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Semicontinuous and batch ozonation combined with peroxymonosulfate for inactivation of microalgae in ballast water

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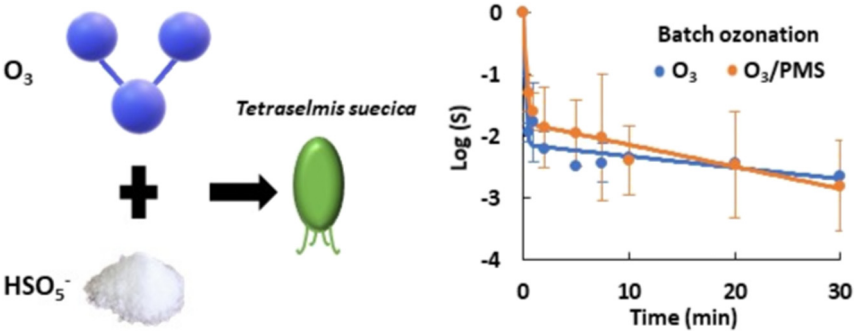
HIGHLIGHTS

- We have tested two methods of ozonation on the microalga *Tetraselmis suecica*.
- Ozone is an effective treatment for inactivation of microalgae in ballast water.
- Batch ozonation is more efficient than semicontinuous ozonation.
- PMS addition improves the inactivation in semicontinuous but not in batch ozonation.
- Three days after the treatment no oxidants were present in ozonated saltwater.

GRAPHICAL ABSTRACT



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ABSTRACT

The Ballast Water Management Convention (BWMC) establishes limits regarding the permissible number of viable organisms in discharged ballast water. Ozone as a ballast water treatment is interesting because it can be generated in-situ and has strong oxidant power. Additionally, some oxidants can be formed in reaction with seawater, especially brominated compounds, that assist in inactivating microorganisms. The objective of this study is to assess the efficacy of semicontinuous and batch ozonation as well as their combination with peroxymonosulfate salt (PMS) as methods to be used to ensure compliance with regulation D2 of the BWMC using *Tetraselmis suecica* as a standard microorganism. Growth modeling method was employed to determine the inactivation achieved by the treatments. The results show that ozone is an effective treatment for accomplishing the D2 of the BWMC. Batch ozonation is more efficient than semicontinuous ozonation probably because of the brominated compounds formed during the ozone saturation of the water. The oxidants that are developed during the ozonation of seawater prolong the residual effect of the treatment throughout the days of storage with practically no presence of them in the ballast tanks at 72 h. The addition of the PMS increases the inactivation in the semicontinuous ozonation, but a threshold concentration of ozone is needed to observe the synergistic effect of both oxidants. No increase is associated with the combination of O_3 and PMS in the case of batch ozonation.

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1. Introduction

The International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWMC) was adopted by the International Maritime Organization (IMO) in 2004 and entered into force in 2017. It has currently been ratified by 86 countries representing approximately 91.12 % of the gross tonnage of the world's merchant fleet (IMO, 2022). The objective of this convention is to avoid the dispersing of species by ballast water between different ecosystems (Bailey, 2015; Carney et al., 2017) that can become invasive species and cause economic, environmental, and social problems (Bax et al., 2003; Gallardo et al., 2016).

A maximum concentration of viable organisms in discharged water is established by the IMO (D2 standards). For organisms between 10 and 50 μm in minimum dimension the maximum is less than 10 viable organisms per mL while for the organisms higher than 50 μm in minimum dimension is less than 10 viable organisms per m^3. For the IMO, viable organisms are the ones able to successfully generate new biological entities (IMO, 2018). To accomplish the D2 standards, the ships usually use Ballast Water Management Systems (BWMS) with the majority of them comprising a filtration and a subsequent disinfection treatment that could be chemical, physical, or even a combination of both (Davidson et al., 2017; Tsolaki and Diamadopoulos, 2010). Although the majority of the approved BWMSs are based on disinfection with ultraviolet radiation (UV), the possibility of repairing DNA damage through photoreactivation processes (Nebot Sanz et al., 2007; Rastogi et al., 2010) should be considered. Blatchley et al. (2018) reviewed published evidence showing that delayed repair after UV disinfection was not a problem. Still, it is interesting to study other treatments such as electrolysis, ozonation, and advanced oxidation that already have some approved BWMSs (Gerhard et al., 2019). The treatments can be applied during a ballasting and/or de-ballasting procedure or even during a voyage.

