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Seasonal variability in irradiance affects herbicide toxicity to the marine flagellate *Dunaliella tertiolecta*

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**INTRODUCTION**

Microalgae are primary producers and play a key role in aquatic ecosystems due to their position at the base of food webs. Hence, toxicants affecting microalgae potentially affect the carrying capacity of marine and freshwater ecosystems (Hylland and Vethaak, 2011). Herbicides are, due to their specific mode of action, amongst the most harmful contaminants for microalgae. Because herbicides act often directly on the photosynthetic machinery, solar radiation is likely a key factor determining the actual toxicity of herbicides in the field. Numerous studies investigated the effect of light stress on individual microalgal species as well as on natural phytoplankton communities (Vassiliev et al., 1994; Buma et al., 2001; Helbling et al., 2001). Photosynthetically Active Radiation (PAR, 400–700 nm) is captured by the light harvesting pigments in the chloroplasts of the microalgae and is used to convert CO₂ and water into carbohydrates during photosynthesis. PAR will be a limiting factor for algal photosynthesis when radiation is low, while excess radiation causes oxidative stress reducing the photosynthetic capacity (Kirk, 2011). Simultaneously, microalgae are exposed to Ultra Violet Radiation (UVR, <400 nm) which has the potential to damage important biochemical molecules (Larson and Berenbaum, 1988). Excess radiation levels of PAR and UVR may result in the formation of Reactive Oxygen Species (ROS) which may lead to a decreased photosynthetic efficiency or even viability loss (Janknegt et al., 2009a). At the same time, light may also affect compound toxicity in several ways. Solar radiation, especially UVR, may cause degradation of the contaminant, creating transformation products with a potential different persistence and modified toxicity to microalgae. Additionally, UVR can change the structure of a compound through photomodification or photosensitization which may enhance toxicity, a phenomenon known as photo-enhanced toxicity, described for PAHs (Gala and Giesy, 1992; Wiegman et al., 2001; Southerland and Lewitus, 2004), PCBs (Ruben et al., 2001; Sargian et al., 2005), pesticides (Guasch and Sabater, 1998; Lin et al., 1999; Pelletier et al., 2006). Considering the described direct and indirect effects of solar radiation on microalgae and contaminants and the fact that intensity of PAR and UVR vary strongly over the year, it is likely that solar irradiance might play an important role in the ultimate toxicity in the field. Consequently, it can be expected that standard toxicity tests may over- or underestimate toxicity of contaminants when compared to the actual toxicity under variable field conditions.

Nevertheless, there has been little attention paid toward seasonal variation in toxicity. Guasch et al. (1997) described a difference in herbicide toxicity between seasons for natural periphyton communities, but as several environmental factors were tested together, it is unclear if the observed difference was caused by a seasonal difference in light conditions. Yet, experimental confirmation on the effect of seasonal variability in solar irradiance on herbicide toxicity is still lacking. Therefore, we aimed to determine herbicide toxicity to microalgae under simulated irradiance conditions mimicking different seasons. Of the total solar radiation 100% of the UVC and 90% of the UVB radiation...
is blocked by stratospheric ozone, while UVA (320–400 nm) is hardly retained (Coldiron, 1992). As a consequence, microalgae in the water are mainly exposed to UVA (315–400 nm) and PAR (400–700 nm). Additionally, it has been demonstrated that photosynthesis of a natural phytoplankton community is more inhibited by UVA than by UVB (Helbling et al., 2001). Consequently, the present study focuses on the effect of UVA and PAR on herbicide toxicity. Subsurface irradiance at our latitude was simulated in a laboratory setup with Light Emitting Diodes (LEDs) of different wavelengths. We selected two model herbicides, diuron and Irgarol®1051 (Cybutryne), which are both described as priority substances under the European Water Framework Directive (2013/39/EU). Toxicity tests (with both single compounds and with a mixture of both) were performed using PAM fluorometry under the mimicked light conditions to determine the effect of different seasonal light conditions on herbicide toxicity.

