Doctoral Thesis

Impact of acclimation and exposure to ultraviolet radiation on the toxicity of pollutants to Chlamydomonas reinhardtii

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Impact of acclimation and exposure to ultraviolet radiation on the toxicity of pollutants to *Chlamydomonas reinhardtii*

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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Summary

In order to protect aquatic ecosystems from the adverse effects of chemical pollutants it is important to understand the factors that can influence their impact on organisms in the environment. Aquatic organisms, such as microalgae, are not only exposed to chemical pollutants, but also to additional environmental stressors, such as ultraviolet radiation (UVR). In combination, chemical and environmental stressors cause so-called multiple stressor effects. Effects resulting from combined exposure to multiple stressors might be predicted from the known effects of each individual stressor. However, multiple stressor effects might also be higher (synergism) or lower (antagonism) than expected, depending on the stressor combination. Stressor effect interactions are believed to be one reason for the limited predictability of multiple stressor effects. Moreover, organisms can acclimate to stressful environmental conditions which may increase or decrease (co-tolerance) their sensitivity toward chemical pollutants and thus change multiple stressor effects.

The aim of this thesis was to shed light on the mechanistic underpinnings of effect interactions between UVR and chemical stressors in aquatic photoautotrophic organisms, also considering UVR acclimation and its impact on chemical toxicity. To this end, exposure experiments were performed using the green alga Chlamydomonas reinhardtii.

Considering the general attenuation of UVR in exposure experiments with algal cultures and the resulting potential for overestimation of UVR sensitivities, in a first study a method was developed to allow for a more accurate estimation of the actual UVA and UVB intensities to which algae are in fact exposed. This method relies on the determination of extinction coefficients for UVA and UVB.

In another study, interactive effects of UVR and chemicals were examined by conducting single and combined exposure experiments. Model substances were selected based on the similarity or dissimilarity of their mode of action (MOA) to that of UVR. To detect effect interactions, experimental data were analysed according to the concept of independent action. Results showed that interactions not only depended on the chemical MOA, but also on the examined endpoint (growth and photosynthesis), the exposure concentration and the exposure duration. As expected, chemicals that affect photosynthesis similar to UVR were shown to interact with UVR effects on photosynthesis, but the type of interaction differed and was synergistic in case of Cd(II) and paraquat and antagonistic in
case of diuron. No interaction on photosynthesis was observed for S-metolachlor, which acts dissimilarly to UVR. However, effects of S-metolachlor and UVR on algal reproduction interacted synergistic. Together, this study indicates that for multiple stressor effects of UVR and chemicals neither a primary MOA of UVR, nor a primary molecular interaction can be assumed, but multiple toxicity pathways should be considered.

In a further study, it was found that *C. reinhardtii* can be acclimated to UVR. Long term pre-exposure experiments revealed temporal dynamics of both UVR effects on growth and photosynthesis and cellular content of UVR absorbing compounds and photosynthetic pigments. UVR acclimation was demonstrated by an increased tolerance of UVR pre-exposed algae toward higher UVR levels, which might in part be explained by the protective function of lutein. UVR acclimated algae were co-tolerant to the photosensitizer rose bengal but not to paraquat. This indicates that under the selected conditions, the dominant mechanism of UVR acclimation and co-tolerance was related to singlet oxygen defence that relies on non-enzymatic antioxidants, rather than the enzymatic antioxidant system which is the primary mechanism for paraquat tolerance.

Concluding, this work contributes to the understanding of multiple stressor effects of UVR and chemical pollutants and UVR induced co-tolerance toward chemicals. It is clear from this work that knowledge of a chemical mode of action provides insufficient grounds for the understanding and prediction of multiple stressor effects and co-tolerance. Instead, detailed knowledge of multiple toxicity and defense pathways is needed. Such pathways should be addressed in multiple stressor studies in order to advance our understanding and predictive capabilities for multiple stressor effects.
Zusammenfassung

Um die aquatische Umwelt vor den negativen Effekten von Schadstoffen schützen zu können, muss man diejenigen Faktoren verstehen, die deren schädlichen Auswirkungen auf die Umwelt beeinflussen. Wasserlebewesen, wie z.B. Mikroalgen, sind nicht nur chemischen Schadstoffen allein ausgesetzt, sondern zusätzlich auch weiteren Umweltstressoren, wie Ultraviolettstrahlung (UVR). In Kombination können chemische Stressoren und Umweltstressoren sogenannte Multiple-Stressor-Effekte verursachen. Die Effekte die aus einer kombinierten Exposition gegenüber mehreren Stressoren resultieren, können gegebenenfalls anhand der Effekte der einzelnen Stressoren erklärt werden, können aber je nach Stressor auch höher (synergistisch) oder niedriger (antagonistisch) sein. Interaktionen von Stressor-Effekten werden als ein Grund für die begrenzte Vorhersagbarkeit von Multiple-Stressor-Effekten angesehen. Darüber hinaus können sich Organismen auch an widrige Umweltbedingungen akklimatisieren was unter Umständen ihre Sensitivität gegenüber chemischen Schadstoffen erhöhen oder vermindern (Ko-Toleranz) kann.


In Anbetracht der unvermeidlichen UVR-Attenuation in Algen Experimenten und der daraus resultierenden Möglichkeit die UVR-Sensitivität der Algen zu überschätzen, wurde in einer ersten Studie eine Methode entwickelt die eine genauere Bestimmung der UVR-Intensität zulässt welcher Algen tatsächlich ausgesetzt sind. Diese Methode beruht auf der Bestimmung von Extinktionskoeffizienten für UVA und UVB.

In einer weiteren Studie wurden die Effekt-Interaktionen von UVR und Chemikalien untersucht, indem Stressoren einzeln und in Kombination getestet wurde. Die verwendeten Modell-Substanzen wurden basierend auf der Ähnlichkeit oder Unterschiedlichkeit ihrer Wirkungsweise (MOA, von engl. *mode of action*) im Vergleich zur Wirkungsweise des UVR ausgewählt. Um Effekt-Interaktionen zu bestimmen wurden die experimentellen Daten auf Grundlage des Konzeptes der Unabhängigen Wirkung analysiert. Die Ergebnisse zeigen, dass Interaktionen nicht nur auf der Wirkungsweise
der Chemikalien beruhen, sondern auch auf dem untersuchten Endpunkt (Wachstum und Photosynthese), der Expositions konzentration sowie der Expositions dauer. Wie vermutet, interagierten die Effekte von Chemikalien welche, ähnlich wie UVR, Photosynthese beeinflussen, aber die Art der Interaktion war unterschiedlich; synergistisch im Fall von Kadmium und Paraquat und antagonistisch im Fall von Diuron. Keine Interaktion ergab sich für S-Metolachlor, welches nicht ähnlich wie UVR wirkt. Indes interagierten die Effekte von S-Metolachlor und UVR auf das Algenwachstum synergistisch. Zusammen genommen weist diese Studie darauf hin, dass für Multiple-Stressor-Effekte von UVR und Chemikalien weder eine primäre Wirkungsweise von UVR, noch eine primäre molekulare Interaktion angenommen werden kann, sondern dass mehrere Toxizitätspfade in Betracht gezogen werden sollten.


Chapter 1:

Introduction
1.1 General introduction

Environmental factors, such as temperature, acidity or ultraviolet radiation (UVR) can modulate the fate and effects of chemical pollutants in aquatic ecosystems. Under stressful environmental conditions, effects of environmental and chemical stressors may jointly cause so-called multiple stressor effects. Organisms can acclimate to environmental stressors which can change multiple stressor effects due to altered sensitivity toward chemical stressors. Corresponding mechanisms are not well understood and conceptual models often fail to predict the net impact of multiple stressors on aquatic biota. Thus, mechanistic approaches are needed to increase our understanding of multiple stressor effects in order to develop predictive capabilities and risk-mitigation strategies. This work addresses the impact of exposure and acclimation to UVR on chemical toxicity in the model green alga *Chlamydomonas reinhardtii*. In a first study, multiple stressor effects of UVR and model chemical stressors were examined. Then also the impact of UVR acclimation on chemical toxicity was examined.

1.2 Multiple stressor effects

Environmental factors can modulate chemical toxicity on the exposure level and on the effect level. For example, acidification or UVR can alter the bioavailability of toxic metals in aquatic systems through modification of toxicant binding organic ligands (Clements et al., 2008; Schwarzenbach et al., 2005). Changes in temperature or nutrient availability may affect toxicokinetic parameters like uptake, biotransformation and elimination of toxicants, that can result in higher internal toxicant concentrations and toxic effects (Heugens et al., 2001). Lastly, even in cases where two organisms contain the same amount of toxicant, stressful environmental conditions may influence an organism’s sensitivity toward the toxicant and vice versa. In this case, the environmental factor causes an additional stress that interacts with toxicant induced stress. The resulting multiple stressor effect may thus be larger (synergism) or smaller (antagonism) than predicted by reference models (see chapter 1.5) based on the effects of the individual stressors. It is assumed that such physiology-based interactions, or simply effect interactions, result from interaction on the toxicodynamic phase (effects on receptor, cellular target, defense, energy budget) (Fischer et al.,
2013b; Holmstrup et al., 2010; Segner et al., 2014). For example, the metabolic cost of osmoregulation under increased salinity can reduce an organism's fitness and tolerance toward toxicant stress (Heugens et al., 2001). Depletion of cellular defence capacities by one stressor may render an organism more sensitive toward another stressor which induces a similar stress. This was demonstrated in *C. reinhardtii*, where the effects of high light and herbicides, which both strongly affect photosynthesis and induce photooxidative stress, interacted synergistically in some cases (Fischer et al., 2010). These examples underline our limited understanding of the mechanisms of stress interactions and indicate the need for mechanism-based multiple stressor studies. In this PhD work, effect interactions of UVR and model chemical stressors were examined.

1.3 Influence of stress acclimation on multiple stressor effects

Organisms and communities can acquire tolerance toward environmental stressors during prolonged exposure which may impact on their sensitivity toward other stressors, including chemical pollutants (Fischer et al., 2013a; Vinebrooke et al., 2004). Stress tolerance can be acquired through selection of sensitive genotypes (adaptation), species (community acclimation) and through activation of physiological and biochemical tolerance mechanisms (acclimation) in individual organisms (Horowitz, 2001; Vinebrooke et al., 2004). Reactive oxygen species (ROS) production is stimulated by a variety of environmental stressors, including UVR, and ROS are implicated as important signalling components in acclimation (Pastori and Foyer, 2002). The sensitivity of acclimated organisms and communities toward other stressors may be unaffected, increased or decreased (co-tolerance), depending on the correlation of sensitivity toward the different stressors (Vinebrooke et al., 2004). Increased sensitivity may result from a fitness trade-off in tolerating multiple stressors, e.g. when metabolic costs associated with the acclamatory response render an organism more sensitive toward other stressors (Clements et al., 2008; Kashian et al., 2007; Larcher, 1995). Co-tolerance may be induced if tolerance mechanisms activated in response to one stressor also protect organisms against other stressors. Some of the mechanisms of co-tolerance between different environmental stressors (Pastori and Foyer, 2002) and between groups of toxicants (Blanck, 2002) are established. For
example, co-tolerance can be expected between chemicals with similar structure or similar MOA (Blanck, 2002). However, relatively few studies have examined co-tolerance between environmental and chemical stressors in photoautotrophic organisms (Table 1.1) and little is known about the underlying mechanisms. Some of the stressors for which co-tolerance was detected are known inducers of oxidative stress, such as cadmium, paraquat and UVB, indicating that the induction of antioxidant defenses during acclimation may be one important mechanisms of co-tolerance. Thus, in the scope of this thesis, we adopt the assumption that co-tolerance between environmental and chemicals stressors can be expected for similarly acting stressors. In the case of UVR, this may apply to those chemicals which induce oxidative stress.

Table 1.1: Examples of induced co-tolerance between environmental and chemical stressors in photoautotrophic organisms.

<table>
<thead>
<tr>
<th>Stressor in pre-exposure</th>
<th>Stressor in challenge exposure</th>
<th>Test system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat</td>
<td>Cadmium</td>
<td><em>Oryza sativa</em></td>
<td>(Chao and Kao, 2010)</td>
</tr>
<tr>
<td>UVR</td>
<td>Cadmium</td>
<td>Periphytic algae</td>
<td>(Navarro et al., 2008)</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herbicide</td>
<td>Cold</td>
<td><em>Chloroella ellipsoidea</em></td>
<td>(Clare et al., 1984)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>UVB</td>
<td><em>Anabaena doliolum</em></td>
<td>(Bhargava et al., 2007)</td>
</tr>
</tbody>
</table>

1.4 Ultraviolet radiation as an environmental stressor for phototrophic organisms in the aquatic ecosystem

UVR is part of the solar radiation that naturally occurs in the aquatic ecosystems and is regarded as an environmental stressor (Häder et al., 2011). Both *in situ* and laboratory studies have demonstrated a negative impact of UVR on organisms from all trophic levels including vertebrates (Blazer et al., 1997), macroinvertebrates (Clements et al., 2008), zooplankton (Leech and Williamson, 2001), and periphytic and planktonic microalgae (Bothwell et al., 1994; Montero et al., 2002; Navarro et al., 2008; Navarro et al., 2007), including *C. reinhardtii* (Hessen et al., 1995; Scott et al., 1999). Due to its higher energy, UVB (280-320nm) is generally more deleterious than UVA (320-400 nm) (Diffey, 2002). Depletion of stratospheric ozone and concomitant increase of UVB that reaches the earth surface has thus raised concerns about the impact on aquatic
ecosystems (Häder et al., 2011). In aquatic ecosystems, the actual UVR exposure of organisms depends strongly on the attenuation characteristics of the water column, which is mainly influenced by dissolved organic matter (DOM) (Morris et al., 1995), particles (Armengol et al., 2003) and organisms, such as phytoplankton (Kirk, 1994). Natural and anthropogenic induced changes in climate and acidification that reduce DOM concentrations are considered to influence UVR exposure of aquatic organisms more strongly than the loss of stratospheric ozone (Pienitz and Vincent, 2000; Williamson et al., 1996). Attenuation of UVR is often considered in ecological studies of UVR, but rarely in ecotoxicological studies; thus limiting the comparability of results on the sensitivity of test organisms to UVR. In the course of the research for this thesis, a method was developed that allows the determination of UVR attenuation in algal cultures.

1.4.1 Effects of UVR on algae

Direct and indirect effects of UVR on algae have been intensely studied because of the great importance of algae as primary producers in the aquatic ecosystem. UVR may indirectly affect algae e.g. by affecting nutrient availability (Anusha and Asaeda, 2008) or grazing pressure (Bothwell et al., 1994). The most important direct UVR target in algae is photosynthesis and the inhibitory effects of UVR on photosynthetic activity and carbon fixation have been extensively described (Franklin et al., 2003; Kataria et al., 2014; Villafane et al., 2003). UVR can also affect a range of other processes in cyanobacteria and algae, including C. reinhardtii, and thus impact on survival, growth, nutrient uptake, motility and orientation, as well as cell differentiation (He et al., 2002; Hessen et al., 1995; Holzinger and Lutz, 2006; Palmer et al., 2002; Scott et al., 1999). At the cellular level, UVR causes toxic effects due to direct interaction with a wide range of biomolecules, or indirectly through the generation of toxic reactive oxygen species (ROS), which are considered key components of UVR toxicity (Franklin et al., 2003; He and Hader, 2002; Vincent and Neale, 2000). UVR absorption by nucleic acids, amino acids or unsaturated fatty acids can directly damage DNA, proteins and membranes, thus inhibiting vital physiological processes (Buma et al., 2003; Vincent and Neale, 2000). For instance, the light reaction of photosynthesis is inhibited through UVR damage at different sites. At the photosynthetic apparatus, UVR primarily damages photosystem (PS) II by interacting with both the water-splitting complex at the site of the D1 protein (electron donor site) and the quinone electron acceptors.
Additionally, photosynthetic pigments may undergo photooxidative degradation upon absorption of UVR (Vincent and Neale, 2000). Together, UVR damage to the photosynthetic apparatus and at other sites can increase the generation of ROS (Franklin et al., 2003; Vincent and Neale, 2000). ROS can also directly be generated when UVR is absorbed by chromophoric groups of biomolecules such as chlorophylls and quinones. The excitation energy can then be transferred to oxygen, thus forming singlet oxygen ($^{1}\text{O}_2$), or to other molecules, thereby forming radicals or including superoxide ($\cdot\text{O}_2^-$), which may subsequently give rise to other ROS including hydrogen peroxide ($\text{H}_2\text{O}_2$) or hydroxyl radicals ($\cdot\text{OH}$). Together, these ROS can react with and damage a wide range of biomolecules and cause toxic effects in algae (He and Hader, 2002; Mallick and Mohn, 2000; Vincent and Neale, 2000).

1.4.2 UVR defence mechanism in algae

Algae can tolerate UVR in their natural habitats without experiencing severe photooxidative damage due a combination of mechanisms tailored toward protection from enhanced radiation and oxidative damage and through repair or de novo synthesis of damaged biomolecules. Motile algae, such as C. reinhardtii, can avoid damaging UVR intensities by migration in the water column (Roy, 2000). Cellular mechanisms that protect algae from UVR involve the accumulation of UVR absorbing compounds (Navarro et al., 2007), such as mycosporine-like amino acids, flavonoids and carotenoids (Kováčik et al., 2010; Scott et al., 1999; Sinha et al., 1998). For example, secondary carotenoids have been shown to shield the chloroplast of the snow alga Chlamydomonas nivalis from UVR exposure (Remias et al., 2005). Also primary carotenoids in the photosynthetic apparatus protect algae from UVR induced damage by multiple mechanisms; either through scavenging of ROS or through quenching of excitation energy after absorption of UVR and after resonance transfer from other excited molecules, respectively (Franklin et al., 2003; Vincent and Neale, 2000). Other important components of the cellular ROS defense are non-carotenoid antioxidants including ascorbate, reduced glutathione and tocopherols, as well as enzymatic antioxidants (Mallick and Mohn, 2000). The latter either directly remove ROS or function to provide reduced antioxidants, e.g. in the ascorbate–glutathione cycle. Relevant enzymes are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and
glutathione-S-transferase (GST) (Gill and Tuteja, 2010; Mallick and Mohn, 2000). The accumulation antioxidants as well as UVR absorbing compounds can be induced in response to UVR, allowing algae to acclimate to higher UVR intensities (Lesser et al., 1996; Tian and Yu, 2009; Vincent and Roy, 1993). UVR damage that occurs despite these protection mechanisms needs to be efficiently repaired. For example, UVB tolerance of the photosynthetic apparatus in microalgae has been associated with a strong capacity for D1 turnover-mediated repair cycle (Xiong, 2001). DNA damage in C. reinhardtii is repaired by UVA/blue light-dependent DNA photolyase (Petersen et al., 1999).

1.5 UVR and chemical pollutants

As for other environmental factors, UVR may affect chemical toxicity on the exposure and on the effect level (Figure 1.1). For example, UVR can modify toxicant binding organic ligands, especially DOM, making the toxicant more bioavailable (Clements et al., 2008). UV-induced photochemical reactions can also affect the speciation and thus toxicity of metals (Zepp et al., 2007). UVR can also transform compounds into photo-transformation products which are either less toxic (Mestankova et al., 2011) or more toxic to algae than their corresponding parent compounds (Schmitt-Jansen et al., 2007). Other compounds, such as polycyclic aromatic hydrocarbons, can be toxic to organisms upon absorption of UVR photon energy and the subsequent production of molecular radicals and ROS (Diamond, 2003). UVR has also been shown to affect internalization of toxicants in algae. For instance, increased toxicity of copper in C. reinhardtii was explained as being a consequence of membrane disruption caused by high UVR and concomitant increase in copper internalization (Cheloni et al., 2014).

While the impact of UVR on the exposure level is fairly well established, relatively little is known about the interaction of UVR and chemicals on the effect level. Exposure of Euglena gracilis to UVR or the ROS generating herbicide paraquat individually resulted in oxidative lipid damage that was strongly increased when algae were exposed simultaneously and diminished in the presence of a ROS scavenger (Palmer et al., 2002). In another study, synergistic effects of UVR and tributyltin on phytoplankton assemblages were suggested to result from similar damaging effects on PSII of the individual stressors (Sargian et al., 2005). These studies suggest that certain chemicals, in particular those which display a MOA that is similar to the MOA of UVR, e.g. through action on similar cellular targets and/or the generation of ROS, may
interact synergistically with UVR on the effect level. However, experimental evidence to support this assumption is scarce and also conflicting results have been produced. For instance, the effects of cadmium interacted synergistically with UVR effects in one study, and antagonistically in another (Prasad and Zeeshan, 2005; Singh et al., 2012). Thus, there is a need to systematically study interactive effects of UVR and chemical pollutants, considering different modes and mechanisms of toxic action, in order to increase our understanding of the mechanisms underlying multiple stressor effects.

Figure 1.1: Possible ways how UVR can affect chemical exposure and effects in aquatic algae such as *C. reinhardtii*. Depicted are the parent chemical (A or $^3A$ to indicate its being in the energetic ground state), transformation products (B and C), toxicant binding ligand (X) and reactive oxygen species (ROS).

1.5.1 UVR induced co-tolerance to chemical stressors

Because algae can induce several physiological and biochemical defence mechanisms during acclimation to UVR stress, it is important to assess the impact of UVR acclimation on the toxicity of chemical pollutants. However, only a few studies examined the co-tolerance between UVR and chemicals in phototrophic organisms. Co-tolerance may be expected if mechanisms that confer tolerance toward UVR also provide protection against the toxic effects of chemicals. Co-tolerance was demonstrated in periphytic algae after a prolonged pre-exposure to UVR that resulted in increased tolerance of the algal community to higher UVR levels and also to
cadmium. (Navarro et al., 2008). The mechanisms of this co-tolerance were not examined. In another study, pre-exposure of the cyanobacterium *Anabaena* to cadmium induced UVB tolerance, and was explained by a cadmium induced antioxidant enzyme activity (Bhargava et al., 2007). Thus, co-tolerance observed by Navarro et al. (2008) might have been due to a similar induction of antioxidative mechanisms during the pre-exposure, in that case by UVR. In the scope of this work, UVR induced co-tolerance is assumed for chemicals which cause toxic effects through mechanisms similar to those of UVR because for such chemicals UVR induced protection mechanisms are also expected to be effective.