Since 1886, ozone has been used as a method to disinfect water, and its utilization as an algae controller in treatment plants or even oxidation of some chemicals are well known (Rice et al., 1981). A problem associated with the use of ozonation is the possible generation of toxic disinfection by-products (DBPs) and especially brominated compounds in the case of seawater because of the high levels of Br^- (Haag and Holgné, 1983; Shah et al., 2015). Different factors affect the formation of bromine compounds during the ozonation but, in the case of seawater, the ozone dose is a key parameter because it is the limiting reactant in the reaction with bromides forming bromine derivates (Jung et al., 2017). The ozonation of seawater also generates other reactive substances such as chlorine, molecular ozone, or hydroxyl radicals, among others, that are important in the inactivation of organisms. It is common to use the Total Residual Oxidant (TRO) concept to refer to the main residual oxidants (chlorine and bromine compounds) resulting from ozonation (Jung et al., 2014; Perrins et al., 2006) because these substances are presented together thus their separate measurement is difficult. In the case of ballast water ozonation, the election of treating during the ballasting procedure is critical because the storage time in the ballast tanks after the treatments affects the TRO concentration and its consequential disinfection effect. A regrowth test period of at least five days after a chemical treatment is mandatory for the approval of a BWMS (IMO, 2018). The storage of the treated ballast water in the tanks is associated with a decay on the TRO that could be practically zero after five days, although the chemistry of the water affects this decay (Perrins et al., 2006; Zhang et al., 2013).

The combination of ozonation with some chemical compounds such as H_2O_2, TiO_2, or PMS in the search for Advanced Oxidation Processes (AOPs) to accelerate or increase the disinfection or decreasing the ozone dose required have been performed (Moreno-Andrés et al., 2020; Rodríguez-Chueca et al., 2015). The generation of radicals ($SO_4^{\bullet -}$ and •OH) by means of a combination of O_3 and PMS (Wu et al., 2019; Yang et al., 2015) and the increase on the disinfection in bacteria and microalgae by the radicals have been demonstrated (Moreno-Andrés et al., 2019; Romero-Martínez et al., 2021).

The objective of the present paper is to check the efficacy of semicontinuous and batch ozonation as well as their combination with PMS as methods to comply with regulation D2 of the BWMC using *Tetraselmis suecica* as a standard microorganism.

2. Material and methods

2.1. Organisms, culture medium, and pre-treatment procedure

The target organism was the chlorophyte microalga *Tetraselmis suecica* (CCMM 03/0202 provided by the Marine Microalgal Culture Collection of the Institute of Marine Sciences of Andalusia). The simplicity of culturing and managing it, its worldwide distribution, the substantial growth rate, and their use as a standard for numerous treatments make it an effective organism for this experiment (Cha et al., 2015; Pećarević et al., 2018; Sun and Blatchley, 2017; Svendsen et al., 2018). Ground saltwater from the Campus of Puerto Real at the University of Cadiz (pH = 7.65; conductivity at 20 °C = 48.9 mS cm^{-1} and salinity = 35.8) autoclaved and enriched with Guillard f/2 medium (Guillard and Ryther, 1962) was employed as the culture medium. Cultures were incubated in a climate room (20 °C) with continuous light (Sylvania Luxline Plus; F36W/T8/865) and a photosynthetically active radiation of 36 μmol photons m^{-2} s^{-1} (QSL-2100 Radiometer, Biospherical Instruments Inc., San Diego, CA, USA) at 20 °C. After dilution, the organisms need time to acclimatize (MacIntyre et al., 2018; MacIntyre and Cullen, 2005). In our experiments, dilution in fresh medium was performed two days before the experiment, as this was enough time to promote acclimation and ensure that cultures were in the exponential growth phase.

2.2. Experimental procedure

2.2.1. Semicontinuous ozonation

Semicontinuous ozonation consists of a reaction system in which the liquid phase is static (batch), and the gas phase (ozone) is dynamic, flowing through the liquid column.

Ozonation assays were conducted in a 2 L reactor with 1.8 L of culture. Ozone was produced by a GZ07 PROY 136 ozonizer (Zonosistem, Spain) and injected at the bottom of the reactor with a porous diffuser. In all the replicates, the inlet and the outlet flow as well as the concentration of the ozone were measured (ozone analyzer BMT 964; BMT MESSTECHNIK GMBH, Berlin) in order to calculate the dose that was transferred to the cultures that were treated. For the replicates of the semicontinuous ozonation, the gas flow rate was maintained at 0.6 NL min^{-1}, and the inlet ozone concentration was 1.80 ± 0.15 g Nm^{-3}. Samples were taken from the reactor at different times between 0 and 15 min. An aliquot of 10 mL was placed in a tube for the purpose of measuring the ozone concentration (and the possible residual oxidants formed) spectrophotometrically (Jenway 7315) with the potassium trisulfonate indigo method (Bader and Hoigné, 1981). Another aliquot of 50 mL was put in an Erlenmeyer flask with 5 mL of sodium thiosulfate ($Na_2S_2O_3$) to neutralize the ozone and other possible residual oxidants (Jung et al., 2014; Tachikawa and Yamanaka, 2014) and subsequently incubated in the climate room. The final liquid volume in all of the experiments exceeded 70 % of the initial volume.

