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Improving the microalgae inactivating efficacy of ultraviolet ballast water treatment in combination with hydrogen peroxide or peroxymonosulfate salt

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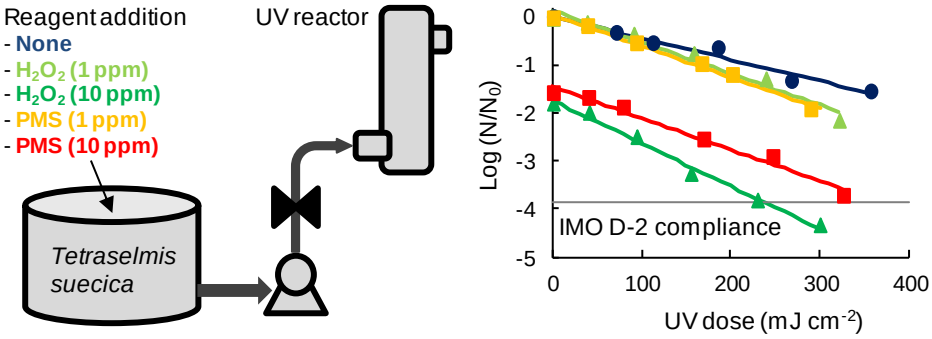
Graphical abstract



Highlights

We have treated *Tetraselmis suecica* with ultraviolet irradiation and reagents

Reduction of viable organisms was determined by growth modeling

Treatment with only UV had a maximum level of inactivation achievable

Addition of hydrogen peroxide or peroxymonosulfate enhanced microalgae inactivation

UV combined with reagents at 10 ppm enabled compliance with international standards

Title

Improving the microalgae inactivating efficacy of ultraviolet ballast water treatment in combination with hydrogen peroxide or peroxymonosulfate salt

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Abstract

Due to the increasing number of ecosystem invasions with the introduction of exogenous species via ballast water, the International Maritime Organization adopted the Ballast Water Convention (BWMC). The BWMC establishes standards for the concentration of viable organisms in a ballast water discharge. Ultraviolet (UV) irradiation is commonly used for treating ballast water; however, regrowth after UV irradiation and other drawbacks have been reported. In this study, improvement in UV treatment with the addition of hydrogen peroxide or peroxymonosulfate salt was investigated using the microalgae *Tetraselmis suecica* as the target organism. Results reported that each of these reagents added in a concentration of 10 ppm reduced the concentration of initial cells by more than 96%, increased the UV inactivation rate, and enabled reaching greater level of inactivation with the treatment. These improvements imply a reduction of the UV doses required for a consistent compliance with the BWMC standards.

Keywords

Ballast water; ultraviolet disinfection; advanced oxidation processes; hydrogen peroxide; persulfate; *Tetraselmis suecica*

1. Introduction

Ballast water is currently a major vector of species exchange between geographic areas that are not naturally connected (David, 2015). The organisms discharged with ballast water can develop in the receptor area and subsequently become invasive species that affect the ecosystem as well as human activities and health (Pimentel et al., 2005). To prevent future impacts due to the exchange of species via ballast water, the International Maritime Organization (IMO) adopted the Ballast Water Management Convention (BWMC) in 2004 which entered into force in September 2017 (IMO, 2004). Similarly, other countries such as the United States developed their own regulations (USCG, 2012). The BWMC establishes standards (D-2) to be achieved in ballast water discharge which is a limit of less than ten viable individuals equal to or larger than 50 μm per cubic meter, less than ten viable individuals shorter than 50 μm and equal to or larger than 10 μm per milliliter, and limitations of the concentration of several fecal bacteria. The BWMC guidelines define the viable organisms as those that have the ability to reproduce (IMO, 2016). On the other hand, the USCG Regulations establish similar concentration limits. Traditionally, the USCG Regulations make reference to living organisms (Čampara et al., 2019), although recently considers viable organisms in the discharge limit (USCG, 2019). To comply with these regulations, ships must be equipped with an approved ballast water treatment system (BWTS). The most of commercial BWTSs are based on a filtration procedure followed by a disinfection treatment.

Ultraviolet (UV) radiation is commonly used as a BWTS (Hess-Erga et al., 2019; IMO, 2020; Lloyd's Register, 2015). Although high doses of UV irradiation can kill the exposed organisms, the bulk of the UV effect damages the DNA by the formation of dimers that inhibit the replication of organisms (Setlow et al., 1963). However, UV treatment has some limitations that must be addressed for its assessment as a feasible BWTS. Firstly, the organisms have different mechanisms for repairing the DNA damage induced by the UV radiation; among these mechanisms, photorepair is the major one (Wen et al., 2019). Secondly, it is known that there is a limit of inactivation that can be achieved by a determined UV device in the meaning that, after a certain level of inactivation, increasing the applied UV dose does not imply increasing the ratio of inactivated organisms (Lambert and Johnston, 2000).

Besides its inactivating effect, UV radiation can also promote the formation of oxidant radical species, resulting in advanced oxidation processes (AOPs). The incidence of UV radiation on hydrogen peroxide (H_2O_2) molecules may generate $\bullet$OH radicals with a high capacity to oxidize organic matter (Legrini et al., 1993; Penru et al., 2012). Similarly, recent studies are focusing on the UV activation of persulfate salts that can result in other oxidant radicals such as $\bullet$OH and $SO_4^{\bullet -}$ (Ghanbari and Moradi, 2017; Xiao et al., 2019). In this case, the final products of the reaction are sulfate and hydrogen ions that are considered harmless for ocean water (Ahn et al., 2013). Persulfate salts are usually applied in the form of sodium peroxydisulfate salt (PDS) or potassium peroxymonosulfate triple salt (PMS). Peroxymonosulfate triple salt is dissociated in water to a strong oxidant permonosulfate (HSO_5^-) (PMS), which can be transformed to the sulfate radical ($SO_4^{\cdot -}$) (Wacławek et al., 2015). Moreover, it is known that dissolved PMS is unstable and reacts with chloride in

seawater leading to the formation of chlorine species (Moreno-Andrés et al., 2019; Wen et al., 2019). The addition of these chemicals prior to UV irradiation is expected to increase the inactivation level of organisms in water by their proper oxidant effect as well as the formation of radicals by their reaction with water matrix ions and activation under UV radiation. Regarding the formation of disinfection by-products, UV disinfection is a physical treatment that, unlike chemical processes (Moreno-Andrés and Peperzak, 2019), does not leave chemical residues in concentrations that could perform a toxic effect (Yang et al., 2020). The combination of UV radiation with oxidants, such as H_2O_2 or persulfate salts may promote the generation of highly oxidant radicals, as hydroxyl or sulfate radicals, with very short lifetime. Besides, these oxidants involve the generation of water and oxygen or the sulfate ion as final products.

Evaluating the inactivation of microalgae organisms is essential for assessing the compliance with the discharge standards since organisms size between 10 and 50 μm are primarily phytoplankters. The organism *Tetraselmis suecica* is considered fairly resistant to the UV radiation and well suited for ballast water validation studies (Montero et al., 2002; Sun and Blatchley III, 2017). Among the different available approaches for determining the phytoplankters viability, the growth phase analysis is lengthy and relatively complex but highly suitable to detect UV effects on the cell ability to reproduce (First and Drake, 2013). In this context, modeling the growth after treatment allows estimating the organisms that were not affected by the treatment as well as those that recover their viability (Romero-Martínez et al., 2016). The ratio of organisms that recover their viability depends greatly on the illumination conditions after irradiation (Romero-Martínez et al., 2020; Sakai et al., 2011). This fact is especially relevant for the ballast water disinfection because the impact

of the treatment depends on whether it is applied during the ballasting or de-ballasting procedures. If the treatment is applied during the ballasting, the organisms will be subjected to a dark period in the ballast tank which increases the loss of viability by the inhibition of the photoreactivation; whereas, if the treatment is applied during the de-ballasting in daylight, the organisms are released into an illuminated environment that promotes the photoreactivation. Therefore, it is important to consider the post-treatment illumination conditions for a comprehensive assessment of the inactivating efficacy by UV-based treatments.