### 1.6 Analysis of multiple stressor effects

Multiple stressor effects can be analyzed on the basis of the same conceptual reference models that are used to analyze the effects of chemicals in mixtures (Table 1.2). The question of effect interaction can be approached by comparing the model prediction of combined effects with experimental data. In case of a match, no-interaction of effects is assumed. In case of larger or smaller than predicted combined effects, synergistic and antagonistic interaction of stressor effects, respectively, is assumed. Since different models may predict different combined effects, conclusions about interaction of stressor effects can also depend on the model selection (Hertzberg and MacDonell, 2002). In the case of chemical mixtures, the selection of the appropriate reference model is usually based on whether chemicals are acting similarly or dissimilarly. Whether this applies also to combinations of environmental stressors, such as UVR, and chemicals stressors is unclear.

<table>
<thead>
<tr>
<th>Model</th>
<th>Effect summation</th>
<th>Concentration addition</th>
<th>Independent action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanistic basis</td>
<td>No</td>
<td>Similar action</td>
<td>Dissimilar action</td>
</tr>
<tr>
<td>Equation</td>
<td>$E_{\text{total}} = E_A + E_B$ (^1)</td>
<td>$TU_{A,B} = TU_A \cdot TU_B = 1$ (^2)</td>
<td>$E_{\text{total}} = E_A + E_B - E_A \cdot E_B$ (^3)</td>
</tr>
</tbody>
</table>

\(^1\) Effects of stressor A ($E_A$) and stressor B ($E_B$) jointly produce a combined effect ($E_{\text{total}}$) toxic unit (TU) = concentration of chemical A divided by the respective effect concentration that is to be predicted (e.g. EC50)

\(^2\)
The effect summation (ES) model assumes that combined stressor effects equal the sum of effects caused by individual stressors. Although this concept has been frequently used, its applicability to chemical mixture and multiples stressor studies has been questioned. The ES model does not consider chemical MOAs and may predict combined effects greater than 100%. Moreover, ES requires a linear relationship between stressor intensity and effects which does not hold for most chemical and environmental stressors due to their often non-monotonic and non-linear dose response relationship, respectively (Berenbaum, 1989; Coors and De Meester, 2008).

In contrast, the concentration addition (CA) (Loewe and Muischnek, 1926) and the independent action (IA) model (Bliss, 1939) are based on pharmacological principles and are frequently applied when analyzing the joint effects of chemicals in mixtures. The basic assumption for CA is that, in a mixture, a chemical can be replaced by an equitoxic concentration of another chemical without affecting the overall toxicity, provided that the chemicals are acting similarly. In a strict sense, CA applies only to chemicals acting competitively on the same molecular target site (Pöch, 1993) but is also widely accepted for chemicals with similar modes of action (Cedergreen et al., 2008). The CA model operates with effect concentrations, which may limit its application to environmental stressors. Still, CA has been applied to combinations of chemical and non-chemical stressors in a toxic unit approach (Cleuvers et al., 2002; Fischer et al., 2010). The IA model on the other hand operates with effect levels and is suggested to apply to dissimilar-acting toxicants (Boedeker et al. 1992). It is based on probability theory of independence, assuming that the effect of one toxicant does not affect the probability for effects by another toxicant. This is expected if toxicants act on different cellular targets (Cedergreen et al., 2008). IA has been recently applied also to multiple stressors (Coors and De Meester, 2008; Fischer et al., 2012; Jensen et al., 2009; Long et al., 2009; Ribeiro et al., 2011) and very few authors have applied and compared both IA and CA (Cleuvers et al., 2002; Fischer et al., 2010; Pestana et al., 2009). In two studies in which both CA and IA were applied, IA either better or equally well predicted multiple stressor effects (Cleuvers et al., 2002; Pestana et al., 2009), suggesting that the examined environmental and chemical stressors acted independently, and that in some cases the difference between CA and IA might be negligible. In one study in which multiple stressor effects of high light and photosynthetic herbicides were examined, neither IA, nor CA, nor ES applied (Fischer et al., 2010). Fischer et al. (2010) suggested that in cases where environmental and
chemical stressors cause similar physiological effects, such stressors might neither act independently nor behave as if having exactly the same target site as assumed for CA. Still, even though the existing reference models many not generally apply to multiple stressors, they are useful tools for hypothesis driven multiple stressor studies. It was suggested that the IA model may be useful to indicate mechanisms underlying interactions of multiple stressor effects (Fischer et al., 2010; Jensen et al., 2009). In this work, the IA model was applied to analyze multiple stressor effect of UVR and chemicals with different MOAs and distinct mechanisms of toxic action.
1.7 Scope of the thesis

The overall aim of this work was to provide an insight into the mechanisms underlying multiple stressor effects of UVR and chemicals in algae. The basic approach was to start at the simplest level by examining stressors individually and then stepwise increase the complexity of the experimental system by combining UVR and chemical in simultaneous exposure experiments and by allowing for UVR defence mechanisms to impact on chemical toxicity in sequential exposure experiments. To provide for a mechanistic insight, chemical stressors were selected to cover a range of different modes and mechanisms of toxic action that were assumed to be more or less similar to those of UVR.

In a first study (chapter 2) effects of model chemical stressors on *C. reinhardtii* in absence or presence of UVR were examined. Tested chemicals were paraquat, diuron, cadmium and *S*-metolachlor (see Chapter 1.7). Experimental data were analysed according to the concept of IA to indicate antagonistic or synergistic interaction of chemical and UVR effects. The experimental design allowed for an examination of effect interactions depending on the chemical’s MOA, different endpoints (growth and photosynthesis), increasing exposure time and chemical exposure concentrations. From these results possible mechanisms of effect interactions were discussed.

The second objective (chapter 3) was to examine mechanisms of UVR acclimation and the impact of acclimation on the sensitivity of *C. reinhardtii* to model chemicals. In this study, tested chemicals were paraquat, diuron and rose bengal (see Chapter 1.7). Algae were first acclimated to UVR by pre-exposing cultures over several days to UVR. Subsequently, UVR pre-exposed and unexposed control cultures were tested for their tolerance toward higher UVR to examine UVR acclimation and to chemicals to examine co-tolerance. Tested chemicals were selected to display a similar mode of action to UVR (inhibition of photosynthesis) but different mechanisms of toxicity. Thus, co-tolerance to one or several of the tested chemicals can inform indirectly about mechanisms of UVR defence and co-tolerance.

In another study (chapter 4), considering the UVR attenuation in exposure experiments with algal cultures, a method was developed that allows the calculation of the UVR dose algae receive in cultures depending on cell density and culture depth.
1.8 Model chemical stressors

In this thesis, interactions of UVR and chemical effects and co-tolerance were examined. Knowledge of the mechanisms of toxic action of UVR and chemicals were used to infer the mechanisms underlying effect interactions and co-tolerance. Inhibition of photosynthesis was assumed to be the main MOA of UVR. ROS mediated UVR damage was assumed to be one of the main toxicity mechanisms underlying this MOA. Mechanisms relevant for UVR acclimation were thus assumed to be related to the prevention of ROS formation and scavenging of ROS, respectively. Model chemicals that were tested in simultaneous or sequential exposure experiments were selected based on their mode and mechanism of action. Effects of chemicals displaying a similar mode of action compared to the MOA of UVR were expected to interact with UVR effects. Co-tolerance of UVR acclimated algae was expected for chemicals which induce effects through the same or similar mechanisms as UVR. The effects of such chemicals were expected to be mitigated by the defense mechanisms that were induced during UVR acclimation. Thus, the following model chemicals were tested in this work (Figure 1.2):

Paraquat (1,1'-dimethyl-4,4'-bipyridylium), also known as Methyl viologen, belongs to the class of bipyridinium herbicides and is an often used model chemical for the study of oxidative stress. Paraquat’s main MOA in plants and algae is the inhibition of photosynthesis. The toxic mechanism involves the transfer of electrons from PSI to molecular oxygen, resulting in the formation of $O_2^-$ (Farrington et al., 1973).

Rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) is frequently used as a model substance for the study of $^{1}O_2$. Rose bengal (RB) is a type II photosensitizer that can absorb UVR or PAR and transfer the excitation energy to molecular oxygen, resulting in the formation of $^{1}O_2$ (Allen et al., 1991; Fischer et al., 2004). $^{1}O_2$ is a strong oxidant species and can directly react with biomolecules, thus damaging e.g. the photosynthetic apparatus, and cause cell death(Krieger-Liszkay et al., 2008).

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) belongs to the phenylurea herbicides. The MOA of diuron (DR) is the inhibition of photosynthesis through blocking of photosynthetic electron flow at PSII. DR binds at the Q$_B$ binding niche of the D1 protein in PSII and thereby interrupts the charge transfer to from Q$_A$ to Q$_B$. Depending on the light conditions, DR may also induce the generation of ROS in
chloroplasts (Hess, 2000), but the potential of DR to cause oxidative damage is much lower compared to PQ and RB (He and Häder, 2002).

**Cadmium** (Cd) is a toxic nonessential heavy metal which enters the aquatic environment from natural and anthropogenic sources (Cullen and Maldonado, 2013). Unlike the selected herbicides, Cd does not have a specific MOA because it binds to various biomolecules, affecting their confirmation and function (Faller et al., 2005). Cd thus inhibits a range of physiological function including photosynthesis and can induce oxidative stress in algae, possibly due to inhibition of antioxidant enzymes (Szivak et al., 2009).

**S-metolachlor** (2-Chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl]-acetamide) belongs to the group of chloroacetanilide herbicides (Böger et al., 2000). Its main MOA is the inhibition of cell division. S-metolachlor inhibits the FAE1-synthase that is required for the elongation of fatty acids (Gotz and Boger, 2004). The resulting lack of very long chain fatty acids (VLCFAs) affects membranes functionality and cell rigidity. Since S-metolachlor does not specifically inhibit photosynthesis is used as chemical stressor that acts dissimilar compared to UVR.

**Figure 1.2:** Structures of chemical stressors used in the present study. Paraquat (CAS 1910-42-5), rose bengal (CAS 11121-48-5), diuron (CAS 330-54-1), cadmium and S-metolachlor (CAS 87392-12-9). Chemical structures retrieved from http://www.sigmaaldrich.com and Wikipedia (public domain).
1.9 References


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Chapter 2:

Multiple stressor effects in *Chlamydomonas reinhardtii* - toward understanding mechanisms of interaction between effects of ultraviolet radiation and chemical pollutants

Based on a version of the following published article:

2.1 Abstract

The effects of chemical pollutants and environmental stressors, such as ultraviolet radiation (UVR), can interact when organisms are simultaneously exposed, resulting in higher (synergistic) or lower (antagonistic) multiple stressor effects than expected based on the effects of single stressors. Current understanding of interactive effects is limited due to a lack of mechanisms based multiple stressor studies. It has been hypothesized that effect interactions may generally occur if chemical and non-chemical stressors cause similar physiological effects in the organism. To test this hypothesis, we exposed the model green alga *Chlamydomonas reinhardtii* to combinations of UVR and single chemicals displaying modes of action (MOA) similar or dissimilar to the impact of UVR on photosynthesis. Stressor interactions were analysed based on the Independent Action model. Effect interactions were found to depend on the MOA of the chemicals, and also on their concentrations, the exposure time and the measured endpoint. Indeed, only chemicals assumed to cause effects on photosynthesis similar to UVR showed interactions with UVR on photosynthetic yield: synergistic in case of Cd(II) and paraquat and antagonistic in case of diuron. No interaction on photosynthesis was observed for S-metolachlor, which acts dissimilarly to UVR. However, combined effects of S-metolachlor and UVR on algal reproduction were synergistic, highlighting the importance of considering additional MOA of UVR. Possible mechanisms of stressor effect interactions are discussed.

2.2 Introduction

Environmental factors can influence the fate and effect of chemical pollutants in the aquatic environment. Changes in environmental factors such as salinity, pH, or ultraviolet radiation (UVR) can influence the bioavailability of toxicants and thus their toxicity towards aquatic organisms (Clements et al., 2008; Rand, 1995). Changes in temperature or nutrition can influence toxicokinetik processes such as uptake or elimination of toxicants (Heugens et al., 2001). Under stressful environmental conditions, such as extreme temperatures, strong predation pressure, or increased UVR, effects of environmental and chemical stressors may jointly cause multiple stressor effects (Eggen et al., 2004; Fischer et al., 2012). Interaction of stressor effects can result in higher (synergism) or lower (antagonism) multiple stressor effects than
predicted by reference models on the basis of the effects of individual stressors, as demonstrated in numerous studies (Crain et al., 2008; Holmstrup et al., 2010). This clearly shows that current conceptual models lack the prediction capacity for interactive multiple stressor effects which is routed in an incomplete mechanistic understanding of effect interactions. It was suggested that effect interactions might occur especially if chemical and non-chemical stressors affect similar physiological processes (Fischer et al., 2013), either through partially similar toxic mechanisms (Fischer et al., 2010) or through action on different but dependent pathways (Crain et al., 2008).

UVR is one stressor which might interact with chemical stressors, causing interactive multiple stressor effects in the aquatic environment. Such interactions might be especially important for photosynthetic organisms, such as algae. Much is known about photoactivated toxicity resulting from direct interaction of UVR and chemicals (Diamond, 2003). However, relatively little is known about the interaction of UVR and chemicals on algal physiology. Absorption of UVR can damage important biomolecules such as proteins, lipids, DNA and pigments directly but also indirectly due to UVR-induced generation of reactive oxygen species (ROS), resulting in the impairment of physiological functions in algae (Cheloni et al., 2014; Häder et al., 2011; Vincent and Roy, 1993). Among the physiological functions, photosynthesis is one of the most sensitive targets and various molecular target sites of the photosynthetic apparatus can be affected by UVB and UVA (He and Hader, 2002; Holzinger and Lutz, 2006; Vass et al., 2005).

Studies that have so far examined combined effects of UVR and different chemicals in aquatic algae and cyanobacteria (Cheloni et al., 2014; Pandey and Rai, 2002; Prasad and Zeeshan, 2005; Sargian et al., 2005; Singh et al., 2012) give limited insight into the mechanisms underlying effect interactions and do not allow the establishment of whether synergistic interactions generally depend on the similarity in the MOA of both stressors. For example, synergistic effects in combined exposures of phytoplankton assemblages to tributyltin and UVB were explained by similar damaging effects on photosystem II (PSII) of the individual stressors (Sargian et al., 2005). Conflicting results were reported for the interaction of effects of UVR and cadmium (Cd(II)), which were synergistic in one study and antagonistic in another (Prasad and Zeeshan, 2005; Singh et al., 2012). These conflicting results might be explained by differences in exposure conditions but also by species specific differences in response to UVR and
Cd(II). Effects of copper and UVR on reproduction of *Chlamydomonas reinhardtii* interacted antagonistically at lower copper and UVR levels, but synergistically at higher UVR levels, indicating that interaction of effects might also depend on stressor levels (Cheloni et al., 2014). In the aforementioned studies, effects of UVR and chemicals were expected to accumulate so as to produce the arithmetic sum of individual effects, which is the basic assumption of the *effect summation* model. However, this model does not have a mechanistic basis and has been criticised for lacking a biological rationale (Fischer et al., 2013; Hertzberg and MacDonell, 2002). More recently, two mechanism based concepts that are usually applied to assess chemical mixture toxicity have also been applied to multiple stressors (Cleuvers et al., 2002; Coors and De Meester, 2008; Fischer et al., 2012; Fischer et al., 2010; Pestana et al., 2009). The *concentration addition* (CA) model (Loewe and Muischnek, 1926) operates with toxic units and assumes that any chemicals in a mixture can be replaced by an equitoxic concentration of another chemical, without changing the toxicity of the mixture, provided both chemicals share the same molecular target site or MOA (Cedergreen et al., 2008). The alternative concept of *independent action* (IA) (Bliss, 1939) is usually applied to mixtures of dissimilarly acting chemicals which are assumed to exert their effects in a statistically independent fashion (Cedergreen et al., 2008). The IA model operates with effect levels and can be directly applied to combinations of chemical and non-chemical stressors, and in contrast to CA, even in the absence of established dose-response relationships (Cleuvers et al., 2002; Fischer et al., 2010). Still, it is largely unknown to what extent these concepts can be applied to combinations of chemical and non-chemical stressors since only a few studies have applied these reference models.

The aim of this study was to shed light on the interaction of UVR and chemical effects in a structured, mechanism-based approach. To do so, we exposed the green alga *Chlamydomonas reinhardtii* to different levels of model chemicals in the absence and presence of a single fixed UVR intensity and assessed effects in a time-dependent manner. Experimental data were analysed according to the IA model. The application of CA was not possible because the required effect concentration equivalents for UVR cannot be determined from single UVR dose experiments (Cleuvers et al., 2002; Fischer et al., 2010). Chemical stressors were selected considering their MOA and their similarity to the MOA of UVR. Additionally, model chemicals were selected to also display different mechanisms of toxic action to enable us to draw conclusions about
possible mechanisms of effect interactions. Thus, the herbicides paraquat (PQ) and diuron (DR) were selected because they both inhibit photosynthesis by specifically acting on the photosynthetic apparatus and, in the case of paraquat, is known to strongly induce the generation of ROS. Cd(II) was selected because it can inhibit photosynthesis, but acts rather unspecific and thus also has other targets which were assumed to be less similar to UVR targets. The herbicide S-metolachlor was selected as a chemical with a dissimilar MOA to UVR: it inhibits algal cell division and does not specifically inhibit photosynthesis. Thus, based on IA, interactive effects were expected for UVR with PQ, DR and Cd(II), whereas UVR and S-metolachlor were expected to act independently.

2.3 Material and Methods

2.3.1 Alga strain and growth conditions
The wild type strain (CC125) of the unicellular freshwater alga *Chlamydomonas reinhardtii* (Chlorophyta) obtained from the Chlamydomonas Genetics Center (Durham, NC, USA), was used as a test organism. Algae were cultured axenically in glass Erlenmeyer flasks in the synthetic freshwater growth medium Talaquil at pH 7.5 (Le Faucheur et al., 2005) with NaNO₃ being substituted by NH₄Cl, since *C. reinhardtii* CC125 cannot utilize nitrate due to a lack of the nitrate reductase. Cultures were agitated at 100 rpm on a High Technology Infors shaker (Infors, Switzerland) and kept at 20°C under a 14:10 h light:dark cycle. Light was provided by cool-white fluorescent lamps (Radium Bonalux super, NL54W/840 white) at 100 ± 10 µmol m⁻² s⁻¹ photosynthetically active radiation (PAR). Algae were acclimated to the described conditions by using successive batch cultures.

2.3.2 UVR exposure setup and conditions
In the UVR exposure setup (Figure S1), algal cultures were exposed in 24-well cell culture plates (Cellstar®, Greiner Bio-One) at 20 °C in a circulating water bath (Lauda,
RC6, Germany). PAR was provided from above by dimmable cool-white fluorescent lamps (Radium Bonalux super, NL54W/840 white). UVR was provided by fluorescent lamps (ARIMED B, 40W, Cosmedico Light, Germany) which were selected based on their suitability for the simulation of solar UVR (Diffey, 2002) (Figure 2.1A). The UVR lamps were arranged above the plates and between the PAR lamps. The UVR intensity was adapted by changing the distance of the lamps to the culture vessels. PLEXIGLAS® (UV 100 0A570, 3mm, Börlin, Switzerland) served as a UVR filter for the control (-UVR) treatment. The resulting irradiation spectra (Figure 2.1A) were measured using a spectroradiometer (JAZ-EL 200, Ocean Optics, 830 Douglas Ave. Dunedin, FL, USA) and integrated for the radiant flux density (Watt/m²) in the range of UVB (280-320 nm) and UVA (320-400 nm) using the Ocean Optics Spectrasuite™ software.

For the UVR exposure, a radiant flux density of approximately 13 W/m² (UVA) and 0.5 W/m² (UVB) respectively at the culture surface was applied. This UVR intensity is comparable to ambient UVR levels and was selected because it partially inhibits reproduction and photosynthesis at levels which allow the detection of additional chemical effects. UVR filters in the control treatment decreased UVA radiation to 0.7 W/m² while UVB was below the detection limit. The UVR filter slightly reduced the PAR (less than 10%). It was adjusted to 100 µmol m⁻² s⁻¹ in the control plate which is in the range of the light intensity required in standard algal growth inhibition tests. For the exposure experiments, which lasted 48 h, UVR was applied twice for 10 h during the two 14 h light periods (Figure 2.1B), thus mimicking a natural irradiation pattern. Culture plates were shaken manually approximately every 2-3 h during the light period to achieve a more homogeneous irradiation of algae. Reproduction and photosynthesis of algae were determined during exposure to chemicals, UVR and the combined exposure to both stressors.

2.3.3 Chemical exposure experiments in absence or presence of UVR

Algae were exposed to increasing concentrations of chemicals alone (chemical-UVR) or in combination with UVR (chemical+UVR). Experimental cultures were prepared by harvesting algae from the pre-culture 2 h after the onset of light and by resuspending them in fresh medium to a culture density of approximately 2.5 x 10⁵ cells/ml in 24-well cell culture plates.
Chemical effects were determined in four independent biological replicates. Each replicate was prepared in duplicate with nine different chemical concentrations in 200 µl Talaquil medium each. Concentrations were selected based on preliminary range-finding experiments to range from 0 to 100 % effect on algal reproduction (see exposure concentration range in Table 2.2). Four wells were filled with 200 µL Talaquil medium without chemical and solvent and served as no-chemical controls on each plate. The remaining two wells were filled with 1 mL Talaquil to serve as blanks. Preliminary experiments showed that there were no significant effects on algal photosynthesis at the maximum solvent concentrations of 0.05% ethanol and 0.01% methanol (Figure S2). Thereafter, 800 µl of algal culture was added so that each well contained 1 mL of algal culture with approximately 2·10^5 cells/mL. Plates were covered with a lid to reduce evaporation and were immediately placed in the UVR exposure setup. A small culture volume was chosen to result in a small culture depth (approx. 4 mm) to minimise effects of algal culture density on the average UVR dose received by the cells (Navarro et al., 2014).

