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RESEARCH

Detection and mitigation of *Paraphysomonas* sp., a chrysophycean responsible for the collapse of industrial *Tisochrysis lutea* cultures

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Abstract

Microalgae are increasingly recognized as a sustainable resource to address challenges associated with climate change and population growth, owing to their capacity to produce high-quality biomass for applications in feed, food, and cosmeceuticals. However, the expansion of microalgae-based industries remains constrained by harmful biological contaminants (HBCs), which can rapidly reduce productivity, often signalled by visible changes in culture appearance (e.g., cell aggregation and discoloration), and may ultimately lead to culture collapse and substantial economic losses. In this study, the grazer *Paraphysomonas* sp. (Chrysophyceae) was identified as the most likely cause of *Tisochrysis lutea* culture collapse in a large-scale production facility using high-throughput amplicon sequencing of the 18S rDNA gene. To enable early detection of this HBC during the scale-up process, a species-specific primer pair targeting the 18S rDNA gene was developed and optimized, allowing detection of *Paraphysomonas* sp. at relative abundances as low as 0.1%. The contaminant was first detected at the final stage of scale-up, in a tubular photobioreactor. To further characterize the impact of the grazer and support mitigation strategies, its feeding behaviour was examined. Laboratory-scale mitigation trials showed that treatment with germanium dioxide (GeO_2) at 1 mg L^{-1} effectively delayed contaminant proliferation and prevented culture collapse without adversely affecting *T. lutea* growth. These findings highlight the value of integrating molecular monitoring with targeted mitigation strategies to improve early detection and management of HBCs in industrial microalgae production systems.

Keywords Microalgae · Industrial cultivation · Harmful biological contaminants (HBCs) · Chrysophyceae · 18S rDNA sequencing · Early detection · *Tisochrysis lutea*

Introduction

Overfishing, the decline of arable land, and the lack of sustainable products are consequences of climate change and human population growth over the past two decades. To address these challenges, new solutions are needed for food, feed, nutraceuticals, and cosmeceuticals (Torres-Tiji et al. 2020). Microalgae represent a promising option for sustainable product development, as they can recycle atmospheric carbon and grow on non-arable land or in wastewater (Levasseur et al. 2020). As photosynthetic organisms, they use light, water, and nutrients to produce high-value compounds. However, the presence of harmful biological contaminants (HBCs) in large-scale production systems remains a serious, yet often overlooked, issue within the scientific community. These contaminants can reduce yields, degrade product quality, or even cause total culture collapse, resulting in significant economic losses (Lam et al. 2018).

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HBCs are generally classified into three groups: competitors (e.g., other photosynthetic microorganisms), parasites (e.g., fungi, viruses), and grazers (e.g., amoeboid and/or flagellated microorganisms) (Lam et al. 2018). Grazers are considered as the most damaging, as they actively feed on microalgae and often produce cysts that can remain dormant until conditions become favourable again (Day 2013). Recently, various approaches have been developed for HBC detection, including optical or electron microscopy, flow cytometry, high-throughput amplicon sequencing, and other molecular methods (Di Caprio 2020). Several physical (e.g., filtration, changes in abiotic factors) and chemical (e.g., growth inhibitors) strategies have been proposed and applied for control following detection (Molina et al. 2019). However, the effectiveness of these mitigation strategies is strongly influenced by the species of both the microalga and the contaminant, as well as the cultivation method used.

Early identification of HBCs is therefore essential to understand and predict their impact on microalgal cultures. This study focuses on the seawater haptophyte *Tisochrysis lutea*, a microalga capable of photo- and mixotrophic growth. Its biomass contains several potentially high value biocompounds, such as polyunsaturated fatty acids (PUFAs) and fucoxanthin, making it a highly sought-after feedstock for aquaculture feed and cosmetic formulations (Mayer et al. 2021). This study aimed to identify the HBC(s) responsible for *T. lutea* culture collapse using high-throughput techniques, including amplicon sequencing targeting the hypervariable V9 region of the 18S ribosomal DNA (rDNA) gene, since most grazers and parasites affecting this haptophyte are eukaryotic. Additionally, to facilitate early detection and control, specific primer pairs were designed, and the feeding behaviour of the HBC(s) was examined through microscopic analysis to support the development of effective mitigation strategies.

Materials and methods

Molecular identification of HBCs

Culture sampling and DNA extraction

To establish a baseline for biological contaminant screening, two sampling procedures were performed (Fig. 1). The first consisted of four different samples: an inoculum sample serving as a negative control (no eukaryotic contamination), an isolated sample from the previous year for comparison with the current sampling, and two reactor samples with *T. lutea* cultures exhibiting high levels of HBCs (Fig. 1A; Table 1). This set of samples was selected for amplicon sequencing, to identify the taxonomic diversity of the biological contaminants present at the industrial scale. They represent independent contamination events occurring in operational photobioreactors and were analysed as case-study observations rather than controlled experiments and were used to design primers for HBC early detection. The second sampling procedure involved a follow-up of the culture to identify where the biological contaminants first appeared (Fig. 1D). Samples of both the culture and the growth medium were collected prior to microalgae inoculation at each step of the *T. lutea* scale-up process (from 5 L to 27 m^3). To replicate the experiment, two scale-ups were monitored in August (culture 1) and September (culture 2) of 2021.

Each sample was harvested into a 50 mL Falcon tube (Fig. 1A and D), centrifuged at 5,752 × *g* for 5 min, and the supernatant was discarded. The resulting biomass was stored at –20 °C prior to DNA extraction, which was performed using the PowerSoil Pro DNA Kit (QIAGEN, Germany) with minor modifications. Briefly, in step 1, Solution CD1 was directly added to the harvested biomass and vortexed to resuspend the cells; the suspension was then transferred to a PowerBead Pro Tube. Additionally, 50 µL of Solution CD6 was added in step 16; after centrifugation, this step was repeated to obtain a final volume of 100 µL. All centrifugation steps were performed at maximum speed (20,000 × *g*). DNA quality and quantity were evaluated using 0.8% agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Scientific).

Amplicon sequencing and taxonomic assignment

The four selected samples from the first sampling procedure were sent to MR DNA (Shallowater, TX, USA) for 100—bp amplicon sequencing of all eukaryotic organisms present (Fig. 1B), using the universal primers euk1391F (5′-GTA CAC ACC GCC CGT C-3′) and EukBr (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) previously described in the literature (Zahedi et al. 2019). These primers target the V9 hypervariable region of the 18S rDNA gene, and sequencing was performed on the Illumina MiSeq platform.

The resulting sequences were clustered into zero-radius operational taxonomic units (zOTUs), representing unique microorganism groups. Each zOTU was analysed using BLASTn (Altschul et al. 1990) to compare sequences against those in the NCBI database. Taxonomic assignments were further confirmed by sequencing the 18S V1–V5 hypervariable regions using the primers 18SUnivFor and 18SUnivRev (Pereira et al. 2011). Phylogenetic analyses were conducted using both the V9 and V1–V5 sequences.

