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**Title:** Combined effects of pharmaceuticals, personal care products, biocides and organic contaminants on the growth of *Skeletonema pseudocostatum*

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## Abstract

Organisms in the environment are exposed to a number of pollutants from different compound groups. In addition to the classic pollutants like the polychlorinated biphenyls, polyaromatic hydrocarbons (PAHs), alkylphenols, biocides, etc., other compound groups of concern are constantly emerging. Pharmaceuticals and personal care products (PPCPs) can be expected to co-occur with other organic contaminants like biocides, PAHs and alkylphenols in areas affected by wastewater, industrial effluents and intensive recreational activity. In this study, representatives from these four different compound groups were tested individually and in mixtures in a growth inhibition assay with the marine algae *Skeletonema pseudocostatum* (formerly *S. costatum*) to determine whether the combined effects could be predicted by models for additive effects; the concentration addition (CA) and independent action (IA) prediction model. The eleven tested compounds reduced the growth of *S. pseudocostatum* in the microplate test in a concentration-dependent manner. The order of toxicity of these chemicals were irgarol > fluoxetine > diuron > benzo(a)pyrene > thioguanine > triclosan > propranolol > benzophenone 3 > cetrimonium bromide > 4-*tert*-octylphenol > endosulfan. Several binary mixtures and a mixture of eight compounds from the four different compound groups were tested. All tested mixtures were additive as model deviation ratios, the deviation between experimental and predicted effect concentrations, were within a factor of 2 from one or both prediction models (e.g. CA and IA). Interestingly, a concentration dependent shift from IA to CA, potentially due to activation of similar toxicity pathways at higher concentrations, was observed for the mixture of eight compounds. The combined effects of the multi-compound mixture were clearly additive and it should therefore be expected that PPCPs, biocides, PAHs and alkylphenols will collectively contribute to the risk in areas contaminated by such complex mixtures.

***Key words:*** *Concentration addition; independent action; algae; microplate test; growth inhibition; pharmaceuticals and personal care products; organic pollutants.*

## 1. Introduction

Organisms in the environment are exposed to a number of pollutants from different compound groups. Even though the environmental concentrations of individual pollutants might be too low to exert an effect on their own, the presence of several similarly acting compounds is expected to induce effects through combined toxicity at concentrations below their individual No Observed Effect Concentrations, NOECs (Backhaus et al., 2011; Kortenkamp, 2008). In addition to the classic pollutants like the polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), alkylphenols, biocides, etc, other compound groups of concern are constantly emerging, and compounds from several of these classes have been found to co-occur in marine waters (i.e. alkylphenols, biocides and pharmaceuticals) (Munaron et al. 2012). One of the compound groups that have received a lot of attention in the last years is pharmaceuticals and personal care products (PPCPs). Most of these compounds are not regulated as pollutants and new PPCPs are continuously developed (Rosi-Marshall and Royer, 2012). The PPCPs are generally introduced to the environment through municipal waste water, and via waste water from hospitals and labs (Daughton and Ternes, 1999; Fent et al., 2006; Kummerer, 2009). The PPCPs and/or their metabolites and transformation products are transported to the seas by the rivers where they contribute to the contaminant load from recreational, shipping, agricultural and industrial activities. The emission of pharmaceuticals from human activities to the environment is expected to increase due to an increase in life expectancy, increase in living standard and affordability of drugs (Kummerer, 2010). Several PPCPs have been shown to be acute toxic to algae (Backhaus et al., 2011; Liu et al., 2011; Nunes et al., 2005), and a relatively large proportion (approx. 30%) of investigated pharmaceuticals are predicted to be potentially very toxic to aquatic organisms (Sanderson et al., 2004). The effect of individual PPCPs have been widely studied (Dave and Herger, 2012; Ellesat et al., 2010; Fent et al., 2006) and mixtures of PPCPs have been studied to a limited extent (Backhaus et al., 2011; DeLorenzo and Fleming, 2008). However few studies have investigated the effect of PPCPs in combination with other relevant contaminants like antifouling biocides, PAHs and industrial compounds.

The mode of action (MoA) of biocides, PPCPs, PAHs and alkylphenols for the growth inhibition in algae are only known for some compounds and encompass both specific toxicity

and narcotic MoA. A majority of toxic compounds are believed to act through a narcotic MoA (baseline toxicity) which is assumed being caused by hydrophobicity-dependent and nonspecific interaction with biological membranes and membrane associated proteins (Mayer and Reichenberg, 2006; van Wezel and Opperhuizen, 1995). Chemicals that have a narcotic MoA are normally sufficiently lipophilic to accumulate in the lipid or the lipid-aqueous interface of biological membranes exerting polar narcosis (narcosis I) or nonpolar narcosis (narcosis II) (van Wezel and Opperhuizen, 1995), and leads to disruption of membrane functions and causes decreased activity and reduced reaction to external stimuli (LeBlanc, 2004). The effective membrane concentrations of baseline toxicants are approximately equal in algae, daphnids and fish (Escher and Schwarzenbach, 2002). Biocides such as irgarol and diuron display a specific toxic MoA through inhibiting the photosystem (PS) II (Jones, 2005). By inhibiting PSII these biocides reduce the photosynthesising organisms' ability to harvest energy and produce carbohydrates, ultimately leading to reduced ability to grow. The primary MoA of pharmaceuticals are usually well known as they are designed to exert a specific therapeutic effect. However, the biological targets for pharmaceuticals are not always present in non-mammalian organisms such as aquatic vertebrates and invertebrates. For instance, the human antidepressant fluoxetine and the beta-blocker propranolol, have previously been shown to be toxic to algae, although the MoA is poorly characterised (Backhaus et al., 2011, Escher et al., 2005).

Even though the MoA of all biocides, PPCPs, PAHs and other organic contaminants are not fully known, it has been observed that compounds causing the same type of effect or having a similar MoA can be additive (Backhaus et al., 2011). The combined effects of chemicals can be studied by application of the two widely used prediction models for additive effects, the concentration addition (CA) and independent action (IA) prediction models. These concepts were first introduced by Loewe and Muischnek (1926, CA) and Bliss (1939, IA), and are based on the assumption that all the compounds in a mixture affect the same endpoint in the same direction, and that the compounds act by similar (CA) or dissimilar (IA) MoA. As the models work as a reference point for additive effects, deviations from the models indicate interactions such as synergy (more than additive effects) and antagonism (less than additive effects). Combined effects of pharmaceuticals or biocides have shown to be mostly additive in algae by following either CA or IA (Backhaus et al., 2011; Cleuvers, 2003; Cleuvers, 2004; Faust et al., 2003). Algae, including the diatoms *Skeletonema costatum* and *Phaeodactylum tricornutum*, are among the most sensitive groups of aquatic species used in regulatory testing

(Bjørnestad et al., 1993). Algal growth inhibition tests are routinely used in ecotoxicity testing of chemicals and environmental samples and international standards and guidelines are available for both freshwater and marine species (ISO, 2006; ISO, 2012; OECD, 2011). To accommodate high-throughput setups, microplate methods using smaller volumes have been developed and used for several algal species (Eisentraeger et al., 2003; Pavlic et al., 2006; Rojickova et al., 1998; Skjelbred et al., 2012; Vendrell et al., 2009).

