Effects of UV-C and Vacuum-UV TiO2 Advanced Oxidation Processes on the Acute Mortality of Microalgae

Eric McGivney[*†1,], Magnus Carlsson[2], Jon Petter Gustafsson[1.3], Elena Gorokhova[4*]

[1]Division of Land and Water Resources Engineering, KTH Royal Institute of Technology, Brinellvägen 28, 100 44 Stockholm, Sweden

[2]Wallenius Water AB, Franzegatan 5, 112 15 Stockholm, Sweden

[3]Department of Soil and Environment, Swedish University of Agricultural Sciences, Uppsala, Sweden

[4]Department of Environmental Science and Analytical Chemistry, Stockholm University, Stockholm, Sweden

*Corresponding author emails: emcgivne@andrew.cmu.edu (Eric McGivney); elena.gorokhova@aces.su.se (Elena Gorokhova)

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Eric McGivney[1‡]*, Magnus Carlsson[2], Jon Petter Gustafsson[1], Elena Gorokhova[3]*

[1]Department of Land and Water Resources Engineering, KTH Royal Institute of Technology, Brinellvägen 28, 100 44 Stockholm, Sweden, [2]Wallenius Water AB, Franzegatan 5, 112 15 Stockholm, Sweden, [3]Department of Environmental Science and Analytical Chemistry, Stockholm University, 109 61 Stockholm, Sweden

*Corresponding authors' e-mail: emcgivne@andrew.cmu.edu (Eric McGivney), elena.gorokhova@aces.su.se (Elena Gorokhova)

‡Current address: Department of Civil and Environmental Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, United States

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ABSTRACT

Advanced oxidation processes/technologies (AOT) that combine a semiconductor, such as titanium dioxide (TiO_2), with a UV source have been used to eliminate microorganisms in various water treatment applications. To facilitate the applicability of this technique, the gain in efficiency from the semiconductor compared to the UV source alone with respect to different target organisms requires evaluation. The primary objective of this study was to determine the effects of TiO_2 and UV wavelength on a freshwater alga, *Pseudokirchneriella subcapitata*, and a marine alga, *Tetraselmis suecica*. For each species, dose-response experiments were conducted to determine the median lethal dose (LC_{50}) of the following treatments: UV-light emitted with a peak of 254 nm, UV-light emitted with a peak of 254 nm in the presence of TiO_2, and UV-light emitted with a peak of 254 nm and 185 nm in the presence of TiO_2. In both species, the presence of TiO_2 significantly increased mortality. Across all three treatments, *P. subcapitata* was more sensitive than *T. suecica*; moreover, the addition of the 185 nm wavelength significantly increased cell mortality in *P. subcapitata* but not in *T. suecica*.

INTRODUCTION

Advanced oxidation processes/technologies (AOT) are a set of technologies that target organic and inorganic compounds indiscriminately via oxidation. There are several forms of AOTs, all of which use the same mechanisms to decompose organic compounds: generation of reactive radical species, decomposition of organic contaminants, and a complete demineralization of materials into inorganic compounds (1). Currently, AOTs are considered to treat ship ballast water as a prevention measure for aquatic invasions (2–5). Ship ballast water is pumped into dedicated ballast tanks or empty cargo holds to increase the draft,

change the trim, regulate stability, or maintain the stress loads of the ship. A complex assemblage of organisms exists in nearly every aquatic system on earth. These organisms, if transported via ballast water of ocean trade vessels, may establish themselves in new environments when discharged from the ship and impact the receiving ecosystems (6). When testing treatment efficacy, special focus is frequently drawn on algae because of their high ecological relevance as a major group of plankton found in ballast waters (7, 8), potential to become invasive and harmful species to receiving waters (9), ability to survive several weeks in the dark (10), and robust DNA-repair systems (11) that enable recovery from UV-exposure (12, 13), which is particularly relevant for UV-based treatment technologies.

