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Cécile Blanchon, Eve Toulza, Christophe Calvayrac, Stanislaw Eichendorff, Marie-Agnes Travers, et al.. Inactivation of two oyster pathogens by photocatalysis and monitoring of changes in the microbiota of seawater: a case study on Ostreid Herpes Virus 1 μ Var and *Vibrio harveyi*. *Chemosphere*, 2024, 346, pp.140565. hal-04305291

HAL Id: hal-04305291

<https://hal.science/hal-04305291>

Submitted on 24 Nov 2023

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Inactivation of two oyster pathogens by photocatalysis and monitoring of changes in the microbiota of seawater: a case study on *Ostreid Herpes Virus 1* μ Var and *Vibrio harveyi*.

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Abstract

The pollution of seawater by both biotic (bacteria, viruses) and abiotic contaminants (biocides, pharmaceutical residues) frequently leads to economic losses in aquaculture activities mostly mortality events caused by microbial infection. Advanced Oxidation Processes (AOPs) such as heterogeneous photocatalysis allow the removal of all organic contaminants present in water and therefore could reduce production losses in land-based farms. Oysters in land-based farms such as hatcheries and nurseries suffer from a large number of mortality events, resulting in significant losses. If photocatalysis has been widely studied for the decontamination, its application for disinfection is still overlooked, especially on seawater for viruses. We therefore studied seawater disinfection using the photocatalysis (UV₃₆₅/TiO₂) method in the context of Pacific oyster mortality syndrome (POMS). POMS has been defined as a polymicrobial disease involving an initial viral infection with *Ostreid Herpes Virus 1*, accompanied by multiple bacterial infections. We investigated the impact of treatment on *Vibrio harveyi*, a unique opportunistic pathogenic bacterium, and on a complex microbial community reflecting a natural POMS event. Viral inactivation was monitored using experimental infections to determine whether viral particles were still infectious after. Changes in the total bacterial community in seawater were studied by comparing UV₃₆₅/TiO₂ treatment with UV₃₆₅-irradiated seawater and untreated seawater. In the case of OsHV-1, a 2-hour photocatalytic treatment prevents POMS disease and oyster mortality. The same treatment also inactivates 80% of viable *Vibrio harveyi* culture (*c.a.* 1.5 log). Since OsHV-1 and *Vibrio harveyi* are effectively inactivated without long-term destabilization of the total bacterial microbiota in the seawater, photocatalysis appears to be a relevant alternative for disinfecting seawater in land-based oyster beds.

Keywords: OsHV-1 μ Var, *Vibrio harveyi*, seawater microbiota, photocatalysis, oyster pathogens, inactivation.

1. Introduction

Contaminated seawater is one of the biggest sources of economic losses in aquaculture (pisciculture, shellfish farming,...) and fishing activity with approximately 60 millions euros losses in 2014 (SE Tunisia) (El Zrelli et al., 2020). Those losses are related to a fish density decrease in the fishing areas due to ecosystem changes (Hernández-Terrones et al., 2015), and especially the contamination of seawater which can negatively affect fish, shellfish, molluscs and algae (Bado-Nilles et al., 2008; Bayen et al., 2007; Gagnaire et al., 2007, 2006) or the rights of to harvest these organisms. At the same time, more intensive farms have been created but, in those places, the higher animal density enhance disease development. In seafood production, diseases are often caused by bacterial and/or virus infections and various microorganisms (algae, fungi, bacteria), and can induce production losses but sometimes those losses can be attributed to biomolecules as toxins or mycotoxins produced by fungi or algae (Gholami et al., 2019; Radcliffe, 2017; Yanong, 2003). Till date, antibiotics remain the most used method to indulge the bacterial infections in aquaculture but this treatment does not protect against virus or toxins and it enhances emergence of resistance to antibiotics (Pérez-Sánchez et al., 2018; You et al., 2021). To eliminate potential pathogens, disinfection procedures are currently applied in closed-loop aquaculture systems, such as filtration of incoming seawater to 20 microns (Cordier et al., 2020; Whittington et al., 2021), chlorination (Ben-Asher et al., 2019), UVC disinfection (Aguilar et al., 2018; Sharrer et al., 2005), disinfection with peracetic acid (Liu et al., 2016) or hydrogen peroxide (Bögner et

al., 2021; Pedersen and Pedersen, 2012), or ozonation (Davidson et al., 2011). These methods can lead to the production of toxic by-products in the water (Chhetri et al., 2019), are expensive due to chemical and energy costs (Badiola et al., 2018) or do not inactivate toxins of biological origin. Therefore, although these seawater treatments are now being applied in the aquaculture sector, there is still a need to develop alternative seawater disinfection methods. In order to inactivate microbial contaminants present in seawater, as well as toxins and other organic contaminants, advanced oxidation processes (AOPs) (Foster et al., 2011), such as heterogeneous photocatalysis, are one of the most promising solutions. As opposed to homogeneous photocatalysis (ozonation or fenton), these require the use of a solid catalyst, which can be reused, and no toxic by-products are produced during the reaction. Nonetheless, while the use of the photocatalytic oxidation reaction of titanium dioxide (TiO_2) for the elimination of organic biotic contamination in freshwater and wastewater has been widely documented (De Pasquale et al., 2021; Kacem et al., 2015; Liga et al., 2011; Malato et al., 2009; Mayer et al., 2015; Yamada, 2013), it remains largely unexplored in the case of seawater, particularly for bacterial and/or viral inactivation (García-Garay et al., 2020; Levchuk et al., 2022, 2019; Rubio et al., 2013). In addition, it should be pointed out that titanium dioxide (TiO_2) is one of the most interesting catalysts for photocatalysis water treatment applications (Malato et al., 2009), as this compound is inert, can be converted into different forms and allows the production of radicals capable of treating chemical compounds as pharmaceutical or herbicide substances and biotic contaminants such as gram-positive and gram-negative bacteria (Brienza, 2016; Kacem et al., 2014). However, using photocatalysis to treat seawater raises a number of challenges, not least because of the presence of salt and other suspended residues that can slow down the inactivation of targeted pathogens (Blanchon et al., 2023).

In seafood production, aquaculture represents one of the fastest-growing food industries with more than 50% of the worldwide production (Ritchie, 2019). The mollusk aquaculture production is dominated by oyster production which is subject to mortality events at every life stage, induced by many *Vibrio* bacteria. During the larval stage oysters are subject to mortality induced by *Vibrio coralliilyticus* or *Vibrio tubiashii* (Richards et al., 2015). Then, juvenils oysters can be subject to mortality induced by *Vibrio alginolyticus* (Yang et al., 2021), *Vibrio tasmaniensis* or *Vibrio crassostreae*. Once adult, oysters can be subject to mortality induce by *Vibrio aestuarianus* (Mesnil, 2022) or *Vibrio harveyi* (Oyanedel et al., 2023; Saulnier et al., 2010). Production losses in aquaculture can also be caused by the accumulation of human vibrio pathogens on oysters such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* (Campbell et al., 2022), which will result in a ban on sales. The Pacific Oyster Mortality Syndrome (POMS) is one of the most prevalent diseases which can kill up to 100% of oysters spat in France every year (ECOSCOPA, 2020). This disease specifically affecting larvae and juvenile oysters is dependent on multiple environmental factors and microbes (De Lorgeril et al., 2018; Petton et al., 2021). We recently evidenced the course of events leading to oyster death when facing POMS (De Lorgeril et al., 2018). POMS is triggered by an early infection with OshV-1 μ Var herpes virus (*Ostreid herpes virus*) which impairs the oyster antibacterial defenses following the infection of oyster immune cells (hemocytes). This immune suppression induces a dysbiosis that enhances the proliferation of opportunistic bacteria, ultimately causing septicemia and the death of the oyster.

The aim of this study was to investigate the potential of UV₃₆₅/TiO₂ seawater treatment for microbial inactivation in the context of oyster mortality. This article attempts to provide new insight into the impact of heterogeneous photocatalytic treatment on two

major classes of aquaculture pathogens, *Vibrio* bacteria and herpes virus considered separately (*Vibrio harveyi* isolate) and on a complex microbial community composed of herpes virus and bacteria released by diseased oysters. The impact of photocatalytic treatment was systematically compared with that of UV₃₆₅ treatment alone (photolysis), in terms of pathogen survival (viable, cultivable *Vibrio* or virus infectivity during experimental infection) and DNA integrity (DNA quantification).

