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RESEARCH

Optimizing *Ulva-Phaeobacter* co-culture: A two-phase light intensity approach for integrated multi-trophic aquaculture applications

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Abstract

Incorporating macroalgae like *Ulva* species into integrated multi-trophic aquaculture (IMTA) enhances sustainability by filtering effluents and provide epiphytic bacteria, that can contribute to disease prevention. Colonizing *Ulva ohnoi* with *Phaeobacter* sp. 4UAC3 is promising for disease control in IMTA systems, but high light intensity impacts *Phaeobacter*'s persistence on *Ulva*. This study investigated the effect of different light intensities and regimes on the ability of *Phaeobacter* sp. 4UAC3·to colonize *U. ohnoi*. Experiments assessed algal growth, water physicochemistry, and microbial profiles using culture techniques and 16S rRNA gene sequencing. Light intensities of 143, 75, 45, and 0 µmol photons m^{-2} s^{-1} were tested on *U. ohnoi* and non-living surfaces inoculated with *Phaeobacter* sp. 4UAC3. Results showed that *Phaeobacter* sp. 4UAC3 colonization on *U. ohnoi* decreased in the light but remained stable in the dark. On non-living surfaces, *Phaeobacter* persisted regardless of light intensity, suggesting light does not directly affect the bacterium. However, light had an impact on microbial community structure on *U. ohnoi*, with genera including *Glaciecola*, *Maribacter* and *Roseobacter* significantly enriched under higher light conditions. This suggests the disappearance of *Phaeobacter* sp. 4UAC3 is in part a result of competition with specific bacteria. Additionally, the chemical microenvironment of the algae influenced by light could be important in *Phaeobacter* disappearance, although further research is required. Finally, optimal co-culture involved alternating dark and low-light (45 µmol photons·m^{-2} s^{-1}) phases, enhancing *Phaeobacter* sp. 4UAC3 maintenance and optimal algal growth. This knowledge will optimize a fish-algae IMTA-RAS using *Ulva* colonized with *Phaeobacter*.

Keywords Microbiome · Macroalgae-bacteria interactions · Microbiome manipulation · Disease prevention · 16S rRNA gene amplicon sequencing

Introduction

Marine aquaculture has experienced a significant growth in recent decades being considered a necessary solution to meet the increasing demand for seafood (FAO 2022) and seaweeds are expected to contribute to that demand (Duarte et al. 2022). However, the intensification of aquaculture operations poses challenges, including the generation of nutrient-rich effluents that, without effective treatment, can contribute to environmental eutrophication (Folke et al. 1994). Additionally, the high-density cultivation in many fish farms make them susceptible to microbial diseases (Sundberg et al. 2016), requiring the use of disinfectants and antibiotics, which potentially leads to the selection of resistant and more virulent pathogens (Schar et al. 2020). With this concern in mind, it is imperative to develop technologies and management practices that ensure an environmentally friendly and sustainable aquaculture.

Different approaches have been explored with integrated multi-trophic aquaculture (IMTA) emerging as a promising solution (Alexander et al. 2016; Granada et al. 2016). This approach involves the co-culture of species from different

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trophic levels, where the waste from one species is used as nutrient by another. These systems offer dual benefits: they mitigate the nutrient load of waste effluent, thereby minimising environmental impacts, while simultaneously diversifying production, enhancing economic resilience and resource utilization (Chopin 2012). Among IMTA systems, those that use the sea lettuce *Ulva* Linnaeus (Ulvophyceae, Chlorophyta) are of interest, as members of this genus efficiently filter effluents with high nutrient uptake rates, especially for nitrogen and phosphorus (Nederlof et al. 2022). *Ulva ohnoi* M. Hiraoka & S. Shimada is a particularly suitable choice for treating effluents from the culturing of Senegalese sole (*Solea senegalensis*) in an IMTA recirculation aquaculture system (IMTA-RAS) due to its adaptability to different culture conditions, rapid growth, vegetative reproduction and the capacity for unattached cultivation (Oca et al. 2019). Moreover, *Ulva* biomass holds significant potential as a valuable resource for both animal and human consumption, and for obtaining high-value products like ulvan (Simon et al. 2022).

Another promising solution to improve the health and productivity of marine organisms in aquaculture is microbiome manipulation (Li et al. 2023). This approach moves away from the traditional "*beat them*" concept, characterized by stringent aseptic and antimicrobial measures to prevent pathogen outbreaks, towards the "*join them*" strategy, focusing on promoting stable and beneficial microbiomes that prevent the emergence of pathogens by niche competition (De Schryver and Vadstein 2014; Bentzon-Tilia et al. 2016; Vadstein et al. 2018). A common intervention to alter microbiome composition is the inoculation of probiotics (Pérez-Sánchez et al. 2018). While most commercial probiotics used in aquaculture are traditionally derived from terrestrial sources (Lakshmi et al. 2013; Lazado et al. 2015), studies suggest that host-associated probiotics, derived from aquatic environments or hosts, may offer better efficacy (Van Doan et al. 2020).

Members of the Rhodobacteraceae, such as *Phaeobacter* spp., are often found in association with marine organisms, including algae (Pujalte et al. 2014; Pintado et al 2023), and are rapid colonizers of surfaces (Dang and Lovell 2002; Dang et al. 2008; Elifantz et al. 2013). This behaviour has been attributed to their ability to form biofilms and to produce antibacterial compounds (Bruhn et al. 2007; Bech et al. 2023). Some strains of the genus *Phaeobacter* produce tropodithietic acid (TDA) (Brinkhoff et al. 2004), which is antagonistic against several fish pathogens, such as *Vibrio* spp. (Planas et al. 2006; D'Alvise et al. 2013). For this reason, *Phaeobacter* species have been studied as potential probiotics in marine fish aquaculture (Sonnenschein et al. 2021). For example, feeding of turbot larvae with *Phaeobacter* strain 27–4 bioencapsulated in the live prey (Planas et al. 2006) or immobilized in biofilters (Prol-García and Pintado 2013) have been found to reduce the mortality when a *Vibrio anguillarum* infection was induced.

Both concepts, IMTA and microbiome manipulation, can be combined in a synergistic approach. Recently, an IMTA-RAS has been proposed using a co-culture of flatfish and *U. ohnoi* colonized with *Phaeobacter* sp. 4UAC3, previously isolated from the microbiome of a wild *Ulva* sp. (Pintado et al. 2023). In this system, *Ulva* removes excess nutrients coming from the fish and also serves as natural niche for *Phaeobacter* sp. 4UAC3, which could act as a biological control agent against disease outbreaks. Indeed, this approach reduced mortality of *V. anguillarum*-infected turbot larvae, without significantly altering the microbiome of *Ulva*. However, during the scale-up of the co-culture process, the concentration of *Phaeobacter* sp. 4UAC3 in *U. ohnoi* tended to decrease when cultured in tanks, with a considerable decrease observed in the last weeks of the experiment, highlighting the need for continuous re-inoculation. This decrease in *Phaeobacter* sp. 4UAC3 was speculated to be due to the increased light intensity used for *U. ohnoi* cultivation in the scale-up (Pintado et al. 2023).

To address this issue, the current study aimed to i) assess the effect of light intensity on the colonization and maintenance of *Phaeobacter* sp. 4UAC3 on the surface of *U. ohnoi*, ii) study the changes in the *U. ohnoi* surface microbiome under different light regimes and assess the impact of this changes on *Phaeobacter* sp. 4UAC3 maintenance and iii) optimize the co-culture of *Phaeobacter* sp. 4UAC3 and *U. ohnoi* by exploring variations in light intensity during different phases of the culturing process. The overall goal is to identify the optimal condition that facilitates the sustainable maintenance of *Phaeobacter* sp. 4UAC3 while promoting efficient algal growth in the IMTA-RAS.