Searching for an improvement of the inactivation by possible AOPs, another series of experiments were conducted similarly but a concentration of 3.25 μM of potassium peroxymonosulfate, PMS ($KHSO_5{\cdot}0.5KHSO_4{\cdot}0.5K_2SO_4$, Oxone©, Sigma-Aldrich) was added. PMA was added at the same moment of initiating the ozonation or thirty seconds later for ensuring that the instantaneous ozone demand or initial ozone demand (IOD) was overcome (Buffle et al., 2006; Hasegawa et al., 2008; Richard, 1994). No significant differences were detected between these two methods of combining the ozone and the PMS. In all the replicates of the combination of ozone and the PMS, the parameters of the ozonizer were kept the same as those with only ozone. Each experiment (ozone combined or not with the PMS) was triplicated.

2.2.2. Batch ozonation

In these assays, the ozonation was performed by adding a previously prepared aliquot (100 mL in 1 L or 50 mL in 1 L, depending on assays) of a water solution saturated in ozone to the culture (Gerrity et al., 2014;

Penru et al., 2013). The parameters of the ozonizer were the same during the ozonation of the water between different replicates (gas flow rate: 0.6 NL min^{-1}; inlet concentration: 3.5 ± 0.71 g Nm^{-3}), and the concentration of the dissolved ozone was monitored by the potassium trisulfonate indigo method. This ensured that all the replicates were performed with ozone-saturated water. Three different water matrices were tested to be saturated in ozone and put in contact with the cultures: distilled water, ground saltwater, and seawater from the Atlantic Ocean collected in the city of Cadiz. The pH (Crison multimeter MM41) and TC-TOC (Shimadzu TOC-L Analyzer; NPOC method) of the three different waters were analysed. The pH of the distilled water was 4 and the pH of the Ground saltwater and Atlantic Ocean water were around 8. The distilled water has no organic carbon while in the other two water matrices it was around 5 mg L^{-1}. Time zero was considered as the time of putting the culture in contact with the saturated ozone water and samples were taken at different times throughout the next 30 min. The procedure with the samples extracted from the reactor was the same as the semicontinuous ozonation (one part of the sample for the culture chamber and the other for measuring the residual ozone and other residual oxidants).

Likewise, as semicontinuous experiments, additional experimental series were performed adding the PMS (3.25 μM) either at the same moment of the ozone-saturated water addition or 30 s after with no significant differences between both procedures. Each experiment (ozone combined or not with the PMS) was triplicated.

2.2.3. Ozone dose calculation

First, the transferred ozone dose (TOD) (mg L^{-1}) was calculated by integrating with Simpson's rule the Eq. (1) (where O_3 is the ozone gas concentration [mg L^{-1}]; Q_g is the gas flow rate [L min^{-1}]; V_{liq} is the ozonized water column volume [L]). Subsequently, by subtracting the dissolved ozone that was measured by the potassium trisulfonate indigo method from the transferred ozone, the concentration of reacting ozone, also referred to as ozone demand, was calculated. Knowing the relationship between the reacting ozone and the inactivation achieved in semicontinuous ozonation affords the possibility of comparing it with the inactivation in batch ozonation.

$$\mathrm{TOD} = \int_0^t \frac{\left([O_3]_{in} - [O_3]_{out}\right) \cdot Q_g}{Vliq}\,\mathrm{dt} \tag{1}$$

The parameter Ct (concentration time; mg O_3 L^{-1}·min) (Eq. (2)) has been also calculated for a better comparison with inactivation from other organisms in the literature.

$$Ct = \int_0^t \left([O_3]_{in} - [O_3]_{out}\right)\mathrm{dt} \tag{2}$$

2.3. Determining the concentration of viable organisms after the treatment

The concentration of viable organisms after the treatment was determined by means of growth modeling in which the growth curves from different samples were fitted according to the logistic model (Rivas-Zaballos et al., 2021; Romero-Martínez et al., 2016, 2020). The total cell concentration was calculated through fluorescence measurements that were previously correlated with determinations with microscopy and a Neubauer chamber (Romero-Martínez et al., 2021). Fluorescence measurements were made with 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. Fluorescence measurements in logarithmic scale, were represented against the time of incubation. Data between the beginning of exponential growth and the end of the incubation were fitted according to the Verhulst logistic model (Peleg et al., 2007; Verhulst, 1830) (Eq. (3) in which N_v: concentration [cells mL^{-1}] of viable organisms at the time equal to t [d]; N_{v0}: initial concentration [cells mL^{-1}] of viable organisms; N_{max}: carrying capacity [cells mL^{-1}]; and r: growth rate [d^{-1}]). By means of a nonlinear regression using the Solver tool (MS Excel), the N_{v0} was calculated as the values that minimize the mean square error, maintaining constant r and N_{max}.

$$N_v(t) = \frac{N_{v0} \cdot N_{max} \cdot e^{rt}}{N_{max} - N_{v0} + N_{v0} \cdot e^{rt}} \tag{3}$$

The survival (S) at certain doses was calculated as the quotient between N_{v0} at that dose and the N_{v0} of the untreated control. The inactivation (dose-survival) curves were fitted according to the most suitable microbiological inactivation model using the GInaFiT tool for MS Excel (Geeraerd et al., 2005) to obtain the kinetics parameters. These were subsequently utilized to compare the different treatment configurations.

