration and toxicity data, it appeared that biocidal compounds may cause adverse effects to aquatic organisms at concentrations as low as $5 \text{ }\mu\text{g.L}^{-1}$ in *C. gigas* (our study). However, preliminary data on environmental concentrations suggested only a minimal risk to marine organisms as effect concentrations are generally 350-fold higher than what has been observed in coastal seawaters.

Toxicity of mixtures

Because aquatic organisms are continuously exposed to complex mixtures of emerging contaminants, there is a need to evaluate mixture toxicity. When two or more chemicals are applied simultaneously to living organisms, the combined effect may result in the addition of toxic effect of one chemical to the other (non-interactive or additive action); or the toxic effects caused by the mixture being significantly less than the sum of the toxic effects of the separate constituents (antagonism model); or the toxic effects caused by the mixture may significantly exceed the sum of effects of the separate components (synergism model) (Stenersen 2004).

First, we showed that the ternary mixture was not the most toxic cocktail, and that the toxicity of all mixtures was in the same range (no statistical difference) for each bioassay. In addition,

the comparative evaluation of the single and joint-toxicity of the chemicals based on the Synergistic Ratio model (Hewlett and Plackett 1959) revealed that the presence of methylparaben generally increased the toxicity of AMPA, with a particularly high potency in *P. subcapitata*. For all bioassay, the toxicity of carbamazepine and methylparaben was also enhanced in presence of each other (synergistic models except in the metamorphosis bioassay). However, the remaining data highlighted that evaluation of joint-toxic effects can be complex and that species-dependent mixture models can appear. As examples, we found that almost all mixtures fitted with a synergistic model in *P. subcapitata*, while these mixtures of molecules agreed with the antagonistic concept in oyster metamorphosis. In addition, the ternary mixture and the cocktail AMPA + CBZ agreed with either synergistic model in algae or antagonism in aquatic invertebrates. Compounds of a heterogeneous mixture often have different toxic sites and dissimilar modes of action such that concentration addition or synergism may not be expected (Broderius et al. 2004). Interestingly, Yang et al. (2008) published that binary mixtures of several antibacterial agents (e.g. triclosan, tylosin, triclocarban, tetracyclines), expecting to have the same mode of action, showed additive, synergistic, and even antagonistic effects in *P. subcapitata*. It has been reported that the types of interactions exhibited by the components of mixtures largely depend on the proportion of the occurrence in the mixtures (Otitolaju 2005). There is a lack of information on joint-toxicity and no agreed methods of dealing with mixtures which lead to difficult comparison between data. However as observed in our study, it was found that most of the mixtures studied, even with chemicals having different modes of action, act antagonistically or together in a synergistic fashion. Recent data reported that *in vivo* and chronic toxicity of pharmaceutical mixtures (10 most detected active pharmaceutical ingredients in the Tama river, Japan) may also deviate from concentration addition and/or independent action to produce synergistic effects (Watanabe et al. 2016). Numerous other examples of synergism

are documented in the literature for mixtures of botanical pesticides, refined petroleum products, crude oil plus dispersants, or trace metals (e.g., Otitoloju 2003; Chukwuand and Okhumale 2009; Al-Jawasim et al. 2015; Ekpendu et al. 2016). Cleuvers (2003) also reported that combinations of various pharmaceuticals revealed stronger effects than expected from the effects measured singly. In addition, the mixture of carbamazepine and clofibrinic acid followed the concept of concentration addition in *D. magna*, while in the macrophyte *Lemna minor*, it acted with independent action. According to Backhaus (2014), the deviation from concentration addition/independent action may appear to be largely confined to mixtures of only a few compounds, whereas multicomponent mixture studies rarely observed synergistic or antagonistic mixture effects. The author suggested that this phenomenon might be explained by buffering against the impact of a few synergistic or antagonistic interactions.

We also showed that joint-action toxicity of the chemicals, and particularly in reference of AMPA and methylparaben, behaved differently in the two *C. gigas* bioassays. The toxicity of the molecules in the mixtures generally agreed with antagonistic models in pediveliger larvae (21 d-old, metamorphosis test); whereas most ratios showed synergistic interactions in D-larvae (36h-old, embryotoxicity test). It could suggest sensitivity difference between life stages and/or differences of mode of action for the chemicals according to the maturity of the animals. In the Pacific oyster, young D-larvae were already found more sensitive to toxicological injuries due to exposure to several herbicides (Mottier et al. 2013) and antidepressants (Di Poi et al. 2014) than older organisms. Indeed, D larvae experience essential and costly developmental processes at this period of life (e.g., formation of morphological axes, rearrangement of cells) which might explain their highest sensitivity.