Figure 2.1 (see next page)
Figure 2.1: Experimental irradiation spectra in Ultraviolet radiation (UVR) exposure (dark area fill [+UVR]) and control condition (white area fill [-UVR]), compared to the standard solar radiation energy spectrum at the earth surface (G173-03) of the American Society for Testing Standards (grey area fill) (A) and exposure scheme (B) showing the duration of the dark:light and UVR exposure phases and the times when endpoints were measured. CN = Cell number; CV = Cell volume; PAR = Photosynthetic active radiation; $\Phi_{PSII}$ = Effective quantum yield of photosynthesis.

2.3.4 Chemicals

All chemicals were obtained from Sigma. Tested chemicals were, paraquat (CAS: 1910-42-5), diuron (CAS 330-54-1), S-metolachlor (CAS 87392-12-9) and cadmium dichloride (CAS 10108-64-2). Stock solutions were prepared using HPLC-grade solvents, methanol for diuron (20 mM), ethanol for S-metolachlor (25 mM), water for paraquat (0.5 mM) and cadmium (100 mM) and stored at 4°C in the dark. The speciation of cadmium in the exposure medium was calculated using the speciation software VMINTEQ (Gustafsson, 2011).

Control experiments were performed to ensure that differences in chemical toxicity in absence or presence of UVR is due to interaction of stressor effects and not due to direct interaction of UVR and the chemical (photomodified toxicity). First, to test the stability of chemicals at the applied exposure conditions, concentrations of the herbicides PQ, DR and S-metolachlor were quantified at the beginning and after 48 h in cell free medium which was either kept in the dark, exposed to PAR+UVR, or PAR-UVR, using high-performance liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS [Finnigan TSQ Quantum Ultra, Thermo Finnigan, CA]). Higher concentrations compared to those used in exposure experiments were used in case of paraquat and DR to account for expected detection limits of the LC-MS/MS system for these herbicides. Overall, herbicide concentrations were not significantly different in UVR exposed and unexposed samples after 48 h (Table S1). To further exclude the possibility that under the selected conditions, UVR transforms herbicides photo-chemically into more toxic photo-transformation products, we tested the toxicity of PQ (0.1 µM), DR (0.18 µM) and S-metolachlor (15.8 µM) which had been pre-exposed in Talaquil growth medium under the experimental conditions for 48 h in absence or presence of UVR, to algal photosynthesis. Pre-exposure of herbicides to
UVR did not alter the toxicity of herbicides to algal photosynthesis significantly (Figure S3).

2.3.5 Endpoint measurements and effect calculation

Algal reproduction and growth was determined by measuring cell number (CN) and cell volume using a CASY cell counter (model TT; Innovatis AG, Germany) operated in the size range of 3 to 18 µm, at the start and the end of the experiment (48 h) and by calculating the total doublings of cells over 48 h using Equation 1.

\[
\text{Algal reproduction} = \log_2 \left( \frac{CN_{48h}}{CN_{0h}} \right)
\]  

(1)

Photosynthesis was determined as the effective quantum yield (\(\Phi_{\text{PSII}}\)) which is a relative measure for the photochemical efficiency of PSII reaction centres under illuminated conditions and a sensitive parameter for stress induced by various environmental or chemical stressors (Nestler et al., 2012; Schreiber, 2004). \(\Phi_{\text{PSII}}\) was experimentally determined by the saturating pulse method using a Maxi-Imaging-PAM chlorophyll fluorometer (Walz, Germany) (Schreiber, 2004). \(\Phi_{\text{PSII}}\) was measured at 3, 7, 27 and 31 hours after the start of exposure (see Figure 2.1B) and after adaptation to an illumination of 82 µmol m\(^{-2}\) s\(^{-1}\) PAR for two minutes. Four to five saturation pulses were applied every 50 s and the maximum fluorescence \(Fm'\) and the steady-state fluorescence \(Fs\) were recorded. \(Fm'\) values were corrected by a factor of 1.05 based on manufacturer recommendations. A minimum of 3 measurements were averaged and \(\Phi_{\text{PSII}}\) was calculated according to (Genty et al., 1989) using Equation 2.

\[
\Phi_{\text{PSII}} = \frac{(Fm' - Fs)}{Fm'}
\]  

(2)

The cellular contents of the photosynthetic pigments chlorophyll \(a\) and total carotenoids were determined in UVR exposed and unexposed algae at the same time-points at which photosynthesis was measured. Algal pigments were extracted from three independently prepared and exposed algal cultures in 80% acetone and measured in triplicates in a photometric microplate assay as previously described (Nestler et al., 2012). The average ratios of total carotenoids to chlorophyll \(a\) were calculated from the ratios in each of the three replicates.
Effects of UVR and of chemicals in UVR exposed and unexposed cultures were calculated as per cent inhibition of algal reproduction and photosynthesis. First, the effects of UVR alone were determined by comparing reproduction and photosynthesis in control cultures (-chemicals/-UVR) and UVR exposed cultures (-chemicals/+UVR). Secondly, the effect of chemicals in absence and presence of UVR were calculated by comparing reproduction and photosynthesis in chemical exposed control cultures and in chemical exposed UVR cultures to their corresponding control cultures (-chemical/- or + UVR). This normalization step yields the chemical parts of the toxicity that can be directly compared for an analysis of effect interactions according to IA (see below) (Fischer et al., 2012). An alternative, but equivalent IA analysis requires the comparison of net combined effects in the combined exposure (calculated by normalizing effects to unexposed control cultures instead of UVR exposed control cultures) with those predicted by the IA model (see Figure S6 and S8) (Pöch et al., 1990).

2.3.6 Evaluation of stressor effect interactions and statistical analysis

The interaction of effects of chemicals and UVR were examined according to the concept of IA following (Fischer et al., 2012; Fischer et al., 2010), who showed that IA holds if the chemical part of toxicity is the same in a single and multiple stressor treatment. Thus, no-interaction between UVR and chemical effects is assumed in case that chemical effects in single exposures (normalized to unexposed controls) equal chemical effects in the combined exposures (normalized to UV exposed controls). For this, chemical effect data from single and combined exposures experiments were fitted to concentration response curves between 0% (bottom) and 100% effect (top) using the program Graph Pad Prism 4 (San Diego, CA, USA), and by applying a four parameter log logistic model with sigmoidal curve and variable Hill slope (hs) according to Equation 3 as previously described (Nestler et al., 2012):

\[ Y = bottom + \frac{top - bottom}{1 + 10^{(\log EC50 - X)/hs}} \]  

where \( Y \) = effect value and \( X \) = the logarithm of the chemical concentration. The program determines the Hill slope, and the EC50 (50% effect on the measured endpoint) of the fitted curve and calculates 95% confidence intervals (CI) for EC50s and slopes as well as the CI-bands for the entire curve. Significant interactions of
chemical and UVR effects can be assumed if EC50s and/or slopes in the chemical+UVR treatment were lying outside the CI for these parameters of the chemical-UVR treatment (Pöch et al., 1990). The F-test was used to calculate p-values for the significance of the difference of EC50s. The EC50s were also used to calculate the so-called index of prediction quality (IPQ). The IPQ value is usually calculated as a relative measure for the exactness of the IA model predictions of effective concentrations (e.g. EC50) (Grimme et al., 1994). In the present study, IPQ values were calculated to quantify the strength of interaction of UVR and chemical effects. IPQ values were calculated from EC50s obtained from concentration response curves in control and UVR exposed cultures according to Equation 4 (modified from Grimme et al., 1994). Equal EC50s result in an IPQ of 0 and indicate no interaction between the effects of the chemical and UVR, whereas an IPQ of e.g. 1 or -1 indicates effect interactions which decreased (synergism) or increased (antagonism) the EC50 in the UVR co-exposures by a factor of 2.

\[
\begin{align*}
- \frac{\text{EC50}_{+\text{UVR}}}{\text{EC50}_{-\text{UVR}}} + 1 & \quad \text{for } \text{EC}_{+\text{UVR}} > \text{EC}_{-\text{UVR}} \\
\frac{\text{EC50}_{-\text{UVR}}}{\text{EC50}_{+\text{UVR}}} - 1 & \quad \text{for } \text{EC}_{-\text{UVR}} > \text{EC}_{+\text{UVR}}
\end{align*}
\]  

(4)

To identify stressor effect interactions over the whole tested concentration range, the CI band of concentration-response curves in control and UVR cultures were compared. Statistically significant effect interactions were assumed in cases where the CI bands were not overlapping. The chemical concentration at the intersection of the CI bands was considered as the lowest concentration for which, based on regression analysis, interactions with the effects of UVR could be detected. Furthermore, a Student’s t-test (unpaired, two-tailed) was performed on the mean chemical effect levels at each tested concentration in UVR exposed and control cultures.

Single and net combined stressor effects were also plotted in one graph following (Pöch et al., 1990) (Figures S6 and S8). For this, net chemical+UVR effect data were fitted to concentration-response curves as described above, with the bottom value corresponding to the average UVR effect, and plotted in the same graph with the concentration response curves for the single chemical. The analysis of effect interactions is generally analogous to the one described above, but requires the comparison of net combined effect levels with those predicted by the IA model. For this
we used the single stressor effect data \( (E_{\text{chemical}} \text{ and } E_{\text{UVR}}) \) to first calculate predicted values for net combined effects \( (E_{\text{net}}) \) based on the equation for IA (adapted from (Bliss, 1939)):

\[
E_{\text{net}} = E_{\text{chemical}} + E_{\text{UVR}} - E_{\text{chemical}} \times E_{\text{UVR}} \quad (5)
\]

Concentration response curves for the predicted net effect data were then plotted in the same graph with the concentration response curves for measured single and net combined stressor effects. Independent action of chemicals and UVR can be assumed when measured combined stressor effects correspond to effects predicted by the IA model, while synergistic and antagonistic effect interactions can be assumed when measured combined stressor effects are higher or lower than predicted.

### 2.4 Results

Algal reproduction, cell size and photosynthetic activity were measured in 48 h exposure experiments with PQ, DR, Cd\(^{2+}\) or S-metolachlor tested in absence or presence of UVR. Where possible, concentration-response relationships were generated from these data for the endpoints reproduction after 48 h (Figure 2.2A-D) and photosynthesis at 3, 7, 27 and 31 h (Figure 2.3). From the resulting concentration-response curves, EC50s and slopes with their corresponding 95% CI were derived for effects on algal reproduction (Table 2.2) and photosynthesis (Table 2.3) and compared to analyse effect interactions. To quantify the strength of interactions, IPQ values were calculated based on the ratios of EC50s determined for chemicals tested in UVR exposed and control algae (Figure 2.4).
2.4.1 Effect of UVR on algal reproduction, photosynthesis, and photosynthetic pigments

The selected UVR intensity for the single and combined exposure experiments resulted in an average inhibition of algal reproduction of 26 ±3%. After 48 h exposed cells displayed a larger volume of 165 ± 23 fl compared to unexposed cells which had an average cell volume of 139 ± 15 fl. Preliminary experiments over 24 h showed that UVR exposure diminished the cell size increase during the light phase and that larger cells at the beginning of the second light phase resulted from a reduced cell division during the dark phase (Figure S4). UVR impacted on photosynthesis in a time-dependent manner with inhibition of ΦPSII decreasing successively from 50 ± 5% after 3h to 30 ± 5% after 31 h (Table 2.1). The cellular content of total carotenoids and chlorophyll a was not significantly different in UVR exposed and control cultures at all measured time-points (Table 2.1). However, there was a trend toward decreasing chlorophyll a levels from 8.4 ± 1.0 fg fl⁻¹ at 3 h to 7.6 ± 0.6 fg fl⁻¹ at 31 h in UVR exposed cultures and increasing chlorophyll a levels from 8.9 ±0.6 at 3 h to 9.9 ±0.8 at 31 h in control cultures. The mean ratio of total carotenoids to chlorophyll a was higher in UVR exposed compared to control cultures at similar values throughout the experiment (Table 2.1).

### Table 2.1: Inhibition of ΦPSII ± SD and the pigment contents (fg) per cell volume (fl) of total carotenoids (cartotal) and chlorophyll a (chl a) in Chlamydomonas reinhardtii at different time points during exposure to UVR and in control cultures (-UVR). Pigment contents and ratios of cartotal and chl a contents with 95% CI obtained from three independent replicates. Asterisks indicate values for which CI are not overlapping.

<table>
<thead>
<tr>
<th>exposure time</th>
<th>ΦPSII inhibition (%) ± SD</th>
<th>- UVR</th>
<th>+ UVR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cartotal [fg fl⁻¹] (95% CI)</td>
<td>chl a [fg fl⁻¹] (95% CI)</td>
</tr>
<tr>
<td>3 h</td>
<td>50 ± 5</td>
<td>3.5 (2.5-4.5)</td>
<td>9.0 (7.6-10.4)</td>
</tr>
<tr>
<td>7 h</td>
<td>43 ± 6</td>
<td>3.6 (3.3-3.8)</td>
<td>9.3 (9.0-9.7)</td>
</tr>
<tr>
<td>27 h</td>
<td>34 ± 7</td>
<td>3.8 (3.1-4.6)</td>
<td>9.8 (6.8-12.7)</td>
</tr>
<tr>
<td>31 h</td>
<td>30 ± 5</td>
<td>3.5 (3.1-3.9)</td>
<td>10.0 (8.0-11.9)</td>
</tr>
</tbody>
</table>

CI = confidence interval; SD = standard deviation; UVR = Ultraviolet radiation; ΦPSII = effective quantum yield of photosynthesis
2.4.2 Chemical toxicity in absence or presence of UVR and identification of effect interactions

The toxicity of chemicals to algal reproduction and photosynthesis in absence or presence of UVR was determined for PQ (0.007-0.43 µM), DR (0.009-1 µM), Cd\(^{2+}\) (0.02-13.4 µM) and S-metolachlor (0.2-50 µM). Preliminary experiments carried out to examine the stability of PQ, DR and S-metolachlor in the presence of UVR indicated that these herbicides were not degraded at the applied UVR intensity (Table S1). Chemicals caused increasing inhibition of reproduction with increasing concentrations in absence and presence of UVR (Figure 2.2A-D; Table 2.2). Values indicating inhibition above 100% were obtained when the cell density after 48 h was below the starting cell density, indicating death and breakdown of cells, as also confirmed microscopically (not shown). Exposure of *C. reinhardtii* to S-metolachlor alone and in combination with UVR for 48 h resulted in a concentration dependent increase of cell size up to 4 and 3 times, respectively (Figure S5). In case of Cd\(^{2+}\), algal cell size decreased with increasing concentrations of Cd\(^{2+}\) in combined exposures only. DR and PQ had only minor effects on cell sizes.

All chemicals, except for S-metolachlor, completely inhibited photosynthesis at the highest tested concentrations in absence or presence of UVR (Figure 2.3; Table 2.3). S-metolachlor alone only slightly inhibited photosynthesis after 7 h and not more than 40% after 31 h at the highest concentration of 50 µM. Toxicity of PQ alone to photosynthesis increased over time with EC50s decreasing from 0.4 µM at 3 h to 0.09 µM at 31 h, whereas toxicity of DR and Cd\(^{2+}\) alone changed little over time (Table 2.3).
Table 2.2: Mean EC50 and Hill slopes with their 95% CI derived from concentration response curves (see Figure 2A-D) of four different chemicals tested in absence (chemical-UVR) and presence (chemical+UVR) of UVR for the endpoint reproduction over 48 h. Values calculated from four independent replicates.

<table>
<thead>
<tr>
<th>Exposure concentration range (µM)</th>
<th>48 h reproduction inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- UVR</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Paraquat 0.007 - 0.43</td>
<td>EC50</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
</tr>
<tr>
<td>Diuron 0.009 – 1</td>
<td>EC50</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
</tr>
<tr>
<td>Cd²⁺ 0.02 - 13.4</td>
<td>EC50</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
</tr>
<tr>
<td>S-metolachlor 0.19 - 50</td>
<td>EC50</td>
</tr>
<tr>
<td></td>
<td>Hill slope</td>
</tr>
</tbody>
</table>

* p < 0.001
CI = Confidence interval; EC50 = 50% effect concentration; UVR = Ultraviolet radiation.

The concentration response curve for the effects of PQ on algal reproduction shifted significantly toward lower concentrations in the PQ+UVR treatment (Figure 2.2A). This shift appeared stronger in the upper part, reflected in a significantly higher slope of the curve for PQ+UVR (Table 2.2). Accordingly, the EC50 for PQ+UVR was significantly (p < 0.05) lower with 0.078 ±0.009 µM compared to 0.11 ±0.01 µM in the control algae (Table 2.2) which corresponded to a calculated IPQ value of 0.35 (Figure 2.4). These data indicate a higher toxicity of PQ when acting in combination with UVR and a synergistic interaction between the effects of PQ and UVR. The comparison of 95% CIs of concentration response curves indicates that the effects of PQ and UVR interacted synergistically at PQ concentrations >0.06 µM (Table S2). In case of photosynthesis, concentration response curves were generally shifted toward lower concentrations in the PQ+UVR treatment (Figure 2.3), indicating a higher PQ toxicity in UVR exposed algae at all time-points and synergistic interactions of PQ and UVR effects also on photosynthesis. The slopes of concentration response curves were not consistently higher or lower in UVR and control algae (Table 2.3). EC50s in the PQ+UVR treatments were significantly (p < 0.05) lower compared to the EC50s in the PQ-UVR treatment at all time-points (Table 2.3). The EC50s of PQ decreased in UVR.
exposed and control algae in a time-dependent manner, but stronger in the PQ+UVR treatment. This was reflected in an increasing difference between EC50s in single and combined exposures with exposure time (Table 2.3). Correspondingly, IPQ values increased over time from 0.3 at 3 h to 0.6 at 31 h, reflecting a time-dependent increase in the strength of interaction between the effects of PQ and UVR (Figure 2.4). The lowest concentration at which synergistic effects on photosynthesis were detected was at >0.02 µM (Table S2).

Figure 2.2: Concentration-response relationships and associated 95% confidence intervals (CI [dashed lines]) for reproduction inhibition of *Chlamydomonas reinhardtii* exposed for 48 h to increasing concentrations of paraquat (A), diuron (B), Cd\(^{2+}\) (C) and S-metolachlor (D) in absence (open circles) and presence (filled circles) of ultraviolet radiation (UVR). Standard deviation of four independent replicates are shown. Synergistic or antagonistic interactions of chemical and UVR effects according to Independent Action model are assumed where chemical effects in UVR exposed algae (chemical+UVR) are greater or lower respectively than the effects of chemicals in control algae. This is assumed when effects are significantly (p < 0.05) different based on point to point comparison (indicated by asterisk) and, based on regression analysis, when CI are not overlapping.
Figure 2.3: (see previous page) Concentration-response relationships and associated 95% confidence intervals (CI [dashed lines]) for the inhibition of the effective quantum yield photosynthesis in *Chlamydomonas reinhardtii* at different time points during 48 h exposure to increasing concentrations of paraquat (PQ), diuron (DR), Cd$^{2+}$ and S-metolachlor in absence (open circles) and presence (filled circles) of ultraviolet radiation (UVR). Data points are mean values with standard deviation of four independent replicates. Synergistic or antagonistic interactions of chemical and UVR effects according to Independent Action model are assumed where chemical effects in UVR exposed algae (chemical+UVR) are greater or lower respectively than the effects of chemicals in control algae. This is assumed when effects are significantly ($p < 0.05$) different based on point to point comparison (indicated by asterisk) and, based on regression analysis, when CI are not overlapping.

The concentration-response curve for the inhibition of algal reproduction in the DR+UVR treatment was significantly shifted to higher concentrations (Figure 2.2B), with a higher (not significant) slope (Table 2.2). The corresponding EC50 for DR+UVR was significantly ($p < 0.001$) higher (0.25 ±0.05 µM) in UVR exposed compared to control algae (0.18 ±0.01 µM), resulting in a negative IPQ of -0.4 (Figure 2.4). These data denote a reduced toxicity of DR in presence of UVR and thus an antagonistic interaction of the effects of DR and UVR which was detected at concentrations > 0.02 µM (Table S2). The net combined effect of DR+UVR on algal reproduction also corresponded to effect levels of DR alone above concentrations causing approximately 30% inhibition of reproduction (Figure S6). In case of photosynthesis, DR toxicity in UVR and control cultures was similar at 3 h, reflected in comparable concentration-response curves (Figure 2.3) with similar slopes (Table 2.3). However, concentration-response curves for DR+UVR subsequently shifted toward higher concentrations and displayed comparable slopes relative to the concentration-response curves for DR-UVR. At 3 h, the corresponding EC50 for DR+UVR was 0.08 µM and only slightly, but significantly ($p < 0.05$), higher than the EC50 of 0.07 µM for DR–UVR (Table 2.3). While the EC50s changed little over time in absence of UVR, they increased with exposure time in the presence of UVR to 0.1 µM at 31 h, indicating a time-dependent decrease of DR toxicity only in the DR+UVR treatment. Due to these changes, IPQ values decreased successively from -0.1 at 3h to -0.5 at 31 h, indicating a time-dependent increase in the strength of antagonistic effect interactions (Figure 2.4). The net combined effects of DR+UVR on algal photosynthesis at 27 and 31 h also corresponded to effect levels of DR alone above concentrations causing approximately 60% inhibition of photosynthesis (Figure S8). Lower toxicity in the DR+UVR treatment
indicates antagonistic interactions of the effects of DR and UVR effects on photosynthesis, which occurred at concentrations > 0.009 µM (Table S2).

