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Hazard and risk of herbicides for marine microalgae

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ABSTRACT

Due to their specific effect on photosynthesis, herbicides pose a potential threat to coastal and estuarine microalgae. However, comprehensive understanding of the hazard and risk of these contaminants is currently lacking. Therefore the aim of the present study was to investigate the toxic effects of four ubiquitous herbicides (atrazine, diuron, Irgarol®1051 and isoproturon) and herbicide mixtures on marine microalgae. Using a Pulse Amplitude Modulation (PAM) fluorometry based bioassay we demonstrated a clear species and herbicide specific toxicity and showed that the current environmental legislation does not protect algae sufficiently against diuron and isoproturon. Although a low actual risk of herbicides in the field was demonstrated, monitoring data revealed that concentrations occasionally reach potential effect levels. Hence it cannot be excluded that herbicides contribute to observed changes in phytoplankton species composition in coastal waters, but this is likely to occur only occasionally.

1. Introduction

Coastal waters are among the most productive ecosystems on the planet (Kaiser et al., 2011). Yet, they also suffer from high contaminant loads due to riverine inputs, land run-off and shipping activities (Hylland and Vethaak, 2011). Moreover, it is expected that the chemical pressure on coastal and estuarine environments will further increase due to the worldwide growth of the human population and the coinciding industrial activities (Laane et al., 2012). Since microalgae form the basis of marine food webs and embody the carrying capacity of marine ecosystems (Hylland and Vethaak, 2011), the potential adverse effect of chemical pressure on coastal and estuarine marine microalgae is of major concern.

The Dutch coastal and estuarine waters are polluted due to their geographical location downstream of large European rivers and several studies demonstrated the presence of a wide variety of contaminants (Quirijns et al., 1979; de Voogt and Laane, 2009; Vethaak et al., 2005), including herbicides (Lamoree et al., 2002). Due to their specific mode of action on the photosynthetic system, herbicides are amongst the most likely contaminants causing toxic effects on microalgae. Atrazine, isoproturon, diuron and Irgarol®1051 (Cybutryne) are widely used herbicides, and listed as priority substances under the European Water Framework Directive as described in Directive 2013/39/EU. They are applied in agriculture, but diuron and Irgarol®1051 are also used as a booster biocide in antifouling paints. Except for atrazine, which is banned in Europe since 2004, the three other herbicides are still allowed as active ingredients in several products in The Netherlands (CTGB).

Due to their specific mode of action herbicides are hazardous to marine microalgae, but the likelihood that they actually cause effects in the field and represent an ecological risk will depend on their concentration in the water. Several studies already showed toxic effects of herbicides and herbicide mixtures on microalgae (Buma et al., 2009; Magnusson et al., 2008; Ma et al., 2006; Knauert et al., 2008; Gatidou and Thomaidis, 2007; DeLorenzo and Serrano, 2006). However, the obtained effect concentrations were often well above environmental concentrations, suggesting a low actual risk of these compounds for marine microalgae. Consequently, changes in phytoplankton composition in the Dutch coastal and estuarine waters during the past decades are mainly ascribed to changes in nutrients (Philippart et al., 2000, 2007; Prins et al., 2012). Yet, since mixtures of compounds at low concentrations as present in the ocean were shown to affect phytoplankton (Echeveste et al., 2010), we question the assumption that herbicides do not contribute to

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changes in coastal and estuarine phytoplankton composition. Therefore the aim of the present study was to assess both hazard and risk of herbicides and herbicide mixtures for marine microalgae. To this purpose the first objective was to determine the compound and species specific toxicity of four herbicides to three common marine microalgal species in laboratory toxicity experiments with photosynthetic efficiency as herbicide specific endpoint. Next, the toxicity of a mixture of the four herbicides was tested to one of the selected test species. To analyse if the microalgae are sufficiently protected by the current environmental legislation, the test species was exposed to the Maximum Allowable Concentration of the Environmental Quality Standard (MAC-EQS) of each of the herbicides as well as to a mixture of the four herbicides all at EQS concentrations. To assess the actual risk of herbicides present in contaminated coastal and estuarine waters for marine phytoplankton, the algae were subjected to maximum field concentrations of the individual herbicides and to a mixture of these four concentrations. Finally, the toxicity of a water sample collected at a rather polluted site in The Netherlands (Hansweert, Western Scheldt) was determined. We expect that this series of consecutive experiments elucidates the potential hazard and risk of herbicides for marine microalgae living in contaminated coastal and estuarine waters.

