# $H_2O_2$ effects on the potential macroalgal culture species *Palmaria palmata* (Linnaeus) Weber & Mohr and *Ulva lactuca* (Linnaeus)

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Front cover photo: *Palmaria palmata* and *Ulva lactuca.* Photo: Helga Øen Åsnes

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# Abstract

Hydrogen peroxide ($H_2O_2$) has been the most used remedy against salmon sea lice (*Lepeophtheirus salmonis*) in Norway since 2013. This remedy has been considered environmentally friendly, because it breaks down to water and oxygen, even though it is a part of reactive oxygen species (ROS) that can cause damage to tissues and cells. Documented half-lives on several days and quick vertical dispersal leaves a time window where it can potentially harm *non-target* organisms, such as macroalgae. Surplus production of dissolved nutrients and organic particles is another challenge for the aquaculture industry in Norway. Multi-trophic aquaculture (IMTA) has been suggested as a solution to this problem, where the production of seaweeds is expected to be an important part of this development. A prerequisite for establishment of such facilities will be that the production of the different species can coexist without affecting each other negatively.

This study has assessed how the commercially interesting red alga *Palmaria palmata* and green alga *Ulva lactuca* are responding to $H_2O_2$ exposure. The state of their photosynthetic apparatus has been mapped by two different methods. The oxygen evolution has been measured during increasing light levels for both species. In addition, fluorescence lifetime and intensity measurements were taken of *U. lactuca.*

Even though there were not always visible signs after exposure, the photosynthetic apparatus in both species were affected. *U. lactuca* showed a higher tolerance towards $H_2O_2$ compared to *P. palmata.* Concentrations equivalent to 10% and 25% of a normal treatment dose in net pens, lowered the estimated maximum photosynthetic efficiency by more than 50% for *P. palmata* and *U. lactuca,* respectively. These differences have been linked to their general tolerance towards environmental stressors and internal ROS scavenging abilities. Comparing these results with measured and estimated concentrations likely to be found in the environment, reveals a potential issue for future IMTA-facilities. Especially when considering that a prerequisite for functional IMTA-facilities is to have the algae in close proximity to the net pens.

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# 1. Introduction

## 1.1 Aquaculture in Norway

The aquaculture industry in Norway mainly consist of monoculture facilities with Atlantic salmon, accounting for about 94% of the total production and reaching 1.3 tonnes in 2017 (Baklien & Steinset 2017; Karlsson-Drangsholt & Nes 2017). In Norway, as well as the rest of the world, this industry is of growing importance (Grefsrud et al., 2018). A sustainable development is therefore critical, not just to maintain production rates, but also for further growth. In December 2017 the Norwegian government published new regulation guidelines (Ministry of Trade, Industry and Fisheries 2015), with the purpose to increase salmon farming *within* the framework of an environmentally sustainable development.

A major environmental issue of finfish aquaculture is the surplus production of dissolved nutrients and organic particles (Alexander et al., 2015). More recently integrated multi-trophic aquaculture (hereafter IMTA) has become more interesting as a way of minimizing negative impact of finfish production on the environment (Ridler et al., 2017). The purpose of IMTA is to cultivate aquatic organisms belonging to different trophic or nutritional levels in the food chain together in the same system (Alexander et al., 2015; Fang et al., 2016). Such a co-culture can consist of finfish, bivalves and seaweeds, where the bivalves feed on faecal particles from the fish and the seaweeds absorbs nutrients (Fang et al., 2016). This contributes to a reduced impact on the natural environment and an increasing total biomass production (Karlsson-Drangsholt & Nes 2017; Strand & Steen 2011). In Norway, it has been discussed if IMTA can be the solution for a more sustainable aquaculture industry, and production of seaweeds is expected to be an important part of this future development (Hancke et at., 2018; Strand & Steen 2011).

The interest for macroalgae, as a source for sustainable biomass, is growing for many different types of industries ranging from food production (both human and animal consumption), pharmaceuticals and fuel (Charrier et al., 2017). The worlds total aquatic plant production in 2016 was 30.1 million tonnes (FAO 2018). The following year, the production in Norway was 149 tonnes (Fauske 2019). Olafsen et al. (2012) estimated that the Norwegian production can increase severely and reach as high as 20 million tonnes by 2050. There are several commercially interesting species in Norway, such *Saccharina latissima*, *Laminaria digitata* and *Alaria esculenta* (kelps), *Porphyra umbilicalis* and *Palmaria palmata* (red algae) and the green alga *Ulva lactuca* (Hancke et al., 2018). All these species are being researched for further

utilization in Norway (Hancke et al., 2018). A prerequisite for future IMTA-facilities and a co-development of finfish aquaculture and industrial macroalgae cultivation, is that they can coexist without affecting each other negatively.

One of the main challenges for the aquaculture industry in Norway today is the infestation of the fish by salmon sea lice (*Lepeophtheirus salmonis*) (Karlsen 2016). Cleaning fish have been used since the beginning of the 1990s, with a substantial increase through the years (Skiftesvik et al., 2016). Since 2016, both non-medical and medical remedies have been used to defeat this parasite. Bath-treatment in warm seawater, freshwater and mechanical removal of the lice are examples of non-medical methods used. Medical remedies used to defeat this parasite are either added in the feed or used in bath-treatments. Substances that inhibit the synthesis of chitin such as flubenzurons are examples of remedies added to the feed (Grefsrud et al., 2018). Hydrogen peroxide ($H_2O_2$) was introduced as a remedy against salmon lice in 1993 and is used in bath-treatments (Andersen & Hagen 2016; Wesenberg et al., 2000).

## 1.2 Hydrogen peroxide and reactive oxygen species in algae

$H_2O_2$ dosages used for delousing in net pens depends on water temperature. During 20 min bath-treatments, at a temperature below eight degrees, the normal dosage is 1700 mg/l $H_2O_2$ (Wesenberg et al., 2000). In Norway, the use of this therapeutant reached its peak in 2015 with 43246 tonnes, declining to 9277 and 6735 tonnes in 2017 and 2018, respectively (Hjeltnes et al., 2019). Bath-treatments are conducted in two different ways. The fish can be treated directly in the net pens with a tarpaulin wrapped around, or the delousing takes place in a well-boat (Andersen & Hagen 2016). When treatment is performed in the net pens, the $H_2O_2$ solution is released to the local environment when the tarpaulin is removed, whilst with the use of a well-boat the solution can be transported and released elsewhere (Andersen & Hagen 2016).

$H_2O_2$ is a part of a group of reactive oxygen species (ROS) that can cause damage to tissues and cells (Aarnes 2012; Potin 2008; Winterbourn 2013). Despite this, it has been considered an environmentally friendly remedy because it breaks down to water and oxygen. Different factors affect the speed of this process, such as the amount of organic compounds in the water, pH and temperature (Andersen & Hagen 2016; Hoddevik 2018; Refseth et al., 2016). The half-life for $H_2O_2$ in seawater increases with temperature (Bruno & Raynard 1994; Fagereng 2016). Bruno & Raynard (1994) found half-lives at 3.5 and 7 days for 8 and 12˚C, respectively, whereas Fagereng (2016) concluded with a half-life of about 7 days at 15˚C. How the solution spreads in the environment is also an important aspect. This depends on both the horizontal and vertical

mixing, affected by factors such as depth, currents, wind and the density distribution in the water column (Hoddevik 2018; Refseth et al., 2016). A field study conducted by Andersen & Hagen (2016), who measured $H_20_2$ concentration inside and in the vicinity of net pens after bath-treatments, found that both vertical and horizontal dispersion depend on currents in the area. Field studies conducted by Fagereng (2016) found quicker vertical dispersion than horizontal. With these documented half-lives of several days and quick dispersal of the solution, this leaves a time window where $H_20_2$ can potentially harm *non-target* organisms, such as macroalgae.

In macroalgae, as in every other photosynthetic organism, the risk of producing ROS is always present (Bischof & Rautenberger 2012). When they are exposed to stressful environmental conditions, such as temperatures beyond their normal range, desiccation or very high light intensities, this risk of internally produced ROS increase (Dring 2005). This can happen through several mechanisms, such as formation of triplet chlorophyll or disruption of the electron transport chain from PSI to ferredoxin (Bischof & Rautenberger 2012). ROS can lead to a breakdown of proteins and nucleic acids, potentially ending with membrane leakages, biochemical dysfunctions and lower photosynthetic efficiency (Bischof & Rautenberger 2012). Such oxidative stress occurs when there is an imbalance between the ROS and the defence mechanisms, which consists of enzymes called antioxidants and nonenzymatic intermediate metabolites, with antioxidative properties (Bischof & Rautenberger 2012; Aarnes 2012). Ascorbate peroxidase (APX) and catalase are examples of enzymes, where the latter breaks down $H_20_2$ to water and triplet oxygen. Carotenoids are examples of nonenzymatic ROS-scavengers, that can break down triplet chlorophyll (Bischof & Rautenberger 2012; Cazzaniga et al., 2019). Despite all this, the algae also produce and release ROS in oxidative bursts to defend themselves against pathogens (Bischof & Rautenberger 2012). This illustrates a very intricate system, where different stress factors can have a severe impact. To understand how different factors can stress the algae, it is vital to get a better insight to their physiological fitness.

## 1.3 Quantifying photosynthetic efficiency

To get an insight into the physiological fitness of algae, photosynthetic performance estimates can be used (Hurd et al., 2014). The photosynthetic efficiency is first and foremost dependent on how much energy – *light* – the algae are exposed to. To determine this, the number of photons of photosynthetic active radiation (PAR), defined as wavelengths between 400-700 nm, are measured (Hurd et al., 2014). The relationship between $O_2$ production and available

PAR gives an insight into the photosynthetic efficiency and the physiological state of the algae (Hurd et al., 2014; kvanteutbytte 2018). A photosynthetic irradiance curve (P-E curve, also called a light response curve) can illustrate the relationship between irradiance and photosynthesis (Hurd et al., 2014). Different photosynthetic parameters can be obtained from such curves and, therefore, this gives a method to quantify the photosynthetic efficiency.

Fluorescence is another parameter that can be used to determine the state of the photosynthetic apparatus. Chlorophylls can release the excess energy absorbed from a photon, by sending out their own – a fluorescence photon (Suhling et al., 2015). The time span from when the energy of the original photon is absorbed to the new photon is released is the *fluorescence lifetime* (Kristoffersen 2018; Suhling et al., 2015). This time span or lifetime depends on the available de-excitation pathways and the direct competition that occurs between them (Kristoffersen et al., 2012). When illuminated, the *fluorescence intensity* (number of fluorescence photons) of a dark-adapted photosynthetic sample also changes over time (Kristoffersen et al., 2016). In a time span of about one second the fluorescence intensity rises before it declines again. This is explained by the amount of available reaction centres to obtain the energy from the light (Maxwell & Johnson 2000). Thus, by measuring the fluorescence lifetime and intensity we obtain unique information about the competitive forces of the de-excitation pathways and therefore, also about the photosynthetic efficiency (Kristoffersen et al., 2012; Kristoffersen 2018).

In this study these methods of quantifying photosynthetic efficiency have been used to give a further insight into how the photosynthetic apparatus of the two species *P. palmata* and *U. lactuca,* is affected by an external stress factor.

## 1.4 study species

### *1.4.1 Palmaria palmata*

***Figure 1*** P. palmata. *Photo: Helga Ø. Åsnes*

*Palmaria palmata* (Linnaeus) Weber & Mohr, 1805, is an abundant marine cold-water red seaweed species distributed in the North Atlantic (Figure 1) (Guiry & Guiry 2019). The pigments phycoerythin and phycocyanin gives them their red colour, concealing their other pigments, chlorophyll *a* and carotenoids, consisting of unique xanthophylls and betacarotene (Guiry 2019b; Cazzinga et al., 2016). They grow in the intertidal and shallow subtidal zone on rocks, mussels

and epiphytic on various other algae species, especially on *Laminaria hyperborea* stipes (Guiry & Guiry 2019). Depending on where they grow, they show high morphological variation. When growing on wave-swept shores and small mussels, they are smaller and narrower compared to the ones growing on kelp or directly on rocks. A key characteristic is the palmate branching and extensions with a finger-like appearance. They have flattened membranous or leathery fonds with a reddish-brown colour, with a small stipe growing from a discoid base. They can become as much as 1 meter long, but have a normal size range from 5-30 cm (Guiry & Guiry 2019).

*P. palmata* has a diplohaplontic heteromorphic lifecycle, where the female gametophyte is microscopic (about 0.1 mm) whilst the male gametophyte and the tetrasporophyte are macroscopic (Hoek et al., 1995; Mayanglambam & Sahoo 2015). The *P. palmata* we see in the shore can be male gametophytes, but mainly these are the tetrasporophytes (Hoek et al., 1995). The female, being small and crust-like, are fertilized by spermatia produced by the male gametophyte, a process taking place in a structure called carpogonium. From the carpogonium the tetrasporophytic plant grow, which in turn produces tetraspores that develops into male and female gametophytes (Hoek et al., 1995).

### *1.4.2 Ulva lactuca*

Recent genetic analyses have revealed that the holotype of *U. lactuca* is actually a tropical *Ulva* species, and that the valid name of *U. lactuca* should therefore be *U. fenestrata* (Linnaeus) Postels & Ruprecht, 1840 (Hughey et al., 2019 *in press*) (Figure 2). Since these results are not published yet, the name *U. lactuca* will be used in this thesis. It is a green alga with a distromatic thallus, meaning that it is made up of two cell layers, and has a blade-like appearance (Ulva lactuca, n.d.). The green colour originates from the pigments chlorophyll *a* and *b*, and in addition it has distinctive xanthophylls (Guiry 2019a). This species is mainly found in the lower intertidal or upper subtidal zone, often growing on floating docks (Ulva lactuca, n.d.).

***Figure 2*** *U. lactuca. Photo: Helga Ø. Åsnes*

*U. lactuca* has a diplohaplontic isomorph life cycle, meaning that the haploid and diploid stage have the same morphology. Two gametes, produced by two separate haploid gametophytes,

fuse together to a zygote. This zygote then develops into a sporophyte which produce spores by meiosis, developing into a new set of gametophytes (Wichard 2015).

## 1.5 Scope of the study

To ensure the sustainable development of the aquaculture industry, further investigation of how different remedies (such as $H_2O_2$) affect the surrounding environment is necessary. This including both species naturally occurring, potential IMTA species and species cultivated separately. This project will focus on two commercially interesting macroalgae species, where both are relevant members of all groups mentioned above. To this day most of the macroalgae production in Norway has been focused on the kelp *Saccharina latissima* (Hancke et al., 2018). Recent studies have shown this species to be highly sensitive towards negative effects of $H_2O_2$ (Haugland et al., 2019). The aim of this master thesis is to get a better insight in how the red alga *P. palmata* and the green alga *U. lactuca* tolerate exogeneous exposure of $H_2O_2$.

This will be done by measuring how this remedy affects the photosynthetic apparatus, by measuring the $O_2$ evolution during increasing light intensities for both species. P-E curves and dose-response curves will then be made to get better insight into how the relationship between the concentration of $H_20_2$ and the physiological response in the algae develops. In addition, fluorescence lifetime and intensity measurements will be done for *U. lactuca,* for a better insight and comparison of results gathered from the different methods.

# 2. Materials and methods

## 2.1 Sampling procedure

The laboratory work for this thesis has been conducted during three different time periods in 2018 and 2019. The sampling sites and time of sampling varied (see Table 1), but the overall sampling method was the same. All specimens were collected by hand, put into transparent plastic bags filled with seawater from the sampling site, and transported in a cooler bag to the lab. At the lab, the algae were rinsed and cleaned for all visible epiphytes.