The objective in this study is determining the loss of viability on the microalgae *Tetraselmis suecica* exposed to UV-C irradiation combined with different concentrations of H_2O_2 and potassium peroxymonosulfate triple salt (PMS) under different regimes of illumination after the treatment. The determination of the inactivation improvement is based on the quantitative comparison of the inactivating effect by the added chemical, the changes in the inactivation kinetic constants, and the changes in the maximum ratio of organisms that can be inactivated without loss of efficacy at higher UV doses.

2. Methods

2.1. Organisms, culture medium and pretreatment procedure

The target organism, chlorophyta *Tetraselmis suecica* CCMM 03/0202 is considered to be well-suited for ballast water validation studies (Sun and Blatchley III, 2017) and was provided by the Marine Microalgal Culture Collection at the Institute of Marine Sciences of Andalusia (CSIC). The culture medium was ground saltwater from the Campus of Puerto Real of the University of Cádiz (pH = 7.65, conductivity at 20ºC = 48.9 mS cm^{-1}, salinity = 35.80 PSS), filtered through 0.45 μm membrane disc filter (Pall Corporation, Port Washington, NY, USA), and subsequently enriched with a Guillard f/2 medium (Guillard and Ryther, 1962). Four methacrylate tanks were filled with 10 L of culture medium and then inoculated with the microalgae at a low concentration of approximately 5000 cells mL^{-1}. Cultures were incubated at a temperature of 20ºC, and illumination was supplied by two LED lamps (Phillips LED tube, 18W, 1600 lm, cool daylight) providing a photosynthetically active radiation of 130 μeinstein m^{-2} s^{-1} (QSL-2100 Radiometer, Biospherical Instruments Inc., San Diego, CA, USA), with a 14:10 light:dark cycle. After three days of incubation, cultures reached a concentration of approximately 7-8 · 10^4 cells mL^{-1} which is greater than the concentration recommended in the Guidelines for Approval of Ballast Water Management Systems (G8) (IMO, 2016). This pre-treatment incubation assures that the treated algae are not subjected to acclimation after their inoculation into new fresh medium (MacIntyre and Cullen, 2005), which may interfere in the calculation of viable organisms after the treatment, as well as assures that cells are treated during their

exponential growth phase. At the time of each assay, the 40 L of algae culture that was previously grown in methacrylate tanks was drawn from the culture chamber and poured into the 50 L plastic tank of the test rig without previous dilution.

2.2. UV device description and dose determination

Samples were irradiated using a continuous flow-through annular UV reactor. The reactor was mounted in a laboratory test rig equipped with a plastic tank of 50 L, a centrifuge pump, a manual valve for regulating the flow rate, and the UV reactor. The laboratory test rig was configured to apply the UV dose by means of one single pass through the reactor. The reactor was equipped with a low Hg-pressure monochromatic UV lamp with total power of 42 W; according to actinometric assays (Vélez-Colmenares et al., 2011), the UV output power at 254 nm was 10.2 W. A complete description of the reactor morphometry, flow features, and UV dose calculation procedure can be found in (Romero-Martínez et al., 2020). In summary, the UV dose was calculated as the product of the mean intensity (I_m) and the theoretical retention time (*TRT*). The I_m was estimated according to the USEPA specifications (USEPA, 2006) and based on reactor morphometry, lamp power, and water transmittance at 254 nm (Equation 1; in which P_{254}: output power at 254 nm, T_Q: transmittance at 254 nm of the quartz sleeve; e: thickness of the quartz sleeve; L: length of the UV lamp; r_R: inner radius of the reactor wall; r_Q: outer radius of the quartz sleeve; T_W: transmittance at 254 nm of the target water). The *TRT* was determined as the quotient between the UV exposed volume and the flow rate. For one determined experimental series, the I_m was calculated using the water transmittance at 254 nm at the time of UV

irradiation, and the different UV doses were subsequently applied by means of variations of the flow rate.

$$I_{\mathrm{m}} = \frac{P_{254} \cdot T_{\mathrm{Q}}^{e}}{2\,L\,\pi^{2}\left(r_{\mathrm{R}}^{2} - r_{\mathrm{Q}}^{2}\right)} \iint_{r_{\mathrm{Q}}}^{r_{\mathrm{R}}} \mathrm{T}_{\mathrm{W}}^{\,r-r_{\mathrm{Q}}} \frac{}{r}\ \mathrm{dx\,dy} \qquad \text{Equation 1}$$

2.3. Measurement of reagents concentration

Two different oxidants were used: hydrogen peroxide (H_2O_2; 30% ultrapure, Scharlau) and potassium peroxymonosulfate triple salt (PMS; $KHSO_5 \cdot 0.5\ KHSO_4 \cdot 0.5\ K_2SO_4$, Oxone© Sigma-Aldrich). Oxidant concentration was monitored spectrophotometrically at 410 nm for H_2O_2 and at 395 nm for PMS according to the protocols proposed by Eisenberg (1943) and Wacławek et al., (2015). A calibration curve was determined for each reagent by preparing dissolutions between 0 and 10 ppm (Fig. S1); the blank for absorbance measurements was the same saltwater as that used for preparing the culture. The concentration of H_2O_2 and PMS was monitored before and after the UV irradiation. According to the previous calibration, determination methods were precise for a concentration higher than 1.36 ppm for H_2O_2 and 2.18 ppm of PMS; therefore, the monitoring of reagents concentration was focused on the samples treated with 10 ppm of reagent.

2.4. Experimental procedure

2.4.1. Addition of reagents and monitoring of the transmittance

Five conditions related with the reagents were tested: absence of reagent (UV only), 1 or 10 ppm of H_2O_2, and 1 or 10 ppm of PMS. Prior to UV irradiation, the reagent was added to the tank containing 40 L of cultured organisms. After the reagent addition and prior to the UV irradiation, the transmittance at 254 nm of the target water was monitored to ensure the different UV doses were applied without variation of the transmittance. The monitoring indicated that the transmittance remained constant from 10 minutes after the reagent addition forward; therefore, the UV irradiation began 10 minutes after the reagent addition. For each experimental series, all samples were taken within the following 20 minutes.

2.4.2. UV irradiation of samples and incubation

The cultures with reagents were subjected to different UV doses by means of one single pass through the reactor at different flow rates (Fig. 1) (Table S1). The UV lamp was turned on 20 minutes before the culture irradiation while tap water was recirculated through the system to allow the lamp to warm and stabilize the irradiance. Samples were taken at the reactor outlet. Low flow rates and thus high UV doses were taken firstly for each experimental series in order to avoid contamination by viable organisms behind afterward the reactor. Between samples, two liters of culture were pumped and wasted to avoid mixing with the previous sample. The control was taken lastly by pumping the culture at the maximum flow rate used in irradiated samples, however, after turning off the UV lamp; control from reach experimental series includes the effect by the reagent in absence of UV irradiation. Additionally, for each experimental series, an aliquot of the target culture was taken before adding the reagent to obtain the non-reagent not UV controls.

