

Controlling of flagellates and ciliates contaminations in *Chlorella* mass culture

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Abstract

Background: Flagellates and ciliates are two common bio-contaminants which frequently cause biomass losses in *Chlorella* mass culture. Efficient and targeted ways are required to control contaminations in *Chlorella* mass cultivation aiming for biofuel production especially.

Results: Five surfactants were tested for its ability to control bio-contaminations in *Chlorella* culture. All five surfactants were able to eliminate the contaminants at a proper concentration. Particularly the minimal effective concentrations of sodium dodecyl benzene sulfonate (SDBS) to completely eliminate *Poterioochromonas* sp. and *Hemiurosomoida* sp. were 8 mg L^{-1} and 10 mg L^{-1} , respectively, yet the photosynthesis and viability of *Chlorella* was not significantly affected. These results were further validated in *Chlorella* mass cultures in 5, 20, and 200 m^2 raceway ponds.

Conclusions: A chemical method using 10 mg L^{-1} SDBS as pesticide to control flagellates or ciliates contamination in *Chlorella* mass culture was proposed. The method helps for a sustained microalgae biomass production and utilization, especially for biofuel production.

Background

Chlorella is a genus of unicellular green microalgae that has long been used as a model organism to study photosynthesis [1]. *Chlorella* biomass is rich in protein, vitamins, and minerals. The success of *Chlorella* mass culture during the late 1940s created a stable *Chlorella* industry, primarily for human nutrition and animal feed [2, 3]. Recently, *Chlorella* is considered a candidate for bioenergy and bioremediation owing to its ability to grow fast, uptake nutrients in wastewaters, and synthesize a large amount of TAGs or carbohydrates in cells [4, 5].

However, the current autotrophic technologies that are used for the mass production of *Chlorella* biomass are facing challenges from biological contamination. Biological contamination occurs frequently in *Chlorella* mass culture in the widely used cultivation systems including circular and raceway ponds [3, 6–8]. Zooplanktonic predators, such as ciliates, rotifers, amoeba, and flagellates, are the most common contaminants as reported in the literature [8, 9]. According to Ma et al's [10, 11] surveys, contamination by the predatory flagellate, *Poterioochromonas malhamensis*, in *Chlorella* culture occurs at any time throughout the year and no matter where the cultures are conducted only when there is air contact. Ciliates are also widely spread and can cause serious problems to microalgae cultivation under broad climate conditions [12]. *Chlorella* cultures are so vulnerable that every “invasion” by these predators might devastate the cultivation. The most direct effect of such contamination is the reduction of biomass yield. For example, the cell density of *Chlorella* has been shown to decrease from $4.0 \times 10^8 \text{ cells mL}^{-1}$ to $1.0 \times 10^8 \text{ cells mL}^{-1}$ within three days, whereas that of the grazer *P. malhamensis* increased to $1.1 \times 10^6 \text{ cells mL}^{-1}$ from $0.1 \times 10^4 \text{ cells mL}^{-1}$ [11]. Moreno-Garrido and Canavate [12] reported that grazing ciliates can visually clarify dense outdoor mass cultures of *Dunaliella salina* within 2 days. Similarly, over 60% of *Chlorella* biomass can be digested in a short time due to the explosive growth of grazers such as

flagellates or ciliates, according to the authors' experiences. Such catastrophic losses are unacceptable. Thus, the control of biological contamination is very important for the mass production of *Chlorella* in open systems.

Biological contaminations are different in their occurrence, development, and contamination mechanisms [6]. Many contaminations have occurred in an associative or sequential manner and interacted with the target microalgae [13]. These factors make the control of biological contamination very complicated. Methods have been suggested to overcome the challenges of biological contamination, such as filtration, changes of the environmental conditions such as medium pH, and use of chemical additives including quinine, formaldehyde, ammonia, and hydrogen peroxide [6, 8, 10, 12]. These methods are helpful in controlling the different types of zooplanktonic contaminants. However, methods such as filtration and changes of medium pH are inefficient to apply in large scale, and chemical additives, for example, ammonia and ammonium bicarbonate, are not applicable in microalgal cultivation where nitrogen limitation is necessary to induce TAG or astaxanthin accumulation since the addition of such chemicals will relieve nitrogen deficiency. Thus more efficient and targeted ways are still required. Wang et al [6] suggested that strain selection (non-susceptibility/resistance to biological pollutants) is the most practicable approach to cope with biological invasions, yet it is very time-consuming because a single algal species is unlikely to excel in all the required characteristics, such as resistance to biological pollutants, rapid growth, high product content, wide tolerance of environmental conditions, and other qualities that facilitate industrial production.

