Aquaculture Reports 21 (2021) 100859

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Inactivation of marine bivalve parasites using UV-C irradiation: Examples of *Perkinsus olseni* and *Bonamia ostreae*

Sergio Fernández-Boo [a,d,*], Clément Provot [b], Cyrielle Lecadet [a], Christophe Stavrakakis [c], Mathias Papin [c], Bruno Chollet [a], Jean-François Auvray [b], Isabelle Arzul [a]

[a] IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) - La Tremblade, SG2M, Laboratoire de Génétique et Pathologie des Mollusques Marins, 17390 La Tremblade, France
[b] SATMAR (Societe Atlantic de Mariculture) - Marais du Caillaud, 17320 Saint-Just-Luzac, France
[c] IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) - Bouin, SG2M, Plateforme Expérimentale Mollusques Marins Bouin, 85230 Bouin, France
[d] CIIMAR (Interdisciplinary Centre of Marine and Environmental Research of the University of Porto), Novo Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos S/N, 4450-208 Matosinhos, Portugal

ARTICLE INFO

Keywords:
Bonamia ostreae
Disease prevention
Pathogen
Perkinsus olseni
UV irradiation

ABSTRACT

Diseases represent a major threat for the bivalve production industry. Their control relies on biosecurity measures to prevent their introduction and limit their spread. When maintained in hatcheries, nurseries and depuration centers, bivalves can become infected from the surrounding water and might release pathogens through wastewater effluents. A major effort was done in controlling the safety of bivalves for human consumption, but, on the other hand, information regarding the resistance of mollusc pathogens to water treatment is scarce. The effect of ultraviolet exposure was tested on two protozoan parasites of marine bivalves, the non-culturable parasite *Bonamia ostreae* and *Perkinsus olseni* culturable in DMEM/HAM's medium. UV exposure experiments were carried out first at the bench scale and then, for *P. olseni*, at a larger scale mimicking depuration plants, hatcheries and nurseries conditions. At the bench scale, our study indicated that up to 40% of *B. ostreae* cells and 85% of *P. olseni* cells died 24 h and 21 days respectively after an exposure to 94 mJ/cm^2 of UV-C. After 40 mJ/cm^2 exposure, *P. olseni* density increased between 15 and 21 days of culture suggesting that the parasite is able to recover from low UV intensity exposure. At large scale, no signs of UV recovery were seen in *P. olseni* cultures, but, at lower intensity (216–244 mJ/cm^2), 15% of the parasites remained alive 21 days post exposure. Finally, a minimum dose of 94 mJ/cm^2 seems required to inhibit proliferation of parasites and 450 mJ/cm^2 to completely kill all parasites. Taken this into account, a dose higher than 450 mJ/cm^2 is suggested to properly treat water to avoid dispersion of bivalve protozoan parasites such as *P. olseni*.

1. Introduction

Effluent water treatment is one of the major concerns in the bivalve production industry, not only for human health consideration but also to decrease the risk of spreading bivalve diseases. Bivalve depuration procedures are well established to prevent human infections whereas recommendations regarding inactivation of bivalve pathogens are lacking.

Bivalves are filter feeder molluscs able to filter more than 3.5 L per hour of sea water (Fournier et al., 2012; Nielsen and Vismann, 2014) and concentrate microorganisms including pathogens present in the water column (Ben-Horin et al., 2018). Depuration systems were established to prevent human infectious diseases associated with the consumption of shellfish and depuration procedures are based on the level of *Escherichia coli* colony forming units (cfu) from 100 g of flesh and intervalvular liquid (CE. No. 854/2004).

Shellfish depuration is an important sector in Europe and relies on more than 1700 depuration plants, among which 80% are located in France (Lee et al., 2010). Almost all depuration plants are open systems, and the regulation about the treatment of effluents depends mainly on the regulation in each country (Lee et al., 2010). By concentrating bivalves from different areas, depuration plants can contribute to

* Corresponding author at: CIIMAR (Interdisciplinary Centre of Marine and Environmental Research of the University of Porto), Novo Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos S/N, 4450-208 Matosinhos, Portugal.
E-mail address: sboo@ciimar.up.pt (S. Fernández-Boo).

https://doi.org/10.1016/j.aqrep.2021.100859
Received 31 May 2021; Received in revised form 2 September 2021; Accepted 4 September 2021
Available online 15 September 2021

disseminate pathogens or toxic microalgae carried by filter feeders (Ben-Horin et al., 2015; Costello et al., 2021).

Several examples of pathogen introductions associated with movements of bivalves have been reported in the literature. Importation of flat oyster *Ostrea edulis* spat from U.S.A. to France and Spain in the 70´s contributed to introduce the haplosporidian parasite *Bonamia ostreae* in Europe (Elston et al., 1986; Peeler et al., 2011). The parasite *Haplosporidium nelsoni* was detected for the first time in 1993 from *Crassostrea gigas* collected in France (Renault et al., 2000) after a possible introduction from Japan and British Columbia (Peeler et al., 2011). The alveolata parasite *Perkinsus olseni* was probably introduced into Portuguese waters by importation of clams in 80´s from Asia (Vilas et al., 2011) and this event led to the dispersion of the parasite through Europe by movements of bivalves.

These examples highlight the importance of regulating bivalve movements especially to avoid introduction of new pathogens or new strains with higher virulence, as occurred in the parasite *P. marinus* (Bushek and Allen, 1996; Carnegie et al., 2021). The legal framework regarding movements of animals is currently provided in the Regulation (EU) 2016/429 and previously in the Council Directive 2006/88/EC. Although movements of animals from disease affected zones to non-affected ones are prohibited, detection of pathogens in previously free zones have been reported. For example, the detection of the parasite *B. ostreae* in Denmark, previously free of the parasite, has not been elucidated and the origin is unclear yet (Madsen and Thomasen, 2015). Also, the detection of OsHV-1 µvar in an oyster farming area in England previously free of the virus was attributed to a release from a depuration center located in the area (Peeler et al., 2011).

Hatcheries and nurseries are also establishments concentrating animals, water treatment is usually in place at the entrance and effluents are generally directly relased in the surrounding waters and could contribute to the spread of diseases. Considering that depuration plants or hactheries-nurseries can unintentionally contribute to the spread of diseases, measures are urgently needed to properly treat their effluents to decrease the risk of disease introduction into a new area. However, information on bivalve pathogens inactivation is very scarce.

UV exposure is the most common water treatment used in hatcheries, nurseries and depuration plants (Lee et al., 2010). It has some advantages in contrast to other treatments including a low maintenance, the lack of production of hazardous by-products and effectiveness in reducing the pathogen viability (Hijnen al, 2006, Leal Diego et al., 2013). UV exposure causes two major classes of mutagenic nucleic acid damage including cyclobutane-pirimidine dimers and 6–4 photoproducts. These lesions prevent DNA replication, and accumulation of pyrimidine dimers is cytotoxic (Bushek and Howell, 2000; Li et al., 2017).

UV treatment effectiveness has been widely tested on bacteria and viruses (Hijnen et al., 2006) and in a lesser concern on protozoan parasites. Almost all of the studies regarding protozoan species were carried out with *Cryptosporidium parvum* or *Giardia* spp., two parasites that cause human gastrointestinal infections by water consumption (Craik et al., 2001; Hijnen et al., 2006; Sunnotel et al., 2007).

Most bivalve pathogens are not culturable, which might explain the few studies carried out to evaluate their susceptibility to UV treatment. *Perkinsus marinus* is an alveolata parasite associated with mass mortalities on *Crassostrea* oysters, and also with infection in clams of the genus *Mya* and *Macoma* (McLaughlin and Faisal, 1998; Dungan et al., 2007). It has a wide distribution all along the east coast of North America, from Maine to Campeche (Mexico) and recently introduced in the Pacific coast of Mexico (Cáceres-Martínez et al., 2008; OIE, 2019), and can be cultivated (Gauthier and Vasta, 1993; La Peyre et al., 1993). The resistance of the parasite to UV treatment was studied *in vitro*. Although low to moderate UV doses (4000–14000 µWs/cm^2; 4–14 mJ/cm^2) could inhibit *P. marinus* proliferation, only higher doses led to parasite mortality (> 28000 µWs/cm^2; > 28 mJ/cm^2) (Bushek and Howell, 2000).