3. Results and discussion

3.1. Modeling of the growth curves

Growth curves were obtained by representing the logarithm of the fluorescence versus the incubation time under ambient light. To facilitate understanding the growth modeling, only the curves of a replicate of the semicontinuous ozonation are shown (Fig. 1). Untreated samples (control) describe a logistic curve with exponential growth followed by a deceleration of it as they became closer to the carrying capacity. The points that are in the exponential phase are aligned if they are represented in logarithmic scale. Points judged not to be in alignment before the beginning of the exponential phase were discarded. In the treated samples, the cell concentration underwent a delay in reaching the exponential growth phase that was proportional to the ozone dose that was applied. In the samples where it was judged that the incubation period ended before the carrying capacity (N_{max}) was reached, the average of the values of the N_{max} of the samples where it was reached was the one applied. The growth rate (r) used is the average of the r of the different samples. The N_{max} and the r were not noticeably affected by the treatments; therefore, the differences between treated samples are related to the decrease in the initial concentration of viable organisms (N_{v0}).

3.2. Inactivation curves and kinetic parameters

The survival (S) at each O_3 demand was calculated as the logarithm of the quotient between the N_{v0} and the N_{v0} of the control. The inactivation curves were obtained by representing the S with respect to the O_3 demand.

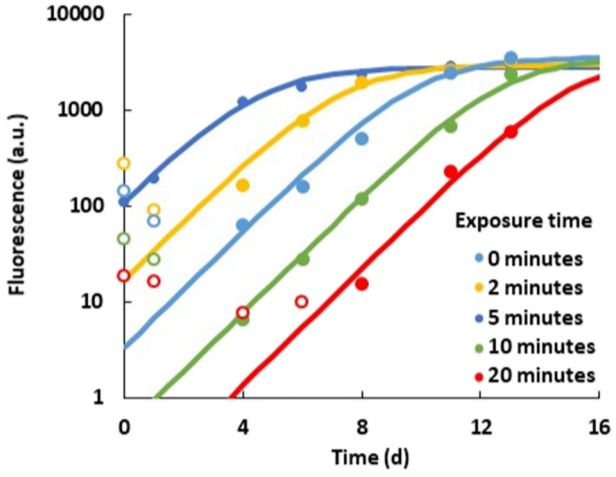

Fig. 1. Growth curves of one of three replicates. Open symbols represent data preceding the logistic section of the growth curve that were not used in modeling.

3.2.1. Semicontinuous ozonation

The *S* in semicontinuous ozonation, only with O_3, followed a log-linear inactivation, characterized by keeping the inactivation rate (*k*) constant throughout the different doses (Eq. (4) in which *S*: survival of organisms; S_0: initial survival of organisms; *k*: inactivation rate [L mg O_3^{-1}]; *D*: O_3 demand [mg L^{-1}]) (Bigelow and Esty, 1920). When O_3 is combined with PMS the *S* followed a log-linear with shoulder inactivation, characterized by a first stage in which there is hardly any disinfection up to a threshold dose (shoulder) followed by a log-linear disinfection stage (Eq. (5); in which *S*: survival of organisms; S_0: initial survival of organisms; *k*: inactivation rate [L mg O_3^{-1}]; *sl*: shoulder length [mg O_3 L^{-1}]; *D*: O_3 demand [mg L^{-1}]) (Geeraerd et al., 2000) (Fig. 2; Table 1). The R^2 provided by the GInaFiT tool was used as a criterion to select the kinetic model that best fit the experimental data (Geeraerd et al., 2005).

$$S(D) = S_0\, e^{-k\cdot D} \tag{4}$$

$$S(D) = S_0 \frac{e^{-k\cdot D}\cdot e^{k\cdot sl}}{1 + (e^{k\cdot sl} - 1)\cdot e^{-k\cdot D}} \tag{5}$$

A possible reason for the shoulder in the O_3/PMS treatment may be because most of the ozone is reacting with the PMS to form the radicals and is therefore not inactivating the microorganisms. This competition effect of the PMS for ozone is overcome by the continued maintenance of ozonation and therefore, from a certain moment, the inactivation is greater due to the ozone and radicals ($SO_4^{\bullet -}$ and •OH) beginning to inactivate the microorganisms (Bai et al., 2021; Moreno-Andrés et al., 2020; Wu et al., 2015; Yang et al., 2015). Therefore, in semicontinuous ozonation, as it can be seen in Fig. 2, a threshold ozone concentration is needed to determine the synergic effect of both oxidants (O_3 and PMS). This synergic effect would allow to decrease the O_3 demand to accomplish with the D2 of the BWMC or increasing the inactivation with the same O_3 demand.

