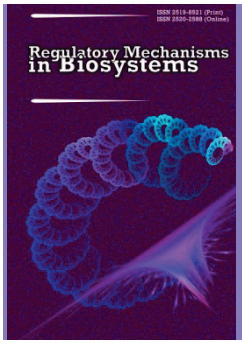

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Evaluating the effect of various treatments on the diatom *Haslea ostrearia* for achieving axenic cultures

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Axenic microalgae cultures are needed for several applications: genetic research, chemical exploration, aquaculture, and the study of interactions between microalgae and bacteria. Eliminating bacterial contamination while preserving the vitality of microalgae remains a considerable challenge. In this study, we focused on *Haslea ostrearia* (Simonsen, 1974), a highly coveted diatom known to produce the valuable blue pigment "marennine", responsible for oyster greening. To establish axenic cultures of the diatom *H. ostrearia*, we cultivated the strain NCC 527 (HoB4) in ASW medium at 19 °C with a 16-hour light and 8-hour dark photoperiod. Various treatments were tested: sonication, centrifugation, filtration, the use of detergent, and antibiotics. We evaluated the diatom response to these treatments and estimated the elimination of bacterial contamination. The results showed that physical treatments were moderately efficient. The sonication treatment effectively eliminated bacterial aggregates, but it also affected cell survival; 15 minutes of sonication reduced bacterial aggregates, but only 21.6% of cells remained motile and alive. Repeated centrifugation two times for 10 minutes allowed a moderate 49.9% reduction of bacteria. Treatment with Triton-X100 had a lethal effect on *H. ostrearia*. Filtration treatments decreased bacterial contaminants by only about 5%. We performed an analysis of variance (ANOVA) to compare diatom densities after treatments and bacterial removal. We also investigated the diatoms' growth after physical treatments and performed a comparison test between densities according to culture days. Repeated centrifugation and supernatant renewal appear to be less aggressive treatments on the diatom, with high bacteria removal without severely impacting the diatom cell density or growth. However, these physical treatments were not enough to produce total bacterial elimination. Combining antibiotic treatment with centrifugation and rinsing proved to be more effective, decreasing bacterial contamination while preserving diatom cells. We controlled the removal of bacterial contamination by using molecular techniques targeting 16S rRNA, along with colony counting and liquid medium inoculation. This work establishes the foundation for the development of an optimal protocol for *H. ostrearia* axenic culture in the future.

Keywords: axenic cultures; growth analyses; decontamination; DNA; molecular test.

Introduction

The demand for microalgal products is growing (Murata et al., 2021). Their rich composition of lipids, proteins, carbohydrates, and pigments (Su et al., 2023) makes them valuable in several domains. In agriculture, they demonstrate an important potential in biostimulants (González-Pérez et al., 2021), in soil fertility enhancement (Abinandan et al., 2019). Microalgae are also essential in aquaculture as feed, in biotechnology, utilized for biofuel production, as ingredients in human food and feed, in cosmetics, and as pollutant removal (Su et al., 2023).

Microalgal cultures are frequently associated with diverse microbial contaminants, including zooplankton, bacteria, fungi, and undesired microalgal species (Ma et al., 2023). Among microalgae, the diatoms are an important group. Characterized by the presence of a siliceous cell wall, the diatoms contribute to approximately 20% of annual carbon fixation (Falciatore et al., 2020) and to primary production in both marine and freshwater ecosystems (Armbrust, 2009). Interactions of diatoms with microbial communities, especially diatom-bacteria interactions, have been considerably explored (Amin et al., 2012; Koedooder et al., 2019). Recent research for understanding those interactions is based on the analysis of the whole microbiome associated with diatoms. The diatom and bacteria have developed specific associations; like in all microalgae, the dynamic microenvironment surrounding cells where those interactions occur is called the "phycosphere" (Di Costanzo et al., 2023). In culture, interactions affect the physiology, growth, and metabolism of diatoms (Cirri & Pohnert, 2019). It can have detrimental effects on growth, productivity (Wang et al., 2013; Baroni et al., 2019), and subsequent applications (Riaño et al., 2012; Zhu et al., 2020). Various methods have been proposed to deal with the imbalance between the desired microalgal strains and the contaminants. Those methods consist of increasing the survival potential of the desired strain (Mooij et al., 2015), changing environmental conditions (Mooij et al., 2015; Novoveská et al., 2023), filtration, and chemical treatment with common pesticides such as Trichlorphon, Buprofezon, or other additives (Novoveská et al., 2023).

The establishment of axenic cultures is important for research on genome sequencing, identifying a biological producer of a bioactive compound, or elucidating the relationship between microalgae and other organisms (Vu et al., 2018). Physical (sonication, centrifugation, and filtration) and chemical (detergent and antibiotics) treatments have been used to accomplish axenization. However, the effectiveness of these protocols can vary depending on the specific microalgae species and the characteristics of the contaminant; adjustments and optimization based on the particular case study are required. In previous studies, the protocols employed for achieving axenic cultures such as *Tetraselmis suecica* (Azma et al., 2010), *Arthrospira* spp. (Sena et al., 2011), *Synedra acus* (Shishlyannikov et al., 2011), and *Thalassiosira rotula* (Mönnich et al., 2020) did not use a single method but a combined approach of various physical procedures, the use of chemical agents and antibiotics to ensure greater eradication of micro-

bial contaminations linked with the strains. Similarly, in the present study, we investigated the effect of different treatments on *Haslea ostrearia* Simonsen, 1974 and the elimination of contaminants.