Proper water treatment is needed to avoid the spread of bivalve pathogens and to guarantee free zone status regarding regulated ones. Considering that very few information is available on the effectiveness of water treatment to inactivate these pathogens, a study using UV-C irradiation was carried out on two bivalve protozoan parasites, *Bonamia ostreae* and *Perkinsus olseni*. The non-culturable haplosporidian parasite *B. ostreae* is notifiable to the European Union (EU) and the World Organization for Animal Health (OIE) and it has been associated with *Ostrea edulis* mass mortality in several European oyster beds since 1979 (Pichot et al., 1980; Elston et al., 1987; Friedman and Perkins, 1994; Arzul et al., 2011; Ramilo et al., 2014; Fernández-Boo et al., 2020). The culturable parasite *P. olseni* is notifiable to the OIE and has been associated with clam and abalone mortality in different parts of the world, especially in Asia (Lester and Davis, 1981; Goggin and Lester, 1995; Villalba et al., 2004; Choi and Park, 2010; El Bour et al., 2012). After testing *in vitro* the effect of a constant UV exposure on both, *B. ostreae* and *P. olseni* suspensions, the effectiveness of UV treatment to inactivate *P. olseni* in conditions similar to depuration plants ones was evaluated.

2. Material and methods

2.1. Perkinsus olseni cultures and viability estimation by light microscopy

P. olseni cultures were obtained from infected *Ruditapes philippinarum* clams collected at Charente Maritime, France (Arzul et al., 2012). Isolated parasites were maintained in Dulbeccós modified Eaglés/Hams F12 (1:2) medium according to Gauthier and Vasta (1995).

Trophozoite density and viability were estimated by counting parasites under a light microscope using a Malassez chamber and neutral red as viability marker (Fig. 1) (Fernández-Boo et al., 2015). UV exposure experiments were done using trophozoite cultures in exponential growth phase.

2.2. Bonamia ostreae isolation and viability estimation using flow cytometry

B. ostreae cells were isolated from infected oysters according to the protocol developed by Mialhe et al. (1988) with some modifications (Prado-Alvarez et al., 2013).

B. ostreae viability was estimated by flow cytometry with an EPICS XL 4 (Beckman Coulter) according to Arzul et al. (2009) using propidium iodide (PI) and fluorescein diacetate (FDA) as mortality and metabolic marker respectively, at 0 and 24 h post UV-exposure. Positive samples of heat inactivated parasites were used to evaluate the efficiency of the markers (Morga et al., 2009). Analysis of the histograms was done using

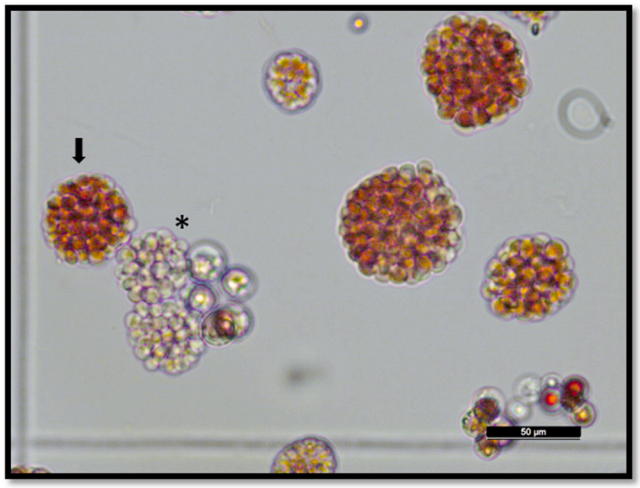

Fig. 1. Schizonts of *Perkinsus olseni* stained with neutral red. Live cells are marked in red (arrow) while dead cells were not stained (*).

Flowing Software version 2.5 (Turku Centre for Technology, Finland).

2.3. Perkinsus olseni and Bonamia ostreae bench scale - UV exposure

2.3.1. Perkinsus olseni UV exposure

Suspensions of parasites were adjusted at $1{\cdot}10^6$ cells/ml with filtered sea water before being exposed to UV for 2 min and up to 60 min (Table 1). An UV lamp (J-121 11283-L106; Light Sources Inc. USA) with 15 cm length and a wavelength of 254 nm (UV-C) was used in order to reach 35 W intensity. The exact irradiation was measured using a light meter. Samples were placed at 43 cm of the UV source in 70 mm Petri dishes without covering, and a volume of 10 ml with light agitation (100 rpm/minute) to ensure that all cells received an equal UV dose. Right after and during 23 days following UV exposure, density and viability of *P. olseni* were evaluated as described above (2.1). Control samples were subjected to the same light agitation but were not exposed to UV. The experiment was carried out twice and all samples were measured in triplicate. (Table 2).

2.3.2. Bonamia ostreae UV exposure

Suspensions of parasites were adjusted at $1{\cdot}10^6$ cells/ml in 0.22 μm filtered sea water before being exposed to the same conditions as described for *P. olseni* (2.3.1). Viability of *B. ostreae* was measured 0 and 24 h post UV exposure using flow cytometry as described above (2.2) (Fig. 2). The experiment was carried out twice and all samples were measured in triplicate.

2.4. Perkinsus olseni large scale – UV exposure

In order to mimic hatchery or depuration plant conditions, *P. olseni* cells were diluted in 100 L of 1 μm- filtered sea water to get a final concentration of $1{\cdot}10^4$ cells/ml. Contaminated water was transferred from one tank to another after a passage through a UV lamp (BIO-UV-1160 HO) with 45 cm length and 87 W intensity. The lamp delivers a minimum dose of 42 mJ/cm^2 irradiation at each passage for a minimal water transmittance of 85% at a 5 m^3/hour flow rate (Table 1) (10 s aprox. at a distance <5 mm from the source) (Fig. 3). In this study, the flow rate was set at 1 m^3/h and the water transmittance was in the range 85–90% before adding *P. olseni* in the seawater. In these conditions, at each passage through the UV reactor, parasites were exposed to a theoretical dose of 216–244 mJ/cm^2.

The experiment was repeated twice with a maximum of 5 passages. At the end of each passage, three 100 ml samples were collected, centrifuged (3000 g, 10 min at room temperature) and *P. olseni* cells were transferred to culture flasks with 5 ml of DMEM/HAMs medium to evaluate their viability and monitor their survival during 21 days as described above (2.1). Additionally, a control consisting of the same *P. olseni* suspension subjected to the same number of passages through the system described in Fig. 3 but without UV exposure was included in each experiment and tested as described above.

Table 1
Time in minutes and intensity in mJ/cm^2 of UV-C used for the bench scale experiments and range of intensity in mJ/cm^2 of UV-C used in the large scale experiment regarding the number of passages through the UV lamp.

In vitro		*In Vivo*	
Time (min)	Intensity mJ/cm^2	Number of passages	Range of intensity mJ/cm^2
2:10	40	1	216–244
5	94	2	432–488
10	188	3	648–732
15	282	4	864–976
20	376	5	1080–1220
30	563.4		
60	1126.8		

2.5. Statistical analysis

All statistical analyses were performed using SPSS (IBM, v.24). Results are expressed as mean ± standard deviation (sd). After verifying normality and variance homogeneity one-way analysis of variance (ANOVA) was carried out. Tukey HSD post hoc test was performed to identify significantly differences among groups with a level of significance of $p \leq 0.05$.

Pearson correlation tests were done to verify the correlation between the intensity of UV and the mortality and esterase activity in *B. ostreae* and mortality in *P. olseni*.

3. Results

3.1. Bonamia ostreae bench scale UV exposure

Control samples, which have not been subjected to UV exposure, showed a mortality rate (percentage of PI positive cells) of 15.92 ± 1.85% and 16.31 ± 1.93% at 0 h and 24 h, respectively (Fig. 4). A correlation was noticed between time or UV intensity and percentage of mortality at time 0 h ($r = 0.986$, $p = 0.002$; $r = 0.949$, $p = 0.014$ respectively) and at time 24 h ($r = 0.921$, $p = 0.027$; $r = 0.921$, $p = 0.027$ respectively). The maximum level of mortality (86.79 ± 4.08%) was recorded 24 h after 60 min of UV exposure (Fig. 4). At time 0 h, significant difference with control samples was only observed after 30 and 60 min of UV exposure whereas at time 24 h, mortality rates were significantly higher than in the control whatever the duration of UV exposure (Fig. 4).

Measures of esterase activities showed a similar pattern to results obtained with PI. Percentages of cells negative for esterase activities (considered as metabolically inactive cells) increased with the duration of UV exposure (Fig. 5). However, differences with the controls were only significant for 30 and 60 min of UV exposure at both times, 0 and 24 h post exposure (Fig. 5).