Multiple alignments were performed using MUSCLE (v3.5) (Edgar 2004) via the phylogeny.fr platform (Dereeper et al. 2008). Phylogenetic inference was performed using the maximum likelihood phylogenetic inference implemented in PhyML (v3.0) (Guindon et al. 2005), with

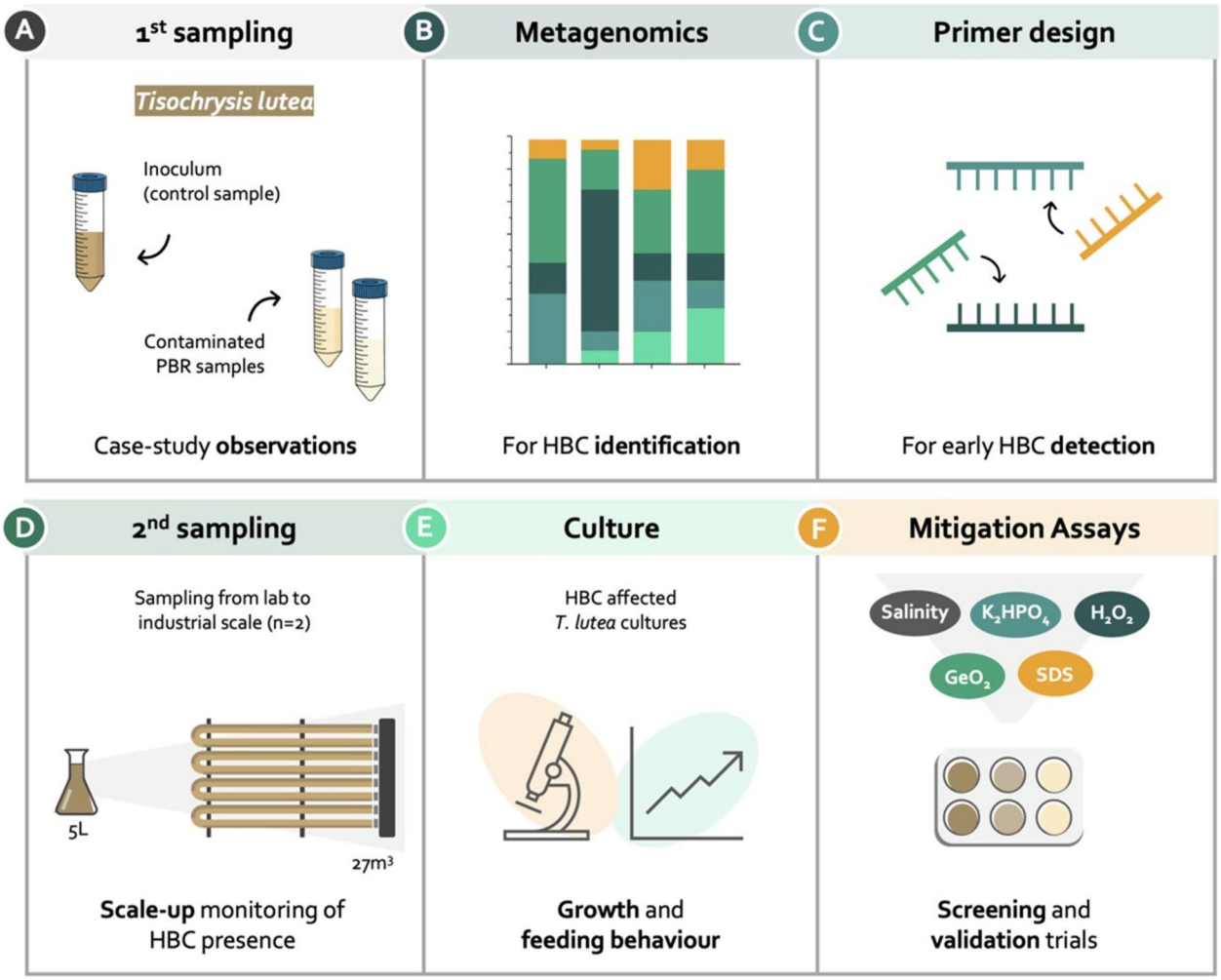

Fig. 1 Schematic overview of the experimental design used for biological contaminant sampling, molecular identification and evaluation of mitigation strategies in *T. lutea* cultures

Table 1 Selected samples for 18S rDNA amplicon sequencing (V9 region)

Species	Sample	Year	Observations
Tisochrysis lutea	1	2021	Inoculum, no HBC detected
	2	2020	Culture from PBR, HBC detected
	3	2021	Collapsed culture from PBR, high HBC levels detected
	4	2021	Collapsed culture from PBR, HBC detected

the best-fit evolutionary model (GTR + Γ; shape parameter = 0.540) selected automatically. Branch support was assessed using a non-parametric SH-like approximate likelihood ratio test (aLRT). Phylogenetic trees were visualized using FigTree (v1.4.3). Morphological confirmation was carried out by comparing microscopic observations of the putative contaminant with published reference micrographs (Scoble and Cavalier-Smith 2014).

Primer design, PCR optimization and sequencing

Following the identification of the collapse-inducing grazer *Paraphysomonas* sp., specific primer pairs Paraphysomonas_1376F (5'- TTA GAG GGA CTT TCG GTG ACT-3') and Paraphysomonas_1549R (5'- TCT ATC CCT AAC ACG ATA CAC A-3') targeting the 18S rDNA gene were designed in-house in this study (Fig. 1C). The

primers were tested on DNA extracted from four commercially available microalgal cultures (*Tetraselmis chui*, *Chlorella vulgaris*, *T. lutea*, and *Phaeodactylum tricornutum*), as well as from three contaminated *T. lutea* cultures from the first sampling procedure (Table 1), under standard PCR conditions.

The optimized PCR conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, with a final elongation step at 72 °C for 10 min. GoTaq G2 Flexi DNA Polymerase (Promega, USA) was used according to the manufacturer's instructions. Briefly, a 20 µL reaction mixture was prepared containing 1 × Colorless GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1 U of GoTaq G2, and 1 µL of DNA template (0.5–2 ng μL^{-1}).

PCR products were visualized by electrophoresis on 1.5% agarose gels stained with GelRed (Biotium, CA, USA). Primer sensitivity was evaluated using serial dilutions of DNA from the most and least contaminated samples, corresponding to a range of 0.1–77% relative read abundance. Primer specificity was verified by Sanger sequencing of samples yielding a single PCR product (Applied Biosystems 3130XL DNA Sequencer, Life Technologies, USA).

Finally, the designed primer pair was used to identify the scale-up step at which the contaminant first appeared (Fig. 1C and D). Briefly, DNA extracted from samples collected during the second sampling procedure (from laboratory to industrial scale) was screened for the presence or absence of PCR amplification using the specific primer pair.

Culture conditions and experimental design

Culture maintenance and monitoring

During the sampling procedure, contaminated *T. lutea* cultures were maintained in 100 mL Erlenmeyer flasks under continuous light (PPFD = 38 µmol photons m^{-2} s^{-1}), with agitation at 140 rpm and an average temperature of 30 ± 5 °C. Every 7 days, cultures were diluted at a 1:50 ratio by transferring the HBC-infected culture (~ 5×10^5 cells mL^{-1}) into fresh *T. lutea* culture medium (~ 5×10^6 cells mL^{-1}). Fresh microalgal cultures were grown in sterile seawater (salinity 33 ppt) enriched with Nutribloom® (Phytobloom, Necton S.A., Portugal). Cultures were maintained in 1 L glass reactors under continuous illumination (PPFD = 140 µmol photons m^{-2} s^{-1}), with continuous bubbling of 0.2 µm-filtered air at 0.4 L min^{-1} and a 24 h photoperiod.