2. Materials and methods

2.1. Test species and culturing conditions

To determine the compound and species specific effects of herbicides, toxicity experiments with four individual herbicides were performed with the marine flagellate *Dunaliella tertiolecta* (Butcher, CCAP 19/27) and the diatoms *Phaeodactylum tricornutum* (Bohlin, CCMP 2558) and *Thalassiosira pseudonana* (Hasle and Heimdal, CCMP 1335). These are commonly used species in ecotoxicological tests on pesticides (PAN pesticide database) and they have a worldwide (*P. tricornutum* and *T. pseudonana*) or European (*D. tertiolecta*) distribution (Algaebase). Mixture toxicity and the effects of EQS, maximum field concentrations and a field sample were evaluated with *D. tertiolecta* only. The test species were batch cultured in artificial seawater. To this purpose, sea salts (Aqua Bio Solutions, Wormerveer, The Netherlands) were dissolved in MilliQ water to obtain a salinity of 33PSU and enriched with f/2 medium (Guillard, 1975) (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands). A light–dark regime of 16:18h at 16 °C with a light intensity of 50 μmol m^{-2} s^{-1} was maintained with fluorescent lamps (F58W/BriteGro2084, Havells Sylvania, Raunheim, Germany). All experiments were performed with exponentially growing cells with a cell density of $1e^6$ cells/mL for *D. tertiolecta* and *T. pseudonana* and $2.5e^6$ cells/mL for *P. tricornutum*.

2.2. Test compounds and toxicity tests

Irgarol®1051 (>97%) was obtained from Ciba Specialty Chemicals Inc. (Basle Switzerland) and atrazine, diuron and isoproturon (PESTANAL® a.s) from Sigma Aldrich (Zwijndrecht, The Netherlands). Stock solutions were made in methanol (ULC/MS grade, Biosolve, Valkenswaard, The Netherlands) and stored at 4 °C. Chemical structure, CAS number and systematic name of the test compounds are mentioned in the supporting information (Tab. SI-1).

Toxicity of the four herbicides to algal photosynthetic efficiency was determined in a short-term algal bioassay using Pulse Amplitude Modulation (PAM) fluorometry. Tests were performed in black polypropylene 96-wells plates (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) and effective photosystem II efficiency (ΦPSII) of the algal suspension was determined after 4.5 h using a WATER-PAM (Heinz Walz GmbH, Effeltrich, Germany). Minimum and maximum fluorescence (F and F'_m respectively) were determined and ΦPSII was calculated as $[F'_m - F]/F'_m$. For a test to be valid, the minimum ΦPSII of unexposed control algae should be at least 0.250 and a ΦPSII inhibition of a fixed concentration of atrazine (95 μg/L) should be 70 ± 10% for all test species.

The effects of the individual herbicides (six concentrations per herbicide, six replicates per concentration and eleven replicates for the control; see also Table SI-2) on the photosynthetic efficiency of the algae were expressed as the 50% effect concentration (EC_{50}). The EC_{50} values were then used to compose an equitoxic mixture of the four herbicides applying the Toxic Unit (TU) concept according to Könemann (1981). The toxicity of the mixture of the four herbicides (six concentrations, six replicates per concentration and eight replicates for the control) was expressed as EC_{50} value and interpreted with the Concentration Addition model according to Könemann (1981). The effects of the EQS concentrations and the current Dutch field concentrations of the herbicides were tested individually ($N = 3$) and in a mixture ($N = 3$). ΦPSII inhibition was expressed as a percentage of the corresponding control (% of control). For the EQS concentration of atrazine, diuron and isoproturon a Maximum Allowable Concentration (MAC-EQS) was available in the Priority Substance Directive (Directive 2008/105/EC) and for Irgarol®1051 the maximum allowable risk (MTR) concentration applied in the Netherlands (described by the RIVM) was used (Table 1). It has to be noted that at the time of the experiments, the new directive (Directive 2013/39/EU) was not available and the previous directive (Directive 2008/105/EC) was used for MAC-EQS concentrations as mentioned in Table 1. As Irgarol (Cybutryne) was added in the newest edition as a priority compound, no MAC-EQS was available. However, the new MAC-EQS (16 ng/L) is close to the used MTR of 24 ng/L (Table1). Monitoring data were used to determine the maximum concentrations of the four herbicides in the water at a rather polluted site (Hansweert, The Netherlands) in 2011 (Waterbase) (Table 1). Next, these experimentally assessed effects of field and EQS concentrations on *D. tertiolecta* were compared with the predicted effects derived from the previously obtained dose response relationships (Fig 1). To this purpose the field and EQS concentrations were used as input (x-value) in the dose response relationships of *D. tertiolecta*. The output (y-value) of the calculations represents the predicted effect on ΦPSII of these concentrations.