The Ct from the maximum time of ozonation (15 min) at semicontinuous assays was 7.8 $mgO_3\ L^{-1}\cdot min$, reaching an inactivation of 3 and 4 orders of inactivation in O_3 and O_3/PMS, respectively. The Ct value needed to reach 3 log reductions with O_3 at 20 °C (temperature common in different studies) is much higher in *T. suecica* (7.80 $mgO_3\ L^{-1}\cdot min$) than in other organisms at the same temperature as enteric viruses (0.40 $mgO_3\ L^{-1}\cdot min$), *Giardia* cysts (0.72 $mgO_3\ L^{-1}\cdot min$) or *Bacillus subtilis* spores (3.1 $mgO_3\ L^{-1}\cdot min$; at 26 °C) or equal to other resistant organisms as *Cryptosporidium* oocysts (7.8 $mgO_3\ L^{-1}\cdot min$) (Balch et al., 2013; Choi et al., 2007; Health Canada, 2004). The high resistance of *T. suecica* to ozone added to the ease of culturing it and its rapid growth make it an interesting organism for ballast water ozonation tests. The addition of the PMS increases the inactivation; however, it needs at least a threshold ozone concentration for the synergic effect of both oxidants to be seen.

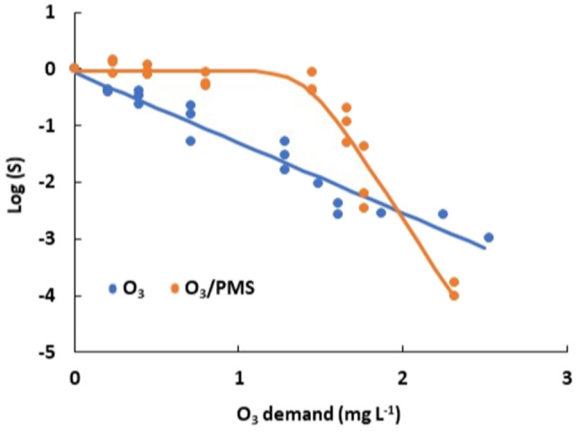

Fig. 2. Inactivation curves from the semicontinuous ozonation. Survival is represented against O_3 demand.

Table 1
Kinetic parameters (mean ± SE) of the inactivation curves from both treatments in semicontinuous ozonation. The R^2 indicates the adjustment of the measured points to the selected inactivation model. k: inactivation rate; sl: shoulder length.

Treatment	Inactivation model	*k* (L mg^{-1} O_3)	*sl* (mg O_3 L^{-1})	R^2	n
O_3	Linear	2.9 ± 0.2	–	0.941	19
O_3/PMS	Log-linear with shoulder	10.2 ± 0.9	1.4 ± 0.1	0.942	21

3.2.2. Batch ozonation

Since a significant transformation of the water matrix in batch ozonation occurs by subjecting it to continuous ozonation until saturation is reached, an assay with three different waters was performed to verify the different responses. The characteristics of the water matrices (distilled water, ground saltwater, and Atlantic Ocean seawater) were described in 2.2.2. An aliquot of 50 mL saturated in ozone was added to 950 mL of a culture of *T. suecica* ($9\cdot10^4 \pm 9\cdot10^3$ cells mL^{-1}), and samples were taken at different times until 10 min. The *S* at batch ozonation in the three water matrices followed a biphasic inactivation, characterized by a decrease in the inactivation rate from (k_1) to (k_2) after an inactivation of a certain proportion (*f*) of organisms is reached (Eq. (6); in which *S*: survival of organisms; S_0: initial survival of organisms; k_1: fast inactivation rate (min^{-1}); k_2: slow inactivation rate (min^{-1}); *f*: ratio of organisms following k_1; *D*: O_3 dose [minutes of exposure]) (Cerf, 1977) (Fig. 3a) The substantial differences between the inactivation of distilled water and the other two waters is consistent with the formation of disinfection by-products (DBP) and especially brominated compounds when seawater or saltwater is ozonized (Jung et al., 2014, 2017; Perrins et al., 2006; Shah et al., 2015; Werschkun et al., 2012). The large drop in the Total Residual Oxidant (TRO) (mg L^{-1} as O_3) in the first 30 s corresponds to the initial ozone demand (IOD) (Buffle et al., 2006; Hasegawa et al., 2008; Richard, 1994). After these initial 30 s, the decrease in the TRO is less. There was a TRO reduction of 86.95 % in distilled water (from 0.10 ± 0.05 to 0.01 ± 0.02 mg L^{-1}), 34.91 % in ground saltwater (from 0.31 ± 0.11 to 0.20 ± 0.002 mg L^{-1}), and 51.38 % in Atlantic Ocean water (from 0.47 ± 0.02 to 0.23 ± 0.06 mg L^{-1}) (Fig. 3b). These data represent the mean and the s.d. from an experiment duplicate. In the case of the distilled water, brominated compounds cannot be formed, thus the signal corresponds to residual ozone and therefore practically all of it is consumed in the first 30 s. Brominated and other possible halogenated compounds formed in the ozonation of the Atlantic Ocean water and ground saltwater prolong the residual effect. First, it was verified the low inactivation of the ozone-saturated distilled water and the absence of significant differences on the inactivation between ground saltwater and Atlantic Ocean water (*p* value >0.1; two-way ANCOVA). It was subsequently decided to continue with the ground saltwater to have all the assays (semicontinuous and batch) with the same water matrix.

$$S(D) = S_0\left[f\cdot e^{-k_1\cdot D} + (1-f)\cdot e^{-k_2\cdot D}\right] \tag{6}$$

Once the ground saltwater was selected, assays were performed to compare the differences between the O_3 and O_3/PMS by adding aliquots of 100 mL of ozone-saturated ground saltwater to 900 mL of *T. suecica* culture.

