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Joint effects of nine antidepressants on *Raphidocelis subcapitata* and *Skeletonema marinoi* : a matter of amine functional groups

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Abstract :

Antidepressants are among the most prescribed pharmaceuticals throughout the world. Their presence has already been detected in several aquatic ecosystems worldwide and their effects on non-target organisms justify the growing concern of both the public and regulatory authorities. These emerging pollutants do not occur as isolated compounds but rather as multi-component mixtures, which may lead to increased adverse effects compared to individual compounds. Freshwater and marine algae seem particularly sensitive to pharmaceuticals, including antidepressants. Studies assessing the toxicity of antidepressant mixture to algae focused mainly on binary mixtures of selective serotonin reuptake inhibitors. In the present experiment, the freshwater algae *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) and the marine diatom *Skeletonema marinoi* were exposed to equitoxic mixtures of 9 antidepressants (fluvoxamine, fluoxetine, sertraline, duloxetine, venlafaxine, clomipramine, amitriptyline, and citalopram) at different concentrations. The growth inhibition was measured.

Results showed that the toxicity of this mixture was higher than the effects of each individual component, highlighting simple additivity or synergistic effects, whereas tested concentrations were below the 10% inhibition concentration (IC10) of each compound. Moreover, the QSAR analysis highlighted that antidepressants would act through narcosis (non-specific mode of action) towards the two species of algae. However, more specific effects can be observed by differentiating compounds with a primary/secondary amine from those with a tertiary amine. These mixture effects on algal species have to be assessed, especially since any impacts on phytoplankton could ultimately impact higher trophic levels (less food, secondary poisoning).

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Highlights

► Additivity and synergism were observed even at concentrations below the IC_{10}. ► Observed mixture toxicity did not always fit with the CA model. ► Polar narcosis would be the mode of action of primary/secondary amines to *Raphidocelis subcapitata*. ► A more specific mode of action is hypothesized to *Skeletonema marinoi*. ► Tertiary amines would act through non-polar narcosis to both algae species.

Keywords : green algae, diatom, mixture, pharmaceutical, QSAR

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1. Introduction

Pharmaceuticals are one of the most frequently detected emerging contaminants in surface waters (Fent et al 2006; Santos et al 2010). Almost three thousand compounds are currently used belonging to diverse therapeutic families. Their increasing consumption and the limited efficiency of sewage treatment plants to completely remove them lead to their continuous emission into surface waters (Fent et al 2006; Santos et al 2010). Their widespread occurrence in aquatic ecosystems raises the question whether pharmaceuticals might pose a risk for exposed non-target organisms (Fent et al 2006). Within the past decade, the number of scientific publications about the assessment of the ecological impact of pharmaceuticals on freshwater and marine ecosystems has significantly increased, concomitantly with improvements in analytical methods and abilities to detect trace levels (e.g. Daughton and Ternes, 1999; reviews by Brooks and Hugett, 2012; Kummerer, 2008; Santos et al 2010). However, knowledge gaps still remain especially concerning marine organisms and mixture toxicities though the usual exposure in the environment is towards pharmaceutical mixtures. In 2004, Henry et al already underlined the importance to investigate the effects of mixtures in future research. Ten years later, in his review Backhaus (2014) reported that knowledge on the impact of pharmaceutical mixtures on aquatic organisms, especially marine organisms, was still severely lacking.

Several types of pharmaceuticals already raise concern due to their toxic effects on aquatic organisms, as highlighted by Minguez et al (2016) in their risk assessment study. Antidepressants are one of these drug classes (Fong and Ford, 2014; Minguez et al 2016). They are used in the treatment of depression and other psychiatric disorders in human by modifying neurotransmission, but the good evolutionary conservation of their therapeutic targets means that they may have physiological effects on non-target aquatic organisms too (Fong and Ford, 2014). An antidepressant classification exists based on their mechanisms of action, and three groups are defined: (1) monoamine oxidase inhibitors (MAOI), (2) selective serotonin reuptake

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inhibitors (SSRI) and (3) serotonin-norepinephrine reuptake inhibitors (SNRI). However, the chemical structure of all organic compounds is also an important parameter to understand their behavior in organisms (McKinney et al, 2000). This is particularly true for organisms not possessing neurotransmitters, like algae, and thus, for which the current classification of antidepressants is irrelevant.

Antidepressants have all in common the presence of an amine functional group participating in the interaction with cellular membranes (Minguez et al 2014), and playing a role both in their physicochemical properties, such as basicity or steric dimension, and in their biological properties by providing a cationic center helping in mimicking or in interfering with biogenic amine neurotransmitters. However, these general properties can change depending on amine subclass (Sánchez and Hyttel, 1999). Antidepressants can thus be separated into primary amines like fluvoxamine, secondary amines like fluoxetine, sertraline and paroxetine, and tertiary amines like venlafaxine and citalopram (Wishart et al 2006). It has to be noted that antidepressants are mainly secondary or tertiary amines (Sánchez and Hyttel, 1999). Despite the key role played by the amine functional group in the biological activity of antidepressants, to our knowledge no studies have tried to link ecotoxicity of antidepressants to aquatic organisms and these subclasses of amines.