In this study we used an algal microplate method with *Skeletonema pseudocostatum* (formerly *S. costatum*), a spring bloom forming diatom found in coastal waters throughout non-polar regions (Kooistra et al., 2008), to investigate the combined effect of pollutants originating from a wide array of environmentally relevant compound groups; PPCPs, antifoulants, PAHs and alkylphenols. The investigated compounds were chosen based on demonstrated presence in the environment (Daughton and Ternes, 1999; Kümmerer, 2010; Schlabach et al., 2009; Thomas and Brooks, 2010), anticipated aquatic toxicity (Sanderson and Thomson 2009) and/or presence on the OSPAR list of chemicals for priority action (OSPAR, 2009). The microplate method has, with a few exceptions, been shown to produce $EC_{50}$ values similar to the flask method after exposure for certain metals, pesticides, pharmaceuticals and environmental samples (Eisentraeger et al., 2003; Pavlic et al., 2006; Rojickova et al., 1998). The small volume, reduced use of laboratory resources and high throughput capacity of the microplate method makes this assay highly attractive for complex studies such as that addressing combined toxicity assessment.

## 2. Materials and methods

### 2.1. Test compounds

The test compounds (table 1) 4-*tert*-octylphenol (OP, cas: 140-66-9), benzo(a)pyrene (BAP, cas: 50-32-8), benzophenone-3 (BP3, cas:131-57-7), cetrimonium bromide (cas: 57-09-0), diuron (cas: 330-54-1), endosulfan (cas: 115-29-7), fluoxetine HCl (cas: 56296-78-7), irgarol 1051 (cas: 28159-98-0), propranolol (cas: 318-98-9), thioguanine (cas: 154-42-7) and triclosan (cas: 3380-34-5) were all from Sigma-Aldrich (St. Louis, MI, US). The chemicals, all with purity ≥96%, were dissolved in dimethylsulfoxide (DMSO) and stored at 4°C when not in use.

## 2.2. *Skeletonema pseudocostatum* microplate test

Growth inhibition tests with *S. pseudocostatum* L.K. Medlin (formerly *S. costatum* Cleve) (NIVA-BAC1; Norwegian Institute for Water Research, Oslo, Norway) were performed in Nunc 96 well plates (Nunc A/S, Roskilde, Denmark). Algal cultures for inoculation were incubated in growth medium 1-4 days prior to the test to ensure that the cultures were in the exponential growth phase. The growth medium was made with 0.45µm filtered (HAWP membrane filter, Millipore Ireland Ltd, Tullagreen, Ireland) sea water collected at 60 m depth from the Outer Oslofjord supplemented with ISO10253 stock solutions (ISO, 2006). Algae concentrations were measured with a Beckman-Coulter Multisizer 3 Coulter Counter (Miami, FL, US) and adjusted to $1*10^4$ cells $mL^{-1}$. Test solutions were prepared by mixing 2 µl of stock solution or solvent (DMSO) with 998 µl growth medium and diluting 1:1 with algae culture ($1*10^4$ cells $mL^{-1}$). The final volume in each well was 200 µl with a nominal algal concentration of $5*10^3$ cells $mL^{-1}$ and a solvent (DMSO) concentration of 0.1%. Nine concentrations plus solvent control were tested in 5 replicates per plate. One replicate without algal inoculum was used to detect fluorescence from the chemical alone. The outer wells of the microplates were filled with 200 µl growth medium without algae to counteract confounding bioassay factors such as edge-specific evaporation from the microplate. The plates were sealed with plate seals (Nunc, Roskilde, Denmark) and incubated in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland) with orbital shaking at 90 rpm, continuous light intensity of 83 ± 6 µmol $m^{-2}s^{-1}$ and temperature of 20 ± 2°C. Fluorescence measurements with a 530 nm excitation filter, bandwidth 25 nm, and a 685 nm emission filter, bandwidth 20 nm, were performed at the start (only controls) and every 24 ± 2 hours with a Cytofluor 2300 (Millipore, Billerica, MA, US). The fluorescence of test solution without algae was subtracted from each replicate. A concentration series of algae were measured with fluorescence, coulter counter and counted manually in a haemocytometer and showed good linear correlation ($r^2$=0.99 and 0.98, respectively). At least three independent experiments were performed for each chemical and mixture. For cetrimonium bromide, a new dilution series with slightly different concentrations were prepared for each individual experiment.

The average growth rate for each sample was calculated from initial fluorescence and fluorescence after 72 hours using the equation (1):

$$\mu = \frac{\ln(N_n) - \ln(N_0)}{t_n} \times 24d^{-1} \quad (1)$$

where $N_n$ is the fluorescence at time $t_n$, $N_0$ is the fluorescence at time zero ($t_0$), $t_n$ is the time at $n^{th}$ measurement. Growth inhibition was calculated as percent of the control.

**2.3. Data analyses and mixture design**

Single compounds and mixtures were screened for growth inhibition in the *S. pseudocostatum* microplate test and the results were modeled with a non-linear regression using a sigmoidal dose-response curve (with variable slope) in the GraphPad prism software version 6 (GraphPad Software Inc., La Jolla, CA, USA) (2). The bottom and top values were fixed at 0 and 100 %, respectively.

$$Y = Bottom + ((top-bottom)/(1+10^{((logEC_{50}-logX)*slope)})) \quad (2)$$

The parameters obtained from the individual concentration-response curves (CRCs, table 2) were used to design the mixtures. Binary mixtures consisting of biocides (irgarol + diuron), pharmaceuticals (thioguanine + fluoxetine), personal care products (triclosan + BP3) and classic organic contaminants (OP + BAP) were tested to assess whether compounds belonging to the same chemical group were acting additive. Binary (thioguanine + triclosan, fluoxetine + BP3) and an eight compound mixture (irgarol, diuron, triclosan, BP3, fluoxetine, thioguanine OP and BPA) of compounds from different chemical groups were tested to determine if the models were robust to more environmentally relevant complex mixtures. The compounds endosulfan, cetrimonium bromide and propranolol were excluded from the mixture design due to effect concentrations were above the water solubility (endosulfan), scattered data and bad curve fit (cetrimonium bromide) and a steep concentration-response curve with no tested concentrations causing effects between 0% and 100% growth inhibition (propranolol). A fixed ratio ray design was chosen, and equi-effective concentrations according to the CA model (3) were calculated based on the ratios of the $EC_{50}$ values.

$$EC x_{(mix)} = \left(\sum_{i=1}^{n} (p_i / EC x_i)\right)^{-1} \quad (3)$$

The $EC x_{(mix)}$ is the total predicted effect concentration of the mixture inducing an effect $x$, $p_i$ is the relative fraction of component $i$ in the mixture and $EC x_i$ is the concentration of substance $i$ needed to induce the effect $x$ when applied alone. In addition, the prediction for IA (4) were calculated for the designed mixtures to define a window of expected additive effects as the MoA of all compounds in algae were not fully known.

$$E_{mix} = 1 - \prod_{i=1}^{n} (1 - E_i) \quad (4)$$

$E_{mix}$ is the effect of a mixture of $n$ compounds and $E_i$ is the effect of substance $i$ when applied singly.