**Table 2.3:** Mean EC50 and hill slopes with 95% CI derived from concentration response curves (see Figures 2.2 and 2.3) of four different chemicals tested after 3, 7, 27 and 31 h exposure in absence (chemical-UVR) and presence (chemical+UVR) of UVR for the endpoint photosynthesis. Values calculated from four independent replicates

<table>
<thead>
<tr>
<th></th>
<th>3 h</th>
<th>7 h</th>
<th>27 h</th>
<th>31 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UVR</strong></td>
<td><strong>Mean</strong></td>
<td><strong>95% CI</strong></td>
<td><strong>Mean</strong></td>
<td><strong>95% CI</strong></td>
</tr>
<tr>
<td>Cd&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC50 (µM)</td>
<td>* 10.9</td>
<td>10.4-11.4</td>
<td>10.4</td>
<td>10.1-10.8</td>
</tr>
<tr>
<td></td>
<td>+ 8.7**</td>
<td>8.4-9.1</td>
<td>8.0**</td>
<td>7.8-8.2</td>
</tr>
<tr>
<td>Hill slope</td>
<td>* 3.2</td>
<td>2.7-3.6</td>
<td>3.9</td>
<td>3.3-4.4</td>
</tr>
<tr>
<td></td>
<td>+ 3.6</td>
<td>3.0-4.1</td>
<td>5.5</td>
<td>4.8-6.3</td>
</tr>
<tr>
<td>Paraquat</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EC50 (µM)</td>
<td>* 0.40</td>
<td>0.38-0.42</td>
<td>0.17</td>
<td>0.16-0.18</td>
</tr>
<tr>
<td></td>
<td>+ 0.30**</td>
<td>0.29-0.32</td>
<td>0.14*</td>
<td>0.13-0.16</td>
</tr>
<tr>
<td>Hill slope</td>
<td>* 3.9</td>
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<td>2.3</td>
<td>2.0-2.7</td>
</tr>
<tr>
<td></td>
<td>+ 2.5</td>
<td>2.2-2.8</td>
<td>2.1</td>
<td>1.6-2.6</td>
</tr>
<tr>
<td>Diuron</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC50 (µM)</td>
<td>* 0.07</td>
<td>0.068-0.074</td>
<td>0.067</td>
<td>0.064-0.069</td>
</tr>
<tr>
<td></td>
<td>+ 0.08*</td>
<td>0.075-0.084</td>
<td>0.097**</td>
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<tr>
<td>Hill slope</td>
<td>* 1.2</td>
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<td>1.3</td>
<td>1.2-1.3</td>
</tr>
<tr>
<td></td>
<td>+ 1.3</td>
<td>1.2-1.4</td>
<td>1.3</td>
<td>1.2-1.4</td>
</tr>
</tbody>
</table>

* p < 0.05  
** p < 0.001  
CI = Confidence interval; EC50 = 50% effect concentration; UVR = Ultraviolet radiation

The concentration-response curves for reproduction inhibition by Cd<sup>2+</sup> in UVR and control cultures were not significantly different (Figure 2.2C), displaying similar slopes and EC50s (Table 2.2). Accordingly, an IPQ value close to zero was calculated (Figure 2.4), indicating no interaction of the effect of UVR and Cd<sup>2+</sup>, and thus, an independent action of both stressors. However, at the lower Cd<sup>2+</sup> concentrations of 3.7 and 5.7 µM, inhibition of algal reproduction was significantly (p < 0.05) lower in presence of UVR than in absence of UVR (Figure 2.2C), indicating an antagonistic interaction of Cd<sup>2+</sup> and UVR effects. When growth inhibition was calculated based on cell volume density
(cell number multiplied by cell volume), Cd\(^{2+}\) toxicity was not significantly different at all tested concentrations in UVR exposed and control cultures (Figure S7). In the case of photosynthesis, concentration-response curves for Cd\(^{2+}\)+UVR were generally shifted toward lower concentrations (Figure 2.3), with no clear trend for the change in slopes (Table 2.3). The corresponding EC50s for Cd\(^{2+}\) were significantly \((p < 0.001)\) lower in UVR exposed algae compared to control algae at all measured time-points (Table 2.3), indicating higher toxicity of Cd\(^{2+}\) in UVR exposed algae and a synergistic interaction Cd\(^{2+}\) and UVR effects on photosynthesis. The difference between EC50 values in UVR and control algae increased slightly over time, reflected in IPQ values increasing from 0.27 at 3 h to 0.42 at 31 h (Figure 2.4). Based on comparison of 95% CIs, effects of Cd\(^{2+}\) and UVR interacted synergistically at concentrations greater than 6.4 µM at 3 h and 1.9 µM at 31 h (Table S2).

The concentration-response curve for the effects of S-metolachlor on algal reproduction in UVR exposed cultures was shifted to lower concentrations (Figure 2.2D) and had a higher (not significant) slope compared to the concentration-response curve in the control cultures (Table 2.2). The corresponding EC50 of S-metolachlor+UVR was significantly \((p < 0.001)\) lower \((4.6 \pm 0.5 \mu M)\) compared to the EC50 \((6.9 \pm 0.5 \mu M)\) for S-metolachlor-UVR (Table 2.2), resulting in a calculated IPQ of 0.5 (Figure 2.4). This indicates higher toxicity of S-metolachlor on reproduction in UVR exposed algae and a synergistic interaction of the effects of both stressors, which occurred at S-metolachlor concentrations >3.2 µM (Table S2). The effects of S-metolachlor on photosynthesis were generally too low for a fitting of concentration-response curves and the calculation of EC50s. However, the effects of S-metolachlor on photosynthesis were generally at comparable levels in UVR and control algae, indicating no interaction of the effects of UVR and S-metolachlor on photosynthesis, except for 7 h at which effects at concentrations ≥3 µM were significantly lower \((p < 0.05)\) in the S-metolachlor+UVR treatment (Figure 2.3). At these concentrations the photosynthetic activity was increased above control levels, resulting in negative values for the inhibition of photosynthesis.
2.5 Discussion

Here we systematically studied multiple stressor effects from single and combined exposures of *Chlamydomonas reinhardtii* to UVR and chemicals with different MOA. The aim was to increase the mechanistic understanding of multiple stressor effects by linking effect interactions with the known mechanisms of toxic action of UVR and the tested chemicals, considering different endpoints (reproduction and photosynthesis), exposure times and exposure concentrations.

2.5.1 UVR effects

Under the experimental UVR exposure conditions, algal reproduction was inhibited by approximately 25%. Experimental evidence supports that the reduced algal reproduction resulted from an inhibited and delayed cell division. First, at 48 h, the
volume of UVR-exposed cells was larger than in control cells. Second, preliminary experiments under similar UVR exposure conditions revealed that UVR-exposed cells increased slower in size during the light phase compared to control cells (Figure S4). Cell divisions in the following dark phase were inhibited in UVR-exposed cells, indicated by a comparably smaller increase in cell numbers at the end of the dark phase. Together, these data suggest that the inhibition of cell division during the dark phase might have been due to the fact that UVR exposed cells did not reach the critical size to initiate cell division. Cell division might have also been inhibited due to UVR induced DNA damage (Holzinger and Lutz, 2006).

At the applied UVR intensity, the effective quantum yield of photosynthesis ($\Phi_{PSII}$) first strongly decreased to approximately 50% of control values but partially recovered during the exposure to approximately 70% of control values after 31 h, suggesting that algae might have induced mechanisms to acclimate to UVR stress. Carotenoids are a diverse group of photosynthetic pigments that can confer protection of PSII against photo-oxidative damage by scavenging UVR induced ROS (Vincent and Roy, 1993). At the conditions applied in this study, the cellular contents of total carotenoids were not increased in UVR exposed algae. However, the ratios of total carotenoids to chlorophyll $a$ were significantly increased, which appears to have resulted from reduced chlorophyll $a$ levels, possibly caused by destruction of chlorophyll $a$ or a reduced synthesis (Vass et al., 2005; Vincent and Roy, 1993). A higher ratio of carotenoids to chlorophyll $a$ likely results in an increased protection of PSII. Moreover, changes may have occurred in the content of different types of carotenoids with different ROS scavenging capacities, without affecting the content of total carotenoids. Other acclimation mechanism to be considered which are known to be induced in UVR exposed algae within hours to days include the $de$ $novo$ synthesis of damaged components of the photosynthetic apparatus, such as the D1 protein, the synthesis of UVR absorbing compounds and an increase of cellular ROS detoxification capacity by increased accumulation of enzymatic and non-enzymatic antioxidants (Cheloni et al., 2014; Häder et al., 2011; Holzinger and Lutz, 2006; Navarro et al., 2007; Singh et al., 2012; Vincent and Roy, 1993).
2.5.2 Interaction of UVR and chemical effects

Paraquat alone inhibited algal reproduction and photosynthesis in *C. reinhardtii* at similar EC50 values as reported in the literature for the same algae (Fischer et al., 2012, 2010; Nestler et al., 2012). The effects of PQ on photosynthesis were time-dependent with EC50 values decreasing from 0.4 µM at 3 h to 0.09 µM at 31 h in absence of UVR, reflecting the mode of toxic action of paraquat. The progressive impairment of photosynthesis upon PQ exposure might reflect the accumulation of oxidative damage caused by PQ induced ROS formation, occurring when cellular ROS quenching capacities are exhausted (Fischer et al., 2010). Paraquat accepts electrons from photosystem I and transfers them to molecular oxygen in a catalytic reaction, leading to the continuous generation of ROS (Farrington et al., 1973). The toxicity of PQ on reproduction and photosynthesis of *C. reinhardtii* was higher than predicted by the IA model when algae were simultaneously exposed to UVR. These results indicate a synergistic interaction of the effects of PQ and UVR with regard to the assumption of independent action between the stressors. However, considering the similarity of the MOA of PQ and UVR, experimental data should also be analyzed by CA to confirm true effect interactions. Since the mechanistic assumption underlying CA does not directly apply to physical stressors such as UVR (Fischer et al., 2010), the ROS produced by both stressors might be considered for CA. Effect interactions, as indicated by IA, occurred particularly at higher PQ concentrations, as shown by a significant shift of the concentration response curve to lower PQ concentrations, and a significantly higher slope. Considering that UVR and PQ can both induce the generation of ROS in algae, increased effects might have occurred due to increased oxidative damage (He and Hader, 2002; Palmer et al., 2002). Despite the fact that the cellular content of total carotenoids did not change significantly with UVR alone, the ROS detoxification capacity of *C. reinhardtii* might have been overwhelmed when algae were simultaneously exposed to higher concentrations of PQ. Indeed, simultaneous exposure of *Euglena gracilis* to UVR and paraquat resulted in synergistically increased oxidative lipid damage that was prevented in the presence of a ROS scavenger (Palmer et al., 2002). The time-dependent increase in the strength of interactive effects of UVR and PQ, as reflected in increasing IPQ values, might thus reflect a faster depletion of cellular antioxidants in the combined exposures.

Diuron inhibited reproduction and photosynthesis in *C. reinhardtii* at similar EC50 values as reported in the literature for the same algae (Fischer et al., 2012; Fischer et
al., 2010; Nestler et al., 2012). Among the tested chemicals, DR had the strongest effect on photosynthesis with maximal effects occurring after a few hours, indicating a fast cellular uptake and distribution of DR to its target site in the chloroplast as also observed in a previous study with C. reinhardtii exposed under similar conditions (Nestler et al., 2012). The low EC50 values reflect the high specificity of DR binding to its target site, the D1 protein in PSII, inhibiting photosynthesis by blocking the photosynthetic electron transfer. The toxicity of DR to algal reproduction and photosynthesis was decreased by UVR, indicating an antagonistic interaction of DR and UVR effects. It was confirmed that the decreased toxicity actually reflects an interaction of stressor effects, because DR was not degraded at the applied UVR conditions as quantified by LC-MS/MS analysis. The high specificity of DR to PSII and the fact that PSII is a primary target for UVR suggests that interactive effects may be explained by the joint action of both stressors on PSII. Above certain concentrations of DR, the net effect levels in combined exposures corresponded to effect levels of DR in single exposures, suggesting that the binding of DR protected PSII against UVR damage. It has previously been demonstrated that UVB driven D1 degradation can be inhibited by DR, presumably because D1 degradation requires the binding of the quinone electron acceptor Q_B to the D1 protein, which can be replaced by DR (Jansen et al., 1993). However, DR acts very fast on photosynthesis and the maximum inhibitory effects of UVR on photosynthesis occurred already at 3h in our study, at which time no clear antagonistic interaction of DR and UVR effects was observed. This might indicate that UVR has affected the action of DR instead, causing the observed time-dependent increase in the strength of antagonistic effect interactions. This was characterized by a progressive shift of the concentration response curve to higher DR concentrations, whereas the curve slopes remained unchanged. A curve-shift with corresponding slopes may generally reflect a modification of a herbicide’s target site, affecting its affinity to a herbicide (Seefeldt et al., 1995). Thus, the corresponding mechanism might involve a UVR induced modification of the Q_B binding site at PSII, lowering the binding efficiency of DR, as shown in Synechocystis sp. (Vass et al., 1999), thereby reducing the effects of DR.

The toxicity of Cd^{2+} alone on reproduction and photosynthesis of C. reinhardtii was, with an EC50 of 8 µM, in the upper range of reported values which range from nM to several µM (Faller et al., 2005; Jamers et al., 2013; Stoiber et al., 2010). Higher effect
concentrations in *C. reinhardtii* were however reported as total Cd(II) (Jamers et al., 2013) and not as the dissolved Cd\(^{2+}\) ion, which is determinant for effects (Sunda and Guillard, 1976). Lower EC50s reported as Cd\(^{2+}\) in *C. reinhardtii* (Stoiber et al., 2010) might be explained by the ten times higher initial cell density in our experiments which is known to reduce metal toxicity in algal bioassays (Moreno-Garrido et al., 2000). At the applied conditions of this study, maximum effects of Cd\(^{2+}\) on photosynthesis manifested within a few hours, reflecting fast cellular uptake of Cd\(^{2+}\). EC50 values were much higher compared to PQ and DR demonstrating the lower specificity of Cd\(^{2+}\) to targets in the photosynthetic apparatus. Cadmium has a high affinity to sulfhydryl groups and binding of Cd\(^{2+}\) to functional groups can affect the conformation and function of various biomolecules, including those with essential functions in photosynthesis as also shown in *C. reinhardtii* (Faller et al., 2005). Furthermore, inhibition of photosynthesis might also have resulted from damage to the photosynthetic apparatus through ROS, which have been demonstrated to accumulate in *C. reinhardtii* exposed to Cd\(^{2+}\) (Szivak et al., 2009). Considering the corresponding concentration-response curves for reproduction, there was no indication for an interaction of Cd\(^{2+}\) and UVR effects. However, at two lower Cd\(^{2+}\) concentrations (3.7 and 5.7 µM), effects were significantly decreased in UVR exposed algae, indicating an antagonistic interaction of effects. Yet, when growth was calculated based on cell volume density, the Cd\(^{2+}\) toxicity was similar in UVR exposed and control algae, suggesting that the effects of stressors interacted specifically on algal cell division. Other than for growth and reproduction, Cd\(^{2+}\) toxicity on photosynthesis was increased at higher concentrations in UVR exposed algae, suggesting a synergistic interaction of Cd\(^{2+}\) and UVR effects on photosynthesis. The ability of Cd\(^{2+}\) to inhibit antioxidant enzymes might have increased the sensitivity of algal photosynthesis to damage caused by UVR induced ROS (Szivak et al., 2009). Cd\(^{2+}\) might have also reduced the ability of algal cells to replace UVR-damaged PSII components, which was shown to be inhibited in *C. reinhardtii* due to competitive binding of Cd\(^{2+}\) to the essential Ca\(^{2+}\) site in PSII (Faller et al., 2005). A decreased ROS defence and damage repair capacity of Cd\(^{2+}\) exposed algae might also explain why the strength of synergistic effects increased slightly over time. Synergistic effects of Cd(II) and UVB related to photosynthesis and growth were reported for the cyanobacterium *Plectonema boryanum* (Prasad and Zeeshan, 2005). These were suggested as being a consequence of increased Cd(II) internalization due to membrane disruption caused by
UVR, which was, however, applied at intensities causing markedly higher effects compared to the present study. In *C. reinhardtii*, exposure to UVR at levels similar to those applied in the present study did not increase membrane permeability (Cheloni et al., 2014).

S-metolachlor alone inhibited reproduction of *C. reinhardtii* at a similar EC50 as previously reported for this alga (Fischer et al., 2012). As expected, photosynthesis was only slightly inhibited by maximum 40% after 31 h exposure to the highest tested concentration. This reflects the MOA of S-metolachlor which inhibits the formation of very long chain fatty acids, leading to the inhibition of cell division and subsequent cell enlargement as observed in this study (Böger et al., 2000). The toxicity of S-metolachlor on photosynthesis was generally similar in UVR exposed and control algae, indicating that both stressors affected photosynthesis through independent mechanisms. Other than for photosynthesis, S-metolachlor effects on reproduction were increased in UVR exposed algae, especially at higher concentrations, indicated by a shift of the concentration response curve to lower S-metolachlor concentrations and a higher slope of the curve. This indicates that effects of both stressors on algal cell division interacted synergistically, suggesting that other targets and toxicity mechanism of UVR and S-metolachlor, which are not related to photosynthesis, need to be considered to explain these results. Exposure of algae to chloroacetamide herbicides, such as S-metolachlor, was reported to cause impaired membrane formation, reduced cell wall stability and inhibition of algal cell division (Böger et al., 2000). Membrane fatty acids can also directly and indirectly be damaged by UVR in the presence of oxygen (Vass et al., 2005). Thus, the observed synergistic effects on algal reproduction might have resulted from aggravated effects of UVR on algal cell membranes which were destabilized by S-metolachlor in combined exposures. S-metolachlor exposed algae might also have a reduced ability to replace UVR damaged fatty acids, resulting in increased inhibition of cell division.

### 2.6 Conclusions

The current study supports the view that the effects of UVR and chemical pollutants can interact in algae, resulting in synergistic and antagonistic multiple stressor effects.
Effect interactions were investigated by applying the IA model, whereas the alternative concept of CA could not be applied, but may be suitable in cases where stressors act through very similar toxic mechanisms, as suggested for UVR and paraquat. Regardless of the applied reference model, the comparison of responses to different chemicals in UVR co-exposed algae, relative to the responses expected for independent action, clearly shows that multiple stressor effects of UVR and chemicals will differ depending on the chemical mode and mechanism of action. Other than expected, effect interactions were not only observed for chemicals acting similar to UVR, but also for the chemical assumed to act dissimilar to UVR. This suggests that UVR affects several targets and physiological processes and the concept of a primary target site of UVR, i.e., the photosynthetic apparatus, cannot be assumed. This conceivably also applies to other environmental stressors for which single primary target sites are unlikely to exist. Thus, the applicability of models designed to predict joined effects of chemicals in mixtures, selected depending on the similarity of the chemicals’ molecular target sites, to multiple stressors might be limited. The current study further shows that interaction of chemical and UVR effects not only depended on the MOA of chemicals, but also on the exposure time, the examined endpoint and exposure concentrations. The latter was especially important for synergistic effect interactions and probably reflects an exhaustion of cellular defence and repair capacities under multiple stressor conditions at higher stressor levels. We suggest that considering such factors in multiple stressor studies can help to better understand the mechanisms underlying stressor effect interactions.

**Acknowledgement**

We would like to thank Enrique Navarro and Holger Nestler for discussion; Kristin Schirmer for discussion and critical reading of the manuscript; René Schönenberger for his help with LC-MS/MS measurements; Peter Gäumann, Andreas Raffainer and Atila Redondo for their help with the construction of the UVR exposure setup.
2.7 References


Supporting Information Chapter 2

Multiple stressor effects in *Chlamydomonas reinhardtii* - toward understanding mechanisms of interaction between effects of ultraviolet radiation and chemical pollutants
Figure S1: Schematic representation of the exposure setup (see also Figure S1, Chapter 3). Algal cultures are exposed in 24 well culture plates at 20°C. All cultures receive photosynthetic active radiation (PAR) from fluorescent lamps, but only cultures which are not covered by an ultraviolet radiation (UVR) filter receive additional UVR.

Figure S2: Mean effective quantum yields of photosynthesis and associated 95% confidence intervals in absence (-UVR) and presence of ultraviolet radiation (+UVR) in Talaquil growth medium without solvent (white bars) and with 0.05 % ethanol (light grey bars) or 0.01 % methanol (dark grey bars). Values obtained from a single experiment with triplicates.
Figure S3: Mean effective quantum yields of photosynthesis and associated 95% confidence intervals in unexposed cultures of *Chlamydomonas reinhardtii* (Ctrl [grey]), or cultures exposed to the chemicals paraquat (0.1 µM [red]), diuron (0.18 µM [green]), S-metolachlor (15.8 µM [blue]) which had been pre-exposed in Talaquil growth medium to ultraviolet radiation (UVR) at exposure conditions (+UVR) or at control conditions (-UVR) for 48 h. Values obtained from a single experiment with triplicates.

Figure S4: Growth of *Chlamydomonas reinhardtii* cultured for 24 h in a light-dark cycle (14:10 h) in absence (blue) or presence (red) of ultraviolet radiation (UVR) at a slightly higher UVR dose (20 W/m² UVA and 0.7 W/m² UVB) than used in the main experiments of this study. Presented are cell numbers (A), volume of cells (B) at different time-points and size distributions of cells measured at 13 h (C) and 24 h (D). Data obtained from a single experiment.
Figure S5: Relative sizes of *Chlamydomonas reinhardtii* cells after 48 h exposure to increasing concentrations of Cd\(^{2+}\), paraquat, diuron and S-metolachlor in absence (white bars) or presence (gray bars) of supplemental ultraviolet radiation (UVR), relative to their corresponding no-chemical controls (either – or + UVR). Mean and standard deviation of four independent replicates are shown.
Figure S6: Concentration-response relationships for the inhibition of reproduction of *Chlamydomonas reinhardtii* exposed for 48 h to increasing concentrations of paraquat (PQ; (A)), diuron (DR; (B)), Cd$^{2+}$ (C) and S-metolachlor (S-Met; (D)) in absence (open diamonds) and presence (filled circles) of ultraviolet radiation (UVR). Presented are single and net combined response levels compared to Independent Action model predictions of combined response levels (black squares and dashed line). Standard deviation of four independent replicates are shown. The asterisks indicate significant differences ($p < 0.05$) between predicted and measured combined effect levels.
Figure S7: Concentration-response relationships and associated 95% confidence intervals (CI) for cell volume density growth (based on the product of cell size and cell number) of *Chlamydomonas reinhardtii* exposed for 48 h to increasing concentrations of Cd\(^{2+}\) in absence (open circles) and presence (filled circles) of ultraviolet radiation (UVR). Effects of Cd\(^{2+}\) in combined exposures are normalized to the UVR exposed no-chemical controls. Mean values and standard deviation of four independent replicates are shown. No-interaction of Cd\(^{2+}\) and UVR effects can be assumed based on the complete overlap of CIs for the two concentration response curves and no significant differences of effect levels at tested Cd\(^{2+}\) concentrations.
**Figure S8.** (see previous page) Concentration-response relationships for photosynthesis inhibition (effective quantum yield) measured in *Chlamydomonas reinhardtii* cultures exposed for 3, 7, 27 and 31 h to increasing concentrations of paraquat (top row), diuron (second row), Cd\(^{2+}\) (third row) and S-metolachlor (bottom row) alone (open diamonds) and in combination (filled circles) with of supplemental ultraviolet radiation (UVR). Presented are single and net combined response levels compared to Independent Action (IA) model predictions of combined response levels (black squares). Mean values and standard deviation of four independent replicates are shown.