Most of antidepressants display octanol-water partition coefficients ($\log K_{OW}$) higher than or equal to 3 (Wishart et al 2006) and thus have the ability to interact with lipid membranes in organisms and bioaccumulate along the food web (Christensen et al, 2007; Besse and Garric, 2010). At the basis of aquatic ecosystems, primary producers such as algal species, seem to accumulate and be particularly sensitive to antidepressants (Brooks et al, 2003; Christensen et al, 2007; Johnson et al, 2007; Minguez et al 2015, 2016). Indeed, according to the classification of EU directive 93/67/EEC (EU, 2003) which classified substances based on their effective concentration affecting 50% of an endpoint (EC_{50}), studied antidepressants, mostly SSRI

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compounds, would be considered from toxic to very toxic to algae (Brooks et al, 2003; Christensen et al, 2007; Johnson et al, 2007; Minguez et al, 2016). Consequently, any disruption of primary producer compartment may have cascading adverse effects on higher trophic levels, and assessing pharmaceutical toxicity to algae is thus of ecological interest.

The aims of the present study was thus (1) to investigate the joint effects of nine antidepressants on growth of two algal species, i.e. the freshwater green alga *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) (Guiry, 2017) and the marine diatom *Skeletonema marinoi*, by applying the Concentration Addition concept (CA) proven to be powerful in predicting mixture toxicity of compounds with similar mode or mechanism of action (e.g. Christensen et al 2007; Henry and Black, 2007); and (2) to identify the compounds driving mixture effects. For that, we exposed the two algal species for 72 hours to different antidepressant mixtures (i.e. a mixture of nine antidepressants, a mixture of primary and secondary amines, and a mixture of tertiary amines) according to guidelines of standardized bioassays.

2. Materials and Methods

2.1. Tested compounds

The nine antidepressants we used for the bioassays were the primary amine, fluvoxamine maleate; secondary amines, fluoxetine HCl, sertraline HCl, paroxetine HCl and duloxetine HCl; and tertiary amines, venlafaxine HCl, clomipramine HCl, amitriptyline HCl, and citalopram HBr (Table 1). All nine were supplied in analytical grade by Kemprotec Limited® (Maltby, Middlsbrough, U.K.). All these antidepressants have been detected in the environment (Roig and D'Aco, 2016).

2.2. Algal growth inhibition assays

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Freshwater algal tests were conducted following the NF EN ISO 8692 (ISO, 2012) guideline using the microalga *Raphidocelis subcapitata* AC152 obtained from Algobank (Caen, France). Marine algal tests were performed following the NF EN ISO 10253 (ISO, 2006) guideline using the diatom *Skeletonema marinoi* AC174 provided by Algobank (Caen, France). All algal growth inhibition tests were conducted at 21 ± 1 °C with continuous shaking at 100 rpm and continuous white light (84 µmol photons/m²/s). Stock solutions were prepared by directly dissolving test compounds in Lefevre-Czarda medium supplemented with trace elements for freshwater assays (AFNOR, 1980) or in f/2 medium (Guillard and Ryther, 1962) for marine assays. Test concentrations were obtained by serial dilutions from stock solutions. To ensure the exposure of alga to the right concentrations, concentrations were quantified according to the method detailed in Minguez et al (2015). Measurements were done only on t0-solutions due to a low volume of solutions available at the end of the exposure. As measured concentrations were within the 95% confidence intervals of respective IC*x* (Table 2; Minguez et al, 2016) and also in the 20-25% range of nominal concentrations, we considered that *R. subcapitata* and *S. marinoi* were exposed to the wanted concentrations. Moreover, under test conditions for algae, antidepressants seem to be stable (Christensen et al 2007). The toxicity tests were performed in 96-well cell culture plates, sealed with a polyester film to avoid evaporation and to isolate each well from the other. Each substance, the medium and the algal inoculums were mixed to obtain an initial algal concentration of 10^4 cells/mL in 0.21 mL of bioassay volume. At least three technical replicates were used per concentration, and the exposure was repeated three times (i.e. biological replicates). The cell density was measured after 72 h of exposure. The results were quantified as average growth rates calculated from cell numbers based on measurements of chlorophyll fluorescence (680 nm, TECAN Infinite® M200 microplate reader). The percentages of inhibition of average specific growth relative to controls were calculated for each concentration. All the controls met the acceptability criteria (i.e. biomass concentration in

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the controls increased by a factor of at least 16 within the test period, and the coefficient of variation of average growth in replicate control cultures did not exceed 15%).

2.3. Calculation of effect concentrations and prediction of mixture toxicity

Non-linear regressions using the Hill equation allowed the calculation of the concentrations inducing 5, 10, 20, 50 or 80% of growth inhibition (*i.e.* IC_5, IC_{10}, IC_{20}, IC_{50} and IC_{80} respectively). These regressions were obtained using the Excel® macro REGTOX (Vindimian 2012) and are shown in Figure S1. The mathematical form of the Hill model used by the macro Excel® Regtox is:

$$f(x) = x^{nH}/(x^{nH} + EC_{50}^{nH})$$

where nH is the Hill coefficient and EC_{50} is the concentration leading to 50% of effects. To assess mixture toxicities, algae were exposed to equitoxic mixtures, where each component in the mixture was present at the same fraction of their own individual toxicity. Ninth of the calculated inhibition concentrations ($IC_5/9$, $IC_{10}/9$, $IC_{20}/9$, $IC_{50}/9$ and $IC_{80}/9$) were used. This implies different molar ratios at each tested concentration, since all antidepressants have different profiles of toxicity (Figure S1). Table 2 shows each inhibition concentrations. If the exposure to the mixture follows the concept of concentration addition, theoretically the combined effect of the nine antidepressants would add up to a total effect of 5, 10, 20, 50 and 80% respectively (Cleuvers, 2004).