2. Materials & Methods

2.1. Catalyst and pilot used for seawater disinfection

In this study, TiO₂ in its photosphere form was used (Joannis-Cassan et al., 2021). These are anatase TiO₂-coated glass microspheres centered on a diameter of 45 μm (range of 5 μm to 85 μm) and with a specific surface of 11.5 m².g⁻¹ (Heydari, 2018). Due to their low density (ρ=0.22 g.L⁻¹), they facilitate the separation of the liquid/solid phases by flotation at the end of the treatment process (Reoyo-Prats et al., 2022). Preliminary tests carried out in freshwater during previous work on the decontamination of pharmaceutical targets concluded that a concentration of 5 g.L⁻¹ of photospheres was optimal to capture all the incident radiation. All photons are absorbed across the thickness of the reactor, so transmittance is zero at the bottom. As the presence of salt in the medium has no influence on photon absorption, this concentration was retained in this study (Plantard and Goetz, 2014). The experiments were conducted in 200 mL beakers (60 cm² surface area) containing seawater and catalyst. The suspension was homogenized with a magnetic stirrer and maintained at 19 ± 1 °C by a water bath. During the experiment, the beakers were subjected to generated artificial UV radiation (Vilbert Lourmat lamp, model VL-300 with an emission spectrum from 300 nm to 400 nm

centered at 365 nm). A flux density of 35 W.m^{-2} was applied for 2 hours and checked at the surface of the batch reactor using a UV sensor (UV Radiation, apogee instruments). Experiments were conducted in parallel to evaluate the effects of photolysis. Photolysis experiments were conducted in a beaker with seawater without a catalyst, maintained at a flux density of 35 W.m^{-2} .

2.2. Disinfection of a bacterial pathogen

2.2.1. Bacterial pathogen: *Vibrio harveyi*

An oyster pathogen, *Vibrio harveyi* Th15_O_G11 modified with a stable Green Fluorescent Protein (GFP) plasmid pFD086 (GFP, TrimR) (Oyanedel et al., 2023) was used to study the disinfection of a single pathogen. This bacterium was cultured in Zobell medium (4 g.L⁻¹ bactopectone, 1 g.L⁻¹ yeast extract in sterile seawater, pH 7.4) with $10 \mu\text{g.mL}^{-1}$ of trimethoprim (T7883, Sigma Aldrich, France) in order to maintain the GFP plasmid at 20 °C until the stationary phase was reached. Then filtered natural seawater (Banyuls, France, seawater filtered at 1 μm , pH 8 and salinity of 35 PSU) was inoculated to reach $10^7 \text{ CFU}/100\text{mL}$ at the beginning of the photocatalysis and photolysis treatments (Supplementary 1). The inoculation was performed directly without separating bacterial cells from the culture media. To avoid contamination of seawater by cultivable bacteria, negative control was carried out by spreading 200 μl on Marine Agar plate before spiking the seawater with *Vibrio harveyi*.

2.2.2. Monitoring

Disinfection was monitored by the enumeration of cultivable bacteria. The culture media was Marine Agar (Marine Broth medium with 15 g.L^{-1} of agar, 76448, Sigma Aldrich, France) with $10 \mu\text{g.mL}^{-1}$ of trimethoprim and all counting were performed in triplicate by spreading 200 μl of treated seawater. This method allows a detection limit of $10^4 \text{ cfu}/100\text{mL}$, corresponding to

99.99% of inactivation (*i.e.* 3 log). The *Vibrio* samples were incubated at 20 °C for 48 h until counting (Supplementary 1). In addition to the trimethoprim screen, the fluorescence of the colonies was checked at 395 nm (Lepro Lampe Torche UV LED) before cultivable bacteria enumeration. The bacterial inactivation curves connected to the seawater disinfection by photocatalysis and photolysis were compared using *Pairwise-t-test* with Benjamini & Hochberg adjusting method, and *Tukey multiple pairwise-comparisons*. Then the disinfection efficiency was estimated by fitting the experimental point to a first-order kinetic model (Equation 1). In this law, the UV dose (Equation 2) was expressed as the quantity of energy received corrected by volume of water in the reactor as for solar treatment (Plantard and Goetz, 2012). It allows to consider the reactor geometry with the irradiated surface / volume of the reactor ratio.

$$\ln\left(\frac{C}{C_0}\right) = -\alpha \cdot Q_{(t)} \quad \text{equation 1}$$

first-order kinetic equation with C and C₀ respectively the bacteria concentration at the sampling time and at the beginning of the experiment in UFC/100mL, Q_(t) the UV dose accumulated since the start of the experiment in kJ.L⁻¹ (equation 2), and α the first order kinetic constant.

$$Q_{(t)} = \frac{S_{irradiated}}{V_{total}} \cdot I \cdot t \quad \text{equation 2}$$

UV dose accumulated since the start of the experiment in kJ.L⁻¹, with S_{irradiated} the irradiated surface of the reactor in m², V_{total} the total volume of the reactor in L and I the flux density received in W.m⁻².

2.3. Disinfection of a seawater microbial complex community

2.3.1. Production of contaminated seawater

The microbial community tested in this work was composed of seawater enriched in virus and bacteria obtained following the experimental infection of juvenile oysters by OsHV-1 μVar, the inducer of the POMS disease. Susceptible pathogen-free juvenile oysters F14V (De Lorgeril et al., 2018) aged of 3.5 months were injected in the adductor muscle with 4.10⁸ Genomic

Unity (UG) of OsHV-1 μ Var virus (injection of 50 μ l of F14-TFF suspension at $7 \cdot 10^6$ UG. μ L⁻¹). 30 *Crassostrea gigas* oysters spats (mean weight 3 g) were put in a 1 L tank to reach an animal density of 90 g of biomass/L. Oysters were kept in oxygenated seawater during 24 h at 21 °C by controlling the room temperature. The contaminated seawater (CSW) produced was directly used for photocatalysis and photolysis treatments (Figure 1a). During experiments, virus inactivation and seawater bacterial microbiota inactivation were analyzed separately.

2.3.2. Oyster mortality monitoring

During the seawater disinfection process, seawater was sampled and filtered at 0.2 μ m (to eliminate bacteria) before injection on oysters. Susceptible pathogen-free juvenile oysters (family F14V aged 3.5 months) initially pathogen-free were injected in the adductor muscle either with 50 μ l of contaminated seawater treated using photocatalysis (UV₃₆₅/TiO₂) or photolysis (UV₃₆₅ alone) or not treated (kept to obscurity) (figure 1a and figure 1b). In addition, non-injected oysters were used as infection control and no mortality occurred in this group during the trial. As previous experiments (Supplementary 2) did not show viral adhesion on TiO₂ catalysis, no adhesion control was performed in this study. Four tank replicates were used with 10 oysters (mean weight of 3g) / 500 mL tanks to reach an animal density of 60 g of biomass/L. Then oysters were put in aerated seawater at 21 °C. Oyster mortality was monitored twice a day for 10 days. Oysters were not fed during the 10 days of monitoring and day/night (10h/14h) cycles were performed.

The nonparametric estimates of the survival function were computed according to Kaplan & Meier (survfit, {survival}) (Therneau, 2023). Survival time started when oysters were injected with treated seawater and stopped after 10 days, when no additional mortality has been observed in the past 48h. The data was read as the number of dead animals within each

condition on each day, the tanks replicated for each condition were considered as one tank for the survival analysis (i.e. $n = 40$ per condition). Survival curves of exposed oysters were plotted and compared among the treatments and their duration. The survival time curves of oysters connected to the seawater disinfection by photocatalysis and photolysis were compared using the Cox regression model (`coxph` and `cox.zph`, {survival}) (Therneau, 2023) and the Log-Rank regression model (`survdiff`, {survival}) (Therneau, 2023). To evaluate viral inactivation, the time required to reach 50% oyster mortality for each treatment (TL50) was calculated from the survival curves. This corresponds to the post-injection time at which 50% of the oysters in the condition have died.

2.3.3. DNA extraction

All seawater samples were stored at $-80\text{ }^{\circ}\text{C}$ until the DNA was extracted. The total DNA of seawater samples was extracted using the blood protocol from Machrey-Nagel NucleoSpin tissue kit (reference 740952). The extractions were realized on $200\text{ }\mu\text{l}$ of seawater with a final elution volume of $30\text{ }\mu\text{l}$. In order to improve DNA extractions, a crushing step of 12 min mechanical lysis with zirconium beads followed by an RNase step was added before the DNA binding step. The DNA extracts were stored at -20°C before analysis.

2.3.4. Metabarcoding analysis

The 16S rRNA gene of bacterial communities was amplified using the variable V3-V4 loops (341F / 805R). Paired-end sequencing was performed on the MiSeq system (Illumina) at the University of Perpignan Via Domitia (Bio-Environment platform, Perpignan University, France). Sequencing data are available at the NCBI-SRA under the accession number PRJNA987558. The microbiome bioinformatics analyses were performed with *QIIME 2 2022.8* pipeline to generate Amplicon Sequence Variant (ASV) (Bolyen et al., 2019). Sequence data

was already demultiplexed so we directly denoised it with *DADA2* using the *---p-pooling-method* and *-p-chimera-method* parameters to pool the sequence between sample for the chimers elimination and the ASV creation. Then the taxonomy affiliation was performed on ASV using a Silva 138 V3V4 classifier (classifier train with the used primers). The phylogenetic tree was created with *FastTree*, using the *mafft* program. After creating the phylogenetic tree, the taxonomic assignment and the ASVs, all the statistical analyses were done using R v4.2.1 (2022-06-23) (Supplementary 3).