The CRC for the experimental data was compared to CA and IA prediction and additive effects were believed to occur if the 95% confidence interval of the CRC for the observed data overlapped with either of the prediction models or if the calculated model deviation ratio (MDR) (5), were within a factor of 2 ($0.5 \leq MDR \leq 2$). The MDRs were calculated by dividing the observed effect concentrations ($EC x_{obs}$) with the predicted effect concentrations ($EC x_{pred}$).

$$MDR = EC x_{obs} / EC x_{pred} \quad (5)$$

## 3. Results

The eleven tested compounds reduced the growth of *Skeletonema pseudocostatum* in a concentration-dependent manner to less than 50% of the control (table 2, figure 1). The observed responses for the eleven compounds were well explained by the applied non-linear regression with $R^2$ values $\geq 0.86$ for all compounds except cetrimonium bromide ($R^2$=0.65) (table 2). Irgarol was the most toxic compound with an $EC_{50}$ of 4.7 nM, and the order of toxicity was irgarol > fluoxetine > diuron > BAP > thioguanine > triclosan > propranolol > BP3 > cetrimonium bromide > OP > endosulfan. The $EC_{50}$ values for the most toxic (irgarol) and the least toxic (endosulfan) compound differed by approximately three orders of magnitude (4.7 nM and 5.9 µM, respectively). Endosulfan induced effects at higher than the reported water-solubility of 0.8 µM (Kegley et al., 2011). The slope of the obtained concentration response-curves differed between the compounds with the steepest slope for

propranolol of -18 and the shallowest for thioguanine of -1.1. The three pesticides, irgarol, diuron and endosulfan exhibited similar slopes of -2.4, -2.3 and -2.3 respectively.

**[Insert Figure 1 here]**

**[Insert table 2 here]**

Six binary mixtures (irgarol + diuron, triclosan + BP3, fluoxetine + BP3, thioguanine + fluoxetine, OP + BAP and thioguanine + triclosan) and an eight compound mixture (irgarol, diuron, triclosan, BP3, fluoxetine, thioguanine OP and BAP) were screened for growth inhibition in the *S. pseudocostatum* microplate test. The microplate test provided reproducible results that were well described by the applied non-linear regression analysis, indicated by $R^2 \geq 0.93$ for all mixtures. Most of the tested binary mixtures (figure 2) were well predicted by both models and MDR values were within a factor of 2 (table 3). The effect of two mixtures (OP + BAP and fluoxetine + BP3) was only predicted by CA, indicative of similar MoA of OP and BAP and of fluoxetine and BP3. The effect of the mixture of irgarol and diuron was better predicted by CA than by IA even though observed effect concentrations were within a factor of two from both prediction models.

**[Insert Figure 2 here]**

**[Insert table 3 here]**

The effect of the eight-compound mixture was positioned in between the two prediction models and was well predicted by IA at the lower mixture concentrations but shifted towards CA predictions at the higher concentrations (figure 3). The positioning of the observed results in the window of additivity defined by the two prediction models indicated that the mixture consisted of compounds displaying a concentration-dependent MoA, e.g. exhibiting dissimilar MoA on low concentrations and similar MoA at high mixture concentrations.

**[Insert Figure 3 here]**

## 4. Discussion

### 4.1. Effects of single compound exposure in the microplate test

Of the 11 tested organic pollutants and PPCPs all but four (OP, BP3, cetrimonium bromide and endosulfan) had $EC_{50}$ levels in the nM range (4.73-797nM). The two biocides irgarol and diuron were among the most potent inhibitors of the growth of *S. pseudocostatum* in our study. This was not surprising as these compounds are designed biocides, and inhibit growth specifically by inhibiting PSII (Jones, 2005). Interestingly, fluoxetine was the second most potent inhibitor of growth of *S. pseudocostatum*. Fluoxetine is a pharmaceutical used in antidepressiva and works by inhibiting serotonin reuptake into presynaptic cells and thereby increases the level of serotonin available for postsynaptic receptors in the synaptic cleft (Hiemke and Härtter, 2000). Fluoxetine is toxic to algae (Neuwoehner and Escher, 2011) and bacteria (Munoz-Bellido et al., 2000), and was observed to be more toxic to algae than to daphnids (Christensen et al., 2007). The mechanism by which fluoxetine is toxic to algae is not fully known, but it has been proposed that fluoxetine act through a narcotic MoA (Neuwoehner and Escher, 2011) and/or potentially by inhibiting cellular efflux pumps as observed in humans (Munoz-Bellido et al., 2000). Benzo(a)pyrene and thioguanine were the fourth and fifth most toxic of the tested compounds, respectively. A transriptomics study by Carvalho et al., (2011) revealed that BAP triggered a change in the lipid metabolism in diatoms, probably by incorporation and perturbation of cellular membranes. In addition, apoptosis was inhibited and the normal progression of the cell cycle was disrupted (indicative of an arrest of the cell cycle). The suggested arrest in the cell cycle progression was consistent with the decreased diatome growth rate (Carvalho et al., 2011) and could also be the MoA for growth inhibition in this study. Thioguanine is an anti-cancer drug which interferes with normal cellular function by incorporation into DNA as replacement for purine bases and subsequently causing increase in DNA strand breaks, triggering apoptotic and cytotoxic pathways (Krynetski et al., 2003; Kverka et al., 2011). The MoAs of the sixth most toxic compound, triclosan, in microalgae might be attributed to baseline toxicity and uncoupling of oxidative phosphorylation (Franz et al., 2008). Inhibition of non-photochemical quenching after exposure to triclosan has also been observed in river biofilms (Ricart et al., 2010). Non-photochemical quenching is a mechanism that is used to dispose of excess energy when the light energy absorption exceeds the capacity for photosynthesis. Inhibition of non-photochemical quenching might lead to damages in the pigments where the non-photochemical quenching takes place (Ricart et al., 2010), ultimately reducing the