Materials and methods

Algal culture

Ulva ohnoi was obtained from a culture maintained at the Centro Interdisciplinar de Química y Biología (Universidade da Coruña, Galicia, Spain). The sporophyte was originally collected at the Parque Natural de las Marismas del Odiel, Huelva, Spain (Alamrousi et al. 2022). For the experiments, algal blades were immersed in sterile seawater (SSW), rinsed, punched into discs (3.14 cm^2) under sterile conditions, and distributed into 6-well plates filled with filter-sterilised (0.22 µm) *Ulva* Culture Medium (UCM).

The UCM, as described in Pintado et al. (2023), was composed of artificial seawater (33 g L^{-1} of Instant Ocean, Aquarium Systems, France) enriched with Guillard's F/2 medium (CellHi F2P, Varicon Aqua, UK) and supplemented with $NaNO_3$ (20 mgN·L^{-1}) to mimic typical N and P levels

in water in a recirculating fish culture system (Oca et al. 2019).

Bacterial culture

Phaeobacter sp. 4UAC3 (CECT 31030) was previously isolated from blades of *Ulva australis* harvested in Tragobe (Cambados), Bay of Arousa, Galicia, Spain (42°31′8"N—8°49′7"W) and selected for its ability to antagonize *V. anguillarum* (Pintado et al. 2023). The strain was stored at −80 °C in Marine Broth 2216 (MB, BD-DIFCO, Spain) with 15% glycerol and cultured following the procedure described in Prol-García et al. (2009). The concentration of *Phaeobacter* sp. 4UAC3 was determined by counting the colonies forming units (CFU) on Marine Agar 2216 (MA, BD-DIFCO, Spain) plates, where it forms distinctive brown colonies (Bruhn et al. 2005).

Experimental designs

Light effect on *Phaeobacter* colonization and maintenance on different surfaces

Experiment 1: *U. ohnoi* surface To study the persistence of *Phaeobacter* sp. 4UAC3 on cultivated *U. ohnoi* under different light intensities, 6-well plates were prepared containing one *U. ohnoi* disc in 10 mL of UCM per well and 100 µL of a 48-h culture of *Phaeobacter* sp. 4UAC3 with a final concentration of 10^7 CFU mL^{-1}. All plates were placed into an incubator (New Brunswick Scientific Excella E24r Temperature-Controlled Benchtop Shaker, USA) at 18 °C under daylight LED panels (44 W – 6000 K), with a light: dark photoperiod of 12 h:12 h and with an orbital agitation of 80 rpm. Four different light intensities were established using shading meshes placed onto the plate lids or a black box: high light (H, 143 µmol photons m^{-2} s^{-1}, no shading mesh), medium light (M, 75 µmol photons m^{-2} s^{-1}, 1 shading mesh), low light (L, 45 µmol photons m^{-2} s^{-1}, 2 shading mesh) and darkness (D, 0 µmol photons m^{-2} s^{-1}, black box) (Fig. 1).

Algae discs were cultivated under these different light conditions for 14 days. At day 5, after the colonization period, the *U. ohnoi* discs were moved to fresh plates with UCM, in order to maintain only the *Phaeobacter* sp 4UAC3 that adhered to the *U. ohnoi* surface, and the medium was further replaced at days 8 and 11. On days 0, 2, 7 and 14, *U. ohnoi* discs ($n=2$) were randomly sampled from each condition for microbial analysis of the *U. ohnoi* surface and the same discs were utilised for growth measurements including, surface area, wet weight, dry weight and specific growth rate (SGR). The SGR was calculated considering the dry weight, with the following formula:

$$\mathrm{SGR}\left(\%\ \mathrm{day}^{-1}\right) = \left(\mathrm{Ln}\left(\frac{X_1}{X_2}\right)\cdot 100\right)/(t_1 - t_2)$$

where X_1 equates to the average of dry weight of collected discs at time one (t_1) and X_2 represents the average of dry weight of collected discs at time two (t_2).

Microbial samples from the surface of *U. ohnoi* collected on days 7 and 14 were selected for 16S rRNA gene sequencing to explore the effects of varying light intensities on the algal surface microbiome during culture.

The UCM in the sampled wells was taken for physicochemical analysis. The pH of the medium was measured with a pH meter (Sension + pH3, HACH, USA). Nitrate was measured following the APHA Method 4500-NO3 (Baird et al. 1992).

Experiment 2: Non-living surfaces

In order to study the direct effect of light on *Phaeobacter* sp. 4UAC3 biofilms, they were grown in two different non-living surfaces (Fig. 1): Glass coverslips (4.84 cm^2) and autoclaved *U. ohnoi* discs (3.14 cm^2). Both supports were sterilized by autoclaving at 121ºC for 20 min. Each type of support was arranged in multi-well plates with 10 mL of UCM and with 100 µL of a 48-h culture of *Phaeobacter* sp. 4UAC3 (as described above). Plates were placed in the culture chamber and exposed to the range of light conditions (H, M, L, D) as described in the previous section. Plates were maintained for 14 days, taking samples randomly on days 0, 2, 7 and 14 from each condition ($n=2$) for *Phaeobacter* sp. 4UAC3 quantification by CFU count plates.

Varying light intensity during culture process of *U. ohnoi* with *Phaeobacter* sp. 4UAC3

With the aim of finding a combination that favoured the best maintenance of *Phaeobacter* sp. 4UAC3 while providing optimal growth of *U. ohnoi*, two experiments were carried out in which light intensity was changed in different successive phases of the culture (acclimatisation, colonization and growth) (Fig. 1). In one experiment (Experiment 3), the light intensity was varied during the colonization phase and high light was used during the growth phase, while in the other (Experiment 4), the light intensity was varied during the growth phase after selecting a light intensity for the colonization phase.

Experiment 3: Varying light during colonization phase *Ulva ohnoi* cultures were maintained for 7 days under low light (acclimatization period), without UCM renewal. At day 7,