Different theoretical indices of mixture toxicity can be found in the literature and apply to quantitative assessment of the deviation of data from the CA model (Altenburger et al, 2003). We analyzed our toxicity data by the sum of toxic units (STU). A toxic unit (TU) is defined as the concentration of a compound present in the mixture with a total effect of *x*% divided by its individual concentration that would alone cause the same *x*% effect. All individual TU values are then summed to obtain the STU parameter. To avoid false positives, the STU limits

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distinguishing a synergistic effect from a simple addition was set at 0.8 (Altenburger et al, 2003). Thus, $0.8 \leq STU \leq 1.2$ indicate perfect fit to the applied model and $STU < 0.8$ indicate underestimation of mixture toxicity, and $STU > 1.2$ indicate antagonism.

To explain the toxicity of the 9-compound mixture, first we used a component-based approach where antidepressants were tested as single compound at the same concentrations as in the mixture. Then, we separated compounds in two groups depending on their amine functions (i.e. presence of primary or secondary amine *vs* a tertiary amine function) (Table 1). The toxicity of these two groups of compounds was assessed at the same concentrations as previously for the mixture of nine compounds.

2.4. QSAR analysis

To highlight the potential mode of action of the antidepressants in mixture, we used single-parameter quantitative structure activity relationship (QSARs) equation based on the octanol-water partition coefficient ($logK_{OW}$). Prior the regression analysis, the toxic concentrations (IC_{50}) of individual compounds were converted to molar concentrations, and experimental $logK_{OW}$ values of each tested compound were retrieved from drugbank (https://www.drugbank.ca/). Linear regressions were calculated using MS-Excel. The quality of the final linear regression was assessed by the squared coefficient of correlation (r^2), the standard error of the regression (s), Fisher's criterion (F) and the p-value.

3. Results

3.1. Toxicity assessment

A mixture of nine antidepressants was screened for growth inhibition of the freshwater algae *Raphidocelis subcapitata* (Figure 1A) and the marine diatom *Skeletonema marinoi* (Figure 1B). There were little or no effects of the nine antidepressants by single-exposure (growth inhibition

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≤ 30%) up to IC_{80}/9. For both species, the effect of the nonary mixture was above the prediction using the CA concept (Figure 1A, B). Calculations of sums of TU (i.e. STU) were done at 20% growth inhibition, since IC_{20} is considered as equivalent to the lowest observed effect concentration (LOEC). STU calculations led to the same conclusion; the mixture of nine antidepressants may be defined by a synergistic effect to *R. subcapitata* and *S. marinoi* (i.e. STU < 0.8) (Table 3). Tested compounds displayed a stronger toxicity to the two algae species in mixture (shift from simple additive effects >20% in Table 3 and lower IC_x in mixture, Table 2).

The two algal species were also exposed to two other mixtures taking into account the amine subclass of each antidepressant (Figure 2A, B): either having primary and secondary amines or having tertiary amines. At the three lowest concentrations, the two amine mixtures were not toxic to *R. subcapitata*, meaning that both amine subclasses were needed to induce the observed toxicity of the 9-antidepressant mixture (Figure 2A). At the IC_{50}/9, the mixture of primary and secondary amines is slightly more toxic than the one composed by tertiary amines. At the highest concentration, both mixtures of amines had the same effect on algal growth with almost 100 % inhibition. According to STU calculations at 20% of growth inhibition for the two mixtures, CA model cannot be rejected (Table 3A). The marine diatom *S. marinoi* responded differently to amine mixtures (Figure 2B). At the three lowest concentrations, the toxicity of tertiary amines followed the toxicity pattern of the nonary mixture (i.e. same effect on growth inhibition). At IC_{50}/9, the tertiary-amine mixture remained more toxic than the mixture composed of antidepressants with primary or secondary amines. Like for *R. subcapitata*, the growth inhibition was almost 100% for the two amine mixtures at the highest concentration. STU calculations also illustrated the different toxicity profiles of the two amine mixtures, with in both cases suggesting the rejection of CA model (Table 3B). STU for the mixture of

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primary/secondary amines indicated antagonistic effects (i.e. STU > 1.2), whereas STU for the mixture of tertiary amines defined strong synergistic effects (i.e. STU << 0.8).

3.2. QSAR analysis

The linear relationship between the ecotoxicity (log IC_{50} in M) to *R. subcapitata* of all the antidepressants and $logK_{OW}$ showed a quite low correlation ($r^2 < 0.8$, equation 1) (Figure S2 A).

$Log(IC_{50}) = -0.62(\pm 0.25)\ logK_{OW} - 2.99\ (\pm 1.03)$ ($r^2 = 0.47$, s = 0.60, n = 9, F = 6.21, p = 0.041) (1)

However, by separating the compounds according to their amine functions, the two considered parameters became strongly correlated (equations 2 and 3, Figure S2 B, C). The main difference between the two equations was the higher intercept value for primary and secondary amine (equation 2).

