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The influence of salinity on the toxicity of selected sulfonamides and trimethoprim towards the green algae *Chlorella vulgaris*

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HIGHLIGHTS

- Sulfamethoxazole and sulfapyridine are the most toxic to *C. vulgaris*.
- Trimethoprim is the least toxic pharmaceutical to selected organism.
- Toxicities were negatively correlated with increasing salinities.
- The effects of tested drugs towards algae are caused by specific mode of action.

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ABSTRACT

This paper presents the investigation of the influence of salinity variations on the toxicity of sulfapyridine, sulfamethoxazole, sulfadimethoxine and trimethoprim towards the green algae *Chlorella vulgaris* after exposure times of 48 and 72 h. In freshwater the EC_{50} values ranged from 0.98 to 123.22 mg L^{-1} depending on the compound. The obtained results revealed that sulfamethoxazole and sulfapyridine were the most toxic, while trimethoprim was the least toxic pharmaceutical to the selected organism. Deviations between the nominal and real test concentrations were determined *via* instrumental analysis to support the interpretation of ecotoxicological data. The toxicity effects were also tested in saline water (3, 6 and 9 PSU). The tendency that the toxicity of selected pharmaceuticals decreases with increasing salinity was observed. Higher salinity implies an elevated concentration of inorganic monovalent cations that are capable of binding with countercharges available on algal surfaces (hydroxyl functional groups). Hence it can reduce the permeability of pharmaceuticals through the algal cell walls, which could be the probable reason for the observed effect. Moreover, for the classification of the mode of toxic action, the toxic ratio concept was applied, which indicated that the effects of the investigated drugs towards algae are caused by the specific mode of toxic action.

1. Introduction

Pharmaceutical residues have been recognized as a continuing threat to environmental stability due to their inherent biological activity and their pseudo-persistency. Thousands of tons of pharmaceuticals are produced annually worldwide, both for human medicine application and veterinary uses. Many pharmaceuticals (such as trimethoprim, enrofloxacin, diclofenac) are not completely metabolized in organisms, therefore a high proportion of them are excreted in an unchanged form. Depending on the type of drug (used in medicine or in veterinary medicine) they can enter the environment *via* different routes. Their occurrence in environmental samples has been reported in recent years in a number of studies [1–4].

Pharmaceuticals are designed to induce effects when used and therefore there is a high probability of them being biologically active towards wildlife species as well [5]. For this reason, data from ecotoxicity tests on different species is relevant to illustrate the several adverse effects that environmental exposure to measured concentrations of these contaminants can have. Although numerous reports covering the toxicity of pharmaceuticals are available, most of them deal with freshwater organisms [1].

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The knowledge of the fate of pharmaceutical residues in the marine environment and the effects that they might pose to marine and estuarine organisms is still quite limited and needs to be expanded for a reliable hazard and risk assessment. Seawater salinity is a critical environmental factor influencing the distribution and maintenance of aquatic wildlife [6,7]. The bioavailability of some toxic substances in the marine environment can differ considerably when compared to freshwater, thus toxicity and its mode of action could also be affected. The accumulation of pharmaceuticals by aquatic organisms in various salinities could also be affected by their osmoregulatory adaptations. Hence, the toxicity may vary with salinity, which was already proved for various classes of inorganic and organic chemicals, such as ionic liquids [8], petroleum hydrocarbons, polycyclic aromatic hydrocarbons, pesticides and metals [7]. There was generally no consistent trend for the toxicity of most organic chemicals in different salinities. Only the toxicity of metals was reported to increase with decreasing salinity [7]. Therefore each group of organic and inorganic toxicants must be studied in detail to gain a full understanding of their prospective fate in the environment. Moreover, pharmaceuticals were not investigated in this very context.

Therefore, the main aim of this study was to evaluate the effects of selected pharmaceutical compounds (sulfapyridine (SPY), sulfamethoxazole (SMX), sulfadimethoxine (SDM), trimethoprim (TMP)—Table 1 [9,10]) towards the marine algae—*Chlorella vulgaris*. Although this green alga is a cosmopolitan species inhabiting fresh, brackish and marine waters, literature data on the toxicity of the investigated pharmaceuticals is limited. The EC_{50} values are only available only for SDM and SMX (11.2 mg L^{-1} for SDM [11] and 6.2 μM for SMX [12]). This species is part of the algal flora of the Baltic Sea, which naturally varies in salinity and is under high anthropogenic influence, including huge discharges of polluted freshwater [8]. As our previous studies proved the presence of several pharmaceuticals in the southern Baltic Sea environment, including those selected for this study [13,14], there is an urgent need to evaluate the ecotoxicological profile of these compounds, especially under environmental conditions.