To better understand the feeding behaviour of the HBC responsible for *T. lutea* collapse, daily micrographs were acquired using a Zeiss Axioscope 5 Z2 microscope at 400 × magnification with ZEN Blue software (v3.3). Microalgal growth was monitored by optical density (OD_{750}) using a Hitachi UH5300 spectrophotometer and cell concentration (CC) was determined using a Neubauer haemocytometer after fixation with acid Lugol's solution (1%). OD_{750} values were correlated with CC to monitor biomass growth throughout the experimental trials (Fig. 1E).

In addition, nitrate concentration in the culture medium was measured spectrophotometrically to assess the physiological conditions of the microalgal cells and distinguish nutrient limitation from grazer-induced decline. Samples were centrifuged for 10 min at 2,000 × *g*, and nitrate concentration was determined by calculating the absorbance difference at 220 nm minus twice the absorbance at 275 nm, using a sodium nitrate calibration curve (Armstrong 1963).

Application of mitigation strategies

In a preliminary assay, various environmental and chemical mitigation strategies were tested on lab-scale *T. lutea* cultures infected by the HBC (Fig. 1F). Environmental treatments consisted of increased salinity, while chemical treatments included germanium dioxide (GeO_2), dipotassium phosphate (K_2HPO_4), sodium dodecyl sulphate (SDS), and hydrogen peroxide (H_2O_2), applied at concentrations based on previous studies (Table 2).

All treatments were conducted in triplicate over a 6-day period under the conditions described above. Growth of both microalgae and HBCs was monitored by microscopy and OD_{750} measurements. Non-contaminated *T. lutea* cultures were used as negative controls, while HBC-infected cultures served as positive controls.

In a validation assay, the most effective treatment (GeO_2 at 1 mg L^{-1}) was further evaluated over a 14-day period in triplicate under the same experimental conditions to validate

Table 2 Procedure and mitigation treatment with different concentrations applied in cultures, based on previous studies

Procedure	Treatments	Concentrations		Unit	Ref
		Minimum	Maximum		
Environmental pressure	Salinity	50	60	ppt	(Nicholls et al. 2003)
Chemical tests	GeO_2	1	2	mg L^{-1}	(Leet 1978)
	K_2HPO_4	2	20	mg L^{-1}	(Lehman 1976)
	SDS	20	40	mg L^{-1}	(Wen et al. 2021)
	H_2O_2	12	24	mg L^{-1}	(Witono et al. 2020)

its mitigation potential. Microalgal and HBC growth were monitored by microscopy, CC, and nitrate concentrations, with the same control treatments described above.

Statistical analysis

Statistical analyses of the mitigation trials were performed using Xlstat Premium (v2022.5.1.1386). Differences among treatments were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test ($\alpha = 0.05$). Graphs were generated using GraphPad Prism (v8.0.2).

Results

Identification of HBCs

Sequencing of the V9 region of the 18S rDNA gene yielded a total of 224 zOTUs. Samples from the first sampling procedure were highly contaminated, and the biological contaminant associated with the collapse of *T. lutea* cultures was present in high relative abundance. While low-abundance taxa may have negative biological effects, the present analysis focused on dominant zOTUs to identify the most likely primary drivers of large-scale culture collapse (Table S1, Supplementary Material).

Using BLASTn with stringent criteria (E-value $< 1 \times 10^{-50}$, identity > 95%), 112 zOTUs were classified, revealing ten potential HBC groups across multiple taxa (Fig. 1). These included Amoebozoa (true amoebae), Bacillariophyceae (diatoms), Bacteria, Cercozoa (microflagellates and amoeboid protists), Ciliophora (ciliates), Ecdysozoa (invertebrates), Euglenozoa (excavates like *Euglena*), Chrysophyceae ("golden" microflagellates), Fungi, and Opalozoa (non-photosynthetic Stramenopiles) (Keeling and Burki 2019). Microscopic observations provided by industrial monitoring were consistent with the taxonomic composition revealed by sequencing.

Bacterial sequences were detected in all samples, with the lowest abundance (~2,700 reads) observed in the inoculum and significantly higher abundances (20,000–30,000 reads) in PBRs (Fig. 2A). Eukaryotic contaminants were absent from the inoculum but were detected at high abundance in PBR samples (2,966 to 11,572 reads) relative to the target microalga. The biological contaminant community composition differed among samples: sample 2 (from the previous year) was dominated by Euglenozoa (zOTU28—50%), while samples 3 and 4 were dominated by Chrysophyceae (zOTU4—77%) and Ciliophora (zOTU9—88%), respectively (Fig. 2) (Table S1, Supplementary Material). These three taxa were selected for further phylogenetic analysis.

Phylogenetic analysis and comparison with published sequences revealed the biological contaminant zOTU28, zOTU4 and zOTU9 belonged to the family Diplonemidae, genus *Paraphysomonas*, and genus *Euplotes*, respectively (Figs. S1, S2 and S3, Supplementary Material). Nevertheless, the most likely candidate for the collapse of *T. lutea* cultures was the chrysophyte (zOTU4). Amplification and sequencing of the 18S V1-V5 region confirmed a close relationship of this HBC with *Paraphysomonas longispina* (JQ967305) and *Paraphysomonas* aff. *longispina* (Z28335), sharing a node with a branch support value of 0.88 (Fig. 3).

Molecular detection of Paraphysomonas sp. *during scale-up*

Following phylogenetic identification, species-specific primers targeting *Paraphysomonas* sp. were designed in house (Fig. 1E). PCR optimization demonstrated high primer specificity and sensitivity, with successful detection of *Paraphysomonas* DNA in samples where the contaminant was represented as only 0.1% of Illumina 18S V9 sequencing reads (Figs. 4 and S4 – Supplementary Material). No amplification was observed in non-target microalgal cultures.

The optimized primer pair was subsequently applied to the samples collected during the second sampling procedure to determine the stage at which *Paraphysomonas* sp. first appeared during scale-up. PCR screening revealed that the HBC was absent from laboratory-scale cultures, flat panel PBRs, and water samples, but was detected in industrial PBRs after five days of cultivation (Table S2, Supplementary Material). This pattern was consistent across both monitored cultures (cultures 1 and 2).

Growth and feeding behaviour of Paraphysomonas sp.

Daily microscopic observations of contaminated laboratory-scale *T. lutea* cultures revealed the distinct stages in the feeding behaviour of *Paraphysomonas* sp. After two days, small (~2 µm), flagellated cells were observed, which were unable to prey on the larger *T. lutea* cells (~6 µm diameter) (Fig. 5A). By day 4, these developed into larger (15–20 µm) flagellated amoeboid forms capable of grazing on multiple microalgal cells (Fig. 5B-E).

Culture collapse occurred by day 6, with no intact *T. lutea* cells remaining. At this stage, amoeboid cells decreased in size (10–15 µm) and exhibited cannibalistic behaviour (Fig. 5F). Following collapse, *Paraphysomonas* sp. abundance declined, leaving cell debris. However, the contaminant was able to re-establish when inoculated into fresh *T. lutea* cultures, suggesting the presence of resistant cyst stages not detectable by routine optical microscopy.