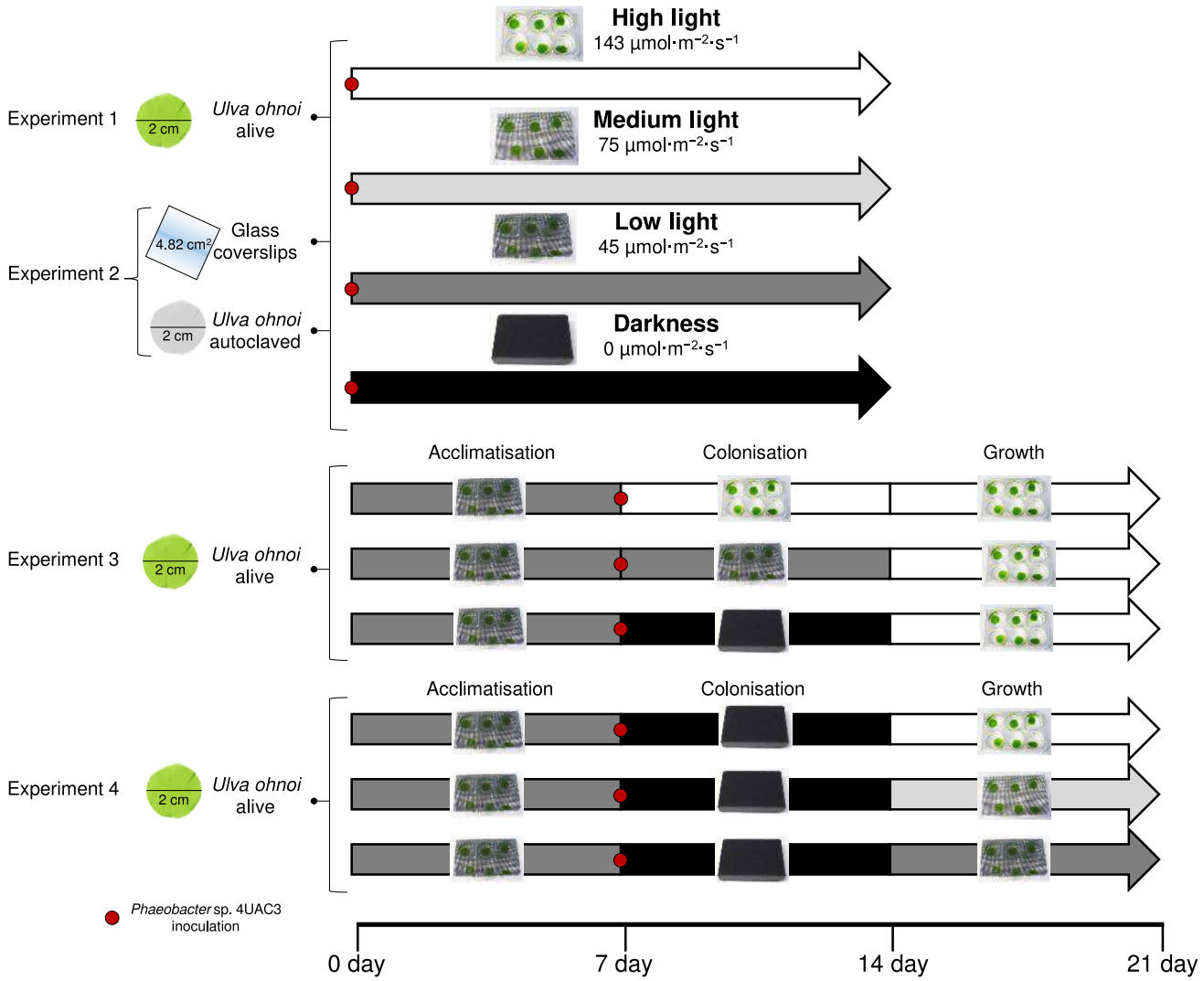


Fig. 1 Experimental overview. The schematic represents the experimental designs that were carried out in this study. The different light intensities used in the cultures (High: 143 µmol photons m^{-2} s^{-1}, Medium: 75 µmol photons m^{-2} s^{-1}, Low: 45 µmol photons m^{-2} s^{-1}, and Darkness) and the surfaces on which *Phaeobacter* sp. 4UAC3 was inoculated (*Ulva ohnoi* alive, glass coverslips and autoclaved *U. ohnoi*) are shown. In experiments 1 and 2 ($n=2$), *Phaeobacter sp.* 4UAC3 was inoculated at time 0 days and the cultures were maintained at a constant light intensity throughout the experiment. In experiments 3 and 4 ($n=3$), *Phaeobacter sp.* 4UAC3 was added at time 7 days, and the light intensity was varied as the culture progressed

the medium was renewed adding the inoculum of *Phaeobacter* sp. 4UAC3 (10^7 CFU·mL^{-1}) in 10 mL of UCM to each well and plates were exposed to either of three different light conditions: high light (H), low light (L) or darkness (D) until day 14 (colonization period). At day 12, the discs were placed into a new plate with 10 mL of fresh UCM, in order to maintain only the *Phaeobacter* sp. 4UAC3 adhered to the *Ulva's* surface. At day 14 all the plates were changed to high light conditions (growth period) and maintained under these conditions, with UCM renewal every three days, until day 21.

Samples of algal discs were taken at 0, 2, 7, 14 and 21 days ($n=3$), for microbial analysis and growth measurements. Microbial surface samples from days 0, 14, and 21 were specifically selected for 16S rRNA sequencing. Water physiochemistry (nitrates consumption and pH) was also determined on these days. Additionally, the algae cultures in the plates were maintained until day 26, and samples for growth analysis were taken at day 22 and 26. Furthermore, images of algae disc cells at day 22 were taken using an optical microscopy with bright field (Nikon SS-Fi1,

Nikon Metrology NV, Belgium) and processed with NIS-Elements BR 2.30 software (Nikon Metrology NV).

Experiment 4: Varying light during growth phase *Ulva ohnoi* discs culture started with the same acclimatization conditions as Experiment 3. During colonization phase all plates were introduced in a black box until day 14. At day 14, the discs were exposed to different light intensities (H, M, L), with UCM renewal every three days, until day 21. Samples were taken at 0, 7, 12, 16 and 21 days ($n = 3$), to determine algae growth, water physicochemistry and microbial analysis, including 16S rRNA sequencing.

Microbial sampling

Ulva ohnoi discs or glass slides were washed thrice with SSW under a laminar flow hood. Then, the top side of individual discs or glass slides were swabbed for one minute using a sterile cotton swab. The swab was placed in a microcentrifuge tube containing 1 mL of SSW and vortexed vigorously for 2 min. The cotton swab was removed, and serial dilutions were plated on MA. An aliquot of suspension was centrifuged at 13,200 × *g* for 15 min and the microbial pellet was stored at − 20 ºC for DNA extraction.

16S rRNA gene amplicon sequencing

DNA from frozen microbial pellets isolated from *U. ohnoi* or glass slide swabs was extracted using INSTAGENE MATRIX (Bio-Rad Laboratories, USA) following the manufacturer´s instructions. To characterise the resulting microbiomes from individual swabs, DNA extracts were subjected to 16S rRNA gene amplification and sequencing as described in Pintado et al. (2023). The primers (NOCHL_F and NOCHL_R) used target the V3-V4 variable region (Thomas et al. 2020) (Supplementary Table 1). The amplified products were checked by electrophoresis and then sequenced on a MiSeq 2 × 300 Illumina sequencer following the manufacturer's instructions at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia). Sequences were deposited in NCBI BioProject: PRJNA1161580.

Bioinformatic and statistical analyses

To assess whether light intensity had a significant impact on the concentrations of *Phaeobacter* sp. 4UAC3 on the algae (CFU counts), as well as the weights and surface areas of *U. ohnoi* discs, a series of statistical tests were conducted. When the data met the assumptions of normality and homoscedasticity, as confirmed by the Shapiro–Wilk and Levene tests, an ANOVA was performed, followed by Tukey's post-hoc test for multiple comparisons. If the data did not meet these assumptions, non-parametric tests, specifically the Kruskal–Wallis test followed by the Nemenyi test, were applied. Due to the small sample size ($n = 2$) in Experiment 1 and 2, no statistical tests can be done and the results should be interpreted with caution. However, these preliminary results were used as the basis for the design of Experiments 3 and 4.

The amplicon sequence data from *U. ohnoi* surface microbiome was processed as described in Pintado et al. (2023). Briefly, Trimmomatic (version 0.38) (Bolger et al. 2014) was utilized to filter low-quality reads truncating them when their quality dropped below 15 in a sliding window of 4 bp or removing them when they were shorter than 100 bp. Paired-end reads were merged if they had a similarity of at least 80% using USEARCH (version 11.0.667) and subsequently filtered out if they were shorter than 370 bp or longer than 440 bp, had low quality (expected error > 1) or more than one ambiguous base (Wemheuer et al. 2017). Amplicon Sequence Variant (ASVs) were generated using the UNOISE3 algorithm implemented in USEARCH and chimeric and singleton sequences were removed using the UCHIME2 algorithm (high confidence mode) (Edgar 2016). ASV sequences were classified using a Bayesian taxonomic classification method BLCA (Gao et al. 2017) (default settings) against the genome taxonomy database (GTDB 207.0) (Parks et al. 2020; Chaumeil et al. 2022).

Data analysis and statistical tests of bacterial community structure were conducted in R version 4.3.1, following similar methodologies described in Pintado et al. (2023). Sampling efficiency and sequencing depth were analysed using rarefaction curves (vegan R package) and the Good's coverage index (QsRutils R package).

The samples were analysed at each time point separately, considering only light as a factor, as the aim of this study is to analyse the effect of light on bacterial communities and the maintenance of *Phaeobacter* sp. 4UAC3. Analysing each time separately, keeping light factor as the only variable, allows us to evaluate how this factor influence the results without the interference of time.