Samples were normalized using the rarefaction method before any statistical analyses at a sample depth of 10 301. Principal coordinate analysis (ordinate, {phyloseq}) (McMurdie and Holmes, 2013) was computed to describe compositions of microbial communities between samples using Bray-Curtis dissimilarities (distance, {phyloseq}) (McMurdie and Holmes, 2013). The impact of seawater treatment on seawater microbiota was tested using ANOVA (anova, {stats}) (R Core Team, 2022) on the Constrained Analysis of Principal coordinates calculated with the Bray-Curtis distance (capscale, {vegan}) (Oksanen et al., 2022). Log Fold Change analysis was performed to analyze changes on bacteria abundance between treatment and control (DESeq, {DESeq2}) (Love et al., 2014) using a adjusted p-value at 0.05. For all analysis, the threshold significance level was set at 0.05.

2.3.5. Quantification of bacteria and virus

Quantifications of OsHV-1 μ Var, total bacteria 16S rDNA and total *Vibrio* 16S rDNA were performed using quantitative PCR (qPCR) with a Roche LightCycler 480 thermocycler (Bio-Environment platform, University of Perpignan, France). The total qPCR reaction volume was 10 μ l including 2.5 μ l of pure DNA extract, 1 μ l of each PCR primer at 5 μ M and 5 μ l of SYBER

Green Takyon Master Mix at 2X (Eurogentec SA). OsHV-1 μ Var specific primer pairs targeted a DNA polymerase catalytic subunit (named DP or ORF100): 5'-ATTGATGATGTGGATAATCTGTG-3' and 5'-GGTAAATACCATTGGTCTTGTCC-3' (Webb et al., 2007). The total bacteria quantification used the same specific primer pairs as for the metabarcoding analysis (see above). The specific primers used for the total *Vibrio* quantifications targeted 567F/680R region of the 16S rDNA: 5'-GGCGTAAAGCGCATGCAGGT-3' and 5'-GAAATTCTACCCCCCTCTACAG-3' (Mansergh and Zehr, 2014). qPCR reactions were performed in triplicate with the following program: 95°C for 3min, followed by 40 cycles of denaturation (95 °C, 10 s), hybridation (60 °C, 20 s) and elongation (72 °C, 30 s). The absolute quantification of the OsHV-1 μ Var DNA copies in the seawater was carried out by comparison of Cp value of sample to Cp value of a standard curve (obtained from a plasmid of the DNA polymerase catalytic subunit) according to Lafont et al. 2017 (Lafont et al., 2017). The results were expressed as the number of OsHV-1 μ Var DNA copies per mL of seawater (UG/mL). The absolute quantification of the total bacteria and total *Vibrio* in the seawater was carried out by comparison of Cp value of sample to Cp value of a standard curve obtained from *Vibrio* genomic DNA of known concentration. The results were expressed as the number of *Vibrio* DNA copies per mL of seawater (CP/mL). The DNA concentration connected to the seawater disinfection by photocatalysis and photolysis was compared using *Pairwise-t-test* with Benjamini & Hochberg adjusting method, and *Tukey multiple pairwise-comparisons*.

3. Results and discussion

3.1. Inactivation of the oyster pathogen *Vibrio harveyi*

The efficiency of photocatalysis and photolysis treatments to inactivate simple and complex microbial communities was evaluated by testing in water either a bacterial pathogen alone or the μ Var OsHV-1 virus present in a complex community composed of seawater microbiota. We began by assessing the inactivation by photocatalysis and photolysis of *V. harveyi*, a bacterium pathogenic to oysters in seawater.

Briefly, sterile seawater was artificially contaminated with *V. harveyi* population in stationary phase before applying photocatalysis and photolysis treatments. Two disinfection experiments were carried out to achieve various levels of seawater decontamination. The first one was a 2 h photocatalytic treatment (UV₃₆₅ associated with TiO₂ catalyst) and the second one was a photolytic treatment (only UV₃₆₅ irradiation without catalyst). The bacterial inactivation was followed by counting the cultivable bacteria during treatment. Preliminary work (Supplementary 4) on the adhesion of *V. harveyi* has shown that under our experimental conditions of pH and salinity, adhesion to the catalyst was negligible in comparison with the duration of the treatment experiments. The inactivation results of *V. harveyi* in seawater are presented as a function of the cumulative UV₃₆₅ dose ($Q_{(t)}$) versus the normalized survival ratio (Figure 2). The raw data was fitted by a first-order model (with or without shoulder) to calculate the constant rate k . Only the UV₃₆₅ treatment exhibited a shoulder in the first 15 minutes of treatment corresponding to a UV₃₆₅ dose of 1 kJ.L⁻¹ and even if authors have already reported evidence of a photoreactivation mechanism of *Vibrio* in freshwater (Das et al., 1981) and in marine water (García-Garay et al., 2020; Moreno-Andrés et al., 2023), no such observation had been observed in our study. The inactivation experiment (figure 2) showed that photocatalysis treatment was much more effective than photolysis in inactivating *Vibrio* cells. Where only 30% (or 0.2 log) of the *bacterium* were inactivated by 2 h of UV₃₆₅ irradiation alone ($\alpha = 0.046 \pm 0.004 \text{ L.kJ}^{-1}$; $R^2 = 0.98$) corresponding to a UV₃₆₅ dose

of 8 kJ.L^{-1} (or $25\,000 \text{ mJ.cm}^{-2}$), almost 80% (or 0.7 log) were inactivated in the presence of TiO_2 catalyst ($\alpha = 0.218 \pm 0.017 \text{ L.kJ}^{-1}$; $R^2 = 0.97$). Most of the photocatalysis disinfection experiments found in the literature ran UVC sources (254 nm) (Rubio et al., 2013; Wu et al., 2011) which have a higher bacteria inactivation power than UVA (365 nm) (Kim et al., 2013) so a higher inactivation rate. Most of those studies compare efficiency of treatment by UV alone or with a catalyst and always found that photocatalysis treatments are the most efficient processes. The UV dose required to obtain the desired level of disinfection has a direct impact on the cost of disinfection (Bogdan et al., 2015). It is therefore valuable to estimate the ratio between the UV dose required for photocatalysis and photolysis to obtain the same level of disinfection. Rubio et al. (2013) reported a value of 38.6 mJ.cm^{-2} to achieve 99.9% disinfection of *Alteromonas sp.* with UV_{254} alone against 25.9 mJ.cm^{-2} with $\text{UV}_{254}/\text{TiO}_2$ which reduces the amount of UV needed by 1.5 (Rubio et al., 2013). In our study, a UV_{365} dose of $25\,000 \text{ mJ.cm}^{-2}$ (8 kJ.L^{-1}) was needed to achieve 30% of the *Vibrio harveyi* inactivation with UV_{365} alone against $3\,000 \text{ mJ.cm}^{-2}$ (1 kJ.L^{-1}) with TiO_2 , allowing to drastically reduce the amount of UV_{365} needed (by 8). As shown for *Vibrio alginolyticus* which is highly resistant to UV_{365} photolysis (UV dose greater than 18 J.cm^{-2} for less than 0.5 log inactivation) (Moreno-Andrés et al., 2023), *V. harveyi* strain tested in our study does not appear to be UV_{365} sensitive as it was shown by the kinetic curves (Figure 2). Indeed, it has already been observed that autochthonous bacteria such as *Vibrio spp.* (*Vibrio owensii*, *Vibrio alfacensis* and *V. harveyi*) in seawater are more resistant to inactivation by SODIS than non-indigenous bacteria such as *E. coli*, *T. coliforms* and *Enterococci* in contaminated drinking water (Levchuk et al., 2019). It is difficult to compare these results with our study, especially when crucial parameters as experimental conditions, bacteria concentration, radiation type and photocatalyst are different. However, given that the light intensity was 48 W.m^{-2} , we can estimate that the UV

dose required for *Vibrio spp.* inactivation obtained in the study by Levchuk, et al. (2019) under UVA and SODIS was greater than that found in the present study.

Given the good efficiency of the photocatalytic treatment obtained under our experimental conditions, it may be considered that this type of process could be applied to the disinfection of aquaculture influents and effluents (Levchuk et al., 2022, 2019; Moreno-Andrés et al., 2020; Villar-Navarro et al., 2019) or ballast water (Moreno-Andrés et al., 2017), provided that further studies on the inactivation of other aquaculture pathogens are carried out.

3.2. Inactivation of a complex microbial community

3.2.1. Effect on OsHV-1 μ Var infectivity

Complex microbial community composed of OsHV-1 μ Var virus associated with a marine bacterial community was treated by photocatalysis and photolysis processes. The treated or untreated seawater was filtered before injection into the oysters, to eliminate the bacterial fraction (Figure 1). Briefly, the contaminated seawater infectivity was followed by evaluating the mortality in oysters, and monitoring of the viral DNA in seawater was also assayed. Three disinfection experiments were carried out to obtain various levels of virus contaminated seawater. The first two experiments involved a 2-hour treatment, one by photocatalysis and the other by photolysis (Figure 1), to achieve a final UV dose of 8 kJ.L^{-1} . A control that had not been subjected to any treatment and had been kept protected from radiation for 2 hours was used. Next, virus-susceptible oysters were injected with the treated or not contaminated seawater from the 3 disinfection experiments. For each treatment, 40 oysters were injected with treated or not treated seawater for 0, 0.5, 1 or 2 hours (respectively 0, 2, 4 and 8 kJ.L^{-1}) and then mortality was followed to assess the suitability of the treatments (Figure 1).