Finally, the toxicity of a field sample collected at a rather polluted site (Hansweert, The Netherlands, November 2011) containing a mixture of unknown compounds was tested ($N = 3$). To this purpose a water sample was concentrated with Oasis HLB and diluted step-wise (1000, 500, 100, 50, 10, 1× concentrated) to determine the concentration factor (CF) at which the photosynthetic efficiency was reduced by 50% (ECf_{50}). Sampling, concentrating and determination of the herbicides concentrations was done according to Booij et al. (2013).

2.3. Chemical analysis

Due to the small test volume in the 96-wells plates and the relatively high detection limit of the chemical analysis of the selected herbicides, a separate test with higher herbicide concentrations was performed to test the relationship between nominal and actual concentrations. To determine the loss of the herbicides during the 4.5 h of exposure, culture medium with *D. tertiolecta* ($1e^6$ cells/mL) was spiked with concentrations (nominal) of atrazine (50 μg/L), diuron (50 μg/L), Irgarol®1051 (10 μg/L) and isoproturon (50 μg/L) dissolved in methanol ($N = 3$). After 4.5 h, algae were separated from the medium by centrifugation of the 96-wells plate at 400 *g* for 15 min (Eppendorf Microcentrifuge 5430) and from each sample two 100 μL subsamples were transferred into two separate vials. To one vial 20 μL of the test compound was added (standard addition) to facilitate the measurement of the nominal concentrations and to the other vial 20 μL of methanol was added. Added concentrations were 33 μg/L for diuron, isoproturon and atrazine and 8 μg/L for Irgarol®1051. Samples were stored at 4 °C and measured within two days. All samples were analyzed with an HPLC Agilent 1290 Infinity system coupled to a micrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electro-spray ionization source (ESI). Chromatographic separation was performed using a Waters (Etten-Leur, The Netherlands) Acquity UPLC BEH column and guard column (2.1 × 30 mm and 2.1 × 5 mm, 1.7 μm respectively). The column was kept at 40 °C. The eluent flow was 0.2 mL/min, and the solvent gradient used was from 20%Methanol/80% MilliQ water containing 0.2% formic acid (v/v/v, solvent A) linear increasing to 100% Methanol containing 0.2% formic acid (v/v, solvent B) in 20 min. The HPLC system was controlled by Hystar software (version 3.2; Bruker Daltonics, Bremen, Germany). The mass spectrometer was controlled by the Compass 1.3 for micrOTOF software package (Bruker Daltonics, Bremen, Germany) and operated in positive ion mode. The capillary voltage was maintained at 4500 V with the end plate offset at −500 V. The pressure for the nebulizer gas (N_2) was set at 4 bar, and the drying gas (N_2) flow rate was 8.0 L/min at a temperature of 200 °C. The full scan mass ranged from m/z 100 to 1000. The data were analyzed by extraction and integration of the accurate mass using Compass Data Analysis Software (version 4.0 SP4; Bruker Daltonics, Bremen, Germany) and actual concentrations after 4.5 h were determined based on standard addition. Formic acid (MS grade) was purchased from Fluka (Sigma–Aldrich, Zwijndrecht, The Netherlands) and methanol (HPLC gradient grade) from J.T. Baker (Deventer, The Netherlands).

Table 1
Environmental Quality Standard (EQS) concentrations (ng/L), field concentrations (ng/L) and test concentrations of the chemical analysis (μg/L) of the four herbicides. For the chemical analysis, the nominal and average actual concentration ± standard deviation are included ($N = 3$).

Compound	EQS (ng/L)	Field concentration[c] (ng/L)	Chemical analysis (μg/L)
Atrazine	2000[a]	6.3	50/55 ± 1
Diuron	1800[a]	33.0	50/56 ± 1
Irgarol®1051	24[b]	4.3	10/10 ± 0
Isoproturon	1000[a]	43.1	50/54 ± 2

[a] Maximum allowable concentration (MAC).
[b] Maximum allowable risk concentration (MTR).
[c] Maximum concentration in the water in 2011 in Hansweert, The Netherlands.