The diatom *H. ostrearia*, is known for producing a valuable blue pigment called marennine, responsible for oyster greening. Increasing the market value of oysters in western France and having an antibacterial, antiviral, antioxidant, antiproliferative, and allelopathic effect, this pigment has also shown a prophylactic effect on bivalve larvae (Falaise et al., 2019). This pigment makes this diatom a promising candidate for many applications, such as aquaculture feed, extraction of natural pigments, and pharmaceutical and biotechnological uses (Gastineau et al., 2014). The attainment of *H. ostrearia* axenic culture is important for various reasons; among them, we can mention that bacterial contamination can interfere with the precise analysis of omic data. Additionally, the presence of bacterial contamination may impact the formation of marennine pigment; removing the bacteria associated with this diatom could reveal important information about the pigment. Therefore, it's necessary to produce bacteria-free diatom cultures suitable for genome extraction and investigate the possibility of maintaining axenic cultures. To produce bacteria-free cultures, we first need to evaluate the potential impact of different treatments on bacterial contamination removal and diatom cell viability. In this study, we explored the effect of treatments previously used for other species on the *H. ostrearia* diatom and evaluated the effect on the *H. ostrearia* strain and bacterial contamination removal.

Materials and methods

Diatom cultivation conditions. The diatom *H. ostrearia*, strain NCC 527, also called HoB4, was obtained from the MMS-Nantes Culture Collection (NCC) as part of the GHaNA project. It was cultivated in the Le Mans University (LMU, France) laboratory in 250 mL Erlenmeyer flasks with a volume of 150 mL ASW medium. The experiments were carried out in triplicate under a photoperiod of 16 hours of light and 8 hours of darkness at a temperature of 19 °C. The initial cell concentration was 5,000 cells/mL, and the culture used for the test was in the exponential development phase. This study was conducted in three different experimental phases. The first phase involved preliminary tests to screen and select the most effective and promising physical treatment methods. In the second phase, the treatments identified as potentially optimal from the preliminary tests were evaluated individually and in combinations. This allowed an assessment of the impact of each promising physical treatment method. The third experimental phase is based on applying the selected optimal physical treatment method in addition to antibiotic treatments. Throughout all experimental phases, two key parameters were controlled: the effects of treatments on the growth and viability of the *H. ostrearia* strain and the efficacy of reducing bacterial contamination. The biomass obtained from the selected protocol was used for DNA extraction.

The effects of treatments on H. ostrearia. After each treatment, the cell motility was evaluated by microscopic observations, and the cell density was measured with a Nageotte chamber. In addition, for the physical treatment, we monitored cell proliferation after treatment to determine the effect of the treatments on the cell's growth capacity.

Axenic culture assessment. To validate the purity of the obtained diatom DNA and verify the absence of bacterial contaminants in the selected protocol, we first inoculated a volume of the culture on solid and liquid media (Doghri et al., 2017). Contaminating bacteria were enumerated as Colony-Forming-Units (CFU) per ml on slide medium, and detected by turbidity appearance in liquid medium. Following that, we employed a molecular approach to detect bacterial genes after extracting DNA. The approach consists of universal ^{16}S PCR amplification to target conserved regions of bacterial ^{16}S rRNA genes. It allows the amplification of bacterial DNA if it is present in the samples. The program uses denaturation for 1 minute at 94 °C, hybridation for 1 minute at 52 °C, and elongation for 1 minute at 72 °C. Electrophoresis gels on a 1.5% agarose was then performed to verify the DNA presence or absence of bacteria. The presence of DNA indicates bacterial contamination.

Different treatments tested to purify the diatom biomass. Several treatments were investigated for the culture of *H. ostrearia*, including sonication, centrifugation and rinsing, filtration, and antibiotic treatment. We performed preliminary tests on the physical treatment, and we selected the least aggressive treatments to minimize the potential damage to the cells while assessing their effects on cell proliferation.

Sonication. A water bath at 90 K cycles per second at a temperature of 30 °C for 10 to 15 minutes was determined to be the most suitable for successfully removing bacterial contaminants from *Tetraselmis suecica* green algae cells without damaging them (Azma et al., 2010). However, for *Synedra acus* subsp. *pennate* diatoms, this treatment led to the disintegration of large bacterial aggregates in the culture but proved ineffective against bacterial contamination (Shishlyannikov et al., 2011). The bacteria remained associated with the cell surface, while the diatoms were often damaged. We evaluated this technique with various exposure times to separate and detach aggregates and contaminants adhering to *H. ostrearia* cells. Cell counting was performed after each test, as well as seeding on agar plates and liquid media and counting bacterial colonies on agar to estimate the effectiveness of this method.

Repeated centrifugation. Repeated centrifugation and rinsing eliminated bacterial contaminants from *Tetraselmis suecica* culture; the number of bacterial colonies was reduced from 180 to 62 colonies after centrifugation and rinsing five times at 2000 RCF for 10 minutes (Azma et al., 2010). Contrary to what was reported, in the removal of contaminants from the culture of *Synedra acus* a pennate diatom, some bacterial aggregates remained in the culture (Shishlyannikov et al., 2011). We experimented with this technique using Falcon tubes, in which we distributed an initial culture under aseptic conditions and centrifuged at 2000 RCF for the following durations: 5, 8, and 10 minutes. Then, we removed the supernatant, replaced the volume with a sterile culture medium, and repeated centrifugation tests.

Treatments including detergent and membrane filtration. The axenic cultures of *Synedra acus*, a pennate diatom (Shishlyannikov et al., 2011), and *Thalassiosira rotula*, a centric diatom (Mönnich et al., 2020) were successfully obtained with procedures implicating filtration through a membrane. The diatoms collected on the membrane were treated with Triton-X 100 (Sigma Aldrich, Germany), and washed by filtration. However, this treatment with *Navicula phyllepta* (Doghri et al., 2017) was ineffective. It reduced bacteria by 40% but affected cell viability as well as the ability to form a biofilm at the bottom of the flask. We have experimented with these methods on *H. ostrearia*. From an exponentially growing stage culture with a density of 87,000 cell/mL, two separate experiments were conducted. The first involved gravity filtration of 40 mL of the initial culture and rinsing with a sterile medium. For the second with 40 mL culture we proceeded to consecutive rinsing with 150 mL of sterile ASW culture medium, 50 mL of sterile ASW containing 20 µg/mL of Triton-X 100 (Sigma Aldrich, Germany), and another rinse with 150 mL of sterile ASW, then the collected diatoms were cultured in a 250 mL Erlenmeyer flask with the addition of antibiotic treatment (1 µL/mL). The membrane filtration porosity was 5 µm.