AOTs are successful at killing and/or inactivating microorganisms due to their ability to use UV light coupled with a semiconductor catalyst, such as titanium dioxide (TiO_2), to generate highly reactive hydroxyl radicals, $^{\bullet}OH$, which interact with cell membranes. With sufficient energy ($\lambda < 387$ nm), TiO_2 absorbs photons, resulting in the generation of an electron-hole pair. These surface electron-hole pairs then react with O_2 or H_2O in the surrounding environment to produce various reactive oxygen species (ROS). When in contact with cell membranes, ROS oxidize membrane-bound polyunsaturated phospholipids, causing the cell to lyse (14). In saline waters, e.g., when treating ballast water that most commonly originates from estuarine and marine environments, the radical generation can be more effective, due to the presence of halide ions, such as Br^- and Cl^- (15). In the presence of $^{\bullet}OH$, halide ions can form reactive halogen species, which are capable of oxidizing organics. With the onslaught of reactive species, organic compounds are eventually mineralized through various chemical reactions such as hydroxylation, hydration, hydrogen abstraction, dihydroxylation, deprotonation, one-electron transfer reactions, and decarboxylation (1).

Many AOTs employ photo ionization via semiconductors to produce ROS. Various metal oxide semiconductors can be used for photocatalytic radical generation; however, TiO_2 is the predominant semiconductor used in industrial water treatment practices because of its efficiency, photostability, durability, and affordability (16). In addition to TiO_2 radical generation, direct UV radiation also contributes to oxidation and disinfection effects of TiO_2/UV based AOTs. In particular, UV-C (280-100 nm) is able to directly damage cellular DNA by dimerizing thymine molecules, thus preventing cell proliferation and even causing cell death (17). Vacuum-UV (VUV, 200-10 nm) is absorbed by water and is thereby capable of generating ROS by breaking the hydrogen bonds in water molecules via hydrolysis (18, 19).

The implementation of TiO_2/UV based AOTs is vast. To evaluate treatment efficacy, most of the studies on TiO_2/UV based AOTs have used easily culturable bacteria, e.g., *Escherichia coli* (14, 20–23) and coliforms, as well as viruses (24), natural organic matter (25–27), and plastics (28). However, to the best of our knowledge, there are no reports comparing the efficiency of 185 nm and 254 nm irradiated TiO_2 on the acute mortality of freshwater and marine algae, i.e., the organisms that are of immediate relevance for various water treatment technologies aiming to eliminate microorganisms from natural and processed waters. Particularly relevant for ballast water and disinfection systems, which employ AOTs, is comparative testing of fresh- and saltwater algae and their relative sensitivity to different AOT components.

This study intends to facilitate the development of UV-based systems and also improve our understanding of algal cell reactivity to irradiation with different wavelengths. Specifically, the primary objective is to determine the combined effects of UV-C (peak λ=254 nm), VUV (peak λ=185 nm) and photocatalytic TiO_2 on freshwater and marine algae. Four

treatments that are of primary interest for the AOT applications were compared: (1) UV with a peak of 254 nm, hereafter $UV_{\lambda=254\ nm}$; (2) UV with a peak of 254 nm in the presence of TiO_2, hereafter $AOT_{\lambda=254\ nm}$; (3) UV with peaks of 185 and 254 nm in the presence of TiO_2, hereafter $AOT_{\lambda=185+254\ nm}$; and in the absence of UV light, hereafter *Dark trial*. To measure treatment efficacy, a cell viability assay was employed using a freshwater alga, *Pseudokirchneriella subcapitata*, and a marine alga, *Tetraselmis suecica*. *Pseudokirchneriella subcapitata* has been recommended by the Organization for Economic Co-operation and Development's (OECD) Guidelines for Testing of Chemicals as a standard species for algal toxicity tests, whereas *T. suecica* is a commonly used saltwater alternative in such tests (29).

MATERIALS AND METHODS

Algal cultures and test media: *Pseudokirchneriella subcapitata* was cultured in MBL medium (30) at 22 ºC under a light intensity of 40 $\mu E/cm^2s^{-1}$. *Tetraselmis suecica* was grown semi-continuously in salt water (Instant Ocean™, Aquarium Systems, 20 g/L) at continuous light with an intensity of 90 $\mu E/cm^2s^{-1}$ (31). These conditions were optimal for exponential growth mode of the cultures (Karin Ek, Stockholm University; personal communication, 2013). Prior to the experiments, cell concentrations were determined using a Spectrex LPC-2000 laser particle counter (Spectrex Corporation, California). All tests with *P. subcapitata* were conducted in aged tap water, whereas in the tests with *T. suecica*, salinity was adjusted to 20 g/L using Instant Ocean™ (see (32) for chemical composition). The aged tap water was prepared by intensive aeration at room temperature for 24 h to remove gaseous chlorine.