The Kaplan-Meier survival curves thus obtained (Figures 3) allowed us to visualize the survival rates and the efficiency of photocatalysis and photolysis treatment to inactivate OsHV-1 μ Var virus. A better oyster survival rate was directly correlated with better virus inactivation (with a lower quantity of virus still infectious). The oyster mortality is caused directly by the injection of still-infectious virus as verified using qPCR on oysters. As showed by blue curve, mortality occurred during the first 80 h on oysters injected with non-treated seawater whereas it started later and to a less rapid rate for oysters injected with treated seawater until there was no mortality at all (see curves in red and green and purple). On one hand, the mortality rate of oysters for the control seawater (seawater without treatment at 0 h and after 2 h) reached 100% (Figure 3a). All the oysters died quickly after 80 hours of monitoring, and no oysters were left alive in the tanks. We also note that the presence of the TiO_2 catalyst does not seem to have a quantifiable impact on the survival rate, indicating that the catalyst alone does not affect the virus and does not induce direct mortality of the oysters (figure 3a). On the other hand, the survival rates of oysters injected with treated seawater was significantly improved (Figure 3b and Figure 3c) and the longer the treatment was, the better the survival rate was. As indicated by statistical analysis (Cox test, p-value = $6e-16$ for UV_{365} treatment vs. control and p-value $< 2e-16$ for $\text{UV}_{365}/\text{TiO}_2$ treatment vs. control), both treatments are significantly different from the control condition. For both oysters injected with seawater treated for long time by photolysis and photocatalysis (1 or 2 hours) part of those oysters were still alive after 10 days (20% to 100% of survival proportion) (Figure 3b, Figure 3c). Photocatalysis ($\text{UV}_{365}/\text{TiO}_2$) was efficient to inactivate viruses, same as, photolysis (UV_{365} alone) alone also decreased virus infectivity but to a lesser extent. Calculating the time needed to achieve 50% oyster mortality for each treatment (TL_{50}) shows that virus inactivation by photocatalysis is twice as effective as by photolysis. In fact, it takes half as long

to achieve 50% mortality in oysters injected with CSW treated for 1 hour by photolysis (96h) as by photocatalysis (192h). Indeed, 2 hours of treatment by photocatalysis of the contaminated seawater allows sufficient inactivation of OshV-1 μ Var to have 100% survival of the oysters whereas exposure to UV₃₆₅ alone only allows a 50% reduction in final oyster mortality (Figure 3b and Figure 3c).

Viral inactivation by photocatalytic process consists in three steps: (1) the viral particle shape distortion, (2) the protein oxidation, and (3) the DNA leakage and damage to the genetic material (Zhang et al., 2019). Quantification of viral DNA (Supplementary 5a) showed that DNA degradation by photocatalytic treatment was not essential to effectively reduce viral infectivity and halt oyster mortality. We observed that the amount of viral DNA present in UV₃₆₅-treated seawater was quite similar to that measured in UV₃₆₅/TiO₂-treated seawater (respectively for UV₃₆₅/TiO₂ and UV₃₆₅ 2h-treatment, $4.23e+03 \pm 1.35e+03$ UG/ μ L and $1.11e+04 \pm 4.47e+03$ UG/ μ L). However, we also observed that oyster mortality was lower for oysters injected with UV₃₆₅/TiO₂-treated seawater than with UV₃₆₅-treated seawater alone. These results are consistent with the data of Bounty et al. (2012) (Bounty et al., 2012) who showed that the increase in viral inactivation by photocatalysis *versus* photolysis was not due to an increase of DNA damages. The 2 hours treatment reduced the OshV-1 μ Var DNA concentration for photolysis treatment and photocatalysis by 95 to 98% respectively whereas in seawater kept out of any UV sources the OshV-1 μ Var DNA was only reduced by 30% (degradation of free DNA during the 2h at obscurity). Several authors have already reported that virus sensibility to photo-oxidative treatment depends on the capsid composition and the nature of the genetic material (Mayer et al., 2015). The OshV-1 μ Var virus is an herpesvirus composed of double-stranded DNA (dsDNA) encapsulated in an enveloped icosahedral capsid of 116 nm diameter (Adamson, 2006; Davison et al., 2005). The virus size,

both in terms of diameter and genome length, plays a role in the virus inactivation by photocatalysis (Mayer et al., 2015), as well as the presence of an enveloped capsid (Bogdan et al., 2015; Mayer et al., 2015; Nakano et al., 2013). The addition of TiO₂ catalyst (the photocatalysis treatment vs photolysis one) enhances the viral inactivation without inducing more DNA degradation but could have target the viral envelope, increasing the reduction of the infectivity.

3.2.2. Effect on a complex *pathobiota* (virus and bacteria)

Both experiments showed that microbial contaminants were efficiently inactivated by the treatment, especially the photocatalytic one. The DNA quantifications of both total *Vibrio* and total bacteria highlighted that DNA was not destroyed by the 2h of treatment while more than 95% of the OsHV-1 μ Var DNA was destroyed by the same treatment (Supplementary 5a). In freshwater some authors have already reported that viruses were the most susceptible organisms to Advanced Oxidative Processes (AOPs) such as photocatalysis (Laxma Reddy et al., 2017; Zheng et al., 2018). This observation seems to be verified both in terms of DNA quantification and in terms of virus infectivity or cultivable GFP *V. harveyi* quantification. The OsHV-1 μ Var infectivity does not induce any oyster mortality after the 2h of photocatalytic treatment whereas only 80% of cultivable GFP *V. harveyi* were inactivated. In addition, the photolytic treatment was less efficient than the photocatalytic one both the OsHV-1 μ Var inactivation and the *V. harveyi* inactivation. The use of TiO₂ with the UV treatment enhanced the formation of OH radical which improved the seawater disinfection but that was not linked to a higher level of DNA damage since the DNA quantification was the same between photolysis and photocatalysis experiments. A better oyster survival rate is directly correlated with better virus inactivation (with a lower quantity of virus still infectious). However, this

reduction in the number of infectious viruses was not correlated with the viral DNA load detected. In fact, the viral DNA load remain the same for photolysis-treated and photocatalysis-treated seawater samples, whereas the infectivity of photocatalysis-treated seawater was lower than that of photolysis-treated seawater. Photocatalytic treatment therefore appears to inactivate viral particles without destroying their DNA. The same observation was already done on virus by Bounty et al. (2012) with adenovirus inactivation in freshwater by UV₂₅₄/H₂O₂ advanced oxidation (Bounty et al., 2012) and by Park et al. (2016) with murine norovirus in solidified agar matrix by UV/TiO₂ processes (Park et al., 2016). On bacteria, Kim et al. (2013) showed that the use of TiO₂ with UV₃₅₂ or UV₃₀₆ treatment induces more damage to DNA than the UV alone including the plasmid DNA linearization (Kim et al., 2013).

3.2.3. Effects of the photocatalysis process on the seawater bacterial community

3.2.3.1. *Microbiota change was linked to seawater treatments*

As the POMS is triggered by an OsHV-1 μ Var oysters infection causing immune-suppression inducing a fatal bacteraemia (Petton et al., 2021), we also analyzed the effect of treatment on whole seawater bacterial communities using the 16S rRNA gene sequencing from samples taken before and during the contaminated seawater treatment (0, 0.5, 1 and 2h). In parallel with the analysis of the total bacteria microbiota analysis, the total bacteria and *Vibrio* loads were quantified using qPCR. All the analyses presented in this section were carried out on contaminated seawater without water filtration, containing both bacteria and viruses (Figure 1).

To investigate the effect of treatment on the dynamics of bacterial microbiota in contaminated seawater, we analyzed total bacterial communities using 16S rDNA metabarcoding during (0.5, 1 and 2 hours) and after the treatment (4, 7 and 10 days). The normalization to a depth of 10 301 sequences per sample allowed us to keep 1 014 ASVs. Given that the total composition of the microbiota is likely to evolve between the end of treatment and the various post-treatment points, we decided to analyze photocatalysis and photolysis effects during and after exposition separately. The principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities computed on samples obtained during the treatment highlighted that seawater microbiota changed between the two treatments and the control (p -value = $7e-04$ for *capscale ANOVA*) (Figure 4) but no significant differences were observed on total bacteria loads between treatments and control (p -value > 0.05 for both *Pairwise-t-test* and *Tukey multiple pairwise-comparisons*) (Supplementary 5b). Photocatalysis-treated samples cluster further apart on the figure 4, revealing that the microbiota composition of photocatalysis-treated CSWs is more affected than that of photolysis-treated CSWs. Furthermore, the longer the treatment, the more important the effect on photocatalysis-treated CSWs. The same taxa were found (Figure 5a), but the proportion of each bacterial genus changed between the UV₃₆₅ treatment, the UV₃₆₅/TiO₂ treatment and the control (seawater kept in the dark during treatment). In the control and UV₃₆₅-treated samples, the relative proportion of bacteria did not change over time, whereas treatment duration had an impact on the relative proportion of bacteria for the photocatalysis treatment (Figure 5a). The DESeq2 analysis allowed us to identify genus significantly modified by the treatment applied by calculating the log₂ fold change value. In UV₃₆₅/TiO₂-treated seawater, ASVs affiliated to the genera *Aesturiibacter* and *Vibrio* were found to be more abundant than in control seawater (log₂ fold change > 0 with adjusted p -