The *S* at batch ozonation in the O_3 and O_3/PMS treatments describes a biphasic inactivation (Eq. (6)) (Fig. 4a). The kinetic constants values from the models are indicated in Table 2. The significant decrease in the TRO and the high inactivation in the first 30 s in both treatments is associated with the IOD (Buffle et al., 2006; Hasegawa et al., 2008; Richard, 1994) (Fig. 4b). Time zero is the moment to add the ozone-saturated ground saltwater to the culture. Each time a sample was taken from the reactor, the TRO present in the sample was stopped by adding $Na_2S_2O_3$. Plotting the TRO decay over a period versus the inactivation achieved over the same period, the survival versus O_3 demand graph is obtained since the TRO has always been calculated in mg L^{-1} as O_3. The high difference between the

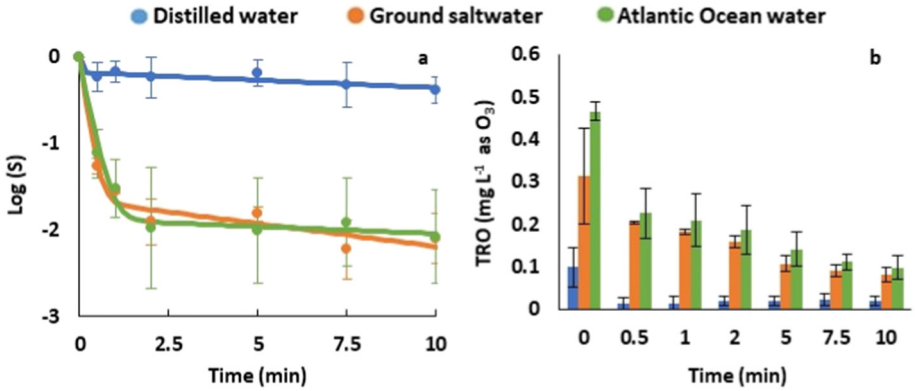

Fig. 3. Three water matrices assays. Two different assays were performed for each water matrix. a) Inactivation curves (Eq. (6)) with survival represented against time of treatment (time zero is the moment of adding the aliquot of ozone-saturated water to the culture) (b) TRO decay over time. Error bars represent the standard deviation between the experiment duplicate.

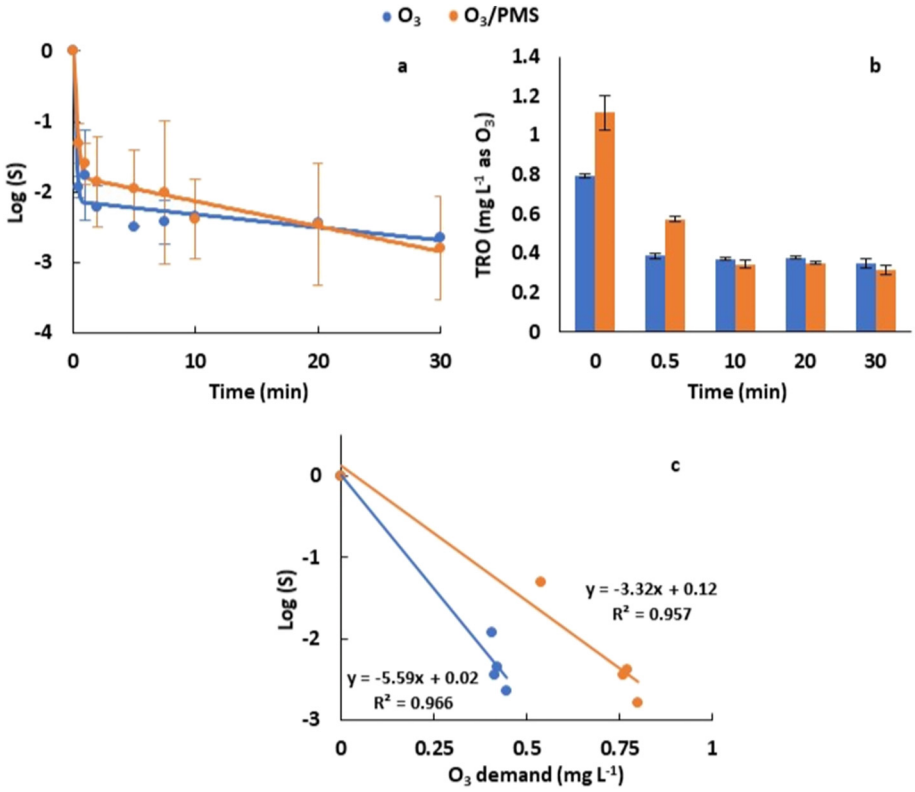

Fig. 4. O_3 and O_3/PMS batch assays. Two different assays were performed for each treatment. a) Inactivation curves with survival represented against time of treatment (time zero is the moment of adding the aliquot of ozone-saturated ground saltwater to the culture) (b) TRO decay over time c) Survival represented with respect to O_3 demand in batch ozonation. Error bars represent the standard deviation between the experiment duplicate.

zero and the other points is due to the IOD where there is always a high consume of O_3 (Fig. 4c).