For every UV dose that was tested and the control, three borosilicate flasks that were previously sterilized were filled with 200 mL of treated culture. The concentration of H_2O_2 and PMS was measured in every sample immediately after the UV irradiation. The 40 L of culture allowed collecting samples subjected to five different UV doses and the control in each experimental series.

2.4.3. Incubation and growth monitoring

Two out of the three sample flasks taken for each UV dose and the control were covered with aluminum foil to avoid their exposure to the environmental light; subsequently, all flasks were introduced and incubated in the culture chamber with similar conditions as those described for the pretreatment incubation. One day after the UV irradiation, the aluminum foil was removed from one of the flasks of every irradiated sample and the control. Five days after the irradiation, the aluminum foil was removed from the remaining covered flasks. The samples exposed directly to the light in the culture chamber would represent a treatment during the de-ballasting procedure whereas the samples subjected to either one or five days of dark post-treatment would represent a treatment during the ballasting procedure and the subsequent storage into the ballast tanks. All samples were then incubated under the light in the culture chamber for a period that was long enough to obtain the microalgae growth curve for each experimental series.

The cell concentration was monitored throughout incubation by means of fluorescence measurements. Fluorescence was measured for every sample once every one or two days using a Microplate Fluorescence Reader (Tecan infinite F200; software Tecan i-control,

1.6.19.2; plate Corning 96 Flat Bottom White Polystyrol) with excitation wavelength of 360 nm, emission wavelength 670 nm, gain of 60, number of flashes of 25, and integration time equal to 20 µs. Four fluorescence measurements were taken from every sample. Concurrently, occasional determinations of the cell concentration were done using a microscope (Leica, DM 750; digital camera Leica, ICC 50 HD) and Neubauer chamber (Blau Brand). In samples treated with reagents, the concentration of H_2O_2 or PMS was measured daily until the reagent concentration decreased below the limit of detection. The possible influence of the chemicals, UV dose, and lengths of dark post-treatment on the correlation between fluorescence and cell concentration was determined by comparing the linear regression lines through the statistical analysis "Further ANOVA for variables in the order fitted" performed with the software Statgraphics Centurion XVII.

2.5. Determining the concentration of viable organisms

Fluorescence values were represented in logarithmic scale against the time elapsed from illumination in the culture chamber to obtain the growth curves. For each experimental series, the growth curves depicted two distinguishable sections: an initial phase in which the fluorescence measurements did not show consistent growth with time followed by a section in which data increase with time according to a logistic curve (Equation 2 in which N_v: concentration of viable organisms at the time equal to t; N_{v0}: initial concentration of viable organisms; N_{max}: carrying capacity; r: growth rate). The modeling assumes: i) the treatment causes the loss of viability (ability to reproduce) of a certain ratio of the initially existing organisms, some of which can be repaired primarily by the photorepair mechanism (Weber, 2005); ii) the total concentration measurements that were obtained through

fluorescence cannot discern whether the organisms are viable or not; although iii) the growth observed in the curves is attributed to the viable organisms including those that were not affected by the treatment as well as those that recovered their ability to reproduce. The complete modeling assumptions and assessment can be found in previous researches (Romero-Martínez et al., 2020, 2019, 2016).

$$N_{\mathrm{v}}(\mathrm{t}) = \frac{N_{\mathrm{v0}} \cdot N_{\mathrm{max}} \cdot \mathrm{e}^{rt}}{N_{\mathrm{max}} - N_{\mathrm{v0}} + N_{\mathrm{v0}} \cdot \mathrm{e}^{rt}} \qquad \text{Equation 2}$$

This logistic section of the curve was modeled using the solver tool of MS Excel to obtain the values of the initial concentration of viable organisms in terms of initial fluorescence (F_{v0}), growth rate (r), and carrying capacity (F_{max}). The values of F_{v0} calculated for each UV dose that was applied were used to obtain the inactivation curve in every experimental series, represented as dose-survival curves. The survival (S) in each sample was estimated as the quotient between the value F_{v0} obtained in that sample and the F_{v0} obtained in the sample that was not subjected to reagent addition, UV irradiation, or dark post-treatment (Equation 3). The different inactivation curves were fitted using the tool for MS Excel GInaFiT (Geeraerd et al., 2005) to inactivation models commonly used in inactivation studies such as the Log-Linear model (Equation 4; in which S: survival at UV dose equal to D; S_0: survival at UV dose equal to 0; k: inactivation rate; D: UV dose) and the Biphasic Model (Equation 5; in which in which S: survival at UV dose equal to D; S_0: survival at UV dose equal to 0; f: ratio of organisms which follow the fast inactivation rate; k_1: fast inactivation rate; k_2: slow inactivation rate; D: UV dose), obtaining the corresponding inactivation parameters. These parameters, calculated for each experimental series, were

compared to evaluate the effect by the different concentration of reagents, focusing on the inactivation due only to the reagent addition (Log (S_0)), the UV inactivation rate (k or k_1), and the existence of maximum levels of inactivation that can be reached by the UV treatment without loss of efficacy which is known as tailing (Log (1-f)).

$$S = \frac{N_{v0}(\text{treated})}{N_{v0}(\text{untreated})} \quad \text{Equation 3}$$

$$S(D) = S_0 \, e^{-k\,D} \quad \text{Equation 4}$$

$$S(D) = S_0 \left[f \cdot e^{-k_1\,D} + (1-f)e^{-k_2\,D}\right] \quad \text{Equation 5}$$

3. Results and discussion

3.1. Determination of the viable organisms concentration

3.1.1. Fluorescence as indicator of the microalgae concentration

The determination of the microalgae concentration by means of the fluorescence measurements was subjected to several facts that establish some conditions affecting the correlation between both fluorescence and concentration. These facts were studied and disclosed in the Supplementary Material (Fig. S2). In conclusion, the relationship between fluorescence and concentration were not affected by the applied treatment (UV, H_2O_2 or PMS) or the dark post-treatment in measurements belonging to the logistic growth section of the growth curves (those that were eventually modeled to determine F_{v0}). The fluorescence measurements and their corresponding values of cell concentration measured with Neubauer plates were subjected to linear regression analysis. A total of 197 pairs fluorescence-concentration with concentrations between 10^4 and $2 \cdot 10^6$ cells mL^{-1} were included in the analysis. The intercept was not significant ($p = 0.918$) and measurements of samples without cells indicated absence of background fluorescence; therefore, the analysis was repeated assuming an intercept equal to zero. The slope was significant ($p < 0.001$) with a value of $1.38 \cdot 10^{-3}$, and R^2 was 0.828 (Fig. 2). The average coefficient of variation was 14.1% for the triplicate concentration measurements and 4.15% for the fluorescence. In this sense, the fluorescence measurements are more precise than the concentration measured with Neubauer plates thus providing smoother growth curves; also, the

determination of the fluorescence was more rapid and not subjected to human error in counting.

3.1.2. Features and modeling of the growth curves

In the most cases, the growth curves depict a logistic curve with an exponential growth phase followed by a deceleration as the concentration reaches higher values (Fig. 3). The growth rate "*r*" and the carrying capacity "F_{max}" do not vary noticeably whereas the differences between samples lie in the time elapsed from the initiation of the illuminated incubation and the beginning of the logistic growth section (this time is commonly known as "lag phase"). A lag phase is absent in non-UV irradiated samples without the addition of chemicals and in those with either 1 ppm of H_2O_2 or PMS. On the contrary, the addition of 10 ppm of either H_2O_2 or PMS caused a growth delay of five days in the absence of UV irradiation which indicates certain damage due to the chemical. In UV irradiated samples, the delay became longer according to the UV dose that was applied.