value < 0.05), while *Croceibacter* and *Pseudophaeobacter* were less abundant (\log_2 fold change < 0 with adjusted p-value < 0.05). (Figure 5b). In seawater treated by UV₃₆₅ alone, only *Pseudoalteromonas* genus was modified and was more abundant in UV₃₆₅ treated seawater than in control seawater (Figure 5b). Although the \log_2 fold change shows a variation in *Vibrio* bacteria in the UV₃₆₅/TiO₂ treated seawater compared to the untreated one, the quantification of total *Vibrio* did not show any significant changes (Supplementary 5c). Bacteria belonging to the *Vibrio* and *Pseudoalteromonas* genera have been shown to be the opportunistic strains associated with POMS disease (Petton et al., 2021). More specifically, numerous *Vibrio* species are involved in oysters production losses both by inducing mortality in oysters (Kim et al., 2013; Oyanedel et al., 2023) or by making them not consumable (Froelich and Noble, 2016). These results are consistent with data found in the literature concerning Vibrios associated with POMS disease during oyster life stages and in particular *i*) during the spat or larval stage with *V. anguillarum*, *V. alginolyticus*, *V. tubiashii*, *V. ostreicida*, *V. coralliilyticus*, *V. neptunius*, *V. bivalvicida* and other *Vibrios* of the *Splendidus* clade (Richards et al., 2015), *ii*) during the juvenile stage with *Vibrio* of the *Splendidus* clade such as *V. splendidus*, *V. tasmaniensis*, *V. cyclitrophicus* or *V. crassostreae* in POMS mortality events (Bruto et al., 2017; De Lorgeril et al., 2018; Lemire et al., 2015) and *V. harveyi* and *iii*) once adult by *V. aestuarianus* (Garnier et al., 2008; Travers et al., 2017).

In conclusion, the 16S rDNA barcoding analyses of the *Vibrio* genera did not allow to show any pattern in the change of the global proportion of the *Vibrio* affiliated ASVs according to the oxidative treatment type or to the treatment duration (data no show). Nevertheless, the taxonomic resolution of the marker used in this study did not provide a reliable identification of the different species within the *Vibrio* genus.

3.2.3.2. Assessment of microbiota resilience at the end of photocatalysis treatment

After the end of the treatment and once the TiO₂ was fully removed by flotation from the seawater, the microbiota shifted towards that of the one of control seawater (Figure 6a). Some bacteria genus remains nevertheless with abundances significantly different from those of the control seawater (Figure 6b). After the catalysis removal, the *BD7-11* and *Roseibacillus* bacteria remain more abundant in UV₃₆₅/TiO₂ treated seawater than in the control seawater (\log_2 fold change > 0) whereas *VadinHA49* bacteria were less abundant. The same trend was observed for UV₃₆₅ treated seawater where *Roseibacillus* bacteria were also more abundant than in the control. Even if some genus showed significant abundance differences, no correlation was observed between seawater bacterial composition and the treatment applied a few days after the end of photo-oxidative treatments (p -value = 0.9725 > 0.05 for *capscale ANOVA*). The total 16S rDNA bacteria quantification was also the same after treatment between treated seawater and the reference seawater (Supplementary 5b). Thus, 10 days after the treatment, the total bacterial community was resilient both in term of quantity and quality by comparison to the control. In the present study, disinfection was not total, and it is quite conceivable that the part of the microbiota still viable after treatment could grow again, restoring a bacterial microbiota closer to that of untreated water. As previously mentioned by Falet et al. (2022), the resilience observed here is an imperative requirement for aquaculture, as pathogen removal must not alter the entire seawater microbiota in order to limit the impact on shellfish and fish development (Fallet et al., 2022).

4. Conclusion

The aim of this study was to apply a photocatalysis process to seawater, either using UV₃₆₅ treatment alone, or using a combination of UV₃₆₅ and titanium dioxide photospheres, in order to assess the microbial inactivation. The feasibility of viral and bacterial inactivation in a context of aquaculture influent and effluent disinfection was investigated as well as the effect

of such treatment on the global bacterial community. The main conclusion of this study is that photo-oxidizing treatments effectively inactivate the *OsHV-1* μ Var virus and the *V. harveyi* bacterium, both considered to be the main pathogens of oysters. More specifically the photocatalysis treatment (UV₃₆₅/TiO₂) was more efficient in inactivating pathogens without inducing more DNA degradation. In addition, our results suggest that treatment by photocatalysis may be a promising technique for the biosecurity of land-based aquaculture facilities, as they demonstrate that under 2-hour treatment conditions, disinfection of μ Var OsHV-1 by photocatalysis was effective in completely inactivating the virus and achieving a 100% survival rate of injected oysters, without observing the installation of POMS. Finally, although the composition of the total bacterial community in seawater changes during photo-oxidative treatment, we nevertheless observed a resilience phenomenon in the seawater microbiota 10 days after treatment, as it recovered in terms of quantity and composition. In the perspective of future application for seawater disinfection, it will be necessary to go a step further by looking at the abiotic compartment and evaluate the potential production of toxic compounds in water. Further studies on microbial inactivation involving different flux densities are underway in the laboratory to model photocatalytic inactivation and propose a short- to medium-term solar photocatalytic solution for land-based aquaculture facilities such as hatcheries or oyster nurseries.

Acknowledgements

We would like to express our sincere gratitude to all the individuals and organizations that have contributed to the publication of this research paper. First, we would like to thank Virginie Le Razavet and Bruno Petton, from the IFREMER Bouin Marine Mollusc Experimental

Platform, for providing us the oysters used for experimental infection in this study. We are also grateful to Betty Faliex and Gael Simon, from the CEFREM laboratory at the Perpignan University, for providing us a place to experiment on oysters. We would also like to thank Pascal Romans and Nancy Trouillard, from the Oceanographic Observatory of Banyuls, for providing us the seawater used for all the experiments. Finally, we would like to thank all the participants in this study for their time and willingness to share their experiences, thanks to Jean-François Allienne from the IHPE and Margot Doberva from the Perpignan University Bioenvironment Platform.

This work received support from the French National Research Agency (ANR) under grant number ANR-10-LABX-22-01-SOLSTICE, the Région Occitanie (PHOTODEPOL, N°19015248), and the University of Perpignan Via Domitia through the Energy Environment Research Federation (FREE) under projects “Lagoon Ecosystem Health and Remediation” and “PHOTOMIC” of the axis “Sustainable processes, management and ecosystem health”. This study is set within the framework of the “Laboratoire d’Excellence (LABEX)” TULIP (ANR-10-LABX-41). This work was also funded by the doctoral school Énergie - Environnement (ED305), University of Perpignan Via Domitia, France.