Here, we report on a simple and efficient chemical method, using surfactant as a single additive, to control the contamination of flagellates and ciliates in *Chlorella* mass culture. Flagellates and ciliates, specifically *Poteriochromonas* sp. and *Hemiurosomoida* sp. in the present study, have several similarities in the context of contamination in *Chlorella* mass culture. They are both unicellular, and can swim and graze on *Chlorella* cells and especially lack a resistant structure outside the plasma membrane [14–16] in comparison to *Chlorella*. These characteristics create possibilities for the targeted control of *Poteriochromonas* and *Hemiurosomoida* without inhibition on *Chlorella* growth. Several surfactants were used as pesticides and their effects on flagellates, ciliates, and *Chlorella* were investigated and compared. The application of this method was also discussed and recommended based on field testing.

Results

Toxic effects of surfactants on grazer growth and reproduction

The successive transfer cultures of the two grazers (*Poteriochromonas* sp. and *Hemiurosomoida* sp.) were established first as described in Methods. Using these successive transfer cultures, the efficacies of the five selected surfactants for controlling *Poteriochromonas* sp. and *Hemiurosomoida* sp. were evaluated. Toxic effects on both *Poteriochromonas* sp. and *Hemiurosomoida* sp. were observed for all five surfactants, namely SDBS, CDEA, SDS, AEO-7, and AES.

As shown in Fig. 1, a greater than 30% increase in cell densities of *Poterioochromonas* sp. were obtained after 24 h cultivation without surfactant addition, suggesting the viability of the *Poterioochromonas* sp. cultures. However, the cell densities decreased in the cultures supplemented with any one of the five surfactants. For example, the cell density of the living *Poterioochromonas* sp. was 2.8×10^4 cells mL⁻¹ in the culture without SDBS addition, yet it decreased to 1.8×10^4 cells mL⁻¹ in the culture supplemented with 3 mg L⁻¹ SDBS and further decreased to less than 100 cells mL⁻¹ with 6 mg L⁻¹ SDBS treatment. No living *Poterioochromonas* sp. were observed microscopically when the SDBS concentration was further increased to 8 mg L⁻¹, which we considered as the complete control of *Poterioochromonas* sp. contamination.

The decreasing trend in *Poterioochromonas* sp. density with increasing surfactant concentration was found for all five tested surfactants, suggesting that they affect the grazer *Poterioochromonas* sp. similarly, however, for each surfactant, the minimal effective concentrations to completely control the contamination were different. SDBS and AEO7 were the most powerful reagents, eliminating *Poterioochromonas* sp. completely at concentrations not lower than 8 mg L⁻¹. Second, the efficacies of CDEA and SDS on *Poterioochromonas* sp. were similar and their minimal effective concentrations were 10 and 12 mg L⁻¹, respectively. AES showed weak efficacy on controlling of *Poterioochromonas* sp., with a minimal effective concentration of 20 mg L⁻¹.