3.2. Perkinsus olseni bench scale UV exposure

No mortality of *P. olseni* cells was observed during the first 7 days of culture following UV exposure whatever the UV intensity was (Fig. 6). At day 15, difference with the control was observed in all the *P. olseni* cultures. However, mortality rate was significantly lower ($p < 0.000$) in *P. olseni* exposed to 40 mJ/cm^2 in comparison with other tested UV intensities. After 21 days, almost all the *P. olseni* cells (>95%) were dead after a UV exposure of or above 188 mJ/cm^2 (Fig. 6).

The monitoring of cell density revealed that contrary to the control, UV exposed cells did not multiply for the 15 first days whatever the UV intensity. Interestingly, cell density increased significantly ($p < 0.001$) between 15 and 21 days in *P. olseni* exposed to 40 mJ/cm^2 (Fig. 7).

3.3. Perkinsus olseni large scale UV exposure

In control samples, mortality of *P. olseni* cells never exceeded 8% (Fig. 8). At day 1, all samples of UV exposed *P. olseni* cells presented higher mortality compared to the control ($p = 0.000$).

Percentages of mortality increased globally over the study period in all the UV exposed *P. olseni* cultures, and the difference between day 1 and day 21 was more important after one and two passages through the UV lamp compared to 3, 4 and 5 passages. At day 21 no significant difference was found between passages 3, 4 and 5 ($p = 0.806$) while after 1 and 2 passages parasites presented significant lower mortalities ($p < 0.039$) than parasites exposed to more than 2 passages.

After 21 days of culture, percentages of *P. olseni* mortality ranged between 69.55 ± 2.69% after one passage and 99.04 ± 0.0% after 5 passages.

While increasing after the second day in control cultures, cell density decreased from the first day in cultures of *P. olseni* exposed to UV.

Table 2
Percentages of PI positive *Bonamia ostreae* cells (mean ± sd) measured by flow cytometry in both UV –C exposure experiments carried out at the bench scale. Results show mortality at time 0 just after exposition and 24 h later. All samples were run in triplicate. Results obtained from the first experiment 24 h after a 20 min UV exposure was excluded from the analysis due to an abnormal mortality in the control sample.

		Exp. 1 - Time 0		Exp. 1 - Time 24		Exp. 2 - Time 0		Exp. 2 - Time 24	
Exposure time	Intensity mJ/cm^2	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
5	94	15.71 ± 0.75	19.26 ± 0.37	17.11 ± 3.38	42.16 ± 10.97	19.71 ± 0.21	22.57 ± 0.09	19.18 ± 0.70	41.01 ± 0.41
10	188	14.85 ± 0.61	18.79 ± 0.04	14.55 ± 1.37	57.66 ± 6.31	16.80 ± 1.07	17.2 ± 0.19	17.67 ± 0.77	40.00 ± 0.05
20	376	14.45 ± 0.01	25.01 ± 0.92	58.24 ± 8.03	59.38 ± 1.05	18.38 ± 0.48	26.00 ± 0.39	17.10 ± 0.56	64.56 ± 0.59
30	563.4	14.24 ± 1.07	43.60 ± 1.67	16.10 ± 0.20	86.28 ± 1.07	14.75 ± 0.38	29.88 ± 0.90	16.73 ± 0.01	72.22 ± 0.25
60	1126.8	14.45 ± 0.44	73.5 ± 0.06	12.43 ± 1.68	89.68 ± 0.42	15.84 ± 0.84	79.42 ± 0.90	15.91 ± 1.82	83.91 ± 0.80

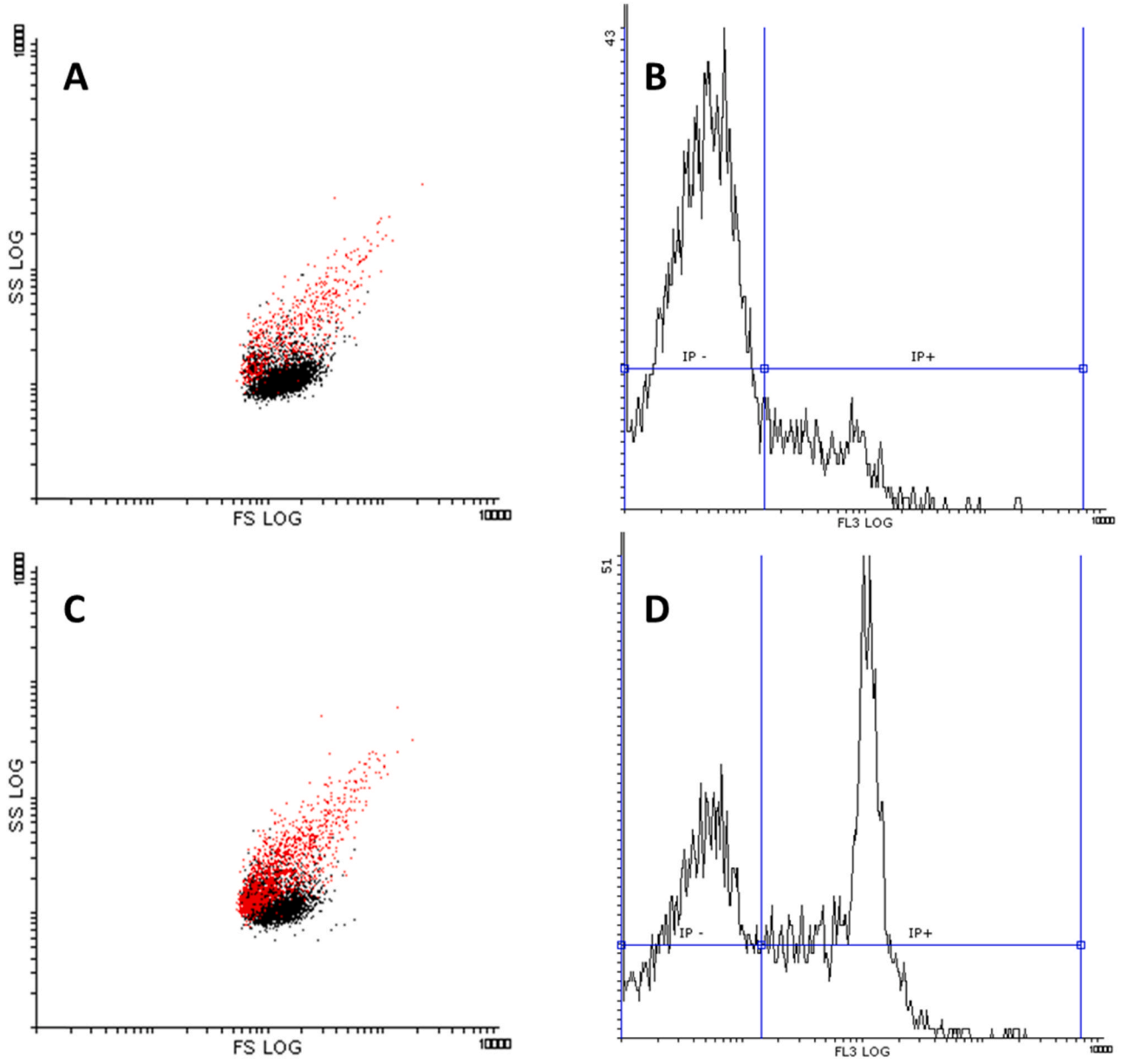

Fig. 2. Flow cytometry Dot Plots (A, C) and histograms (B, D) from *Bonamia ostreae* cells non-exposed (A, B) and exposed to 30 min UV (C, D) at day 0. Dead cells (IP+) are marked in red in the Dot Plot section and showed in region "IP+ " at both histograms.

Almost no cells (1743.5 ± 936.7 cells/ml) were seen in cultures 21 days after one passage in the UV reactor (UV emission in the range 216–244 mJ/cm^2), corresponding to an inactivation higher than 85.46%. (Fig. 9, Table 3). Interestingly, after 21 days, cell density did not show difference between cultures of parasites exposed from 2 to 5 passages ($p = 0.649$) whereas a significantly higher density was observed in cultures of parasites exposed to one unique passage through the UV reactor ($p = 0.036$). It is also remarkable that at day 1, cell density decreased by half after just one passage compared to control, and up to 96.97% after 5 passages (Table 3).