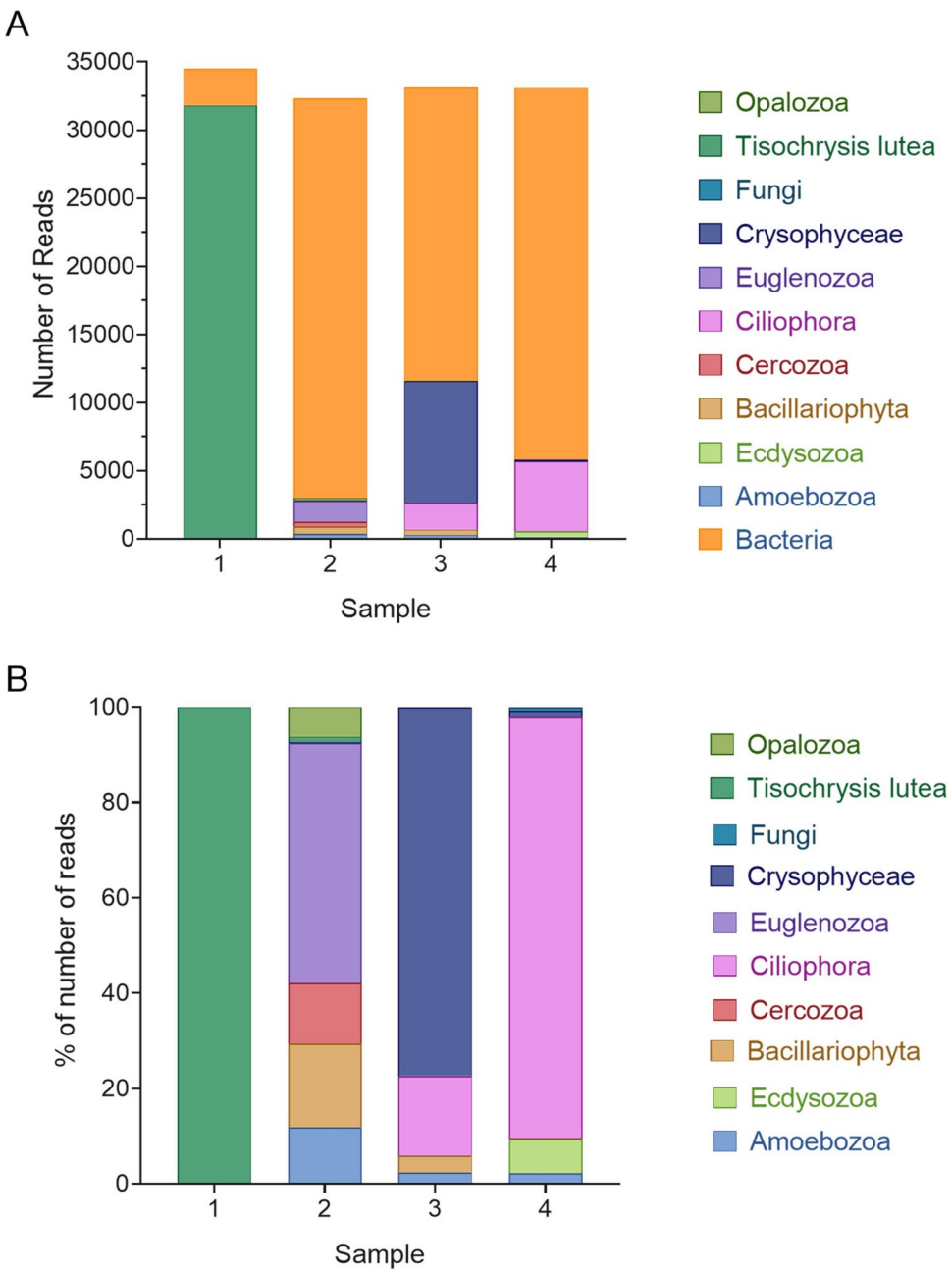

Fig. 2 Abundance of relevant taxa obtained by 18S amplicon sequencing of the V9 region in inoculum (sample 1) and contaminated samples of *T. lutea* (sample 2, 3 and 4). Taxonomic assignments are shown as total number of reads with bacteria (**A**) or as percentage of number of reads without bacteria (**B**)

Evaluation of mitigation trials

Preliminary assay

Following confirmation of the biological impact of *Parahysomonas* sp. on *T. lutea* cultures, several environmental and chemical mitigation strategies were evaluated for their ability to control the HBC while maintaining microalgal growth. Culture colour changes, from brown (healthy) to yellow (contaminated) to transparent (collapsed), were used as a qualitative indicator of the culture condition (Fig. 6).

The negative control (non-contaminated *T. lutea* culture) exhibited a lag phase between days 0 and 2, followed by exponential growth between days 2 and 3, stationary phase by day 6, and reaching a final concentration of approximately 2×10^7 cells mL^{-1} (Fig. 7A). Cultures remained dark brown throughout the experiment (Fig. 6A). In contrast, the positive control (HBC-infected *T. lutea* culture) showed no significant increase in *T. lutea* cell concentration. Flagellated amoeba-like cells were observed from day 2 onward, and the culture turned transparent by day 6 (Fig. 6C). Optical density–based estimates overestimated residual cell concentration due to light scattering by debris and HBC cells,