Selected treatment methodology. In all protocols used for strain decontamination, antibiotic treatment is necessary and is always utilized. Following the evaluation of physical treatments, a combination of methods was selected to achieve maximal decontamination while preserving *H. ostrearia* growth. As a final decontamination step, successive antibiotic treatments were applied to further reduce bacterial contamination. We conducted three antibiotic treatments at a 48-hour interval, followed by centrifugation and removal of the supernatant containing dead bacteria and spores. Batch cultures were initiated with the same cell density of 25,000 cells per milliliter. Each batch contained sufficient culture volume to obtain the same biomass weight at the end (Fig. 1). We first performed an antibiotic treatment for all Erlenmeyer flasks. After every 48 hours (incubation period), the supernatant was replaced, and the antibiotic treatment was added at a concentration of 10 µL of antibiotic for ml of culture medium for all batches, followed by biomass collection and azote flushing and storage for the first batch. The antibiotic treatment used is a mix of Fisher antibiotics composed of penicillin, streptomycin, and amphotericin B. We proceed to similar procedures with the second and third batches. The bacterial contamination and cell density were controlled for each Erlenmeyer from the batch culture.

gDNA extraction. For the DNA extraction, we employed the CTAB protocol as mentioned in Gabed et al. (2022). The most interesting method

that eliminates bacterial contamination while preserving the diatom strain was selected; the final gDNA of HoB4 obtained from this technique was utilized for bacterial assessment. The DNA quantity and quality were verified with Nanodrop 2000 (Thermo Fisher Scientific).

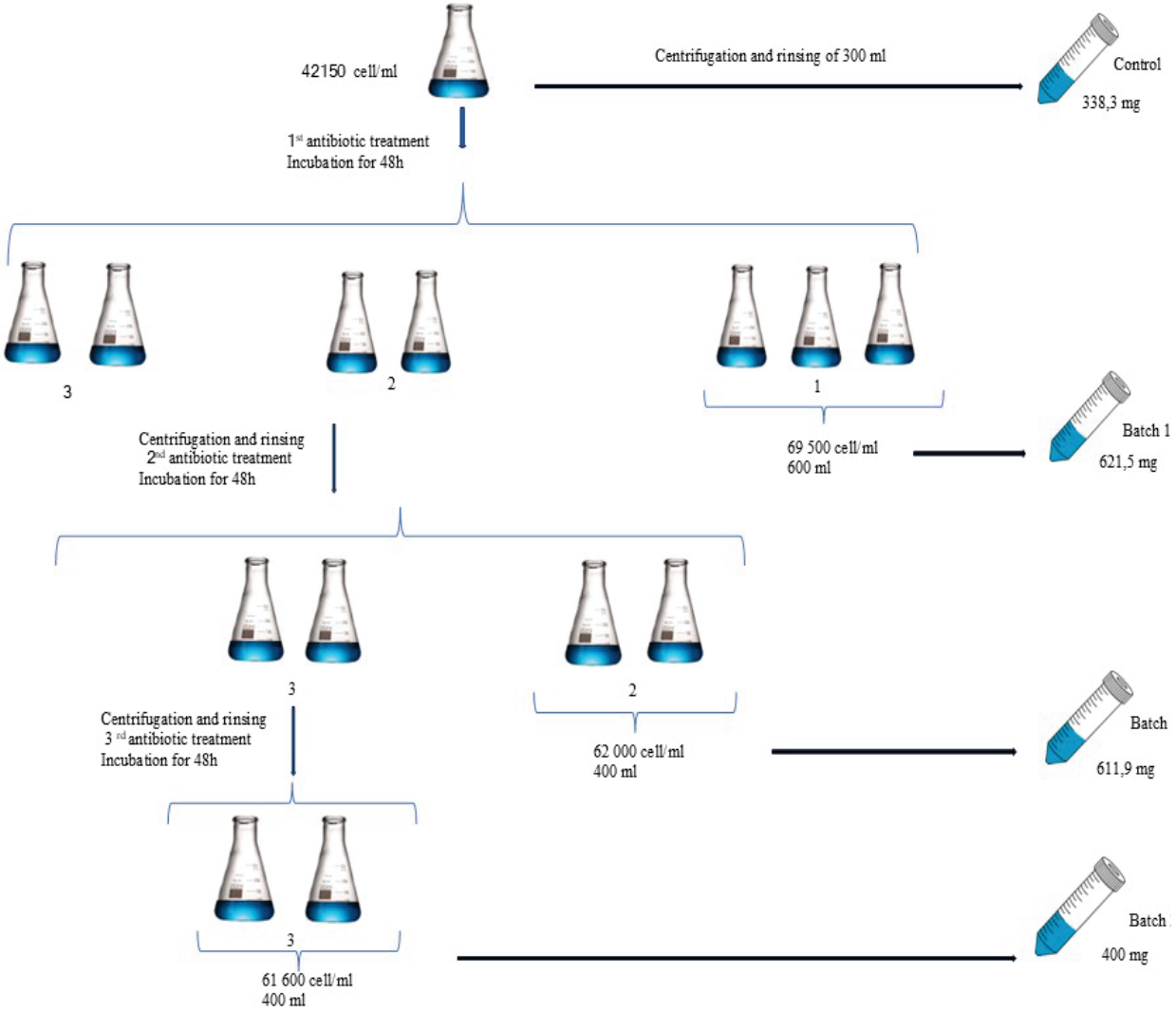

Fig. 1. Experimental protocol and results scheme for obtaining axenic cultures of *Haslea ostrearia* through successive antibiotic treatments and renewal of the supernatant by centrifugation

Statistical analyses. All data are presented as means ± standard deviations of three replicates. For the determination of significant differences between diatom growth after different physical treatments, a two-way analysis of variance (ANOVA) was performed (factors: day, density) with an Honest Significant Difference (HSD) Tukey's test for the post_hoc testing. For other statistical analyses, we used a one-way ANOVA. Tests were performed using GraphPad Prism (version 10.0.0 for Windows). Based on the statistical analysis performed, the lower case letters in the figures indicate significant differences between treatments, those sharing the same letter are not significantly different from each other, while treatments with different letters are considered statistically significantly different.