***Table 1*** *Sampling sites and dates for the two species.*

| Date | Species | Location | Latitude | Longitude |
|---|---|---|---|---|
| 13.03.18 | *Palmaria palmata* | Algrøyna, Fjell | 60° 21’36.26878’’N | 4° 56’38.24395’’E |
| 22.03.18 | *Palmaria palmata* | Syltøy, Fjell | 60° 18′58.16137″N | 4° 59′7.04061″E |
| 15.10.18 | *Ulva lactuca* | Espegrend, Bergen | 60° 16’11.06061’’N | 5° 13’18.99584’’E |
| 29.11.18 | *Ulva lactuca* | Espegrend, Bergen | 60° 16’11.06061’’N | 5° 13’18.99584’’E |
| 15.01.19 | *Ulva lactuca* | Espegrend, Bergen | 60° 16’11.06061’’N | 5° 13’18.99584’’E |
| 25.02.19 | *Ulva lactuca* | Espegrend, Bergen | 60° 16’11.06061’’N | 5° 13’18.99584’’E |
| 16.03.19 | *Palmaria palmata* | Syltøy, Fjell | 60° 18’58.16137’’N | 4° 59’7.04061’’E |

## 2.2 Procedure for exposing the algae to $H_2O_2$

The Norwegian Medicines Agency (Legemiddelverket) have defined recommended bath-treatment doses for the use of $H_2O_2$ in the fish farms. The normal treatment for fish at temperatures below 8 °C is 1.7 kg $H_2O_2$ per $m^3$ water (1700 mg/l) in approximately 20 minutes (Wesenberg et al., 2000). This recommendation has been the basis for the concentrations used in this study and, therefore, the concentration of 1700 mg/l $H_2O_2$ was defined as the 100% dose. To assess how $H_2O_2$ affects the two species of algae, they were exposed to different concentrations for one hour. The exposure time of one hour was chosen because (even though the fish are only treated for 20 min), the mentioned half-life of $H_2O_2$ and dispersal in the environment imply that it stays in the surrounding water longer, and thereby exposing the algae longer (Bruno & Raynard 1994; Fagereng 2016).

The method for exposure was the same for all the experiments conducted. The solutions were made by diluting a $H_2O_2$ solution to different treatment concentrations by adding filtrated seawater. The amount of seawater needed was first prepared, and thereafter the appropriate volume of $H_2O_2$ was added with a syringe or a pipette, before stirring gently. The algae

specimens were deployed individually in each beaker. After one hour exposure, the algae were rinsed in fresh seawater, ensuring no remnants of $H_2O_2$ left on the alga surface.

## 2.3 Assessing lethal effects

The first part of the experiments was to assess the lethal doses of $H_2O_2$. This was done in 1 L beakers, each assigned a specific concentration, including a control containing seawater (Table 2). A total of ten specimens of *P. palmata* were exposed to each concentration. Due to fewer available algae, only five *U. lactuca* were used. After the exposure, the specimens were kept in 1 L beakers with fresh seawater throughout the next week under a 12:12 light regime. The water was changed daily, and the algae were kept at a temperature of about 9 °C (Figure 3).

***Table 2*** *By exposing ten* P. palmata *and five* U. lactuca *specimens to $H_2O_2$ dilutions for one hour, lethal effects where assessed. The dilutions were based on a common bath-treatment dose (being 1700 mg/l) used during salmon sea-lice infestation in net pens.*

| Concentration (mg/l $H_2O_2$) | Dilution of bath-treatment (%) | Concentrations used on *P. palmata* | Concentrations used on *U. lactuca* |
|---|---|---|---|
| Seawater (control) | 0 | X | X |
| 85 | 5 | | X |
| 170 | 10 | X | X |
| 475 | 25 | X | X |
| 850 | 50 | X | X |
| 1250 | 75 | X | X |
| 1700 | 100 | X | X |

No standardized method exists for determining the time of mortality in macroalgae, which makes this a challenging task. Haugland et al. (2019), who worked with *S. latissima,* concluded that the algae had to be under observation for at least seven days after exposure. Because of these results, the aim was to monitor them for one week. This resulted in an observation period of seven days for *U. lactuca*, but it was shortened to six days for *P. palmata* due to practical issues. During this week, changes in colour, smell and consistency of the thallus were registered. The results of this initial lethal experiment were then used to decide treatment doses in the sublethal experiment.

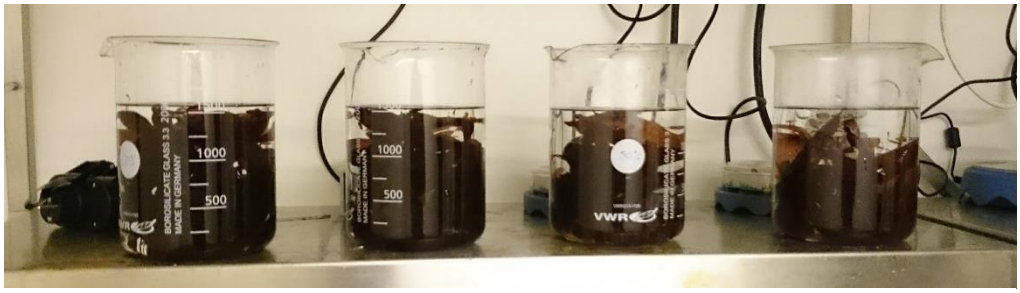



***Figure 3*** *Illustration of how the exposure and observation was done, here with* P. palmata. *Each beaker containing ten speciemens.*

## 2.4 Assessing sublethal effects

### *2.4.1 Samples and lab setup*

To examine sublethal effects, specimens were first exposed to $H_2O_2$ and subsequently incubated, where measurements were taken to assess their photosynthetic ability. This was based on the method described by Haugland et al. (2019). The algae were held in a storage tank with flowing seawater before and between the exposure and incubations. This water had the same temperature and salinity as the water they were exposed to during the incubations, and the tank was held under a 12:12 light regime (Figure 4).

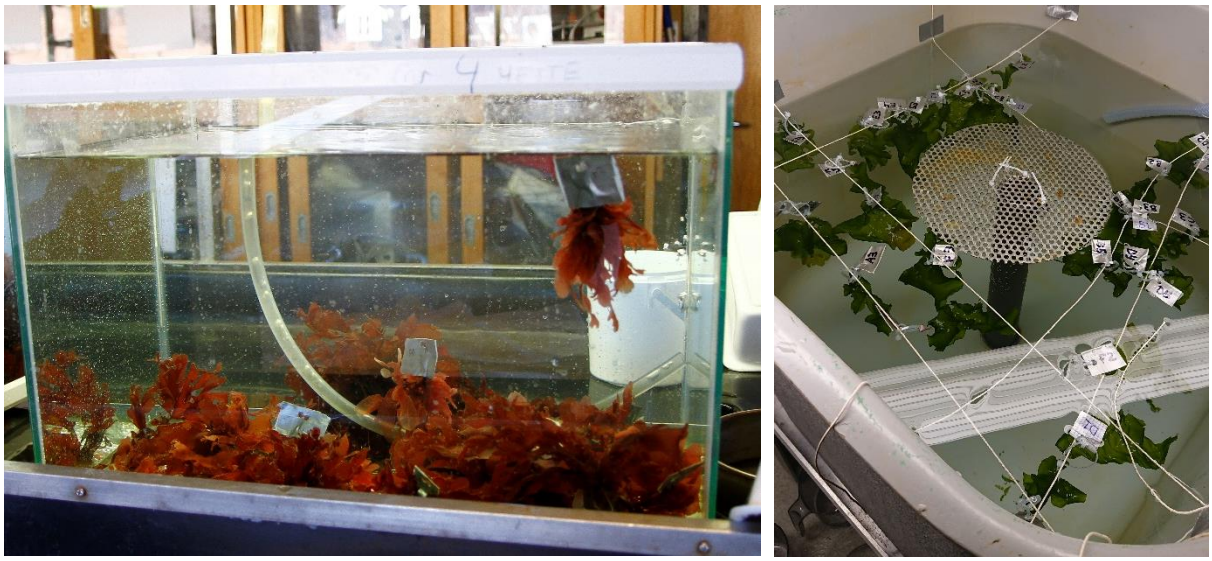


***Figure 4*** *The tanks with flowing seawater that the algae were held in before and between exposure and incubations*. P. palmata *(left) and* U. lactuca *(right).*

By randomly picking specimens from the tank, they were individually labelled and given numbers defining their treatment. Every specimen with label number one was treated with the strongest concentrations and the ones with the highest label number were treated with the lowest. Both species were treated with four different concentrations of $H_2O_2$, and one control treatment with seawater (Table 3). For each concentration there were several replicates, five for *P. palmata* and six for *U. lactuca*, giving a total of 25 and 30 specimens, respectively. Each alga was incubated three times, immediately-, one day- and about two weeks after exposure (day 0, day 1 and day 11/14). They were placed in the incubation chambers immediately after they were rinsed (for $H_20_2$), resulting in a time span of only a few minutes before the day 0 measurements started. Five specimens were incubated at the same time, giving a total of 15 incubations for *P. palmata* and 18 for *U. lactuca.* The total incubation time for the two species were approximately five and nine hours for *P. palmata* and *U. lactuca* respectively, with preparations and exposures in addition. This made it possible to conduct two incubations a day with *P. palmata* and one with *U. lactuca*, resulting with 18 and 26 days to complete the

experiment. The first specimens of the two species to be exposed to $H_2O_2$ where kept in the lab for two (*P. palmata*) and four days (*U. lactuca*). Each incubation contained one individual from each treatment, to avoid that time spent in the lab would affect the results in a systematic way. For each incubation the placement of specimens in different chambers were randomized.

***Table 3*** *By exposing* P. palmata *(n=five) and* U. lactuca *(n=six) specimens to $H_2O_2$ dilutions for one hour, sublethal effects where assessed. The dilutions were based on a common bath-treatment dose (being 1700 mg/l) used during salmon sea-lice infestation in net pens.*

| Concentration (mg/l $H_2O_2$) | Dilution of bath-treatment (%) | Concentrations used on *P. palmata* | Concentrations used on *U. lactuca* |
|---|---|---|---|
| Seawater (control) | 0 | X | X |
| 85 | 5 | | X |
| 170 | 10 | X | X |
| 475 | 25 | | X |
| 850 | 50 | X | X |
| 1250 | 75 | X | |
| 1700 | 100 | X | |

The photosynthetic ability was determined by measuring oxygen level over gradually increasing light levels. During the incubations the algae were placed in separate transparent and gas tight chambers (height:10 cm, length:15 cm, width:4 cm). These were then placed in a big tank (height:22 cm, length: 200cm, width: 25cm) filled with fresh seawater (Figure 5). This system was developed to ensure that the algae were exposed to the same stable environment. During the incubations each chamber was sealed, and only connected to the surrounding water by a water pump system. This system was used to renew the water in the chambers between light levels, avoiding that the oxygen content got too high (or low), thereby affecting the following performance of the algae. All probes and pipes were connected through holes in the lid, and parafilm was used to seal it properly to ensure no water flow during the measurements.

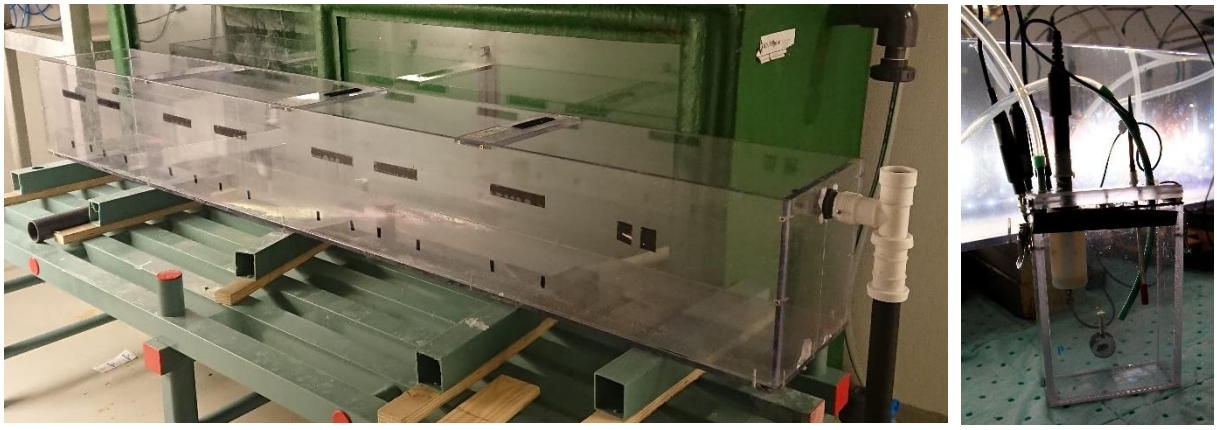


***Figure 5*** *The big tank (left) where the six small incubation chambers where placed (right). The incubation chamber here showed with its lid and the connected probes and pipes.*

### *2.4.2 Instrumentation and measurements*

Fresh weight and pictures of the algae were taken before and after the experiment (day 0 and day 11/14), for *U. lactuca* this was also done at day seven. When weighing, the method for removal of excess water was standardized to ensure as comparable conditions as possible. Pictures of each alga were taken on millimetre paper. Because volume measurements of the incubated specimens had not been done, new specimens had to be gathered in the spring of 2019. Then 20 weight and volume measurements were taken for each species, giving a recalculation constant used to estimate the volume of the algae used in the experiment. These volume measurements were done by measuring displacement volume of thalli in a cylinder beaker filled with seawater, after weighing each thallus.

During an incubation the oxygen, temperature and salinity were measured. The oxygen content in each chamber was measured with an OXY-10 mini PreSens precision sensor, logging four times a minute. The temperature was measured in the big tank during both experiments, but with different methods. During the incubations with *P. palmata* a Vernier extra-long temperature probe (TPL-BTA) was used, logged with a Vernier LabQuest® 2 also measuring four times a minute. Because of very fluctuating temperature readings when using this equipment during the incubations of *U. lactuca* a WTW Digital precision meter (Multi 3410 with a TetraCon 925 IDS sensor) was used to measure the temperature. This was done between each light level. The salinity was also measured between each light level with the WTW Digital precision meter. During the experiment with *P. palmata* the salinity was not measured, because of lacking equipment.

The different light levels were determined based on the light levels used by Haugland et al. (2019) and the light range of the equipment. These levels were decided during the preparations, and by using a Vernier PAR sensor (PAR-BTA) to measure the light inside the incubation chambers, when filled with water. This measured the photon flux density (PFD), which is the number of photons hitting an area per second (μmol/ $m^2$ * s). Measurements were taken for each chamber at all the different light levels, to ensure as similar light conditions as possible. No light measurements were taken during the incubations. For the experiment conducted with *P. palmata* the light source used was two led tube lights (230 V and 13W) and dimmable flexible RGB LED strips (230V). These were taped directly to the tank (Figure 6). In addition to turning the two separate led tubes on and off, a remote control connected to the LED strips was used to obtain the right light levels. Due to difficulties with getting high enough light

intensities this system was modified for the next round of experiments. During incubations conducted with *U. lactuca*, one LED lamp (V-light E14 dimmable HALED bulb; 230 V, 7 W) was used for each chamber. To obtain the different light levels a dimmer and density screens were used.

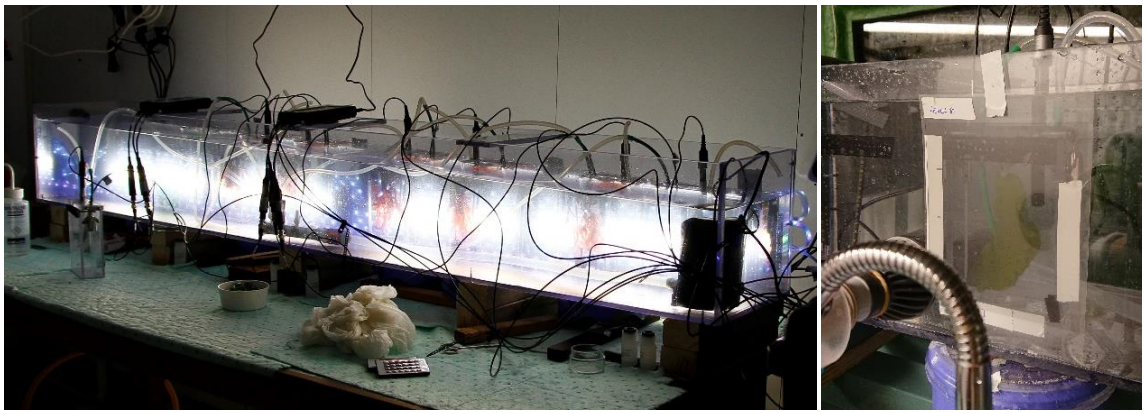


***Figure 6*** *The tank with the connected light sources used with* P. palmata *(left) and the one LED lamp with a density screen used with* U. lactuca *(right).*

All incubations started with a dark period, followed by higher and higher light intensities. The intensity and number of different light levels varied between the two species, depending on possible light levels and how fast the oxygen changed (Table 4). The aim for each light level was to observe a change in oxygen level above 5%. This limit was set to ensure that changes were caused by reactions in each test individual, and not just fluctuations in the measurements. Because of the lack of priori knowledge of the reaction of the algae to the $H_2O_2$, this change had to be observed in untreated algae. For the experiment conducted with *P. palmata* these time intervals were decided on the first incubation following the changes registered in the chamber with the control alga. When doing the experiment with *U. lactuca* the time intervals were decided by doing a test run with only untreated algae. For both species, the time spent at one specific light level was the same for each incubation, but the length of time at the different levels in one incubation varied. This was because it took shorter time to reach a 5% change at higher light intensities than at the lower ones.