photosynthetic capacity of the cell. Propranolol with an $EC_{50}$ for growth inhibition in *S. pseudocostatum* of 797 nM is a sympatholytic non-selective beta blocker used to treat anxiety, hypertensives, vasoconstriction and arrhythmia in human patients (Drugbank, 2013). Propranolol has been shown to be toxic to algae (Backhaus et al., 2011), and a narcotic MoA have been proposed (Neuwoehner and Escher, 2011). In addition, propranolol has been shown to reduce the photosynthetic capacity and efficiency of algae indicative of irreversible damages to the photosynthetic apparatus (Bonnineau et al., 2010). This damage could possibly be a result of oxidative stress, as an intermediate radical is formed during the photolysis of propranolol (Liu and Williams, 2007). Benzophenone was the fourth least toxic of the tested compounds with an $EC_{50}$ of 1.1 µM. Rodil et al., (2009) found that the observed algal toxicity of BP3 was 42 times higher than that predicted based on baseline toxicity, and conclude that this is indicative of a more specific MoA of this substance to algae. In addition, the more polar properties of BP3 assume permeation through the algae membranes and might cause additional effects to the organism (Rodil et al., 2009). The third least toxic compound, cetrimonium bromide is accumulated in the mitochondrial matrix by a membrane potential driven uptake mechanism that may lead to toxicity by a collapse of the mitochondrial membrane potential (Bragadin and Dell'Antone, 1996). In addition, cetrimonium bromide may induce mitochondria-mediated apoptosis (Ito et al., 2009), which may reduce the growth of algae. 4-*tert*-octylphenol with an $EC_{50}$ for growth inhibition of *S. pseudocostatum* of 5.6 µM has previously been shown to inhibit the growth of algae, decrease the ratio of variable and maximal fluorescence, cause thickening and wrinkling of the cell wall matrix, and increase the number of starch granules with a reduced size (Zhou et al., 2013). An indication of an effect on the PSII primary photochemical events and inactivation of PSII reaction centres has also been observed (Perron and Juneau, 2011). The least toxic of the tested compounds, endosulfan, has been shown to inhibit PS II activity in cyanobacteria (Prasad et al., 2011).

The observed results were comparable to previous reported $EC_{50}$ levels as all but one (BAP) of the obtained $EC_{50}$ values were within one order of magnitude from previously reported data, and the $EC_{50}$ of four of the tested compounds (irgarol, diuron, fluoxetine, BP3) were within a factor of 2 from previous observed results (Cleuvers, 2005; DeLorenzo and Fleming, 2008; Djomo et al., 2004; Gatidou and Thomaidis, 2007; Rodil et al., 2009; Sanderson and Thomsen, 2009). The $EC_{50}$ of BAP was more than one order of magnitude higher in our study than previously reported for algae incubated for 7 days in glass flasks (Djomo et al., 2004), a

discrepancy that might be explained by the different species, test systems, and test duration used in these studies. As exposure concentrations were not verified by chemical analysis in the present study, differences between reported values and data obtained in our study could also be related to reduction in exposure concentration compared with the nominal concentration. Benzo(a)pyrene has a high logKow (5.97) and might adsorb to the plastic surface of the microplate well. Differences in adsorption of highly hydrophobic compounds due to different surface area to volume ratios between the microplate test and tests performed in glass vessels might also contribute to the observed discrepancy between $EC_{50}$ for BAP in this study and previous reported data. A general loss of exposure concentration due to hydrophobicity and solubility limitations is expected for compounds with a logKow above 4 (OECD, 2000), and chemicals with a logKoW > 3 have been observed to be less effective in the microplate assay than in traditional algal assays (Riedl and Altenburger, 2007). Although the microplate assay may potentially underestimate the effect of chemicals with logKow higher than 3, the individual compounds are expected to behave in a similar way when dosed in mixtures and thus accurately reflect the combined effects.

### 4.2. Combined effects

Acute toxicity to aquatic organisms by single human pharmaceuticals are unlikely to occur as environmental concentrations are 100-1000 times lower than acute effect concentrations and is only relevant in case of spills (Fent et al., 2006). However, PPCPs can add to the effect of other algae-toxic compounds like biocides, PAHs and alkylphenols as shown in this study. The present study clearly showed that complex mixtures of PPCPs (fluoxetine, thioguanine, triclosan and BP3), a PAH (BAP), an alkylphenol (OP) and biocides (irgarol and diuron) had additive effects on the growth inhibition of *S. pseudocostatum*. The results are in agreement with previous reported data on combined toxicity of mixtures of pharmaceuticals (Christensen et al., 2007; Cleuvers, 2003; Cleuvers, 2004), PPCPs (Backhaus et al., 2011; DeLorenzo and Fleming, 2008), biocides (Faust et al., 2003; Porsbring et al., 2010) and in a multi-compound mixture of priority pollutants (Walter et al., 2002). Although hormesis has been observed at the lower concentrations of a mixture of PPCPs, the higher concentration effects have been shown to follow the CA prediction (Backhaus et al., 2011).

The binary mixture of irgarol and diuron was clearly additive and was best estimated by the CA model in our study. This was expected as they have the same mode of action, i.e. inhibition of PSII (Jones, 2005). Synergistic, antagonistic and additive effects have previously

been reported for mixtures of these two compounds on photosynthetic organisms (Chesworth et al., 2004; Koutsaftis and Aoyama, 2006). The effect on growth inhibition of the binary mixtures of fluoxetine and BP3, and of OP and BAP were additive according to the CA model and could not be predicted successfully by the IA model. These results are indicative of a similar MoA of the 2 compounds BP3 and fluoxetine and of the 2 environmental compounds OP and BAP. The MoA of fluoxetine and BP3 is not fully known but have been suggested to be through a specific MoA rather than just by baseline toxicity, possibly involving inhibition of cellular efflux pumps for fluoxetine (Munoz-Bellido et al., 2000). By assuming a similar MoA of these two compounds it can be hypothesised that BP3 have a specific, not yet identified, MoA on the algae which is in agreement with previous results obtained by Rodil et al. (2009) where a higher than baseline toxicity of BP3 was observed. The compounds OP and BAP can be expected to act by a narcotic MoA based on previously observed data (Carvalho et al., 2011; Zhou et al., 2013). However, other specific MoA have also been observed for these two compounds, including disruption of the cell cycle by BAP (Carvalho et al., 2011) and indications of inhibition of PSII by OP (Perron and Juneau, 2011). As the binary mixture of these two compounds (BAP and OP) followed the concept of CA, it can be hypothesized that the primary MoA of these two compounds is through a narcotic MoA, and that the other proposed specific MoA only contribute to a limited extent to the effects on the growth of *S. pseudocostatum*. The binary mixtures of triclosan and BP3, of thiogunaine and fluoxetine, and of thioguanine and triclosan were also found to be additive, but no distinction between the concept of CA or IA could be made as the MDRs where within a factor of 2 from both models for most of the effect range (table 3).

The combined effect of the mixture of the eight selected compounds were additive as the observed effects were positioned between the two prediction models, sometimes referred to as the “window of additivity” (Altenburger et al., 2003; Faust et al., 2003). Interestingly, the observed effects were explained by the IA predictions at the lower concentrations and the CA predictions at the higher concentrations, indicating a shift from dissimilar MoAs at lower concentrations to a similar MoA at the higher concentrations. The 8-compound mixture includes compounds presumed to both act through an unspecific, specific and unknown MoA. Based on this information a position of the observed results between the two prediction models would be expected due to a combination of compounds with similar and dissimilar MoAs. However, the concentration-dependent shift from IA to CA with increasing concentrations has not been reported, nor properly characterized previously. At low

concentrations it is believed that the compound-specific MoAs are dominating the toxicity (van Wezel and Opperhuizen, 1995), whereas it can be speculated that an increase in the exposure concentration will gradually increase the contribution of unspecific MoA such as narcosis (baseline toxicity) and therefore cause a shift from IA to CA as baseline toxicity is known to be concentration additive (Mayer and Reichenberg, 2006). Alternatively, increasing the compound concentrations is expected to affect a higher number of biological targets and toxicity pathways that may affect each other (Hoffmann et al., 2006). Activation of converging toxicity pathways may thus lead to departure from independently acting MOAs and lead to a shift from IA to CA with increasing concentrations. As characterization of the MoA of the test compounds and mixtures of these in *S. pseudocostatum* was beyond the scope of this study, future studies to reveal the mechanistic rationale for a shift from IA to CA is clearly warranted.