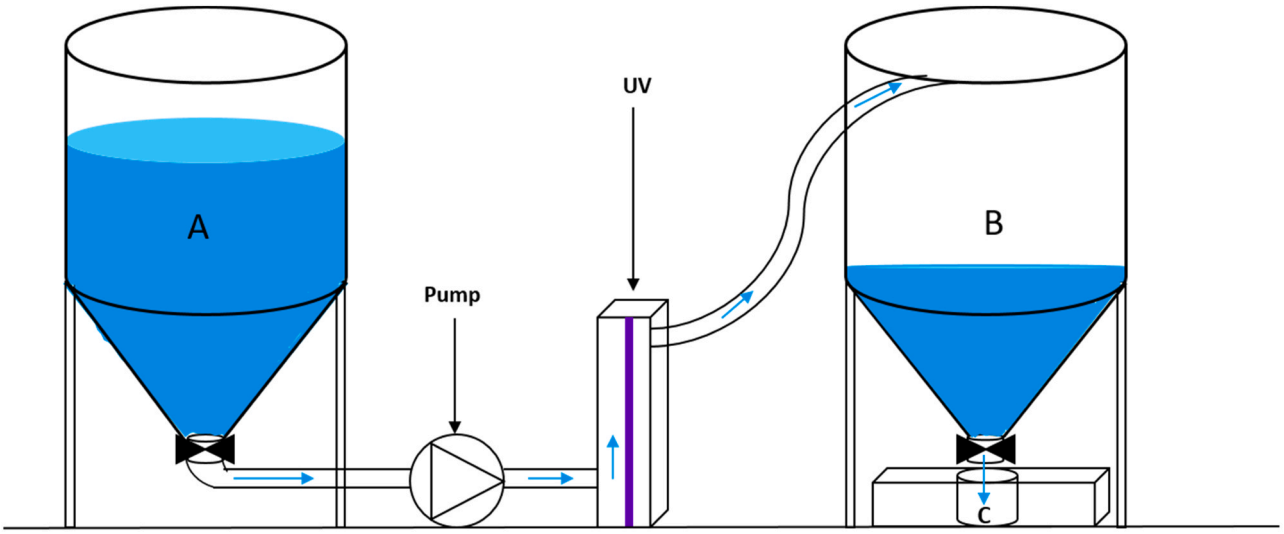

Fig. 3. Experimental setup used to evaluate the effect of UV-C exposure on *Perkinsus olseni* parasite suspensions circulating through the system including the UV lamp (45 cm and 87 W intensity) between 1 and 5 passages through UV lamp. At the end of each passage, a sample of 100 ml was collected in compartment C to measure mortality rate and cell density in DMEM/HAM's medium during 21 days. The control consisted of *P. olseni* cells which have circulated through the system with the UV lamp off.

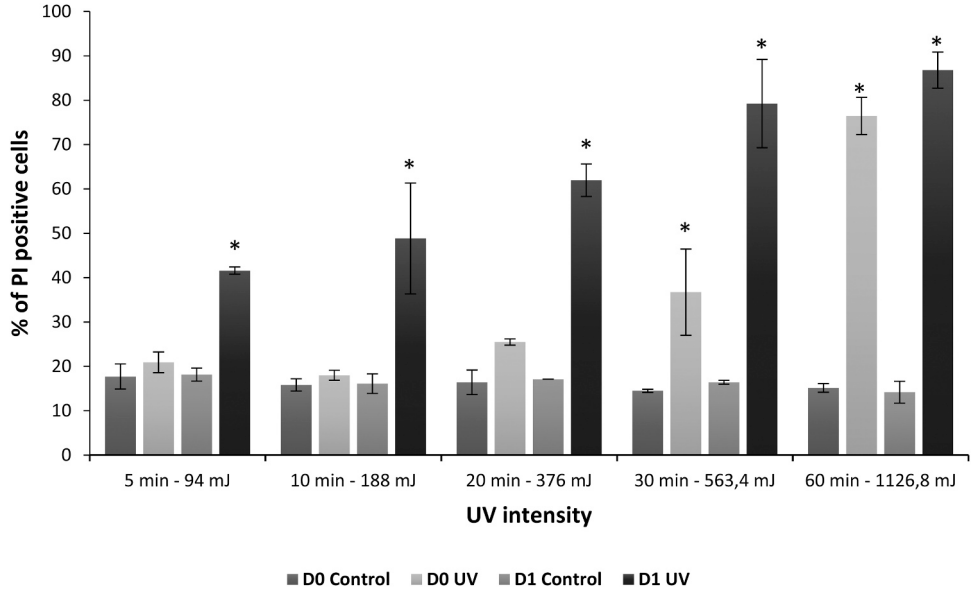

Fig. 4. Percentages of PI positive *Bonamia ostreae* cells at 0 (D0) and 24 h (D1) with (UV group) or without (control) UV-C exposure depending on duration in minutes-intensity in mJ/cm^2. Results are expressed as mean ± sd (n = 6). Asterisk indicates significant differences at 0 or 24 h between control and UV exposure parasites.

4. Discussion

Most of the information available on UV protozoan parasite inactivation concern resistant parasite stages present in drinking water including cyst from *Giardia lambia* and *Giardia intestinalis* or oocysts from *Cryptosporidium parvum* (Leal Diego et al., 2013; Einarsson et al., 2015). For example it has been shown that oocysts from *C. parvum* and *Toxoplasma gondii* present high resistance to UV and up to 100 mJ/cm^2 is needed to be inactivated (Morita et al., 2002; Ware et al., 2010). Additionally, some parasites like *G. intestinalis* can recover from irradiation below 100 mJ/cm^2 (Einarsson et al., 2015).

In contrast, information about the effect of UV on protozoan parasites infecting marine bivalves is very scarce. Ford et al. (2001) showed that 30 mJ/cm^2 were enough to prevent oysters from infection with the parasites *Haplosporidium nelsoni* and *P. marinus* in hatcheries. Also, Lester and Hayward (2005) found that 60 mJ/cm^2 UV-C can prevent up to 90% of the *P. olseni* hypnospores but, up to 480 mJ/cm^2 or higher irradiation is needed to completely inactivate all hypnospores evidencing the great resistance of the parasite to disinfection measures. However, most of the effluent water treatments currently applied in the bivalve industry are calibrated in order to inactivate viruses and bacteria to ensure food safety in depuration plants or animal health within hatcheries and nurseries. Irradiation of 40 mJ/cm^2 is enough to inactivate enteric viruses in depuration molluscs plants (Hijnen et al., 2006; Garcia et al., 2015; Pilotto et al., 2019) while at least 72 mJ/cm^2 are needed to inactivate enteric bacteria and up to 1800 mJ/cm^2 are required against heterothrophic bacteria in salmonid aquaculture production systems (Sharrer et al., 2005). These UV doses are higher than

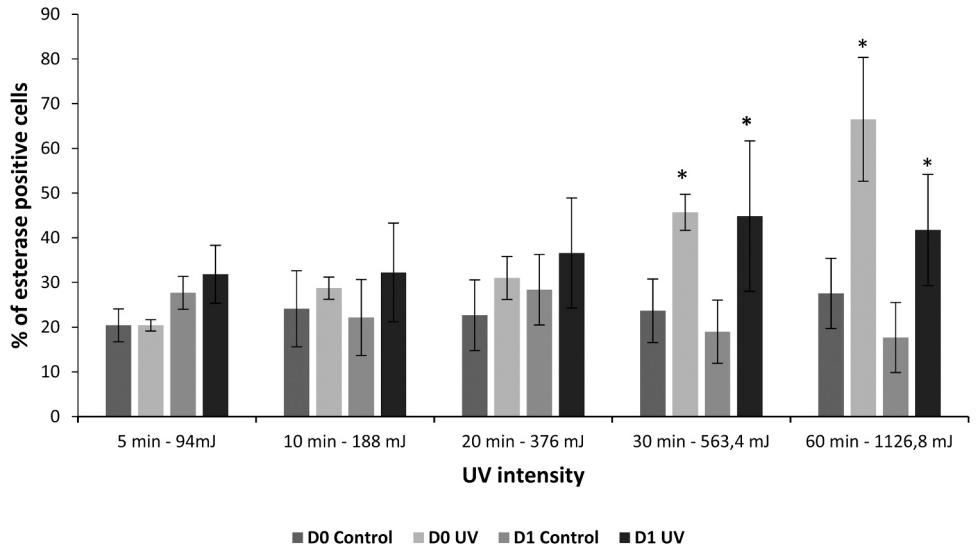

Fig. 5. Percentages of *Bonamia ostreae* cells negative for non-specific esterase activities at 0 (D0) and 24 h (D1) with (UV group) or without (control) UV-C exposure depending on duration in minutes-intensity in mJ/cm^2 Results are expressed as mean ± sd (n = 6). Asterisk indicates significant differences at 0 or 24 h between control and UV exposure parasites.