***Table 4*** *Light levels measured in PAR and length of each level for incubations conducted with* P. palmata *and* U. lactuca.

| *Palmaria palmata* | | | *Ulva lactuca* | | |
|---|---|---|---|---|---|
| Light level | Measured PAR (µmol photons $m^{-2}$ $s^{-1}$) | Time intervals (min) | Light level | Measured PAR (µmol photons $m^{-2}$ $s^{-1}$) | Time intervals (min) |
| 1 | 0 | 60 | 1 | 0 | 120 |
| 2 | 14 | 30 | 2 | 12 | 90 |
| 3 | 35 | 30 | 3 | 26 | 40 |
| 4 | 50 | 30 | 4 | 46 | 30 |
| 5 | 61 | 25 | 5 | 60 | 30 |
| 6 | 75 | 25 | 6 | 90 | 20 |
| 7 | 110 | 25 | 7 | 124 | 20 |
| 8 | 177 | 15 | 8 | 160 | 20 |
| - | - | - | 9 | 203 | 20 |
| - | - | - | 10 | 232 | 20 |
| - | - | - | 11 | 349 | 20 |

### *2.4.3 Data processing*

The oxygen production/consumption rate was calculated for each alga at the different light levels, giving µmol $O_2$ produced per gram fresh weight per hour (µmol $O_2$ / g FW * h). These values were calculated by converting the measured oxygen content in each chamber over time (given in %) to $pO_2$. This was done after a method by Benson & Krause (1980; 1984). The barometric pressure was adjusted for relative humidity and vapour pressure and was multiplied by the solubility coefficient and the amount of water in the chamber (disregarding the calculated volume of the alga). These values were then corrected by the measured changes in the empty control chamber.

Linear regression was used to obtain the oxygen production (or change) per gram fresh weight per hour, by plotting the evolution of µmol $O_2$ against time (h) divided by the fresh weight of the alga given in grams (g). These values were then plotted against the light intensity (µmol photons /$m^2$ s) used in the experiment giving photosynthetic-irradiance curves for the three different time periods. By fitting a linear model to the measurements taken at the first light level (dark) the respiration rate was estimated for the different treatments.

The package *drc* in R for dose-response analyses was used to make dose-response curves and finding the best model fit for the results (R Core Team 2017; Ritz et al., 2015). For both species

a four-parameter log-logistic function was the best fitted model, with $P_{max}$ as the only variating variable. The autocorrelation that occurred because of repeated measurement on the same specimens on different light levels were not accounted for in this model. The "comParm" function was used to compare $P_{max}$ between the different treatments.

## 2.5 Assessing fluorescence lifetime and fluorescence intensity

An additional experiment was conducted with *U. lactuca*, measuring both the fluorescence lifetime and intensity after exposure to $H_2O_2$. Earlier studies have concluded with an increase in both fluorescence parameters, when photochemistry is decreased (Kristoffersen et al., 2016; Maxwell & Johnson 2000). Therefore, these measurements were included both to investigate if they could provide more information about the state of the photosynthetic apparatus, and to see if they coincide with the other results. A general description of these methods will be considered first, before the lab procedures.

### *2.5.1 Theory behind Fluorescence-lifetime imaging microscopy (FLIM)*

To measure the fluorescence lifetime, the method fluorescence-lifetime imaging microscopy (FLIM) was used. This is a method where a laser pulse is used to excite a part of the sample, consisting of many cells, before measuring the decay of the fluorescence intensity. Short optical laser pulses give the precision needed to measure this decay, with a resolution of nanoseconds accuracy (Suhling et al., 2015). Each laser pulse gives measurements that provide an image of the sample. The fluorescence decay can be collected by several methods, but the standard is to obtain them from each pixel in this image by time-correlated single photon counting (TCSPC) (Suhling et al., 2015). The FLIM setup consists of a laser pulse, a scanning microscope, a single photon counting detector (such as a detection photomultiplier tube - PMT), and a TCSPC card connected to a computer (Figure 7).

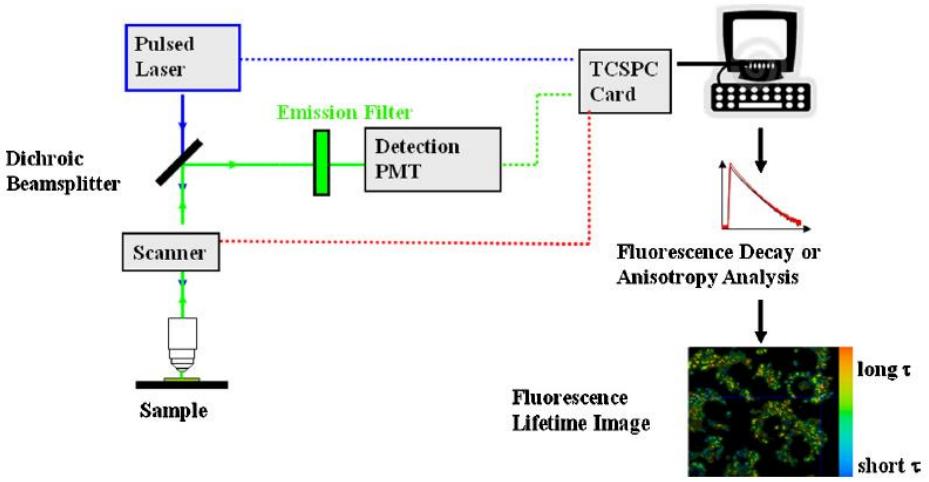



***Figure 7*** *A time-correlated single photon counting (TCSPC)-based scanning fluorescence-lifetime imaging microscopy (FLIM) set-up obtain from Suhling et al. (2015). The pulsed laser, scanning microscope and the detection photomultiplier tube (PMT) all synchronized to the time-correlated single photon counting (TCSPC) card, for accurate measurements of the fluorescence lifetime. The computer compiles the data and makes a fluorescence lifetime histogram, showing the lifetime decay over time. Further making a fluorescence lifetime image, composed of the fluorescence lifetime from each pixel from the obtained measurements.*

For every laser pulse, a *start* signal is sent to the TCSPC system. When the laser pulse hits the sample and the fluorescence is scattered back, the single photon counting detector (the PMT) registers the photon and sends a *stop* signal to the TCSPC system. These start and stop signals are gathered and a fluorescence lifetime histogram is made, showing the fluorescence lifetime decay in the sample. Hence, it shows how long the chlorophylls stay in the excited state before returning to the stable state.

The time span between each laser pulse and the wavelength is decided based on the target fluorophore in the sample, in our case the chlorophylls. A certain amount of energy must be attained before a chlorophyll molecule enters the excited state. By focusing the laser pulse, using a method called *two photon excitations*, it is possible to excite the sample with two photons with a lower energy (longer wavelength) instead of one higher energized photon. This is advantageous both because it leads to less strain in the rest of the sample and because only the area where the laser pulse is focused will obtain enough energy to emit fluorescence photons, hence reducing noise (Kristoffersen 2018).

### *2.5.2 Theory behind Fluorescence intensity*

It takes less than a second for the fluorescence intensity to reach its peak, and this phase is described as the OJIP phase. This phase is divided into four parts, the origin (O), being the minimum initial intensity level, two intermediate intensities (JI) and at last the intensity peak (P) (Kristoffersen et al., 2016). By investigating this development different photosynthetic

parameters can be obtained, such as minimum ($F_0$), maximum ($F_m$) and variable fluorescence ($F_v$) (Maxwell & Johnson 2000).

### *2.5.3 Samples and lab setup*

Prior to the exposure the specimens were acclimatized in one, five and eleven days and kept in 1L beakers filled with filtrated seawater of about 9 °C. The water was changed daily, and they were kept under a 12:12 light regime, until the commence of each experiment. Due to technical issues and equipment availability the time before the experiment started varied from one to 11 days. Before exposure, individual alga was randomly picked from the beaker. Due to their small size, they were all individually exposed in 45 ml conical centrifuge tubes (Figure 8). This was also to minimize the time difference from exposure to measurements, between the different individuals. After exposure they were kept separate, in the same types of tubes filled with seawater, until all measurements were done. This, due to both their small size, making it difficult to label them individually and because it led to less strain on the algae, not having to move them around. They were kept in these separate tubes until all measurements were taken.

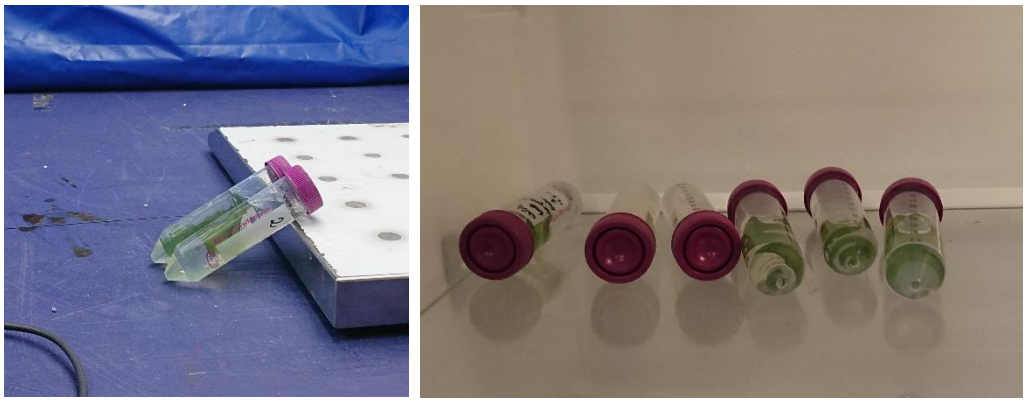


***Figure 8*** U. lactuca *being individually exposed in 45 ml conical centrifuge tubes before assessing fluorescence lifetime (left). Between the day 0 and day 1 measurement the algae were held in individually tubes in a fridge (right).*

To be able to apply a low enough concentration of $H_2O_2$ in such small volumes, these were made by further diluting 10% and 20% solutions (except for the 50% solution). The concentrations used in this experiment were chosen based on the results from the sublethal experiments (see Table 5). Because of the results from the first series of experiment were conducted in December 2018, additional experiments using higher concentrations of $H_2O_2$ were conducted in January 2019, using the same procedure.

***Table 5*** *By exposing* U. lactuca *(n=3) specimens to $H_2O_2$ dilutions for one hour, the fluorescence lifetime was measured. The dilutions were based on a common bath-treatment dose (being 1700 mg/l) used during salmon sea-lice infestation in net pens.*

| December 2018 | | January 2019 | |
|---|---|---|---|
| Concentration (mg/l $H_2O_2$) | Dilution of bath-treatment (%) | Concentration (mg/l $H_2O_2$) | Dilution of bath-treatment (%) |
| Seawater (control) | 0 | 212.5 | 15 |
| 85 | 5 | 340 | 20 |
| 127.5 | 7.5 | 850 | 50 |
| 170 | 10 | - | - |
| 212.5 | 12.5 | - | - |
| 255 | 15 | - | - |

### *2.5.4 Instrumentation, measurements and data processing*

The fluorescence lifetime and fluorescence intensity measurements were done according to Kristoffersen et al. (2016). Measurements were taken both immediately after (day 0) and 24H after exposure (day 1). Both types of measurements were taken by cutting a piece of the alga placing it in either the microscope or in the Aquapen. Three specimens were exposed, for one hour, to each concentration of $H_2O_2$. Three replicates were taken for each specimen for the lifetime measurement and two for the intensity, resulting in nine and six measurements per concentration. During the lifetime measurements the laser (excitation) wavelength was initially set to 860-nm, but because of some difficulties with the equipment some samples were excited with 800-nm or 830-nm. When lowering the wavelength, the exposure time was lowered as well from 3 to 2 minutes, because of the increasing intensity. An Aquapen-C AP-C 100 fluorometer (Photon Systems Instrument, Czech Republic) was used to measure this fluorescence intensity.

The raw data was processed by Arne S. Kristoffersen, as done in Kristoffersen et al. (2016). This resulted in fluorescence lifetime values, in addition to the PSII maximum quantum yield ( $\frac{F_V}{F_M} = (\frac{F_m - F_0}{F_m})$) and $\frac{F_M}{F_0}$ ratio, calculated from the Aquapen results. Then, the significance difference between the different treatments and control was tested in R (R Core Team 2017).

# 3. Results

## 3.1 *Palmaria palmata*

### *3.1.1 Lethal effects*

The aim of the lethal experiment was to determine lethal doses of $H_2O_2$ by observing the algae over several days and registering changes in appearance, smell and structure. The results from this experiment conducted with *P. palmata* gave neither visible effects on the algae in the control group, nor the two groups exposed to the lowest $H_2O_2$ concentrations, being 170 mg/l and 475 mg/l (Table 6). The group exposed to the highest concentration (1700 mg/l $H_2O_2$) showed signs of bleaching after six hours. At concentrations of 1250 mg/l and 850 mg/l $H_2O_2$ the algae started to show sign of exposure after 24h and five days, respectively, with bleached areas and a rotten smell. The visible effects varied between these two concentrations, but after six days all of them had discoloration, being green and yellow patches in the lamina. Some specimens also had a rotten smell and a flaccid lamina structure.

***Table 6*** *lethal effects on* P. palmata*, n = 10 specimens for each concentration. NVE (no visible effects), W (Whitened areas), S (rotten smell), F (flaccid lamina structure), Dis (discoloration – green/yellow patches). In the table new signs of the H2O2 impact on one or more specimens are shown on the first day of emergence.*

| Concentration ( $H_2O_2$ mg/l) | Dilution of bath-treatment (%) | Acute | 6h | 24h | Day 3 | Day 5 | Day 6 |
|---|---|---|---|---|---|---|---|
| Seawater (control) | 0 | NVE | NVE | NVE | NVE | NVE | NVE |
| 170 | 10 | NVE | NVE | NVE | NVE | NVE | NVE |
| 425 | 25 | NVE | NVE | NVE | NVE | NVE | NVE |
| 850 | 50 | NVE | NVE | NVE | NVE | Dis | - |
| 1250 | 75 | NVE | NVE | W, S | - | Dis | - |
| 1700 | 100 | NVE | W | S | F | Dis | - |

### *3.1.2 Sublethal effects*

In the sublethal experiment the state of the photosynthetic apparatus was investigated after exposure to different concentrations of $H_2O_2$. The concentrations were based on the results from the lethal experiment. One method used was to make PI-curves, illustrating the oxygen production at increasing light levels. The PI-curves for *P. palmata* showed an immediate difference in in photosynthetic efficiency between the different treatments of $H_20_2$ (Figure 9 – day 0) when compared with the control. The three different incubation periods (day 0, day 1 and day 11) all showed the same pattern with a decreasing oxygen production when exposed to an increasing $H_2O_2$ concentration. The PI-curves for the groups exposed to the three highest $H_20_2$ concentrations were similar and did not exceed zero at all, meaning that there was no oxygen production, hence no photosynthetic activity. At day 11 these values were negative, meaning that these algae were consuming oxygen during all light levels. Specimens from the

control group showed higher individual differences compared to specimens in the $H_2O_2$ exposed groups at all time points. In addition, all PI-curves reached a point where the oxygen production no longer increased with increasing PAR.