Results

Preliminary tests for evaluating physical and chemical treatments and selecting potential methods for effective bacterial elimination and cell preservation in H. ostrearia culture. Throughout the preliminary experiments conducted in the present study, it was shown that exposing *H. ostrearia* cells to sonication reduced bacterial contamination. However, it also had a simultaneous effect on cell motility and survival. We started the experiment with a 73,600 cell/mL culture density. After an exposure of 15 minutes, only 21.6% of the cells remained motile and alive. At the same time, we observed a reduction of bacterial aggregates under microscopy. This treatment could be utilized for removing the bacterial cells that adhered to the cell surface at a frequency of 90 K cycles per second for a duration of 5 to 10 minutes. The centrifugation, repeated twice during 10 minutes reduced bacterial colonies up to 49.9%, from 1431 ± 310 cells to 717 ± 69 cells. This procedure was employed in the next experiments as a step to remove contaminants detached from *H. ostrearia* cells after sonication treatment. The use of the tangential flow membrane filtration system and rinsing it with a sterile medium with 30 µm filter porosity does not retain diatoms but retains aggregates. However, a 5 µm filter reduced contamination by 5.4%; it reduced bacterial contamination from 1431 ± 310 cells to 1354 ± 313. Although Triton-X 100 (Sigma Aldrich, Allemagne) has a lethal effect on *H. ostrearia* cells, this treatment proved to be ineffective for our strain.

Effect of physical treatments on the cell density of H. ostrearia. For this experiment, the initial cell density was 13000 cell/mL represented by the control in Figure 2. After treatments, the cell density indicates that the effect on *H. ostrearia* varied greatly. The sonication treatments for 5 and 10 minutes, the combined treatments of 5 minutes sonication and centrifugation and rinsing twice, and centrifugation and rinsing twice did not show significant differences in their effect on the diatom. They are the most similar to the densities measured in the control, followed by the 10 minutes sonication and centrifugation test, which shows a lower density than the first similar group but is also closest to that measured in the control. The filtration showed lower densities, with 5 minutes of sonication and filtration and only filtration did not present a significant difference. The lowest density was reported in the 10 minutes sonication and filtration.

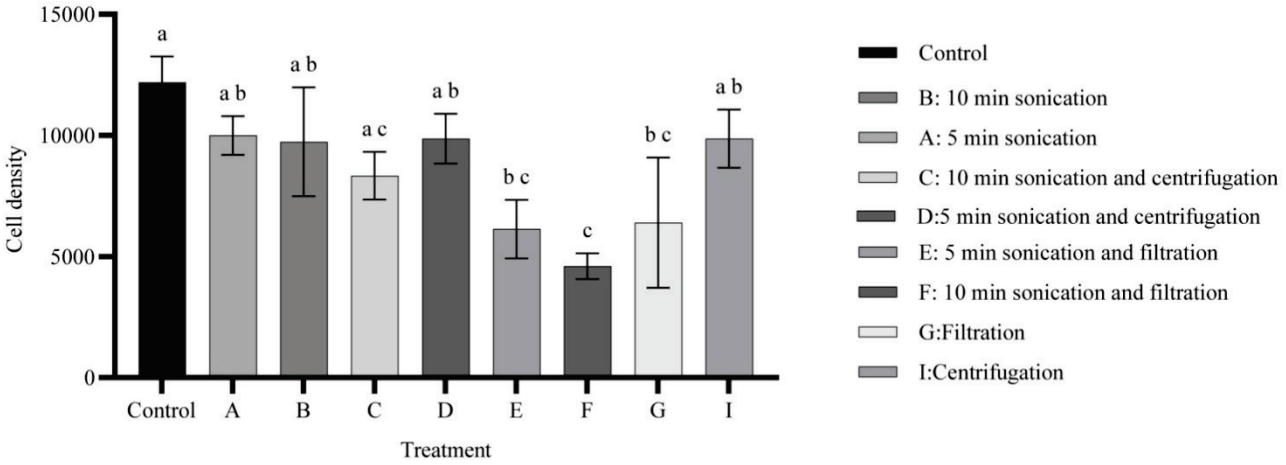

Fig. 2. Effect of physical treatment on cell density, different letters indicate samples that are significantly ($P < 0.05$) different from one another according to Tukey's Honest Significant Difference (HSD) Test