Toxic effects of the five surfactants on *Hemiurosomoida* sp. were also observed (Fig. 2). The viability of *Hemiurosomoida* sp. was shown by an increased in cell densities, which were more than 40% higher in comparison to the initial density in the culture without surfactant supplementation. *Hemiurosomoida* sp. densities decreased significantly after surfactants addition. Taking the SDBS treatment as an example, almost 60% decrease in the *Hemiurosomoida* sp. density, from 1.6×10^3 cells mL⁻¹ to 680 cells mL⁻¹, was obtained when 4 mg L⁻¹ SDBS was supplemented into the culture. A further increase in the SDBS concentration (10 mg L⁻¹) led to the complete elimination of *Hemiurosomoida* sp. and no living cells were observed under the microscope. The general trends of decreasing cell densities with increasing surfactant concentrations were also detected for the five surfactants. However, the efficacies against *Hemiurosomoida* sp. were not the same as that for *Poterioochromonas* sp. The most powerful one was AEO7, which eliminated *Hemiurosomoida* sp. at a concentration of 8 mg L⁻¹. The next ones were SDBS and CDEA, the minimal effective concentrations of which were 10 mg L⁻¹ and 15 mg L⁻¹, respectively. The complete elimination of *Hemiurosomoida* sp. by AES was only obtained at 30 mg L⁻¹. A substantial difference was observed in SDS, which had a minimal effective concentration of 12 mg L⁻¹ for *Poterioochromonas* sp., but at least 35 mg L⁻¹ SDS was needed to completely eliminate *Hemiurosomoida* sp.

Effects of the five surfactants on *Chlorella* growth

Chlorella pyrenoidosa XQ-20044 was cultured under different concentrations of the five surfactants to evaluate the surfactant effects on cell growth, photosynthetic activity, and viability. Data of the SDBS

exposure experiment are shown in Fig. 3 as an example; other data concerning CDEA, SDS, AES, and AE07 are provided in Additional file 1.

The time courses of the *Chlorella* biomass DW showed no significant difference when the SDBS concentration was less than 20 mg L⁻¹ (Fig. 3a). The biomass DW of the culture having no SDBS supplementation reached 0.72 g L⁻¹ on day 3, with an average growth rate of 0.84 d⁻¹. Smaller but insignificant biomass DW (0.67 g L⁻¹) and growth rate (0.82 d⁻¹) were obtained in the culture with 20 mg L⁻¹ SDBS supplementation. However, the biomass DW was only 0.41 g L⁻¹ with a significantly decreased growth rate of 0.66 d⁻¹ when the SDBS concentration was further increased to 40 mg L⁻¹.

The photosynthetic activity of *Chlorella* (Fig. 3b) showed that in comparison to the SDBS-free culture, the changes in the photochemical yield of *Chlorella* cells were very small after 3 days of exposure to 20 mg L⁻¹ SDBS. The ratio between variable fluorescence and maximum fluorescence (F_V/F_M) of *Chlorella* was 0.72 in the SDBS-treated (20 mg L⁻¹) culture in the present study. This value fell into the general F_V/F_M range of dark-adapted green microalgae [17], suggesting that the photosynthetic activity of *C. pyrenoidosa* XQ-20044 was not influenced by SDBS at concentrations lower than 20 mg L⁻¹.

FDA staining (Fig. 3b, 3c) clearly showed membrane integrity and viability of the *Chlorella* cells, with similar fluorescein fluorescence intensities in both the SDBS-treated (20 mg L⁻¹) and the contrast culture. All of the above results suggested that *Chlorella* biomass yield may be reduced due to over exposure to SDBS, but the influences of SDBS was negligible at a concentration not higher than 20 mg L⁻¹.

Application of sodium dodecyl benzene sulfonate (SDBS) as a pesticide to control flagellates and ciliates grazing on *Chlorella* in raceway pond

The SDBS surfactant was further tested outdoors to validate the laboratory data. According to its performance in the raceway ponds, more technical details with respect to its outdoor application are discussed.

According to our observation, naturally occurring contaminations of *Poteriochromonas* sp. or *Hemiurosomoida* sp. can be observed generally on days 2–4 of a newly inoculated *Chlorella* culture in an outdoor raceway pond (unpublished results). This trend was successfully mimicked by the addition of *Poteriochromonas* sp. or *Hemiurosomoida* sp. “seeds” into the *Chlorella* culture ponds (Fig. 4). 18S rDNA based metagenomic data for identification of the contaminating species can be seen in Additional file 2. As soon as continued increases in grazer densities were observed for 3–4 days, for example, the grazer *Hemiurosomoida* sp. increased continually from 1.0 × 10⁵ cells L⁻¹ on the 4th day to 2.7 × 10⁵ cells L⁻¹ on the 5th day and 6.4 × 10⁵ cells L⁻¹ on the 6th day, and further increased to 1.4 × 10⁶ cells L⁻¹ the next day, the cultures were treated with 10 mg L⁻¹ SDBS to control *Hemiurosomoida* sp. or *Poteriochromonas* sp., and the other parallel cultures allowed contaminations to develop.