Figures

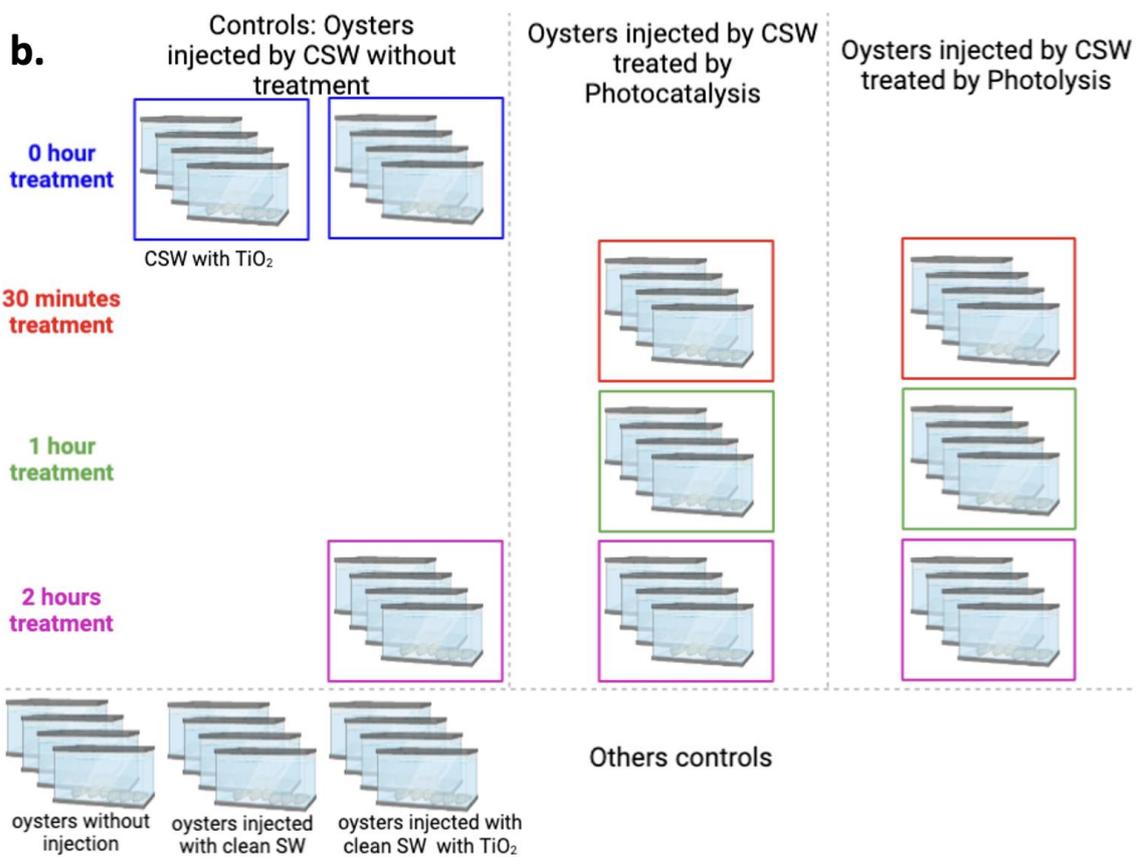
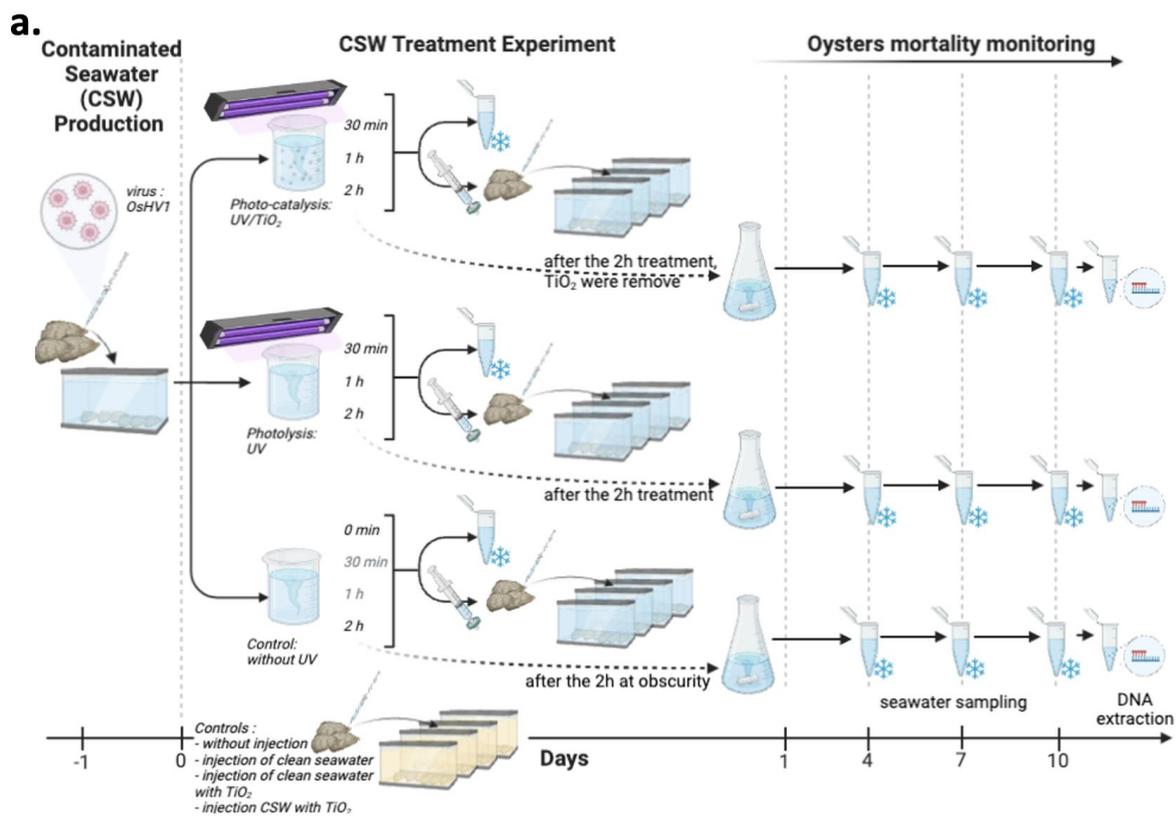


Figure 1: (a) experimental design for the OshV-1 μ Var virus and seawater total bacterial community inactivation with (b) the detail of oyster injections. DNA extracts were used for the barcoding analysis and also for the total bacteria, total *Vibrio* bacteria and OshV-1 μ Var quantification by qPCR.

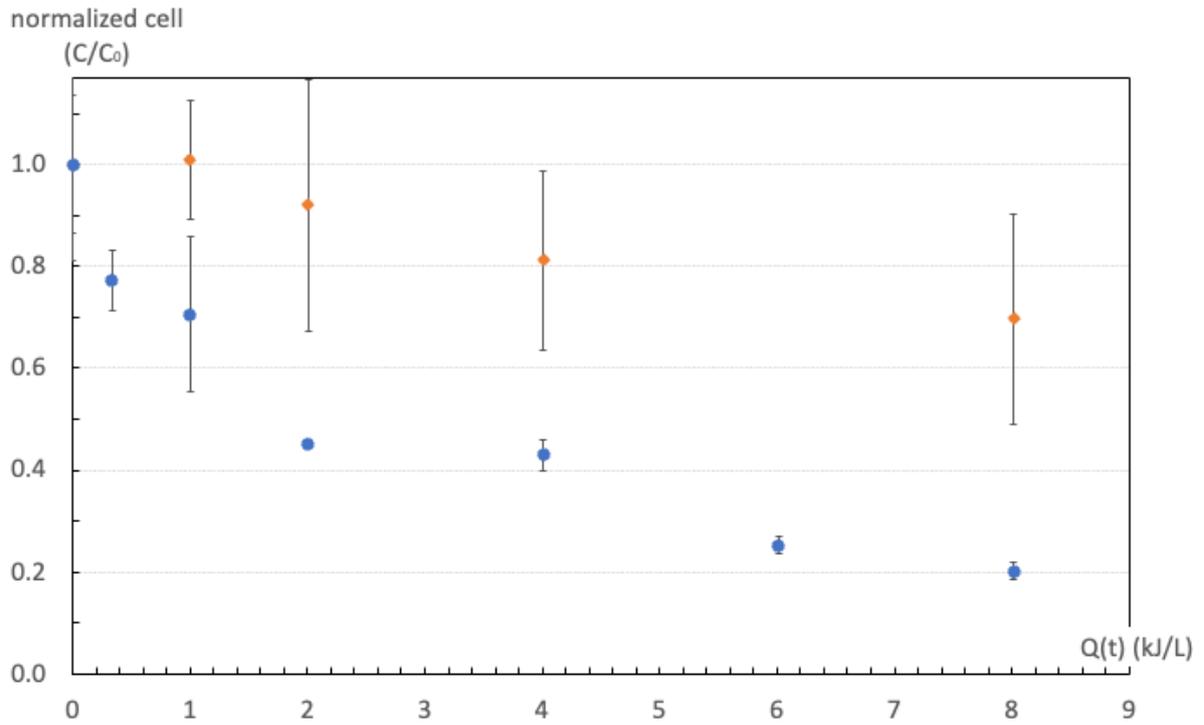
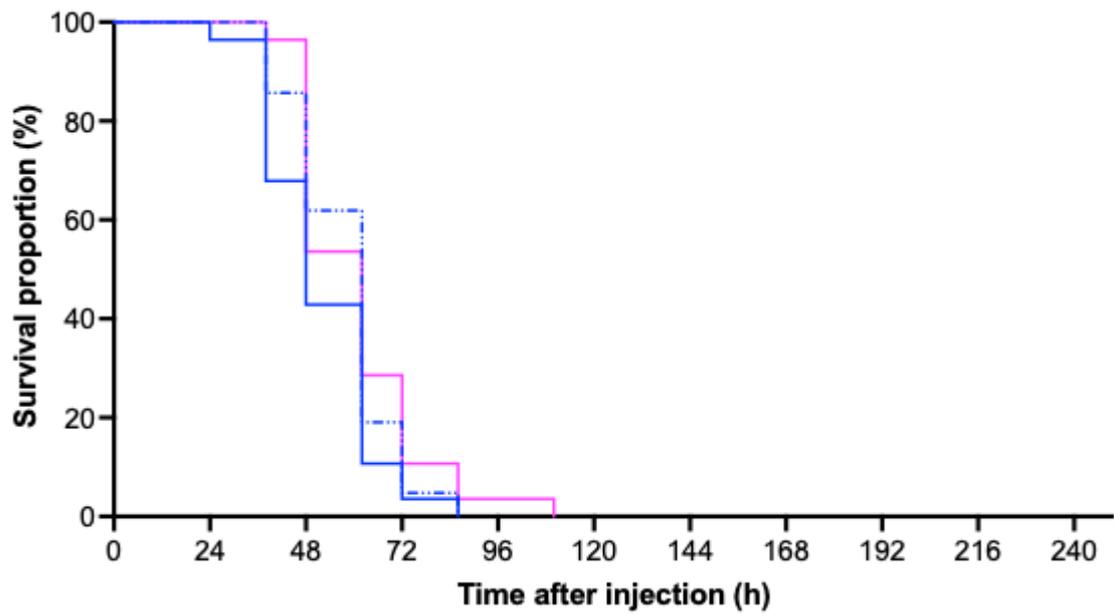
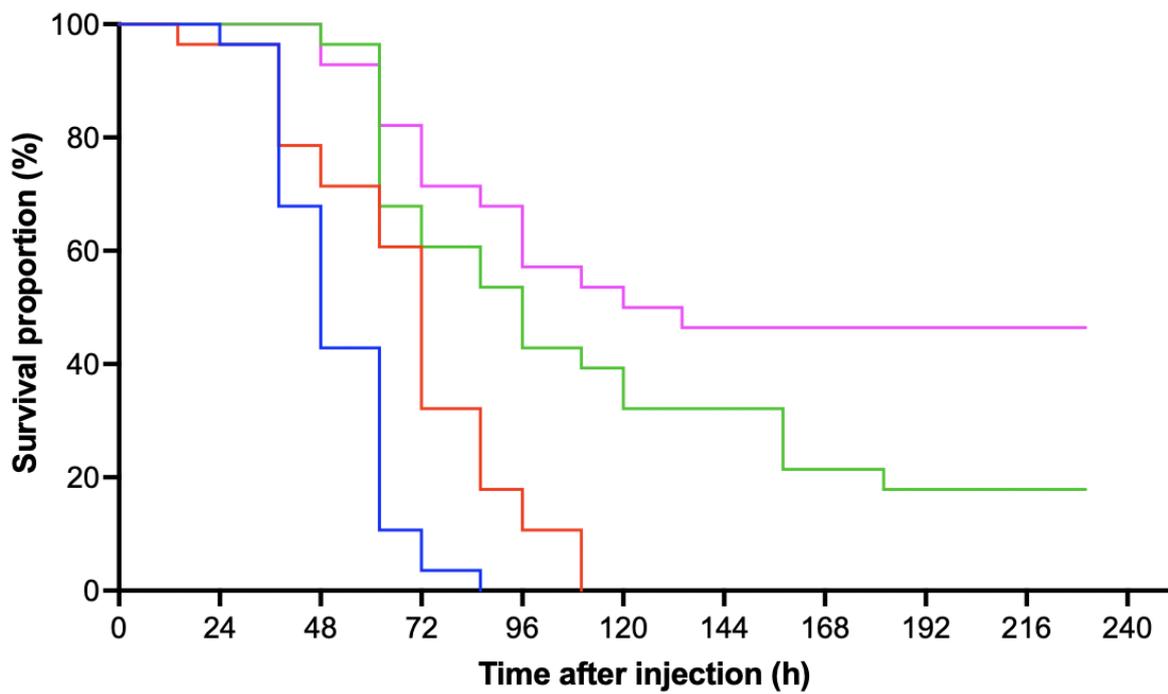


