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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 *Skeletonema 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.

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

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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, 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, 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 *S. 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; Kummerer, 2010; Schlabach et al., 2009; Thomas and Brooks, 2010), anticipated aquatic toxicity (Sanderson and Thomsen, 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. S. 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 0.45 μm 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