As shown in Fig. 4, cell densities of the grazers *Poterioochromonas* sp. and *Hemiurosomoida* sp., increased regularly for 3 or 4 days. The target microalgae *C. pyrenoidosa* XQ-20044 also showed a quick increase in cell density (indicated by Chl *a* content) during this period because the grazer populations were not large enough to have a significant grazing effect on *Chlorella*. The increase in grazer densities continued thereafter in the cultivations without SDBS addition, with the majority of grazers swallowing plenty of *Chlorella* cells and enclosing in their bodies. When the densities of *Poterioochromonas* sp. and *Hemiurosomoida* sp. reached approximately 3.6×10^7 cells L⁻¹ and 6.4×10^5 cells L⁻¹, respectively, the *Chlorella* density decreased due to grazing. By comparison, almost all the *Poterioochromonas* sp. and *Hemiurosomoida* sp. cells disintegrated and disappeared in one day in the cultivations with SDBS addition (10 mg L⁻¹) on the 6th day and 7th day, respectively, with the *Chlorella* growth kept as normal.

Overall, the final *Chlorella* biomass concentration reached 0.6 g L⁻¹ after a 12-day cultivation applying SDBS pesticide. It was only 0.26 g L⁻¹ if the *Poterioochromonas*-contamination was not controlled and 0.17 g L⁻¹ if the *Hemiurosomoida*-contamination was not controlled (Fig. 5). These data suggest that by applying 10 mg L⁻¹ SDBS as a pesticide to control *Poterioochromonas* sp. or *Hemiurosomoida* sp. contamination, the reduction in *Chlorella* biomass yield, which was estimated to be greater than 60% owing to grazer contamination, can be avoided. Actually, economic loss caused by biological contamination was much bigger than expected because the residual *Chlorella* biomass could only be used as low-quality raw materials when no effective steps were taken to manage the contaminations. The working concentration of SDBS (10 mg L⁻¹) was slightly higher than the minimal effective concentration to eliminate *Poterioochromonas* sp. in the laboratory. This was to simplify the application that using one uniform concentration to control both *Poterioochromonas* and *Hemiurosomoida* contaminations.

SDBS pesticide was also applied in 20 and 200 m² cascade cultures of *Chlorella* at October 2019 (Fig. 6). Two rounds of contamination naturally occurred during the process, both of which were *Poterioochromonas* sp. contaminations. The first round of *Poterioochromonas* contamination was observed early on the 2nd day in the 20 m² pond. The cell density of *Poterioochromonas* increased gradually from 7.6×10^4 cells L⁻¹ to 1.1×10^6 cells L⁻¹ on the 3rd day, and then drastically increased over the following days with densities on the 4th and 5th days reaching 8.1×10^6 cells L⁻¹ and 2.8×10^7 cells L⁻¹, respectively. During this time, the *Chlorella* density was not significantly influenced because the grazer density was relatively low. SDBS addition (10 mg L⁻¹) on the 5th day resulted in a sharp decrease in *Poterioochromonas* sp. density and the grazer was rarely observed over the following days. The alga could still grow and continuously increase its biomass owing to the robust effects of the SDBS pesticide. A cell density of 11.1 mg Chl *a* L⁻¹ (0.42 g DW L⁻¹, alternatively 8.4 g m⁻² d⁻¹) was observed on the 10th day. The biomass yield of *Chlorella* was comparable to those previously reported [18, 19]. On the 10th day, the culture was scaled up into a 200 m² raceway pond and four days later the second round of *Poterioochromonas* contamination was observed. The development of the second round of

contamination was very similar to the previous one observed in the 20 m² pond. SDBS pesticide (10 mg L⁻¹) successfully eliminated *Poterioochromonas* sp. once again, without damaging *Chlorella* growth.