$Log(IC_{50}) = -0.45(\pm 0.11)\ logK_{OW} - 4.12\ (\pm 0.45)$ ($r^2 = 0.84$, s = 0.15, n = 5, F = 15.92, p = 0.028) (2)

$Log(IC_{50}) = -0.79(\pm 0.15)\ logK_{OW} - 1.81\ (\pm 0.66)$ ($r^2 = 0.92$, s = 0.31, n = 4, F = 24.59, p = 0.038) (3)

These two relationships (observed toxicities) can be compared with QSAR-predicted baseline toxicities calculated according to equation 17 from Villain et al (2014) (log ($1/IC_{50}$ in M) = 0.45 $logK_{OW}$ + 3.08) (Figure S2 B, C). Primary / secondary amines displayed higher toxicity (i.e. differences between the two curves higher than 1, Figure S2 B), whereas toxicity of tertiary amines can be predicted by baseline toxicity (differences between the two curves less than 1, Figure S2 C).

For *Skeletonema marinoi*, by considering all the antidepressants together or only the primary/secondary amines, a low or no correlation between log(IC_{50}) and $logK_{OW}$ was observed

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(equations 4 and 5, Figure S2 D, E); whereas a strong correlation was found between the toxicity of tertiary amines and their octanol-water partition coefficients (equation 6, Figure S2 F).

$Log(IC_{50}) = -0.88 (\pm 0.36) logK_{OW} - 3.11 (\pm 1.41)$ ($r^2 = 0.46$, s = 0.86, n = 9, F = 5.98, p = 0.044) (4).

$Log(IC_{50}) = -0.13 (\pm 0.61) logK_{OW} - 6.38 (\pm 2.45)$ ($r^2 = 0.016$, s = 0.82, n = 5, F = 0.05, p = 0.84) (5).

$Log(IC_{50}) = -1.27(\pm 0.26) logK_{OW} - 1.11 (\pm 1.12)$ ($r^2 = 0.92$, s = 0.52, n = 4, F = 22.84, p = 0.041) (6)

4. Discussion

The joint toxic effects of pharmaceutical mixtures remain today an important issue for a more reliable hazard and risk assessment. Algal species are good biological models to assess mixture toxicities, since they are important components in aquatic ecosystems and any impacts on these primary producers may lead to knock-on effects on higher trophic levels. In the present study, we highlighted additive and even synergistic effects of a mixture of nine antidepressants to a freshwater green alga *R. subcapitata* and a marine diatom *S. marinoi*, whereas tested concentrations were below IC_{10} of each compound. The IC_{10}-values are considered as equivalent to no observed effect concentrations (NOEC) (Chapman et al, 1996; Warne and van Dam, 2008). The QSAR analysis highlighted at least for tertiary amine a non-specific MOA for the two algae species.

When applying a whole-mixture approach with a known composition as defined by Backhaus (2014), the assessment of the toxicity of each mixture component is one step in the understanding of the overall toxicity. The common pattern for pharmaceutical mixtures is to display a higher toxicity than their individual components, independently of the chemical nature of these components, the exposed organisms or the studied endpoints (reviewed by Backhaus,

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2014). This toxicity is precisely the origin of environmental concerns. Indeed, several authors report this higher toxicity towards aquatic organisms from binary to more complex mixtures of dissimilarly acting pharmaceuticals (e.g. Backhaus et al, 2000a; Cleuvers, 2003; DeLorenzo and Fleming, 2008; Dietrich et al, 2010; Fent et al, 2006; Flaherty and Dobson, 2005) as well as mixtures of compounds with similar mode of actions (e.g. Backhaus et al, 2000b; Christensen et al, 2007; Henry and Black, 2007).

Herein, we investigated the toxicity of the whole mixture as a combination of the individual effects of 9 antidepressants, and by grouping compounds according to their amine function. The higher toxicity of the whole mixture compared to effects of individual components can be defined either by simple addition or by synergism. Since the mixture was composed by nine antidepressants sharing similar pharmacological mode of action, a concentration addition model (CA) was expected (Cleuvers, 2003). However, for the two algal species, observed IC_xs for the mixture were well below those expected according to CA model. This is translated by STU < 0.8 suggesting synergistic effects. Deviations from CA-predicted mixture toxicities have been already reported in the literature but mainly for binary mixtures of pharmaceuticals. For example, synergistic effects to *R. subcapitata* have been observed for mixtures of antibiotics (Christensen et al, 2007; Eguchi et al, 2004) or binary combinations of anticancer drugs (Brezovšek et al, 2014). However, most of the studies performed with *R. subcapitata* exposed to antidepressant mixture concluded that CA cannot be rejected (Christensen et al, 2007; Henry and Black, 2007; Johnson et al 2007). This study mainly focused on mixture of SSRIs, and especially ones with secondary amines. By grouping compounds according to their amine function, CA-model fitted well with observed results. This result suggests that each compound within a sub-group of amines share an identical mode of action. Studies assessing the toxicity of pharmaceutical mixtures on marine algae are still scarce, and tested mixtures were often composed by dissimilarly acting compounds. In these cases, CA model was a good predictor of

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mixture toxicities (Backhaus et al, 2011; DeLorenzo and Fleming, 2008; Petersen et al, 2014). Herein, CA is rejected for all the studied mixtures on *S. marinoi*, suggesting that the amine function is not the only parameter explaining antidepressant toxicity. Further studies are thus needed to better understand the observed synergistic effects of antidepressants on *S. marinoi*, especially effects of tertiary amines.