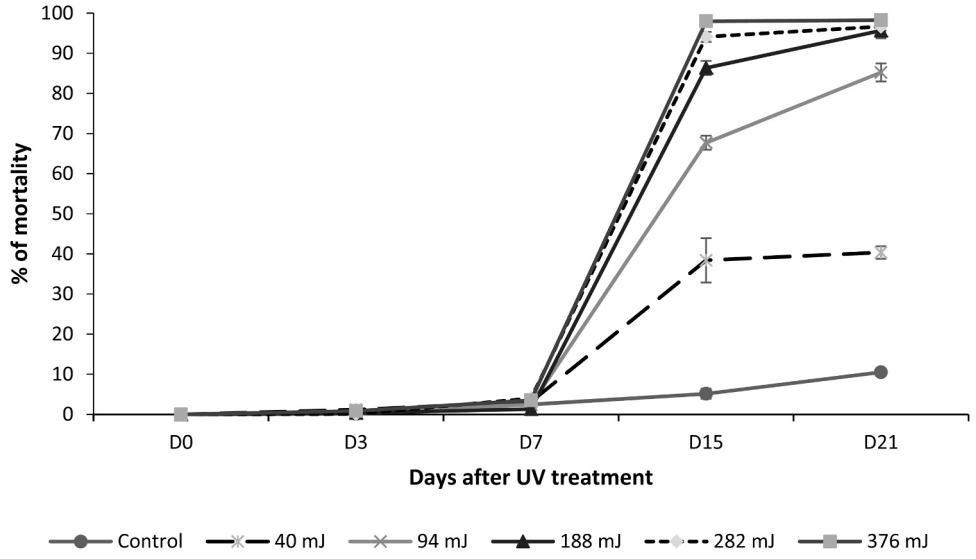

Fig. 6. Percentages of mortality of *Perkinsus olseni* cells after different UV-C exposure intensities and following culture in DMEM/HAMS medium during 21 days. Results are expressed as mean ± sd (n = 6). Control samples were parasites not exposed to UV-C irradiation. All treatments were statistically different from control at day 15 and day 21.

the 30 mJ/cm2 dose typically recommended (Wedemeyer, 1996; Liltved and Cripps, 1999). These differences are explained by the capacity of bacteria to aggregate and the presence of solid dissolve matter in water (Sharrer et al., 2005).

Considering that most of regulated bivalve diseases are due to protozoan parasites and the lack of data regarding their inactivation, we have established a two scale experimental approach to determine the minimum UV irradiance needed to inactivate two bivalve pathogens notifiable to the OIE, *B. ostreae* and *P. olseni*. The later can be maintained and multiplied *in vitro* in DMEM/Ham's medium (Casas et al., 2002; Robledo et al., 2002). Although it is possible to maintain, for about two days, *B. ostreae* cells isolated from highly infected oysters in filtered sea water complemented with antibiotics (Arzul et al., 2009; Mérou et al., 2020), the lack of culture limits the possibility to evaluate the effect of UV in experimental conditions mimicking conditions used in hatcheries or depuration plants.

We have thus tested in a first step the effect of UV at the bench scale using different fixed doses of UV on high density parasite suspensions. The viability of *P. olseni* was evaluated by light microscopy using neutral red and the viability of *B. ostreae* was evaluated by measuring mortality and esterase activities by flow cytometry. The different methods used for viability evaluation were due to the difficulty to evaluate the viability in *P. olseni* cultures by flow cytometry; the characteristic schizonts from *P. olseni* need to be disrupted before injection in the flow cytometer. This step can damage the cells and biasing the results. In a second step, we have investigated the effect of different UV doses at large scale in an experimental setup mimicking conditions used in hatcheries-nurseries and depuration plants. Such conditions require a high number of parasite cells which could only be obtained for *P. olseni*.

In the bench scale experiment, a different susceptibility to UV exposure was observed between both tested parasites. While *B. ostreae* presented a higher mortality 24 h after UV exposure, no mortality was

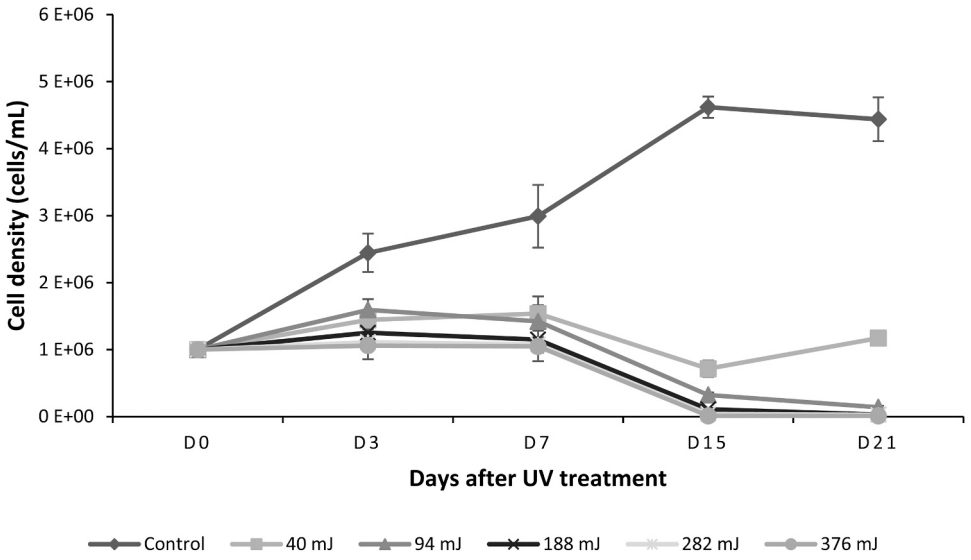

Fig. 7. Cell culture density (cell/ml) of *Perkinsus olseni* after different UV-C exposure intensities and following culture in DMEM/HAMS medium during the next 21 days. Results are expressed as mean ± sd (n = 6). Control samples were not subjected to UV light. All treatments were statistically different from control at all times.

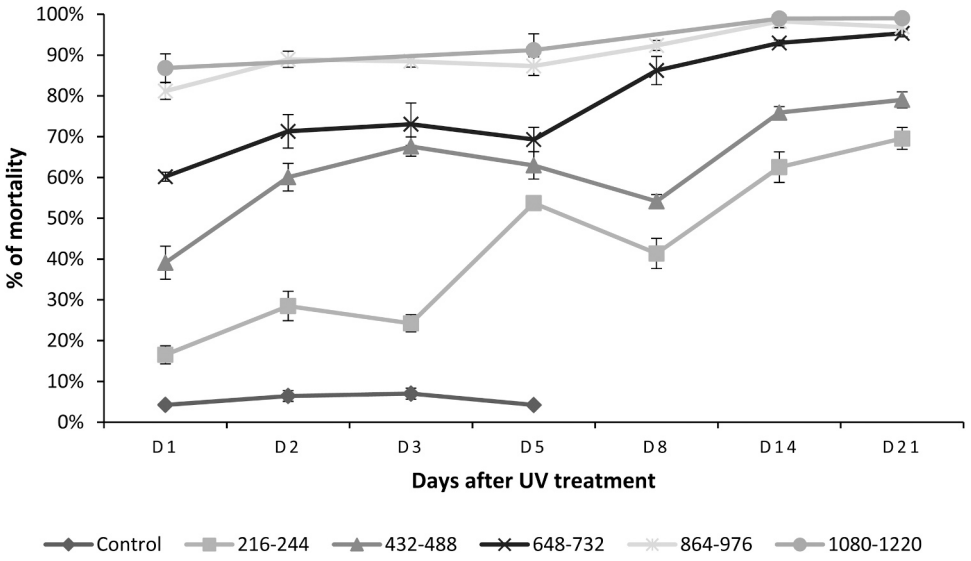

Fig. 8. *P. olseni* mortality rate (%) evaluated from cultures in DMEM/HAMS medium during 21 days after 1–5 passages in our large-scale setup (corresponding from 216 to 1220 mJ/cm^2 UV-C light). Results are expressed as mean ± SEM (n = 6). Control samples correspond to *P. olseni* passed through the system without UV exposure. Due to bacterial contamination, our control cultures were not monitored after day 5. All treatments were statistically different from the control at all times.

recorded in *P. olseni* cultures during the first 7 days.

Although mortality was significantly higher in UV-exposed *B. ostreae* compared to the control after 1 day whatever the tested UV dose, mortality rates above 80% were only reached after a UV exposure above 563.4 mJ/cm^2.