Discussion

Surfactants as novel pesticide for controlling biological contamination in *Chlorella* culture

In the present study surfactants were used as pesticide to control ciliates and flagellates contaminations in *Chlorella* culture. Among the selected five surfactants, SDBS, SDS, and AEO7 met the basic requirements of a pesticide for the control of *Poterioochromonas* sp. and *Hemiurosomoida* sp. in *Chlorella* culture. First, the complete control (elimination) of the two grazers could be achieved by the addition of any one of the tested surfactants at a proper concentration (Figs. 1 & 2). Second, the surfactants SDBS, SDS, and AEO-7, which eliminated the two grazers at the minimal effective concentrations, had little effects on *Chlorella* growth. Particularly, the minimal effective concentrations of SDBS for the complete elimination of *Poterioochromonas* sp. and *Hemiurosomoida* sp. were as low as 8 mg L⁻¹ and 10 mg L⁻¹, respectively. However, SDBS concentrations as high as 20 mg L⁻¹ had no effect on photosynthetic activity, cell membrane integrity, and biomass accumulation of *C. pyrenoidosa* XQ-20044 (Fig. 3).

For other two tested surfactants CDEA and AES, the minimal effective concentrations for complete control of *Poterioochromonas* sp. were 10 mg L⁻¹ and 20 mg L⁻¹, respectively. These effective concentrations did not significantly affect the growth of *Chlorella*. But the minimal effective concentrations of CDEA and AES for complete control of *Hemiurosomoida* sp. were as high as 15 mg L⁻¹ and 30 mg L⁻¹, respectively, which inhibited the growth of *Chlorella* significantly. So, if CDEA and AES were used to control *Hemiurosomoida* sp. contamination in *Chlorella* culture, the biomass of *Chlorella* might be reduced for some extents.

Possible mechanisms of SDBS pesticide for controlling bio-contaminations

Previous studies regarding the aquatic toxicity of anionic surfactants [20–23] showed that green algae were more tolerant to anionic surfactant (such as SDBS) exposure compared to invertebrates including daphnia, ciliates, flagellates, and bacteria. These results were consistent with those of the present study. Such differential tolerance between *Chlorella* and the two grazers provide evidence that these surfactants can be used as pesticides to control contamination in *Chlorella* mass cultures.

One remaining question is why did the surfactants only eliminate grazers such as *Poterioochromonas* sp. and *Hemiurosomoida* sp. rather than *Chlorella*? Why is there a different tolerance? Microscopical observation at 24 h after the addition of surfactants showed that the grazers decreased in numbers or even disappeared from the *Chlorella* culture. In fact, these changes occurred in less than 10 min after the addition of surfactants. Continuous microscopic monitoring (Additional file 3) revealed that the grazer cells, whether it was *Poterioochromonas* sp. or *Hemiurosomoida* sp., disintegrated shortly once the SDBS

concentration got close to the minimal effective concentration. However, the free-living *Chlorella* cells that were not swallowed retained their morphological and physiological integrity (Fig. 3).

Surfactants (or 'surface active agents') are organic compounds that can modify the solution properties both within the bulk of the solution and at the solid/water interface [24], and they have been recognized as having certain cytotoxicity [21, 22, 25]. Cell membranes are the primary target for the toxicological effects of surfactants on cells, which are known to be loss of cell viability and cell lysis [26, 27]. So, surfactants such as SDBS, caused the disintegration of *Poterioochromonas* sp. and *Hemiurosomoida* sp. cells in the present study. *Poterioochromonas* and *Hemiurosomoida* are unicellular organisms that lack a rigid or resistant structure (they are composed of insoluble non-hydrolysable biopolymers) outside the plasma membrane [14–16]. These cells were so sensitive that the lipid bilayers were disrupted immediately when enough surfactants were available in the medium.

One of the cell structures that differs *Chlorella* from the two grazers (*Poterioochromonas* sp. and *Hemiurosomoida* sp.) is its cell wall. Numerous species of green microalgae including *C. pyrenoidosa* have a two-layer cell wall with a classical polysaccharidic layer that is proximal to the cytoplasmic membrane and a thin outer layer [28, 29]. The outer layers are often trilaminar organized (termed as the trilaminar sheath, TLS) and are composed of insoluble non-hydrolysable biopolymers exhibiting an unusually high resistance to non-oxidative chemical degradation [30, 31]. In a study concerning bio-toxicity of environmental chemicals, Gwenaél Corre et al [28] found that the presence of a TLS in *C. emersonii* was associated with a very high resistance to anionic (DBS) and nonionic (TX-100) detergents at all growth stages and the net photosynthesis was not significantly affected in that species. This is also the reason why the photosynthetic capacity and viability of *C. pyrenoidosa* were not significantly affected by 20 mg L⁻¹ SDBS in the present study. The TLS of *C. pyrenoidosa* may have worked as a protective structure against SDBS.