MATERIALS AND METHODS
SIMULATING SOLAR RADIATION
Light regimes were simulated in a controlled laboratory setup based on the average light intensity in spring and autumn in The Netherlands. To this purpose, the average light intensity in spring (21 March–21 June 2002–2008) and autumn (21 September–21 December 2002–2008) was obtained from the Royal Netherlands Meteorological Institute resulting in an average intensity of 202 and 53 W/m² for spring and autumn, respectively. The average daily light periods were 14:57 h:min and 9:34 h:min, respectively (Royal Netherlands Meteorological Institute). In addition, the solar spectrum was measured outside (Amsterdam, The Netherlands) at the end of May during clear sky with a USB4000 Fiber Optic Spectrometer (Ocean Optics, Dunedin, Florida, USA). This spectrum was normalized to the average calculated PAR intensities of 202 and 53 W/m² to obtain a spring and autumn light profile. Since phytoplankton is not exposed to the irradiance levels at the surface, the experimental irradiance levels were attenuated to 10% to simulate subsurface irradiance. The attenuation level was chosen to match the conditions at our field sites in the coastal zone of the North Sea with a mixed water column of ca. 30 m and a light attenuation co-efficient Kd of roughly 0.6 m-1 (Delft3D-GEM for the North Sea). The 10% irradiance level of the normalized spectrum (Figure 1, solid lines), was simulated in the laboratory with five LEDs emitting light peaking at wavelengths of 450, 475, 522, 632, and 685 nm, corresponding to the absorbance maximum of the main algal pigments. An additional LED with a peak at 370 nm was used to simulate UVA. A total of 16 clusters of six LEDs provided homogeneous light during the experiments. The spectra for both spring and autumn light conditions, hereafter referred to as SPRING and AUTUMN, are depicted in Figure 1 (dashed lines).

TEST SPECIES AND CHEMICALS
All tests were performed with the marine flagellate Dunaliella tertiolecta (Butcher, CCAP 19/27) which was cultured in Erlenmeyer flasks on artificial seawater medium. Artificial seawater (33%) was obtained by dissolving sea salts (Aqua Bio Solutions, Wormerveer, The Netherlands) in de-ionized water (MilliQ) and this was enriched with f/2 medium (Guillard, 1975) (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Toxicity tests were performed using diuron (CAS: 330-54-4, analytic standard, Sigma Aldrich, Zwijndrecht, The Netherlands) and Irgarol®1051 (CAS: 28159-98-0, >97%, Ciba Specialty Chemicals Inc., Basle, Switzerland). Stock solutions of both compounds were made in methanol (ULC/MS grade, Biosolve, Valkenswaard, The Netherlands). All presented concentrations are nominal concentrations. Culturing and toxicity experiments were performed under SPRING and AUTUMN light conditions at a constant temperature of 13 ± 0.5°C.

TOXICITY TEST
To investigate the algal growth rate under the different irradiance regimes, cell densities of acclimatized D. tertiolecta were counted daily in a Bürker counting chamber. These data were used to
determine the exponential growth phase under both light regimes and all toxicity experiments were performed with light adapted, exponentially growing populations. The growth rates of *D. tertiolecta*, determined by linear regression of ln-transformed cell densities were 0.8 and 0.3 (day$^{-1}$) for SPRING and AUTUMN, respectively.

The toxic effects of the Irgarol®1051 and diuron (five concentrations per herbicide, six replicates per concentration and five replicates for the control) on the photosynthetic efficiency of the algae was determined for SPRING and AUTUMN. To this purpose, Pulse Amplitude Modulation (PAM) fluorometry bioassays were performed in which the effective photosystem II efficiency (ΦPSII) 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 $[F_m-F]/F_m$. The PSII inhibition was expressed as percentage of the corresponding solvent control (% of control). The effect on the photosynthetic efficiency of the algae was expressed as the 50% effect concentration (EC50). Next to the experiments with diuron and Irgarol individually, toxic effect of both herbicides was determined in a mixture for SPRING as well as AUTUMN. The Toxic Unit (TU) concept was applied for the composition of an equitoxic mixture of both herbicides, based on the individual EC50 values. The toxicity of this mixture (tested for eight concentrations) was determined using the same bioassay as described above and the results were interpreted according to the Concentration Addition model (Könemann, 1981).

The actinic light used to determine ΦPSII of these PAM measurements consisted of LEDs of 632 nm (chlorophyll a fluorescence) which were identical to the 632 nm LEDs used to simulate solar radiation. The same light intensity of these LEDs was used during exposure and measurement to maintain the required simulated field relevant irradiance.

**DATA ANALYSIS**

The log-logistic dose-response model described by Haanstra et al. (1985) was used to determine the 50% reduction (EC50) in ΦPSII and was calculated as $y = c/(1 + e^{(\log(b) - \log(a))})$, where $y$ is the ΦPSII (% control), $x$ is the concentration of the toxicant (μg/L), $a$ is the EC50 value (μg/L), $b$ is the slope of the curve, and $c$ is the ΦPSII of the control. Likelihood ratio tests were applied to compare effect concentrations of SPRING and AUTUMN ($\chi^2 > 3.84$, $df = 1$, $p < 0.05$). The analyses were all performed with SPSS (IBM SPSS Statistics 20).