Infection with *B. ostreae* is harmful for the production of flat oysters. Several restoration programs are currently undertaken in some European countries, such as the Native Oyster Restoration Alliance (NORA) (Pogoda et al., 2019), which considers producing oysters in hatcheries for restocking. Usually combination of bag or cartridge filters and sand filters are used to reduce the incoming particles to around 5 μm (Breese and Malouf, 1975; Ford et al., 2001; Arndt and Wagner, 2003; Wallace et al., 2008). Filtration, together with UV treatment at higher doses, should be used to avoid entrance of *Bonamia* sp. Results suggest that a UV dose higher than 563.4 mJ/cm^2 of UV is needed to inactivate completely the parasite in tested conditions. However, the lack of *B. ostreae* culture did not allow to evaluate the effect of UV longer than 24 h and verify the minimum dose required to completely inactivate the parasite. It was also described that temperatures of 25 °C and low salinity can be lethal for *B. ostreae* (Arzul et al., 2009), so the combination of UV with higher temperatures/low salinities could have a stronger effect on the parasite.

Concerning *P. olseni*, the bench scale experiment showed that up to 188 mJ/cm^2 were necessary to inactivate up to 96% of the parasites 21 days after UV treatment. On the other hand, results suggest a complete inhibition of the replication at a moderate UV dose of 94 mJ/cm^2. Results obtained on *P. marinus* (Bushek and Howell, 2000) showed 80% of mortality after an UV exposure of 465 mJ/cm^2 and inhibition of replication at lower doses (4–14 mJ/cm^2). Based on our results, *P. olseni* seems to be more resistant to UV than *P. marinus*.

The monitoring of *P. olseni* density gave results consistent with the evaluation of mortality rates. However, interestingly, the density of *P. olseni* exposed to 40 mJ/cm^2 almost doubled between 15 and 21 days post UV exposure indicating a possible photoreactivation of the cells.

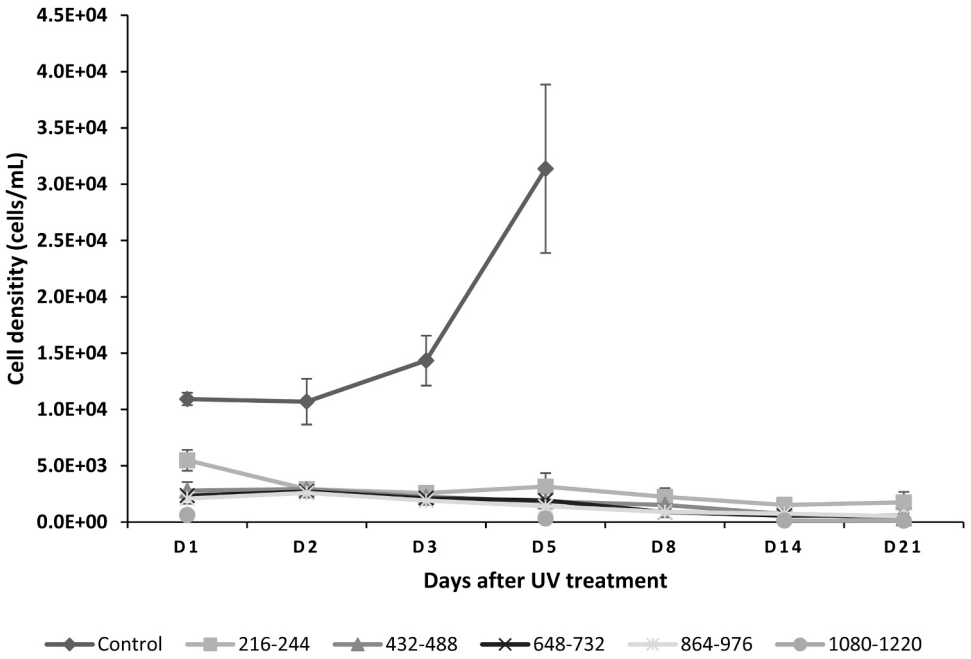

Fig. 9. Density of *P. olseni* in cells·ml^{-1} evaluated from cultures in DMEM/HAMS medium during 21 days after 1–5 passages in our large-scale setup (corresponding to 216–1220 mJ/cm^2 UV-C light). Due to bacterial contamination, our control cultures were not monitored after day 5. Results are expressed as mean ± sd (n = 6). All treatments were statistically different from the control at all times.

Table 3
Percentages of cell density reduction of *Perkinsus olseni* when compared with control samples at day 1 post UV exposure in the large scale experiment. Results are presented as mean ± sd.

Days	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5
1	50.53 ± 1.04	71.26 ± 4.44	71.28 ± 10.17	75.12 ± 8.08	96.97 ± 0.55
2	71.96 ± 1.80	71.84 ± 1.81	73.92 ± 1.68	75.22 ± 1.59	100 ± 0.00
3	75.23 ± 1.59	78.35 ± 1.39	79.66 ± 1.31	81.67 ± 1.18	100 ± 0.00
5	73.61 ± 3.43	79.48 ± 5.23	77.35 ± 7.14	81.88 ± 7.35	98.46 ± 0.31
8	78.52 ± 1.38	85.45 ± 0.94	91.60 ± 0.54	91.35 ± 0.56	100 ± 0.00
14	86.66 ± 0.78	93.64 ± 0.13	94.52 ± 0.35	92.40 ± 0.98	99.34 ± 0.12
21	85.46 ± 2.01	96.12 ± 1.02	94.48 ± 0.35	93.83 ± 1.95	99.39 ± 0.10

The mechanisms of photoreactivation have been investigated in other protozoan species (Oguma et al., 2001; Rochelle et al., 2005) suggesting that after UV treatment some cells are able to recover, repairing the damage of UV in the DNA.

In the large scale experiment, water flow was adjusted at 1000 liter per hour in order to deliver a UV dose of 216–246 mJ/cm^2 at each passage through the UV lamp with a water transmittance around 85–90%. However, in real water treatment conditions, it is important to distinguish the dose emitted with regard to the dose received by the targeted microorganisms which may, at each passage through the UV reactor, be protected from UV rays by other particles present in the water to be treated. This precision is important in view of the high concentrations used in this study. Ford et al. (2001) previously suggested that 30 mJ/cm^2 after water filtration at 1 μm was enough to prevent infections of *C. virginica* oysters larvae and juveniles by the protozoan parasites *H. nelsoni* and *P. marinus* in hatchery facilities. Similarly, in our study, sea water was filtered at 1 μM prior to be contaminated with *P. olseni*.

Contrary to the bench scale experiment, *P. olseni* presented mortality as soon as one day after the UV exposure whatever the number of passages through the UV lamp. The distance to the UV lamp combined with the lower density in the large scale experiment (10^6 vs 10^4 cells/ml) and also the high dose received in each passage might explain the higher mortality. Mortality rates increased from 16.52% to 86.84% with the UV dose while cell density showed a reduction of 50.53–96.97% compared to the control suggesting that most of *P. olseni* cells are inactivated as soon as one day after UV exposure.

More than 94% (2 $\log_{10}$: from 1.10^4 to 1.10^2 cel/ml) of the parasites died after 15 days of culture after a UV exposure around 700 mJ/cm^2 (corresponding to 3 passages through the lamp). As demonstrated by the monitoring of cell density, number of live cells was lower than 100 cells/ml 21 days after an exposure around 500 mJ/cm^2 (corresponding to two passages through the lamp). No sign of recovery of *P. olseni* cells was seen after 21 days at any tested UV dose.

Although in our approach we have not tested the ability of UV exposed *P. olseni* to infect hosts, our results suggest that in tested conditions a high UV dose is needed to inactivate *P. olseni*. At the bench scale, a UV dose of 563 mJ/cm^2 was needed to kill 80% of the cells, and a lower dose (432–488 mJ/cm^2) was required to reach the same percentage of mortality in the larger scale tests. This difference could be explained by the initial parasite concentration which was two log higher at the bench scale compared to the larger scale test.

Additionally, whatever the tested UV dose no bacteria proliferation was observed in cells cultures except in the control cultures, which presented contamination with bacteria after day 5 (data not shown). That means that UV exposure has probably inactivated all bacteria within a single pass through the system.

Differences observed between both, the bench and the large scale studies; highlight the need to carry out studies as close as possible to the conditions used in hatcheries or depuration plants. These studies would

need to be complemented by testing different conditions including combinations of temperature, salinity and also turbidity as limiting factors to reach the minimum dose of UV to inactivate pathogens.