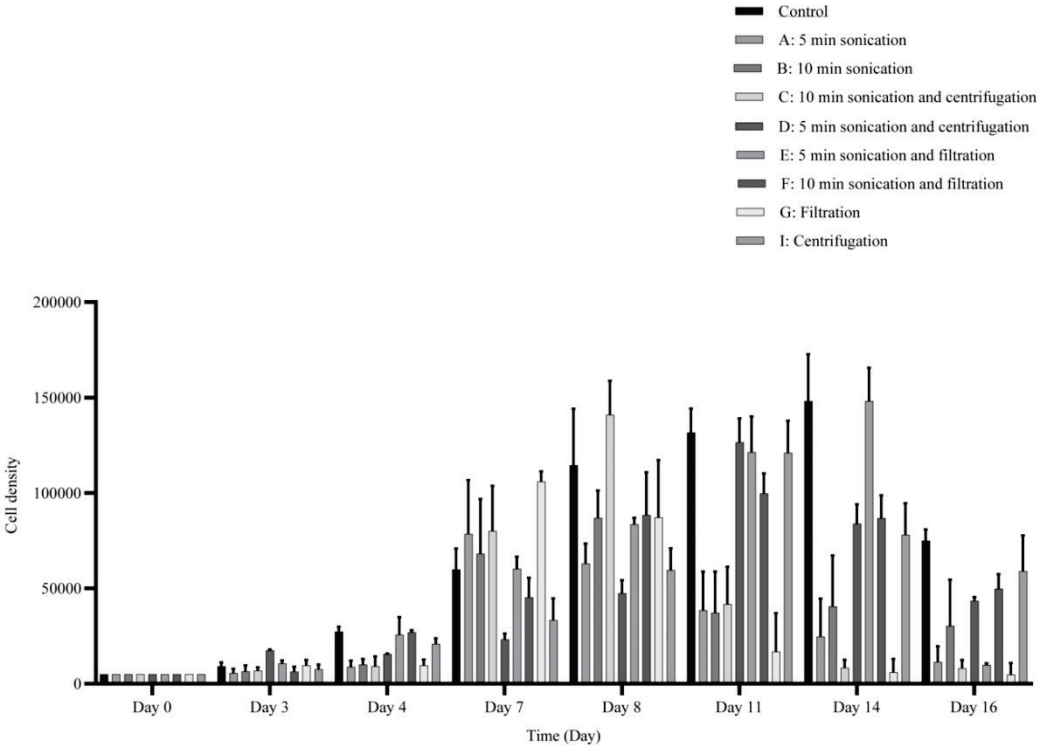

Fig. 3. Growth of *Haslea ostrearia* after physical treatments

Effects of physical treatments on H. ostrearia growth. We inoculated the cells subjected to physical treatments at a density of 5,000 cells/mL in a fresh medium. The experiment was conducted in triplicate for each treatment, and the culture densities were recorded on the following days of cultures 3, 4, 7, 8, 11, 14, and 16 (Fig. 3) indicates the densities as mean ± SD. We performed a multi-comparison test to determine if there were significant differences in growth with the control. The difference was considered statistically significant when the P-value was lower than 0.05 (Table 1). The control cultures start the exponential growth phase from the 7th day with the plateau phase between the 11th and 14th days, and are in the decline phase on the 16th day.

Compared to the growth of the control, the cells that underwent sonication for 5 and 10 minutes had densities most similar to the control, as noted for treatments E and F respectively, 5 minutes sonication and filtration and 10 minutes sonication and filtration. On the 4th, 7th, and 8th days, the cells were in the exponential growth phase, where we observed a rapid increase in density reaching the 8th day (114.5 ± 29.6) x 10^3 cell/mL for the control, (84.0 ± 3.3) x 10^3 cell/mL for 5 minutes sonication and filtration, and (88.4 ± 22.4) x 10^3 cell/mL for 10 minutes sonication and filtration. Then there was a stationary phase until the 14th day where the recorded densities remained approximately similar, although there was a significant difference with 10 minutes of sonication and filtration, whose density was (80.8 ± 8.2) x 10^3 cell/mL compared to that of the control (148.2 ± 24.5) x 10^3 and (148.1 ± 17.5) x 10^3 cell/mL for the 5 minutes sonication and filtration treatment, noting that this density remains the highest and closest to the control compared to the other treatments on day 14. The growth of the diatoms was slowed for treatments of 5 minutes of sonication and centrifugation, and only centrifugation. There was a significant difference with the control on days 7 and 8, respectively. Culture from the treatment of 5 minutes sonication and centrifugation entered the exponential phase on the 7th day to reach (126.6 ± 12.5) x 10^3 cell/mL on the 8th day, then entered the stationary phase with a recorded density on day 14 of (83.9 ± 10.1) x 10^3 cell/mL. I with densities similar to D enters the exponential phase on the 4th day, but the density increases very slowly to reach (59.6 ± 11.4) x 10^3 cell/mL on the 8th day. Then there was the plateau phase, with a density of (121.1 ± 16.8) x 10^3 cell/mL on the 11th day and (78 ± 16.6) x 10^3 cell/mL on the 14th day.

For the treatments of 5 minutes sonication, 10 minutes sonication, 10 minutes sonication, and centrifugation, the highest densities were recorded on the 7th and 8th days. A significant difference from the control appears on the 11th day for the three treatments as well as on the 8th day for the 5 minutes sonication treatment.

Cultures that underwent filtration showed a significant difference from the control on the 7th day, with a higher density of (106 ± 5.3) x 10^3 cell/mL compared to (59.9 ± 10.9) x 10^3 cell/mL for the control, suggesting a precocious exponential growth. For cultures that underwent treatments; 5 minutes sonication, 10 minutes sonication, 10 minutes sonication and centrifugation, and filtration, the highest densities were recorded on the 7th and 8th days, contrary to control cultures that did not undergo treatment and whose highest densities were noted from the 8th to the 14th day.