Figure 2 : Photocatalysis kinetics of *Vibrio harveyi* inactivated by UV₃₆₅ alone (♦) or inactivated by UV₃₆₅/TiO₂ (●). C/C_0 is the survival ratio along with the standard error of the means ($n = 3$)

a.



b.



c.

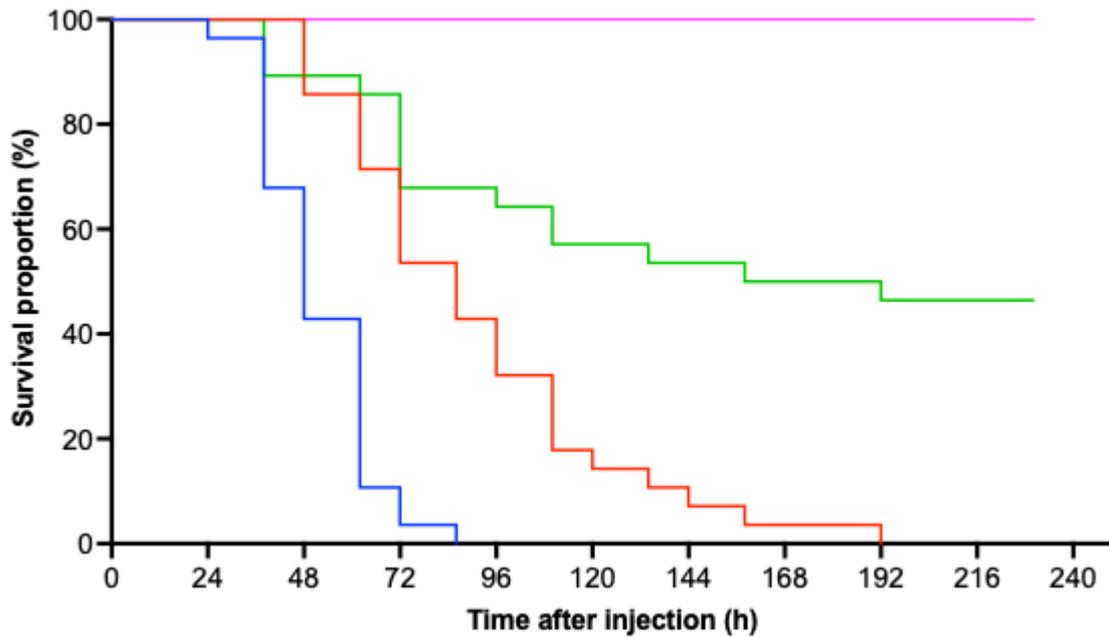


Figure 3 : Survival curves for oysters injected by (a) seawater without any treatment, (b) seawater disinfected by UV₃₆₅ only, and (c) seawater disinfected by UV₃₆₅/TiO₂ treatment. — CSW without treatment, — CSW after 30min of treatment, — CSW after 1h of treatment, — CSW after 2h of treatment or 2h kept out of UV for the control, and - - - CSW with TiO₂ particles

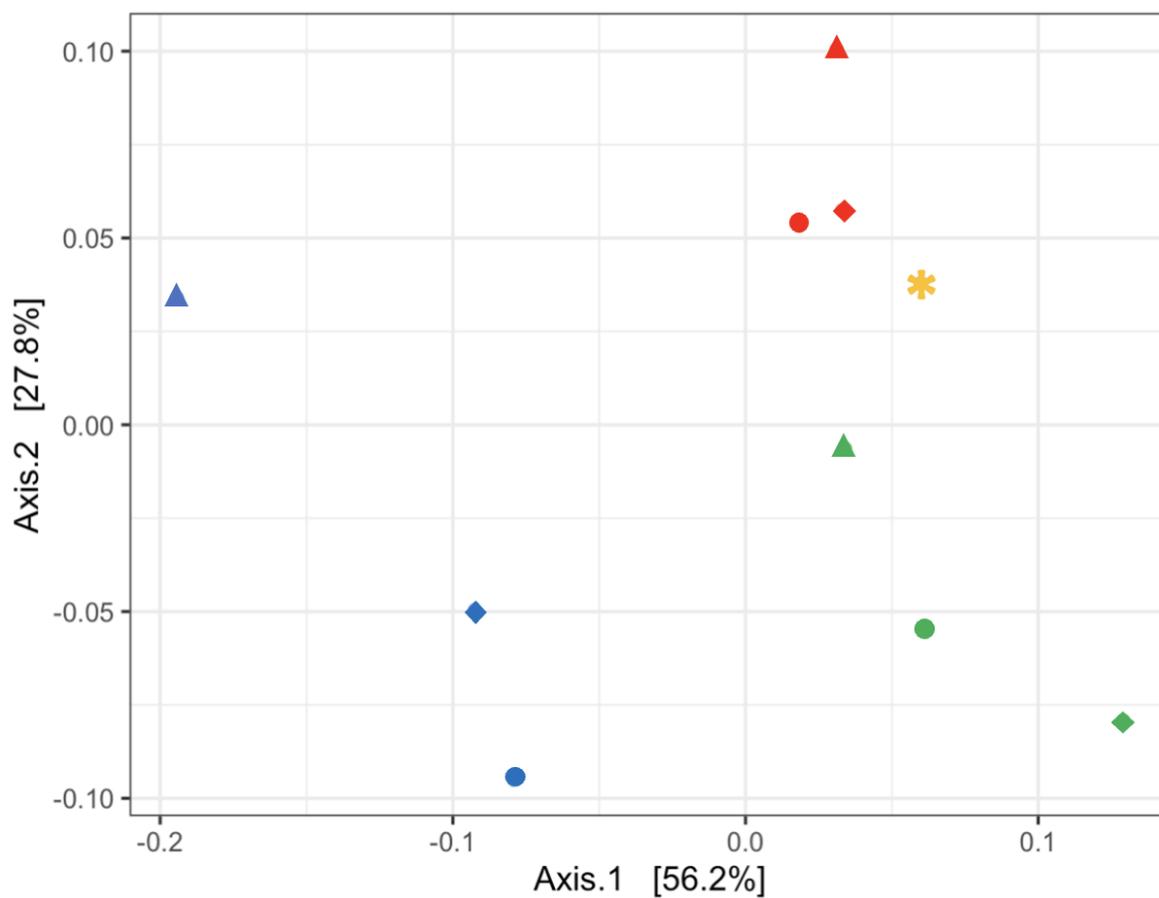
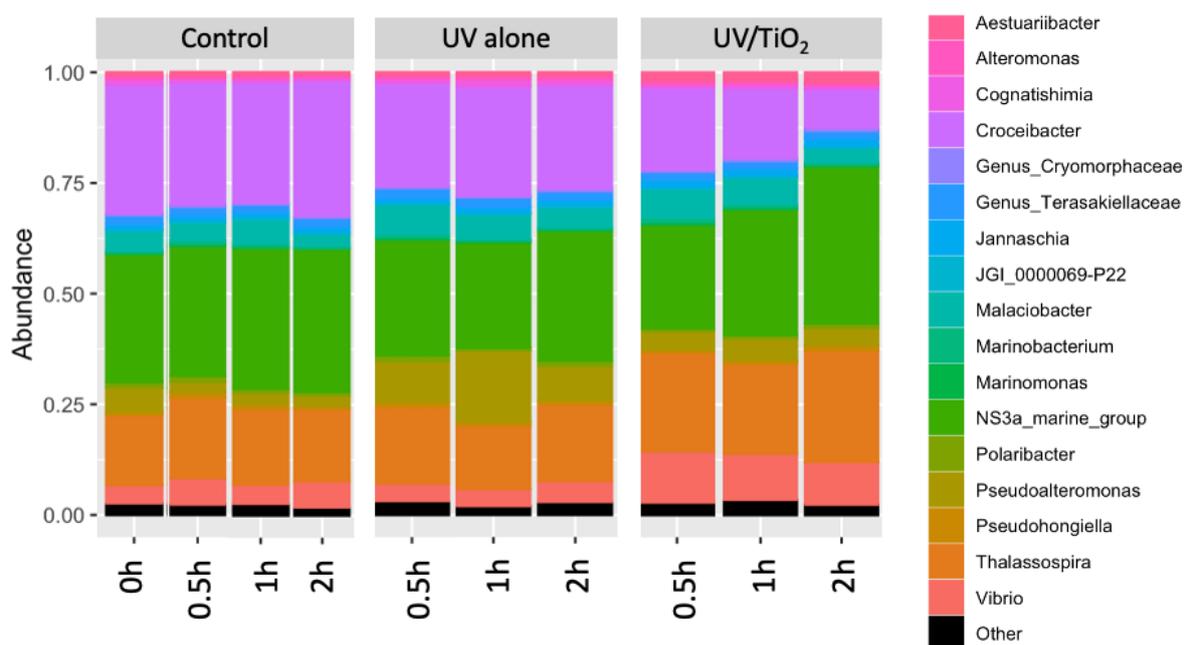