Conclusions

As in their natural habitat, organisms are exposed not to a single chemical but simultaneously or sequentially to multiple mixtures of compounds, the present study investigated single and joint-effects of binary and ternary mixtures of several emerging substances. In summary, the results showed heterogeneity of the acute and sub-chronic toxicity of the tested molecules with the biocidal compounds (triclosan, methylparaben) being particularly toxic to freshwater and marine organisms contrary to the pesticide AMPA. Based on the EC_{50} values, acute effects seemed to be unlikely since the median toxicity concentrations were at minimum 10-fold higher than actual environmental levels. However, significant combination effects of substances can occur even if the toxicity of the single substances is low. In our study, the toxicity of binary and ternary mixtures was not different but the mixtures were much more toxic than the chemicals singly, and eventually led to synergistic or antagonistic interactions. Our data were preliminary, and repeated testing and careful chemical analyses are necessary to confirm that these interactive effects occur consistently at these exposure concentrations. However, the realisation that a potentiation of toxicity could result when pollutants occur in mixtures underscores the need for an awareness of cocktail effects for better ecological risk assessment. In addition, our study also confirmed a higher sensitivity of bivalve larvae to chemicals compared to freshwater species (green algae, daphnia), and therefore highlights the importance of considering *C. gigas* bioassays in addition to regulatory standard tests in ecotoxicological studies.

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Caption for Figure

Figure 1. Location of the two seawater collection sites (geographical coordinates: $49^{\circ}20'38''\text{N}$ $0^{\circ}34'19''\text{W}$ and $49^{\circ}20'45''\text{N}$ $0^{\circ}31'99''\text{W}$) for the quantification of emerging pollutants on the Northwestern French coast (Normandy, France). These sites are important regional oyster and cockle harvesting areas and are illustrated by the drops.



Table 1. Concentration quantification of emerging pollutants belonging to three chemical classes (pharmaceuticals, biocides, pesticides) in seawater at two shellfish production sites (Meuvaines 49°20'38"N 0°34'19"W; Ver-sur-Mer 49°20'45"N 0°31'99"W, Normandy, France).

Compound class	Type	Linear molecular formula	Sampling site	
			Meuvaines [C] (ng.L ⁻¹)	Ver s/Mer [C] (ng.L ⁻¹)
<i>Pharmaceuticals</i>				
Atenolol	beta-blocker	C ₁₄ H ₂₂ N ₂ O ₃	< <i>lq</i> (0.5)	< <i>lq</i> (0.5)
Carbamazepine	antiepileptic	C ₁₅ H ₁₂ N ₂ O	0.96	1.01
Diclofenac	NSAID*	C ₁₄ H ₁₁ Cl ₂ NO ₂	< <i>lq</i> (0.5)	< <i>lq</i> (0.5)
Ibuprofen	NSAID*	C ₁₃ H ₁₈ O ₂	< <i>lq</i> (1.0)	< <i>lq</i> (1.0)
Ketoprofen	NSAID*	C ₁₆ H ₁₄ O ₃	1.56	< <i>lq</i> (0.5)
Metoprolol	beta-blocker	C ₁₅ H ₂₅ NO ₃	< <i>lq</i> (0.5)	< <i>lq</i> (0.5)
Oxazepam	anxolytic	C ₁₅ H ₁₁ ClN ₂ O ₂	1.47	1.74
Sulfamethoxazole	antibiotic	C ₁₀ H ₁₁ N ₃ O ₃ S	1.47	1.60
Venlafaxine	antidepressant	C ₁₇ H ₂₇ NO ₂	1.45	0.81
<i>Biocides</i>				
Benzylparaben	preservative	C ₁₄ H ₁₂ O ₃	< <i>lq</i> (1.0)	< <i>lq</i> (1.0)
Butylparaben	preservative	C ₁₁ H ₁₄ O ₃	< <i>lq</i> (1.0)	< <i>lq</i> (1.0)
Ethylparaben	preservative	C ₉ H ₁₀ O ₃	3.00	1.56
Isobutylparaben	preservative	C ₁₁ H ₁₄ O ₃	< <i>lq</i> (1.0)	< <i>lq</i> (1.0)
Isopropylparaben	preservative	C ₁₀ H ₁₂ O ₃	< <i>lq</i> (1.0)	< <i>lq</i> (1.0)
Methylparaben	preservative	C ₈ H ₈ O ₃	18.59	3.64
Propylparaben	preservative	C ₁₀ H ₁₂ O ₃	2.95	0.87
Triclocarban	antimicrobial	C ₁₃ H ₉ Cl ₃ N ₂ O	1.75	0.18
Triclosan	antimicrobial	C ₁₂ H ₇ Cl ₃ O ₂	14.23	4.02
<i>Pesticides</i>				
Glyphosate	organophosphate	C ₃ H ₈ NO ₅ P	< <i>lq</i> (0.05)	< <i>lq</i> (0.05)
Aminomethylphosphonic acid (AMPA)	glyphosate degradate	CH ₆ NO ₃	< <i>lq</i> (0.05)	< <i>lq</i> (0.05)
Glufosinate	organophosphate	C ₅ H ₁₂ NO ₄ P	< <i>lq</i> (0.05)	< <i>lq</i> (0.05)

with < *lq* = limit of quantification (concentration limit in ng.L⁻¹).