**Table S1.** Mean herbicide concentrations with 95% CI in cell free growth medium at the beginning (0 h) and after 48 h exposure to 100 mol m\(^{-2}\) s\(^{-1}\) PAR in absence (PAR-UVR) or presence of ultraviolet radiation (PAR+UVR), or in the dark, measured by HPLC-MS/MS. Values obtained from four replicates

<table>
<thead>
<tr>
<th>time</th>
<th>0 h</th>
<th>48 h</th>
<th></th>
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<td>-</td>
<td>dark</td>
<td>PAR-UVR</td>
<td>PAR+UVR</td>
<td></td>
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<tr>
<td>mean herbicide concentration (µM) (95% CI)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Paraquat</td>
<td>9.49 (9.23 - 9.75)</td>
<td>10.3 (9.73 - 10.9)</td>
<td>9.98 (9.41 - 10.6)</td>
<td>9.93 (9.36 - 10.5)</td>
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<tr>
<td>Diuron</td>
<td>18.7 (18.4 - 19.0)</td>
<td>19.5 (17.4 - 21.7)</td>
<td>19.1 (18.4 - 19.7)</td>
<td>19.1 (18.7 - 19.5)</td>
<td></td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>3.55 (3.03-4.06)</td>
<td>3.98 (3.86- 4.10)</td>
<td>3.35 (3.05-3.65)</td>
<td>3.42 (2.91-3.92)</td>
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</tr>
</tbody>
</table>

CI = Confidence interval; HPLC-MS/MS= high-performance liquid chromatography coupled to a tandem mass spectrometry; PAR = photosynthetic active radiation; UVR = Ultraviolet radiation

**Table S2.** Lowest chemical concentrations (µM) at which effects on growth and photosynthesis in *Chlamydomonas reinhardtii* interacted with the effects of UVR, based on calculated chemical concentrations at intersections of 95% CI bands of fitted concentration response curves (compare to Figures 2.2A-D and 2.3) for paraquat, diuron, Cd\(^{2+}\) and S-metolachlor tested in UVR exposed and control algae

<table>
<thead>
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<th>Photosynthesis</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td>3 h</td>
<td>7 h</td>
<td>27 h</td>
<td>31 h</td>
<td></td>
</tr>
<tr>
<td>Paraquat</td>
<td>0.065</td>
<td>0.022</td>
<td>0.11</td>
<td>0.026</td>
<td>0.028</td>
</tr>
<tr>
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<td>0.022</td>
<td>0.023</td>
<td>0.0091</td>
<td>0.010</td>
<td>0.0091</td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>no intersection</td>
<td>6.4</td>
<td>6.4</td>
<td>5.1</td>
<td>1.9</td>
</tr>
<tr>
<td>S-metolachlor</td>
<td>3.2</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

CI = Confidence interval; n.a.= Not applicable; UVR = Ultraviolet radiation
Chapter 3:

Acclimation of *Chlamydomonas reinhardtii* to ultraviolet radiation and its impact on chemical toxicity

A version of this chapter is submitted to *Aquatic Toxicology*

Muris Korkaric, Mao Xiao, Renata Behra, Rik I. L. Eggen
3.1 Abstract

The toxicity of chemical pollutants can be modulated under stressful environmental conditions, such as increased temperature, salinity or ultraviolet radiation (UVR), due to the interaction of effects during simultaneous stressor exposure. However, organisms may acclimate to such conditions by activation of physiological and biochemical defense mechanisms. In sequential exposures, organisms acclimated to environmental stressors may display an increased sensitivity or co-tolerance towards chemical pollutants. It has been suggested that co-tolerance might be expected for similar acting stressors due to common defense mechanisms. To test this for combinations of UVR and chemical stressors, we first acclimated the model green alga *Chlamydomonas reinhardtii* to UVR and subsequently compared the sensitivity of UVR pre-exposed and control algae towards chemicals. Selected chemicals all act on photosynthesis and thus share a common physiological target, but display distinct toxicity mechanisms. Results showed that UVR pre-exposure for four days partially inhibited algal growth and photosynthesis, but also increased algal tolerance to higher UVR levels, confirming UVR acclimation. HPLC analysis of algal pigments indicated that UVR acclimation might in part be explained by the protective function of lutein while the contribution of UVR absorbing compounds was less clear. Challenge exposure to chemicals in the absence of UVR showed that acclimated algae were co-tolerant to the photosensitizer rose bengal, but not to the herbicides paraquat and diuron, suggesting that the fast physiological and biochemical defense mechanisms that conferred tolerance of algae towards higher UVR levels were related to singlet oxygen defense. The presented study suggests that knowledge of the molecular toxicity mechanisms of chemicals, rather than their general physiological target, is needed in order to predict co-tolerance between environmental and chemical stressors.

3.2 Introduction

Aquatic ecosystems are threatened by exposure to both chemical and environmental stressors such as increasing temperatures, acidity or ultraviolet radiation (UVR). The observation that chemical and environmental stressors frequently act on aquatic biota in a non-additive manner has led to a growing number of studies on the interactions between multiple stressors (Crain et al., 2008; Eggen et al., 2004; Holmstrup et al.,
2010). Most of these studies focused on simultaneous stressor exposures while studies deploying sequential stressor exposure to examine the impact of adaptive responses on chemical toxicity are comparably scarce (Fischer et al., 2013). It is known that organisms and communities can acclimate to stressful environmental conditions, which may positively (co-tolerance) or negatively impact on their tolerance towards chemical pollutants (Vinebrooke et al., 2004). It has been suggested that co-tolerance might be expected for similar acting stressors due to the action of common defense mechanisms. In aquatic algae, this is indeed supported by the demonstration of co-tolerance between high light and metal stress, both known to be able to cause oxidative damage (Nielsen and Nielsen, 2010). On the other hand, co-tolerance has also been demonstrated between predation and herbicides, assumed to be acting dissimilarly (Fischer et al., 2012). Thus, and in order to increase the understanding of co-tolerance between environmental stressors and chemical toxicity there is a need for more systematic study design to determine which and how environmental factors can influence the impact of chemical pollutants on aquatic algae.

UVR is an environmental stressor, which can negatively impact organisms in aquatic ecosystems, and to which organisms can acclimate (Häder et al., 2011; Williamson, 1995). In photoautotrophic organisms, such as microalgae, photosynthesis is regarded as one of the most sensitive physiological targets for UVR damage. (Hideg et al., 2002; Vass et al., 2005). Mechanistically, UVR can damage components of the photosynthetic apparatus directly or through the generation of reactive oxygen species (ROS), such as singlet oxygen (¹O₂), superoxide anion (·O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (·OH) (He and Häder, 2002; Vincent and Neale, 2000). In addition to repair mechanisms, algae possess several physiological and biochemical mechanisms to reduce UVR damage. Accumulation of UVR absorbing compounds, such as mycosporine like amino acids, flavonoids, or carotenoids can reduce the amount of UVR reaching sensitive cellular targets (Navarro et al., 2007; Sinha et al., 1998). Carotenoids and non-carotenoid antioxidants, together with enzymatic antioxidants, function to prevent excessive ROS formation upon UVR exposure (Hideg et al., 2013; Mallick and Mohn, 2000). In addition to protection from UVR damage, UVR defense mechanisms are expected to alleviate toxicity of chemicals which act similar to UVR, such as ROS inducing chemicals. This has been demonstrated by co-tolerance between UVR and cadmium
in periphytic algae that were acclimated to UVR (Navarro et al., 2008). However, it remained unclear which defense mechanisms were responsible for the observed co-tolerance because UVR acclimation of periphytic algae involves changes on the community level in addition to inducible physiological defense mechanisms. Moreover, while cadmium has a strong inhibitory effect on photosynthesis and can increase the generation of ROS similar to UVR, it acts rather unspecific and thus limits the interpretation of results concerning the mechanisms underlying co-tolerance (Szivak et al., 2009). Thus, there is a need to study co-tolerance between UVR and chemicals in single algal species for chemicals with specific mechanisms of toxic action.

The aim of this study was to examine the acclimation of the model green alga \textit{C. reinhardtii} to UVR and its co-tolerance to chemicals in relation to their mechanism of action. Thus, co-tolerance of UVR-acclimated algae to chemicals which inhibit photosynthesis, but which differ in their mechanisms of toxic action, was tested. Rose bengal is a photosensitizer which specifically generates $1O_2$. Paraquat acts on the site of the photosystem I (PS I) by the catalytic generation of $\cdot O_2^-$. The herbicide diuron inhibits photosynthesis by blocking the photosynthetic electron transport at PS II and displays a lower potential for the generation of ROS compared to rose bengal and paraquat. Co-tolerance to one or several of the selected chemicals in UVR acclimated algae can inform indirectly about common toxicity mechanisms of chemicals and UVR and about UVR defense mechanisms in \textit{C. reinhardtii}.

### 3.3 Material and Methods

#### 3.3.1 Alga strain and growth conditions

The wild type strain (CC125) of the unicellular freshwater alga \textit{Chlamydomonas reinhardtii} (Chlorophyta) obtained from the Chlamydomonas Genetics Center (Durham, NC, USA), was used as a test organism in this study. Algae were cultured axenically in glass Erlenmeyer flasks in the synthetic freshwater growth medium Talaquil at pH 7.5 (Le Faucheur et al., 2005) with NaNO$_3$ being substituted by NH$_4$Cl, since \textit{C. reinhardtii} CC125 cannot utilize nitrate due to a lack of the nitrate reductase. Algae were cultured in a shaking incubator (Infors HT Multitron,
Bottmingen, Switzerland) at 90 rpm, 23 °C and continuous illumination at 125 ± 10 µmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation (PAR) provided by fluorescent lamps (Osram LUMILUX L 15 W/840). Algae were acclimated to the described conditions by using successive batch cultures.

### 3.3.2 Chemicals and stock solutions

Chlorophyll a (CAS 479-61-8), chlorophyll b (CAS 519-62-0), lutein (CAS 127-40-2), β-carotene (CAS 7235-40-7), α-tocopherol (CAS 10191-41-0) and trans-β-Apo-8′-carotenal (CAS 1107-26-2) for reversed phase high pressure liquid chromatography (RP-HPLC) were purchased as analytical grade standards from Sigma-Aldrich (Burgdorf, Switzerland). Working solutions were prepared in 90 % acetone (v/v).

Chemicals for challenge exposure experiments were paraquat (PQ; CAS 1910-42-5), diuron (DR; CAS 330-54-1) and rose bengal (RB; CAS 632-69-9). RB was obtained from Fluka (Buchs SG, Switzerland), PQ and DR from Sigma-Aldrich (Burgdorf, Switzerland). Stock solutions were prepared using HPLC-grade solvents, water for RB (1 mM) and PQ (0.5 mM), and methanol for DR (20 mM) and stored at 4 °C in the dark.

### 3.3.3 Pre-exposure of algae to UVR

In order to acclimate algae to UVR, algal cultures were continuously exposed to UVR for up to 96 h in UVR-transparent 75 mL borosilicate-vessels covered with a borosilicate lid (Duran®). The UVR exposure setup is shown in Figure S1. UVR was provided from above the experimental cultures by fluorescent lamps (ARIMED B, 40 W, Cosmedico Light, Germany). The irradiation conditions for the pre-exposure were selected based on preliminary experiments to allow for an UVR acclimation of algae. Thus, the selected condition for the UVR acclimation were 18 W/m$^2$ (UV-A) and 0.8 W/m$^2$ (UV-B) (Table S1), representing environmentally realistic UVR intensities. Experimental irradiation spectra were measured and integrated as previously described (Korkaric et al., Chapter 2) and are reported in Figure S2. UVR filters (Plexiglas™ 0A00 HC) in the control treatment limited UV radiation to 0.9 W/m$^2$ (UV-A) while UVB was below the detection limit. Cool-white fluorescence lamps (Radium Bonalux super, NL54W/840 white) provided 124 ±5 µmol m$^{-2}$ s$^{-1}$ PAR in the UVR cultures and, due to attenuation by the UVR filter, a slightly lower PAR of 116 ±4
µmol m$^{-2}$ s$^{-1}$ in the control culture. PAR was measured with a PAR quantum sensor connected to a SpectroSense 2 meter (Sky Instruments, UK). Magnetic stirrers were used to constantly mix cultures during exposures. A circulating water bath (Lauda, RC6, Germany) was used to maintain cultures at a constant temperature of 23 °C during exposures. Cultures were diluted every 24 h in fresh medium to assure sufficient nutrient supply. For this, cells were pelleted by 10 minutes centrifugation at 4000 rmp and resuspended in Talaquil to $2 \times 10^5$ cells/mL.

**3.3.4 Photosynthetic pigments and UVR-absorbing compounds**

Changes in cellular contents of UVR-absorbing compounds, photosynthetic pigments and α-tocopherol were examined during UVR pre-exposure. For this, between 10 and 15 mL of algal culture of known cell-density were filtered onto glass fibre filters (Whatman GF/F), immediately frozen in liquid N2 and stored at -80 °C.

UVR-absorbing compounds were determined after ethanol extraction according to Navarro et al. (2007). For this, 1.5 mL of 90 % (v/v) ethanol was added to frozen samples which were then boiled at 85 °C for 10 minutes and stored in the dark at 4 °C for 14 h. Pigment extracts were filtered (PTFE 0.22 µm) and the absorbance of extracts were measured relative to a reference (GF/F filter extracted in ethanol and filtered with PTFE membrane) using a Cary 100 UV–Vis spectrophotometer (Agilent, Santa Clara, CA, USA) in the range from 210 to 800 nm (1 nm steps) and normalized to an absorbance of 1 at 665 nm. The area below the normalized spectra in range of UV-B (280–315 nm), UV-A (315-400 nm) was obtained by integration. The resulting UV ratio is a dimensionless number, representing a ratio between the absorbance capacities of the UVR-absorbing compounds per absorbance unit of chlorophyll a (Navarro et al., 2007).

For the determination of algal pigments and α-tocopherol, samples were extracted for 24 h in the dark at 4 °C with 90 % acetone (v/v) containing 2.5 µg/mL of trans-β-apo-8′-carotenal as internal standard. Extracts were filtered (PTFE 0.22 µm) and 20 µL were analysed according to previously published methods (García-Plazaola and Becerril, 1999). Analysis was performed with an Agilent 1200 system (Wilmington, DE, USA) consisting of a RP-HLPC coupled to a photo diode array (DAD) and a fluorescence detector (FLD). Analytes were separated on a C18 column (EC 250/4
Nucleosil 100-5 C18, 250 × 4 mm, 5 µm; Macherey-Nagel, Germany) at a flow rate of 1.2 mL/min and linear gradient from 100 % solvent A (acetonitrile/methanol/0.1 M Tris-HCl pH 8.0; 84/2/14, v/v/v) to 100 % solvent B (methanol/ethyl acetate; 68/32, v/v) within 16 min followed by 100 % solvent B for 7 min. Pigments were detected by DAD at 445 nm, α-tocopherol was detected by FLD with excitation at 295 nm and emission at 340 nm and identified according to retention time and absorption spectra. A chromatogram of an extract is presented in Figure S4. Chlorophyll a (Chl a), chlorophyll b (Chl b), lutein and β-carotene were quantified using external calibration curves for the respective standards (Figure S5). Loroxanthin, cis-neoxanthin, violaxanthin and antheraxanthin were quantified using the calibration curve of lutein. Pigment concentrations were normalized to the average algal cell volume in the respective sample. For quality control, the internal standard concentrations in all samples (n=46) were determined by an external calibration curve showing a low coefficient of variation (%CV = 1.5).

### 3.3.5 Tolerance of UVR pre-exposed algae to higher UVR levels

To test whether UVR pre-exposure increased UVR tolerance of algae, short term challenge exposures of 6 h to higher UVR levels were performed. For this, pre-exposed and control cultures were harvested from UVR pre-exposures after 24, 48, 72 and 96 h by centrifugation and resuspended in fresh culture medium to a density of approximately 2 × 10⁵ cells/mL. UVR challenge exposures were performed in a setup similar to that used in the pre-exposure, with UVR and PAR being provided by an Osram HTC400-241 bulb. Applied UVR intensities were high UVR (hUVR: 24 ± 6 W m⁻² UVA, 2.5 ± 1 W m⁻² UVB, 250 ±1 µmol m⁻² s⁻¹ PAR) and very high UVR (vhUVR: 28 ± 8 W m⁻² UVA, 5.3 ± 2 W m⁻² UVB and 265 ±3 µmol m⁻² s⁻¹ PAR). These intensities corresponded to 33% and 55% increase of UVA and 225% and 588% increase of UVB irradiance, respectively, compared to UVA and UVB in pre-exposure. To achieve vhUVR, the UVR lamp was fixed at a distance of 65 cm to the culture surface. Culture vessels covered with a polystyrene plastic lid received hUVR. For the determination of photosynthesis, which was selected as endpoint, an aliquot of 1 mL was withdrawn every 30 minutes during the challenge exposure, transferred to a 24 well plate and directly measured as described below (Chapter 3.3.7). Increased tolerance of UVR pre-exposed algae compared to controls was interpreted
as successful UVR acclimation.

### 3.3.6 Co-tolerance of UVR pre-exposed algae to chemicals

UVR pre-exposed algae were assessed for co-tolerance to chemicals by comparing sensitivity of pre-exposed and control algae to increasing concentrations of rose bengal, paraquat and diuron in absence of UVR. Experimental cultures were prepared by harvesting algae after 48 h of UVR pre-exposure and by resuspending in fresh medium to a culture density of approximately $2.5 \times 10^5$ cells/mL. Experimental cultures were exposed to chemicals in 24-well cell culture plates (Cellstar®, Greiner Bio-One) as previously described (Korkaric et al., Chapter 2) and placed in a shaking incubator under pre-culturing conditions for up to 24 h. Algal reproduction was determined over 24 h and photosynthesis was measured after 1, 3 and 6 h of exposure. Survival was examined after 3 h of exposure by spotting an aliquot of algal cultures onto a Tris acetate phosphate medium agar plate and inspecting the formation of colonies after 10 to 14 days.

### 3.3.7 Endpoint measurements and effect calculation

Algal growth and reproduction was determined by measuring cell number (CN) and cell volume (CV) using a CASY cell counter (model TT; Innovatis AG, Germany) in the size range of 3 to 18 µm, at various timepoints during exposure experiments. From these data, total biovolume (fL cell volume/mL culture volume) were calculated as the products of CN and CV (Nestler et al., 2012). Where needed, it is indicated whether growth refers to changes in total biovolume over time, or to changes in cell density (cells/mL culture volume) over time (reproduction). To quantify biovolume growth, yields were determined by subtracting total biovolumes after dilution of cultures (0, 24, 48, 72 h) from total biovolumes of culture after 23 h of exposure (23, 47, 71, 96 h). Effects of chemicals on algal reproduction were calculated based on the reduction of doublings of cell densities over 24 h as previously reported (Korkaric et al., Chapter 2).

Photosynthesis was determined as the *maximum* $(F_{v}/F_{m})$ and the *effective quantum yield* $(\Phi_{PSII})$ which are relative measures for the photochemical efficiency of PSII reaction centres after dark adaption and under illumination, respectively. For each algal sample, photosynthetic yields were determined in duplicates of 1 mL each in 24 well plates by a saturating pulse method using a Maxi-Imaging-PAM chlorophyll
fluorometer (Walz, Germany) (Schreiber, 2004). Algae were adapted to dark for 7 min and to an illumination of 114 µmol m$^{-2}$ s$^{-1}$ PAR for 30 seconds for the determination of Fv/Fm and $\Phi_{\text{PSII}}$, respectively. Subsequently, three saturation pulses were applied every 40 s in order to record the maximum (Fm and Fm') and baseline fluorescence (F$_0$ and Fs). Fm and F$_0$ are measured in dark and Fm' and Fs in light adapted algal, respectively. The average of three measurements was used for the calculation of Fv/Fm and $\Phi_{\text{PSII}}$ according to equation (1) $Fv/Fm = Fm - F_0/Fm$ and (2) $\Phi_{\text{PSII}} = (Fm' - Fs)/Fm'$ (Genty et al., 1989).

### 3.3.8 Statistical analysis

In a first step, a one-way analysis of variance (ANOVA) was performed using GraphPad Prism software (version 5.0) to test whether UV-ratios and pigment content in control algae changed over time in control algae. A Dunnett's Multiple Comparison Test was used to identify timepoints at which data significantly deviate from the mean. Based on this analysis, the 3 and 6 h timepoints were omitted in the next step, the two-way analysis of variance (ANOVA) to test for a significant effect of UVR exposure (control and UVR exposure), exposure times (24 to 96 h) and its interaction on UV ratios and pigment contents. In case of a significant result, a post-hoc comparison was performed using the Bonferroni-test. All comparisons were examined at the significance level of 0.05.

Effects of chemical exposure were calculated as percent inhibition of algal growth or photosynthesis relative to controls (either UVR pre-exposed or unexposed cultures) and fitted to concentration response curves as previously described (Korkaric et al., Chapter 2). From these concentration response curves the effective concentrations causing 50 % effect on the measured endpoints (EC50s) and Hill slopes with their respective 95 % confidence intervals (CI) were calculated (Table 3.2 and S5). Co-tolerance to chemicals was assumed in case were the CI bands of the concentration response curves and EC50s determined for UVR-pre-exposed cultures and control cultures were not overlapping.

### 3.4 Results

Cultures of *C. reinhardtii* were acclimated to UVR by exposing algae for up to 96 h to UVR (pre-exposure). During this time, effect of UVR on algal growth and
photosynthesis were assessed. Impact of UVR on UVR-absorbing compounds and pigment composition were measured to investigate possible mechanisms involved in the adaptive response of algae to UVR. Acclimation to UVR was tested by examining tolerance of algal photosynthesis in pre-exposed and control algae towards higher UVR intensities. Co-tolerance was examined by comparing the sensitivity of UVR acclimated and control algae towards chemicals with different toxicity mechanisms.