One further step in the understanding of mixture toxicities is to identify which compound(s) is/are the driver(s) of the overall mixture toxicity. Interactions between several pharmaceuticals can be seen as either toxicokinetic interactions (e.g. interactions in uptake or metabolism of the compounds) and/or toxicodynamic interactions (i.e. interactions at the target site). The risk of interactions between several compounds is then a function of both the number of compounds and their chemical structures. More antidepressants are used at the same time, more the risk of interactions between them increases. Moreover, the chemical structure of the compounds influences both their toxicokinetics and toxicodynamics, which can lead to different effects. However, the specific mechanism of toxicity of antidepressants to algae is not yet known. That's why we decided to gather antidepressants according to their amine functional group. Indeed, this amine group plays a key role in the physico-chemical properties of antidepressants (Sánchez and Hyttel, 1999). To our knowledge, this is the first time that amine functional groups are considered in mixture toxicities.

Patterns of toxicity towards the two algal species can be described by three phenomena taking place (1) at the three lowest concentrations, (2) at $IC_{50}/9$ and (3) at the highest concentration. Results obtained on *R. subcapitata* showed a coalition of primary/secondary amines and tertiary amines to lead to the overall mixture toxicity at the three lowest tested concentrations, since neither the primary/secondary amine mixture nor the tertiary amine mixture were toxic alone. Between $IC_{20}/9$ and $IC_{50}/9$, a threshold was reached and both amine mixtures displayed significant toxicities producing an increase in the overall mixture toxicity.

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At the highest concentration, whatever the mixture considered, algal growth was almost completely inhibited. In principle, organic compounds can act as narcotics (non-specific MOA) and accumulate in membranes. QSAR equations using $\log K_{OW}$ are often used to predict this minimum toxicity, and two MOAs are distinguished, i.e. the so-called non-polar and polar narcosis, the latter being slightly more toxic (Verhaar et al 1992). Herein, we found a strong correlation between ecotoxicity of antidepressants to *R. subcapitata* and $\log K_{OW}$ ($r^2 > 0.8$ in equation 2 and 3). As the observed toxicities are given as concentrations in the media, they depend on both the uptake of antidepressants by algae and the affinity of the tested compounds to their site of action in algae. Knowledge about toxicokinetics of antidepressants is not yet available in algae. Thus, the observed strong correlation between IC_{50} and $\log K_{OW}$ suggests either narcotic MOA or a similar MOA with a passive and lipophilicity-driven uptake. By comparing observed toxicities with QSAR-predicted baseline toxicities, relationships between IC_{50} and $\log K_{OW}$ indicate narcosis for tertiary amines and a specific MOA for primary / secondary amines. Mechanistic studies are needed to fully understand the difference of MOAs between the two types of amines. According to the biopharmaceutical drug disposition classification system (BDDCS) (Benet et al, 2011), all antidepressants belong to classes 1 or 2 for compounds with high permeability. Indeed, they display structural characteristics, i.e. lipophilic aromatic group, an amino group and an alkyl chain, helping to reach an optimum interaction with biological membranes. However, the presence of a cell wall and the composition of cell membranes in algae, which differ from cells in higher animals, may influence the strength of the interaction. Thus, the classification of antidepressants for algae might be different. Some antidepressants can have a more specific MOA depending on their ability to cross biological membranes. In our study, the toxicity level of the two types of amines differed as illustrated by equation intercepts. The higher intercept value and a great difference between baseline QSAR-expected and observed toxicity for primary and secondary amines

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(|intercept| = 4.12) suggests a stronger toxicity, and so an effect through a polar narcosis. Fluoxetine is one of the most studied of these secondary amines for which physiological modes of action have been highlighted for different model organisms (e.g. Minguez et al 2014; Neuwoehner et al 2009). For example, by exposing *R. subcapitata* to this antidepressant, Neuwoehner et al (2009) have shown that this secondary amine exerted baseline toxicity but was also able to disturb the membrane-protein interfaces in a non-specific way which added to the overall effect. In contrast, tertiary amines would act through a non-polar narcosis (|intercept| = 1.81). The QSAR equations that we obtained were based on few compounds (4 or 5 depending on the type of amines), thus one could wonder whether these equations are reliable to identify antidepressant modes of action. Nonetheless, Villain et al (2014) found similar equations for the two kinds of narcosis by using a broader set of toxicity data for 336 chemicals with also non-specific MOA and quantile regression analyses, which support our findings. Toxicity pattern to *S. marinoi* differed from *R. subcapitata*. Indeed, at the three lowest concentrations, the overall mixture toxicity was only related to the effects of the four tertiary amines. From QSAR analysis on these amines, a non-polar narcosis was recorded for this antidepressant group (equation 6, strong correlation between $Log(IC_{50})$ and $LogK_{OW}$, and low intercept). Moreover, the absence of correlation between primary/secondary amine toxicities and their $LogK_{OW}$ suggest a more specific MOA.