Before performing the statistical analysis, the ASV corresponding to *Phaeobacter* sp. 4UAC3 was identified using the "blastn" function and removed from the data set to exclude the variance caused by its abundance (Bech et al. 2023). Count data were normalized using the DESeq2 package (Love et al. 2014) and were used for measurements of diversity indices, as well as in distance-based analyses and plots. To analyse the bacterial community structure, Bray–Curtis similarity coefficients were calculated using normalized abundances of the ASV sequences (square root transformed data) and the resulting similarity matrix was visualized using non-metric multi-dimensional scaling (nMDS).

The effect of light intensity on the overall community composition in each time point was assessed with permutational multivariate analysis of variance (PERMANOVA)

using Bray–Curtis similarity with 999 random permutations ("adonis" function in the R "vegan" package) (Anderson 2001) and data dispersion assessed using Permutational Multivariate Analysis of Dispersion (PERMDISP) (Warton et al. 2012).

Measures of alpha diversity, including richness, Shannon and Simpson diversity indices, were calculated using the vegan package (Oksanen et al. 2022). Analysis of variance (ANOVA) followed by Tukey's pairwise comparison test was used to assess statistical significance (p-value ≤ 0.05) between groups adjusted by the Benjamini–Hochberg procedure.

The R package "mvabund" was used to determine which ASVs were affected by light intensity at each time point. Each ASV was treated as individual variable and analysed using a separate univariate generalized linear models (GLMs) with a negative binomial distribution (Wang et al. 2012). The model assumes a quadratic mean–variance relationship and a log-linear response. This assumption was confirmed, as the mean–variance relationship showed no patterns in the Residuals vs. Fitted plot, and the quantile–quantile (QQ) plot displayed a straight line, indicating a good model fit. ASVs with a p-value ≤ 0.05 were considered significantly affected by the different factors considering in each experiment. Spearman correlation was performed on the abundance of significantly affected ASVs to further explore their relationships with light intensity.

Results

The effect of light on *Ulva-Phaeobacter* culture: Experiments 1 and 2

Phaeobacter sp. 4UAC3 colonization and maintenance

Phaeobacter sp. 4UAC3 (Fig. 2A-D) on living *U. ohnoi* discs (Experiment 1) reached a maximum concentration at day 2 for all light intensities, with the highest concentrations occurring in dark conditions (6.49 ± 0.85 Log_{10}(CFU cm^{-2})) compared to high (4.65 ± 0.698 Log_{10}(CFU cm^{-2})), medium (4.63 ± 0.289 Log_{10}(CFU cm^{-2})) and low light

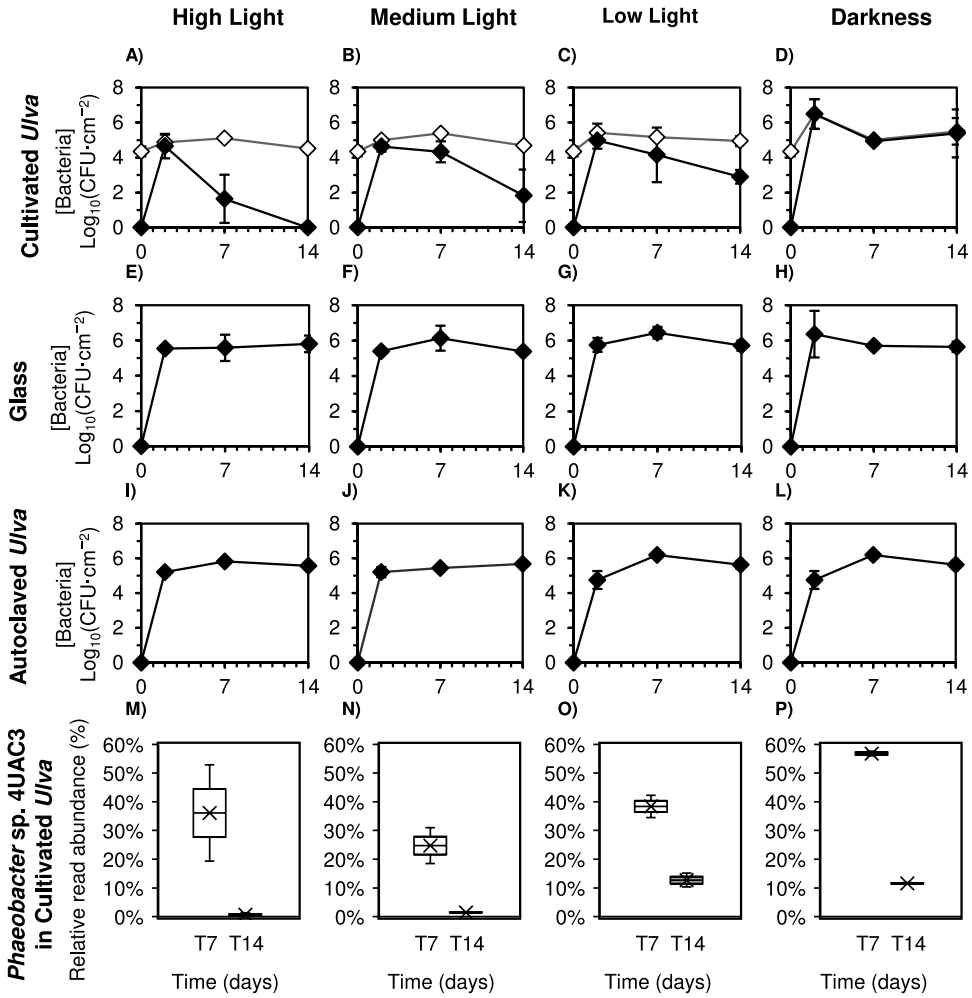


Fig. 2 Total culturable bacteria and *Phaeobacter* on varied surfaces under different light intensity conditions. Variations in total culturable bacteria (white diamonds) and *Phaeobacter* (black diamonds) counts on cultured *Ulva ohnoi* discs (A-D), cover glass slips (E–F) and autoclaved *Ulva ohnoi* discs (I-L) over a 14-day culture period. Also, P*haeobacter* sp. 4UAC3 ASV relative read abundance in Cultivated *Ulva* (M-P) is represented as boxplot under different light intensities and at days 7 and 14 (The Tx_ notation represents the culture times when the samples were collected). Light conditions include: High Light (A, E, I, M), Medium Light (B, F, J, N), Low Light (C, G, K, O), and Darkness (D, H, L, P). These measurements were conducted using marine agar culture medium. Each data point represents the mean (n = 2) at different sampling times (0, 2, 7, and 14 days), with error bars indicating standard deviations

(4.97 ± 0.468 Log_{10}(CFU cm^{-2})). Total culturable bacteria (Fig. 2A-D) showed stable and similar values ($\approx 10^5$ CFU·cm^{-2}) under all light conditions. A decrease of *Phaeobacter* sp. 4UAC3 was observed throughout the experiment, especially in high light samples, where *Phaeobacter* sp. 4UAC3 was not detected at day 14. Conversely, in darkness *Phaeobacter* sp. 4UAC3 remained at almost constant concentrations of ~ 5.38 ± 1.369 Log_{10}(CFU mL^{-1}). In contrast, there was no impact of light at any time point on *Phaeobacter* sp. 4UAC3 colonization when cultivated on either glass (Fig. 2E-H) or autoclaved *U. ohnoi* discs (Fig. 2 **I-L**), with concentrations of *Phaeobacter s*p. 4UAC3 reaching between 5.1–6.4 Log_{10}(CFU cm^{-2}).

Algae growth and nutrient consumption

Ulva discs grown at different light intensities showed continuous growth, except for those kept in darkness. By the end of the experiment, dry weight reached similar values across different light intensities (H: 20.10 ± 0.99 mg, M: 18.65 ± 1.48 mg, L: 21.95 ± 0.21 mg), and surface area was slightly higher under medium light (14.86 ± 0.483 cm^2) compared to high (9.69 ± 2.33 cm^2) and low light (9.08 ± 0.76 cm^2, Fig. 3).