Moreover, as standard toxicity bioassays do not take into account variable environmental conditions, this study reports for the first time in literature the influence of salinity on the biological activity of selected pharmaceuticals on *C. vulgaris*. The toxic effects were tested in fresh water (0 practical salinity unit, PSU) and in water of different salinities – 3, 6 and 9 PSU – reflecting the range encountered in the Baltic Sea and the range in which *C. vulgaris* is present. The next aim of this study is to determine the mode of toxic action of the selected compounds based on the calculated toxic ratio.

2. Materials and methods

2.1. Chemicals

Sulfapyridine was purchased from Serva (Weissensberg, Germany). Sulfamethoxazole, sulfadimethoxine, trimethoprim, chemicals used for preparing the culturing medium, potassium dichromate and 2,4-dichlorophenol (all of analytical reagent grade) were purchased from Sigma–Aldrich (Steinheim, Germany). Salts (all of analytical reagent grade) used for preparing the synthetic seawater, ammonium acetate (NH_4Ac) and acetic acid were purchased from Chempur (Piekary Śląskie, Poland). Acetonitrile (ACN) (HPLC—grade) was obtained from POCH S.A. (Gliwice, Poland).

The test algae were batch-cultured in Bold's Basal Medium (BBM) [15]. The 35 PSU synthetic seawater was prepared according to the recipe of Lyman and Fleming [16]. The salinity used for the tests (3, 6 and 9 PSU) was made up by the proper dilution of synthetic seawater with BBM.

2.2. Batch culture

In this study a synchronized unicellular green algae *C. vulgaris* culture (strain 211–11b, SAG (Culture Collection of Algae), Universität Gottingen, Germany) was used. The stock culture was grown under photoautotrophic conditions at 20 °C (±0.5 °C) in an inorganic, sterilized BBM medium (pH 6.4–6.7) with saturating white light (22–33 klx, Lumilux Daylight L 36 W-11 and Lumilux Interna L 36 W-41, Osram, Berlin, Germany). The cells were aerated with 1.5 vol.% CO_2 and synchronized using a 14–10 h light–darkness cycle. The stock culture was diluted every day to a cell density of 5×10^5 cells mL^{-1} and it was acclimatized for 10 days before each test. This test is a modified version of the assay described by Altenburger et al. [17]. and its sensitivity is comparable to the standardized 72 h test [18].

2.3. Reproduction inhibition assay

The toxicity tests started with autospores. The algae were exposed to the test substances for 48 and 72 h. The endpoint of this assay was the inhibition of algal reproduction, measured as the inhibition of population growth. Cell numbers were determined with a CASY® Cell Counter, Model TTC (Roche Innovatis AG, Reutlingen, Germany). All the tests were performed in sterilized glass tubes, where the algae were stirred throughout the 72 h test period. The temperature and the light–darkness cycle were the same as for the stock culture. The only exception was the source of the CO_2—here 150 μL of $NaHCO_3$ solution was added to each test tube. All details are described in Refs. [19,20].

First, all the substances were tested in a range finding test (five concentrations, two replicates); then, the toxicity of each substance was determined for five concentrations per substance in two parallel replicates. At least five controls were used for each assay. These data were pooled for analysis. Growth inhibition was calculated using the cell counts of the treated samples in relation to the untreated controls. The observed EC_{50}'s generally lie in the range of the values found in ring tests referred to in the corresponding guideline (OECD 201). The data presented fulfill the validation criteria: the specific growth rate was >0.92 day^{-1} and the coefficient of the variation of average specific growth rates was during the whole test period in replicate control cultures <10%. Moreover, in order to verify the conditions of the test and prove the quality of the obtained data the toxicity of standard reference compounds (potassium dichromate and 2,4-dichlorophenol) towards *C. vulgaris* was determined. The obtained results showed that for 2,4-dichlorophenol the EC_{50}(96 h) = 7.9 mg L^{-1} and for potassium dichromate the EC_{50}(24 h) = 0.29 mg L^{-1}, EC_{50}(72 h) = 0.23 mg L^{-1}, EC_{50}(96 h) = 0.39 mg L^{-1}. The results were in agreement with the literature data [21,22], which proves that these tests were conducted properly. It enabled us to use them for the evaluation of the ecotoxicity of the selected pharmaceuticals.

2.4. Effect data modeling and statistical analysis

All the experiments were performed four times. Dose–response curve parameters and plots were obtained using the drift package (version 0.05-95) for the R language and environment for statistical computing (www.r-project.org) (R Development Core Team, 2010).

A one-way analysis of variance (ANOVA) with the Tukey HSD post-hoc test was used to determine significant differences between the EC_{50} values of SDM, SMX and SPY obtained for *C. vulgaris* grown at different salinities (0–9 PSU). Significant differences were accepted at $P \leq 0.01$. The normality and homogeneity of