The marine algae *S. pseudocostatum* is found in coastal waters throughout the non-polar regions of the world (Kooistra et al., 2008). This makes it a relevant test species as most marine pollution originates from land-based sources, and coastal areas will most likely contain a mixture of PPCPs, biocides and classic organic pollutants like PAHs and alkylphenols. Growth inhibition of algae due to combined effects of contaminants may have implications on the aquatic ecosystem as algae are important for carbon fixation in oceans, providing food and oxygen to the aquatic ecosystem. In addition, algae are an important pathway for the accumulation of lipophilic water-borne contaminants and can serve as a source of contaminants to organisms at higher trophic levels (Dann and Hontela, 2011). Combined effects of organic pollutants might influence the structure and function of algal communities as have been observed after exposure to triclosan and two other PPCPs (Wilson et al., 2003). This might possibly lead to shifts in the nutrient processing capacity and food web structure, and effects on zooplankton associated with macrophytes through loss of habitat and food has been observed in a mesocosm study after exposure to irgarol (Mohr et al. 2008).

Assessment of combined effects of chemicals from these and other compound groups by use of prediction models for additive effects (CA and IA) is becoming increasingly important in protecting the aquatic environment against undesired effects. Use of prediction models such as CA and IA to assess combined effects have proven successful in several studies, and in this study we showed that these models are also applicable for assessment of combined effects of

a diverse group of chemicals from different compound groups with both known and unknown MoA.

## 5. Conclusion

The combined effects of PPCPs, biocides, PAH and alkylphenol were tested on the growth inhibition of *S. pseudocostatum* in microplates. The combined effects of the binary mixtures used in this study were additive, and the effects were well estimated by CA and/or IA. The 8-compound mixture of irgarol, diuron, thioguanine, fluoxetine, triclosan, BP3, BAP and OP, followed the IA predictions at the lower concentrations and the CA predictions at the higher total mixture concentrations, indicative of a shift from dissimilar to similar MoA. The shift from IA to CA is possibly linked to an increased number of activated targets and/or pathways leading to a higher possibility of the compounds to be involved in the same pathways, and/or a shift from a specific MoA at low concentrations to a general narcotic MoA at higher concentrations. This study show that PPCPs will contribute to the chemical load and increase the risk of adverse effects on marine organisms like *S. pseudocostatum* in coastal areas that are also contaminated with other organic pollutants like antifoulants, PAHs and alkylphenols.

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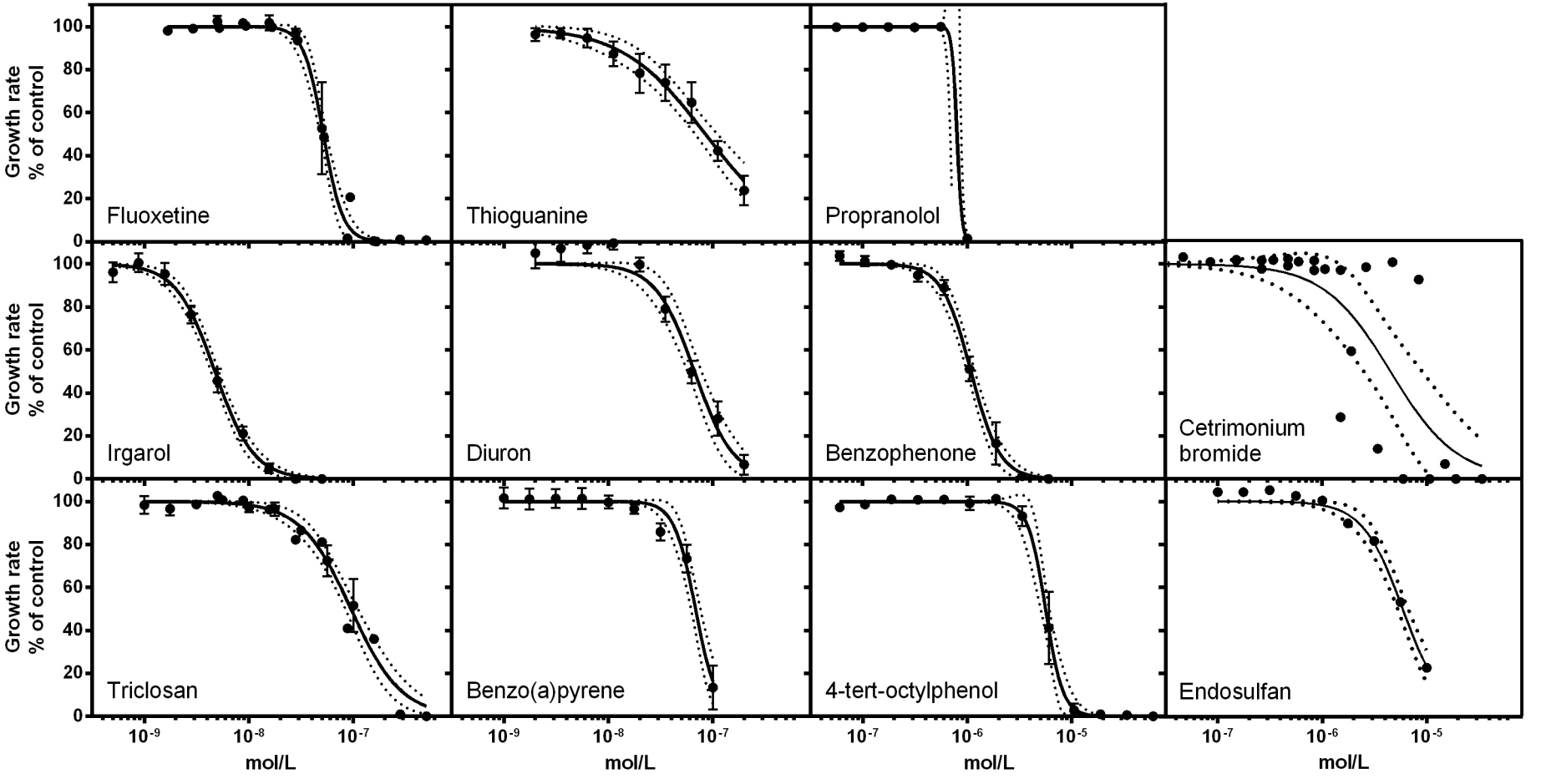
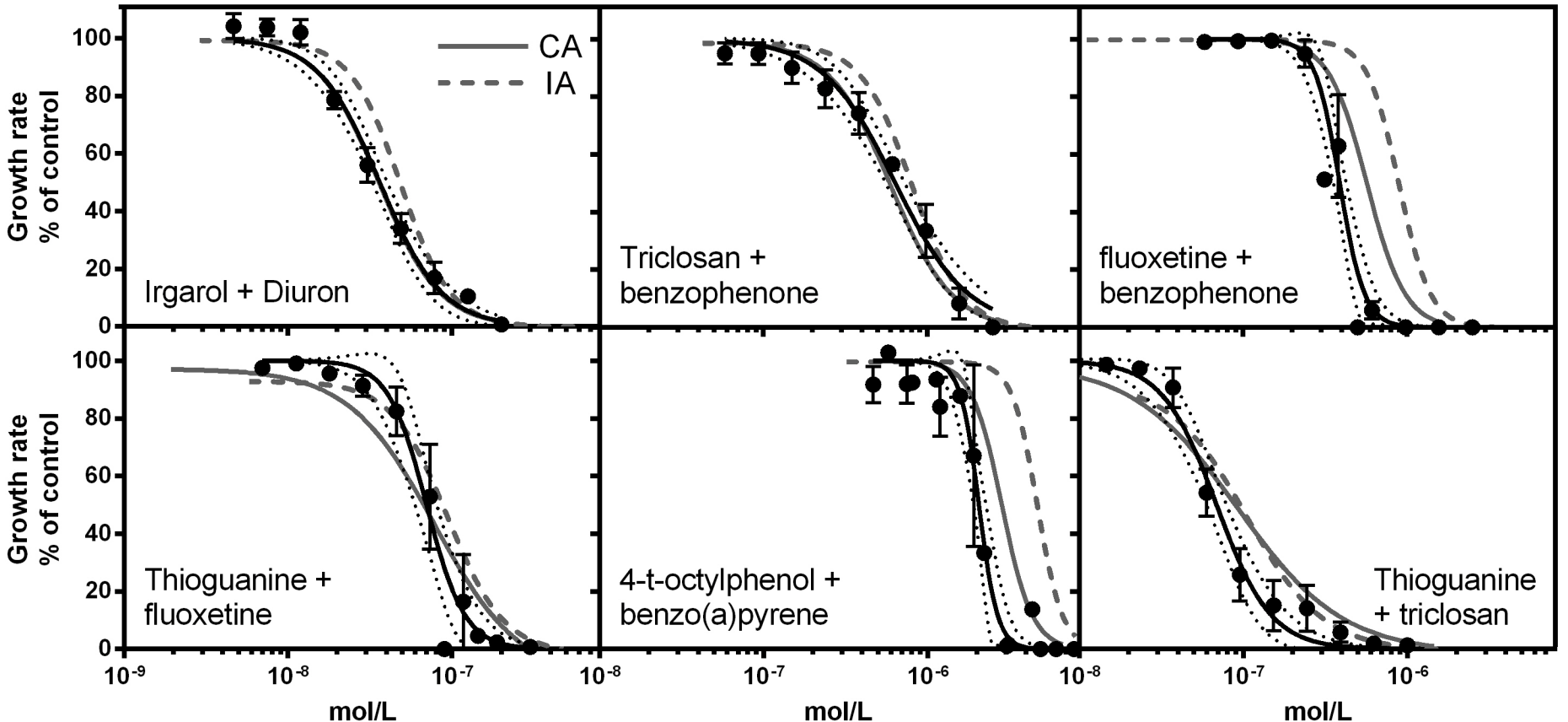
## Figure Legends