### 3.4.1 Algal growth and photosynthesis during UVR pre-exposure

Algal reproduction was almost completely inhibited in exposed cultures during the first day of UVR pre-exposure from 0 to 23 h (Figure 3.1A). Afterwards, reproduction in UVR exposed and control cultures were similar during the second and fourth day, but diminished during the third day of UVR pre-exposure. In comparison, algal growth in terms of total biovolume (fL cell volume / fL culture volume), was diminished during the first three days of the UVR pre-exposure to approximately 72 %, 89 % and 78 % relative to controls, respectively, but comparable on day four (72 to 96 h) (Figure 3.1B). Comparing growth data for day one shows that that UVR exposed algae were growing primarily in size during the first day of exposure.

The maximum quantum yield of photosynthesis (Fv/Fm) in control cultures remained unchanged during the experimental time, whereas the effective quantum yield of photosynthesis (ΦPSII) slightly decreased from a maximum value of 0.44 at 6 h to 0.35 at 96 h (Figure 3.1C).
Figure 3.1: Growth and photosynthetic activity of *C. reinhardtii* during pre-exposure to UVR for up to 96 h, indicated by changes in algal cell density (A), total biovolume (fL cell volume/mL culture volume) (B) and effective ($\Phi_{\text{PSII}}$, left y-axis) and maximum (Fv/Fm, right y-axis) quantum yield of photosynthesis (C), respectively, in unexposed control and UVR exposed algae. Algal cultures were diluted at 23, 47 and 71 h in fresh Talaquil medium to a starting cell density of $2 \times 10^5$ cells/mL. Mean values obtained from a minimum of three independent experiments with associated 95% confidence intervals are shown.
In UVR exposed algae, Fv/Fm rapidly decreased within 3 h to values 38 % below values in controls and remained at these lower levels until the end of the UVR pre-exposure. In case of ΦPSII, values decreased by 55 % within the first 3 h of UVR exposure and subsequently recovered from 6 to 24 h to values 35 % below controls and remained at similar values until the end of the pre-exposure. Taken together, after initial dynamics of Fv/Fm and ΦPSII in UVR exposed algae, values remained constant at levels approximately 30 % below values in control cultures.

3.4.2 UVR-absorbing compounds and pigment composition during UVR acclimation

UVA and UVB ratios were assessed during UVR pre-exposures, representing the total absorbance of algal cell extracts in the UVA and the UVB spectrum, respectively, relative to the absorbance of Chl a. There was an initial dynamic of UVA ratios, which decreased in control algae and increased in UVR exposed algae within the first 3 h of the experimental time (Figure 3.2A). After 3 h, UVA ratios decreased and subsequently stabilized in UVR exposed algae at constant values that were higher compared to values in controls. UVB ratios showed similar initial dynamics during, with higher UVB ratios in UVR exposed algae at 3 h, but UVB ratios in exposed cultures stabilized from 24 to 96 hours at values which were similar to values in controls (Figure 3.2B). One-way ANOVA with Dunnett's Multiple Comparison Test of both UVA and UVB-ratios in controls showed that values at the 3 h and 6 h timepoints significantly deviated from the mean values at the start of the experiment, indicating that an experimental factor other than UVR affected UV ratios in controls especially at these timepoints (Table S2). Assuming that this factor would similarly affect UVR exposed algae, these timepoints were omitted from statistical analysis and regarded as non-representative for the long-term changes in response to UVR that were of interest to this study. Statistical analysis with two-way ANOVA indicated that overall, UVR exposure, but not exposure time and their interaction, significantly affected UVA and UVB rations from 24 h to 96 h (Table 3.1). Post hoc testing showed that UVA ratios were significantly higher at 48 h and 96 h in UVR exposed compared to control algae, while UVB ratios were not significantly increased in exposed algae at any of the tested timepoints.
Figure 3.2: Changes in UVA ratios (A) and UVB ration (B) during pre-exposure of *C. reinhardtii* to ultraviolet radiation (UVR) for up to 96 h. UV ratios reflect the absorption capacity of algal pigments for UVA and UVB relative to the absorbance of chlorophyll a. Mean values with standard deviation obtained from three independent experiments are shown. Significant effects of UVR exposure, exposure times (24, 48, 72, 96 h) and their interaction on UV ratios were examined by two-way analysis of variance (Table 3.1).

Pigment analysis by HPLC showed that the content of all pigments decreased slightly in control cultures within the first 6 h of exposure but subsequently recovered to values similar to initial contents and remained constant up to 96 h (Figure 3.3). In UVR exposed algae, the content of all pigments, except for lutein and violaxanthin, also decreased in the first 6 h. From 24 to 96 h, UVR exposed algae displayed lower cellular content of Chl a, Chl b, β-carotene, cis-neoxanthin and violaxanthin compared to control algae, whereas the content of antheraxanthin and lutein was
higher in UVR exposed algae. The content of loroxanthin was similar in UVR exposed and control algae throughout the exposure. As for UV-ratios, one-way ANOVA indicated that the 3 h and 6 h timepoints were non-representative for the overall changes in pigment contents over longer timescales and were thus excluded in the subsequent statistical analysis (Table S2). Two-way-ANOVA showed that UVR significantly affected the content of all pigments except for loroxanthin from 24 h to 96 h (Table 3.1). Exposure time had no effect on the cellular content of pigments, except antheraxanthin. Careful inspection of data shows that this is most likely due to an outlier at 72 h (Figure 3.3). Alpha-tocopherol was neither detected in control nor in UVR exposed algae, possibly due to insufficient amounts that were below the detection limit of the applied method.

![Figure 3.3: Mean pigment content (fg/fL cell volume) with standard deviation in UVR exposed and control algae during UVR pre-exposure for up to 96 h determined by HPLC. Values obtained from a minimum of three independent experiments. Significant effects of UVR exposure, exposure times (24, 48, 72, 96 h) and their interaction on pigment contents were examined by two-way analysis of variance (Table 3.1).](image-url)
Table 3.1: Two-Way ANOVA results for the effect of UVR, exposure time and their interaction on UVA and UVB ratios (Figure 3.2) and pigment contents (Figure 3.3) examined at 24, 48, 72 and 96 h in UVR exposed and control algae.

<table>
<thead>
<tr>
<th></th>
<th>UVR¹</th>
<th>Time²</th>
<th>UVRxTime²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p value</td>
<td>F</td>
</tr>
<tr>
<td>UV ratios</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UVA ratio</td>
<td>48</td>
<td>&lt; 0.001</td>
<td>1.8</td>
</tr>
<tr>
<td>UVB ratio</td>
<td>6.1</td>
<td>0.025</td>
<td>0.4</td>
</tr>
<tr>
<td>Pigments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>38</td>
<td>&lt; 0.001</td>
<td>3.0</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>23</td>
<td>&lt; 0.001</td>
<td>1.9</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>75</td>
<td>&lt; 0.001</td>
<td>0.9</td>
</tr>
<tr>
<td>Lutein</td>
<td>191</td>
<td>&lt; 0.001</td>
<td>3.0</td>
</tr>
<tr>
<td>Loroaxanthin</td>
<td>0.9</td>
<td>0.338</td>
<td>2.7</td>
</tr>
<tr>
<td>cis-Neoxanthin</td>
<td>9.5</td>
<td>0.007</td>
<td>0.3</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>54</td>
<td>&lt; 0.001</td>
<td>1.9</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>21</td>
<td>&lt; 0.001</td>
<td>4.1</td>
</tr>
</tbody>
</table>

¹ one degree of freedom
² three degrees of freedom

3.4.3 Tolerance of pre-exposed algae to higher UVR

Exposure of *C. reinhardtii* to high UVR (hUVR) decreased maximum quantum yields of photosynthesis (Fv/Fm) in control and UVR pre-exposed algae in a time dependent manner (Figure 3.4A). Fv/Fm was completely inhibited in control algae after 6 h, whereas a residual photosynthetic activity remained in UVR pre-exposed algae, independent of the duration of the pre-exposure. Fitting Fv/Fm data to an exponential decay model yielded two main results. First, the goodness of fit (r²) of Fv/Fm values was always higher in control cultures (r² ranging from 0.86 to 0.97) compared to pre-exposed cultures (r² ranging from 0.62 to 0.80) (Table S4). Second, the rate constant k (h⁻¹) for the exponential decay of Fv/Fm were consistently lower in UVR pre-exposed algae (k 0.20 to 0.49) compared to control algae (k 0.43 to 0.79), indicating a higher tolerance of photosynthesis in UVR pre-exposed algae (Table S4). Rate constants did not decrease further with longer UVR pre-exposure times from
24 h to 96 h, indicating a fast UVR acclimation during pre-exposure. When UVR pre-exposed algae were cultured for 24 h in absence of UVR under control conditions, Fv/Fm values in these cultures decreased similarly to controls when algae were challenged with hUVR (Figure S6), indicating that UVR acclimation was transient.

Upon exposure of *C. reinhardtii* to very high UVR (vhUVR) photosynthetic activity was completely inhibited in control and UVR pre-exposed algae within the first 30 minutes (not shown). Nonetheless, changes in the baseline chlorophyll fluorescence (F₀) were recorded during vhUVR exposures because this parameter is a direct indicator for photoinhibition and damage to thylakoid membranes and PSII reaction centres. F₀ increased under vhUVR in control and UVR pre-exposed cultures up to a maximum value and subsequently decreased below the starting value. The time point at which maximum F₀ was reached differed between control and pre-exposed cultures. In control cultures, maximum F₀ occurred between 1 and 2 h of the challenge exposure, while in pre-exposed cultures, maximum F₀ occurred after approximately 3 h of exposure to vhUVR, independent of the length of UVR pre-exposure.

**Figure 3.4:** Maximum quantum yield of photosynthesis (Fv/Fm) upon exposure to hUVR¹ (A) and baseline chlorophyll fluorescence (F₀) upon exposure to vhUVR² in *C. reinhardtii* after different UVR pre-exposure times (24, 48, 72 and 96 h). Fv/Fm data fitted to a first order exponential decay function. Error bars represent the standard error of the mean obtained in two independent experiments with one biological replicate each.

¹hUVR = 24 ± 6 W/m² UVA, 2.5 ± 1 W/m² UVB at 250 ±1 µmol m⁻² s⁻¹ photosynthetic active radiation (PAR)
²vhUVR = 28 ± 8 W/m² UVA, 5.3 ± 2 W/m² UVB at 265 ±3 µmol m⁻² s⁻¹ PAR
3.4.4 Co-tolerance of UVR pre-exposed algae to chemicals

To test whether pre-exposure of algae to UVR had an impact on the tolerance of algae to chemical stressors, reproduction, survival and photosynthesis of 48 h pre-exposed and control algae was determined for rose bengal (RB, 0.75-10 µM), paraquat (PQ, 0.007-0.4 µM) and diuron (DR, 0.009-1 µM). All chemicals caused concentration-dependent inhibition of algal reproduction (Figure 3.5) and photosynthesis (Figure 3.6). Corresponding EC50 are presented in Table 3.2. After 6 h, all chemicals completely inhibited photosynthesis at the highest tested concentrations. In case of PQ, toxicity to photosynthesis increased in control cultures over time, as indicated by decreasing EC50s for ΦPSII from 0.23 µM at 1 h to 0.027 µM at 6 h, whereas toxicity of RB and DR changed little over time (Table 3.2). Exposure of control algae to RB and PQ for 3 h were lethal at concentrations of 3.2 µM and 0.24 µM, respectively, whereas there was no visible effect of diuron on the survival of C. reinhardtii at all tested concentrations (Figure 3.5D).

Table 3.2: Mean EC50 with 95 % CI derived from concentration response curves (see Figure 3.5 and 3.6) for rose bengal, paraquat and diuron for the endpoint effective (ΦPSII) and maximum (Fv/Fm) yield of photosynthesis after 6 h of exposure and growth over 24 h, tested in C. reinhardtii pre-cultured for 48 h in absence (controls) or presence of ultraviolet radiation (UVR). Values calculated from three independent experimental replicates.

<table>
<thead>
<tr>
<th></th>
<th>Rose Bengal</th>
<th>Paraquat</th>
<th>Diuron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl</td>
<td>UVR</td>
<td>Ctrl</td>
</tr>
<tr>
<td>ΦPSII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.95</td>
<td>2.45***</td>
<td>0.027</td>
</tr>
<tr>
<td>(1.87-2.04)</td>
<td>(2.32-2.59)</td>
<td>(0.026-0.028)</td>
<td>(0.032-0.038)</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.08</td>
<td>3.29***</td>
<td>0.031</td>
</tr>
<tr>
<td>(1.99-2.17)</td>
<td>(3.17-3.42)</td>
<td>(0.029-0.032)</td>
<td>(0.033-0.039)</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.02</td>
<td>2.92***</td>
<td>0.025</td>
</tr>
<tr>
<td>(1.93-2.11)</td>
<td>(2.76-3.09)</td>
<td>(0.023-0.027)</td>
<td>(0.021-0.027)</td>
</tr>
</tbody>
</table>

ΦPSII and Fv/Fm after 6 h exposure; growth determined after 24 h exposure
In UVR pre-exposed algae, sensitivity to RB was reduced compared to control algae, indicated by significant right shifts of corresponding concentration response curves for reproduction (Figure 3.5A), photosynthesis (Figure 3.6) and significantly higher EC50s for these endpoints (Table 3.2). Moreover, UVR pre-exposed algae survived exposure to RB concentrations (4.2 and 5.6 µM) that were lethal in control cultures. Tolerance of *C. reinhardtii* to RB was also increased after a shorter UVR pre-exposure time of 3 h, and was comparable to the tolerance to RB after 48 h of UVR pre-exposure (Figure S8), indicating fast induction of co-tolerance to RB. However, when 48 h UVR pre-exposed algae were cultured for 24 h in absence of UVR, tolerance to RB was similar to the tolerance of control algae, as indicated by corresponding survival and concentration response curves for photosynthesis inhibition (Figure 3.7).

In case of PQ, there was no visible difference in the survival of PQ exposure in UVR pre-exposed and control cultures, and concentrations above 0.24 µM were lethal in both cases (Figure 3.5D). Furthermore, sensitivity of UVR pre-exposed and control algae to the effects of PQ on reproduction was similar, indicated by corresponding concentration response curves (Figure 3.5B) and EC50 values (Table 3.2). In case of photosynthesis, pre-exposed algae displayed a slightly reduced sensitivity to PQ compared to controls, indicated by small shifts of concentration response curves toward higher concentrations (Figure 3.6) and significantly higher EC50 values (Table 3.2).

In case of DR, UVR pre-exposed algae displayed a higher sensitivity of reproduction compared to controls, indicated by a significant left shift of the concentration response curve (Figure 3.5C), with a significantly lower EC50 of 0.35 µM in pre-exposed algae compared to 0.48 µM in control cultures. UVR pre-exposed and control algae displayed, however, comparable tolerance toward the inhibitory effects of DR on photosynthesis, indicated by corresponding concentration response curves (Figure 3.6) and EC50 values (Table 3.2).
Figure 3.5: Concentration-response relationships and associated 95% confidence intervals (CI [dashed lines]) for growth inhibition of *Chlamydomonas reinhardtii*, pre-cultured for 48 h in absence (controls) or presence of ultraviolet radiation (+UVR) and subsequently challenged for 24 h to increasing concentrations of rose bengal ([RB] A), paraquat ([PQ] B) and diuron ([DR] C). Error bars represent standard deviation of the mean obtained in three independent experiments, each prepared in duplicate. Aliquots were spotted onto TAP agar medium after 3 h of exposure to assess viability (D).
Figure 3.6: Concentration-response relationships and associated 95 % confidence intervals (CI [dashed lines]) for the inhibition of photosynthesis in *Chlamydomonas reinhardtii*, pre-cultured for 48 h in absence (controls) or presence of ultraviolet radiation (+UVR) and subsequently challenged to increasing concentrations of rose bengal (top row), paraquat (middle row) and diuron (bottom row). Photosynthesis as effective ($\Phi_{\text{PSII}}$) and maximum quantum yield (Fv/Fm) after 1 and 6 h of exposure to chemicals. Error bars represent standard deviation of the mean obtained in three independent experiments prepared in duplicate.
Figure 3.7: Concentration-response relationships and associated 95% confidence intervals (CI [dashed lines]) for the inhibition of the maximum quantum yield photosynthesis (Fv/Fm) in Chlamydomonas reinhardtii. Algae were first pre-cultured in absence (controls) or presence of ultraviolet radiation (+UVR), then cultured in absence of UVR for 24h before being exposed to increasing concentrations of rose bengal for 6 h. Error bars represent standard deviation of the mean obtained in three independent experiments, each prepared in duplicate. (A). Aliquots were spotted onto TAP agar medium after 3 h of exposure to assess viability (B).
3.5 Discussion

In the presented study, we systematically examined UVR acclimation in the alga *C. reinhardtii* and its impact on chemical toxicity. The aim was to better understand mechanisms of co-tolerance by linking changes in the tolerance of UVR-acclimated algae towards chemicals with known toxicity mechanisms.

3.5.1 Effects of UVR during long term exposure of algae

Selected conditions for UVR acclimation, which were similar to natural UVR regarding intensity, partially inhibited algal growth and photosynthesis during long term exposure for up to four days. The effects on algal growth were characterized by temporal dynamics. Reproduction in UVR exposed algae was almost completely inhibited on day one but returned to control levels on day two and four. The strong initial inhibition of reproduction can be explained by the inhibitory effect of UVR on cell volume growth, resulting in the delay of cell division, as shown in preliminary experiments (Figure S3) and as previously reported (Korkaric et al., Chapter 2). UVR induced DNA damage has also been reported as one reason for the inhibition of cell division (Buma et al., 2003).

Effects of UVR on photosynthesis of *C. reinhardtii* were also characterized by temporal dynamics. Effects to photosynthetic activities manifested quickly with the strongest inhibitory effect to both $\Phi_{PSII}$ and Fv/Fm after 3 h, reflecting the rapid photoinhibitory effects of UVR (Sfichi-Duke et al., 2008; Vincent and Roy, 1993). Our data indicate the action of different photo-inhibitory mechanisms during UVR pre-exposure. The reduction of Fv/Fm reflects slowly reversible photoinhibition (qI) which is mainly caused by damage to PSII and degradation of the PSII protein subunit D1 (Barber and Andersson, 1992; Jahns and Holzwarth, 2012). Reduction of $\Phi_{PSII}$ can also reflect, in addition to qI, fast reversible (during dark adaptation) non-photochemical quenching mechanisms (NPQ), typically induced under high-light conditions. Corresponding mechanisms are energy dependent quenching, state transition, and zeaxanthin dependent quenching that involves enzymatic de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin (for a review see Jahns
et al. 2012). The fact that the inhibition of ΦPSII was relatively stronger (55 % at 3 h) than the inhibition of Fv/Fm (38 % at 3 h) during the first hours of UVR exposure, most likely reflects a transient induction of one or several of these NPQ mechanisms as an early response to UVR. The later similar and constant inhibition of ΦPSII and Fv/Fm may reflect a fast established equilibrium of UVR damage as well as UVR defense and repair mechanisms (Heraud and Beardall, 2000), suggesting UVR acclimation during pre-exposure. The action of repair mechanisms, especially for PSII, is supported by the full recovery of photosynthetic activity within 24 h in absence of UVR (Figure S7)

3.5.2 Algae acclimate to UVR stress

Increased tolerance of pre-exposed cultures to higher UVR levels than applied during pre-exposure confirmed acclimation of algae. Increased tolerance to the photoinhibitory effects of high UVR (hUVR) was evident after 24 h and did not further increase as indicated by smaller rates of Fv/Fm decay in pre-exposed algae. Under even stronger UVR (vhUVR), photosynthetic activity was completely inhibited in all cultures within minutes, but the baseline fluorescence (F0) increased more slowly in pre-exposed algae and reached maximum values later compared to control. This indicates higher resilience of thylakoid membranes and PSII against UVR damage (Osmond, 1994; Vass et al., 1992). The increased tolerance to higher UVR was, however, lost when pre-exposed algae were cultured in the absence of UVR for 24 h (Figure S6). Together, these results support the view that UVR acclimation was mediated through rapidly inducible and reversible physiological and biochemical defense mechanisms. In addition to repair, known mechanisms against photoinhibition might include structural and functional alterations in PSII and accumulation of enzymatic and non-enzymatic antioxidants including photo-protective carotenoids to either prevent excessive ROS formation or to detoxify ROS that have formed as a result of UVR exposure (Krieger-Liszkay et al., 2008; Sfichi-Duke et al., 2008; Vincent and Roy, 1993).

In this study, increased UVR tolerance might have been partially conferred by UVR absorbing compounds, including carotenoids. Increased UV ratios in pre-exposed algae reflect higher cellular contents of UV absorbing compounds relative to the contents of Chl a, presumably reducing the amount of UVR that can affect sensitive targets, such as PSII (Navarro et al., 2007). However, due to a decrease of Chl a
content in UVR pre-exposed algae, it is not possible to infer on the contribution of UVR absorbing compounds to the increased UVR tolerance. HPLC pigment analysis showed that, in addition to Chl \( a \), also the cellular contents Chl \( b \), \( \beta \)-Car, cis-neoxanthin and violaxanthin decreased during UVR pre-exposure, which may reflect a UVR induced pigment destruction or reduced synthesis (Holzinger and Lutz, 2006). As expected for the non-saturating light intensities during pre-exposures, zeaxanthin was not detected in control algae and was also not detected in UVR exposed algae (Polle et al., 2001). Thus, the decrease in violaxanthin content in UVR exposed algae most likely reflects changes in the pool size, rather than UVR induced activation of zeaxanthin dependent NPQ. On the other hand, the cellular content of lutein was fast and markedly increased in UVR pre-exposed algae (by approximately 80 % from 24 h to 96 h), suggesting its role in UVR acclimation. Other than in higher plants, lutein, rather than zeaxanthin, was shown to be the most important xanthophyll for NPQ during high light stress in \( C. \) reinhardtii (Niyogi et al., 1997) and was also shown to increase in the green alga \( S. \) obliquus upon exposure to UV-B (Sfichi-Duke et al., 2008). The main photo-protective function of lutein is the quenching of excited triplet chlorophyll (\( ^3 \text{Chl} \)) and singlet oxygen (\( ^1 \text{O}_2 \)), which is mainly formed from \( ^3 \text{Chl} \) (Ramel et al., 2012; Triantaphylidès et al., 2008), suggesting that the generation of these excited species was increased under the selected UVR pre-exposure conditions.