Table 1
Growth of *Haslea ostrearia*, expressed as "cells per milliliter" after physical treatments (x ± SD, n = 3, duration of experiment – 16 days)

Groups	Day 0 (10^3)	Day 3 (10^3)	Day 4 (10^3)	Day 7 (10^3)	Day 8 (10^3)	Day 11 (10^3)	Day 14 (10^3)	Day 16 (10^3)
Control	5 ± 0	9.1 ± 2.0	27.3 ± 2.5	59.9 ± 10.9	114.5 ± 29.6	131.7 ± 12.6	148.2 ± 24.5	75.0 ± 5.8
A	5 ± 0	5.6 ± 2.2	8.8 ± 3.3	78.5 ± 28.2	62.9 ± 10.5*	38.5 ± 20.3*	24.7 ± 20.0*	11.4 ± 8.3*
B	5 ± 0	64.7 ± 3.2	10.0 ± 2.9	68.1 ± 28.7	87.0 ± 14.2	37.2 ± 21.6*	40.5 ± 26.7*	30.3 ± 24.3*
C	5 ± 0	68.7 ± 1.7	9.1 ± 5.2	80.1 ± 23.6	141.0 ± 17.7	41.7 ± 19.7*	8.4 ± 4.1*	8.1 ± 4.9*
D	5 ± 0	17.5 ± 0.5	15.6 ± 0.2	23.3 ± 3.0*	47.5 ± 6.8*	126.6 ± 12.5	83.9 ± 10.1*	43.5 ± 1.9
E	5 ± 0	10.6 ± 1.6	25.7 ± 9.3	60.3 ± 6.3	83.6 ± 3.3	121.4 ± 18.7	148.1 ± 17.5	9.9 ± 0.9*
F	5 ± 0	6.3 ± 2.6	26.9 ± 1.1	45.3 ± 10.3	88.4 ± 22.4	99.8 ± 10.5	80.8 ± 8.2*	49.8 ± 7.7
G	5 ± 0	9.7 ± 9.7	9.7 ± 3.0	106.0 ± 5.3*	87.2 ± 30.0	16.9 ± 20.1*	6.0 ± 6.9*	4.7 ± 6.1*
I	5 ± 0	7.6 ± 7.6	20.9 ± 20.9	33.4 ± 11.3	59.6 ± 11.4*	121.1 ± 16.8	78.0 ± 16.6*	59.0 ± 18.7

Note: "*" indicates densities that are significantly ($P < 0.05$) different from the control within the column of the table according to the results of ANOVA with Bonferroni's corrections. A: 5 minutes sonication, B: 10 minutes sonication, C: 10 minutes sonication and centrifugation, D: 5 minutes sonication and centrifugation, E: 5 minutes sonication and filtration, F: 10 minutes sonication and filtration, G: filtration, I: Centrifugation.

Estimation of bacteria elimination through physical treatments. Figure 4 illustrates the effect of different physical treatments on the number of bacterial colonies. The results show that treatments of 10 minutes of sonication and centrifugation, 10 minutes sonication and filtration are significantly similar and more effective in reducing bacteria. More similar to this group of treatments, the treatments: 10 minutes of sonication, 5 minutes of sonication and centrifugation, 5 minutes sonication and filtration, and centrifugation, are equally effective, showing a lower efficiency in bacteria reduction but also performing well.

The highest presence of bacterial colonies was observed for the 5 minutes sonication treatment, followed by the filtration treatments. For all physical treatments, there was a growth of bacterial colonies on the solid medium, and the inoculated liquid medium also became turbid.

Effect of the optimized protocol on the cell density of H. ostrearia. From all physical treatments, the centrifugation repeated twice showed better preservation of the diatom cell (Fig. 2) and a moderate elimination of bacteria (Fig. 4). We proposed a protocol that combined this physical treatment with antibiotic treatment to complete bacteria removal. The details of this experiment are explained in Figure 1. From an initial culture of 42,150 cell/mL density, we initiated fresh cultures with a density of 25,000 cell/mL. Seven Erlenmeyer flasks of 250 mL were inoculated and filled up to 200 mL. From the initial culture considered the control for this experiment, we extracted 300 mL and proceeded with centrifugation, rinsing, biomass collection by centrifugation, and then a rapid freeze using liquid nitrogen. The Erlenmeyer flasks were separated into three groups. Batch 1 is comprised of three flasks with a combined volume of 600 mL, specifically intended for one antibiotic treatment. Batch 2 consisted of two flasks with a total volume of 400 mL, which were used to evaluate two antibiotic treatments. Batch 3 consisted of 400 mL of liquid divided into two flasks intended for the experiment, including three antibiotic treatments.

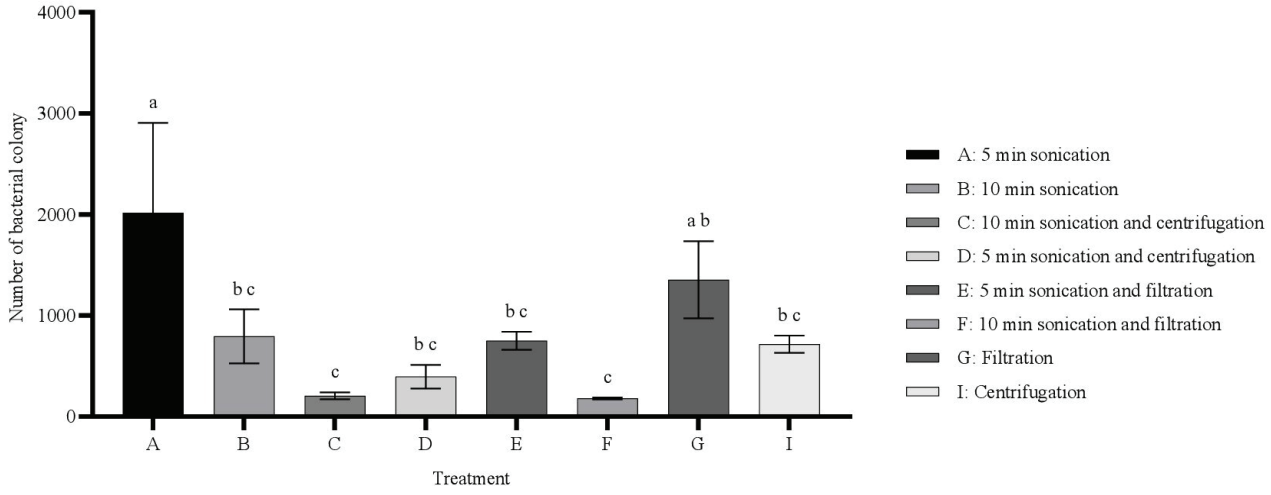

Fig. 4. Number of bacterial colonies after physical treatments: different letters indicate samples that are significantly ($P < 0.05$) different from one another according to Tukey's Honest Significant Difference (HSD) Test