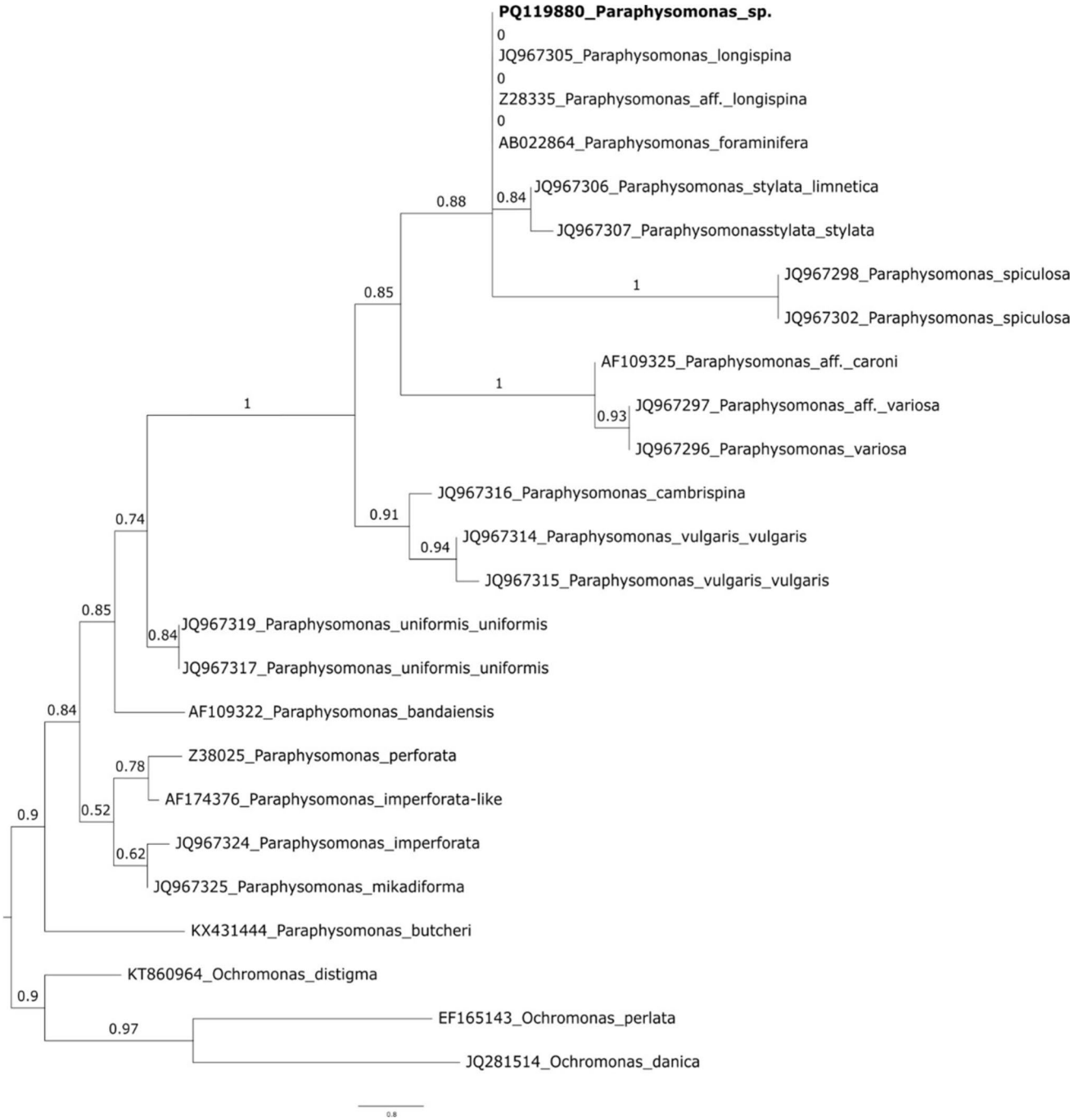

Fig. 3 Maximum likelihood (PhyML) 18S phylogenetic tree of the Chrysophycean HBC present in highly contaminated cultures of *T. lutea* (**PQ119880**). Branch support values were estimated by a non-parametric SH-like approximate likelihood ratio test. Several 18S rDNA sequences belonging to the genus *Ochromonas* have been added to determine the root node by means of an outgroup

resulting in an apparent final concentration of ~ 4×10^6 cells mL^{-1}.

At a salinity of 60 ppt, contaminated cultures exhibited growth comparable to the negative control, with no significant differences in cell concentration (Fig. 7B). Microscopy revealed only a small number of flagellated cells, and cultures retained a dark brown colour (Fig. 6A). At 50 ppt, however, a significant decrease in cell concentration was observed by day 6 (~ 7×10^6 cells mL^{-1}), and *Paraphysomonas* sp. cells were detected microscopically.

Treatment with GeO_2 at both 1 and 2 mg L^{-1} prevented culture collapse, with no significant differences in *T. lutea* cell concentration relative to the negative control and no observable presence of the contaminant (Fig. 7C). In

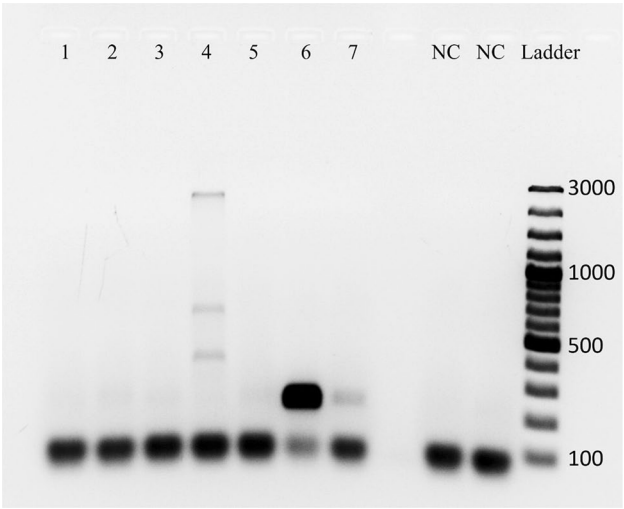

Fig. 4 Amplification products of *Paraphysomonas* sp. 18S rDNA using the primer pair Paraphysomonas_1376F/Paraphysomonas_1549R. 1) *Tetraselmis chui*; 2) *Chlorella vulgaris*; 3) *Tisochrysis lutea* and 4) *Phaodactylum tricornutum* 5) Sample 2 (Culture from PBR, HBC detected); 6) Sample 3 (Collapsed culture from PBR, high HBC levels detected); and 7) Sample 4 (Collapsed culture from PBR, HBC detected). Negative control (NC) and 100 bp ladder (Ladder)

contrast, K_2HPO_4 at 2 mg L^{-1} did not prevent contaminant development and resulted in a significant decrease in cell concentration. At 20 mg L^{-1}, cell concentrations were significantly higher than in the negative control across all time points; however, microscopic examination revealed stressed, smaller microalgal cells and substantial cell detritus. The elevated cell concentration estimated from OD_{750} measurements was therefore attributed to light scattering by debris rather than true biomass increases (Fig. 7D).

SDS and H_2O_2 treatments showed no inhibitory effect on *Paraphysomonas* sp. growth, with cultures exhibiting dynamics similar to the positive control throughout the experiment (Fig. 7E, F). Flagellated amoeba-like cells were observed after three days, and cultures became transparent by day 6 (Fig. 6C).

Overall, GeO_2 at 1 mg L^{-1} and high salinity (60 ppt) effectively controlled *Paraphysomonas* sp. while maintaining *T. lutea* growth. Given the potential physiological stress associated with repeated salinity increases, GeO_2 was selected for further evaluations.

Validation assays

The efficacy of GeO_2 at 1 mg L^{-1} was further evaluated (Figs. 8 and 9). The negative control (non-contaminated *T. lutea* culture) exhibited sustained exponential growth, increasing from 4×10^6 to 2×10^7 cells mL^{-1} by day 14, with consistent nitrate uptake (~2 mM). Positive control (HBC-infected *T. lutea* culture) grew comparably to negative control until day 4, but collapsed by day 7 (Fig. 8), coinciding with a significant increase of *Paraphysomonas* sp. cell concentration (6×10^6 cells mL^{-1}) and a minimal nitrate uptake (< 1 mM), relative to the negative control (Fig. 9).

GeO_2-treated cultures without *Paraphysomonas* sp. showed no significant differences from negative control. In GeO_2-treated cultures inoculated with the HBC, *T. lutea* cell concentration did not differ significantly from the negative control until day 10, indicating a delay of contaminant-induced collapse by approximately seven days (Fig. 8). However, by day 14, a significant decrease in *T. lutea* cell concentration was observed, accompanied by contaminant overgrowth. Nitrate consumption in these cultures did not differ significantly from that of the negative control (Fig. 9).

Discussion

Identification and characterization of Paraphysomonas sp.

Molecular approaches such as amplicon sequencing are valuable tools for detecting biological contaminants because they do not require prior knowledge of contaminant identity and allow simultaneous monitoring of contaminant communities and microalgal cultures (Carney et al. 2014). In this study, NGS-based analysis revealed the presence of multiple eukaryotic taxa potentially associated with HBC events. Although primers targeting the eukaryotic 18S rDNA gene were used, bacterial and archaeal sequences were also amplified, likely due to primer universality and sequence similarity, as previously reported (Pawlowski et al. 2011; Kounosu et al. 2019). As the focus of this study was eukaryotic grazers, the bacteriome was not analysed further.