### 3.5.3 Co-tolerance of UVR acclimated algae toward chemicals

UVR acclimated algae displayed an increased tolerance only towards rose bengal, despite the fact that all tested chemicals strongly inhibit photosynthesis and thus display a physiological target similar to UVR. These results suggest co-tolerance of algae to UVR and rose bengal, which might be explained by the induction of defense mechanisms during UVR pre-exposure that are effective in mitigating both the toxic effects of UVR and of rose bengal.

The toxicity of paraquat (PQ), diuron (DR), and rose bengal (RB) in control algae were comparable with data reported in the literature for the same alga (Fischer et al., 2010; Korkaric et al., 2015; Ledford et al., 2007; Nestler et al., 2012). In case of PQ, a slight co-tolerance in algae pre-exposed to UVR for 48 h was observed only during the first hours of the challenge exposure for the endpoint photosynthesis, but not for
survival and growth over 24 h. However, at early times also the photosynthetic activity was reduced in pre-exposed algae (Figure S7), lowering the flux of electron to PSI. Considering the mechanism of toxicity for PQ that involves the diversion of electrons from the photosynthetic electron transfer chain to molecular oxygen at the site of PSI, giving rise to superoxide radicals (·O$_2$) (Farrington et al., 1973), reduced electron flux might have resulted in an apparent co-tolerance. The presented data suggest that UVR defense mechanisms specific to ·O$_2$ detoxification, most importantly the superoxide dismutases (SOD), were not induced. This most likely reflects that excess ·O$_2$ generation is expected to occur especially at high UV-B levels (Agrawal et al., 2009; Tian and Yu, 2009). However, it cannot be excluded that ·O$_2$ was transiently produced and SOD transiently induced during UVR pre-exposure.

As in the case of PQ, no co-tolerance was detected for DR. Similar to UVR, DR specifically reduces the photosynthetic activity. This mechanism involves binding of DR to the D1 protein at PSII and the blocking of electron transfer from QA to QB (Dan Hess, 2000). In a previous study, DR was found to interact antagonistically with UVR in simultaneous exposures (Korkaric et al., Chapter 2). UV-induced modulation of the QB binding site at PSII, lowering its affinity towards DR, was discussed as a possible mechanism. Another explanation could be that binding of DR raises the midpoint potential of the QA/QA$-$ redox couple, which lowers the yield of $^{1}$O$_2$ production at PSII and thus oxidative damage (reviewed in Krieger-Liszakay et al. 2008). Tolerance mechanisms of plants and algae towards PSII inhibitors, such as DR, are not associated to antioxidative defense, but rather to the metabolic detoxification of the compound itself or mutational modifications of the PSII target (Gronwald, 1997; Hirschberg and McIntosh, 1983; Ryan et al., 1981). As expected, none of these mechanisms were induced during the UVR pre-exposure experiment.

Co-tolerance towards RB for all examined endpoints was detectable after 48 h of UVR pre-exposure and also after a shorter pre-exposure of 3 h. The toxicity of RB derives from its photosensitizing properties. RB transfers excitation energy to molecular oxygen, resulting in the formation of toxic $^{1}$O$_2$ (Allen et al., 1991). In chloroplasts, singlet oxygen is mainly detoxified by lipophilic antioxidants like α-tocopherol and carotenoids such as lutein (Triantaphylidès et al., 2008). In this study, cellular contents of lutein strongly increased after 48 h UVR pre-exposure, and to a
lesser extent after 3 h, suggesting that increased lutein levels might have allowed for a higher tolerance of pre-exposed algae towards RB induced $^1$O$_2$. However, induced tolerance to RB has also been observed in the absence of increased lutein levels in *C. reinhardtii* (Ledford et al., 2007), suggesting that additional mechanisms need to be considered. Other $^1$O$_2$ tolerance mechanisms might be related to the function of GPXH, a thioredoxin-dependent peroxidase and GSTS1, a glutathione-S-transferase (Fischer et al., 2009). In *C. reinhardtii*, the expression of GPXH and GSTS1 is rapidly upregulated upon exposure to RB (Fischer et al., 2005; Ledford et al., 2007) and UVR (Cheloni et al., 2014). Taken together, these studies and the present study provide evidence for a common toxic mechanism of UVR and RB, most likely the generation of $^1$O$_2$, and a common cellular response pathway in *C. reinhardtii*. As shown here, and before by Ledford et al. (2007), this response is fast and transient, as also supported by the observed loss of RB co-tolerance when UVR pre-exposed algae were cultured in absence of UVR for 24 h prior to RB challenge.

### 3.6 Conclusion

The co-tolerance to RB and the absence of co-tolerance to DR and PQ suggest a specific role of $^1$O$_2$ in UVR toxicity and of $^1$O$_2$ detoxification mechanisms in acclimation of algae to UVR. These results imply that for multiple stressor research knowledge of the physiological target of pollutants (e.g. photosynthesis) is not sufficient to predict co-tolerance, but rather detailed knowledge of the mechanism of toxic action is required.

### Acknowledgement

We would like to thank Eawag for funding and René Schönenberger and Adrian Ammann for help with HPLC measurements.
3.7 References

Agrawal, S., Singh, S., Agrawal, M., 2009. Ultraviolet-B induced changes in gene expression and antioxidants in plants. Advances in botanical research 52, 47-86.


Korkaric, M., Behra, R., Fischer, B.B., Junghans, M., Eggen, R.I., 2015. Multiple stressor effects in Chlamydomonas reinhardtii—towards understanding mechanisms of interaction between effects of ultraviolet radiation and chemical pollutants. Aquatic Toxicology.


Supporting Information Chapter 3

Acclimation of *Chlamydomonas reinhardtii* to ultraviolet radiation and its impact on chemical toxicity
Figure S1: UVR exposure setup used for UVR acclimation experiments. The same setup was used in the study reported in Chapter 2.
Figure S2: Experimental irradiation spectra in UVR pre-exposure (A) and UVR challenge exposure experiments (B) compared to the standard solar radiation energy spectrum at the earth surface (G173-03) of the American Society for Testing Standards (Reference). Detailed irradiation spectra in the UVR range between 280 and 400 nm (C).
Figure S3: (see previous page): Growth of *Chlamydomonas reinhardtii* cultured continuously for 36 h in absence (green) or presence (red) of ultraviolet radiation (UVR) at conditions corresponding to the UVR pre-exposure. Presented are mean cell numbers (A) and volumes of cells (B) at different time-points and size distributions of cells measured at different times from 0 to 36 h. Error bars represent standard error of the mean obtained from a single experiment with duplicates.

![Figure S3](image)

Figure S4: Representative chromatogram of acetone (90 % v/v) extracts of *C. reinhardtii* obtained using reverse-phase high performance liquid chromatography (HPLC). Absorption peaks correspond to loroxanthin (retention time (RT) on x-axis = 6.58 min), cis-neoxanthin (RT = 6.89 min), violaxanthin (RT = 7.97 min), lutein (RT = 11.49 min), trans-β-Apo-8′-carotenal ([internal standard] RT = 15.49 min), chlorophyll b (RT = 16.55 min), chlorophyll a (RT = 18.07 min) and β-carotene (RT = 22.85 min).
Figure S5: Standard curves for chlorophyll a, chlorophyll b, lutein, β-carotene, a-tocopherol and trans-β-Apo-8′-carotenal measured with reverse-phase high performance liquid chromatography (HPLC).
Figure S6: Maximum quantum yields of photosynthesis in *C. reinhardtii* exposed to 24 ± 6 W/m² UVA, 2.5 ± 1 W/m² UVB at 250 ± 1 mol m⁻² s⁻¹ photosynthetic active radiation (PAR) for 6 h. Algae were pre-exposed to UVR for 24 h (A), 48 h (B), 72 h (C) and 96 h (D) or cultured under control conditions for these times (Control) and subsequently cultured in absence of UVR under control conditions for 24 h. Single, unrepeated experiment.
Figure S7: Photosynthesis during 24 h recovery of *C. reinhardtii* in absence of UVR after 3 h or 48 h pre-exposure to UVR relative to effective quantum yield of photosynthesis (Φ_{PSII}) in previously unexposed control cultures. Mean values with 95% confidence intervals obtained in three independent experiments, each prepared in duplicate.

Figure S8: Concentration-response relationships and associated 95% confidence intervals (CI [dashed lines]) for the inhibition of the maximum quantum yield photosynthesis (Fv/Fm) in *Chlamydomonas reinhardtii*. Algae were cultured in absence (controls) or pre-exposed in presence of ultraviolet radiation (+UVR) for 3 h before being exposed to increasing concentrations of rose bengal for 6 h. Error bars represent standard deviation of the mean obtained in three independent experiments, each prepared in duplicate. (A). Aliquots were spotted onto TAP agar medium after 3 h of exposure to assess viability (B).
Table S1: Irradiation intensities applied in experiments of this study. Irradiation spectra are presented in Figure S2.

<table>
<thead>
<tr>
<th>UVR exposure</th>
<th>PAR mol m$^{-2}$ s$^{-1}$</th>
<th>UVA W/m$^2$</th>
<th>UVB W/m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>116 ±4</td>
<td>0.9 ±0.04</td>
<td>n.d.</td>
</tr>
<tr>
<td>UVR</td>
<td>124 ±5</td>
<td>18.1 ±1.8</td>
<td>0.77 ±0.1</td>
</tr>
<tr>
<td>challenge exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high UVR (%) increase</td>
<td>250 ±1</td>
<td>24 ± 6</td>
<td>2.5 ± 1</td>
</tr>
<tr>
<td>very high UVR (%)</td>
<td>265 ±3</td>
<td>28 ± 8</td>
<td>5.3 ± 2</td>
</tr>
</tbody>
</table>

n.d. = not detected

Table S2: One-Way analysis of variance (ANOVA) results for UVA and UVB rations (Fig. 3.2) and pigment contents) and results of Dunnett’s Multiple Comparison Test to examine differences of values during experimental time (3, 6, 24, 48, 72 and 96 h) with initial values (0 h) in control algae.

<table>
<thead>
<tr>
<th></th>
<th>One-way ANOVA</th>
<th>Dunnett’s Multiple Comparison Test</th>
</tr>
</thead>
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<tr>
<td></td>
<td>F</td>
<td>P value</td>
</tr>
<tr>
<td>UV ratios</td>
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</tr>
<tr>
<td>UVA ratio</td>
<td>3.209</td>
<td>0.029</td>
</tr>
<tr>
<td>UVB ratio</td>
<td>5.117</td>
<td>0.004</td>
</tr>
<tr>
<td>Pigments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>12.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>10.27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>10.39</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lutein</td>
<td>6.294</td>
<td>0.002</td>
</tr>
<tr>
<td>Loroxanthin</td>
<td>14.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>cis-Neoxanthin</td>
<td>0.9680</td>
<td>0.477</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>2.837</td>
<td>0.044</td>
</tr>
</tbody>
</table>

1) Not performed for antheraxanthin due to missing time zero value
2) Significance levels: ns (not significant) p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001
Table S3: Pigment content as per cent of control in UVR exposed *C. reinhardtii* cultures during UVR pre-exposure for 3 and 48 h. Results are shown as mean values with standard deviation.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Relative pigment content (%)</th>
<th>3 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>15 ±6</td>
<td>-15 ±1</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>19 ±6</td>
<td>-13 ±2</td>
<td></td>
</tr>
<tr>
<td>β-carotene</td>
<td>3 ±45</td>
<td>-21 ±2</td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>39 ±7</td>
<td>83 ±12</td>
<td></td>
</tr>
<tr>
<td>Loroxanthin</td>
<td>17 ±3</td>
<td>-7 ±13</td>
<td></td>
</tr>
<tr>
<td>cis-Neoxanthin</td>
<td>-0.7 ±4</td>
<td>-82 ±21</td>
<td></td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>28 ±8</td>
<td>-45 ±15</td>
<td></td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>n.d.</td>
<td>160 ±124</td>
<td></td>
</tr>
</tbody>
</table>

n.d. = not detected

Table S4: rate constant (K) of maximum quantum yield obtained from fitting yield data during challenge exposure to hUVR (see Fig. 3.4) to exponential decay function. $R^2$ indicates the goodness of the fit.

<table>
<thead>
<tr>
<th>UVR pre-exposure time</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>K [h⁻¹]</td>
<td>0.45</td>
<td>0.20</td>
<td>0.43</td>
<td>0.26</td>
</tr>
<tr>
<td>% of Ctrl</td>
<td>44 %</td>
<td>60 %</td>
<td>39 %</td>
<td>62 %</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.86</td>
<td>0.62</td>
<td>0.91</td>
<td>0.80</td>
</tr>
</tbody>
</table>
Table S5: Mean EC50 and hill slopes (p) with their 95 % CI derived from concentration response curves (see Fig. 3.5 and 3.6) for rose bengal, paraquat and Diuron tested in control cultures of *C. reinhardtii* and cultures pre-exposed to UVR for 48 h for the endpoint effective ($\Phi_{PSII}$)and maximum (Fv/Fm) yield of photosynthesis and growth over 24 h. Values calculated from four independent replicates.

<table>
<thead>
<tr>
<th>Time</th>
<th>Rose Bengal</th>
<th>Paraquat</th>
<th>Diuron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl</td>
<td>48 h UVR pre-exposed</td>
<td>Ctrl</td>
</tr>
<tr>
<td>1 h</td>
<td>$\Phi_{PSII}$</td>
<td>EC50 2.04</td>
<td>4.32* 0.23 0.47* 0.053 0.065*</td>
</tr>
<tr>
<td>6 h</td>
<td>$\Phi_{PSII}$</td>
<td>EC50 1.95</td>
<td>2.45* 0.027 0.035* 0.075 0.082</td>
</tr>
<tr>
<td></td>
<td>Fv/Fm</td>
<td>EC50 2.08</td>
<td>3.29* 0.031 0.036* 0.082 0.099</td>
</tr>
<tr>
<td>24 h</td>
<td>growth</td>
<td>EC50 2.02</td>
<td>2.92* 0.025 0.024 0.48 0.35*</td>
</tr>
</tbody>
</table>
Chapter 4:

Ultraviolet radiation dose calculation for algal suspensions using UVA and UVB extinction coefficients

Based on a version of the following published article:

4.1 Abstract

Although the biological importance of ultraviolet light (UVR) attenuation has been recognised in marine and freshwater environments, it is not generally considered in in-vitro ecotoxicological studies using algal cell suspensions. In this study, UVA and UVB extinction were determined for cultures of algae with varying cell densities, and the data were used to calculate the corresponding extinction coefficients for both UVA and UVB wavelength ranges. Integrating the Beer-Lambert equation to account for changes in the radiation intensity reaching each depth, from the surface until the bottom of the experimental vessel, we obtained the average UVA and UVB intensity to which the cultured algal cells were exposed. We found that UVR intensity measured at the surface of *Chlamydomonas reinhardtii* cultures led to an overestimation of the UVR dose received by the algae by 2 to 40 times. The approach used in this study allowed for a more accurate estimation of UVA and UVB doses.

4.2 Introduction

4.2.1 UVR measurement in ecotoxicological studies

Ozone decline during the 1980s and 1990s increased the amount of ultraviolet radiation (UVR) reaching the Earth’s surface (McKenzie et al., 2007), raising concern about the impacts of UVR on biological systems (Andrady et al., 2005). Since then, a huge research effort has focused on assessing and predicting UVR impacts on human and natural ecosystems at different biological scales (Andrady et al., 2005).

Because of the importance of algae and macrophyta as primary producers in aquatic environments, the effects of UVR effects on these organisms has been studied extensively (Navarro et al., 2007; Pessoa, 2012). In aquatic environments, numerous materials absorb and scatter light, contributing to vertical light attenuation. These include dissolved substances (Maloney et al., 2005; Scully et al., 2000), particles (Armengol et al., 2003; Piazena and Hader, 1994) and organisms (Kirk, 1994). Vertical light attenuation processes result in a decrease of the intensity and changes in the light spectrum. While light attenuation is routinely considered in ecological studies of UVR (V-Balogh et al., 2009), it is not often considered in ecotoxicity studies using algae or
other aquatic organisms (Dong et al., 2007; Roos and Vincent, 1998). In such studies, the responses of organisms are generally related to doses of UV that are calculated using measurements of UV intensity at the surface of experimental vessels or liquid media. While UVR extinction may be irrelevant under certain conditions (i.e., low cell densities, low depth of experimental vessels and high media transparency), the cell concentrations required for laboratory studies are expected to significantly attenuate UVR intensity (Fujiki and Taguchi, 2002a; Kirk, 1975a). Clearly, in that way, doses received by algae cells during UVR exposure can be overestimated, leading researchers to conclude that the observed effects occur at lower doses than the real ones (Gaberscik et al., 2002; Germ et al., 2002; Helbling et al., 2006; Hodoki and Ohbayashi, 2005; Martinez et al., 2000).

### 4.2.2 Integrative approaches for PAR modelling and UVR extinction assessment

Standardised procedures have been proposed for calculating the vertical attenuation coefficients for photo-synthetically active radiation (PAR) models based on phytoplankton suspensions (Kirk, 1975a, b). In the case of UVR, recent studies have demonstrated that simple laboratory measurements allow for establishing reliable relationships between concentrations of optically active substances (such as chlorophyll, dissolved organic matter and total suspended solids) and the underwater UVR light climate in natural systems (V-Balogh et al., 2009).

These approaches are based on the theoretical assumption that light passing through a dilute suspension of cells should obey Beer-Lambert’s law (V-Balogh et al., 2009). Accordingly, optical density is proportionate to the number of cells (Duysens, 1956; Stokes, 1975). Nonetheless, other factors, such as cell size, cell shape and intracellular pigment concentration, can modify light attenuation in a cell suspension (Kirk, 1975a, b). Even if the major factors that determine light extinction characteristics in cell suspensions are identified, the specific absorption of light is not a linear function of either pigment concentration or cell size (Fujiki and Taguchi, 2002b). The non-linearity of light absorption results from the “package effect”, which represents the decreased light absorption of pigments contained in particles relative to the absorption of the same pigments in solution (Kirk, 1975a). Nevertheless, studies of various
phytoplankton species have shown significant relationships between cell size and volume and light absorption, indicating that morphological and population parameters may be useful for developing improved models that link biological and optical properties (Fujiki and Taguchi, 2002a). At present, there is a lack of methodological approaches for laboratory studies that consider UVR extinction.

4.2.3 Objectives of the study

The goal of this study was to improve the measurement of UVR intensity in algal cultures under agitation (assuming that the cells would be moving throughout the entire medium in the vessel) by calculating UVR extinction coefficients. A spectroradiometer that provides a spectral power distribution (power per unit area per unit wavelength) was used. We also tested a radiometer equipped with simpler UVA and UVB integrative sensors. UVA and UVB extinction were measured using different algal densities of two strains of *Chlamydomonas reinhardtii* and *Synechocystis sp.* to test the robustness of our proposed approach. The extinction coefficients ($k$) for UVA and UVB were calculated and modelled as a function of OD (the optical density, a proxy for number of cells). We demonstrated that our approach was a rapid method for using light absorption to estimate $k_{\text{uva}}$ and $k_{\text{uvb}}$ with algal cell suspensions.

4.3 Material and Methods

4.3.1 Algal cultures

The experiments were performed in various labs over various time periods, using the cultures available at each lab: *Chlamydomonas reinhardtii*, CC-125 (Chlamydomonas Resource Center, Univ. Minnesota, MN 55108, USA), and 137C+ 83.81 (Institute for Plant Physiology (University of Göttingen, Germany). The two strains of this eukaryote algae showed differences in a few genome sequences (Maul et al., 2002). The culture medium was prepared according to the procedures described by Le Faucheur et al. (Le Faucheur et al., 2005). The algae were grown in a HT Multitron (Infors, Bottmingen, Switzerland) at 25°C with continuous illumination of 120 μMol photons sec$^{-1}$ cm$^{-1}$ (Philips Coolwhite TLD 15 W fluorescent lamps) and shaken at 90 rpm. For cell number and cell volume measurements, a 200 μL sample of the cell suspension was added to a final volume of 4 mL Isoton II diluent solution (Beckman Coulter) and
then counted using a Z2 Coulter Particle Counter (Beckman Coulter, Nyon, Switzerland) within the 2.7-10.64-μm size range.

Table 4.1: Details of the six experimental setups (first column). UVR refers to the type of lamp used: HTC for halogen and fluo for fluorescence tubes; Measu. indicates the device used: spectro for the spectroradiometer and radio for the integrative radiometer; algae indicates the species; Vessel indicates the type of vessel used for measuring UVA/UVB extinction; Exp. indicates the use of different cultures (real replicates) and the sequence in which the experiments were performed; OD(750) indicates the optical density at 750 nm; and the k_uva, k_uvb and their respective r² values are also shown (k units are cm⁻¹). Also exhibited is the chlorophyll a concentration for selected experiments, expressed both as chlorophyll per volume (chl a) and as chlorophyll per cell (pg chl cell⁻¹). These last values have been calculated using the values obtained using the cell coulter (data not shown). The last two columns show the Z values at which 99% of the incident UVA and UVB have been attenuated. The last two rows in the table show the models used for calculating k as a function of OD by the method described in Chapter 4.4.2.

<table>
<thead>
<tr>
<th>UVR</th>
<th>Measu.</th>
<th>Algae</th>
<th>Vessel</th>
<th>Exp.</th>
<th>OD(750)</th>
<th>K_uva</th>
<th>r²</th>
<th>K_uvb</th>
<th>r²</th>
<th>pg chl cell⁻¹</th>
<th>Z₉₉% UVA</th>
<th>Z₉₉% UVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>fluo spectro</td>
<td>CC125 boro</td>
<td>1</td>
<td>0.5895</td>
<td>1.247</td>
<td>0.87</td>
<td>1.088</td>
<td>0.99</td>
<td>2.04</td>
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<tr>
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Models for k_uva

\[ k = 2.01 \cdot \text{OD}_{750} - 0.09 \quad (r^2 0.97) \]

Models for UVB

\[ k = 5.36 \cdot \text{OD}_{750} - 0.19 \quad (r^2 0.70) \]

\[ k = 0.18 \ln(\text{OD}_{750}) + 0.56 \quad (r^2 0.91) \]

\[ k = 0.31 \ln(\text{OD}_{750}) + 0.79 \quad (r^2 0.96) \]
Figure 4.1: Distribution of spectral power of the UV lamps used in this study. At left: ARIMED fluorescent lamps (showing the 260-400 nm range). At right: OSRAM HTC bulbs (showing the 250-450 range and the UVB region, in black). See also Figure 2.1 and Figure S2 in Chapter 3.