The specific growth rate (SGR) based on dry weight (Fig. 3) of *U. ohnoi* discs grown in light conditions had a maximum value on day 2 for medium (18.32% day^{-1}) and low light (16.15% day^{-1}) and on day 7 for high light (13.57% day^{-1}). Then, a decreased of SGR was observed in all cases.

The pH (Supplementary Fig. 1) of UCM in samples from *U. ohnoi* grown in light conditions remained above pH 9.0 for the first 7 days, decreasing on day 8 to pH 8.6. In contrast the pH of samples maintained in dark conditions, fell to pH 8.5 or below within 2 days. The consumption of nitrates (Supplementary Fig. 1) in *U. ohnoi* cultivated in light conditions was similar for all light intensities, with levels reducing from 20 to below 10 mgN-NO_3^- L^{-1} in all cases (with some days below 1 mgN-NO_3^- L^{-1}). In samples where *U. ohnoi* were kept in the dark no or little nitrate consumption was observed.

Bacterial community analysis on cultivated *U. ohnoi*

The ASV corresponding to the inoculated *Phaeobacter* sp. 4UAC3 (Fig. 2 **M-P**) showed a similar trend in each tested condition as observe with the CFU counts (Fig. 2). As for the bacterial community, Alpha diversity seems to be similar between the light intensities used at the different sampling times according (Supplementary Fig. 2). Based on the nMDS plot, the bacterial community structure of samples kept in the dark showed a tendency to cluster further apart than those kept the light (Supplementary Fig. 3).

Optimizing *Ulva-Phaeobacter* co-culture based on light regime

Experiment 3: Effect of light intensity during *Phaeobacter* sp. 4UAC3 colonization

Phaeobacter *sp. 4UAC3 colonization and maintenance* In Experiment 3, where light was varied during *Phaeobacter* sp. 4UAC3 colonization (from 7 to 14 days), total culturable bacteria (Fig. 4) showed stable and similar values under all light conditions. In contrast, the concentrations of *Phaeobacter* sp. 4UAC3 differed according to the light conditions used during its colonization. For example on day 14 (Fig. 4) *Phaeobacter* sp. 4UAC3 concentrations were lower in *U.*

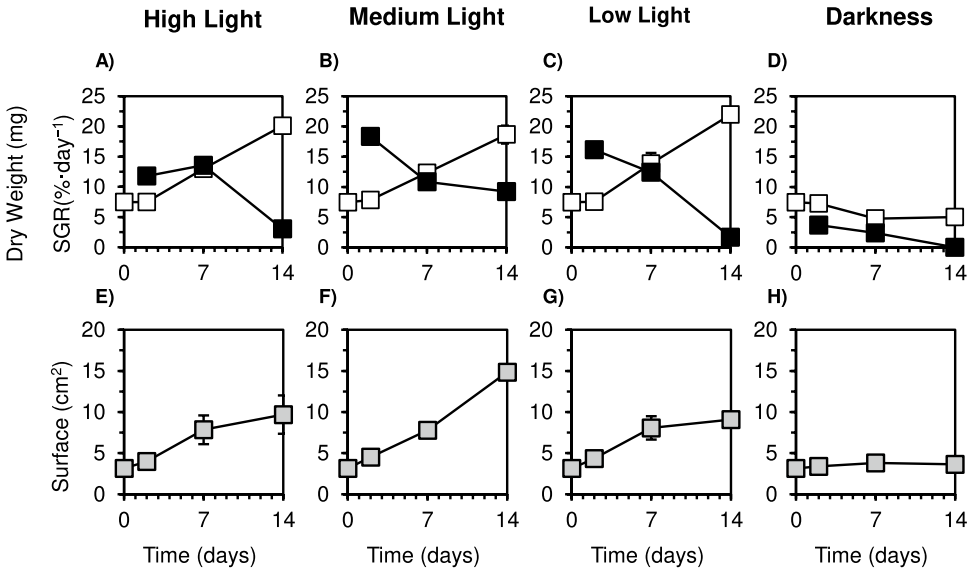


Fig. 3 Growth of *Ulva* discs cultured under different light intensities from Experiment 1. Dry weight (mg, white squares, A-D), specific growth rate based on dry weight (SGR, %·day^{-1}, black squares, A-D) and surface (cm^2, grey squares, E–H) of the algal discs over time of Experiment 1. The different light intensities correspond to: High Light (A, E), Medium Light (B, F), Low Light (C, G), and Darkness (D, H). Each data point in the graph for dry weight and surface represent an average ($n = 2$) and the error bars represent the standard deviations