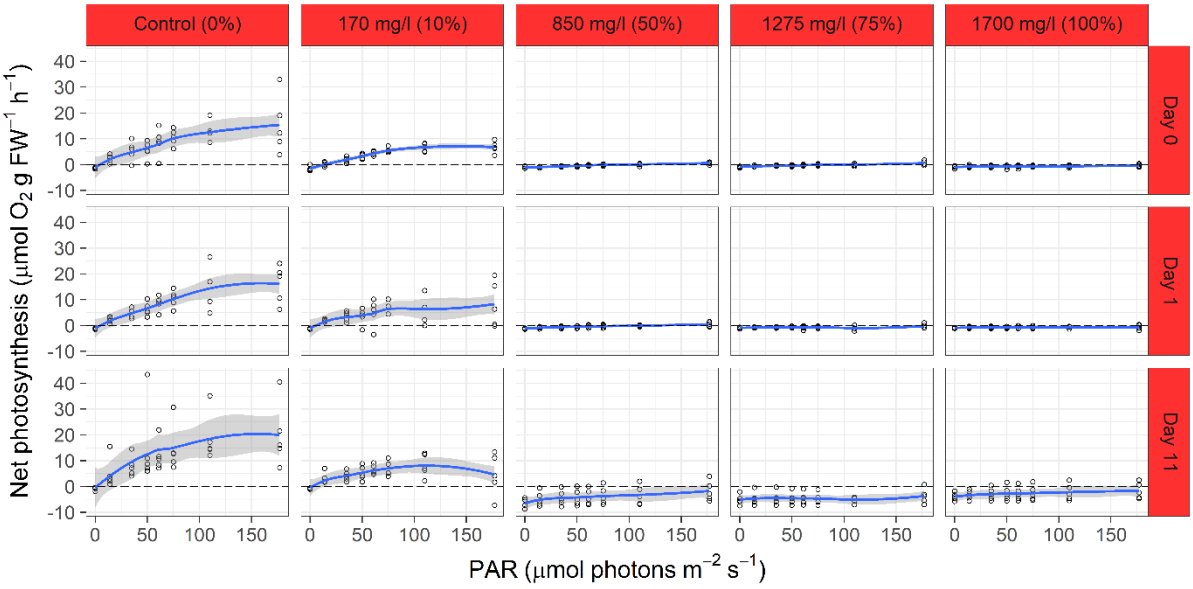



***Figure 9*** *Photosynthesis-irradiance curves for the red alga* P. palmata. *Measurements taken at different time points after exposure. Here showing the average production for n=5 specimens per concentration. PAR: photosynthetic active radiation. FW: fresh weight.*

The dose-response curves represented an average for the five specimens per concentration, with $P_{max}$ as the only parameter varying between the treatments (Figure 10). The control group showed a maximum $O_2$ production below 20 µmol g $FW^{-1}h^{-1}$ right after and the day after exposure (day 0 and day 1), whereas 11 days after exposure these values were between 20 and 30 µmol $O_2$ g $FW^{-1}h^{-1}$. The algae exposed to the lowest dose (170 mg/l $H_2O_2$) had an oxygen production between five and ten µmol $O_2$ g $FW^{-1}h^{-1}$ at all incubations. The specimens from the three groups exposed to the highest concentrations (850, 1250 and 1700 mg/l $H_2O_2$) did not produce any oxygen, and therefore never exceed zero in the dose-response curves.

The estimated $P_{max}$ values for the different treatment groups, from the model that gave the best data fit is shown in Table 7. These results show that through the entire experiment there were significant differences in the estimated $P_{max}$ values for the control group and the $H_2O_2$ exposed algae. The control group always had an estimated production above 22 µmol $O_2$ g $FW^{-1}h^{-1}$. The group of algae exposed to 170 mg/l $H_20_2$ had highest estimated production the day after exposure, being 14 µmol $O_2$ g $FW^{-1}h^{-1}$. At the two next incubations this group resulted with values at about 11 µmol $O_2$ g $FW^{-1}h^{-1}$ (day 1 and day 11). The three groups exposed to the

highest $H_20_2$ concentrations (850, 1250 and 1700 mg/l), never exceeded 1.5 μmol $O_2$ g $FW^{-1}h^{-1}$ and were not significantly different from each other.

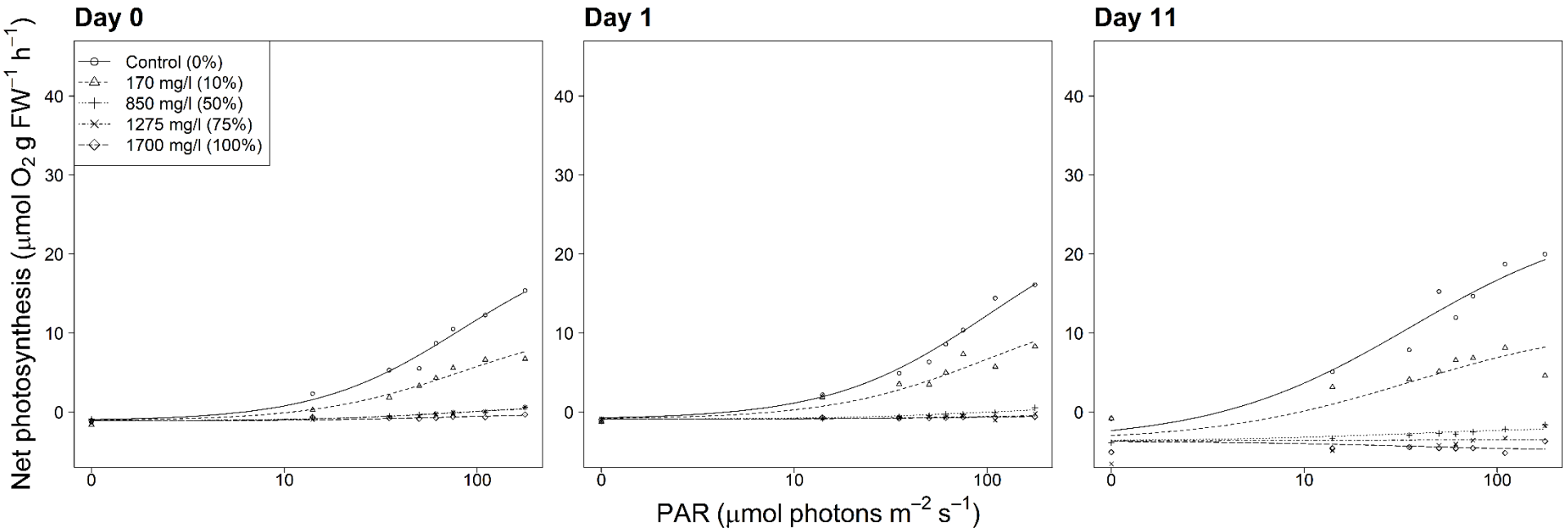



***Figure 10*** *Dose-response curves for the red alga* P. palmata *exposed to different* $H_2O_2$ *concentrations for one hour. Measurements taken at different time points after i after exposure. Showing the average production for n=5 algae per concentration. PAR: photosynthetic active radiation. FW: fresh weight*

***Table 7*** *Estimated $P_{max}$ values (µmol $O_2$ g $FW^{-1}h^{-1}$) for the red alga* P. palmata *at different time points after exposure to different $H_2O_2$ concentrations. Showing the standard error for the value and the p-value when comparing the different treatments with the control group exposed to seawater (0 mg/l $H_2O_2$). The estimates and SE rounded to three decimal places. *significantly different from the control.*

| Treatment dose Mg/l $H_2O_2$ | Day 0 | | | Day 1 | | | Day 11 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Estimate | SE | P- value | Estimate | SE | P- value | Estimate | SE | P- value |
| 0 | 22.008 | 6.385 | | 25.509 | 10.601 | | 25.178 | 9.253 | |
| 170 | 11.302 | 3.503 | 0.0009* | 14.361 | 6.267 | 0.0178* | 11.288 | 4.898 | 0.0044* |
| 850 | 1.027 | 1.102 | 0.0004* | 0.916 | 1.473 | 0.0133* | -3.451 | 1.381 | 0.0022* |
| 1250 | 1.087 | 1.117 | 0.0004* | -0.222 | 1.283 | 0.0132* | -4.977 | 1.435 | 0.0022* |
| 1700 | -0.092 | 0.965 | 0.0004* | -0.375 | 1.266 | 0.0132* | -1.762 | 1.514 | 0.0023* |

The linear model fitted to the measurements taken during the first light level (in the dark) estimated the respiration rate ($R_d$) for the different treatments (Table 8). These results showed an oxygen consumption of the control algae descending from -1.2 to -0.8 during the three incubations (µmol $O_2$ g $FW^{-1}$ $h^-1$). The measurements from day 0 showed no significant difference in respiration rate of the control specimens compared to the ones exposed to $H_2O_2$. The first significantly different estimate was from the day 1 incubations, which was between the control group and the group exposed to 1250 mg/l $H_2O_2$. At day 11 the three groups exposed to the three highest concentrations consumed a significantly higher amount of oxygen than the control group.

***Table 8*** *Estimated $R_d$ (µmol $O_2$ g $FW^{-1}h^{-1}$) values for the red alga* P. palmata *at different time points after exposure to different $H_2O_2$ concentrations for one hour. Showing the standard error for the value and the P-value when comparing the different treatments with the control group exposed to seawater (0 mg/l $H_2O_2$). The estimates and SE rounded to three decimal places. *significantly different.*

| Mg/l $H_2O_2$ | Day 0 | | | Day 1 | | | Day 11 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Estimate | SE | P- value | Estimate | SE | P- value | Estimate | SE | P- value |
| 0 | -1.264 | 0.246 | | -1.179 | 0.092 | | -0.844 | 0.619 | |
| 170 | -0.338 | 0.348 | 0.344 | -0.079 | 0.129 | 0.5482 | 0.049 | 0.875 | 0.9560 |
| 850 | 0.133 | 0.348 | 0.707 | 0.033 | 0.129 | 0.7994 | -5.716 | 0.875 | $2.31^{-6}$ * |
| 1275 | 0.381 | 0.348 | 0.287 | 0.301 | 0.129 | 0.0309* | -4.237 | 0.875 | $9.94^{-5}$ * |
| 1700 | 0.182 | 0.348 | 0.592 | 0.223 | 0.129 | 0.1005 | -3.051 | 0.875 | 0.0023* |

Pictures were taken of all specimens before exposure and 11 days after. The first signs of impact on specimens that were exposed to $H_2O_2$ were bleached areas on the lamina and a rotten smell,

just as in the lethal experiment. The three groups exposed to the highest concentrations (1700, 1250 and 850 mg/l $H_2O_2$) showed the same signs of effect, being a high degree of discoloration of the lamina after 11 days (Figure 11 - A). The control group and the group exposed to 170 mg/l showed no signs of effect after 11 days (Figure 11 - B and C).


***Figure 11*** *Effects after one-hour exposure to different concentrations of $H_2O_2$ on lamina of* P. palmata. *Pictures taken before exposure (left) and 11 days after (right). Exposed to 850 mg/l (A), 170 mg/l (B) and a control (C)*

Weight measurement were also taken before and 11 days after exposure. During this time period the specimens exposed to 1700, 1250 and 850 mg/l $H_2O_2$ had a significant decrease in biomass ($p < 0.02$) (Figure 12). The control group and the group exposed to 170 mg/l $H_2O_2$ showed no significant change in biomass ($p > 0.4$). 11 days after exposure there was a significant difference between the biomass of the specimens in the control group, compared to the ones exposed to the three highest concentrations ($p < 0.0003$). At the same time there were no significant differences when comparing the control group to the specimens exposed to the lowest concentration of $H_20_2$ ($p > 0.6$).

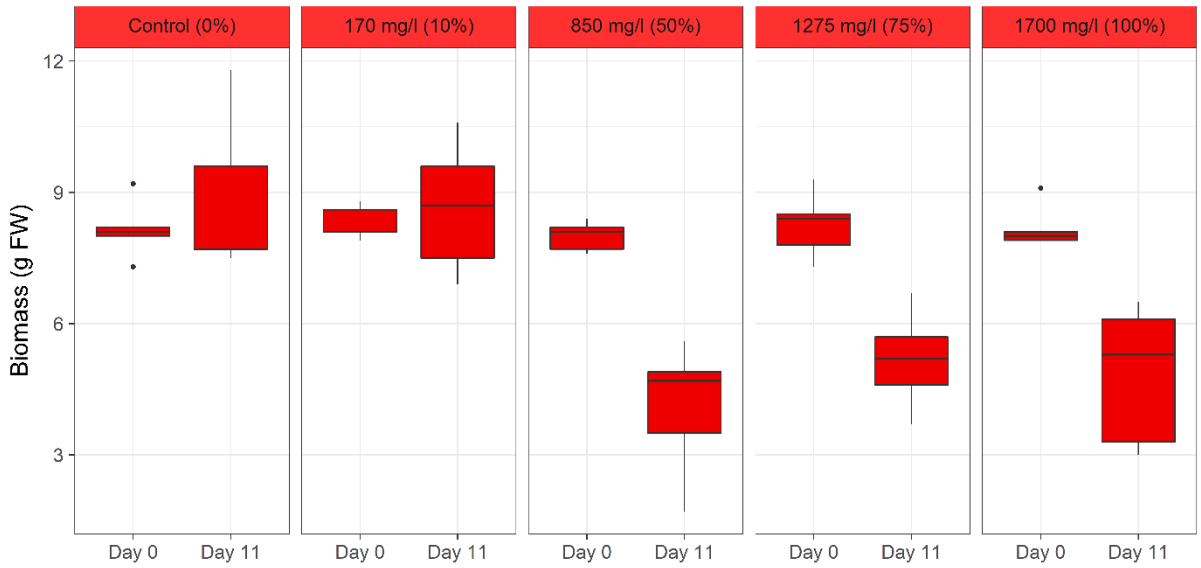



***Figure 12*** *Change in biomass for the red alga* P. palmata *exposed to different* $H_2O_2$ *concentrations for one hour. Measurements taken right before exposure and 11 days after for n=5 algae per concentration. FW: fresh weight.*

## 3.2 Ulva lactuca

### *3.2.1 Lethal effects*

The lethal effects experiment was conducted in the same way with *U. lactuca* as it was with *P. palmata* (except for other concentrations), trying to determine lethal doses of $H_2O_2$ by observing the algae over several days. The results from this experiment gave no visible effects on the algae in the control group, exposed to seawater. After seven days some of the specimens in each treatment group had visible effects (Table 9).

24 hours after exposure the first sign of the treatment was observed, being a rotten smell, in specimens exposed to 425 mg/l and higher $H_2O_2$ concentrations. The first visible sign of the treatment was faded coloration, and this appeared two days after exposure for individuals exposed to 850 mg/l, 1275 mg/l and 1700 mg/l. After five days some of the algae exposed to the lower concentrations (170 mg/l and 85 mg/l $H_2O_2$) also showed visible signs. After one week, all the individuals exposed to the highest concentrations (1700 mg/l, 1275 mg/l and 850 mg/l $H_2O_2$) appeared to be dead, because of high degree of discoloration and a very flaccid lamina structure.

***Table 9*** *Lethal effects on* U. lactuca. *NVE (no visible effects), S (rotten smell), F (flaccid lamina structure), Fa (faded coloration), Dis (discoloration – green/yellow patches). n=5 for each concentration * Some of the individuals, others where NVE. In the table new signs of the $H_2O_2$ impact on one or more individuals are shown on the first day of emergence.*

| Concentration (mg/l $H_2O_2$) | Dilution of bath-treatment | Acute | 24h | Day 2 | Day 3 | Day 4 | Day 5 | Day 7 |
|---|---|---|---|---|---|---|---|---|
| Seawater (control) | 0% | NVE | NVE | NVE | NVE | NVE | NVE | NVE |
| 85 | 5% | NVE | NVE | NVE | NVE | NVE | F, Fa* | - |
| 170 | 10% | NVE | NVE | NVE | NVE | NVE | F, Fa* | - |
| 425 | 25% | NVE | S | F | Fa | - | - | - |
| 850 | 50 % | NVE | S | Fa | Dis | - | - | Dead |
| 1275 | 75% | NVE | S | F, Fa | Dis | - | - | Dead |
| 1700 | 100% | NVE | S, F | Fa | Dis | - | - | Dead |

### *3.2.2 Sublethal effects*

One of the methods to investigate the sublethal effects of $H_2O_2$ was to make PI-curves. The PI-curves from the measurements for *U. lactuca* from day 0, showed an inverse correlation between $H_2O_2$ concentration and oxygen production (Figure 13). Because of an outlier the PI-curve for the 170 mg/l treatment group consists of five specimens at day 1 and day 14. The individual difference between specimens within each treatment group was higher when exposed to lower $H_2O_2$ concentration. All PI-curves (from day 0) reached saturation, where the oxygen production no longer increased. The measurements taken the day after (day 1) showed similar results, but the control group still had an increasing trend in net photosynthesis at the highest light level. After two weeks (day 14) the PI-curves for the three lowest concentrations and the control showed that they had (in average) a higher net photosynthetic production compared with the acute measurements (day 0). The PI-curve for the highest concentration showed that there was in average no net photosynthesis at day 1 or day 14, but one individual was still showing signs of photosynthetic activity.