Following the inoculation of the 7 flasks, antibiotic treatment was applied for all batches by adding 1 mL of antibiotic per 100 mL of culture. After 48-hour of incubation, all batches underwent centrifugation and rinsing. In addition to this step, we used centrifugation to collect the batch 1 biomass. The other batches received a second antibiotic treatment. 48 hours later, we repeated the same technique. The biomass of the second batch was collected, and the others were treated with a third antibiotic. 48 hours after that, the third batch was rinsed and collected. All obtained biomasses (control, batches 1, 2, and 3) were used for DNA extraction. The following biomass weights were obtained: Control: 338.3 mg; Batch 1: 621.5 mg; Batch 2: 611.9 mg; Batch 3: 400 mg. One antibiotic treatment combined with centrifugation allowed for better preservation of cell density. There was no significant difference between two and three antibiotic treatments (Fig. 5).

Antibiotic treatment and bacteriam elimination. Bacterial contamination was measured by inoculation on solid and liquid mediums 48 hours after each treatment. The colony counting on the solid medium was done 48 hours after incubation. For Batch 1, a single Petri dish from the triplicates uncemented contained two colonies. For Batch 2, one colony appeared on one of the triplicate Petri dishes, and no colonies appeared in Batch 3. The liquid medium showed turbidity starting from the 3rd day for Batch 1, from the 5th day for Batch 2, and from the 9th day for Batch 3. The statistical test does not indicate a significant difference in the number of bacteria for the three treatments (Fig. 6).

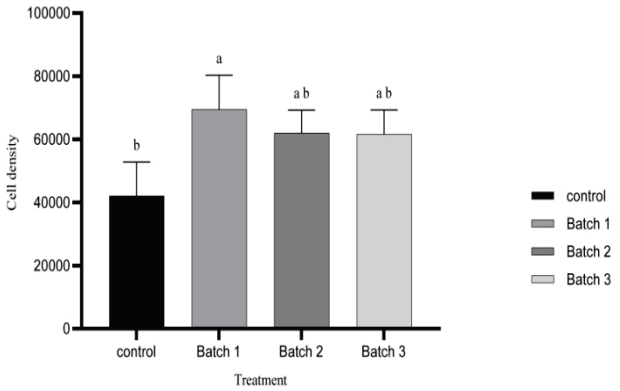

Fig. 5. Cell density of cultures under antibiotic treatments: different letters indicate samples that are significantly ($P < 0.05$) different from one another according to Tukey's Honest Significant Difference Test

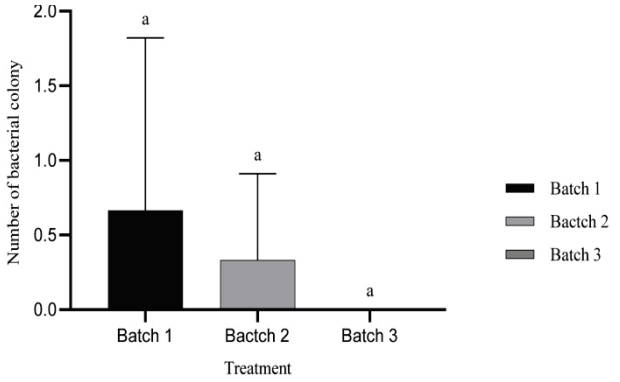

Fig. 6. Bacterial removal and significant difference between the different shoots of antibiotic treatments: different letters indicate samples that are significantly ($P < 0.05$) different from one another according to Tukey's Honest Significant Difference Test

gDNA extraction. The biomass subjected to selected treatments was used for DNA extraction. The following table summarizes the extraction results (Table 2).

Table 2
Details of DNA extraction for the treated biomass

DNA obtained from treated biomass	Concentration, µg/µL
Control	90.00
Batch 01: One antibiotic treatment	121.33
Batch 02: Two antibiotic treatments	71.86
Batch 03: Three antibiotic treatments	17.20

Axenic culture assessment. We perform 16 PCR to confirm the absence of bacteria. For the DNA obtained from the optimized protocol, we observed the migration of PCR products on a 1% agarose gel (Fig. 6).

Fig. 7. 16S PCR for control of bacterial contamination, the PCR products migration on 1% agarose gel: M – DNA ladder; "-" – negative control; "+" – positive control; *0* – untreated culture; *1* – Batch 1 with one antibiotic treatment; *2* – Batch 2 with two antibiotic treatments; *3* – Batch 3 with three antibiotic treatments

Discussion

We tested the effects of different treatments on the diatom *H. ostrearia* and evaluated the reduction in bacterial contamination. We conducted this research intending to produce bacteria-free diatom cultures appropriate for genome extraction and investigating the possibility of maintaining axenic cultures. Different techniques were used previously for the preparation of axenic microalgal culture. We utilized a combined approach; physical methods, integrating chemical agents, antibiotics, and sometimes needed to develop new methods. The treatment with detergent allowed the establishment of axenic strains of *Synedra acus* (Shishlyannikov et al., 2011) and *Thalassiosira rotula* (Mönnich et al., 2020). The identical treatment with the same concentrations proved to have a lethal effect on *H. ostrearia*. The physical treatments of sonication, repeated centrifugation, filtration, and rinsing have shown efficacy in earlier experiments, acting on the cell surface and removing adhered bacteria for sonication (Azma et al., 2010), reducing bacterial contaminants for centrifugation and filtration (Azma et al., 2010; Shishlyannikov et al., 2011). Those methods effectively reduced bacterial contamination in *H. ostrearia* culture (Fig. 4), but their effectiveness varied. The sonication separates and detaches aggregates. The combined treatments of 10 minutes of sonication and centrifugation, and 10 minutes of sonication and filtration are the most effective for eliminating bacteria, followed by treatments involving only sonication for 10 minutes, 5 minutes of sonication with centrifugation and filtration, or only centrifugation. The filtration and sonication for 5 minutes of treatment are the least effective for bacterial elimination. Nevertheless, colony growth persists after all treatments. Regarding the effect on the cell density (Fig. 2), milder treatments such as only sonication for the duration of 5 and 10 minutes, the short duration sonication combined with centrifugation, and only centrifugation with rising have little impact on the cell density compared to the control. This suggests that these moderate physical treatments do not significantly damage the diatom cells. In contrast, filtration, and especially the combined treatment of 10 sonications with filtration, reduced significantly the cell density. The filtration retains cells on the filter, cells remain trapped causing cell density diminution. In examining the cell growth after physical treatment (Table 1), we observe that most treatments slow down the growth of *Haslea ostrearia* compared to the control, exhibiting a delay in the exponential phase and lower maximum densities. This is particularly true for the most aggressive treatments (10 minutes of sonication and filtration). Interestingly, cultures subjected to sonication, filtration and only filtration, exhibit similitude with the control for the combined treatments and early exponential growth for only filtration.