The term "lag phase" could be confusing since it may suggest that the cells do not reproduce within this period, due for instance, to their acclimation after inoculating them into new clean medium (MacIntyre and Cullen, 2005). However, the lag phase can be also an artifact due to the lack of precision by some determination techniques, especially at low cell concentration; this fact is evident in this study in which the detection of the logistic growth phase was detected earlier using fluorescence measurements with respect to concentration measurements with Neubauer plates (Romero-Martínez et al., 2020). Moreover, the experiments in this study were designed to avoid microalgae dilution prior to

the treatment, and authors such as MacIntyre et al. (2018) did not observe a growth delay due to the UV irradiation beyond the time required for the organisms to be repaired which occurs in the hours immediately subsequent to the irradiation (Hull et al., 2017). Thus, the lag observed in growth curves is compatible with the reduction of the concentration of viable organisms due to the treatment.

The comparison of the values of growth rate "*r*" between the different samples showed slight differences that were not related with the applied UV dose and the chemicals that were added, thus considered as random noise, reporting an average value of "*r*" equal to 0.651 d^{-1}. This value of "*r*" was kept fixed, and then the values of F_{v0} and F_{max} were calculated for every growth curve. In some cases, the incubation was completed before the concentration reached the stationary phase, and then the F_{max} used in the model was the one calculated for the control. This fact did not affect the calculation of F_{v0} since this was not sensitive to the changes of F_{max}. To evaluate the goodness of fitting of the data to the logistic model, the experimental values of fluorescence were compared with their respective estimated values by means of a linear regression analysis (Fig. S3).

3.2. Inactivation curves and kinetic parameters

3.2.1. Features of the inactivation curves

Inactivation curves were obtained as the dose-response profiles, representing the survival (*S*) against the applied UV dose, for every chemical treatment and length of dark post-treatment (Fig. 4). According to the average initial cell concentration of $7.27 \cdot 10^4$ cells mL^{-}

^{1} (minimum of $5.80 \cdot 10^{4}$ cells mL^{-1} and maximum of $9.14 \cdot 10^{4}$ cells mL^{-1}), the BWMC D-2 standard of 10 viable individuals per milliliter is achieved with an inactivation level of 3.86 orders of magnitude (3.76 and 3.96 orders of magnitude respectively for the minimum and maximum initial concentration), represented as a continuous horizontal line in the Fig. 4. At low concentration of survivor organisms, the stochastic scattering due to the low concentration of organisms implies that the calculated survival data are not reliable. According to the growth curves (Fig. 3), the relationship between the UV dose applied and the time required for detecting the logistic growth phase breaks down in samples which take more than 15 days to exhibit detectable growth. Applying the logistic model and considering the growth rate "*r*" equal to 0.651 d^{-1}, used to determine the values of F_{v0}, the absence of detectable growth after 15 days indicates that the concentration of viable organisms in the sample is less than 0.25 cells mL^{-1}. Since the average initial concentration was $7.27 \cdot 10^{4}$ cells mL^{-1}, a value of 0.25 cells mL^{-1} represents an inactivation of 5.46 orders of magnitude. This level of inactivation, represented as a horizontal dashed line in the Fig. 4, was considered as the limit for obtaining reliable survival data. In this sense, the threshold at which the results lack of reliability exceeds 1.60 orders of magnitude the level of inactivation required to comply with the BWMC D-2 standards.

According to the experimental precepts in this study, survival data represent the ratio of organisms that retained or recovered their viability after the treatment. The inactivation curves obtained matched with either log-linear or biphasic models. All samples without dark post-treatment exhibited log-linear inactivation whereas the samples subjected to one or five days of dark post-treatment exhibited biphasic inactivation. The log-linear model maintains a constant inactivation rate (*k*) throughout the experimental range of UV doses.

On the other hand, biphasic inactivation is characterized by a decrease of the inactivation rate from (k_1) to (k_2) once inactivated in a certain ratio (f) of organisms; this fact is known as tailing (Lambert and Johnston, 2000). Causes of tailing may be several and are a matter of debate (Hijnen et al., 2006). Previous studies treating fecal bacteria *Escherichia coli* and *Enterococcus faecalis* reported tailing after the inactivation of between 3.38 and 4.24 orders of magnitude of the initial concentration when the irradiation was applied using a collimated beam reactor whereas tailing was absent or it existed at higher levels of disinfection when using a flow through reactor such as the one utilized in this study (Romero-Martínez et al., 2014). In this sense, the appearance of tailing is dependent on the type of UV device that is used. Tailing can also represent an artifact in the determination of very low concentrations of organisms due to stochastic scattering when the organisms are sparse (Frazier et al., 2013; Miller et al., 2011).

3.2.2. Effect of H_2O_2, PMS and dark post-treatment on the microalgae viability

The loss of viability due to the reagents addition and the dark post-treatment in absence of UV irradiation is given by the parameter Log (S_0) (Table S2). This parameter is calculated by modeling and indicates the survival at a UV dose equal to 0, that is, the y-intercept of the dose-response curve. The values of Log (S_0) indicated no noticeable loss of viability from the addition of 1 ppm of H_2O_2 or 1 ppm of PMS in absence of UV irradiation and without dark post-treatment (Fig. 5). On the contrary, the addition of 10 ppm of H_2O_2 and the addition of 10 ppm of PMS reduced the concentration of viable cells by 1.74 and 1.45 orders of magnitude respectively, that is, the reagents inactivated 98.2% and the 96.5% of the initial concentration of viable organisms. On the other hand, the correlation between

Log (S_0) and the length of the dark post-treatment was significant ($p = 0.006$) and strong ($R_2 > 0.999$) only in the case of samples without a reagent addition although the five days of dark storage poses a reduction of only 26.9% of the viable organisms. In the rest of cases, the correlation between Log (S_0) and the length of the dark post-treatment was not significant ($p > 0.05$). In this context, the data reported a lack of consistent inactivation due to the dark post-treatment in absence of UV or chemical treatment, as reported in previous studies (Romero-Martínez et al., 2020). The presence of relatively high uncertainty in the calculation of Log (S_0) for the series of samples subjected to five days of dark post-treatment is remarkable, especially in the samples treated with 10 ppm of either H_2O_2 or PMS. This is due to the stochastic scattering of data from UV doses of 40 mJ cm^{-2} and 80 mJ cm^{-2} forward, respectively, for H_2O_2 and PMS. Nevertheless, despite the high uncertainty, the values of Log (S_0) calculated by modeling matched with the experimental values of survival of non irradiated samples with a maximum deviation of 5.6%.

3.2.3. Effect of H_2O_2 and PMS on tailing after UV radiation

The inactivation curves obtained in this study exhibited either absence of tailing, tailing at low inactivation levels, or tailing at high inactivation levels with erratic values of survival data. The absence of tailing was observed in samples without dark post-treatment. In these cases, tailing is expected at doses greater than the experimental range that was tested since it makes no sense that the inactivation surpasses those calculated for one and five days of dark post-treatment. Tailing at low inactivation levels appeared in all data series of samples subjected to one day of dark post-treatment as well as samples treated with UV only, 1 ppm of H_2O_2, and 1 ppm of PMS and five days of dark post-treatment. In these cases, tailing

begins, on average, after the inactivation of 3.48 orders of magnitude of the organisms that survive to the reagent addition according to the values of –Log(1-*f*). Lastly, tailing at high inactivation levels was observed in the series of samples treated with either 10 ppm of H_2O_2 or 10 ppm PMS and subjected to five days of dark post-treatment. The change from the fast inactivation rate (k_1) to slow inactivation rate (k_2) had place after the inactivation of 4.80 orders of magnitude of the organisms that survive to the addition of 10 ppm of H_2O_2 and 5.53 orders of magnitude in the case of 10 ppm of PMS. In these cases, the combined effect of both the reagent and the UV irradiation led to inactivation levels such that the scarcity of survivor organisms caused high stochasticity in the survival data with respect to the UV dose that was applied.