Exposure experiments: Algal suspensions were diluted to 2,000-4,000 cells/mL in 16 L of water and added to the pump-driven circulation system (Fig. 1). The UV lamps were turned on at least 5 min prior to running all experiments to avoid dose rate gradients during the

incubation, except for in the *Dark trial*. The circulation pump, which was off while the UV lamp was warming up, was turned on as soon as the algae were added. The circulation apparatus, powered by a Temag pump, had a flow rate of 26.3 L/min, a total volume of 16 L, and employed a quartz sleeve surrounding the UV-lamp (AOT5, Wallenius Water AB, Sweden). Two different UV radiation-emitting lamps (LightTech Lamp Technology, Ltd.) were used in the exposure experiments: (1) a 42 W low-pressure lamp with a peak emission occurring at 254 nm (UV-C), and (2) a 42 W low-pressure lamp with peak emission wavelengths at 254 nm and 185 nm (VUV). It was assumed that the circulation created a homogenous reactor. Two treatment columns, in which the lamps were located, were used to implement TiO_2 addition: one of the columns was uncoated stainless steel, the other was stainless steel coated with anatase TiO_2. To determine the effects of TiO_2 and the VUV addition, the following treatments were applied: (1) exposure to 254 nm light in the stainless steel column ($UV_{\lambda=254\ nm}$), (2) exposure to 254 nm light with TiO_2-coated stainless steel ($AOT_{\lambda=254nm}$), and (3) exposure to 254 and 185 nm light with TiO_2-coated stainless steel ($AOT_{\lambda=185\ +\ 254\ nm}$). The UV dose was calculated to be 27.0 and 28.6 mJ/cm^2 in the $UV_{185+254}$ and UV_{254} lamps, respectively, and was estimated for each treatment by multiplying by the exposure time. As a negative control to test the effect of TiO_2 only, a Dark trial was performed by circulating the algae in the system containing the TiO_2-coated column in the absence of light. Separate trials for *P. subcapitata* and *T. suecica* were conducted with three treatments and a control. The algae were sampled over a time series, *t*, of 0, 1, 2, 4, 8, 16, and 32 minutes by taking 1.5 mL samples directly after passing through the pump, at the inlet stream leading to the carboy container. All trials were done in duplicate or triplicate. Samples were stored in the dark for no more than 1 h before the Live/Dead analysis.

UV/VUV dose: UV and VUV dosage was measured in the circulation tank using a UV-sensor (D-Si131-LP, ZED EVG, Germany) and UV-monitor (Pro 11DPI-1). UV dose is

linearly related to the recirculation time and was calculated to be 27.0 and 28.6 mJ/cm^2 per minute from the VUV (185/254 nm) and UV (254 nm) lamps, respectively. These values were measured while the lamps were in a quartz sleeve (consistent with exposure experiment). Both lamps had the same electrical power, i.e., 42 W.

Live/Dead analysis: Cell viability was estimated using TO-PRO-1 iodide, a fluorescence dye (MW 645, excitation wavelength 509 nm, emission wavelength 533 nm), obtained from Molecular Probes Inc. (Eugene, OR), to stain the algae according to (33). TO-PRO-1 is a monomeric cyanide dye with a single cationic side chain that enters and stains cells with compromised membranes, binding to the nucleic acids. The TO-PRO-1 iodide stock solution was diluted with 20% NaCl (1:9 v/v) for use with *T. suecica* or with distilled water for *P. subcapitata* and stored at -20 ºC in the dark. Samples were centrifuged for 2 minutes at 13,000 rpm (~12,000 g). The supernatant was carefully poured off, leaving about 50 µL of suspension along with the algal pellet, in the bottom of the tube. To each sample, 5 µL of the respective TO-PRO-1 iodide stain solution were added, the pellet was gently resuspended. The samples were then incubated at room temperature in the dark for 20-40 minutes. After incubation, the stained samples were visually observed and counted (at least 200 cells per sample) using an epifluorescence Nikon Eclipse-Ti inverted microscope system with a standard long-pass blue filter (530-600 nm) at 400× magnification. Cells that showed any sign of a stained nucleus were considered non-viable, while viable cells exhibited only red chlorophyll autofluorescence (Figs 2 and 3).

Data analysis and statistics: Dose-response data were normalized using Abbott's correction (34):

$$P_N = \left(\frac{P_T - P_C}{100 - P_C}\right) \times 100 \qquad \text{(eq. 1)},$$

where P_N is the normalized percentage of non-viable cells, P_T is the observed percentage of non-viable cells at time point, t, and P_O is percentage of non-viable cells in the untreated, Dark trial.