The addition of PMS does not significantly increase inactivation, according to statistical analysis to compare regression curves (p value >0.1; two-way ANCOVA). When ozone concentrations are low, PMS is more involved, but when ozone concentrations are high, its effect is diluted because PMS competes with O_3. The combination of both oxidants, at least when ozone concentrations are high, is not advisable since inactivation does not increase.

Table 2
Kinetic parameters (mean ± SE) of the inactivation curves from both treatments in batch ozonation. The R^2 indicates the adjustment of the measured points to the selected inactivation model. k_1: fast inactivation rate; k_2: slow inactivation rate.

Treatment	Inactivation model	k_1 (min^{-1})	k_2 (min^{-1})	R^2	n
O_3	Biphasic	10.7 ± 2.9	0.04 ± 0.02	0.924	10
O_3/PMS	Biphasic	6.4 ± 1.1	0.08 ± 0.01	0.970	9

At the end of the experiment, a sample was taken and, without stopping the TRO, kept in darkness for five days before placing it in the culture chamber attempting to mimic the standard storage in a ballast tank (a regrowth test period of at least five days after a chemical treatment is mandatory for the approval of a BWMS) (IMO, 2018). After 17 days in the culture chamber, no growth was detected which means that the inactivation was greater than 7 log reductions which affirmed that there was total disinfection. This residual toxicity of the ozone treatment can increase disinfection even with

a lower dose of ozone if the treatment is carried out as a ballasting procedure. This is due to having some days of storage of the water in the ballast tank although the amount of organic matter and the concentration of organisms influence the decrease in the TRO and therefore the associated disinfection (Jones et al., 2006; Kureshy et al., 1999; Perrins et al., 2006).

3.3. Decay of the oxidants after the treatment

In turn, a decrease in the TRO throughout the five days of dark storage was studied. A culture of *T. suecica* was treated with O_3 and O_3/PMS to follow the decay on the TRO over a long period of time. Three days after the treatment, the concentration was practically zero, and no signal was detected on the fourth day after the treatment (Fig. 5). This decay in the TRO seen in other studies with seawater (Oemcke and Van Leeuwen, 2005; Perrins et al., 2006; Wright et al., 2010) and even tested in ballast tanks (Park et al., 2010) allows the inactivation to be prolonged for a time after the treatment but avoids the discharge of disinfection by-products into the environment during the de-ballasting procedure.

4. Semicontinuous versus batch ozonation: application on ships

To comply with the D2 standards from the BWMC (< 10 viable organisms per millilitre) and considering the initial concentration of the experiments (mean ± SE) different log reductions would be needed. For semicontinuous ozonation, 3.87 and 3.65 log reductions are needed for O_3 ($6.7 \cdot 10^4 \pm 6.7 \cdot 10^3$ cells mL^{-1}) and O_3/PMS ($4 \cdot 10^4 \pm 8.6 \cdot 10^3$ cells mL^{-1}) respectively. For batch ozonation, 3.36 and 3.26 log reductions are needed for O_3 ($2.3 \cdot 10^4 \pm 1.2 \cdot 10^3$ cells mL^{-1}) and O_3/PMS ($1.8 \cdot 10^4 \pm 2 \cdot 10^3$ cells mL^{-1}) respectively. With the D_n parameter (dose required to achieve n log reductions), the O_3 demand required to reach the different log reductions in different treatments can be compared. After analysing the inactivation that is achieved with each method of ozonating water, it was determined that batch ozonation is more efficient (Table 3).