Figure 1. Growth rate of *Skeletonema pseudocostatum* expressed as % of control after exposure to pharmaceuticals (fluoxetine, thioguanine, propranolol), biocides (irgarol, diuron), personal care products (benzophenone, cetrimonium bromide, triclosan), organic pollutants (benzo(a)pyrene, 4-*tert*-octylphenol, endosulfan). Results (●) are shown as mean values ± standard deviation of 3 independent studies. The concentration-response curves with 95% confidence interval were modelled by non-linear regression using a sigmoidal concentration-response curve with variable slope. The data for endosulfan was based on 1 study.

Figure 2. Growth rate of *Skeletonema pseudocostatum* as % of control after exposure to different binary mixtures of pharmaceuticals, personal care products, biocides and organic contaminants. Results (●) are shown as mean values ± standard deviation of 4 independent studies. The concentration-response curves with 95% confidence interval were modelled by non-linear regression using a sigmoidal concentration-response curve with variable slope. The grey solid and broken lines are the concentration addition and independent action predictions, respectively. The data for the mixture of 4-*tert*-octylphenol + benzo(a)pyren were based upon 3 independent studies.

Figure 3. Growth rateof *Skeletonema pseudocostatum* as % of control after exposure to a mixture of 8 compounds; irgarol, diuron, triclosan, benzophenone, thioguanine, fluoxetine, 4-*tert*-octylphenol and benzo(a)pyrene. Results (●) are shown as mean values ± standard deviation of 3 independent studies. The concentration-response curves with 95% confidence interval were modelled by non-linear regression using a sigmoidal concentration-response curve with variable slope. The grey solid and broken lines are the concentration addition and independent action predictions, respectively.


Growth rate
% of control
100
80
60
40
20
0
Fluoxetine
Thioguanine
Propranolol
Irgarol
Diuron
Benzophenone
Cetrimonium
bromide
Triclosan
Benzo(a)pyrene
4-tert-octylphenol
Endosulfan
10-9
10-8
10-7
10-6
10-5
mol/L


CA
IA
Growth rate
% of control
Irgarol + Diuron
Triclosan +
benzophenone
fluoxetine +
benzophenone
Thioguanine +
fluoxetine
4-t-octylphenol +
benzo(a)pyrene
Thioguanine
+ triclosan
mol/L
mol/L
mol/L