Our results suggest that the MOA of antidepressants towards algae would be narcosis associated or not to a more specific MOA, but the toxicity patterns were different between the two species. The compounds were 5 to 10-fold more toxic to *S. marinoi* than to *R. subcapitata*. The pH of culture media can be an important parameter influencing the toxicokinetics (uptake, concentration, distribution and elimination) of pharmaceuticals (Neuwoehner and Escher, 2011). The pH of the LC medium used to grow *R. subcapitata* was 7 whereas the pH of the f/2 medium used for *S. marinoi* was 8. This slight difference is enough to increase the neutral

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fraction of antidepressants and therefore to facilitate their entry into algal cells (Neuwoehner and Escher, 2011). Moreover, antidepressants as basic lipophilic compounds are also known to be trapped by acidic subcellular compartments (Daniel and Wójcikowski, 1997). Unlike the green alga *R. subcapitata*, the exoskeleton of the diatom *S. marinoi*, called the frustule, consists in two valves of silica and organic matter. During cell cycle, silica formation imposes special steps consisting of forming new silica valves inside a specialized vesicle, the silicon deposition vesicule (SDV) (Vrieling et al 1999). This SDV is assumed to be acidic (Gordon and Drum, 1994). Due to their physico-chemical properties antidepressants could be trapped in the SDV. Consequently, any disturbance of the silica fixation in the SDV will prevent the formation of two daughter cells, and growth will be inhibited. Samorì et al. (2011) have observed a similar difference of sensitivity between two diatoms *S. marinoi* and *Phaeodactylum tricornutum* exposed to 1-butyl-3- methylimidazolium chloride, the latter species being more sensitive. Like antidepressants, this compound is associated to a positive formal charge. In their publication, authors hypothesized that difference of sensitivity would be related to silica, *P. tricornutum* requiring silica to grow.

In this study, algae were exposed to equitoxic mixtures leading to significant growth inhibition even at the lowest tested concentration. Although pollutants are rarely all present in aquatic ecosystems at concentrations with the same effects, additive and synergistic effects may be expected. Any impacts on phytoplankton populations could ultimately impact higher trophic levels (less food, secondary poisoning). One needs to focus especially on marine ecosystems, since marine diatoms seem more sensitive and more specific MOAs are possible than in freshwater ones. As recommended by Ankley et al (2007), we should read across the therapeutic MOA of pharmaceuticals to understand their MOA in non-target organisms. One possibility will be to take into account chemical structures, since in a sense each functional group of a compound plays a role in its toxicokinetics and toxicodynamics.

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Figure Captions

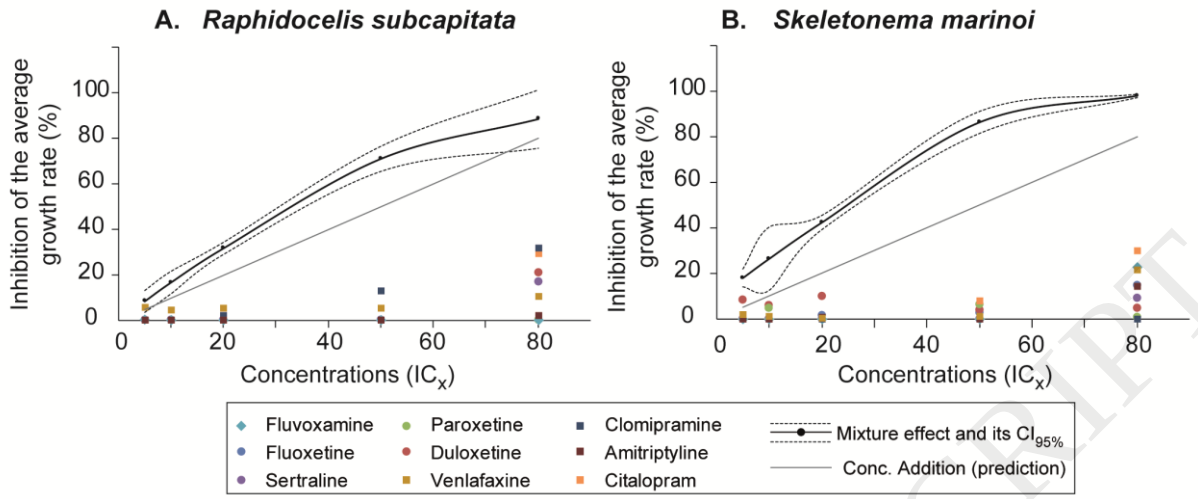
Figure 1. Effect on algal growth rate of nine antidepressants after exposure to single compounds or the mixture. Effect on the freshwater green algae *Raphidocelis subcapitata* (A) and on the marine diatom *Skeletonema marinoi* (B). The mixture toxicity predicted by the concentration addition model is indicated by a grey line.