Among all detected zOTUs, three dominant candidates were selected for further analysis: a euglenozoan (zOTU28), a chrysophyte (zOTU4), and a ciliate (zOTU9). Euglenozoans are colourless biflagellated protists capable of phagotrophic feeding on microalgae and are rarely bacterivorous (Kostygov et al. 2021). However, microscopy did not reveal organisms morphologically consistent with *Diplonema*-like flagellates, and this taxon was therefore not further investigated. Ciliates of the genus *Euplotes* are known grazers of microalgae such as *Chlorella vulgaris*, *Dunaliella salina*, and *Nannochloropsis salina*, and have been reported to induce rapid culture collapse (Day et al. 2017). Nonetheless, industry-based microscopic observations indicated that ciliates appeared only after the onset of culture decline, suggesting that they were secondary opportunists rather than primary agents of collapse. Additionally, ciliates are generally susceptible to several mitigation strategies, including

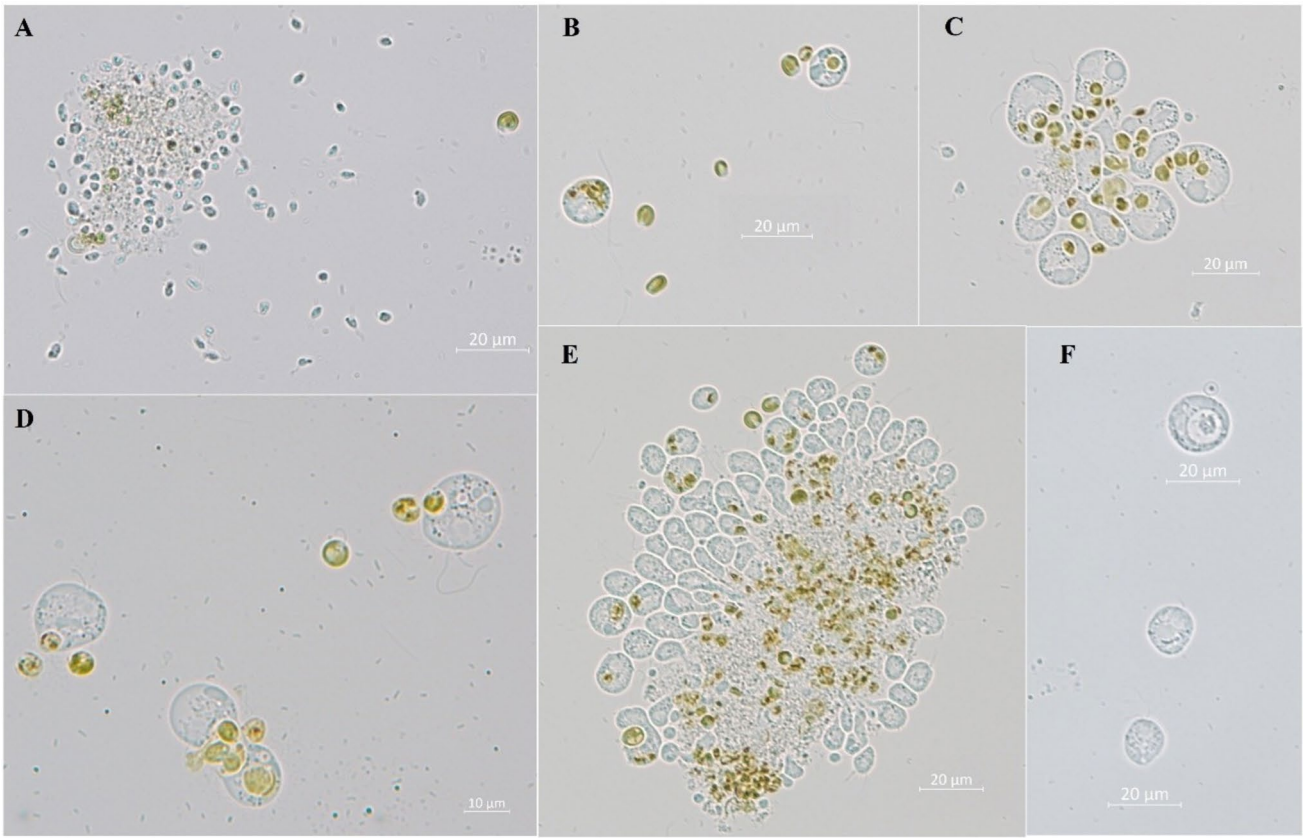

Fig. 5 Different morphological stages of the flagellated *Paraphysomonas* sp., feeding on *T. lutea*: small, flagellated cells (**A**); large, flagellated amoeba-like cells feeding on microalgae (**B**, **C**, **D** and **E**); and medium flagellated amoeba-like cells feeding on themselves (**F**). Images captured using differential interference contrast (DIC)

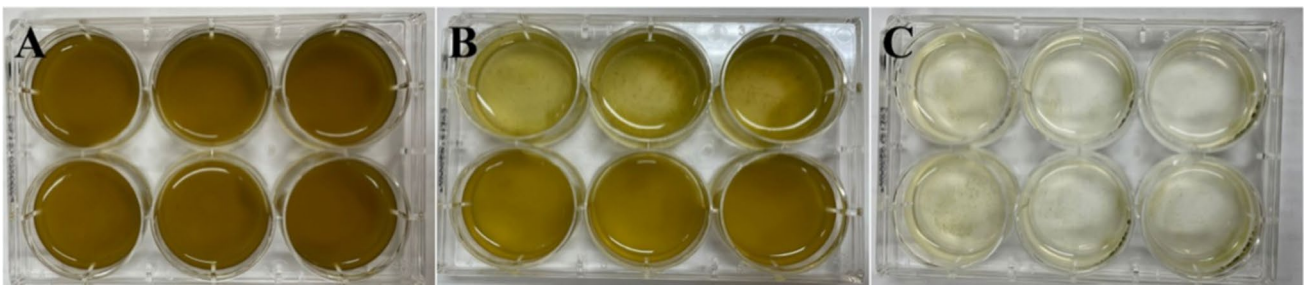

Fig. 6 6-well plates trials with contaminated *T. lutea* cultures. **A**) healthy culture inoculated with *Paraphysomonas* sp. (day 0), **B**) culture at its early stages of collapse (day 3) and **C**) fully collapsed culture (day 6)

quinine sulphate and SDS (Molina-Grima et al. 2022), making them less problematic in applied settings.

In contrast, chrysophytes of the genus *Paraphysomonas* are colourless chrysomonads capable of forming cysts and bearing silica scales that are typically only visible by electron microscopy (Scoble and Cavalier-Smith 2014). This genus is a known grazer of both bacteria (e.g., *Vibrio natrigens*) and haptophyte microalgae such as *T. lutea* and *Pavlova lutheri* (Davidson and John 2001). When both prey types are present, *Paraphysomonas* preferentially ingests phytoplankton. Based on phylogenetic analysis, feeding behaviour, and microscopic observations, *Paraphysomonas* sp. was identified as the most likely causative agent of *T. lutea* culture collapse.