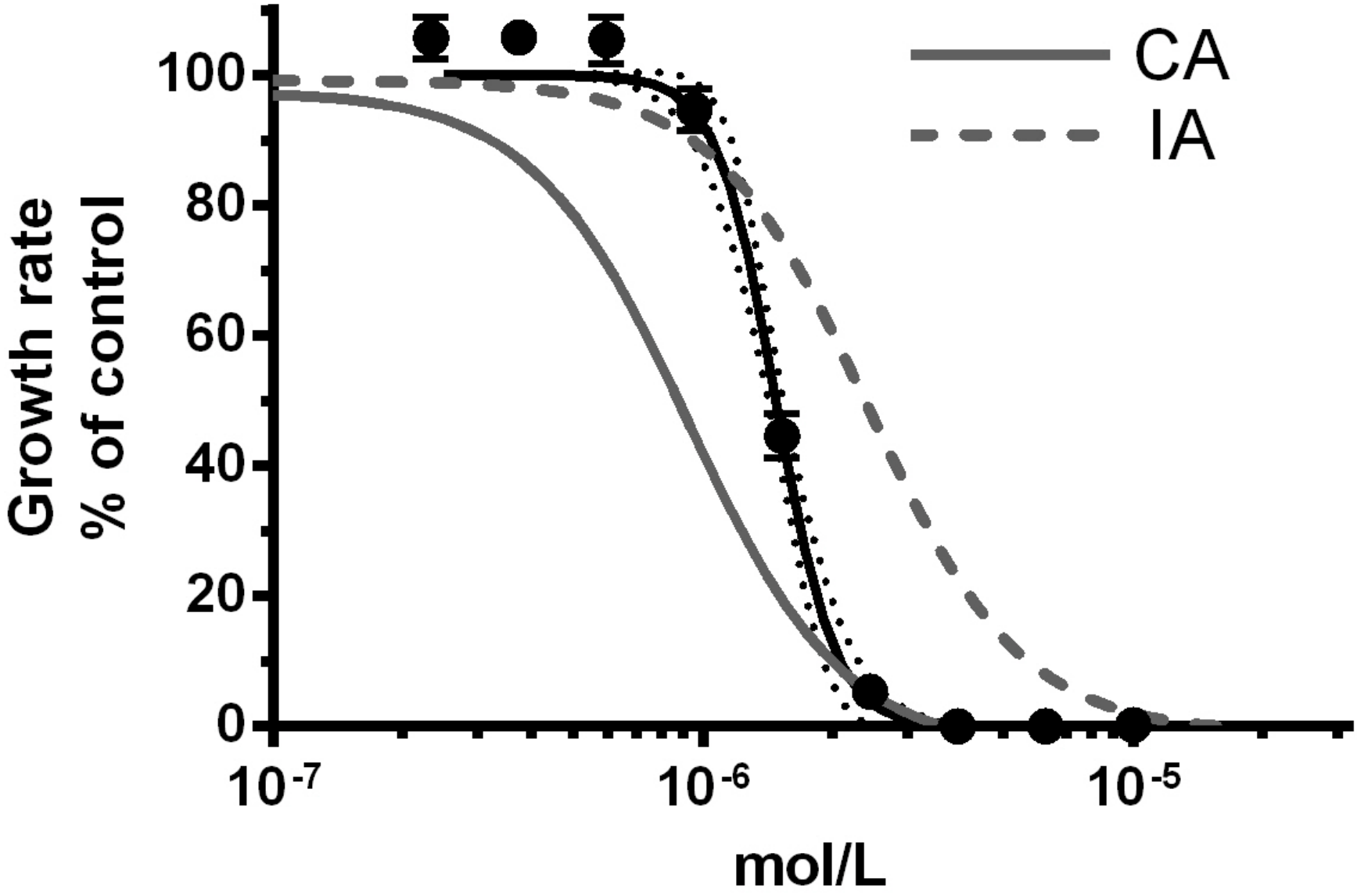
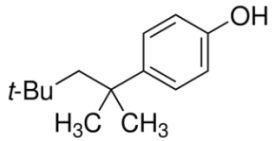
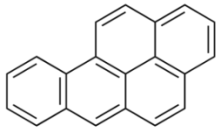
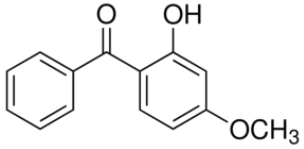
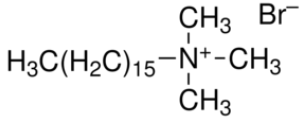
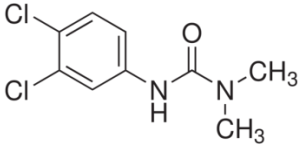
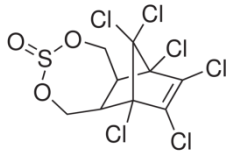
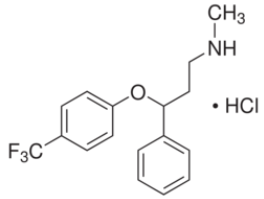
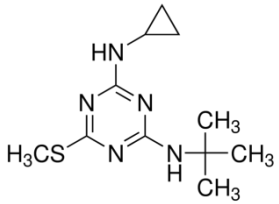
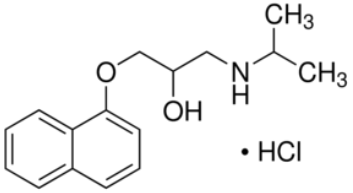
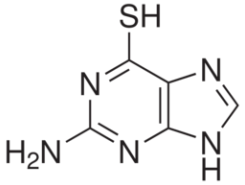
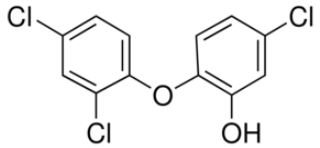

CA
IA
Growth rate
% of control
100
80
60
40
20
0
10-7
10-6
10-5
mol/L

Table 1. Chemical structure, use and mode of action of the test chemicals

| Compound | Log KoW | Structure | Use/Origin | Main Mode of action |
|---|---|---|---|---|
| **4-*tert*-octylphenol** cas: 140-66-9 | 3.7[a] |  | Various chemical industrial applications. Chemical intermediate mainly used to make phenolic resins for tire production (Brooke et al., 2005) | Estrogen receptor agonist (Tollefsen et al., 2008) |
| **Benzo(a)pyrene** cas: 50-32-8 | 5.97[a] |  | Byproduct of incomplete combustion or burning of organic materials (wood, gasoline, cigarettes etc) (EPA, 2007) | Aryl-hydrocarbon receptor agonist, metabolite form DNA-adducts (EPA, 2007) |
| **Benzophenone 3** cas: 131-57-7 | 3.79[c] |  | Used in sunscreens and cosmetics (SCCP, 2008) | Estrogenic, anti-estrogenic, anti-androgenic (Kunz and Fent, 2006) |
| **Cetrimonium bromide** cas: 57-09-0 | 3.18[a] |  | Antiseptic agent (Thefreedictionary.com, 2013) Ingredient in personal care products (Lush, 2013) | Mitochondria-mediated apoptosis (Ito et al., 2009) |
| **Diuron** cas: 330-54-1 | 2.64[b] |  | Herbicide (Kegley et al., 2011). Used as an anti-fouling agent in boat paints (Voulvoulis et al., 2002) | Inhibition of photosystem II (Jones, 2005) |
| **Endosulfan** cas: 115-29-7 | 3.83[a] |  | Organochlorine insecticede used in agriculture (Kegley et al., 2011) | Blocking of GABA-gated chlorine channels (Canadian Council of Ministers of the Environment, 2010) |

| | | | | |
|---|---|---|---|---|
| **Fluoxetine**<br>cas: 56296-78-7 | 1.8[a]<br>4.09<br>(Neuwoehner and Escher, 2011) |  | Antidepressant used against major depressive disorder, bulimia, obsessive-compulsive disorder, panic disorder etc. in humans (Daughton and Ternes, 1999; Drugs.com, 2013) | Selective serotonin reuptake inhibitor (Hiemke and Härtter, 2000) |
| **Irgarol**<br>cas: 28159-98-0 | 3.9[a] |  | Used as an anti-fouling agent in boat paints (Voulvoulis et al., 2002) | Inhibition of photosystem II (Jones, 2005) |
| **Propranolol**<br>cas: 318-98-9 | -0.45 (pH2)[c]<br>2.90<br>(Neuwoehner and Escher, 2011) |  | Beta-blocker used against anxiety, hypertensives, vasoconstriction, arrhythmia (Drugbank, 2013) | non-cardioselective beta-adrenergic antagonist (Drugbank, 2013) |
| **Thioguanine**<br>cas: 154-42-7 | 0.13[b] |  | Cancer drug used in treatment of leukemia (Vora et al., 2006) | Incorporates into DNA as replacement for purine bases (Kverka et al., 2011) |
| **Triclosan**<br>cas: 3380-34-5 | 4.7[a] |  | Antibacterial agent (Daughton and Ternes, 1999) | Targets the Fab1 component of bacterial fatty acid synthesis (Heath et al., 1999) |