Figure 2. Mixture toxicities to the freshwater green algae *Raphidocelis subcapitata* (A) and the marine diatom *Skeletonema marinoi* (B). Algae were exposed to a mixture of nine antidepressants (i.e. Mixture), to a mixture of antidepressants with primary and secondary amines (i.e. Amines I/II), or to a mixture of tertiary amines (i.e. Amines III). Mean ± confidence intervals 95%

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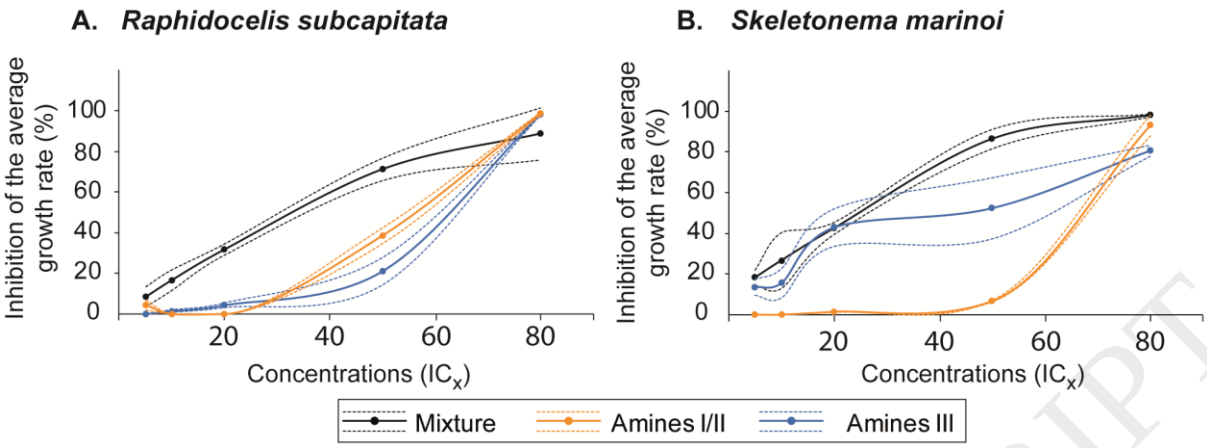
Fig 1



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Fig 2



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Table 1. Characteristics of tested antidepressants. The octanol-water partition coefficients $LogK_{OW}$ were obtained from Drugbank database (experimental data).

Table 2. Concentrations of the nine antidepressants (µg/L) leading to 5, 10, 20, 50 and 80% of growth inhibition (IC_x), following freshwater and marine algal growth inhibition tests with *Raphidocelis subcapitata* and *Skeletonema marinoi* (ISO, 2006, 2012). The corresponding IC_x values in the mixture of 9 compounds are given between brackets.

Table 3. Predicted toxic units (TU) following Concentration Addition model and experimental TUs define for each antidepressant as the ratio between its observed IC_{20} in mixture and its IC_{20} when alone, for *Raphidocelis subcapitata* (A) and *Skeletonema marinoi* (B). Shift from simple additive effects is calculated as: 1 – (Experimental TU_i / Predicted TU_i), when CA is rejected (i.e. for STU < 0.8: synergistic effects and, STU > 1.2: antagonistic effects). Positive shifts mean that compounds are more toxic in mixture than expected, and inversely in the case of negative shifts.

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Tables

Table 1. Characteristics of tested antidepressants. The octanol-water partition coefficients $LogK_{OW}$ were obtained from Drugbank database (experimental data).

Compounds	CAS number	Description [a]	Subclass of amine	$LogK_{OW}$ [b]
Fluvoxamine	61718-82-9	SSRI	Primary	2.8
Fluoxetine	56296-78-7	SSRI	Secondary	4.1
Sertraline	79559-97-0	SSRI	Secondary	5.1
Paroxetine	78246-49-8	SSRI	Secondary	3.6
Duloxetine	136434-34-9	SNRI	Secondary	4.2
Venlafaxine	99300-78-4	SNRI	Tertiary	2.8
Clomipramine	17321-77-6	TCA	Tertiary	5.2
Amitriptyline	549-18-8	TCA	Tertiary	4.9
Citalopram	59729-32-7	SSRI	Tertiary	3.5

[a]SSRI: Selective serotonin reuptake inhibitor, SNRI: selective serotonin-norepinephrine reuptake inhibitor, TCA: Tricyclic antidepressant

[b]Drugbank database (august 2017): https://www.drugbank.ca/

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Table 2. Concentrations of the nine antidepressants (µg/L) leading to 5, 10, 20, 50 and 80% of growth inhibition (IC_x), following freshwater and marine algal growth inhibition tests with *Raphidocelis subcapitata* and *Skeletonema marinoi* (ISO, 2006, 2012). The corresponding IC_x values in the mixture of 9 compounds are given between brackets.