Synechocystis PCC 6803 wild type (Cyanophyta) was obtained from the Pasteur culture collection (Institute Pasteur, France). The culturing procedure was similar to that described for C. reinhardtii, but a culture medium (BG-11) was used (Kim et al., 2011) and the illumination was reduced to approximately 40 μMol photons sec⁻¹ cm⁻¹. This photosynthetic prokaryote was selected to test the reliability of the experimental approach with smaller sized algae. The word algae will be used throughout this text to refer to both the prokaryote and the eukaryote species.

Experimental batch cultures were prepared by transferring an inoculum of algae in the exponential growth phase at a starting density of approximately 6×10⁵ cells mL⁻¹. Cell densities (see details in Table 4.1) were obtained by centrifuging (3000 rpm, 10 min) the experimental batch cultures and then resuspending the cell pellet until the desired density was reached.

For the estimation of the chlorophyll a, algal cells from sampled aliquots were centrifuged at acceleration 16,000 x g, resuspended in 80% cold acetone and incubated for 5 min on ice in the dark. Following another centrifugation step, the supernatants were transferred into a transparent 96-well plate and absorption was measured at wavelengths 750, 663, 647, and 470 nm for calculating concentrations according to a previous development (Nestler et al., 2012).
4.3.2 UVR intensity measurements

Two different systems were used. The first system consisted of a borosilicate beaker placed under a fluorescent lamp (Arimed B, 40 W; Cosmedico Light, Germany) with an emission maximum between 330 and 350 nm (Figure 4.1). Because borosilicate is transparent to UVR radiation, the beaker walls were wrapped with black tape to prevent UVR light from entering from a lateral surface. The spectroradiometer (JAZ-EL 200, Ocean Optics, 830 Douglas Ave., Dunedin, FL, USA) was placed under the beaker and a full spectrum scan was recorded that represented the UVR intensity on the bulk surface. The beaker was then filled step-by-step with the appropriate volume of algal culture (of a certain cell density), such that with each step the culture depth (z) increased by 1 cm. UVR intensity was recorded after a few seconds (3-5 s), as soon as the UVR values had stabilised and prior to sedimentation of the cells. New aliquots were added using a 5 mL pipette, which provided sufficient flow to mix the entire liquid column. This assured a homogeneous light pathway for UVR from the surface of the liquid to the sensor in the bottom of the beaker for each sample. This method had been previously applied successfully to measure irradiance extinction in a volume of water that was too shallow to use a radiometer (V-Balogh et al., 2009) by placing a “vessel” on top of a sensor and repeatedly refilling the vessel with water.

Each recorded spectrum was integrated at a radiant flux density (Watt m⁻²) in the range of UVB (280-320 nm) and UVA (320-400 nm) and plotted against z (Figure 4. 2) using Ocean Optics Spectrasuite™ software. The ranges for energy integration were chosen to fit the ranges of the integrative sensors of Solar Light that were used in the second measuring system.

The second system used to quantify UVR intensity consisted of a PMA2100 Radiometer (Solar Light Co., Oak Line, USA) equipped with two sensors (a UVA-2110 WP sensor integrating energy in the 320-400 nm wavelength range and a UVB-2106 WP sensor integrating energy in the 280-320 nm range). The sensors were placed 55 cm away from a UV lamp equipped with either an Osram HTC400-241 bulb (Figure 4.1) or fluorescent tubes (Arcadia D3 Reptile Lamp T5, 39 W, 12% UVB, 30% UVA). The algal suspensions were added to the upper part of a 50-mL Uthermöl chamber fixed over the UVR sensors. The upper part of the chamber was a tube (95 mm in length, 25 mm in diameter) made with plastic that was opaque to UVR wavelengths. A bit of silicon was used around the bottom of the tube where the tube made contact with
the glass surface of the sensors to avoid leaching of the cell suspension. UVR intensity measurements were made before sequentially adding 5-mL suspension aliquots to completely fill the column. Each aliquot increased the height of the suspension column by 1 cm, thus allowing UVA and UVB intensity data to be plotted as a function of depth.

**Figure 4.2:** Diagram showing measurement setup and data processing. The method was applied to standardised algal cultures (1). A dilution bank of the cultures is prepared (2). Extinction coefficients are measured by sequentially adding fixed volumes of each algal dilution to a column exposed to UVR (3). Each time additional volume is added to the column, the depth of the column increases, and UVA and UVB intensity is recorded. The resulting UVR intensity data are plotted against depth and the Beer-Lambert equation is used to obtain the extinction coefficient ($k$) for UVA and UVB (4). The $k$ values were used to calculate the average intensity of UVR received by a cell suspension (6). It is also possible to plot all $k$ values calculated as a function of the number of cells, or OD (5) and then fit these values to an appropriate model. The model could be used to estimate the $k$ of a given algal suspension.
4.3.3 Experimental design

To test the robustness, feasibility and usefulness of this new methodological approach, various experimental setups that combined different algal strains or species and measuring systems were tested (Table 4.1). To examine the role of cell density in UVR extinction, three different (i.e., independently developed) cultures of *C. reinhardtii* CC125 at varying cell densities were exposed to UVR using the fluorescent tubes previously described. UVR extinction was measured in a borosilicate beaker using a spectroradiometer (Exp. 1, 2 and 8). See details of the whole experimental setup in Figure 4.2. To assess the influence of different UV measuring devices on extinction calculations, an integrative radiometer was used (Exp. 7). *C. reinhardtii* (137C+ 83.81) was used in Experiments 3, 4, 5 and 6). UVR extinction was measured in a Uthermöl chamber using HTC lamps and the integrative radiometer. The potential effects of using different UVR lamps, measuring devices and vessels were tested in Experiment 9. Finally, to test the influence of smaller-sized algae on UVR extinction, *Synechocystis PCC 6803* wild type (Institute Pasteur, France) was used (Exp. 10). In this case, UVR extinction was measured in borosilicate beakers using the PMA2100 Radiometer.

4.3.4 Statistics and modelling

The UVA and UVB extinction data from all experiments were adjusted to the Beer-Lambert equation (Equation 1) using Sigma Plot 12.5 (Systat Software Inc., San Jose, USA). Excel was used for fitting the linear models. To assess the range of cell density for which our approach was most robust, $r^2$ squared modelling terms were used (see Figure 4.3C).

$$I_z = I_0 \cdot e^{-zk}$$

*Equation 1:* Beer-Lambert’s equation of light extinction. $I_z$ represents the light intensity at depth $z$ (cm); $I_0$ is the intensity at the surface of the bulk suspension; and $k$ the extinction coefficient.
Figure 4.3: Graph A shows UVA extinction as a function of depth for four different cell densities. Values at 0 cm represent UVR intensity on the bulk surface. Graph B shows the correlation between OD$_{750}$ and number of cells. The data showed a good fit ($r^2 = 0.94$) to a polynomial cubic equation (OD = $-0.0035 + 2.8E-7$ cells $- 4.2E-14$ cells$^2 + 2.8E-21$ cells$^3$). Numbers (1 to 9) represent the different experiments (see Table 4.1). The black line represents the fitted model, and the long dashed grey lines are the 95% confidence intervals for that model. Graph C represents the $r^2$ fitted values of the Beer-Lambert equation for different values of OD (used as a proxy for cell density). The black dotted horizontal line represents $r^2 = 0.95$. White points represent $k_{UVB}$ values and black points represent $k_{UVA}$ values, regardless of the device used for measurement. The black line is the adjusted curve ($f(x) = y_0 + (a/x) + (b/x^2)$ with a $r^2$ of 0.75), with the 95% confidence intervals used as the criteria for selecting the applicability range of the method.
4.3.5 UVA and UVB extinction coefficients

The coefficients $k_{\text{uva}}$ and $k_{\text{uvb}}$ were estimated by using the UVA and UVB intensity values from all experiments in the Beer-Lambert equation. All $k$ values are shown in Table 4.1. Later, the resulting UVA and UVB extinction coefficients were represented as a function of the OD$_{750}$ of the cell suspension (used as a proxy of cell density) and adjusted to a polynomial equation (see details in Figure 4.3B). This was designed to eventually allow for the estimation of extinction coefficients based on the OD$_{750}$ of the cell suspensions (details in Chapter 4.4.2).

4.3.6 Calculating average UVA and UVB intensity received by algal cultures

The Beer-Lambert equation, integrated from depth 0 to $m$ (expressed in cm, with $m$ being the total depth of the solution), was used to calculate the average energy received by an algal cell in a cell suspension presenting a certain $k_{\text{uva}}$ or $k_{\text{uvb}}$ and exposed to $I_0$ (i.e., UV intensity) at the surface of the liquid (Equation 2). The resulting value (intensity integration) was divided by depth (expressed in cm to maintain homogeneity in the units). This value (the average UVR intensity received by a cell in the suspension) was multiplied by exposure time to obtain the UVR dose received by the algal cells.
Equation 2. The Beer-Lambert equation integrated for depths 0 to \( m \) (expressed in cm, with \( m \) being the total depth of the solution) to allow for calculating the average energy received by each cell in a given cell suspension with the coefficient \( k_{\text{uva}} \) or \( k_{\text{uvb}} \) and a UV intensity \( I_0 \) at the surface of the liquid.

### 4.4 Results and discussion

#### 4.4.1 UVR extinction coefficients

UVB and UVA radiation intensity decreased exponentially with increasing culture depth in all experiments (Figure 4.3A), following the Beer-Lamberts equation (Eq. 1). This behaviour was independent of the device used to measure UVR, cell density or the type of vessel used. The extinction coefficients \( k_{\text{uva}} \) and \( k_{\text{uvb}} \) showed a linear relationship with cell density (measured as OD\(_{750}\)), as shown in Figure 4.4. Accordingly, the highest \( k_{\text{uva}} \) and \( k_{\text{uvb}} \) values were obtained for the suspensions with the highest cell density (Table 4.1).

Values for \( k_{\text{uva}} \) ranged from 0.0076 to 1.86 cm\(^{-1}\), whereas \( k_{\text{uvb}} \) ranged from 0.010 to 3.79 cm\(^{-1}\). The higher values of UVB extinction compared to UVA extinction were
consistent with the weaker absorption of UVA by biomolecules present in cells (Tyrrell and Keyse, 1990). The $k$ values determined in our study were in the range of those determined for water with comparable concentrations of chlorophyll a. The $k$ value for wavelengths under 400 nm was between 1-16 m$^{-1}$ (Leech et al., 2005) for a freshwater lake, between 1-2 m$^{-1}$ for water with a chl-a concentration of 0.5-0.6 µg l$^{-1}$ and approximately 1 m$^{-1}$ for water with a chlorophyll a concentration of 0.3 µg l$^{-1}$ (Novales-flamarique et al., 1992). In our study, cell suspensions with chlorophyll concentrations between 0.4-0.8 µg l$^{-1}$ had a $k_{\text{UVA}}$ between 2 and 4 m$^{-1}$.

Figure 4.4. Extinction coefficients for UVA (A1 and B1) and UVB (A2 and B2) calculated using a spectroradiometer (A1 and A2) or an integrative radiometer (B1 and B2) and represented as a function of the OD. Numbers represent the different experiments (see Table 4.1). For the purposes of comparison, the linear models of A1 and A2 are shown as lines in B1 and B2.
The use of different UVR measuring devices affected the dose calculations, depending on the UV range selected (see Figure 4.4). As shown in Figure 4.4, the $k_{uva}$ values were quite similar, regardless of the device used. The cultures that clearly differed from the others in terms of UVR dose (Cultures 5 and 6) were those with the highest cell densities. These differences can be attributed to the fact that the sensors on the integrative spectroradiometer only record part of the incident energy (approximately 64%). This effect was even more apparent for UVB radiation than for the UVA range. For these cultures, $k_{uvb}$ was between 30 and 80% higher than $k_{uva}$. This difference reflected the higher extinction of UVB (i.e., lower penetration in the water column) in the cell suspensions (see $k$ values in Figures 4.4-A1 vs. A2).

Regarding the application of this approach to differently sized algae, Figure 4.4 shows that extinction coefficients from experiment 10 (using *Synechocystis* sp.) exhibit similar values and trends to other experiments using *Chlamydomonas* and the same measuring setup (exp. 10). The content of pigment per cell (Table 4.1) did not show any kind of influence on the extinction coefficients; correlations resulted in $r^2 = 0.03$ for $k_{uva}$ and 0.01 for $k_{uvb}$.

For long-term UVR exposure, time-dependent variability in $k$ is an important factor to consider. High UVR intensities may cause bleaching of cell pigments or cell death, thereby modifying $k$ values. Therefore, when using the proposed method for long-term studies, time-dependent variation in $k$ should be assessed and integrated into the calculations. In our case, exposures up to two hours did not modify the $k$ values (results not shown).

### 4.4.2 Assessing the cell range for which OD750 is a reliable estimator of $k_{uva}$ and $k_{uvb}$

Experiments focusing on the effects of UVR on algal cultures use homogeneous algal populations that have similar physiological states, pigment contents and size. Based on this practice and the previously described rationale for PAR studies (see Chapter 4.2.2), UVR extinction under controlled conditions would vary only with cell density or any of its proxies, such as OD$_{750}$ (see Figure 4.4). Therefore, data for $k$ and OD$_{750}$ have been fitted to a polynomial equation (see details in Figure 4.3B). Eventually, this
model could allow for the estimation of extinction coefficients based on the OD\textsubscript{750} of a cell suspension.

The $r^2$ values for all extinction curves were represented as a function of the OD\textsubscript{750} and fitted to a polynomial inverse second order equation (see details in Figure 4.3). This approach identified the OD values for which the experimental data showed a good fit to the Beer-Lambert model. That values ranged from OD\textsubscript{750}$= 0.07$(defined the by the intersection of the $r^2=0.95$ and the upper confidence interval of the polynomial model) to OD\textsubscript{750}$=1$. At these elevated OD, extinction curves start worsening the fitting to Beer-Lambert model. That OD range corresponds to a cell density range of 2.5E5 to 3.2E6 cells mL$^{-1}$ \textit{(C. reinhardtii)}. Finally, we noted that the lowest cell densities showed a poor fit to the Beer-Lambert equation (i.e., lower $r^2$ values). This point will be further discussed below in “Practical considerations” at 4.4.4.

\subsection*{4.4.3 Comparison of UVR doses calculated using different approaches}

We calculated the UVR dose received by a cell suspension as a function of cell density using three methods: a) measuring the UVR intensity reaching the surface of the cell suspension, b) calculating $k_{\text{uva}}$ and $k_{\text{uvb}}$ using a spectroradiometer (more accurate) and c) calculating $k_{\text{uva}}$ and $k_{\text{uvb}}$ using a radiometer (less accurate). UVR intensity reaching the cell suspension was 9.54 and 1.56 mW cm$^{-2}$ for UVA and UVB, respectively, for 1 hour in a beaker (10 cm depth). For these calculations, we used data from Experiment 1 and Experiment 2 (see Table 4.1 for details), and the doses are shown in Figure 4.5. Without considering UVR extinction, UVR dose was not dependent on cell density (black points on Figures 4.5A and 4.5B). In contrast, UVR dose based on $k$ was dependent on cell density and had lower values. UVA dose was overestimated by 1.4 to 20 times, depending on the cell density. The overestimation was higher for the highest cell densities. UVB dose was overestimated by 1.8 to 39 times. Even if the radiometer measurements underestimated UVR dose compared to the spectroradiometer measurements, the values obtained with the radiometer method provided a more realistic estimate of UVR does than the estimates obtained by measuring UVR intensity at the surface of the liquid.
Figure 4.5: Comparison of UVA (A) and UVB (B) calculated doses received by algal suspensions of varying cell densities based on different approaches. Surface measurements (surf.) are compared to methods based on $k$ calculations using either a spectroradiometer (spect.) or radiometer (rad.). Results are represented as a function of the OD and cell density.

4.4.4 Practical considerations

At high cell densities, UVB radiation fell below the detection limit at $z > 1$; and the extinction coefficients were calculated using only two data points ($z = 0$ cm and $z = 1$ cm). Accordingly, in these cases almost all UVA and UVB light (99%) was attenuated within the first centimetres ($z < 10$ cm) of the suspensions (see details in Table 4.1, last
two columns). It is thus important not to use cell densities higher than those leading to the complete UVR attenuation.

Another effect observed with the beakers was that at \( z > 4 \), UVA and UVB intensities slightly increased. This was due to the “lense effect” of the beaker walls, but could only be observed under very transparent media conditions, such as in the samples with very low cell densities. This may also help explain the poor fit of the data to the Beer Lambert equation for these samples. When the beaker was almost full, more light was diverted toward the sensor that, at a lower culture depth, would have been absorbed by the walls of the beaker (or more likely, by the black tape with which the beaker was wrapped). For that reason, some of the data points were excluded from the model fitting. This effect would most likely be specific to each particular experimental setup, depending on the distance of the lamp from the vessel and the type of vessel used. For this reason, and the differences that may arise from using the different UVR measuring devices in the market, it is always necessary to calibrate the experimental setup used (see Chapter 4.4.1).

The similarity between \( k \) values calculated in this study and those measured in real environments showed that our approach as described above provides much more realistic calculations of UVR dose than those based on UVR intensity at the surface of the liquid. The accuracy of \( k \) calculations may be improved by (i) considering sensor shape corrections (e.g., the use of common plane sensors versus the spherical sensors used in open waters), and/or (ii) by applying calibrations for the different media encountered by the light pathway from the lamp to the sensor. With such improvements, the differences between \( k \), determined in the laboratory and that determined in the real environment should then be small. Nevertheless, the simplicity and affordability of the our approach, and its robustness (shown using different measuring setups, different algal species and different pigment concentrations) result in more precise UVR dose response calculations, allowing for comparing results among various natural and laboratory studies. This experimental approach would also be useful for cell suspensions other than photosynthetic ones.
Acknowledgments

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4.5 References


Chapter 5:

Outlook
5.1 Outlook

Multiple stressor effects of UVR and chemical pollutants in aquatic organisms have not been studied extensively. This thesis demonstrates that UVR and chemical pollutants do not generally act independently on \textit{C. reinhardtii}, indicating effect interactions. Moreover, \textit{C. reinhardtii} was shown to acclimate to UVR in part by modulating cellular carotenoid contents. The detected co-tolerance of UVR acclimated algae toward rose bengal indirectly suggests $^{1}\text{O}_2$ defense as a mechanism of UVR acclimation and co-tolerance. Still, questions regarding the mechanisms of effect interactions and co-tolerance remain.

\textit{Why were UVR acclimated algae not co-tolerant to paraquat?}

Co-tolerance to paraquat was expected based on evidence in the literature that antioxidant enzymes play a role in UVR defense in plants and algae (Bhargava et al., 2007; Vincent and Roy, 1993) and are the primary mechanism of paraquat resistance (Ye and Gressel, 2000). The UVR pre-exposure condition is most likely the reason for the absence of co-tolerance to paraquat. Studies that report increased antioxidant enzyme activity mostly applied higher UVR intensities, especially in the UVB range, which is likely to increase the generation of ROS (Hideg et al., 2013). Moreover, exposure time might also be a crucial factor. Tian et al. (2009) showed that enzyme activities in \textit{Dunaliella salina} were only temporarily induced, whereas levels of non-enzymatic antioxidants remained high throughout prolonged UVR exposure. Thus, co-tolerance patterns could be examined following pre-exposures under UVR conditions that vary regarding the applied UVR intensity and exposure time. Ideally, such experiments would be complemented by the quantification of intracellular ROS (especially $^{1}\text{O}_2$ and $\text{O}_2^-$) and enzyme activities (especially SOD and APX).

\textit{Predictability of combined effects of UVR and chemicals by the concentration addition model}

In this work, the \textit{independent action} (IA) model used to analyse experimental data provided strong indications for effect interactions of UVR and chemicals. In the case of chemical mixtures with unknown MOA, interactions are usually assumed when mixture toxicity deviates from both IA and \textit{concentration addition} (CA) predictions. Thus, in a next step, interactions could also be examined by using the CA model. The dilution principle behind CA cannot directly be applied to non-chemical stressors such
as UVR, but this limitation can be overcome by a toxic equivalence approach as demonstrated by Fischer et al. (2010). This would require the experimental determination of full UVR dose response relationships for UVR for the endpoints of interest. Chemical toxicity would need to be tested at each of the selected UVR intensities. From these experiments, EC$_{50}$ for each tested chemical at each UVR intensity and the effective values (EV) for UVR and thus toxic units (for EV$_{50}$) are calculated. Finally, a 50% effective-values isobole would be calculated for each endpoint and compared with the measured data to analyse effect interactions according to CA (Fischer et al., 2010). The required data could conveniently be obtained from the experiments proposed above. In addition, one could attempt to apply the dilution principle of CA by considering concentrations of intracellular ROS that are produced by UVR and selected chemicals alone and in combination.

How can we dig deeper into the mechanisms of UVR and chemical effect interactions?

This work shows that knowledge of toxic mechanisms and defense mechanisms is needed for the understanding of multiple stressor effects of UVR and chemicals, UVR acclimation and co-tolerance. Molecular approaches, such as targeted gene expression analysis by qPCR, could provide a more direct and detailed insight into the corresponding mechanisms, e.g. the regulation of antioxidant pathways. This was recently done for a set of genes involved in the antioxidant defense of *C. reinhardtii* to investigate multiple stressor effects of high light (including UVR) and copper (Cheloni et al., 2014). Non-targeted transcriptomic approaches (e.g. using microarray or RNA-sequencing) may prove even more useful because they would in addition allow the examination of other response pathways, or unique stress responses under multiple stressor conditions that cannot be extrapolated from the response under single stressor conditions. For example, UVR and paraquat may jointly deplete energy resources and thus cause adverse effects in pathways which are not detectable in individual UVR and paraquat exposures. Gene expression profiles in *C. reinhardtii* have been obtained for all stressors examined in this work, except for S-metolachlor and Diuron, but only in single exposures with UVR (Barthelemy, 2014), rose bengal (Fischer et al., 2005), paraquat (Jamers and De Coen, 2010), cadmium (Simon et al., 2008). The application of transcriptomic approaches to multiple
stressors could benefit from the existing conceptual framework that was developed for their application to the study of mixture toxicity (Altenburger et al., 2012).

5.2 References


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