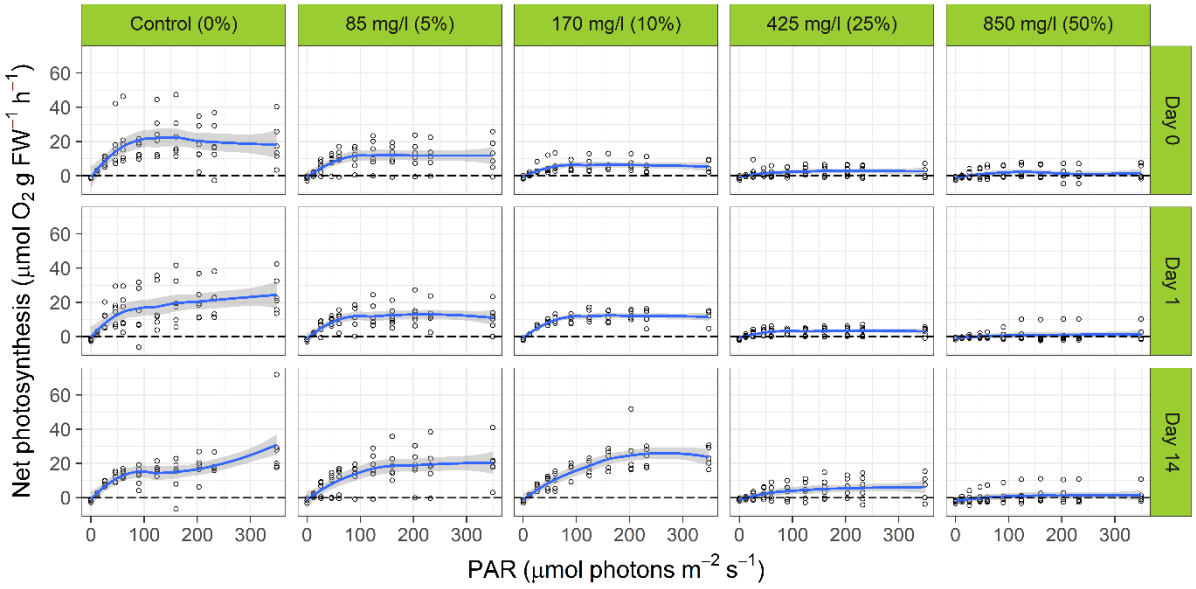



***Figure 13*** *Photosynthesis-irradiance curves for the green alga* U. lactuca *at different time points after exposure to different $H_2O_2$ concentrations for one hour. Here showing the average production for n=6 algae per concentration, except for 10% where n=5 at day 1 and day 14. PAR: photosynthetic active radiation. FW: fresh weight. The outliners for the group exposed to 850 mg/l for day 1 and day 14 was the same individual.*

The dose-response package in R (drc) was used to make dose-response curves and estimating the maximum photosynthetic rate ($P_{max}$). These curves represent an average for the five specimens per concentration, with $P_{max}$ as the only parameter varying between the treatments. The dose-response curves showed a clear difference between the different treatments (Figure 14), and illustrate that $P_{max}$ was reached for all the treatment groups at day 0 and day 1. It also becomes evident when studying curves for day 14 that these do not reach the same flattened curvature, indicating that $P_{max}$ was not reached.

The estimated $P_{max}$ values for the different treatments showed a significantly different maximum $O_2$ production right after exposure for all treatments compared with the control group (Table 10). The $P_{max}$ value for the control group increased from around 20 µmol $O_2$ g $FW^{-1}h^{-1}$ to above 35 µmol $O_2$ g $FW^{-1}h^{-1}$ during the two weeks in the lab. All groups of algae exposed to $H_2O_2$ had also an increase in maximum oxygen production during this period. At both day 0 and day 1 all treated groups had a significantly lower estimated $P_{max}$ value, compared with the control group. After 14 days, the two groups exposed to the two lowest concentrations had estimated $P_{max}$ values higher than the control group, but these were not significantly higher.

**Table 10** *Estimated $P_{max}$ ($\mu$mol $O_2$ g $FW^{-1}h^{-1}$) values for the green alga* U. lactuca *at different time points after exposure to different $H_2O_2$ concentrations for one hour. Showing the standard error for the value and the P-value when comparing the different treatments with the control group exposed to seawater (0 mg/l $H_2O_2$). The estimates and Se rounded to three decimal places. *significantly different from the control group.*

| Mg/l $H_2O_2$ | Day 0 | | | Day 1 | | | Day 14 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Estimate | SE | P- value | Estimate | SE | P- value | Estimate | SE | P- value |
| 0 | 20.285 | 1.011 | | 21.764 | 1.874 | | 35.922 | 11.856 | |
| 85 | 11.477 | 0.918 | $4.031x10^{-12}$* | 13.538 | 1.349 | $5.343x10^{-9}$* | 33.200 | 10.873 | 0.3057 |
| 170 | 6.224 | 0.953 | $< 2.2x10^{-16}$* | 13.181 | 1.385 | $5.905x10^{-9}$* | 39.596 | 12.820 | 0.1828 |
| 425 | 2.464 | 0.860 | $< 2.2x10^{-16}$* | 3.880 | 0.947 | $< 2.2x10^{-16}$* | 10.684 | 4.343 | 0.0023* |
| 850 | 1.543 | 0.857 | $< 2.2x10^{-16}$* | 1.192 | 0.896 | $< 2.2x10^{-16}$* | 3.261 | 2.547 | 0.0018* |

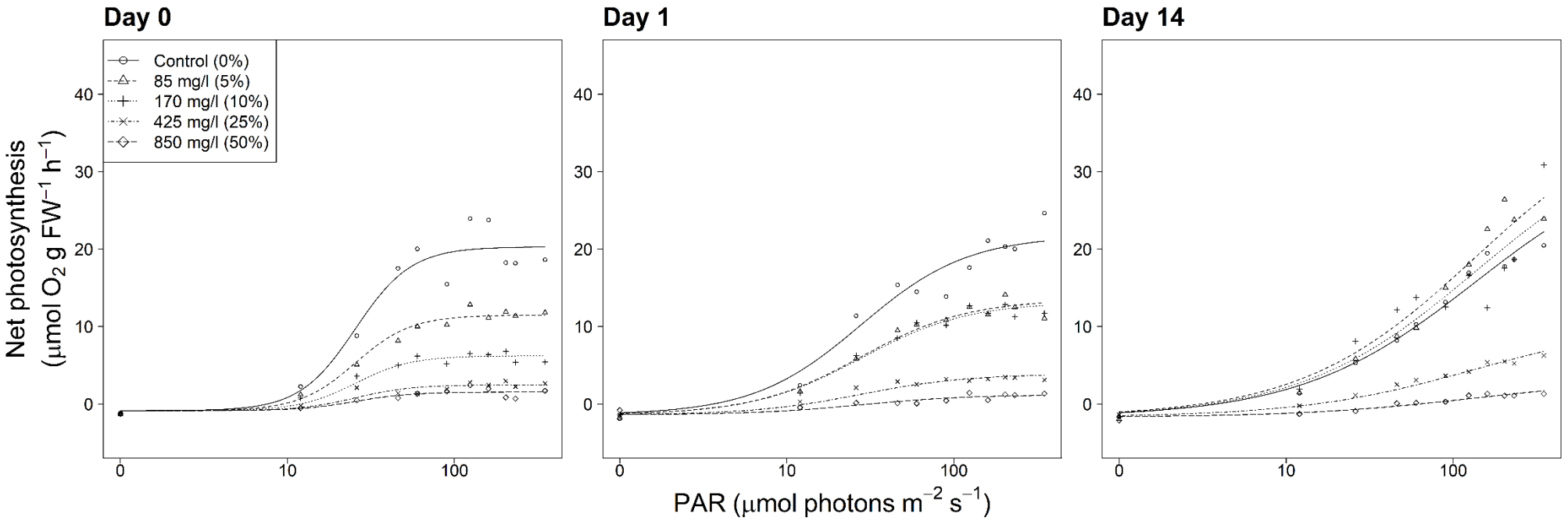



***Figure 14*** *Dose-response curves for the green alga* U. lactuca *at different time points after exposure to different* $H_2O_2$ *concentrations for one hour. The plot showing the result from the best fitted model, being a log-logistic model where only the parameter maximum asymptote varied. Here showing the average production for n=6 algae per concentration together in the same plot, except for the 170 mg/l treatment group were n=5 for day 1 and day 14. PAR: photosynthetic active radiation. FW: fresh weight*

The estimated values for the dark respiration (Rd) for the different algae groups showed an oxygen consumption above 1.2 μmol $O_2$ g $FW^{-1}h^{-1}$ at the three different incubations for the control group (Table 11). The estimates from the day 0 incubations resulted in an oxygen consumption for all the different algae groups, but with no significant difference from the control group. The estimates from day 1 showed positive values for the $H_2O_2$ exposed groups, indicating an oxygen production, but only the group exposed to the highest concentration (850 mg/l) were significantly different from the control. After two weeks in the lab all exposed groups had a lower oxygen consumption than the control group, but none where significantly different.

**Table 11** *Estimated $R_d$ ($\mu mol\ O_2\ g\ FW^{-1}h^{-1}$) values for the green alga* U. lactuca *at different time points after exposure to different $H_2O_2$ concentrations for one hour. Showing the standard error for the value and the P-value when comparing the different treatments with the control group exposed to seawater (0 mg/l $H_2O_2$). The numbers rounded to four decimal places. *significantly different*

| Mg/l $H_2O_2$ | Day 0 | | | Day 1 | | | Day 14 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Estimate | SE | P- value | Estimate | SE | P- value | Estimate | SE | P- value |
| 0 | -1.200 | 0.291 | | -1.896 | 0.208 | | -1.690 | 0.296 | |
| 85 | -0.155 | 0.411 | 0.7092 | 0.136 | 0.294 | 0.6477 | 0.033 | 0.418 | 0.937 |
| 170 | -0.007 | 0.431 | 0.9875 | 0.441 | 0.309 | 0.1658 | -0.283 | 0.439 | 0.525 |
| 425 | -0.001 | 0.411 | 0.9990 | 0.476 | 0.294 | 0.1192 | 0.299 | 0.418 | 0.481 |
| 850 | -0.090 | 0.411 | 0.8290 | 1.104 | 0.294 | 0.0010* | -0.424 | 0.418 | 0.321 |

As with the lethal effect experiment, the first visible signs of $H_20_2$ exposure were faded green coloration of the lamina, followed by patches of yellow (discoloration). Of the six individuals exposed to the two highest concentrations five of them had severe pigmentation loss and such flaccid lamina structure that they disintegrated (Figure 15, A and B). The remaining one from each concentration still had parts of the lamina that looked healthy (Figure 16). All algae exposed to 170 and 85 mg/l $H_2O_2$ and the control group looked healthy through the two weeks (Figure 15 – C).

During this time period, the control group and the group exposed to 170 mg/l $H_2O_2$ showed no significant weight change ($p > 0.4$). The control algae had a significant increase in biomass during this period ($p = 0.000967$), as well as the algae exposed to 85 mg/l and 170 mg/l $H_2O_2$ ($p = 0.01078$ and $p = 0.0358$ respectively) (Figure 17). Both these groups had one individual representing an outlier in the dataset after 7 and 14 days. None of the three groups exposed to the highest concentrations had a significant change in biomass during the last week (from 7 to

14 days). The two groups of algae exposed to the highest $H_20_2$ concentrations (425 mg/l and 850 mg/l) resulted in no significant weight change at all but had a decreasing trend.

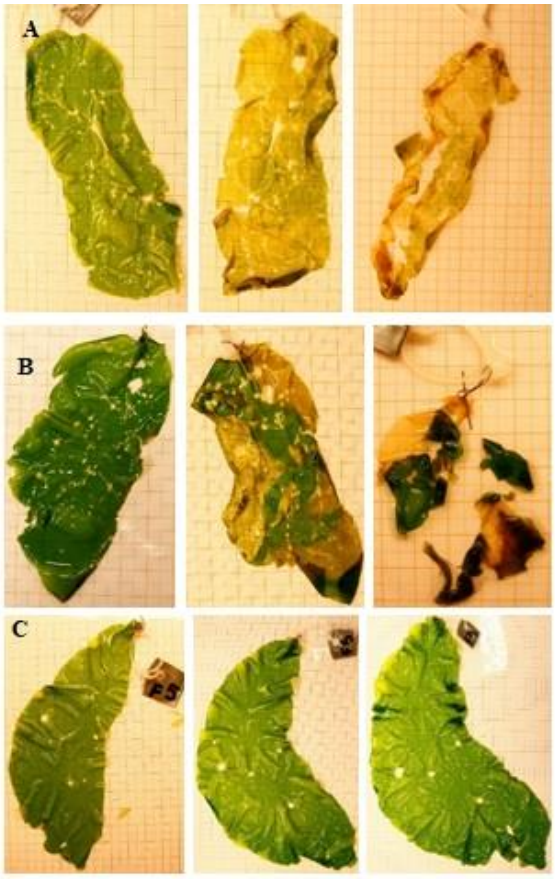



***Figure 15*** *Effects after one-hour exposure to different concentrations of $H_2O_2$ on lamina of* U. lactuca. *Pictures taken before exposure (left), 7 days after (middle) and 14 days after (right). Exposed to 850 mg/l (A) and 425 mg/l (B)*


***Figure 16*** *Effects after one-hour exposure to different concentrations of $H_2O_2$ on lamina of* U. lactuca. *Pictures taken before exposure (left), 7 days after (middle) and 14 days after (right). Exposed to 850 mg/l (A), 425 mg/l (B) and a control-alga exposed to seawater (C)*

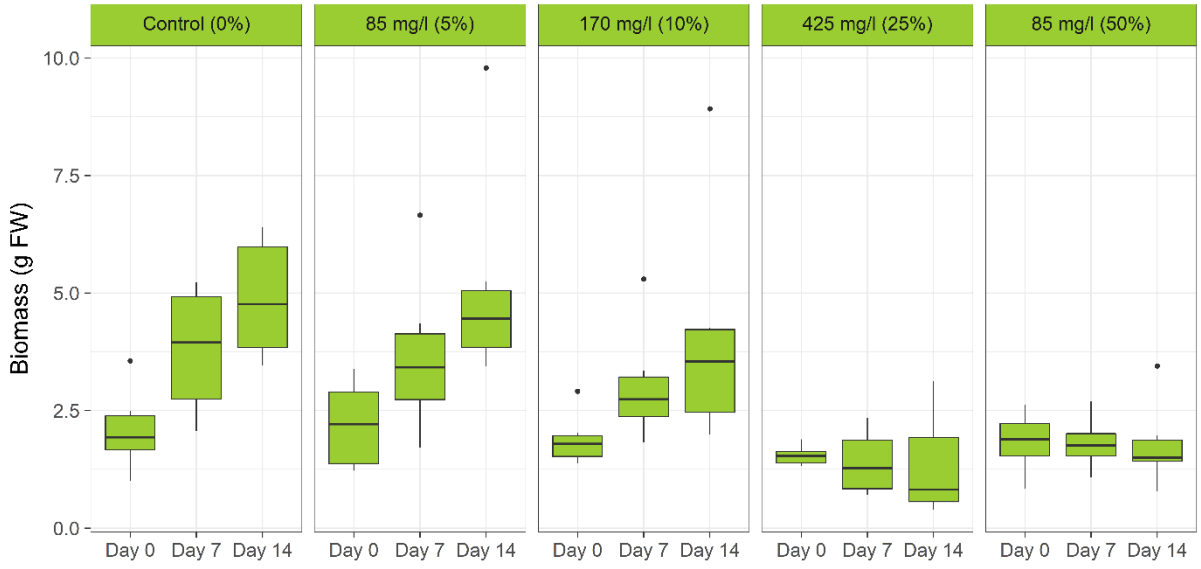



***Figure 17*** *Change in biomass for the green alga* U. lactuca *exposed to different $H_2O_2$ concentrations for one hour. Measurements taken right before exposure, 7 days- and 14 days after for n=6 algae per concentration. FW: fresh weight.*

### *3.2.3 Fluorescence lifetime and intensity*

The fluorescence lifetime of specimens of *U. lactuca* exposed to different concentrations of $H_2O_2$ was obtained by using a fluorescence-lifetime imaging microscopy set-up. The results from these measurements, being an average for each concentration, showed both significantly higher and lower lifetimes for the different treatments (Table 12). All the measurements resulted in two lifetime components for the fluorescence lifetime data. The short component ($\tau_1$) had a relative amplitude between 70-85%, which made it the main component and the most interesting to discuss. The results from day 0 showed longer fluorescence lifetimes for specimens exposed to the lowest $H_2O_2$ concentrations, compared with the control, except for the group exposed to 85 mg/l that had a significant reduction. The specimens exposed to the two highest $H_2O_2$, also resulted with significant lifetime reductions compared to the control. The two groups of specimens exposed to 255 mg/l $H_2O_2$ gave opposite results, where the first group showed an increasing trend in lifetimes (though with a very high standard deviation) and the last group a decreasing one.