The species-specific primer pair developed in this study exhibited high specificity and sensitivity for the detection of *Paraphysomonas* sp. Application of this molecular tool during scale-up revealed that the contaminant first

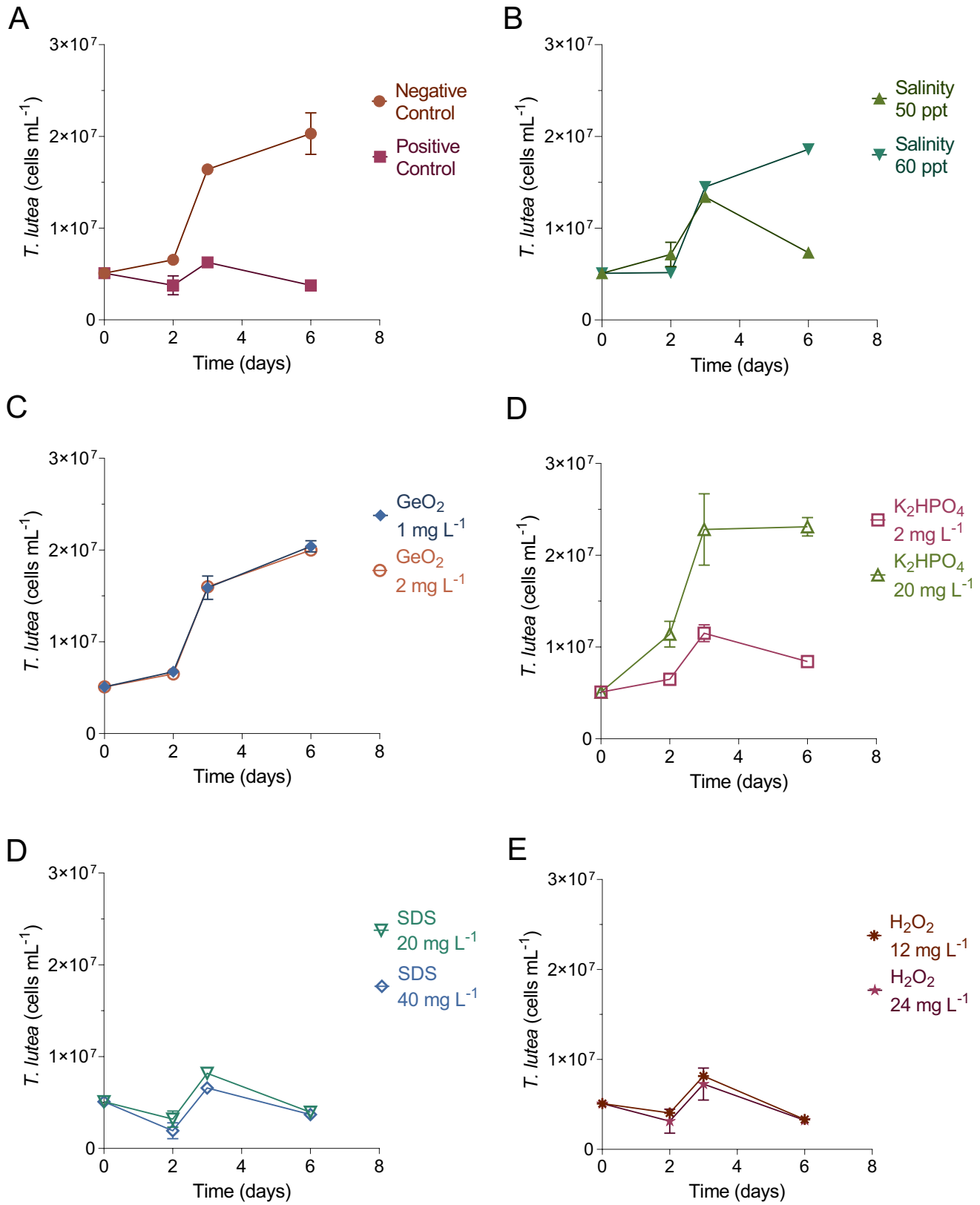

Fig. 7 Growth curve of non-contaminated *T. lutea* culture (negative control) and HBC-infected *T. lutea* culture (positive control) without any treatment applied (**A**); and growth curve of the HBC-containing culture treated with higher salinity (**B**), GeO_2 (**C**), K_2HPO_4 (**D**), SDS (**E**) and H_2O_2 (**F**) to avoid culture collapse. $n = 3$, mean ± SD

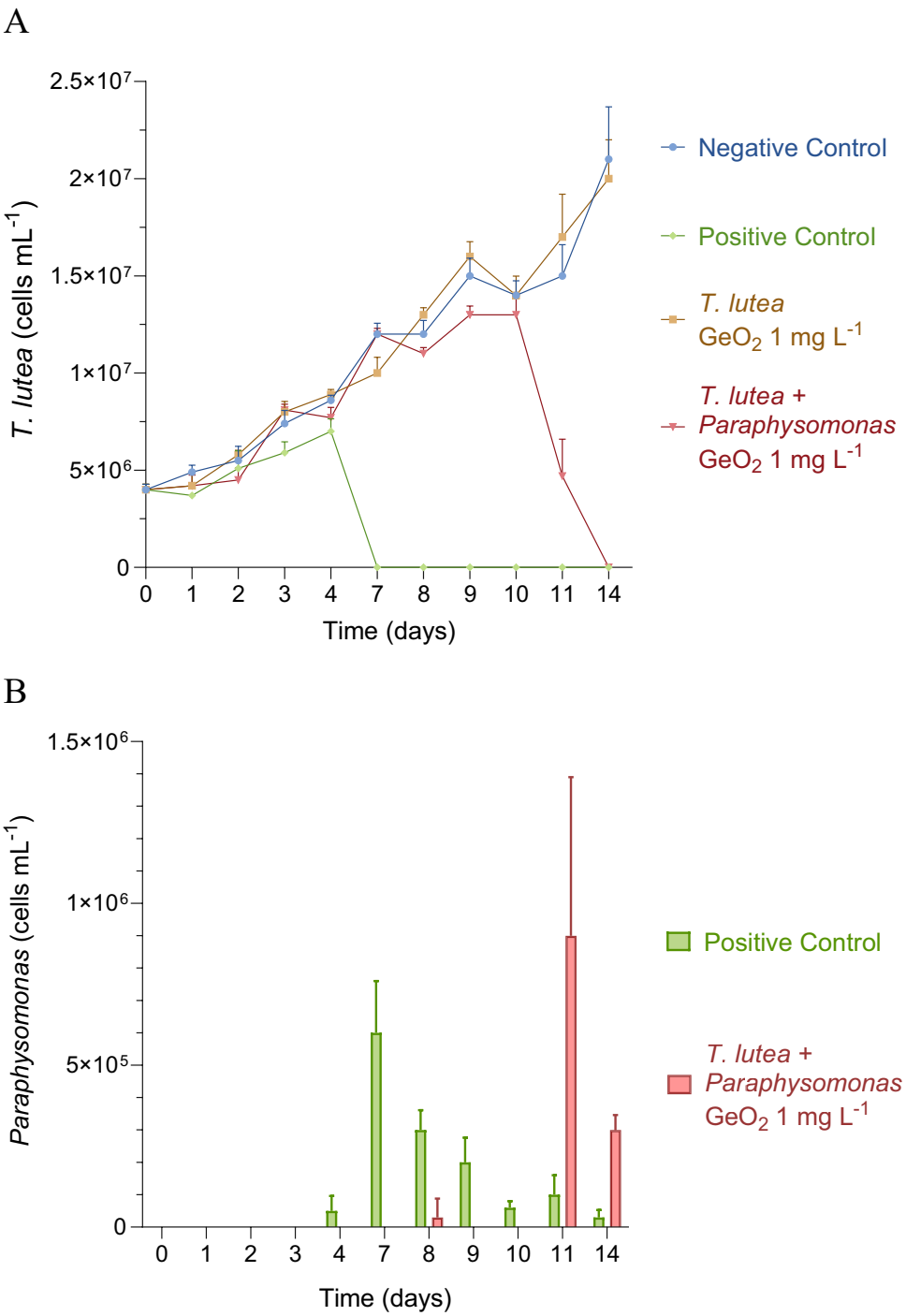

Fig. 8 Growth curve using cell count of *T. lutea* cells (**A**) and *Paraphysomonas* sp. cells (**B**) over 14 days of cultivation, with non-contaminated *T. lutea* culture (negative control) and HBC-infected *T. lutea* culture (positive control) as controls. GeO_2 was applied to the uncontaminated (*T. lutea*) and the HBC-containing culture of *T. lutea* (*T. lutea* + *Paraphysomonas*). $n = 3$, mean ± SD

appeared in industrial photobioreactors a few days after inoculation. This suggests that *Paraphysomonas* sp. may persist as resistant cysts within industrial systems, surviving routine cleaning procedures and excysting under favourable growth conditions. Despite the closed nature of photobioreactors, contamination can occur through air, water, or incomplete sterilization, particularly for organisms with amoeboid behaviour and resistant morphological stages (Zhu et al. 2017).