*NSAID = NonSteroidal Anti-Inflammatory Drugs.

Table 2. Toxicity of the chemicals for each endpoint evaluated by the values of EC₁₀ (equivalent to the No Observed Effect Concentration), EC₂₀ (equivalent to the Lo Observed Effect Concentration) and EC₅₀ (effective concentration that affects 50 % of the population) calculated on nominal concentrations by the ExcelTM macro REGTOX.

Test species	Endpoint	Pesticides			Antimicrobial preservatives						Psychotropic drugs					
		AMPA			Methyl-paraben			Triclosan			Carbamazepine			Venlafaxine		
		EC _x (mg.L ⁻¹)			EC _x (mg.L ⁻¹)			EC _x (mg.L ⁻¹)			EC _x (mg.L ⁻¹)			EC _x (mg.L ⁻¹)		
	10	20	50	10	20	50	10	20	50	10	20	50	10	20	50	
Marine species:																
<i>C. gigas</i>	36 h-embryotox	38.55	42.68	50.78	8.18	11.07	18.57	5x10 ⁻³	0.01	0.04	5.27	8.20	17.48	0.45	0.57	0.87
	24 h-metamorph	27.08	39.80	76.90	1.24	2.46	7.88	0.09	0.15	0.34	10.78	14.22	22.83	0.04	0.09	0.31
Freshwater species:																
<i>P. subcapitata</i>	72 h-growth	85.05	> 100	> 100	20.34	24.92	35.25	0.01	0.01	0.03	0.60	6.15	> 100	10*	17*	47.58*
<i>D. magna</i>	48 h-immobil	> 100	> 100	> 100	27.01	31.57	41.23	0.21	0.21	0.23	> 100	> 100	> 100	68*	89*	141.3*

*data from L. Miguez (personal communication).

Table 3. Toxicity of the mixtures evaluated for each endpoint by the values of EC₁₀ (equivalent to the No Observed Effect Concentration), EC₂₀ (equivalent to the Lo Observed Effect Concentration) and EC₅₀ (effective concentration that affects 50 % of the population) calculated on nominal concentrations by the ExcelTM macro REGTOX. The unit X corresponds to the environmental concentration given in Table 1. There was no statistical difference in toxicity between the four mixtures for each bioassay (Kruskal-Wallis tests).

Test species	Endpoint	Mixture 1 AMPA + MP + CBZ			Mixture 2 MP + CBZ			Mixture 3 AMPA + CBZ			Mixture 4 AMPA + MP		
		EC _x (X)			EC _x (X)			EC _x (X)			EC _x (X)		
		10	20	50	10	20	50	10	20	50	10	20	50
Marine species:													
<i>C. gigas</i>	36 h-embryotox	7,465	14,322	43,629	6,476	22,673	193,112	25,177	36,932	71,097	147.0	769.0	12,981
	24 h-metamorph	29,970	57,498	175,157	32,298	113,076	963,100	77,633	113,880	219,227	19,353	36,816	158,124
Freshwater species:													
<i>P. subcapitata</i>	72 h-growth	49.4	708.6	67,227	617.5	8,045	647,754	66.0	988.0	100,893	13.3	81.4	1,793
<i>D. magna</i>	48 h-immobil	241,853	252,573	272,012	515,496	641,031	930,445	256,635	267,513	287,187	99,999	105,528	115,697

AMPA = aminomethylphosphonic acid, MP = methylparaben, and CBZ = carbamazepine.

Table 4. Synergistic ratios (SR) of binary and ternary mixtures calculated according to the synergistic ratio model (Hewlett and Plackett 1959) for each endpoint, where $SR = 1$ describes that the joint action is additive; $SR < 1$ describes antagonism and $SR > 1$ describes synergism.

Test species	Endpoint		Mixture 1	Mixture 2	Mixture 3	Mixture 4
			AMPA + MP + CBZ	MP + CBZ	AMPA + CBZ	AMPA + MP
Marine species:						
<i>C. gigas</i>	36 h-embryotoxicity	CBZ	0.77	4.76	0.49	-
		AMPA	2.24	-	1.43	7.55
		MP	0.82	5.06	-	2.76
	24 h-metamorphosis	CBZ	0.25	1.25	0.21	-
		AMPA	0.85	-	0.70	0.94
		MP	0.09	0.43	-	0.10
Freshwater species:						
<i>P. subcapitata</i>	72 h-growth	CBZ	9.35	26.52	6.46	-
		AMPA	3.83	-	2.65	143.95
		MP	1.01	2.86	-	37.95
<i>D. magna</i>	48 h-immobilization	CBZ	0.71	5.66	0.70	-
		AMPA	0.71	-	0.70	1.67
		MP	0.29	2.33	-	0.69

AMPA = aminomethylphosphonic acid, MP = methylparaben, and CBZ = carbamazepine.