To determine the treatment effect on *P. subcapitata* and *T. suecica*, the normalized log dose-response data were plotted and median lethal concentration values (LC_{50}), with corresponding 95% confidence intervals, were determined using the Hill equation with standard slope (eq. 2):

$$P_N = \frac{100}{1+10^{(\log LC_{50} - x)}} \quad \text{(eq. 2)},$$

where x is logarithm of the dose; the calculations were done in GraphPad Prism 6 software (GraphPad Software, Inc., 2013).

A two-way analysis of variance (two-way ANOVA) was applied to the LC_{50} values to determine whether the observed response was affected by the treatments, algal species, or by both. After the interaction effect proved to be significant, an unpaired t-test evaluated treatment effect for each species.

RESULTS

Dark trial results

Circulating through the embedded TiO_2 column in the absence of light (*Dark trial*), showed no observable effect of time on cell viability in either *P. subcapitata* or *T. suecica*, and thus no LC_{50} values were determined. After 32 minutes of circulation, the mortality values did not exceed 1.3% and 1.6% for *P. subcapitata* or *T. suecica*, respectively. This was not significantly different than mortality values when algae were circulated through the stainless steel column (data not shown).

Effects of TiO_2 addition

Dose response curves for *P. subcapitata* and *T. suecica* under four different treatments were used to calculate LC_{50} values (Figs 4 & 5). The LC_{50} values for *P. subcapitata* treated with $AOT_{\lambda=254\ nm}$ and $UV_{\lambda=254}$ were 189 mJ/cm^2 and 236 mJ/cm^2, respectively (Fig. 5). The LC_{50} values for *T. suecica* treated with $AOT_{\lambda=254\ nm}$ and $UV_{\lambda=254}$ were 253 mJ/cm^2 and 353 mJ/cm^2, respectively (Fig. 5). Thus, in both species, the LC_{50} values indicate a significantly higher algal mortality in the presence of TiO_2. Moreover, they also show that *P. subcapitata* is more sensitive to the addition of TiO_2 than *T. suecica* (Table 1).

Effect of VUV addition

The LC_{50} values for *P. subcapitata* treated with $AOT_{\lambda=185+254\ nm}$ and $AOT_{\lambda=254\ nm}$ were 89 mJ/cm^2 and 189 mJ/cm^2, respectively. The LC_{50} values for *T. suecica* treated with $AOT_{\lambda=185+254\ nm}$ and $AOT_{\lambda=254}$ were 275 mJ/cm^2 and 253 mJ/cm^2, respectively. The interaction effect in the two-way ANOVA was significant ($F_{1,5} = 32.88$; $p = 0.002$); hence, an unpaired t-test was used for the univariate comparisons. The addition of VUV in the AOT treatment had a significant effect on LC_{50} in *P. subcapitata* ($t_3 = 6.62$, $p < 0.007$) but not in *T. suecica* ($t_2 = 1.10$, $p > 0.3$).

DISCUSSION

The addition of TiO_2 increased treatment efficacy as indicated by the significant decrease in LC_{50} values in both algae species exposed to $AOT_{\lambda=254\ nm}$, compared to $UV_{\lambda=254\ nm}$. These results are supported by previous studies which indicated that $^{\bullet}OH$ generation, a product of UV irradiated TiO_2, contributes to the decomposition of organics by promoting peroxidation

of cells polyunsaturated phospholipid components in the lipid membrane (14, 35, 36). In the absence of light (Dark trial), there was no observed toxicity, and thus presumably no photocatalytic radical generation. While it has been shown that suspended TiO_2 nanoparticles can have negative effects on *P. subcapitata* by entrapping cells when exposed for 72 to 96 h (37, 38), we observed no cell mortality in either of the test species in the short-term exposure ($\leq$ 32 min) in the flow-through system with embedded TiO_2, in the absence of light. Therefore, the effect of TiO_2 was attributed to the increased ROS production in the system. However, ROS generation and accumulation was not measured in this study.

The addition of VUV significantly decreased the LC_{50} value of *P. subcapitata*. VUV's ability to generate $^{\bullet}OH$ in the process of water via hydrolysis likely creates this effect. VUV-initiated hydrolysis is efficient in generating hydroxyl radicals in aqueous solution, and thus effective for the inactivation of microorganisms (39). However, the effect was not detectable in *T. suecica*, indicating greater tolerance in this marine alga compared to the freshwater *P. subcapitata*.