Previous studies have shown that *Paraphysomonas* species can graze on a wide range of phytoplankton and bacteria and, under food-limited conditions, even exhibit cannibalistic behaviour (Goldman and Caron 1985). The ability to form species-specific stomatocysts allows these organisms to survive adverse environmental conditions and facilitates global dispersal (Nicholls and Wujek 2003). Together with their small size, heterotrophic metabolism, and rapid growth, these traits likely contribute to the difficulty of early

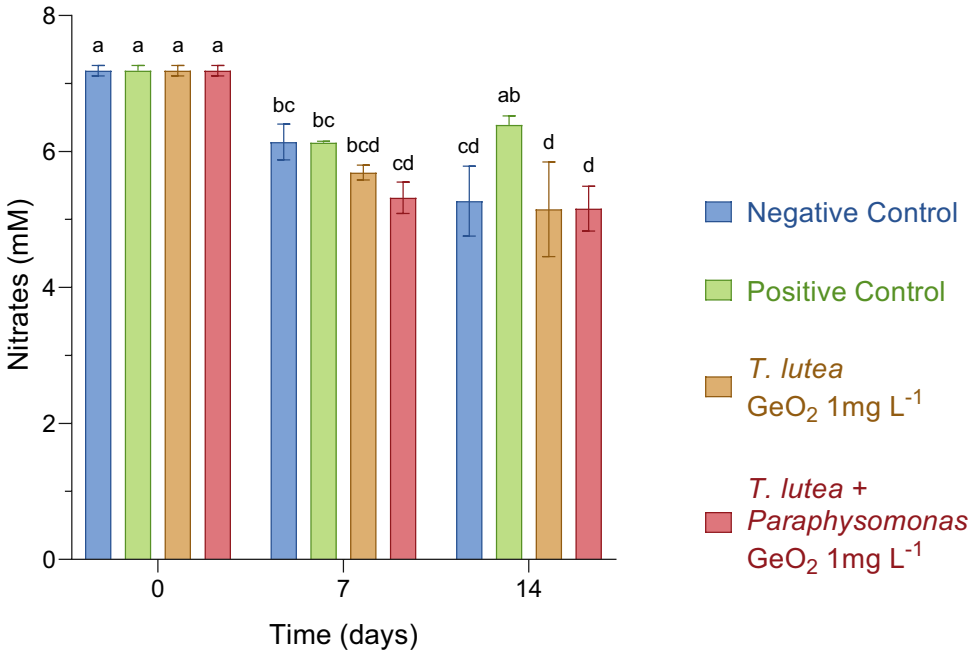

Fig. 9 Nitrate concentration (in mM) of non-contaminated *T. lutea* culture (negative control) and HBC-infected *T. lutea* culture (positive control). GeO_2 was applied to the culture of *T. lutea* only (*T. lutea*), and cultures with *T. lutea* contaminated with *Paraphysomonas* sp. (*T. lutea* + *Paraphysomonas*). $n = 3$, mean ± SD. Different letters over the bars indicate significant differences between samples ($p < 0.05$, ANOVA Tukey)

detection in large-scale cultures. Consequently, molecular monitoring approaches represent a valuable strategy for early detection of such grazers, enabling timely intervention before irreversible culture collapse occurs.

Effectiveness of mitigation strategies

The objective of the mitigation trials was to contaminate healthy *T. lutea* cultures under controlled conditions and evaluate environmental and chemical strategies capable of limiting grazer proliferation while preserving algal growth. This approach allowed direct translation of biological insights into applied solutions relevant for large-scale cultivation systems. Culture colour changes were used as a qualitative indicator of contamination status, reflecting reductions in microalgal biomass during grazing events. Similar colour shifts have been reported in other algal systems, where grazing leads to chloroplast degradation, reduced pigmentation, and lower cell densities (Ma et al. 2018). Quantitative measurements, including cell concentration and nutrient uptake, complemented these visual observations.

Elevated salinity effectively suppressed the HBC proliferation, consistent with previous reports indicating that most *Paraphysomonas* species are primarily freshwater organisms, with relatively few marine representatives thriving above 35 ppt (Nicholls and Wujek 2003). In contrast, *T. lutea* is a halotolerant species capable of growing across a wide salinity range (35–75 ppt), explaining its resilience under increased salinity conditions observed in this study (Ishika et al. 2017).

GeO_2 also proved effective in delaying culture collapse. It is naturally present in aquatic environments and can substitute for silicon in organisms with silica-based structures. It has been shown to inhibit diatom growth by interfering with silica metabolism and cell division (Lewin 1966). Given that *Paraphysomonas* sp. cells possess silica scales, incorporation of GeO_2 may disrupt scale formation or interfere with cellular processes such as DNA polymerase activity, thereby limiting population growth (Leet 1978).

K_2HPO_4 exhibited inhibitory effects on *Paraphysomonas* sp. at higher concentrations; however, these concentrations also negatively affected *T. lutea*, limiting its applicability as a selective mitigation strategy. Similarly, SDS and H_2O_2, despite reported effectiveness against other microbial contaminants (Witono et al. 2020; Wen et al. 2021), were ineffective against *Paraphysomonas* sp., likely due to the protective role of silica scales shielding the cell membrane from chemical damage.

Both elevated salinity and GeO_2 delayed *Paraphysomonas*-induced collapse by approximately one week without significantly impairing *T. lutea* growth. However, repeated application of environmental stress through salinity increases may not be feasible in continuous cultivation systems, as it could eventually compromise algal performance. In contrast, chemical mitigation using GeO_2 may allow repeated applications upon contaminant detection. Similar strategies involving periodic chemical treatments have been successfully applied in other algal systems to control grazers (Méndez and Uribe 2012; Pradeep et al. 2015). Overall, these findings indicate that GeO_2 represents a promising mitigation option for extending *T. lutea* culture viability, although optimization of treatment frequency and long-term effects needs further investigation.

Conclusion

This study demonstrates that 18S rDNA amplicon sequencing is an effective approach for detecting diverse eukaryotic taxa in industrial *T. lutea* cultures. The HBC responsible for culture collapse was identified as *Paraphysomonas* sp. (Chrysophyceae), a grazer exhibiting pronounced morphological plasticity, which may contribute to its persistence in large-scale cultivation systems and its capacity to prey on microalgae.

Molecular detection using a newly designed species-specific primer pair provided a sensitive early-warning tool, enabling detection of *Paraphysomonas* sp. at very low abundances that are not readily observable by microscopy. In parallel, laboratory-scale mitigation trials showed that treatment with 1 mg L^{-1} GeO_2 effectively delayed contaminant proliferation and culture collapse without adversely affecting *T. lutea* growth.

Together, these results highlight the value of integrating molecular monitoring with targeted mitigation strategies to improve early detection and management of grazers in large-scale microalgal production, contributing to more resilient and economically viable cultivation systems.

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Data availability Data can be made available upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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