Figure 4 : Change in the microbial composition inducing by the photo-oxidative treatment (PCoA representation of the Bray-Curtis dissimilarity), (*) initial seawater (seawater before treatment), and the seawater at different exposition time (●) after 0.5 h, (◆) after 1 h, and (■) after 2 h, for (●) the references seawater (seawater without UV_{365}), (●) the seawater treated by UV_{365} , and (●) the seawater treated by UV_{365}/TiO_2 .

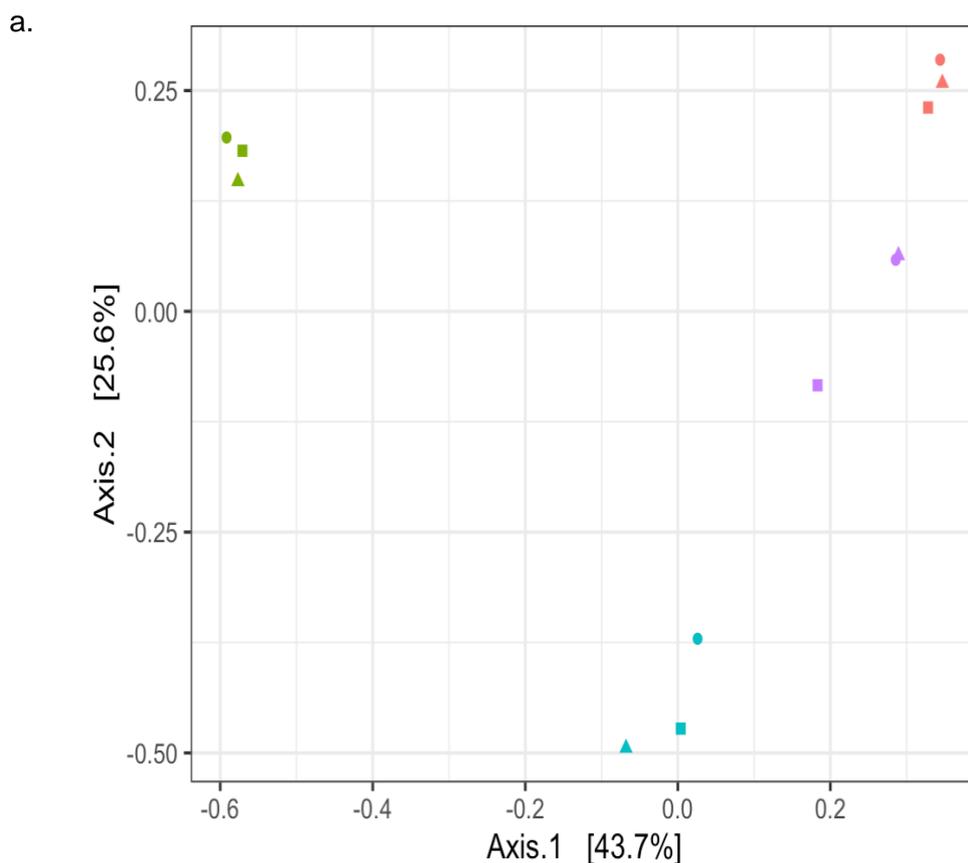
a.



b.

Genus	ASV id	Log ₂ Fold Change
<u>UV/TiO₂ treatment vs Control</u>		
<i>Aestuariibacter</i>	6cc2f88029d0da77a3ca104f2ba66d1d	1.267 (p _{adj} = 0.025)
	498793998a76d2da5987e21bc3d6c395	0.958 (p _{adj} = 0.011)
	2ea23ff77adaa9792ef01d3cc2aff00e	3.281 (p _{adj} = 0.023)
<i>Croceibacter</i>	58a8b0198b359e6eebeb182d3dfd367e	-1.242 (p _{adj} = 8.8e-04)
<i>Pseudophaeobacter</i>	0ef41f0d34ecf6dd1ad5a65934c64446	-1.520 (p _{adj} = 0.023)
<i>Vibrio</i>	69b5ff80eb6e4c005bc1cdd361091c4c	0.968 (p _{adj} = 0.012)
	e137d44214f795b6f81b842ffbb09785	1.409 (p _{adj} = 0.029)
<u>UV treatment vs Control</u>		
<i>Pseudoalteromonas</i>	44b14eda9d335426706b1ab37407bced	1.984 (p _{adj} = 0.012)

Figure 5: Bacteria genus modified according to the seawater photo-oxidative treatment applied (a) genus composition of the 17 more abundant genus, (b) genus significantly modified, log₂ fold change (with a p-value adjust < 0.05) of photo-oxidative treatment compared to untreated seawater (kept out of any UV₃₆₅ irradiation)



b.

Genus	ASV id	Log ₂ Fold Change
<u>UV/TiO₂ treatment vs Control</u>		
<i>vadinHA49</i>	6039762db2d5077b52dfcca5705dc427	-23.678 (p _{adj} = 3.57e-08)
(<i>Planctomycetota</i>)	e0c86b41e39439b4da07e788d97ad8c0	-24.711 (p _{adj} = 7.97e-09)
<i>BD7-11</i>	0865eafea28ebc73911cd6a23bb9e7ee	25.471 (p _{adj} = 3.74e-09)
(<i>Planctomycetota</i>)		
<i>Roseibacillus</i>	4871b4a3b38bfece10cccfa648820929	20.078 (p _{adj} = 1.12e-05)
<u>UV treatment vs Control</u>		
<i>Taeseokella</i>	3d04a3aeb7c7aaab28a5ed6df926370e	-21.053 (p _{adj} = 5.98e-06)
<i>Roseibacillus</i>	4871b4a3b38bfece10cccfa648820929	20.888 (p _{adj} = 5.98e-06)

Figure 6: Bacteria genus modified according to the time after the photo-oxidative treatment was applied (a) PCoA representation of the Bray-Curtis dissimilarity (●) at the end of the treatment, (●) 4 days, (●) 7 days, and (●) 10 days after the end of the treatment, for (●) the references seawater (seawater without UV₃₆₅), (▲) the seawater treated by UV₃₆₅, and (■) the seawater treated by UV₃₆₅/TiO₂, (b) genus significantly modified, log₂ fold

change (with a *p*-value adjust < 0.05) of photo-oxidative treatment compared to Control (seawater without treatment)

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