**RESULTS**

Clear dose-response relationships of the effect of Irgarol and diuron on ΦPSII of *D. tertiolecta* were obtained for SPRING and AUTUMN (Figure 2). The observed toxicity was significantly ($p < 0.05$) higher for SPRING (EC50: 0.8 and 3.6 μg/L) compared to AUTUMN (EC50: 1.3 and 4.8 μg/L) for Irgarol and diuron, respectively. The same pattern, with herbicides having a higher toxic effect in SPRING compared to AUTUMN, was observed for the binary mixture of the herbicides (Figure 3). The EC50 of the equitoxic mixture for SPRING was significantly lower (0.8 TU, 95% C.I. 0.7–0.9) than 1 TU, indicating a more than additive effect for the mixture of both herbicides. In contrast, the EC50 of the mixture for AUTUMN was significantly higher (1.2 TU, 95% C.I. 1.1–1.3) than 1 TU, indicating a less than additive effect.

**DISCUSSION**

We clearly demonstrated a significant difference in the toxicity of Irgarol and diuron to *D. tertiolecta* cultures under simulated spring and autumn irradiance conditions, thereby providing the first experimental confirmation that seasonal variation in irradiance indeed affects herbicide toxicity to microalgae. Results of previous studies on the toxicity of both Irgarol and diuron to microalgae are recently reviewed by Suresh Kumar et al. (2014). It was found that Irgarol, used in antifouling paints on ship hulls, is highly toxic to individual microalgal species as well as to microalgal communities (e.g., Bérad et al., 2003; Devilla et al., 2005; Gatidou and Thomaidis, 2007; Buma et al., 2009; Sjollema et al., 2014). Negative effects of diuron, used in agriculture and antifouling paints, on individual microalgal species and communities have also been described (e.g., Gatidou and Thomaidis, 2007; Magnusson et al., 2008; Knauert et al., 2009; Pesce et al., 2010;
Sjollema et al., 2014). From these studies it can be concluded that Irgarol is in general more toxic to microalgae compared to diuron (Gatidou and Thomaidis, 2007; Sjollema et al., 2014), which was confirmed by the present study. However, the majority of the toxicity tests of Irgarol and diuron are performed under standardized laboratory conditions, using fluorescent tubes as a light source. As the present study demonstrated that irradiance conditions affect the toxicity of these herbicides, the question remains how these reported toxicity levels relate to the actual conditions affect the toxicity of these herbicides, the question remains how these reported toxicity levels relate to the actual conditions under field conditions. The observed short term seasonal-specific effect on \( \Phi_{PSII} \) of these herbicides are indicative for the ecological relevant chronic effect on growth (Magnusson et al., 2008; Buma et al., 2009) and must therefore be taken into account when determining toxic pressure of contaminants in aquatic and marine ecosystems.

Although the differences in toxicity between seasons are relatively small in the present laboratory study, the observations on single herbicides are corroborated by their joint effects under different irradiance regimes. Yet, our observations urge to develop more insight in field relevant irradiance conditions. Algae in the field are exposed to a complex and dynamic light regime, using fluorescent tubes as a light source. As the present study demonstrated that irradiance conditions affect the toxicity of these herbicides, the question remains how these reported toxicity levels relate to the actual risk of these compounds under field conditions. The observed short term seasonal-specific effect on \( \Phi_{PSII} \) of these herbicides are indicative for the ecological relevant chronic effect on growth (Magnusson et al., 2008; Buma et al., 2009) and must therefore be taken into account when determining toxic pressure of contaminants in aquatic and marine ecosystems.

The toxic pressure of herbicides in the field will mainly depend on the timing of (1) concentrations of the compounds, (2) presence of algae, and (3) the irradiance spectrum and total light intensity. Additional multi-stress factors which might interfere with the toxicity of the herbicides are nutrient limitation (Hall et al., 1989; Guasch et al., 2004) and temperature (Chalifour and Juneau, 2011). A combination of timing as well as the presence of additional multi-stress factors will determine the herbicide toxicity in the field, while the ultimate effect on the ecosystem will also depend on the ability of the microalgae to recover from damage by solar radiation (Janknegt et al., 2009b) and/or contaminants (Buma et al., 2009; Magnusson et al., 2012). Therefore, standard toxicity test may over- or underestimate the toxic effect when performed under controlled laboratory conditions, thereby misjudging the potential hazard of these compounds in the field.

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