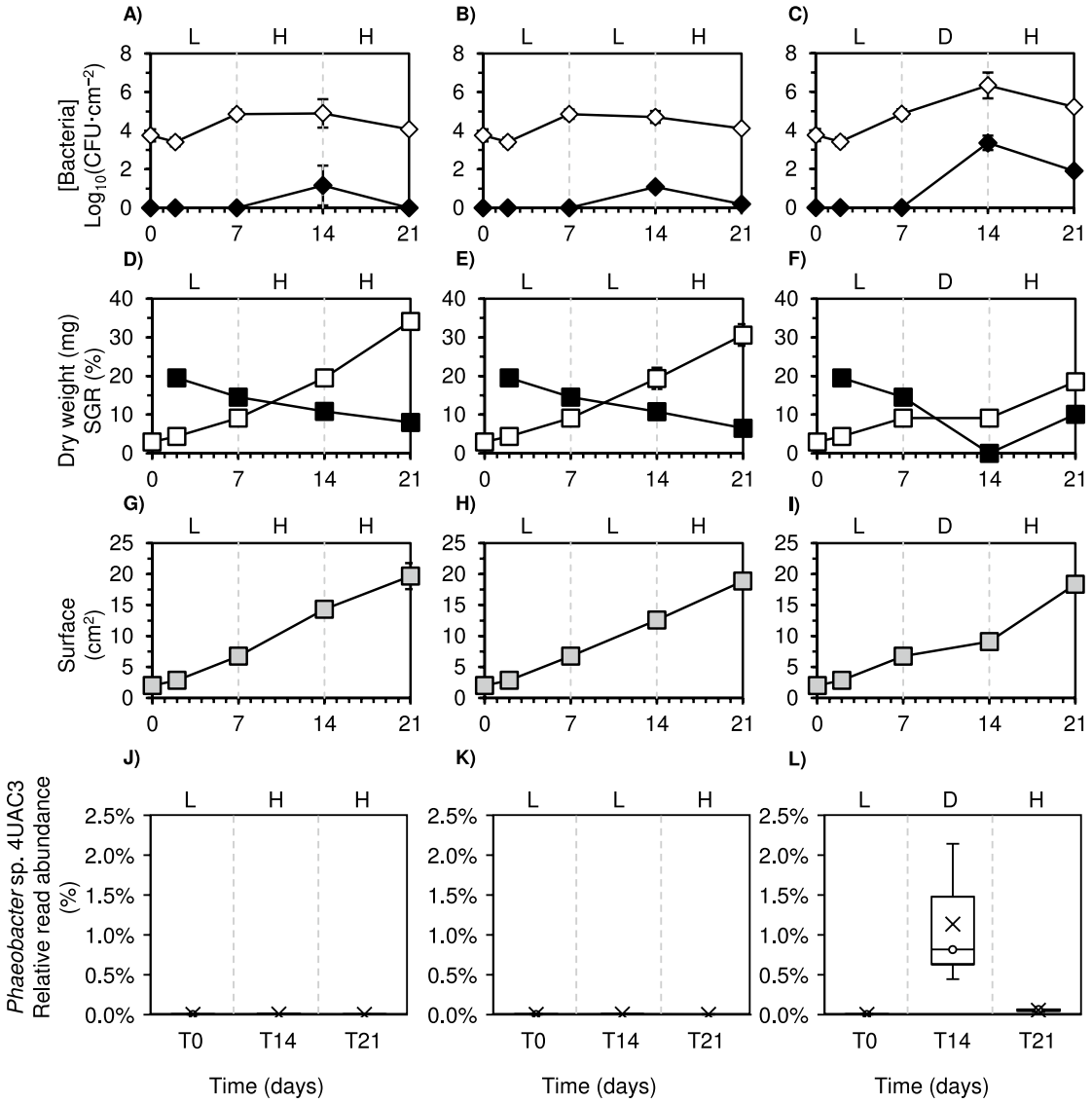


Fig. 4 Total bacteria and detection of Phaeobacter on the surface of *Ulva ohnoi* discs and algal growth parameters in Experiment 3. Changes in the number of total bacteria (white diamonds) and *Phaeobacter* bacteria (black diamonds) on *Ulva ohnoi* discs as well as algal growth parameters including the dry weight (mg, white squares), specific growth rates (SGR, % day^{-1}, black squares) and surface area (cm^2, grey squares), and relative read abundance (%) of *Phaeobacter* sp. 4UAC3 (white box plot) in Experiment 3, where light intensity was changed during colonization phase, are shown. The different light intensities used were: High (H), Low (L), and Darkness (D). The Tx_ notation represents the culture times when the samples were collected. The different phases of the experiments are separated by discontinue grey lines and they were: Acclimatization (from 0 to 7 days using L), Colonization (when *Phaeobacter* sp. 4UAC3 was inoculated, from 7 – 14 days testing different light intensities: H, L and D) and Growth (14 to 21 days using H). Each data point represents an average ($n = 3$) and error bars indicate standard deviations

ohnoi exposed to light during the colonization phase (< 2 Log_{10}(CFU) cm^{-2}), whereas in those where *Phaeobacter* sp. 4UAC3 colonization was carried out in the dark, numbers reach over 3 Log_{10}(CFU) cm^{-2}. By day 21, *Phaeobacter* was not detected by plate count in samples where *Phaeobacter* sp. 4UAC3 colonization occurred under high light conditions. For low light conditions, the concentration of *Phaeobacter* sp. 4UAC3 on the *U. ohnoi* discs was below 1 Log_{10}(CFU) cm^{-2}, whilst samples that were colonized with *Phaeobacter* sp. 4UAC3 under dark conditions numbers of *Phaeobacter* sp. 4UAC3 remained at 1.9 ± 0.2 Log_{10}(CFU) cm^{-2}.

Although analysis at day 14 did not have enough statistical power to detect the differences among light intensities, at day 21 statistical support was found for an improved maintenance of *Phaeobacter* sp. 4UAC3 when colonization of *U. ohnoi* occurs in the dark rather than at high light (p-value = 0.020, Supplementary Table 2).

Algae growth and nutrient consumption The algal dry weight and surface area increase (Fig. 4) was linear for samples exposed to high and low light intensity. As expected, the *U. ohnoi* kept in the dark during colonization experienced an arrest in growth (days 7 – 14), however recovered in the subsequent growth phase (days 14 – 21) with light achieving a SGR similar to the other light conditions (10%·day^{-1}, Fig. 4). The final dry weight values at day 21 for each condition were: 34.15 ± 0.48 mg for the algae colonized with high light, 30.63 ± 2.79 mg for low light and 18.53 ± 1.21 mg for darkness. At day 21, dry weight of *U. ohnoi* discs exposed to high light during colonization were significantly higher than discs maintained in darkness (p-value = 9.11×10^{-8}, Supplementary Table 3), however the statistical analysis of surface area did not find a significant difference in that period of time (Supplementary Table 4). Although the algae exposed to high light during the colonization phase had a higher dry weight than the other conditions, visual observations detected a higher amount of bleached areas (Supplementary Fig. 4 D) compared to the other light conditions, and in fact, after 26 days, it was observed that the dry weights of *U. ohnoi* discs (Supplementary Fig. 4 E) exposed to high light during colonization were significantly reduced (p-value = 0.011, Supplementary Table 5). The algae subjected to darkness during colonization showed less shrinkage of their cytoplasm (Supplementary Fig. 4 C) than the other light conditions (Supplementary Fig. 4 A and B). This observation influenced the decision to implement a colonization period under darkness in Experiment 4, as the algae exhibited better cellular conditions and achieved satisfactory growth when afterwards being exposed to light.

As observed in Experiment 1, when *U. ohnoi* discs were exposed to different light intensities, the nitrates were almost completely consumed before the UCM change, and pH values increase above 9. In contrast samples that remained in darkness did not show any nitrate consumption and the pH remained below 8.5 (Supplementary Fig. 5).

Analysis of bacterial communities The ASV corresponding to inoculated *Phaeobacter* sp. 4UAC3 was detected by 16S rRNA sequencing at day 14 and 21 (Fig. 4). Samples exposed to darkness during colonization phase showed the highest relative read abundance (1.13 ± 0.89%). There was no support for an effect of light on the alpha diversity of bacterial communities associated with *U. ohnoi* (Supplementary Fig. 6, Supplementary Table 6). Results of an nMDS ordination (Supplementary Fig. 7) showed that the structure of bacterial communities at time 0 and at time 14 for communities that were colonized by *Phaeobacter* sp. 4UAC3 under dark condition grouped differently from other samples. PERMANOVA at time 14 (Supplementary Table 7) indicated significant differences between the bacterial communities exposed to different light regimes, however, pairwise comparisons did not identify which conditions were different. At day 21 all samples grouped equally based on the nMDS (Supplementary Fig. 7), and PERMANOVA did not find support for a significant differences between samples (Supplementary Table 7). The results of the *mvabund* analysis at day 14 (Supplementary Fig. 8) indicated that 8 ASVs were significantly affected by light intensity used at colonization phase, and these ASVs belonged to the genera *Aliiglaciecola*, *Alloprevotella*, *Corynebacterium*, *Glaciecola*, *Maribacter*, *Pelagibacterium*, *Roseobacter* and *Streptomyces*, which were all positively correlated with light intensity. At day 21, 4 ASVs were significantly affected by light intensity belonged to the genera *Boseongicola* and *UBA6002* (Gammaproteobacteria), which were positively correlated with light intensity, and *Fabibacter* which was negatively correlated with light intensity (Supplementary Fig. 8).

Experiment 4: Varying light during *U. ohnoi* growth phase

***Phaeobacter* sp 4UAC3 colonization and maintenance** In Experiment 4, where colonization was conducted in darkness and light was varied during the growth phase (from 14 to 21 days), total culturable bacteria and *Phaeobacter* sp. 4UAC3 maintained a similar concentration on *U. ohnoi* surface across the different light conditions. A decrease of *Phaeobacter* sp. 4UAC3 appeared earlier (at 16 days) in high and medium light treatments, which was statistically significant (p-value = 0.020). *Phaeobacter* sp. 4UAC3 showed a longer maintenance in low light conditions, with a slight decrease at the end of the growth phase (day 21), reaching similar levels as the other light conditions (Fig. 5 and Supplementary Table 8).

***U. ohnoi* growth and nutrient consumption** *Ulva ohnoi* discs showed no growth in the colonization phase in darkness, but they resumed growth upon exposure to light from day 14 to day 21 (Fig. 5). At day 16, significant differences in dry weight and surface area were observed between the light conditions, being higher in discs growth with medium light (Supplementary Table 9 and Supplementary Table 10). By day 21, while statistical analysis of dry weight did not find support for significant differences and remained across all light conditions. Only the surface area showed significant variation among all of them, being slightly lower in discs growth with low light (17% and 25%), compared with high and medium, respectively. (Supplementary Table 9 and Supplementary Table 10). As in the previous experiments, nitrate consumption was observed only when algae were exposed to light and pH values increased above 9 in those cases (Supplementary Fig. 9).