Tailing may prevent a BWTS from complying with the BWMC D-2 standards, even considerably increasing the applied UV dose. This fact is not quite relevant for treating natural seawater which contains relatively low concentrations of organisms except in cases of algal blooms in which microalgae concentration increases up to $1.15 \cdot 10^5$ cells mL^{-1} (Lee et al., 2013; Villacorte et al., 2015). However, tailing becomes especially relevant in the treatment of water that is affected by algae blooms and also in land based testing for Type Approval, which requires an initial concentration of no less than 10^3 viable organisms per mL and a recommended concentration of 10^4 viable organisms per mL, according to the Guideline 8 recommendation (IMO, 2016). Therefore, the addition of 10 ppm of either H_2O_2 or PMS are expected to be beneficial in cases in which the high concentration of organisms in the water does not allow achieving the BWMC D-2 standards even when considerably increasing the applied UV dose.

3.2.4. Effect of H_2O_2 and PMS on the UV inactivation kinetic constants

The inactivation kinetic constants indicate the dependence of survival with respect to the UV dose that is applied. For simplification, the values of both k in a log-linear model and k_1 in a biphasic model will be referred as k for comparison purposes. The value of k in absence of any reagent and dark post-treatment was 0.010 ± 0.001 cm^2 mJ^{-1} (Fig. 6). Values of k in literature for *T. suecica* under similar conditions and measured by methods focused on viability, such as growth modeling, most probable number, or real-time polymerase chain reaction, reported values of k between 0.019 and 0.084 cm^{-2} mJ^{-1} (L. Liu et al., 2016; Lundgreen et al., 2019; Sun and Blatchley III, 2017). Therefore, the value of k obtained in this study is slightly lower although in accordance with other previous studies. The addition of reagents increased the values of k with respect to the treatment with UV only in all cases of samples without dark post-treatment (Fig. 6). The greater improvement was achieved by the addition of 10 ppm of H_2O_2 which doubled the value of k; in the case of 10 ppm of PMS, the increase of k was by a factor of 1.47; this increase in k was additional to the inactivation due to the organism exposure to the reagent. For one and five days of dark post-treatment, the values of k on samples subjected to UV increased from 0.010 ± 0.001 up to 0.038 ± 0.005 and 0.095 ± 0.027 cm^2 mJ^{-1} in the absence of chemical treatment, respectively. This increase can be attributed to the combination of UV irradiation and dark storage which prevents the microalgae from photoreactivation (Romero-Martínez et al., 2020; Sakai et al., 2011). For one day of dark post-treatment, only the addition of 1 ppm of H_2O_2 caused a noticeable increase of k with respect to the treatment with only UV. For five days of dark post-treatment, the uncertainty in the calculation of k increases because only two or three survival data on the experimental series are before the change of slope (Fig. 4).

However, the increase of *k* is observable in the curves for the addition of 1 and 10 ppm of H_2O_2 and 1 ppm of PMS. Therefore, the impact of the reagents on *k* was, in general, less important in the cases of one and five days of dark post-treatment since the lack of photoreactivation increases the inactivation achieved by UV only treatment.

3.2.5. Action mechanisms and evolution of H_2O_2 and the PMS

Two lines of action can be distinguished for H_2O_2 and PMS reagents: their proper oxidant effect and the formation of oxidant radicals by the incidence of the UV-C; both effects are expected to show synergic mechanisms with the UV-C irradiation. The H_2O_2 concentration measured 30 minutes after the addition of 10 ppm reported a slight decline of 0.86 ppm in non-irradiated samples. The increasing UV doses that were applied caused slight shrinkage on the H_2O_2 concentration which was measured immediately after the irradiation according to a significant ($p = 0.004$) linear regression with a slope equal to $-1.06 \cdot 10^{-3}$ ppm cm^2 mJ^{-1} and R^2 of 0.956 (Fig. S4- left). This implies a reduction of the 3.44% of the concentration of H_2O_2 at the higher UV dose tested of 298.8 mJ cm^{-2} and exposure time of 9.98 s in comparison with the non irradiated samples. This slight reduction on the H_2O_2 concentration in concordance with the applied UV dose can be attributed to homolysis of the H_2O_2 under the UV-C light with quantum yield (ϕ) of approximately 0.5 in radical generation and molar absorption coefficient (ε) of 18 M^{-1} cm^{-1} (Li et al., 2017).

The photolysis of H_2O_2 is produced with a constant rate of 10^{-3} s at wavelength of 254 nm (Li et al., 2017; Wang et al., 2020) which could explain the low consumption of H_2O_2 due to the UV irradiation. Additionally, anions present in water such as bicarbonate, carbonate,

chloride ,and bromide can scavenge the •OH radicals (Wang et al., 2000, 2020). In this context, although the formation of •OH radicals increased the values of "*k*" with respect to only UV (Fig. 6), it was limited by the short exposure time (below ten seconds) which characterizes the single pass through-flow UV reactors and the brief life of the radicals. On the contrary, the major inactivating effect by the H_2O_2 can be attributed to the oxidation of the organic matter (Mikutta et al., 2005) which was evident after the addition of 10 ppm and shows synergic effects with the UV irradiation. The presence of oxidants such as H_2O_2 in the bulk implies the presence of exogenous sources of reactive oxidant species (ROS) that can inflict damages to the membrane cells, but these oxidants can also diffuse into the cell and possibly imbalance the internal ROS equilibrium which can induce programmed apoptosis in algal cells (Giannakis et al., 2016; D. Liu et al., 2016; Pulgarin et al., 2020; Wong et al., 2003). This balance is normally regulated by enzymes such as catalase (CAT), superoxide dismutase (SOD), or glutathione peroxidase (GPX) which might be damaged by UV radiation so the regular function could be damaged after irradiation as well as other functional compounds in the photosynthetic system (Li et al., 2020; Pardieck et al., 1992; Sigaud-Kutner et al., 2005). After one day of incubation, the concentration of H_2O_2 decreased in all samples but especially in non-irradiated samples. Because the relatively low rate of reaction by H_2O_2 (Neyens and Baeyens, 2003), the cells can reproduce in non-irradiated samples to a certain extent, increasing the organic matter content by the cell growth and the releasing of their flagella and their outer thecae during reproduction (Gonzalez et al., 2015). Additionally, the antioxidant enzymes CAT, SOD, and GPX can be damaged in UV irradiated cells and thus their capacity to eliminate H_2O_2 is impaired. Therefore, the UV irradiation, even in low dosages, delays the depletion of the H_2O_2.

Eventually, the concentration of H_2O_2 in all samples decreased until values below the limit of detection after two days of incubation.

The concentration of PMS registered a noticeable reduction immediately after its addition to the algae culture from 10 until 3.94 ppm in non-irradiated samples (Fig. S4 - right). This shrinkage in PMS concentration is consistent with its known instability, especially at basic pH (Guan et al., 2011) as well as favored by the easy reaction with organic and inorganic compounds that are present in the solution (Wang and Wang, 2018; Yang et al., 2018). After the UV irradiation, the concentration of remaining PMS ranged between 2.61 and 2.91 ppm; these values indicated shrinkage of PMS due to the UV irradiation although a direct relationship between PMS concentration and UV dose was not addressed as observed in the case of H_2O_2 irradiation. Similar to the H_2O_2, the most of the microalgae inactivation due to the PMS addition was observed after the addition of 10 ppm due to its proper oxidant effect and the oxidant species generated by the reaction of the PMS with chemical species in the water matrix, primarily the formation of Cl· from the chloride ions present in salt water (Xiao et al., 2019). Eventually, the concentration of PMS decreased until values were below the limit of detection in all samples after one day of incubation.