The day after exposure (day 1) the lowest concentrations (up to 212.5 mg/l $H_2O_2$) had an increasing trend in fluorescence lifetimes, where two of them were significantly higher than the control group. The day 1 lifetime results for the two-measurement series conducted with 255 mg/l $H_2O_2$ showed the same decreasing trend, with one series significantly lower. The two highest $H_2O_2$ (340 mg/l and 850 mg/l $H_2O_2$) concentration resulted with significantly lower fluorescence lifetimes at day 1. In addition to this, the value for each specimen in each group

was also calculated, for both days (appendix 2). This revealed some individual differences, but with the same overall patterns as the average values showed.

***Table 12*** *Chlorophyll α lifetime data for the green alga* U. lactuca *exposed to different concentrations of $H_2O_2$ and a control group exposed to seawater. Measurements taken right after exposure and the day after. Including the short and long lifetime components, $\tau_1$ and $\tau_2$ and the short lifetime components relative amplitude, $\alpha_1$. All values including ± standard deviation. The P-value showing a comparison with the control. * measurements taken in the second period in the lab in January.*

| Mg/l $H_2O_2$ | Day 0 | | | | Day 1 | | | |
|---|---|---|---|---|---|---|---|---|
| | $\tau_1$ (ps) | P – value | $\tau_2$ (ps) | $\alpha_1$ (%) | $\tau_1$ (ps) | P – value | $\tau_2$ (ps) | $\alpha_1$ (%) |
| Control | 415 ± 49 | | 1042 ± 90 | 78 ± 2 | 448 ± 71 | | 1173 ± 273 | 79 ± 5 |
| 85 | 362 ± 58 | 0.0099 | 1114 ± 170 | 79 ± 2 | 487 ± 98 | 0.2131 | 1210 ± 169 | 75 ± 6 |
| 127.5 | 528 ± 34 | $8.34x10^{-6}$ | 1690 ± 219 | 75 ± 4 | 519 ± 63 | 0.0271 | 1606 ± 314 | 76 ± 3 |
| 170 | 438 ± 85 | 0.3521 | 1451 ±221 | 81 ± 2 | 524 ± 61 | 0.0183 | 1525 ± 119 | 78 ± 6 |
| 212.5 | 485 ± 46 | 0.0044 | 1399 ± 208 | 76 ± 2 | 499 ± 74 | 0.1141 | 1196 ± 146 | 77 ± 5 |
| 255 | 427 ± 113 | 0.6199 | 1187 ± 270 | 72 ± 6 | 397 ± 65 | 0.1087 | 1115 ± 206 | 77 ± 2 |
| 255* | 324 ± 30 | 0.0031 | 1249 ± 149 | 80 ± 7 | 375 ± 53 | 0.0287 | 1180 ± 190 | 70 ± 3 |
| 340* | 315 ± 55 | 0.0001 | 1337 ± 202 | 83 ± 4 | 348 ± 59 | 0.0023 | 1220 ± 145 | 84 ± 4 |
| 850* | 308 ± 30 | $4.47x10^{-5}$ | 1150 ± 173 | 77 ± 6 | 289 ± 28 | $1.27x10^{-5}$ | 1169 ± 212 | 85 ± 3 |

The fluorescence intensity measurement taken with the Aquapen, investigating the OJIP phase, resulted with highly variable estimates (Table 13). Neither the measurements taken right after exposure (day 0) or the ones taken at day 1, showed any clear patterns between the different treatments for the maximum fluorescence over the minimum fluorescence ratio ($F_m/F_0$). The variable fluorescence over maximum fluorescence ($F_v/F_m$) did not either show any patterns between the different treatments at any of the two time points. The two different measurements series conducted with the 255 mg/l $H_2O_2$ concentration also gave very different results. The calculated ratios for each specimen in each group showed some individual differences, but the same overall pattern as the other results (appendix 3).

***Table 13*** *Fm/Fo and Fv/Fm ratios calculated from measurements of OJIP fluorescence of chlorophyll α. Measurements taken of the green alga* U. lactuca *exposed to different concentrations of $H_2O_2$ and a control group exposed to seawater. *measurements taken in the second period in the lab in January.*

| Mg/l $H_2O_2$ | Day 0 | | Day 1 | |
|---|---|---|---|---|
| | Fm/Fo | Fv/Fm | Fm/Fo | Fv/Fm |
| Control | 3.99 | 0.75 | 4.23 | 0.76 |
| 85 | 4.22 | 0.76 | 4.31 | 0.77 |
| 127.5 | 2.33 | 0.54 | 2.76 | 0.63 |
| 170 | 1.54 | 0.34 | 1.81 | 0.45 |
| 212.5 | 3.18 | 0.68 | 4.08 | 0.74 |
| 255 | 3.68 | 0.72 | 3.85 | 0.74 |
| 255* | 1.56 | 0.36 | 1.68 | 0.40 |
| 340* | 1.76 | 0.43 | 1.78 | 0.44 |
| 850* | 2.00 | 0.50 | 2.16 | 0.52 |

# 4. Discussion

$H_2O_2$ has been the most used remedy against salmon sea lice (*L. salmonis*) in Norway since 2013 (Hjeltnes et al., 2019). Recent studies have revealed that the kelp *S. latissima* is highly sensitive toward this remedy (Haugland et al., 2019). This study assessed how the red alga, *P. palmata* and the green alga, *U. lactuca* respond to exposure. The state of their photosynthetic apparatus was mapped using two different methods. The oxygen evolution was measured during increasing light levels for both species. In addition, fluorescence lifetime and intensity measurements were taken of *U. lactuca.* This information is important, not only to assess how this remedy can affect the algae in their natural habitat, but also important for procedures at IMTA facilities. Because a functional IMTA facility will require the macroalgae to be placed relatively close to the net pens, in order to be able to utilize the dissolved nutrients, hence also close to the emissions source of $H_2O_2$.

## 4.1 Uncertainties of the results and sources of error

When macroalgae are affected by external stress factors their photosynthetic efficiency is lowered, and the production of ROS increase (Dring 2005). This is an important aspect to remember when deliberately stressing algae and using oxygen production as a tool to measure this effect. Potential additional stress factors that can have affected the algae in this study are related to transfer, acclimation and handling of the algae during the experiments.

During the sublethal experiment there were no standardized acclimation period. Before the experiment started *P. palmata* and *U. lactuca* were held in the lab for two and four days, respectively. The time span between the first and last specimens were exposed were six days for *P. palmata* and 11 days for *U. lactuca.* This resulted in both varying acclimation periods and total time spent in the lab, which may have influenced the specimen's photosynthetic activities differently. The temperature of the seawater was not known when collecting the algae in the field, potentially this could have led to a higher stress factor on the first exposed algae, compared to the ones who were acclimated longer. This could explain why there were such big individual differences between the specimens in the control group, when looking at the PI-curves (Figure 9, Figure 13 and Appendix 1). For the comparison between the different treatment groups, each incubation contained one individual exposed to each concentration, thus accounting for the different acclimation periods.

When considering the results from the fluorescence experiments (both lifetime and intensity), lack of a standardized acclimation period might have been more decisive for the results. It took

three measurements series to conclude these analyses, where all replicates of one treatment were included in the same series. This resulted in one acclimation period for each treatment, except for the concentration measured in two series (255 mg/l $H_2O_2$), with two different acclimation periods. Another potential uncertainty with these measurements is the fact that the results show an average for each concentration, not takin into account that they were measured on different specimens. Therefore, an average for each specimen was also calculated, even though not all cells in a tissue necessarily gives the same response (appendix 2 and appendix 3). Overall, the Aquapen measurements gave very variable results, with no clear trend following the different treatments (table 13). In addition, the two series of measurements conducted on the same concentration were not comparable, which indicates that the difference in acclimation period can have affected the results. This makes it difficult to conclude anything in relation to the different treatments, and it is reasonable to assume that other stressors than $H_2O_2$ have had a severe impact on the results. The Aquapen results will therefore not be discussed further. The fluorescence lifetime measurements showed more of a trend (table 12). Even though, because of the difficulties to determine other stressors, only the day 1 lifetime measurements will be discussed further.

To be able to construct both the PI- and dose-response curves, controlling the amount of light that the algae were exposed to was fundamental. Therefore, designing the light system was a substantial part of the preparations in the lab, despite this some uncertainties followed. With the available equipment it was not possible to ensure the exact same light intensity throughout the whole chamber. The defined light levels were therefore an average of the measured light values. The light setup used with *P. palmata* resulted in a relatively high variance within each chamber, but this was improved with the lab setup used with *U. lactuca.* Because of this, efforts were made to obtain the same average intensity in each chamber, insuring as similar light conditions as possible. This making the results from the different chambers comparable. Another aspect of uncertainty, when considering light conditions were the potential for self-shading. In order to achieve high enough oxygen changes within a reasonable time span, more than one blade of *P. palmata* had to be used. This can potentially have resulted in a lower production per gram algae than normally, because of self-shading. This was not the case for *U. lactuca* where one blade produced enough oxygen.

The other decisive part of making the PI- and dose-response curves were the oxygen measurement. Controlling the different parameters affecting how oxygen dissolves in seawater were therefore important. The lab setup intention, with the big water tank containing all the

small incubation chambers, was to ensure that the algae were exposed to the same stable physical environment. Potential differences between the five incubations chambers containing the algae were accounted for by randomizing the algae placement separately for each incubation. Oxygen solubility in seawater is mainly affected by water temperature (Boyer et al., 1999). Therefore, good temperature control was very important during the incubations. Even though the temperature was not logged four times a minute during the incubations of *U. lactuca*, the measurements taken showed very stable results. Sometimes during an incubation, the oxygen content in the chamber got so high that the pump system did not sufficiently renew the water during the break between light levels. When this happened the lid on the chamber had to be lifted to increase the water flow out of the chamber. On some occasions this led to a sudden stagnation in the oxygen production of the individual, before it increased again after a few light level exposures. This can potentially have resulted in an extra stress factor for the algae or led to measurements errors.

## 4.2 Sublethal effect on photosynthesis

The results from the sublethal effect experiment showed a clear effect on the photosynthetic apparatus after exposure to $H_2O_2$ for both *P. palmata* and *U. lactuca*. The estimated $P_{max}$ values resulted with an acute reduction of the photosynthetic efficiency for both species, where all exposed groups were significantly different from the controls at day 0 and day 1. After 11 and 14 days the $P_{max}$ values and PI-curves implied that the three highest concentrations for *P. palmata* (above 850 mg/l) and the two highest for *U. lactuca* (above 425 mg/l) damaged the algae beyond repair. The result for the lowest treatment group for *P. palmata* (170 mg/l) resulted with no visible effect of exposure, but with a $P_{max}$ value below 45% of the control after 11 days. Neither off the two lowest concentrations for *U. lactuca* (85 mg/l and 170 mg/l) showed any visible effects and resulted in estimated $P_{max}$ values that were not significantly different from the control, after 14 days.

These results support earlier findings that macroalgae species are affected by $H_2O_2$ and other ROS in varying degrees (Aguilera et al., 2002; Collén & Pedersén 1996; Dummermuth et al., 2003; Haugland et al., 2019). The decrease in photosynthetic efficiency in *P. palmata* coincided with an earlier study conducted by Dummermuth et al. (2003), who measured the maximum quantum yield ($F_v/F_m$) of *P. palmata* after a 30 minutes exposure to $H_2O_2$. They also tested 170 mg/l, which resulted in a 50% decrease compared with the $F_v/F_m$ value of the control. This indicate that the results from this experiment are valid, despite some potential sources of error. The immediate response in *U. lactuca* has been documented on another *Ulva* species before,

*Ulva rigida*, where concentrations above 102 mg/l resulted in an acute inhibition of photosynthesis (Collén & Pedersén 1996). Another species that has been investigated is *S. latissima*, who showed a severe acute reaction, with an estimated 50% reduction in $P_{max}$ when exposed to 27.8 mg/l $H_2O_2$ (Haugland et al., 2019).

After 15 days the estimated $P_{max}$ values for *U. lactuca* specimens exposed to the three lowest concentrations (85 mg/l, 170 mg/l and 425 mg/l), were comparable with the control group, indicating that the response to high light intensities had recovered. For *P. palmata* the reduction in $P_{max}$ remained after 11 days, for the lowest concentration (170 mg/l), this implied that the photosynthetic apparatus was severely harmed, not able to return to its prior state. *U. lactuca* specimens exposed to the same concentrations did not show such a prolonged effect (at day 14), indicating a higher tolerance towards oxidative stress.

Differences in tolerance towards ROS is naturally linked to the different species antioxidative capacities. An important aspect that affects the oxygen-reactive scavenging system in a species is their exposure towards stressors in their environment (Bischof & Rautenberger 2012). Especially high light intensities and UV light induce ROS production (Bischof & Rautenberger 2012; Dring 2005). Other environmental factors have also been found to induce physiological stress (hence also production of ROS) in intertidal seaweeds such as, desiccation, freezing and wounding production (Bischof & Rautenberger 2012; Dring 2005). A good defence system against these ROS is therefore crucial for these seaweeds to be able to survive in an ever-changing environment (Davison & Pearson 1996). This coincides with research that have found that the activity of the antioxidative enzyme SOD vary between algal groups in relation to depth distribution (Aguilera et al., 2002). Because of this, when explaining an algal species tolerance towards oxidative stress, their respective habitats and natural exposure to environmental stress is relevant. *S. latissima* grows in the subtidal zone (Andersen et al., 2011) while *P. palmata, U. lactuca* and *U. rigida* is usually found in the high subtidal (Guiry & Guiry 2019; Ulva lactuca n.d.; Slejkovec et al., 2006). This may partly explain the low tolerance towards $H_2O_2$ in *S. latissima* compared to the other species. Another factor may be that the activity of antioxidative enzymes is low in brown alga in comparison to other taxa (Aguilera et al., 2002).

Another factor that affects the antioxidative abilities of the different algae is their pigmentation (Pangestuti & Se-Kwon 2011). Carotenoids, a type of pigment found in all algae, act as both light harvesters and ROS-scavengers (Pangestuti & Se-Kwon 2011). They are divided into two types, carotenes or xanthophylls, and are participating in different photoprotective mechanisms through nonphotochemical quenching (Cazzinga et al., 2016). One of these mechanisms are the

different types of xanthophyll cycles (six in total), who all are a part of the plants antioxidative defence (Latowski, Kuczyńska & Strzałka 2011). These xanthophyll cycles exist in green algae, but not in red algae (Sagert & Schubert 2000; Müller, Li & Niyogi 2001). This can be another a part of the explanation for the difference between *U. lactuca* and *P. palmata* oxidative defence.

An overall higher activity of antioxidative enzymes is shown for green algae, compared to red and brown (Aguilera et al., 2002), which suggests that *U. lactuca* can have a higher tolerance to stressors. $H_2O_2$ is not only directly harmful to the algae, but can initiate formation of hydroxyl radicals (OH) which are extremely reactive (Bischof & Rautenberger 2012; Collén & Davison 1999). The algae have no defence against these radicals, which make the reduction of $H_2O_2$ of high importance (Collén & Davison 1999). The enzyme ascorbate peroxidase (APX) is the main scavenger of $H_20_2$ in the chloroplasts (Collén & Davison 1999). Another ROS scavenging enzyme is catalase (CAT) that split $H_2O_2$ to water and oxygen (Bischof & Rautenberger 2012), in the peroxisomes (involved in the abiotic stress response in algae) (Hu et al., 2012). Differences in the activity of antioxidative enzymes such as these, will naturally affect a species tolerance towards exposure of $H_2O_2$.

Whilst *P. palmata* showed a delayed effect of $H_2O_2$ exposure on the respiration rate ($R_d$) (effect showed at day 11), no effect was observed in *U. lactuca* after two weeks of recovery. These results coincide with earlier research indicating that respiration is less sensitive towards $H_2O_2$ compared to photosynthesis (Haugland et al., 2019; Collén & Pedersén 1996). It has also been stated that $H_2O_2$ mainly oxidizes enzymes that is crucial for photosynthesis and not for respiration (Asada 1992, cited in Haugland et al., 2019). Under general stress it has been observed that the $R_d$ usually increase in seaweeds (Davison et al., 1996; Andersen et al., 2011). This coincide with the results for *P. palmata*¸ indicating that even though $H_2O_2$ might not directly affect the respiration it leads to an imbalance in the algae.