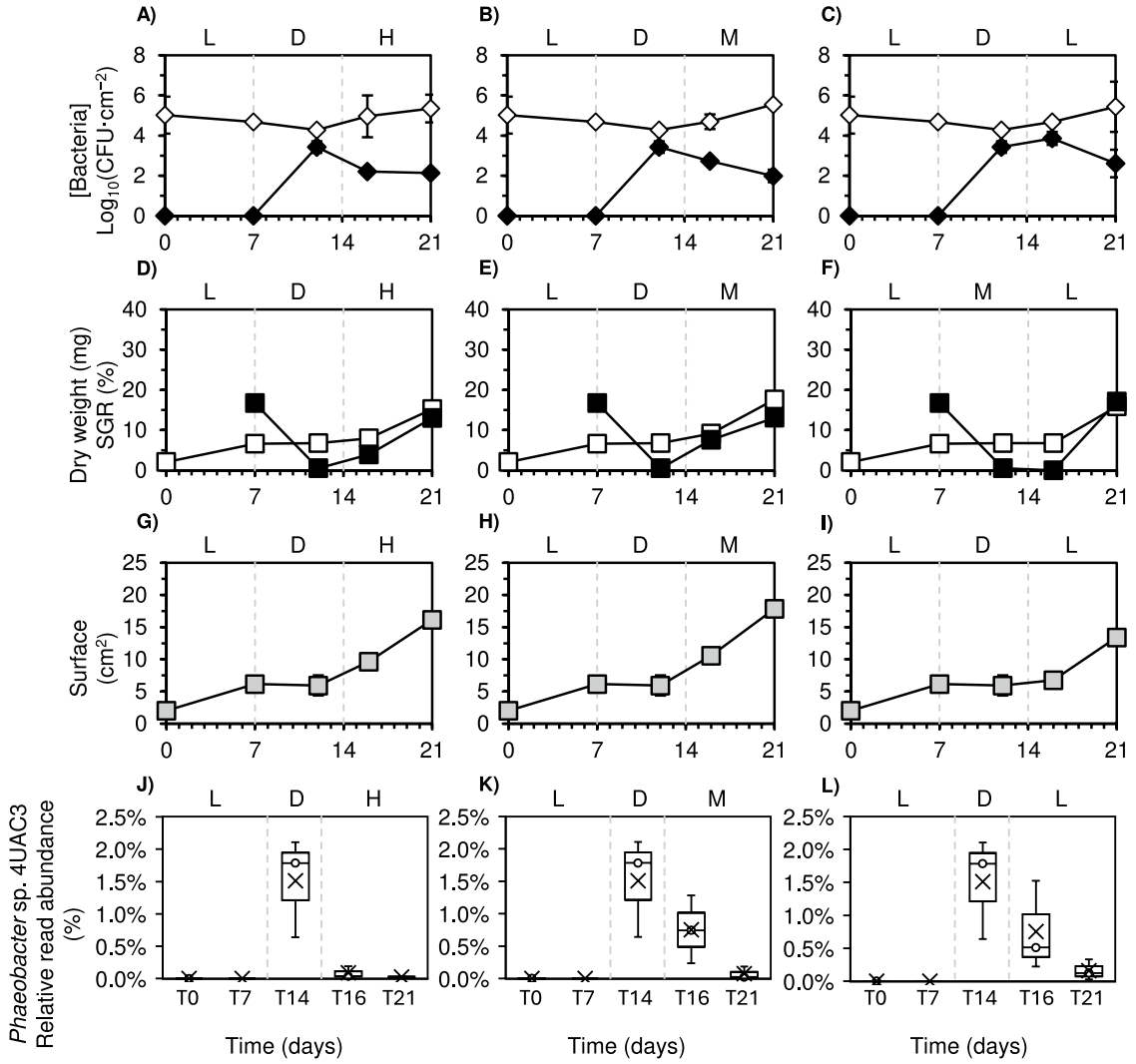


Fig. 5 Total bacteria and detection of Phaeobacter on the surface of *Ulva ohnoi* discs and algal growth parameters in Experiment 4. Changes in the number of total bacteria (white diamonds) and *Phaeobacter* bacteria (black diamonds) on *Ulva ohnoi* discs as well as algal growth parameters including the dry weight (mg, white squares), specific growth rate (SGR, % day^{-1}, black squares) and surface area (cm^2, grey squares) and relative read abundance (%) of *Phaeobacter* sp. 4UAC3 (white box plot) in Experiment 4, where light intensity was changed during growth phase, are shown. The different light intensities used were: High (H), Medium (M) Low (L), and Darkness (D). The different phases of the experiments are separated by discontinue grey lines and they were: Acclimatization (from 0 to 7 days using L), Colonization (when *Phaeobacter* sp. 4UAC3 was inoculated, from 7 – 14 days using D) and Growth (14 to 21 days testing different light intensities: H, M and L). The Tx_ notation represents the culture times when the samples were collected. Each data point represents an average ($n = 3$) and error bars indicate standard deviations

Analysis of bacterial communities Sequencing results showed that *Phaeobacter* sp. 4UAC3 (Fig. 5) was detected on day 12, with the highest relative read abundance values, as well as on days 16 and 21 where the relative read abundances decreased drastically. Overall, alpha diversity (Supplementary Fig. 10) displayed no significant differences between different light conditions on days 16 and 21 (Supplementary Table 11). The inverted Simpson index on day 16 exhibited significant differences between the light intensity, being higher in low light intensities compared to the others. While the nMDS plot (Supplementary Fig. 11) showed close clustering of bacterial communities across most samples, exceptions were noted for samples at day 0 and day 12. Despite these trends, PERMANOVA analysis revealed no significant differences in bacterial communities among light conditions on days 16 and 21, or even when compared with samples from day 12 (Supplementary Table 12). *Mvabund* analysis at day 16 detected only 5 ASVs to be significantly affected by light intensity, belonging to the genera *Empedobacter*, *Meiothermus*, *Tateyamaria*, and *Pseudomonas*, which were positively correlated with light intensity and *GCA-2402195* (Rickettsiaceae), which was

negatively correlated with light intensity. At day 21, 8 ASVs were identified as significantly affected by light intensity and belonged to the genera *Neptunochlamydia*, *Streptomyces*, *Halomonas*, *Caldicellulosiruptor*, *Parageobacillus* and *Pseudomonas*, which were positively correlated with light intensity, and *Alteromonas* and another *Halomonas*, which were negatively correlated with light intensity (Supplementary Fig. 12).

Discussion

Previous results showed that *Phaeobacter* sp. 4UAC3 on *U. ohnoi* tended to decrease with each step of the up-scaling process, which was speculated to be due increased light intensity in each up-scale step (Pintado et al. 2023). Our results confirm that *Phaeobacter* sp. 4UAC3 is reduced from the surface of *U. ohnoi* cultured at high light intensities. Nevertheless, light has no direct negative impact on *Phaeobacter* sp. 4UAC3 as it remained at a constant concentration on non-living surfaces regardless of light conditions. Therefore, the reduction of *Phaeobacter* sp. 4UAC3 is either due to the physiological response of *U. ohnoi* to light and/or due to an effect on other microbiome members, which in turn could indirectly impact *Phaeobacter* sp. 4UAC3 (González and Moran 1997; Kessler et al. 2018; Califano et al. 2020; Henriksen et al. 2022; Morris et al. 2022).

Algal photosynthesis is strongly influenced by light causing, for example, fluctuations in pH, oxygen (O_2), and dissolved inorganic carbon (DIC) in the surrounding environment (Middelboe and Hansen 2007; Hurd et al. 2011; Layton et al. 2019). Algal photosynthesis reduces DIC, raises pH, and increases O_2, while in the dark respiration elevates DIC, lowers pH, and reduces O_2. These variations occur in boundary layers formed on the macroalgae's surface at scales of micro- to millimetres, creating distinct chemical microenvironments compared to the surrounding seawater (Hendriks et al. 2017). These variations in the microenvironment or phycosphere may particularly affect *Phaeobacter* sp. 4UAC3. It has been demonstrated that photosynthesis by *Ulva intestinalis* can elevate the pH in rock pools to levels as high as pH 10 (Björk et al. 2004), and although most *Phaeobacter* species can tolerate pH levels ranging from 6 to 9, they usually have optimal growth within a pH range of 7 to 8 (Supplementary Table 13). This could explain the reduction of *Phaeobacter* sp. 4UAC3 under light exposure, where in our study the pH rose above 9.0.