3.3. Assessing the feasibility of the application of H_2O_2 and PMS to improve UV-based BWTSs

The feasibility of a BWTS depends on its biological efficacy as well as technical, economic, and environmental factors. Within the Type Approval (Guideline 8) procedure, the land based test supposes a challenge for the biological efficacy of the BWTS since it

requires achieving the BWMC D-2 standards from a minimum concentration of 10^3 cells mL^{-1} and a recommended concentration of 10^4 cells mL^{-1} (IMO, 2016). The effect of the combination of either H_2O_2 or PMS, UV irradiation, and different lengths of dark post-treatment can be summarized with the parameter $D_{IMO\ D2}$, that is, the UV dose required to achieve the level of inactivation of 3.86 orders of magnitude, which reduce the initial concentration of $7.27 \cdot 10^4$ cells mL^{-1} to 10 viable individual per milliliter (Fig. 7).

An important fact to be considered is the possibility of treatment during the ballasting and/or de-ballasting procedures. Due to photoreactivation, UV doses greater than 400 mJ cm^{-2} are recommended for a treatment applied during the de-ballasting with the absence of dark post-treatment (Olsen et al., 2016). The addition of 10 ppm of H_2O_2 reduced the $D_{IMO\ D2}$ to 238.0 mJ cm^{-2}, although the low consumption of the H_2O_2 after the treatment (Fig. S4 - left) suggests the necessity for a relatively long contact time which may be insufficient for a treatment applied during the de-ballasting. The addition of 10 ppm of PMS reduced the $D_{IMO\ D2}$ to a lesser extent to 368.3 mJ cm^{-2}, but the reduction of the concentration (Fig. S4 - right) and the decreasing of the fluorescence immediately after the treatment indicated that the impact of the PMS is more immediate in comparison with the H_2O_2. The time of contact with the reagent that was required for effective inactivation of organisms will be studied in future experiments using neutralizer chemicals after the irradiation. In the case of a treatment applied during the ballasting, the relevance of the addition of the reagents depends on the time for the sample is subsequently within the ballast tanks. With one day of dark post-treatment, the addition of the reagents became more relevant in all cases. Although the treatment with UV only reported a value of "*k*" that was 3.7 times greater with respect to the absence of dark post-treatment, the presence of tailing prevented

fulfilling the D-2 standards. The addition of 1 ppm of H_2O_2 or PMS did allow complying with the D-2 standards with UV doses of 259.7 and 294.9 mJ cm^{-2}, respectively. In the case of only UV, 1 ppm of H_2O_2 and 1 ppm of PMS, the tail runs close to $D_{IMO\ D2}$; therefore, small variations on the initial concentration of organisms or even stochastic variations can determine the positive or negative compliance with D-2. The values of $D_{IMO\ D2}$ were considerably reduced by the addition of 10 ppm of H_2O_2 or PMS in samples subjected to one day of dark post-treatment to values below 100 mJ cm^{-2}. Reducing the values of $D_{IMO\ D2}$ implies a reduction of the UV lamps required and allows higher operation flow rate. With five days of dark post-treatment, the values of $D_{IMO\ D2}$ were reduced in all cases. The inactivating efficacy by the UV only treatment increased due to the dark storage preventing the microalgae from photoreactivation thus the addition of the reagents did not noticeably reduce the values of $D_{IMO\ D2}$ with respect to UV only. However, the inactivation due to the addition of 10 ppm of H_2O_2 or PMS eliminates the risk derived from the occurrence of tailing before achieving the D-2 standards.

The D-2 compliance achieved with a treatment during the ballasting procedure provides the possibility of avoiding the re-treatment during the de-ballasting. Although the phytoplankton organisms have mechanisms to survive prolonged dark periods (Carney et al., 2011; Jochem, 1999; Ribeiro et al., 2011), the lack of available light within the ballast tanks prevents the cells from reproducing. To explore the possibility of dismissing the treatment during the de-ballasting, it is necessary to consider that some phytoplankton species can show heterotrophic behavior (Llario et al., 2019; Lu et al., 2017). Although *T. suecica* is able to demonstrate heterotrophic behavior under certain conditions (Azma et al., 2011; Jo et al., 2004), the growth monitoring did not register any increase on the cell

concentration throughout the dark post-treatment. Fecal bacteria *Escherichia coli* and intestinal enterococci can also survive within the ballast tanks, although they are not able to reproduce in non-enteric host environments, and their survival is limited (Winfield and Groisman, 2003). It is also known from previous studies that UV doses below 60 mJ cm^{-2} combined with 10 ppm of H_2O_2 implies the inactivation of six orders of magnitude of the initial concentration (Moreno-Andrés et al., 2019, 2016). In this sense, the treatment used in this study also assures the D-2 compliance for target water with concentrations of fecal bacteria up to than 10^8 CFU 100 mL^{-1} without the requirement of re-treating during the de-ballasting. Heterotrophic bacteria can develop within the ballast tanks (Hess-Erga et al., 2010); however, these organisms are not involved in the D-2 compliance. The zooplankton organisms are a concern for assuming that the compliance in ballasting implies compliance in de-ballasting since they can grow and reproduce without major limitations within the ballast tanks if food is available. In this sense, further studies using known heterotrophic and mixotrophic algae within the 10-50 μm range such as *Heterosigma akashiwo* (Clough and Strom, 2005) and zooplankton as target organisms are recommended to determine whether compliance in ballasting implies compliance in de-ballasting.

Tankers and other large ships with a high dependence on ballast water could have a capacity up 95 000 m^3 in their ballast tanks (ABS, 2011); therefore, 950 kg of pure reagent is required for applying a treatment using 10 ppm of H_2O_2 or PMS as studied in this research. Regarding the costs of the reagents, it is remarkable that the H_2O_2 is less expensive than PMS with reference values for a kilogram of pure reagent of 1.50 USD/kg and 2.20 USD/kg, respectively (Wacławek et al., 2017). On the other hand, treatments that use active substances require extra steps in the approval procedure to ensure the safety for

ships, human health, and the aquatic environment (BWMC Guideline 9). In this regard, the PMS has some desirable features with respect to H_2O_2 since PMS depletion occurs one day after the addition of 10 ppm whereas the H_2O_2 remains for two days. Although the H_2O_2 provided better performance for microalgae inactivation, the election between both of them is subjected to operational criteria.

This study was focused on adverse scenarios with a relatively resistant target organism (Sun and Blatchley III, 2017) and initial cell concentration exceeding what is recommended in the G-8 for land based testing of BWTSs. Next steps on this research consider the treatment of species assemblages, as well as the treatment under different conditions of salinity and concentration of dissolved organic carbon, particulate organic carbon, and total suspended solids. As this study was performed using high salinity water, decreasing the salinity would imply a lower scavenging of oxidant radicals by dissolved ions and thus the treatment may become more effective (Liu et al., 2012; Moreno-Andrés et al., 2017). On the other hand, increasing the levels of organic carbon and suspended solids may reduce the water transmittance and increase the demand of oxidant (Gregg et al., 2009); therefore, these variations should be taken into account in future experiments focused on the approximation to the conditions of land based testing for BWTSs.