An increase in respiration together with a decrease of photosynthesis will naturally lead to a negative carbon budget, which would imply no growth in an alga. This is demonstrated by the significant decrease in biomass observed for *P. palmata* specimens exposed to the three highest concentrations of $H_2O_2$. The *U. lactuca* specimens exposed to the highest concentrations did not show a significant change in biomass, but there were severe changes in the lamina structure of these specimens. This was also the case for the *P. palmata* specimens exposed to the highest concentrations. Flaccid lamina structure and loss of pigment concentration after exposure to

$H_2O_2$ was observed in *S. latissima* by Haugland et al. (2019) and they suggested membrane leakage, oxidation of pigments and cell death as likely explanations.

The fluorescence lifetime measurements conducted with *U. lactuca* support the hypothesis that the photosynthetic apparatus was severely affected by exposure to $H_2O_2$ (Table 12). Due to potential other stressors that might have affected the results from day 0, such as handling during exposure, it is most interesting to discuss the results from day 1. The higher fluorescence lifetimes for the specimens exposed to the lowest concentrations is in correspondence with earlier studies that shows an increase in lifetime when photochemistry is decreased (Kristoffersen et al., 2016). A decrease in photochemistry initiates a reduction in de-excitation pathways for the light energy, followed by an increased fluorescence lifetime (Kristoffersen et al., 2016). The significantly lower lifetimes for the specimens exposed to the highest concentrations of $H_2O_2$ indicates that the photosynthetic apparatus in the cells were too damaged to function properly. Accounting for the uncertainties connected to these measurements and small samples size it is difficult to draw a conclusion from this small pilot study, but the results indicate that this is an applicable method to investigate the state of the photosynthetic apparatus in macroalgae.

## 4.3 Risk of exposure to hydrogen peroxide

Due to the results from the lethal study, the $H_2O_2$ concentrations used in the present study were different for the two species. *P. palmata* was exposed to concentrations ranging from 170 mg/l to 1700 mg/l and *U. lactuca* to a range between 85 mg/l and 850 mg/l. The probability for these two algae species to be exposed to these concentrations of $H_2O_2$ in their natural environment, is difficult to determine due to lack of valid models for dispersal and dilution.

Three studies on how $H_2O_2$ disperses and dilutes in Norwegian fjord systems have been conducted by Andersen & Hagen (2016), Fagereng (2016) and Refseth et al. (2016). The highest concentrations measured in the field study conducted by (Andersen & Hagen (2016) were between 600-700 mg/l $H_2O_2$ and found from the edges to eight meters from the net pens, above 15 meters depth. Considering the low current speed in the area where this was conducted the concentrations of $H_2O_2$ was as expected. The current speed in the areas measured by Fagereng (2016) was also relatively weak. The highest values observed were between 720 mg/l and 780 mg/l $H_2O_2$ depths between 0 and 10 m. These concentrations persisted 15-25 minutes after the tarpaulin had been removed. In general, the highest concentrations were found within five meters from the net pens and in the upper ten meters of the water column. It is important to emphasise that most measurements showed much lower values than this. The model used by

Refseth et al. (2016) was made specially to simulate currents in areas with an irregular coastline. The model indicates that an area of one km radius surrounding the net pens could have concentrations of 300 mg/l $H_2O_2$, but concentrations of 100 mg/l could be detected within a two km radius and could remain high for almost two hours. Refseth et al. (2016) stated that the chemical breakdown of $H_2O_2$ is less important than the diluting effect. They all concluded that there was a higher horizontal than vertical dispersal, but that the opposite may occur in highly mixed water column.

When comparing the concentrations used in this study with results of probable and measured values, one can argue that some of the treatment concentrations were much higher than they will ever be after bath treatments under natural conditions. Considering the risk of being exposed in their natural habitat, this is the case for the two highest concentrations used with *P. palmata* (1250 mg/l and 1700 mg/l). Assessing the risk for exposure at IMTA facilities, the highest concentration of *U. lactuca* and the middle one for *P. palmata* (850 mg/l) is more likely to occur given the usually short distance from seaweed culture structures to fish cages. For seaweeds and kelps to be able to utilize the dissolved nutrients resources in IMTA facilities they have to be placed relatively close to the net pens (Strand & Steen 2011). Which entails that they realistically can be exposed to concentrations reaching almost 800 mg/l.

Aquaculture facilities often treat one of several cages at a time and often have repeated treatments of the whole farm. This can result in several releases of $H_2O_2$ a day and several days in a row. Fagereng (2016) found a trend of increasing concentrations in the environment after such repeated treatments. In addition, both field studies revealed leakages of $H_2O_2$ during treatments (Fagereng 2016; Andersen & Hagen 2016). This reveals that organisms in the surrounding environment can be exposed for a substantial amount of time. Especially macroalgae species in IMTA facilities can potentially be exposed both during and after treatments in addition to an increasing concentration as the sea lice treatments are repeated.

## 4.4 Conclusion

The results from this study shows that exposure to $H_20_2$ can have a severe effect on the photosynthetic apparatus of both *P. palmata* and *U. lactuca*, even though there are no visible signs of stress. The sub-lethal effects experiments showed that concentrations down to 10% of normal treatment doses in net pens had a severe acute response in both species. Though, after two weeks the *U. lactuca* specimens were no longer affected, indicating a higher tolerance than *P. palmata*. These differences are probably due to species specific stress tolerance and presence of oxidative scavengers. For both species the $P_{max}$ and $R_d$ results is in line with earlier studies

indicating that respiration is less sensitive compared to photosynthesis, towards oxidative stress. The fluorescence intensity measurements showed no clear pattern after exposure, indicating the need the for more a higher sample size. The fluorescence lifetime measurements also had some fluctuating results, but showed the same pattern of exposure as the sublethal experiment. This illustrates that this is an applicable method to investigate the state of the photosynthetic apparatus in macroalgae.

When comparing the results with earlier measured and modelled concentrations in the local environment, this study reveals that emissions of $H_20_2$ from aquaculture facilities can potentially harm these two macroalgae species. Both within their natural environment and especially when considering IMTA facilities. A prerequisite for future IMTA facilities is that the macroalgae must be placed relatively close to the net pens in order to be able to utilize the dissolved nutrients. This also entails that they will be in the area where the highest $H_20_2$ concentrations have been found. This illustrates that for such facilities to thrive, a holistic approach is necessary, taking all organisms into account when using remedies such as $H_20_2$.

# 5. References


Aarnes, H 2012, *oksygen - reaktive oksygenforbindelser*, store norske leksikon. Available from: https://snl.no [26 March 2019].

Aguilera, J, Dummermuth, A, Karsten, U, Raimund, S & Wiencke, C 2002, 'Enzymatic defences against photooxidative stress induced by ultraviolet radiation in Arctic marine macroalgae', *Polar Biology*, vol. 25, pp. 432–441.

Alexander, KA, Angel, D, Freeman, S, Israel, D, Johansen, J, Kletou, D, Meland, M, Pecorino, D, Rebours, C, Rousou, M, Shorten, M & Potts, T 2015, 'Improving sustainability of aquaculture in Europe: Stakeholder dialogues on Integrated Multi-trophic Aquaculture (IMTA)', *Environmental Science and Policy*, vol. 55, pp. 96–106.

Andersen, GS, Steen, H, Christie, H, Fredriksen, S & Moy, FE 2011, 'Seasonal Patterns of Sporophyte Growth, Fertility, Fouling, and Mortality of Saccharina latissima in Skagerrak, Norway : Implications for Forest Recovery', *Journal of Marine Biology*, vol. 2011.

Andersen, PA & Hagen, L 2016, *Fortynningsstudier – Hydrogenperoksid, september 2016*, Aqua kompetanse as.

Baklien, AT & Steinset, TA 2018, *Akvakultur*, statitisk sentralbyrå. Available from: https://www.ssb.no/fiskeoppdrett. [5 April 2018].

Benson, BB & Krause, D 1980, 'Isotopic Fractionation of Helium During Solution: A Probe for the Liquid State", *Journal of Solution Chemistry,* vol. 9, pp. 895-909.

Benson, BB & Krause, D 1984, 'The concentration and isotopic fractionation of oxygen dissolved in freshwater and seawater in equilibrium with the atmosphere", *Limnology and Oceanography,* vol. 29, pp. 620-632.

Bischof, K & Rautenberger, R 2012,'Seaweed Responses to Environmental Stress: Reactive Oxygen and Antioxidative Strategies', in *Seaweed Biology*, eds C Wiencke & C Bischof, Springer-Verlag Berlin Heidelberg, vol. 219, pp. 109-132.

Boyer, T, Conkright, ME & Levitus, S 1999, 'Seasonal variability of dissolved oxygen, percent oxygen saturation, and apparent oxygen utilization in the Atlantic and Pacific Oceans', *Deep-Sea Research I,* vol. 46, pp. 1593–1613.

Bruno, DW & Raynard, RS 1994, 'Studies on the use of hydrogen peroxide as a method for the control of sea lice on Atlantic salmon', *Aquaculture International,* vol. 2, pp. 10-18.

Cazzaniga, S, Bressan, M, Donatella, C, Agostini, A & Dall'Osto, L 2016, 'Differential Roles of Carotenes and Xanthophylls in Photosystem I Photoprotection", *Biochemistry,* vol. 55, pp. 3636-3649.

Charrier, B, Abreu, MH, Araujo, R, Bruhn, A, Coates, JC, De Clerck, O, Katsaros, C, Robaina, RR & Wichard, T 2017, 'Furthering knowledge of seaweed growth and development to facilitate sustainable aquaculture', *New Phytologist*, vol. 216, pp. 967–975.

Collén, J & Davison, IR 1999, 'Reactive Oxygen Metabolism in Intertidal *Fucus* spp. (Phaeophyceae).', *Journal of Phycology*, vol. 35, pp. 62–69.

Collén, J & Pedersén, M 1996, 'Production, scavenging and toxicity of hydrogen peroxide in the green seaweed ulva rigida', *European Journal of Phycology*, vol. 31, pp. 265–271.

Davison, IR & Pearson, GA 1996, 'Stress tolerance in intertidal seaweeds', *Journal of Phycology*, vol. 32, pp. 197–211.

Dring, M 2006,'Stress Resistance and Disease Resistance in Seaweeds: The Role of Reactive Oxygen Metabolism', *Advances in Botanical Research*, vol. 43, pp.175–207.

Dummermuth, AL, Karsten, U, Fisch, KM, König, GM & Wiencke, C 2003, 'Responses of marine macroalgae to hydrogen-peroxide stress', *Journal of Experimental Marine Biology and Ecology*, vol. 289, pp. 103–121.

Fagereng, MB 2016, *Bruk av hydrogenperoksid i oppdrettsanlegg; fortynningstudier og effekter på blomsterreke ( Pandalus montagui )*. MSc. Thesis, University of Bergen.

Fang, J, Zhang, J, Xiao, T, Huang, D & Liu, S 2016, 'Integrated multi-trophic aquaculture (IMTA) in Sanggou Bay, China', *Aquaculture Environment Interactions*, vol. 8, pp. 201–205.

FAO 2018, *The state of the world fisheries and aquaculture 2018 - meeting the sustainable development goals*. Rome.

Fauske, M 2019, *Akvakulturstatistikk alger*, Directorate of Fisheries, Available from: https://www.fiskeridir.no/Akvakultur/Statistikk-akvakultur/Akvakulturstatistikk-tidsserier/Alger [8 May 2019].

Grefsrud, ES, Glover, K, Grøsvik, BE, Huda, V, Karlsen, Ø, Kristiansen T, Kvamme, BO, Mortensen, S, Samuelesen, OB, Stien, LH & Svåastad, T (red.) 2018, *Risikorapport norsk fiskeoppdrett 2018,* Fisken og havet, særnr. 1-2018.

Guiry, MD & Guiry, GM 2019, *Palmaria palmata (Linnaeus) F.Weber & D. Mohr*, algaebase. Available from: http://www.algaebase.org [30 March 2019].

Guiry, MD 2019a, *Chlorophyta: Green algae*, The seaweed site: information on marine algae, Available from: http://www.seaweed.ie/algae/chlorophyta.php [31 May 2019].

Guiry, MD 2019b, *Rhodophyta: Red algae*, The seaweed site: information on marine algae, Available from: http://www.seaweed.ie/algae/rhodophyta.php [31 May 2019].

Hancke, K, Bekkby, T, Gilstad, M, Chapman, A & Hartvig, C 2018, *Taredyrking – mulige mijøeffekter, synergier og konflikter med andre interesser I kystsonen*, NIVA-report 7265-2018.

Haugland, BT, Rastrick, SPS, Agnalt, A, Husa, V, Kutti, T & Samuelsen, OB 2019, 'Mortality and reduced photosynthetic performance in sugar kelp *Saccharina latissima* caused by the salmon-lice therapeutant hydrogen peroxide', *Aquaculture Environment Interactions*, vol. 11, pp. 1–17.

Hjeltnes, B, Jensen, BB, Bornø, G, Haukaas, A & Walde, CS (red.) 2019, *Fiskehelserapporten 2018*, Veterinærinstituttet 2019.

Hoddevik, B 2018, *Hva vet vi om hydrogenperoksid?*, Institute of marine research. Available from: https://www.hi.no/hi/nyheter/2018/september/hva-vet-vi-om-hydrogenperoksid [27 March 2018].

Hoek, C, Mann, D & Jahns, HM 1995, *ALGAE - An introduction to phycology*, Cambridge university press.

Hu, J, Baker, A, Bartel, B, Linka, N, Mullen, RT, Reumann, S & Zolman, BK 2012, 'Plant Peroxisomes : Biogenesis and Function', *The plant Cell*, vol. 24, pp. 2279–2303.

Hughey, JR, Maggs, CA, Mineur, F, Jarvis, C, Miller, KA, Shabaka, SH & Gabrielson, PW 2019, 'Genetic analysis of the Linnaean *Ulva lactuca* (Ulvales, Chlorophyta) holotype and related type specimens reveals name misapplications, unexpected origins, and new synonymies', *Journal of Phycology*, in press.

Hurd, CL, Harrison, PJ, Bischof, K & Lobban, CS 2014, *Seaweed Ecology and Physiology*, second edi. Cambridge university press.

Karlsen, Ø 2016, *Lakselus*, Institute of marine research. Available from: http://www.imr.no/temasider/parasitter/lus/lakselus/nb-no [9 April 2018].

Karlsson-Drangsholt, A & Van Nes, S 2017, *Miljøkosekvensanalyse: Integrert havbruk i Norge*, Belona.

Kristoffersen, AS 2018, 'Fotosyntese og fluorescens', *Naturen*, no. 4, pp. 157–162.

Kristoffersen, AS, Hamre, B, Frette, Ø & Erga, SR 2016, 'Chlorophyll a fluorescence lifetime reveals reversible UV-induced photosynthetic activity in the green algae Tetraselmis', *European Biophysics Journal*, vol. 45, pp. 259–268.

Kristoffersen, AS, Svensen, Ø, Ssebiyonga, N, Erga, SR, Stamnes, JJ & Frette, Ø 2012, 'Chlorophyll a and NADPH fluorescence lifetimes in the microalgae haematococcus pluvialis (chlorophyceae) under normal and astaxanthin- accumulating conditions', *Applied Spectroscopy*, vol. 66, pp. 1216–1225.

*Kvanteutbytte* 2018, UiO institutt for biovitenskap. Available from: https://www.mn.uio.no/ibv/tjenester/kunnskap/plantefys/leksikon/k/kvanteutbytte.html [10 March 2019].

Latowski, D, Kuczyńska, P & Strzałka, K 2011, 'Xanthophyll cycle – a mechanism protecting plants against oxidative stress', *Redox report*, vol. 16, pp. 78-90.

Linnaeus, C 1753, *Linnaeus, C. (1753) Species plantarum, exhibentes plantas rite cognitas ad genera relatas cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas.* Stockholm.

Maxwell, K & Johnson, GN 2000, 'Chlorophyll fluorescence - A practical guide', *Journal of Experimental Botany*, vol. 51, pp. 659–668.