As another option, *Phaeobacter* sp. 4UAC3 could be affected by reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) generated by photosynthesis (Fridovich 1978). *Ulva* species produce notably higher levels of H_2O_2 compared to other macroalgae (Twigg et al. 2020), potentially as a defence mechanism against epiphytes and endophytes (Collén et al. 1995) and at levels sufficiently high to have a physiological and ecological effect on bacterial activity (Twigg et al. 2020). Studies have also reported that the production of H_2O_2 in *Ulva rigida* increased with higher light levels (Collén et al. 1995, 1996) and elevated pH (Collén et al. 1995), reaching concentrations of 1.2 µmol g^{-1} FW h^{-1} (pH 8.2) or 5.0 µmol g^{-1} FW h^{-1} (pH 9) in seawater at 700 µmol photons·m^{-2}·s^{-1} irradiance (Twigg et al. 2020). Although no minimum inhibitory concentration of H_2O_2 has yet been described for *Phaeobacter* species, *Phaeobacter* sp. strain Y4I has been reported to be sensitive to H_2O_2 and non-viable cells were observed when biofilms were exposed to 75 mM H_2O_2 for 10 min (Cude et al. 2012).

The reduction of *Phaeobacter* could also be influenced by other metabolites produced by *U. ohnoi* under high light conditions, in fact, recent results (Qui-Minet et al. 2024) show that light regime significantly altered the metabolite production of a co-culture of *U. ohnoi* and *Phaeobacter* sp. 4UAC3. For instance, p-coumaric acid is an essential compound in the synthesis of polyphenols in photosynthetic organisms and is one of the most abundant phenols found in *Ulva* extracts (Douma et al. 2021; Ibrahim et al. 2021). Recently, p-coumaric acid has been shown to induce an oxidative response in *Phaeobacter inhibens*, being toxic at concentrations of 2.5 mM (Wang et al. 2022). It is possible that the production of this compound by *Ulva* could be enhanced by high light intensity, and therefore exert a detrimental effect on *Phaeobacter* sp. 4UAC3. Future work should consider the impact of light on the physical and chemical properties of *U. ohnoi* and the mechanisms by which these changes impact *Phaeobacter* sp. 4UAC3 colonization and viability.

In addition to the physicochemical changes in *U. ohnoi* due to light exposure, the maintenance of *Phaeobacter* sp. 4UAC3 could be indirectly impacted by changes in the surrounding microbiome. Studies on microalgae have described that the disappearance of inoculated *Phaeobacter* in co-cultures may be due to competition with other microbiome members (Henriksen et al. 2022; Morris et al. 2022). In our study, ASVs belonging to genera *Glaciecola*, *Maribacter* and *Roseobacter* tended to increase in relative read abundance when *U. ohnoi* was exposed to light. These genera have been extensively described in different studies on the *Ulva* microbiome. For instance, *Glaciecola* is abundant in *Ulva* cultures (Califano et al. 2020) and possesses enzymes capable of degrading polysaccharides such as ulvan (Mondal and Ohnishi 2020). *Maribacter* is a genus associated with morphogenesis processes in *Ulva* (Kessler et al. 2018) and *Roseobacter* is associated with the degradation of volatile organic compounds such as dimethylsulphoniopropionate (DMSP) produced in algae (González and Moran 1997). While there is no evidence that these genera have direct antagonistic activity against *Phaeobacter* species,

we hypothesize that their increased abundance with light results in competitive exclusion of *Phaeobacter* sp. 4UAC3. Higher light intensity likely stimulates *Ulva* growth, involving changes of morphology and leading to increased production of organic matter and compounds like ulvan (Ibrahim et al. 2022) or DMSP (Karsten et al. 1990; Kessler et al. 2018), which in turn could support the proliferation of these bacterial genera over *Phaeobacter* sp. 4UAC3.

Although the exact reason for the light-dependent disappearance of *Phaeobacter* sp. 4UAC3 on the surface of *Ulva* remains unclear, light is an important factor and therefore needs to be considered when developing strategies to optimise *Ulva-Phaeobacter* co-culture. *U. ohnoi* requires medium to high light intensities to facilitate growth, biomass generation and nutrient reduction when used as a natural biofilter to treat fish culture effluents (Neori et al. 2003; Msuya et al. 2006). For *Phaeobacter* sp. 4UAC3, darkness is the best condition to persist on *U. ohnoi*. The strategy we propose for the maintenance of *Phaeobacter* sp. 4UAC3 on *U. ohnoi* is to combine different light intensities during the process of co-culture, such as colonization phase in the dark for 7 days and a algal growth with low light for 7 days. This can not only increase *Phaeobacter* sp. 4UAC3 colonization and maintenance, without affecting the production of algae biomass, but also prevents bleaching and photosynthetic stress. Other strategies to improve the maintenance of *Phaeobacter* on the *Ulva* surface is to reduce the acclimatisation period. It was decided to include an acclimatisation period in Experiments 3 and 4 to reduce the stress of the algae due to the transport and disc cutting. In these experiences there was an acclimatisation period of 7 days at low light, and a maximum colonisation of *Phaeobacter* of less than 4 $LOG_{10}(CFU\ cm^{-2})$, whereas in Experiment 1, where *Phaeobacter* was inoculated directly without acclimatisation period, concentrations of more than 5 $LOG_{10}(CFU\ cm^{-2})$ were reached within a few days of inoculation, even at high light intensities. This is also noticeable in the relative read abundances of 16S rRNA sequencing. One explanation for these observations could be that after 7 days of growth in the light, the available niches on the algal surface are occupied. On the other hand, it is also possible that in that time the holobiont has created a chemical microenvironment on its surface influenced by low light which could also hinder the establishment of *Phaeobacter*, from pH, ROS or other metabolites such as the p-coumaric acid already discussed above. Thus, it is proposed to reduce the acclimatisation time of the algae. Additionally, it should be noted that the assays condition used here limited algal movement, thus potentially increasing stress, and we expect that growth in scale-up tanks with aeration will provide self-shading reducing the level of stress. Future work will need refine those dark-versus-light condition in full IMTA-RAS implementation.

In conclusion, the results of this study show that *Phaeobacter* sp. 4UAC3 disappears from the surface of *U. ohnoi* as the light intensity increases and demonstrate that this is not due to direct influence of light on the bacterium. The composition of epiphytic communities of *U. ohnoi* are influenced by light, and certain taxa were significantly promoted under high light intensities, which could be involved in *Phaeobacter* sp. 4UAC3 displacement. To fully understand this dynamic and its causal relationship with the disappearance of *Phaeobacter* sp. 4UAC3, further studies are required. These studies should also consider the impact of light on the alga's chemical micro-environment. We also demonstrate that co-culturing with alternating darkness and low light periods can achieve the algae growth and *Phaeobacter* sp. 4UAC3 maintenance. This knowledge will lay the foundation for optimising a fish-algae IMTA-RAS based on the use of *Ulva* colonized with *Phaeobacter*.

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Authors' contributions Gonzalo Del Olmo: Formal analysis, Investigation, Software, Visualization, Writing—original draft, Writing—review & editing Patricia Ruiz: Conceptualization, Investigation, Writing—review & editing Jadranka Nappi: Formal analysis, Software, Writing—review & editing Torsten Thomas: Formal analysis, Resources, Writing—review & editing Suhelen Egan: Formal analysis, Resources, Writing—review & editing Javier Cremades: Conceptualization, Funding acquisition, Resources, Supervision, Writing—review & editing José Pintado: Conceptualization, Funding acquisition, Resources, Supervision, Writing—original draft, Writing—review & editing.

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Declarations

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

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