4. Conclusions

In this study, the addition of H_2O_2 or PMS was investigated as a way to improve the UV treatment of ballast water. The inactivating effect of various concentrations of each reagent in combination with different UV doses and lengths of dark post-treatment on the cell viability were tested on the algae *T. suecica*. The addition of 1 ppm of either H_2O_2 or PMS did not cause inactivation by the addition of the reagent; although the UV inactivation rate increased, tailing at high UV doses prevented consistent compliance with the D-2 standards. The addition of 10 ppm of H_2O_2 inactivated more than 98% of the initial algae concentration and increased the UV inactivation rate, especially in the case of the absence of dark post-treatment (simulating a treatment during de-ballasting) and one day of dark post-treatment (simulating a treatment during ballasting followed by a one day stay in a ballast tank). The addition of PMS also inactivated more than the 96% of the initial algae concentration and increased the UV inactivation rate in the absence of dark post-treatment. The exposure to the reagent elevated the maximum level of inactivation that was reachable by the treatment, allowing consistent compliance with BWMC D-2 standards with a UV dose below 400 mJ cm^{-2} in the absence of dark post-treatment and below 150 mJ cm^{-2} with one day of dark post-treatment. PMS has some desirable features with respect to H_2O_2 since PMS depletion occurs one day after the addition of 10 ppm whereas the H_2O_2 remains for two days. Although the H_2O_2 provided better performance for microalgae inactivation, the election between both of them is subjected to operational criteria.

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Figure(s)

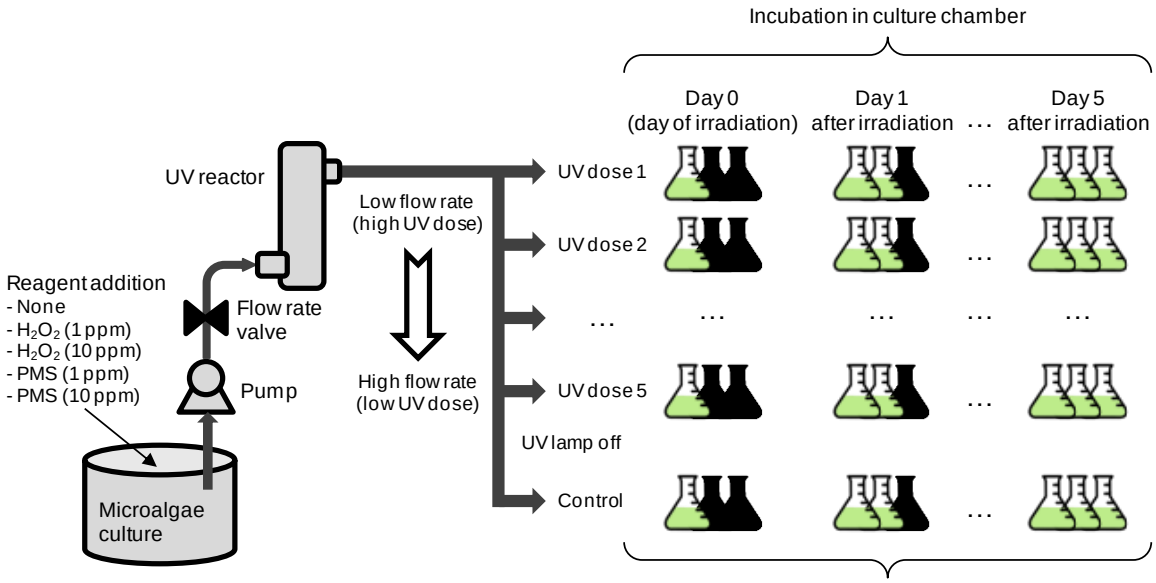

Figure 1. Experimental procedure and parts of the laboratory test rig

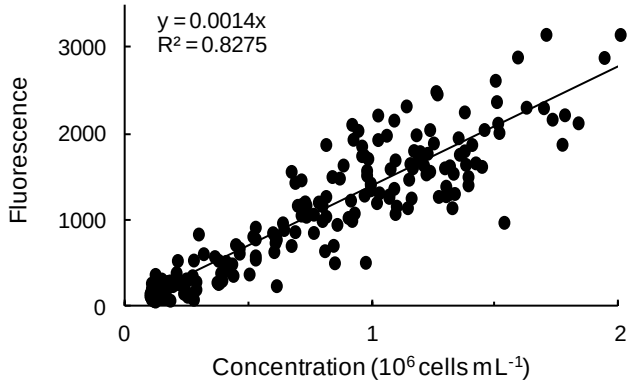

Figure 2. Linear correlation between fluorescence and cell concentration measured with microscopy, after removing the data subjected to variations not related with the concentration. n = 197.

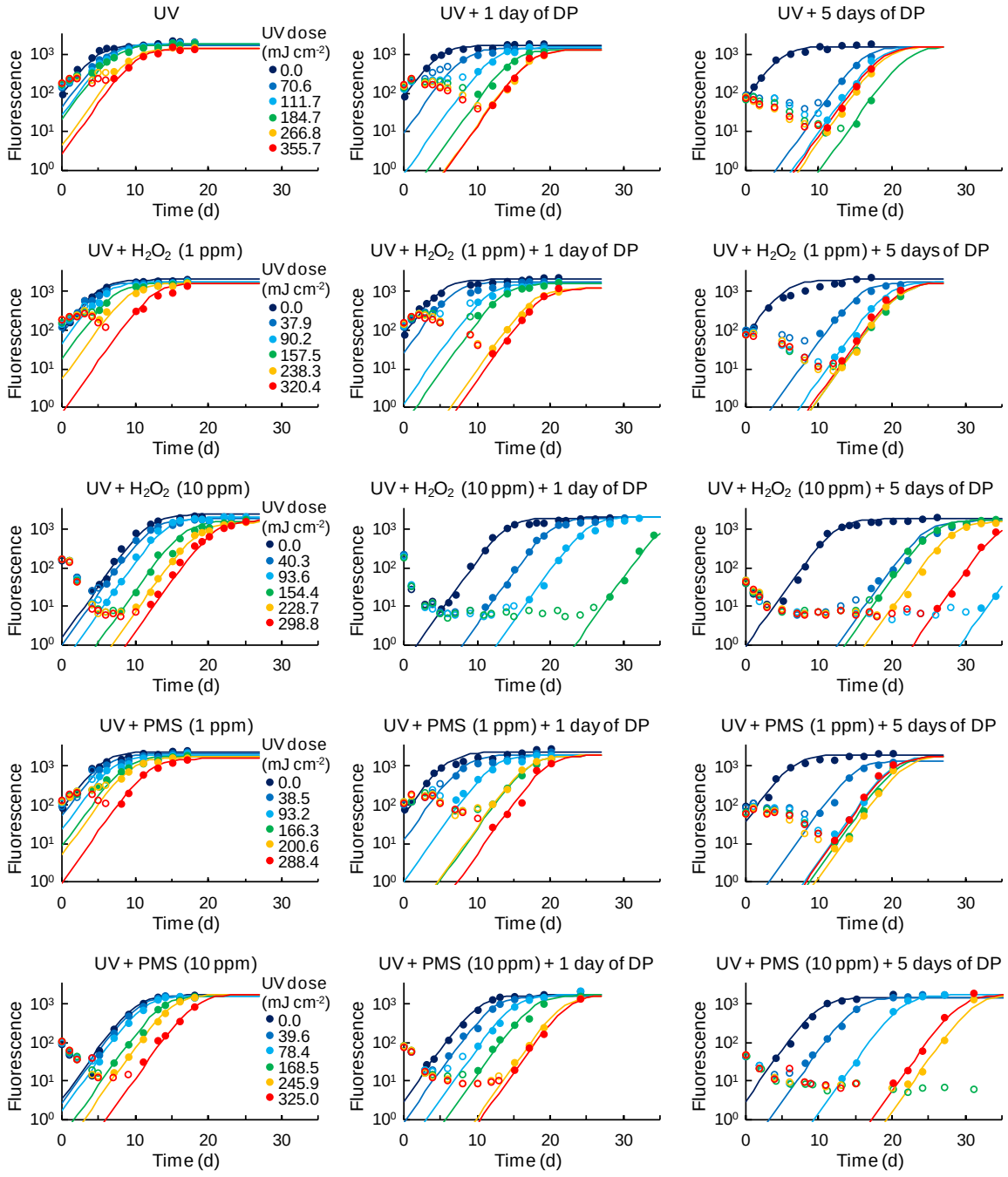

Figure 3. Growth curves based on the fluorescence measurements (in arbitrary units of fluorescence). Curves start at the time of the exposure of samples to the light in the culture chamber. Open symbols represent data preceding the logistic section of the growth curve that were not used in modeling. DP: length of the dark post-treatment.