Moreover, our results suggest that compared to human protozoan parasites, higher UV dose is required to inactivate bivalve ones such as *P. olseni* (Morita et al., 2002; Ware et al., 2010).

Taking into account our results and the lack of cell cultures for most of the protozoan parasites infecting bivalves, *P. olseni* appears as an interesting model species to evaluate the different inactivation systems including UV irradiation in the bivalve industry and, finally, establish biosecurity measures to prevent the introduction and avoid the dispersion of bivalve pathogens into and from depuration plants or hatcheries and nurseries.

CRediT authorship contribution statement

Sergio Fernández-Boo: Data curation, Writing – original draft. **Clément Provot**: Investigation, Data curation. **Cyrielle Lecadet**: Investigation, Data curation. **Christophe Stavrakakis**: Conceptualization, Methodology, Writing – review & editing. **Mathias Papin**: Methodology, Writing – review & editing. **Bruno Chollet**: Investigation, Data curation, **Jean-François Auvray**: Methodology, Writing – review & editing. **Isabelle Arzul**: Conceptualization, Methodology, Writing – review & editing.

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.

Acknowledgements

The Région of Poitou Charentes and Ifremer supported this research. The authors thank the team of the hatchery facilities of Ifremer - La Tremblade for their help in the maintenance of oysters for *Bonamia ostreae* isolation. This work was also supported by the project "Cragiamp" with reference PTDC/BIA-BQM/30232/2017, co-financed by COMPETE 2020, Portugal 2020 and the European Union through the ERDF. This research was supported by national funds "through FCT – Foundation for Science and Technology" within the scope of UIDB/0443/2020 and UIDP/04423/2020.

References

Arndt, R.E., Wagner, E.J., 2003. Filtering *Myxobolus cerebralis* Triactinomyxons from contaminated water using rapid sand filtration. Aquac. Eng. 29 (3), 77–91.

Arzul, I., Gagnaire, B., Bond, C., Chollet, B., Morga, B., Ferrand, S., Robert, M., Renault, T., 2009. Effects of temperature and salinity on the survival of *Bonamia ostreae*, a parasite infecting flat oysters *Ostrea edulis*. Dis. Aquat. Org. 85 (1), 67–75.

Arzul, I., Langlade, A., Chollet, B., Robert, M., Ferrand, S., Omnes, E., Lerond, S., Joly, J. P., Francois, C., Garcia, C., 2011. Detection of *Bonamia ostreae* in larvae of *Ostrea edulis*. J. Shellfish Res. 30 (2), 483-483.

Arzul, I., Chollet, B., Michel, J., Robert, M., Garcia, C., Joly, J.P., Francois, C., Miossec, L., 2012. One *Perkinsus* species may hide another: characterization of *Perkinsus* species present in clam production areas of France. Parasitology 139 (13), 1757–1771.

Ben-Horin, T., Bidegain, G., Huey, L., Narvaez, D.A., Bushek, D., 2015. Parasite transmission through suspension feeding. J. Invertebr. Pathol. 131, 155–176.

Ben-Horin, T., Burge, C.A., Bushek, D., Groner, M.L., Proestou, D.A., Huey, L.I., Bidegain, G., Carnegie, R.B., 2018. Intensive oyster aquaculture can reduce disease impacts on sympatric wild oysters. Aquac. Environ. Interact. 10, 557–567.

Breese, W.P., Malouf, R. E., 1975. Hatchery manual for the Pacific oyster. Oregon State University Sea Grant College Program ORESU-H-75–002.

Bushek, D., Allen, S., 1996. Races of *Perkinsus marinus*. J. Shellfish Res. 15 (1), 103–107.

Bushek, D., Howell, T.L., 2000. The Effect of UV Irradiation on *Perkinsus marinus* and its Protential Use to Reduce Transmission via Shellfish Effluents. NRAC Publication. No.00-008.

Cáceres-Martínez, J., Vásquez-Yeomans, R., Padilla-Lardizábal, G., del Río, Portilla, M. A., 2008. *Perkinsus marinus* in pleasure oyster *Crassostrea corteziensis* from Nayarit, Pacific coast of México. J. Invertebr. Pathol. 99 (1), 66–73.

Carnegie, R.B., Ford, S.E., Crockett, R.K., Kingsley-Smith, P.R., Bienlien, L.M., Safi, L.S.L., Whitefleet-Smith, L.A., Burreson, E.M., 2021. A rapid phenotype change in the pathogen *Perkinsus marinus* was associated with a historically significant marine disease emergence in the eastern oyster. Sci. Rep. 11 (1), 12872.

Casas, S.M., La Peyre, J.F., Reece, K.S., Azevedo, C., Villalba, A., 2002. Continuous in vitro culture of the carpet shell clam *Tapes decussatus* protozoan parasite *Perkinsus atlanticus*. Dis. Aquat. Org. 52, 217–231.

Choi, K.S., Park, K.I., 2010. In: Ishimatsu, A., Lie, H. –J. (Eds.), Review in the Protozoan Parasite *Perkinsus olseni* (Lester and Davis 1981) Infection in Asian waters. Coastal Environmental and Ecosystem Issues of the East China Sea, pp. 269–281.

Costello, K.E., Lynch, S.A., O'Riordan, R.M., McAllen, R., Culloty, S.C., 2021. The importance of marine bivalves in invasive host–parasite introductions. Front. Mar. Sci. 8, 147.

Craik, S.A., Weldon, D., Finch, G.R., Bolton, J.R., Belosevic, M., 2001. Inactivation of *Cryptosporidium parvum* oocysts using medium- and low-pressure ultraviolet radiation. Water Res. 35 (6), 1387–1398.

Dungan, C.F., Reece, K.S., Hamilton, R.M., Stokes, N.A., Burreson, E.M., 2007. Experimental cross-infections by *Perkinsus marinus* and *P. chesapeaki* in three sympatric species of Chesapeake Bay oysters and clams. Dis. Aquat. Org. 76 (1), 67–75.

Einarsson, E., Svärd, S.G., Troell, K., 2015. UV irradiation responses in *Giardia intestinalis*. Exp. Parasitol. 154, 25–32.

El Bour, M., Dellali, M., Boukef, I., Lakhal, F., Mraouna, R., El Hili, H.A., Paillard, C., Klena, J., 2012. First assessment of Perkinsosis and brown ring disease co-infection in *Ruditapes decussatus* in the North Lake of Tunis (southern Mediterranean Sea). J. Mar. Biol. Assoc. U. Kingd. 92 (7), 1579–1584.

Elston, R.A., Farley, C.A., Kent, M.L., 1986. Occurence and significance of bonamiasis in European flat oysters *Ostrea edulis* in North America. Dis. Aquat. Org. 2, 49–54.

Elston, R.A., Kent, M.L., Wilkinson, M.T., 1987. Resistance of *Ostrea edulis* to *Bonamia-ostreae* infection. Aquaculture 64 (3), 237–242.

Fernández-Boo, S., Villalba, A., Cao, A., 2015. Cell proteome variability of protistan mollusc parasite *Perkinsus olseni* among regions of the Spanish coast. Dis. Aquat. Org. 113 (3), 245–256.

Fernández-Boo, S., Gervais, O., Prado-Alvarez, M., Chollet, B., Claverol, S., Lecadet, C., Dubreuil, C., Arzul, I., 2020. Is pallial mucus involved in *Ostrea edulis* defenses against the parasite *Bonamia ostreae*? J. Invertebr. Pathol. 169, 107259.

Ford, S.E., Xu, Z., Debrosse, G., 2001. Use of particle filtration and UV irradiation to prevent infection by *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) in hatchery-reared larval and juvenile oysters. Aquaculture 194 (1), 37–49.

Fournier, J., Dupuy, C., Bouvy, M., Couraudon-Réale, M., Charpy, L., Pouvreau, S., Le Moullac, G., Le Pennec, M., Cochard, J.-C., 2012. Pearl oysters *Pinctada margaritifera* grazing on natural plankton in Ahe atoll lagoon (Tuamotu archipelago, French Polynesia). Mar. Pollut. Bull. 65 (10–12), 490–499.

Friedman, C.S., Perkins, F.O., 1994. Range extension of *Bonamia ostreae* to Maine, USA. J. Invertebr. Pathol. 64 (3), 179–181.

Garcia, L.A.T., Nascimento, M.A., Barardi, C.R.M., 2015. Effect of UV light on the inactivation of recombinant human adenovirus and murine norovirus seeded in seawater in shellfish depuration tanks. Food Environ. Virol. 7 (1), 67–75.