[a]Value from MSDS sheet, [b]obtained from ALOGPS, [c]obtained from http://pubchem.ncbi.nlm.nih.gov

Table 2. Growth inhibition in *Skeletonema pseudocostatum* exposed to organic contaminants

| Compound | $EC_{50}$[a] growth inhibition (mol/L) | Slope[a] | Goodness of fit ($R^2$)[a] |
|---|---|---|---|
| 4-*tert*-octylphenol | $5.6E^{-6}$ ($5.2E^{-6}$-$6.1E^{-6}$)[b] | -5.2 | 0.965 |
| Benzo(a)pyrene | $6.8E^{-8}$ ($6.2E^{-8}$-$7.5E^{-8}$) | -4.3 | 0.905 |
| Benzophenone 3 | $1.1E^{-6}$ ($1.0E^{-6}$-$1.2E^{-6}$) | -3.2 | 0.974 |
| Cetrimonium bromide | $4.5E^{-6}$ ($2.5E^{-6}$–$8.0E^{-6}$) | -1.4 | 0.646 |
| Diuron | $6.7E^{-8}$ ($5.7E^{-8}$-$7.8E^{-8}$) | -2.3 | 0.929 |
| Endosulfan[c] | $5.9E^{-6}$ ($5.3E^{-6}$-$6.6E^{-6}$) | -2.3 | 0.985 |
| Fluoxetine | $5.2E^{-8}$ ($4.8E^{-8}$-$5.5E^{-8}$) | -4.6 | 0.962 |
| Irgarol | $4.7E^{-9}$ ($4.4E^{-9}$-$5.2E^{-9}$) | -2.4 | 0.981 |
| Propranolol | $8.0E^{-7}$ ($6.3E^{-7}$-$1.0E^{-6}$) | -18.0 | 0.998 |
| Thioguanine | $8.5E^{-8}$ ($6.7E^{-8}$-$1.1E^{-7}$) | -1.1 | 0.858 |
| Triclosan | $9.5E^{-8}$ ($8.3E^{-8}$-$1.1E^{-7}$) | -1.8 | 0.924 |

[a]$EC_{50}$, slope and $R^2$ values are obtained from the fitted concentration-response curves. [b]Values in brackets show the 95% confidence intervals. [c]Values for endosulfan are based on one test only.

Table 3. Model deviation ratios (MDRs) at different effect levels (% growth) for the seven tested mixtures

| | irgarol + diuron | | triclosan + benzophenone | | fluoxetine + benzophenone | | thioguanine + fluoxetine | | 4-*tert*-octylphenol + benzo(a)pyrene | | thioguanine + triclosan | | 8-compound mixture | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $EC_{50}$=3.63E$^{-8}$ mol/L | | $EC_{50}$=6.37E$^{-7}$ mol/L | | $EC_{50}$=3.88E$^{-7}$ mol/L | | $EC_{50}$=6.99E$^{-8}$ mol/L | | $EC_{50}$=2.05E$^{-6}$ mol/L | | $EC_{50}$=6.87E$^{-8}$ mol/L | | $EC_{50}$ =1.48E$^{-6}$ mol/L | |
| % Growth | MDR[a] CA | MDR IA | MDR CA | MDR IA | MDR CA | MDR IA | MDR CA | MDR IA | MDR CA | MDR IA | MDR CA | MDR IA | MDR CA | MDR IA |
| 95 | **1.00** | **1.59** | **1.08** | **1.75** | **1.10** | **1.96** | 0.24 | n.a | **1.08** | **1.95** | 0.40 | n.a. | 0.21 | **0.71** |
| 90 | **1.00** | **1.55** | **1.06** | **1.67** | **1.19** | 2.04 | 0.41 | **0.62** | **1.15** | 2.04 | **0.57** | **0.53** | 0.31 | **0.90** |
| 80 | **0.99** | **1.50** | **1.01** | **1.52** | **1.28** | 2.14 | **0.60** | **0.95** | **1.23** | 2.12 | **0.78** | **0.96** | 0.40 | **1.12** |
| 70 | **0.99** | **1.41** | **0.98** | **1.41** | **1.35** | 2.19 | **0.73** | **1.08** | **1.29** | 2.18 | **0.96** | **1.14** | 0.47 | **1.30** |
| 60 | **0.99** | **1.37** | **0.95** | **1.33** | **1.41** | 2.24 | **0.85** | **1.17** | **1.33** | 2.22 | **1.13** | **1.28** | **0.53** | **1.45** |
| 50 | **0.98** | **1.33** | **0.92** | **1.25** | **1.46** | 2.29 | **0.96** | **1.25** | **1.38** | 2.27 | **1.30** | **1.39** | **0.59** | **1.61** |
| 40 | **0.98** | **1.28** | **0.90** | **1.18** | **1.52** | 2.33 | **1.08** | **1.33** | **1.43** | 2.31 | **1.50** | **1.50** | **0.66** | **1.78** |
| 30 | **0.98** | **1.24** | **0.87** | **1.10** | **1.58** | 2.38 | **1.21** | **1.41** | **1.48** | 2.36 | **1.75** | **1.62** | **0.73** | **1.99** |
| 20 | **0.98** | **1.19** | **0.83** | **1.01** | **1.66** | 2.45 | **1.35** | **1.50** | **1.55** | 2.42 | 2.08 | **1.77** | **0.82** | 2.27 |
| 10 | **0.97** | **1.11** | **0.77** | **0.88** | **1.79** | 2.54 | **1.48** | **1.63** | **1.65** | 2.51 | 2.66 | **1.99** | **0.96** | 2.74 |
| 5 | **0.97** | **1.04** | **0.70** | **0.77** | **1.90** | 2.62 | **1.48** | **1.71** | **1.75** | 2.59 | 3.20 | 2.17 | **1.06** | 3.19 |

[a] the model deviation ratios (MDRs) were calculated by dividing the observed effect concentration by the effect concentration predicted by CA (MDR CA) or IA (MDR IA). Effect concentrations were calculated from the non-linear regression curve fits. Bold text indicates were MDRs are within a factor of two.