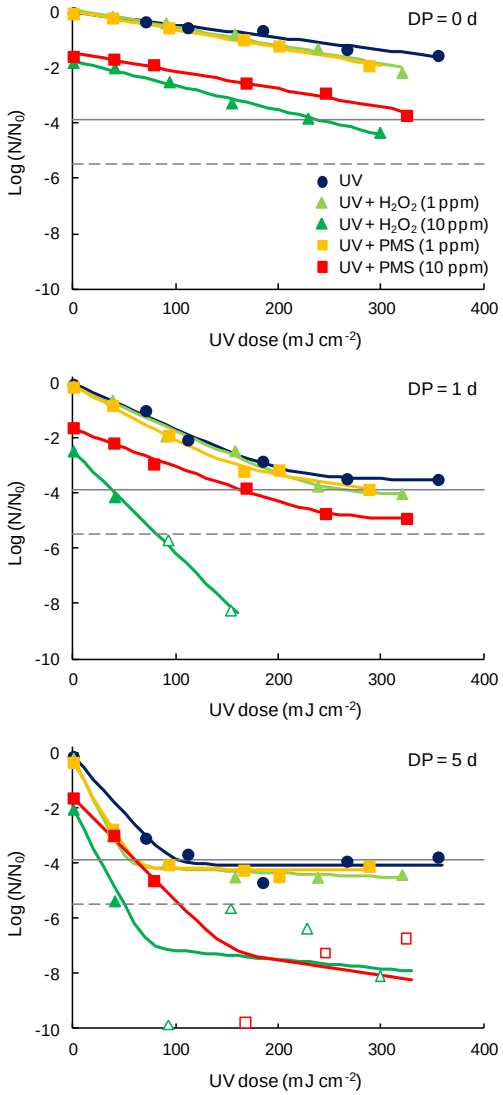

Figure 4. Inactivation curves for every experimental series representing the survival with respect to the UV dose applied. Continuous horizontal line represents the level of inactivation required to achieve the BWMC D-2 standards, calculated as the logarithm of the quotient between the discharge limit of ten viable individuals per milliliter and the average initial concentration of $7.27 \cdot 10^4$ cells mL^{-1}; dashed horizontal line represents the limit to obtain precise measurements of viable organism concentration. Data below this limit are subjected to high stochastic scattering and represented with empty symbols. DP: length of the dark post-treatment.

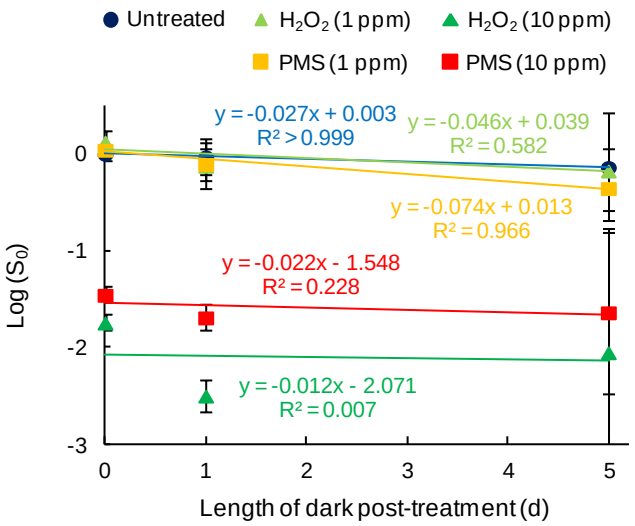

Figure 5. Effect of the different reagents and lengths of dark post-treatment on the survival in absence of UV irradiation (S_0). Error bars represent the standard error.

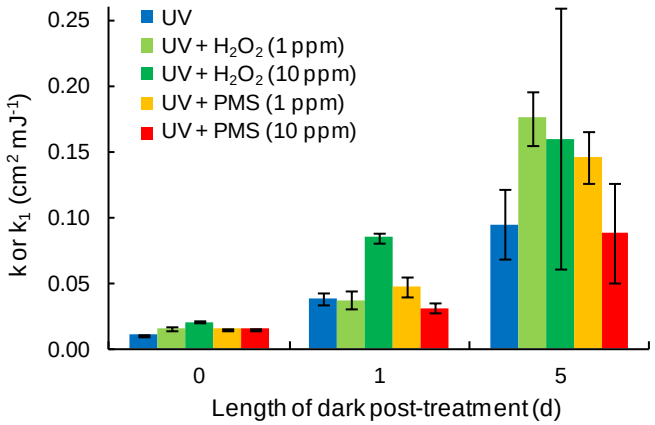

Figure 6. Improvement on the UV inactivation rate by the addition of the different concentrations of reagents. Error bars represent the standard error.

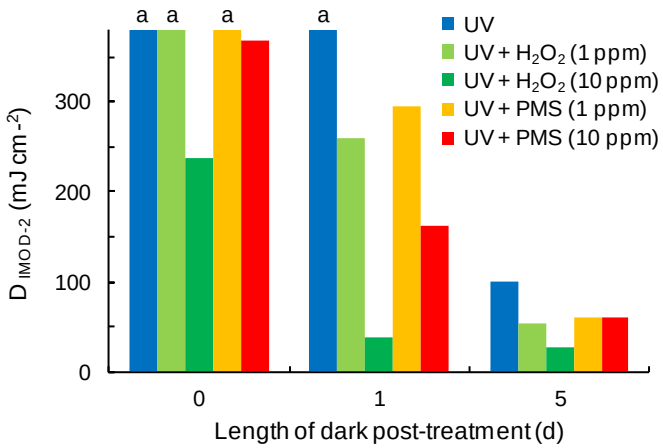

Figure 7. UV doses required to achieve BWMC D-2 standards ($D_{\text{IMO D-2}}$) under different concentrations of reagents added and different length of dark post-treatment. The "a" letter over the column indicates that the BWMC D-2 standards were not reached within the experimental range of UV doses tested.

*Declaration of Interest Statement

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Credit Author Statement

Leonardo Romero-Martínez: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization, Writing - Review & Editing. **Ignacio Rivas-Zaballos:** Investigation, Validation, Writing - Review & Editing. **Javier Moreno-Andrés:** Conceptualization, Methodology, Investigation, Validation, Resources, Writing - Review & Editing, Project administration, Funding acquisition. **Ignacio Moreno-Garrido:** Methodology, Resources, Writing - Review & Editing. **Asunción Acevedo-Merino:** Conceptualization, Methodology, Formal analysis, Resources, Writing - Review & Editing, Supervision, Project administration. **Enrique Nebot:** Conceptualization Methodology, Formal analysis, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.