The use of antibiotic treatments for the obtaining of axenic microalgae is cited in all previous works, to obtain axenic cell cultures of marine microalgae *Chrysotila roscoffensis* (Liu et al., 2023), the diatom *Synedra acus* (Shishlyannikov et al., 2011), the diatom *Thalassiosira rotula* (Mönnich et al., 2020), and *Arthrospira* spp. cyanobacteria (Sena et al., 2011) were used as a single treatment or combined with other techniques. In a previous study of the bacterial community structure of the marine diatom *H. ostrearia* (Lépinay et al., 2016), the axenic culture of *H. ostrearia* was obtained with antibiotic treatment and incubating culture for 7 days, then the supernatant was removed, and the biofilm washed with fresh medium and left for 7 days of culture without treatment before applying a second antibiotic treatment. It was considered that bacteria concentration was drastically reduced compared to the non-treated culture, but without details on the quantification, the culture issue from those two antibiotic treatments was called "axenic" and used for the study experiments. In accordance with those results, our study showed that the use of antibiotic treatments reduced effectively bacterial contamination from the culture. Statistically, no significant differences in bacterial removal were revealed between the three treatments, but the bacterial densities number was reduced to no colony on the slide medium for the third treatment (Fig. 6). The cell growth was not affected by the treatment for one antibiotic treatment. We started the experiment with 42,150 ± 10,671 cell/mL, represented by control in Figure 5. After the first treatment, the cell density increased, reaching 69,500 ± 10,759 cell/mL. No significant similarity was detected by statistical tests between the two densities, indicating that there was growth and the antibiotic didn't affect the cells' capacity to grow. After

two and three treatments, the densities were, respectively, 62,000 ± 7,211 and 61,600 ± 7,725 cell/mL. Similar to those of the first antibiotic treatment, they remain lower and are also close to the starting density, though still higher. This indicates that the effects of the antibiotic and rinsing by centrifugation are more pronounced in these two latter treatments compared to the initial.

In this work, it appears that antibiotic treatment with centrifugation and rinsing is a more promising approach for DNA extraction and genome sequencing. It demonstrates efficacy in obtaining presumed cultures free of bacterial contamination. To obtain a higher yield after extraction, it would be necessary to perform the antibiotic treatment on a larger volume of culture to obtain a greater quantity of biomass or to use one antibiotic treatment. Alternatively, several bioinformatic tools can be employed to detect and clean a genome assembly from residual bacterial contaminant sequences, such as Blast+ (Camacho et al., 2009), BlobToolKit (Challis et al., 2020), AlienTrimmer (Criscuolo & Brisse, 2013), DeconSeq (Schmieder & Edwards, 2011), HoCoRT (Rumbavicius et al., 2023) and other tools, and pipelines. For further studies on *H. ostrearia* that require maintaining axenic strain cultures, this protocol can be used, but it needs to be repeated to maintain a lower bacterial contamination.

Obtaining the axenic culture of the diatom *H. ostrearia* proved to be a significant challenge in this study. An important obstacle in our work was the fragility of the diatom and the precise evaluation of cell counts during the treatments. For homogenizing the cultures, we used a magnetic stirrer and a shaking balance to ensure an equal distribution of cells throughout the volume. Counting using the Nageotte chamber is a commonly used method, but becomes more challenging due to the effects of the treatment, potentially damaging or lysing cells. Given that we conducted multiple treatments simultaneously, it was difficult to perform the counts while keeping cells fresh and motile after treatments. Another difficulty is the association between *H. ostrearia* cells and contaminating bacterial cells. Many of the bacterial contaminants appeared to tightly adhere within the extracellular matrix surrounding the diatom cell "phycosphere" (Di Costanzo et al., 2023). This intimate interaction made it complicated to separate without causing significant damage or loss of diatom viability. Strategies like sonication, centrifugation, and filtration treatment were moderately successful in dislodging bacteria, but also impaired diatom growth to varying degrees.

Conclusion

The contaminants present in *H. ostrearia* culture can differ from one culture to another. In this study, we highlighted the effects of different physical treatments on *H. ostrearia* and found that these treatments can effectively act against contamination; sonication for disaggregation, centrifugation for gravity-based separation and rinsing of cultures, and filtration for retaining larger aggregates and rinsing cells. The experiment's findings showed the possibility of obtaining axenic culture with antibiotic treatments and rinsing by centrifugation. Other physical methods were able to decrease bacterial contamination, but they proved insufficient for the total removal of bacterial contamination. Furthermore, these combined approaches frequently fail to preserve the integrity of diatom cells. Antibiotic treatment combined with centrifugation eliminates the bacterial contamination the most efficiently. Thus, this work establishes the foundation for the development of an optimal protocol for *Haslea ostrearia a*xenic culture in the future.

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