Ozone generation was not considered to have an influence in these experiments. While ozone may be produced in the atmosphere under VUV irradiation (185 nm), it cannot be produced through the irradiation of dissolved oxygen. The circulation tank set-up was designed in such a way as to not let air bubbles flow into the UV sleeve unit as any air bubbles would rise to the surface rather than be pumped from the bottom of the tank.

Phytoplankton use different strategies for both protecting against UV light and repairing cellular damage. To minimize ROS-induced damage, antioxidant enzymes (40, 41), UV-absorbing pigments, e.g., mycosporine-like amino acids (42), carotenoid and scytonemin (43); hydroxyl radical scavengers, e.g., dimethylsulphoniopropionate and dimethylsulphide

(44) are produced; as well as DNA reparation proteins, e.g., photolyase (45). Specifically, *T. suecica* has shown the ability to adapt to and tolerate extreme solar-UV exposure (46). It is likely that these same defense/repair mechanisms are used to cope with higher UV energies, such as UV-C and VUV employed in the UV-based water treatment technologies. Moreover, the diurnal rhythm/biological clock of a cell has been shown to influence the production of the abovementioned radical defense enzymes (47–49). It should be noted that *T. suecica* was cultured under constant UV light at 90 $\mu E/cm^2s^{-1}$, while *P. subcapitata* was grown at less than half the UV dosage, 40 $\mu E/cm^2s^{-1}$. These conditions were optimal for growth of the algal strains used in the experiment (K. Ek, Stockholm University, personal communication, 2013), which implies that *T. suecica* requires higher light levels for maximal growth, but also indicates that it is more resilient to photodamage. This difference in culture environment may also have pre-adapted the *T. suecica* cells and caused them to upregulate production of protective enzymes and pigments, which could explain *T. suecica*'s relative resilience, compared to *P. subcapitata* under the UV/AOT treatments.

It is also interesting to note the difference in chlorophyll intensities between the two algal species at similar UV doses (Figs 2 & 3). This difference may be related to (1) species-specific decline in fluorescence reflecting a dynamic interaction between ROS-induced damage and repair, (2) the difference in composition and amount of photosynthesizing pigments that have varying levels of both autofluorescence (i.e., the tone and intensity of the red color in Figs. 2 & 3) and light sensitivity, and (3) the distribution and total amount of non-nuclear DNA in algal cells, including mitochondrial and chloroplast DNA, and the resulting distribution of green fluorescence. A higher number of mitochondria and longer exposure time would result in TO-PRO-1 binding to the mitochondrial and chloroplast DNA, which would generate a stronger green fluorescence that will, at least to some extent, mask

chlorophyll autofluorescence. It is likely that all these mechanisms contribute to the observed disappearance of the red fluorescent signal from the photosynthetic pigments, which is more pronounced in the *P. subcapitata* compared to *T. suecica*. One can speculate that the difference in the light intensity used for stock cultures and empirically identified as optimal for algal growth, is reflecting higher sensitivity of the photosynthetic machinery in *P. subcapitata* to light in general. On the other hand, this also implies that pigments in *T. suecica* could have been pre-acclimated to higher light levels and respond to the UV treatment more actively. In line with that, a rapid (<30 min) increase in intracellular carotenoids and a marked decline in chlorophyll *a* has been reported in *T. suecica* exposed to UVB radiation (50). This could potentially contribute to the greater stability of the red pigment in *T. suecica*, but we are not aware of similar pigment responses reported for *P. subcapitata*. Finally, comparative studies show that the photosynthetic system in prasinophyceae and, in particular, *T. suecica*, has a higher tolerance to UV compared to green algae (51).

Comparing the efficiency of 185 and 254 nm irradiated TiO_2 on the acute mortality of freshwater and marine algae is particularly relevant for ballast water effluent. Cargo ships traverse waters of different ecosystems containing vastly diverse chemical and biological compositions. Moreover, in an effort to quell economical and ecological damage due to ballast-tank-enabled biological invasions, the United Nations International Maritime Organization (IMO) has adopted a convention that aims to manage and control ships' ballast water and sediments by instituting industry standards and practices (52). These standards will require ships to treat both saline and fresh water organisms to acceptable effluent levels. The results from this study may aid water treatment engineers in designing on-board disinfection system that meet the IMO's ballast water performance standards.