Mayanglambam, A & Sahoo, D 2015, 'Red Algae' in *The Algae world*, eds D Sahoo & J Seckbach, Springer Dordrecht, p. 223.

Ministry of Trade, Industry and Fisheries 2015, *Forutsigbar og miljømessig bærekraftig vekst i norsk lakse- og ørretoppdrett (*Meld. St. 16 (2014-2015). Available from: https://www.regjeringen.no/no/dokumenter/meld.-st.-16-2014-2015/id2401865/ [29 August 2018].

Müller, P, Li, XP & Niyogi, KK 'Non-photochemical quenching. A response to excess light energy', *Plant physiology*, vol. 125, pp. 1558-1566.

Olafsen, T, Winther, U, Olsen, Y, & Skjermo, J 2012, *Value created from productive oceans in 2050*, DKNVS and NTVA, pp. 1–83.

Pangestuti, R & Se-Kwon, K 2011, 'Biological activites and heatlh benefit effects of natural pigments derived from marine algae', *Journal of functional foods*, vol. 3, pp. 255-266.

Potin, P 2008, 'Oxidative Burst and Related Responses in Biotic Interactions of Algae', in

*Biotic Interactions of Algae*, CD Amsler (ed), Springer-Verlag Berlin Heidelberg, pp. 245-271.

R Core Team 2017, *R: a language and environment for statistical computing*, version 3.4.3, R foundation for Statistical Computing, Vienna.

Refseth, GH, Sæther, K, Drivdal, M, Nøst, OA, Augustine, S, Camus, L, Tassara, L, Agnalt, A-L & Samuelsen, OB 2016, *Miljørisiko ved bruk av hydrogenperoksid Økotoksikologisk vurdering og grenseverdi for effekt*, Akvaplan-niva.

Ridler, N, Wowchuk, M, Robinson, B, Barrington, K, Chopin, T, Robinson, S, Page, F, Reid, G, Szemerda, M, Sewuster, J & Boyne-Travis, S 2007, 'Integrated multi-trophic aquaculture (IMTA): A potential strategic choice for farmers', *Aquaculture Economics and Management*, vol. 11, pp. 99–110.

Ritz, C, Baty, F, Streibig, JC, Gerhard, D 2015 *Dose-Response Analysis Using R*, PLOS ONE, 10(12) e:0146021.

Sagert, S & Schubert, H 2000, 'Acclimation of *Palmaria palmata* (Rhodophyta) to light intensity: comparison between artificial and natural light fields', *Journal of phycology*, vol. 36, pp. 119-1128.

Skiftesvik, AB, Mortensen, S & Bjelland, RM 2016, *Bruk av rensefisk – muligheter og begrensninger*, Havforskningsrapporten, pp. 26-28

Slejkivec, Z, Kapolna, E, Ipolyi, I & van Elteren, JT 2006, 'Arsenosugars and other arsenic compounds in littoral zone algae from the Adriatic Sea', *Chemosphere*, vol. 63, pp. 1098–1105.

Strand, Ø & Steen, H 2011, 'Integrert multitrofisk akvakultur i Norge', *Havforskingsrapporten*, pp. 16–17.

Suhling, K, Hirvonen, LM, Levitt, JA, Chung, PH, Tregidgo, C, Le Marois, A, Rusakov, DA, Zheng, K, Ameer-Beg, S, Poland, S, Coelho, S, Henderson, R & Krstajic, N 2015, 'Fluorescence lifetime imaging (FLIM): Basic concepts and some recent developments', *Medical Photonics*, vol. 27, pp. 3–40.

*Ulva lactuca*, n.d, Norwegian Seaweeds. Available from: http://seaweeds.uib.no/?art=503. [30 March 2019].

Wesenberg, GR, Bredal, W, Vågen, I & Reinnel, H 2000, *Terapianbefaling : Behandling mot lakselus i oppdrettsanlegg*, Statens legemiddelkontroll (SLK).

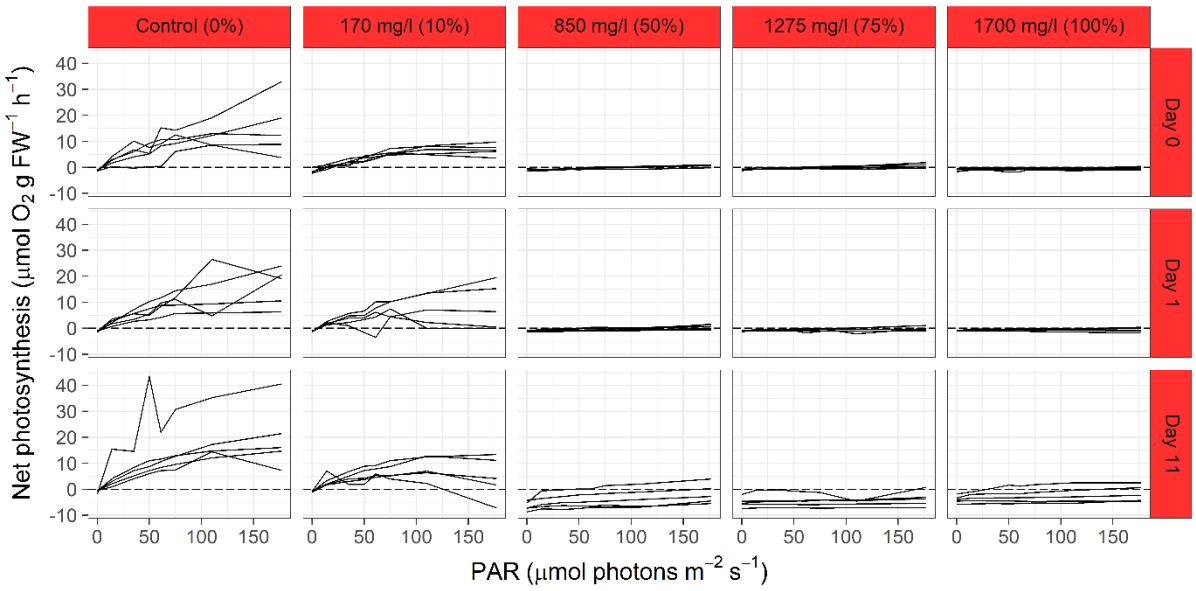
Wichard, T 2015, 'Exploring bacteria-induced growth and morphogenesis in the green macroalga order Ulvales ( Chlorophyta )', *Frontiers in plant science*, vol. 6.

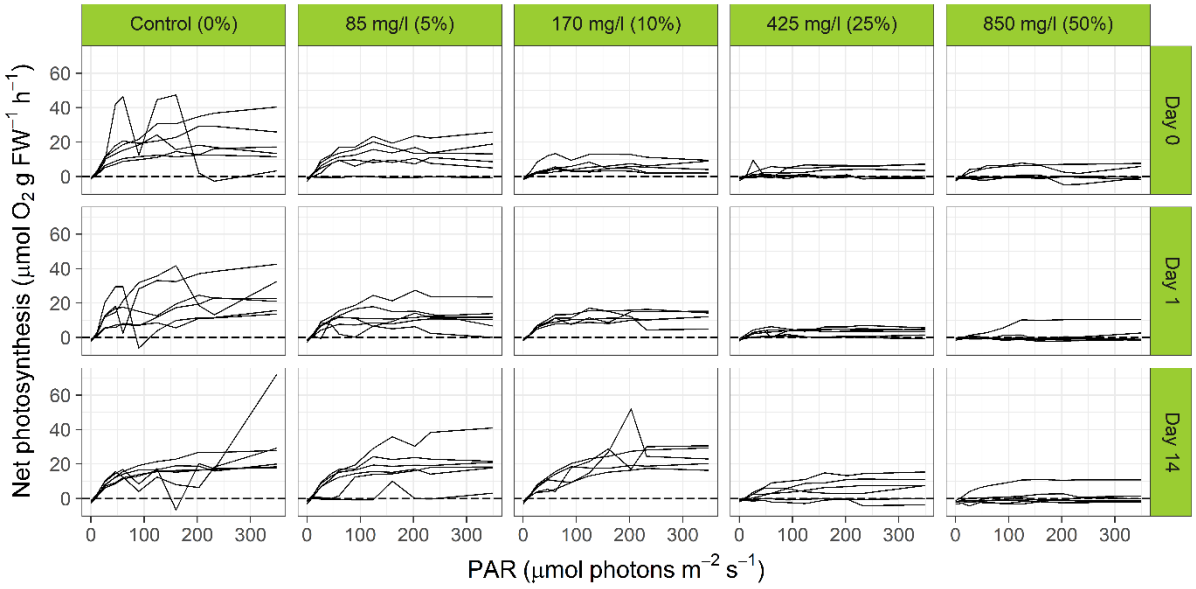
Winterbourn, CC 2013, 'The biological chemistry of hydrogen peroxide', *Methods in Enzymology*, vol. 528, pp. 3-25.

# 6. Appendices

## Appendix 1

Individual PI-curves for each specimen in each treatment for the two species *P. palmata* (red) and *U. lactuca* (green).



## Appendix 2

Chlorophyll a lifetime results for the green alga *U. lactuca*, showing the average per specimen (n=3 for all groups, except the control). Including the short and long lifetime component $\tau_1$ and $\tau_2$ and the short lifetime components relative amplitude ($\alpha_1$). All values including ± standard deviation. The P-value showing a comparison with the control. Lifetimes higher than the control and significant values are marked with bold. * significantly different from the control.

| Mg/l $H_2O_2$ | Day 0 | | | | Day 1 | | | |
|---|---|---|---|---|---|---|---|---|
| | $\tau_1$ (ps) | P – value | $\tau_2$ (ps) | $\alpha_1$ (%) | $\tau_1$ (ps) | P – value | $\tau_2$ (ps) | $\alpha_1$ (%) |
| Control | 415 ± 49 | | 1042 ± 90 | 78 ± 2 | 448 ± 71 | | 1173 ± 273 | 79 ± 5 |
| 85 | 346 ± 48 | 0.0009* | 1100 ± 143 | 79 ± 2 | 536 ± 81 | 0.0166* | 1287 ± 123 | 78 ± 4 |
| 85 | 428 ± 66 | 0.6853 | 1326 ± 77 | 80 ± 2 | 450 ± 83 | 0.9564 | 1088 ± 139 | 73 ± 10 |
| 85 | 345 ± 39 | 0.0261* | 944 ± 45 | 79 ± 2 | 419± 142 | 0.5558 | 1200 ± 290 | 73 ± 3 |
| 127.5 | 508 ± 30 | 0.0041* | 1571 ± 80 | 71 ± 3 | 560 ± 75 | 0.0108* | 1499 ± 225 | 79 ± 1 |
| 127.5 | 543 ± 38 | 0.0001* | 1640 ± 188 | 78 ± 3 | 469 ± 32 | 0.6227 | 1371 ± 207 | 73 ± 1 |
| 127.5 | 535 ± 36 | 0.0003* | 1858 ± 289 | 77 ± 1 | 529 ± 54 | 0.0616* | 1948 ± 162 | 77 ± 1 |
| 170 | 501 ± 114 | 0.0077* | 1668 ± 293 | 79 ± 3 | 573 ± 51 | 0.0049* | 1550 ± 165 | 82 ± 4 |
| 170 | 379 ± 42 | 0.2420* | 1354 ± 11 | 82 ± 2 | 486 ± 56 | 0.3704 | 1515 ± 55 | 74 ± 5 |
| 170 | 434 ± 57 | 0.5576 | 1332 ± 54 | 81 ± 1 | 514 ± 57 | 0.1258 | 1510 ± 158 | 79 ± 6 |
| 212.5 | 535 ± 32 | 0.0003* | 1575 ± 160 | 77 ± 1 | 514 ± 97 | 0.1239 | 1199 ± 177 | 81 ± 3 |
| 212.5 | 466 ± 40 | 0.1055 | 1249 ± 169 | 76 ± 4 | 466 ± 87 | 0.6789 | 1134 ± 186 | 76 ± 5 |
| 212.5 | 454 ± 5 | 0.2218 | 1372 ± 195 | 75 ± 3 | 516 ± 51 | 0.1167 | 1256 ± 93 | 73 ± 6 |
| 255 | 373 ± 113 | 0.1742 | 1006 ± 244 | 71 ± 8 | 372 ± 50 | 0.0777 | 954 ± 86 | 75 ± 3 |
| 255 | 547 ± 65 | $7.10x10^{-5}$ | 1490 ± 119 | 69 ± 2 | 371 ± 91 | 0.0740 | 1207 ± 302 | 79 ± 1 |
| 255 | 362 ± 46 | 0.0926 | 1065 ± 92 | 76 ± 5 | 448 ± 21 | 0.9938 | 1184 ± 111 | 78 ± 2 |
| 255* | 307 ± 8 | 0.0050* | 1234 ± 279 | 87 ± 1 | 375 ± 83 | 0.0926 | 1314 ± 207 | 80 ± 3 |
| 255* | 316 ± 9 | 0.0093* | 1223 ± 30 | 77 ± 1 | 395 ± 5 | 0.2871 | 1550 ± 51 | 78 ± 1 |
| 255* | 377 | 0.4610 | 1335 | 71 | 362 ± 46 | 0.0485* | 1193 ± 69 | 76 ± 3 |
| 340* | 348 ± 47 | 0.0756 | 1499 ± 81 | 82 ± 2 | 377 ± 19 | 0.1017 | 1362 ± 94 | 84 ± 1 |
| 340* | 350 ± 41 | 0.0401* | 1451 ± 94 | 80 ± 3 | 390 ± 23 | 0.1774 | 1186 ± 76 | 80 ± 3 |
| 340* | 258 ± 11 | $3.22x10^{-6}$ | 1115 ± 104 | 87 ± 2 | 276 ± 5 | 0.0002* | 1112 ± 107 | 88 ± 1 |
| 850* | 311 ± 35 | 0.0067 | 1348 ± 45 | 84 ± 3 | 308 ± 5 | 0.0066* | 1450 ± 60 | 86 ± 2 |
| 850* | 321 ± 41 | 0.0034* | 1118 ± 137 | 77 ± 4 | 260 ± 11 | $4.64x10^{-5}$ | 1052 ± 97 | 86 ± 1 |
| 850* | 294 ± 18 | 0.0002* | 1051 ± 171 | 72 ± 5 | 315 ± 6 | 0.0099* | 1065 ± 160 | 82 ± 2 |

## Appendix 3

$F_m/F_o$ and $F_v/F_m$ ratios showing the average from each *U. lactuca* specimen measured. *measurements taken in the second period in the lab in January.

| Mg/l $H_2O_2$ | Day 0 | | Day 1 | |
|---|---|---|---|---|
| | Fm/Fo | Fv/Fm | Fm/Fo | Fv/Fm |
| Control | 3.99 | 0.75 | 4.23 | 0.76 |
| 85 | 4.51 | 0.78 | 3.93 | 0.75 |
| 85 | 3.95 | 0.75 | 4.53 | 0.78 |
| 85 | 4.19 | 0.76 | 4.47 | 0.77 |
| 127.5 | 2.85 | 0.64 | 3.18 | 0.69 |
| 127.5 | 2.45 | 0.60 | 2.90 | 0.65 |
| 127.5 | 1.67 | 0.40 | 2.19 | 0.54 |
| 170 | 1.69 | 0.41 | 1.80 | 0.44 |
| 170 | 1.38 | 0.26 | 1.75 | 0.43 |
| 170 | 1.53 | 0.35 | 1.87 | 0.47 |
| 212.5 | 2.80 | 0.64 | 4.18 | 0.76 |
| 212.5 | 3.60 | 0.72 | 3.64 | 0.73 |
| 212.5 | 3.13 | 0.67 | 4.42 | 0.75 |
| 255 | 4.31 | 0.76 | 4.14 | 0.76 |
| 255 | 3.71 | 0.73 | 3.72 | 0.73 |
| 255 | 3.02 | 0.67 | 3.70 | 0.73 |
| 255* | 1.58 | 0.37 | 1.98 | 0.50 |
| 255* | 1.56 | 0.36 | 1.59 | 0.37 |
| 255* | 1.54 | 0.35 | 1.49 | 0.33 |
| 340* | 1.70 | 0.41 | 1.87 | 0.46 |
| 340* | 1.76 | 0.43 | 1.71 | 0.42 |
| 340* | 1.83 | 0.45 | 1.76 | 0.43 |
| 850* | 1.98 | 0.49 | 2.52 | 0.60 |
| 850* | 2.01 | 0.50 | 1.96 | 0.48 |
| 850* | 2.02 | 0.50 | 1.99 | 0.49 |