Gauthier, J., Vasta, R., 1993. Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus*. J. Invertebr. Pathol. 62, 321–323.

Gauthier, J., Vasta, R., 1995. *In vitro* culture of the eastern oyster parasite *Perkinsus marinus*: optimisation of the methodology. J. Invertebr. Pathol. 66, 156–168.

Goggin, C.L., Lester, R.J.G., 1995. Perkinsus, a protistan parasite of abalone in Australia: a review. Mar. Freshw. Resour. 46, 639–646.

Hijnen, W.A.M., Beerendonk, E.F., Medema, G.J., 2006. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. Water Res. 40 (1), 3–22.

La Peyre, J.F., Faisal, M., Burreson, E., 1993. *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostea virginica*. J. Eukariotic Microbiol. 40 (3), 304–310.

Leal Diego, A.G., Dores Ramos, A.P., Marques Souza, D.S., Durigan, M., Greinert-Goulart, J.A., Moresco, V., Amstutz, R.C., Micoli, A.H., Romeu Cantusio, N., Célia Regina Monte, B., Regina Maura Bueno, F., 2013. Sanitary quality of edible bivalve mollusks in Southeastern Brazil using an UV based depuration system. Ocean Coast. Manag. 72, 93–100.

Lee, R., Lovatelli, A., Ababouch, L., 2010. Bivalve depuration: fundamental and practical aspects. FAO - Fisheries technical paper 511: 153.

Lester, R.J.G., Davis, G.H.G., 1981. A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. J. Invertebr. Pathol. 37, 181–187.

Lester, R.J. G., Hayward, C. J., 2005. Control of Perkinsus disease in abalone. Final Report for FRDC project no. 2000/51. Fisheries Research and Development Corporation, Australian Government, and The University of Queensland - Marine Parasitology, Brisbane.: 50.

Li, G.-Q., Wang, W.-L., Huo, Z.-Y., Lu, Y., Hu, H.-Y., 2017. Comparison of UV-LED and low pressure UV for water disinfection: photoreactivation and dark repair of *Escherichia coli*. Water Res. 126, 134–143.

Liltved, H., Cripps, S.J., 1999. Removal of particle-associated bacteria by prefiltration and ultraviolet irradiation. Aquac. Res. 30 (6), 445–450.

Madsen, Lone, Thomasen, Helena H.E., 2015. First detection of *Bonamia ostreae* in native flat oysters from the Limfjord in Denmark. 17th International Conference on Diseases of Fish And Shellfish: Abstract book (pp. 92-92). European Association of Fish Pathologists, O-084.

McLaughlin, S.M., Faisal, M., 1998. *In vitro* propagation of two *Perkinsus* species from the softshell clam *Mya arenaria*. Parasite 5, 341–348.

Mérou, N., Lecadet, C., Pouvreau, S., Arzul, I., 2020. An eDNA/eRNA-based approach to investigate the life cycle of non-cultivable shellfish micro-parasites: the case of

Bonamia ostreae, a parasite of the European flat oyster *Ostrea edulis*. Microb. Biotechnol. 13 (6), 1807–1818.
Mialhe, E., Bachere, E., Chagot, D., Grizel, H., 1988. Isolation and purification of the protozoan *Bonamia ostreae*, a parasite affecting the Flat Oyster *Ostrea edulis* L. Aquaculture 71 (4), 293–299.
Morga, B., Arzul, I., Chollet, B., Renault, T., 2009. Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*. Fish. Shellfish Immunol. 26 (6), 836–842.
Morita, S., Namikoshi, A., Hirata, T., Oguma, K., Katayama, H., Ohgaki, S., Motoyama, N., Fujiwara, M., 2002. Efficacy of UV irradiation in inactivating *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 68 (11), 5387–5393.
Nielsen, P., Vismann, B., 2014. Clearance rate of *Mytilus edulis* (L.) as a function of current velocity and mussel aggregation. J. Shellfish Res. 33 (2), 457–463.
Oguma, K., Katayama, H., Mitani, H., Morita, S., Hirata, T., Ohgaki, S., 2001. Determination of pyrimidine dimers in *Escherichia coli* and *Cryptosporidium parvum* during UV light inactivation, photoreactivation, and dark repair. Appl. Environ. Microbiol. 67 (10), 4630–4637.
OIE , 2019. Infection with Perkinsus marinus. OIE - Manual of diagnostic tests for aquatic animals. Chapter 2.4.6.
Peeler, E.J., Oidtmann, B.C., Midtlyng, P.J., Miossec, L., Gozlan, R.E., 2011. Non-native aquatic animals introductions have driven disease emergence in Europe. Biol. Invasions 13 (6), 1291–1303.
Pichot, Y., Comps, M., Tigé, G., Grizel, H., Rabouin, M.A., 1980. Recherches sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis* L. Rev. Trav. Inst. Pêches Marit. 43 (1), 131–140.
Pilotto, M.R., Souza, D.S.M., Barardi, C.R.M., 2019. Viral uptake and stability in *Crassostrea gigas* oysters during depuration, storage and steaming. Mar. Pollut. Bull. 149, 110524.
Pogoda, B., Brown, J., Hancock, B., Preston, J., Pouvreau, S., Kamermans, P., Sanderson, W., von Nordheim, H., 2019. The Native Oyster Restoration Alliance (NORA) and the Berlin Oyster recommendation: bringing back a key ecosystem engineer by developing and supporting best practice in Europe. Aquat. Living Resour. 32, 13.
Prado-Alvarez, M., Chollet, B., Couraleau, Y., Morga, B., Arzul, I., 2013. Heat Shock Protein 90 of *Bonamia ostreae*: Characterization and possible correlation with infection of the flat oyster, *Ostrea edulis*. J. Eukaryot. Microbiol. 60 (3), 257–266.
Ramilo, A., González, M., Carballal, M.J., Darriba, S., Abollo, E., Villalba, A., 2014. Oyster parasites *Bonamia ostreae* and *B. exitiosa* co-occur in Galicia (NW Spain): spatial distribution and infection dynamics. Dis. Aquat. Org. 110 (1–2), 123–133.
Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Gérard, A., Burreson, E. M., 2000. Haplosporidiosis in the Pacific oyster *Crassostrea gigas* from the French Atlantic coast. Dis. Aquat. Org. 42 (3), 207–214.
Robledo, J.A.F., Nunes, P.A., Cancela, M.L., Vasta, G.R., 2002. Development of an in vitro clonal culture and characterization of the rRNA gene cluster of *Perkinsus atlanticus*, a protistan parasite of the clam *Tapes decussatus*. J. Eukariotic Microbiol. 49, 414–422.
Rochelle, P.A., Upton, S.J., Montelone, B.A., Woods, K., 2005. The response of *Cryptosporidium parvum* to UV light. Trends Parasitol. 21 (2), 81–87.
Sharrer, M.J., Summerfelt, S.T., Bullock, G.L., Gleason, L.E., Taeuber, J., 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. Aquac. Eng. 33 (2), 135–149.
Sunnotel, O., Snelling, W.J., McDonough, N., Browne, L., Moore, J.E., Dooley, J.S.G., Lowery, C.J., 2007. Effectiveness of standard UV depuration at inactivating *Cryptosporidium parvum* recovered from spiked pacific oysters (*Crassostrea gigas*). Appl. Environ. Microbiol. 73 (16), 5083–5087.
Vilas, R., Cao, A., Pardo, B.G., Fernández, S., Villalba, A., Martínez, P., 2011. Very low microsatellite polymorphism and large heterozygote deficits suggest founder effects and cryptic structure in the parasite *Perkinsus olseni*. Infect., Genet. Evol. 11, 904–911.
Villalba, A., Reece, K.S., Ordás, M.C., Casas, S.M., Figueras, A., 2004. Perkinsosis in molluscs: a review. Aquat. Living Resour. 17, 411–432.
Wallace, R.K., Waters, P., Rikard, F.S., 2008. Oyster Hatchery Techniques. Southern Regional Aquaculture Center - SRAC publication,, p. 4302.
Ware, M.W., Augustine, S.A.J., Erisman, D.O., See, M.J., Wymer, L., Hayes, S.L., Dubey, J.P., Villegas, E.N., 2010. Determining UV inactivation of *Toxoplasma gondii* oocysts by using cell culture and a mouse bioassay. Appl. Environ. Microbiol. 76 (15), 5140–5147.
Wedemeyer, G., 1996. Physiology of Fish in Intensive Culture Systems. Springer Science & Business Media.