CONCLUSION

In both studied species, the addition of TiO_2 in AOT $_{\lambda=254}$ treatment resulted in higher algal mortality, compared to $UV_{\lambda=254}$. Positive effects on treatment efficacy were also observed when VUV light was added as a part of the irradiation. However, the effects were dependent on the algal species used as test organisms. Therefore, in future studies and treatment efficacy testing, it is essential to include test species from distant phylogenetic groups grown in various conditions (particularly, light levels) as they may differ in their capacity to cope with ROS-induced damage. The actual mechanisms that contributed to cell mortality were not studied, but are assumed to be due to radical production and ROS-induced oxidative damage. Our findings also highlight the applicability of AOT/UV for the elimination of phototrophic microorganisms from fresh and marine waters, which could be considered in various applications, such as ballast water treatment.

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Table 1. Two-way ANOVA results; effects of TiO_2 addition (treatment; $AOT_{\lambda=254\ nm}$ vs. $UV_{\lambda=254}$) and species (*Pseudokirchneriella subcapitata* vs. *Tetraselmis suecica*) on algal LC_{50}. SS=sum of squares, DF= degrees of freedom, MS= mean squares, F(DFN, DFD)= an F distribution with its two parameters: degrees of freedom numerator (DFn) and degrees of freedom denominator (DFd), where DFn= *# of groups* -1 and DFd= *total number of subjects* - *# of groups*.

	SS	DF	MS	F (DFn, DFd)	*p*-value
Interaction	0.001152	1	0.001152	F (1, 4) = 1.723	0.2595
TiO_2 addition	0.02928	1	0.02928	F (1, 4) = 43.80	0.0027
Species	0.04560	1	0.04560	F (1, 4) = 68.22	0.0012
Residual	0.002674	4	0.0006685		

FIGURE CAPTIONS

Figure 1. A photo (A) and simplified schematic (B) of the circulation treatment system with TiO_2 coated stainless steel UV column. The path of the circulation system is as follows: (1) from the 16 L carboy container with an outlet located at the bottom of the tank. From the

bottom of the carboy, through a plastic tube to the top of the UV lamp treatment column (2), which empties through an outlet in the bottom of the column, then through another plastic tube into the pump (3), where it is then pumped back through an inlet hole at the top of the carboy container and the cycle repeats.

Figure 2. Images of stained (TO-PRO-1 Iodide) *P. subcapitata* under fluorescence microscope. Cells that showed any sign of a stained nucleus were considered dead, while viable cells exhibited only red chlorophyll autofluorescence. These samples were exposed to $AOT_{\lambda=254\ nm}$ (28.6 mJ/cm^2 per min) for (A) 1 minute (28.6 mJ/cm^2), (B) 2 minutes (57.2 mJ/cm^2), (C) 4 minutes (114.4 mJ/cm^2), (D) 8 minutes (228.8 mJ/cm^2), (E) 16 minutes (457.6 mJ/cm^2), and (F) 32 minutes (915.2 mJ/cm^2).

Figure 3. Images of stained (TO-PRO-1 Iodide) *T. suecica* under fluorescence microscope. Cells that showed any sign of a stained nucleus were considered dead, while viable cells exhibited only red chlorophyll autofluorescence. These samples were exposed to $AOT_{\lambda=254\ nm}$ (28.6 mJ/cm^2 per min) for (A) 1 minute (28.6 mJ/cm^2), (B) 2 minutes (57.2 mJ/cm^2), (C) 4 minutes (114.4 mJ/cm^2), (D) 8 minutes (228.8 mJ/cm^2), (E) 16 minutes (457.6 mJ/cm^2), and (F) 32 minutes (915.2 mJ/cm^2).

Figure 4. Dose-response curves for (A) *P. subcapitata* and (B) *T. suecica* treated with $AOT_{\lambda=185+254\ nm}$, $AOT_{\lambda=254\ nm}$, and $UV_{\lambda=254\ nm}$. Data are shown as mean values (n = 3 and 2 for *P. subcapitata* and *T. suecica*, respectively), error bars show minimal and maximal values. Models are fitted using eq. 2.

Figure 5. LC_{50} (mean ± SD) for *P. subcapitata* and *T. suecica* treated with $AOT_{\lambda=185+254\ nm}$, $AOT_{\lambda=254\ nm}$, and $UV_{\lambda=254\ nm}$.