	Raphidocelis subcapitata					*Skeletonema marinoi*				
Compounds	IC_5	IC_{10}	IC_{20}	IC_{50}	IC_{80}	IC_5	IC_{10}	IC_{20}	IC_{50}	IC_{80}
Fluvoxamine	237.3 (26.7)	340.9 (33.5)	505.0 (43.3)	988.7 [a] (70.8)	1935.7 (148.0)	45.0 (4.7)	57.0 (5.4)	73.8 (6.3)	114.5[a] (8.3)	177.9 (11.8)
Fluoxetine	68.6 (7.7)	90.4 (9.1)	121.8 (11.1)	202.9 [a] (16.2)	338.2 (28.3)	4.1 (0.5)	7.3 (0.7)	13.9 (1.0)	48.2 (1.9)	138.5 (4.5)
Sertraline	58.4 (6.5)	74.3 (7.6)	96.4 (9.0)	150.7 [a] (12.5)	235.6 (20.4)	28.5 (3.0)	34.7 (3.4)	42.9 (3.9)	61.8 (4.9)	88.9 (6.6)
Paroxetine	227.1 (25.5)	293.9 (29.9)	388.9 (35.9)	627.6 [a] (51.0)	1012.8 (86.2)	38.3 (4.0)	51.3 (4.7)	70.4 (5.7)	121.1[a] (8.0)	208.4 (12.3)
Duloxetine	68.8 (8.0)	105.8 (10.4)	168.7 (14.0)	374.7 [a] (24.9)	832.0 (58.6)	0.7 (0.1)	0.9 (0.09)	1.2 (0.1)	1.9[a] (0.1)	3.1 (0.2)
Venlafaxine	5.7 x 10^3 (600.4)	9.8 x 10^3 (850.3)	17.4 x 10^3 (1260.2)	47.6 x 10^3 (2680.3)	132.4 x 10^3 (8294.8)	1.7 x 10^3 (160.1)	2.4 x 10^3 (200.1)	3.5 x 10^3 (256.8)	6.9 x 10^3 (407.4)	13.5 x 10^3 (737.6)
Clomipramine	95.4 (10.7)	141.8 (13.8)	218.1 (18.2)	455.5 [a] (31.3)	951.1 (70.2)	1.8 (0.2)	2.0 (0.2)	2.5 (0.2)	3.3 (0.3)	4.4 (0.4)
Amitriptyline	211.6 (23.8)	288.3 (28.8)	403.7 (35.9)	714.9 [a] (54.6)	1268.0 (102.4)	15.8 (1.7)	20.4 (1.9)	27.1 (2.3)	43.8[a] (3.1)	70.9 (4.5)
Citalopram	405.9 (46.0)	727.2 (66.2)	1369.4 (100.2)	4039.8 (221.4)	11918.4 (725.4)	213.2 (22.2)	264.7 (25.2)	334.7 (29.1)	499.8 (38.0)	746.4 (52.8)

[a] from Minguez et al (2016)

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Table 3. Predicted toxic units (TU) following Concentration Addition model and experimental TUs define for each antidepressant as the ratio between its observed IC_{20} in mixture and its IC_{20} when alone, for *Raphidocelis subcapitata* (A) and *Skeletonema marinoi* (B). Shift from simple additive effects is calculated as: 1 – (Experimental TU_i / Predicted TU_i), when CA is rejected (i.e. for STU < 0.8: synergistic effects and, STU > 1.2: antagonistic effects). Positive shifts mean that compounds are more toxic in mixture than expected, and inversely in the case of negative shifts.

A. *Raphidocelis subcapitata*

	Mixture 9 compounds		Mixture Amines I / II		Mixture Amines III	
Compounds	Predicted TU_i	Experimental TU_i	Predicted TU_i	Experimental TU_i	Predicted TU_i	Experimental TU_i
Fluvoxamine	0.111	0.086 (+0.218)	0.200	0.200	-	-
Fluoxetine	0.111	0.091 (+0.173)	0.200	0.173	-	-
Sertraline	0.111	0.094 (+0.145)	0.200	0.164	-	-
Paroxetine	0.111	0.092 (+0.164)	0.200	0.169	-	-
Duloxetine	0.111	0.083 (+0.245)	0.200	0.222	-	-
Venlafaxine	0.111	0.072 (+0.345)	-	-	0.250	0.299
Clomipramine	0.111	0.084 (+0.236)	-	-	0.250	0.231
Amitriptyline	0.111	0.089 (+0.191)	-	-	0.250	0.196
Citalopram	0.111	0.073 (+0.336)	-	-	0.250	0.325
STU = Σ TU_i		0.764		0.928		1.051

B. *Skeletonema marinoi*

	Mixture 9 compounds		Mixture Amines I / II		Mixture Amines III	
Compounds	Predicted TU_i	Experimental TU_i	Predicted TU_i	Experimental TU_i	Predicted TU_i	Experimental TU_i
Fluvoxamine	0.111	0.073 (+0.336)	0.200	0.186 (+0.070)	-	-
Fluoxetine	0.111	0.040 (+0.636)	0.200	0.462 (-1.310)	-	-
Sertraline	0.111	0.079 (+0.282)	0.200	0.175 (+0.125)	-	-
Paroxetine	0.111	0.066 (+0.400)	0.200	0.210 (-0.050)	-	-
Duloxetine	0.111	0.072 (+0.347)	0.200	0.193 (+0.035)	-	-
Venlafaxine	0.111	0.059 (+0.463)	-	-	0.250	0.075 (+0.700)
Clomipramine	0.111	0.089 (+0.189)	-	-	0.250	0.097 (+0.612)
Amitriptyline	0.111	0.070 (+0.364)	-	-	0.250	0.083 (+0.668)
Citalopram	0.111	0.076 (+0.309)	-	-	0.250	0.086 (+0.656)
STU = Σ TU_i		0.624		1.227		0.341