Contemplating what the IMO establishes as the minimum concentration of organisms (1000 cells mL^{-1}) to test the efficiency of the BWMS to achieve the D2 of the BWMC, it was established that 2 log reductions (D_2) would be required. To achieve D_2, a higher ozone demand was required when O_3 was combined with PMS in both semicontinuous and batch ozonation, but these O_3 demands were lower in the case of batch ozonation. The greater efficiency in batch ozonation would be related to the formation of other oxidants, especially brominated compounds when the ground saltwater was saturated in ozone. In the case of semicontinuous ozonation, although the combination of the PMS with ozone increases the inactivation, the threshold concentration of ozone necessary to observe the synergistic effect of both oxidants would discourage its use in the treatment of ballast water with concentrations of organisms equal to or less than 1000 cells mL^{-1}. If the concentrations of organisms were equal to or greater than 10,000 cells mL^{-1} for which three or more inactivation orders would be necessary, its combination with ozone would be advisable to reduce the demand for ozone and therefore energy consumption. In the case of batch ozonation, the absence of an increase in inactivation due to the combination of O_3 and PMS allows us to rule out the use of PMS, thus avoiding the economic expense of buying the chemical as well as the risk of transporting it in the ship. Semicontinuous and batch ozonation could be accomplished in ballast tanks on the ships. If semicontinuous ozonation is employed by means of diffusers inside the ballast tank, an issue is how to get homogeneous distribution of the ozone and oxidants in all of the water from the ballast tank (Herwig et al., 2006). In batch ozonation (tank with saturated water), a different tank would be needed for containing the ozone-saturated water. A high ozone concentration would be required because it would be diluted in the enormous volume of the ballast tanks. One way of increasing the concentration of the saturated water would be to increase the pressure for a more effective transfer of the ozone into the water. One problem of batch ozonation is the demand for large amount of space (ballast water management system and tank for saturated water) which is a problem in many ships. For avoiding that, a possible mode of treating the ballast water would be to deviate a fraction of the ballasting water to be saturated in ozone and subsequently send it to the ballast tanks to be mixed with the rest of ballast water. In this case, no tank would be needed, however, a very rapid transfer of ozone to the water would be required so high inlet ozone concentration and high pressures would be necessary. Working with high ozone partial pressure, it is possible to reach a dissolved ozone concentration of approximately 50 mg L^{-1} (White, 2010). Considering that 0.8 mg L^{-1} of O_3 in the seawater is sufficient (Fig. 4b), consequently, saturating only 1/63 of the total ballast water will be needed. The residual effect of the Total Residual Oxidant (TRO) throughout the days of storage in the ballast tanks will contribute with the microorganisms' inactivation. However, those oxidant compounds have a brief life span thus, when the de-ballasting will be done, there will hopefully be no toxic effect.

Table 3
Ozone demand (mgO_3 L^{-1}) needed to reach the different log-reductions (D_1-D_4). D_{IMO} indicates the inactivation required for complying with the D2 standards considering the initial concentration of the treated culture.

Treatment	D_1	D_2	D_3	D_4	D_{IMO}
O_3 (Semicontinuous)	0.75	1.56	2.37	3.18	3.07
O_3/PMS (Semicontinuous)	1.62	1.85	2.08	2.31	2.23
O_3 (Batch)	0.18	0.36	0.54	0.72	0.60
O_3/PMS (Batch)	0.34	0.64	0.94	1.24	1.02

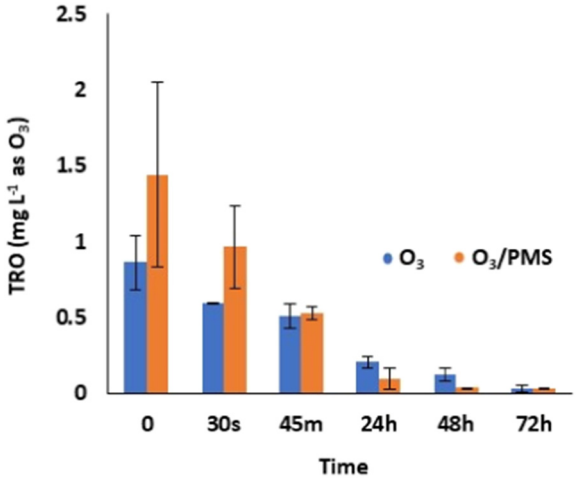

Fig. 5. Decrease on the TRO in the days following the ozonation experiment, on samples in darkness trying to mimic ballast tank conditions. Error bars represent the standard deviation between the experiment duplicate.

5. Conclusions

The objective of this work was to verify the efficiency of semicontinuous and discontinuous ozonation as well as its combination with the PMS as methods to comply with the BWMC D2 regulation using *Tetraselmis suecica* as a model.

The growth modeling of the treated cultures is a suitable method for evaluating the efficacy of the treatment. The use of organisms such as *T. suecica* with a high growth rate is desirable for determining the concentration of viable organisms after treatment with shorter incubation times.

Batch ozonation is more efficient than semicontinuous ozonation because, in the former, the saturation of the marine water produces the formation of other oxidants, especially brominated compounds. In semicontinuous ozonation, a threshold ozone concentration is necessary to achieve the synergistic effect of O_3 and PMS while in batch ozonation

no synergistic effect of both oxidants has been observed. This is likely because, until that level, the ozone is reacting with the PMS to form radicals ($SO4^{\bullet-}$ and •OH), and there is less ozone inactivating the organisms.

The residual effect of ozone and the oxidants formed during the ozonization of seawater allows the inactivation of the organisms to continue during the days following the treatment. At the same time, however, the decrease in the concentration of the Total Residual Oxidant (TRO) over time ensures the safety of the water during the de-ballasting procedure.

CRediT authorship contribution statement

Ignacio Rivas-Zaballos: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing - original draft, Visualization, Writing - review & editing.

Leonardo Romero-Martínez: Investigation, Data curation, Validation, Writing - review & editing.

M. Eugenia Ibáñez-López: Investigation, Validation, Writing - review & editing.

José L. García-Morales: Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Declaration of competing interest

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.

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