Applications of SDBS pesticide in *Chlorella* mass culture

SDBS is one of the most commonly used anionic surfactants for cleaning application, degreasing preparations, and emulsion polymerization [32]. This surfactant is easy to manufacture, store, transport, and handle [32], making the production industry and consumption market well-developed. It is forecasted that the world surfactant market will grow from the 2018 level to \$66.4 billion by 2025 [33]. As one of the most commonly used surfactants, the current price of SDBS is only \$1.4 per kilogram. Owing to its near-universal application, SDBS and its variants are also the most researched and documented, especially in terms of their fate in the environment. SDBS is generally regarded as a biodegradable surfactant and its degradation rate may be as high as 97–99% under aerobic conditions [34]. We harvested the SDBS-treated *Chlorella* in other study to find if there was residual SDBS in the harvested *Chlorella* biomass, and no SDBS was detected. Our previous studies have shown that the surfactants such as SDBS are unable to induce changes in algal lipid synthesis [35, 36]. Therefore, it will be very cheap, convenient, and safe to use SDBS as a new pesticide in microalgal mass cultivation, especially for biofuel production. The surfactant SDBS directly acts on the unprotected plasma membrane of the grazers, therefore, the efficacy

of SDBS as a pesticide may be general, and it might be possible to apply SDBS in control of contaminations caused by other grazers in *Chlorella* mass culture.

Avoiding target biomass reduction is a necessary principle for biological contamination control in microalgal cultivation. Grazer reproduction and *Chlorella* biomass loss are both becoming faster and bigger with the extension of time for a contaminated *Chlorella* cultivation. So, early detection and treatment are crucial for minimizing algal biomass reduction. From this point of view, 10 mg L⁻¹ SDBS should be added as soon as grazers are observed microscopically to prevent further reproduction of grazers. Since cell densities of the grazer *Poterioochromonas* and *Hemiurosomoida* are relatively low at this time, 10 mg L⁻¹ SDBS would be adequate for completely eliminating the contaminants.

The tolerable SDBS concentration for *C. pyrenoidosa* is 20 mg L⁻¹, which is at least two times that of the minimal effective concentration for eliminating the grazers *Poterioochromonas* sp. and *Hemiurosomoida* sp. Such a difference is very helpful for outdoor application. Even the SDBS pesticide is required once again in a short time, the *Chlorella* will not be affected negatively. Besides, the surfactant is inevitably degraded by bacteria and fungi in the open culture, resulting in a decreased effective term. Fortunately the disintegration effect of SDBS on grazers will not be significantly weakened because the grazers can be eliminated in less than one day.

Conclusions

All five selected surfactants were effective for eliminating *Poterioochromonas* sp. and *Hemiurosomoida* sp. contamination in the laboratory. Further studies indicated SDBS (10 mg L⁻¹) is an efficient pesticide to control the contaminations without damaging *Chlorella*. One of the principles for SDBS pesticide application is early detection and treatment of contaminations. The surfactant SDBS directly acts on the unprotected plasma membrane of the grazers; therefore, the efficacy of SDBS as a pesticide may be general. The authors expect a broad spectrum of anti-bio contaminations to be developed using the method outlined in the present study.

Methods

Chemicals used for the control of biological contaminants

Five surfactants, specifically sodium dodecyl benzene sulfonate (SDBS, CAS NO. 25155-30-0), coconut diethanolamide (CDEA, CAS NO. 68603-42-9), sodium dodecyl sulfate (SDS, CAS NO. 151-21-3), fatty alcohol polyoxyethylene ether (AEO-7, C12, CAS NO. 68131-39-5), and alcohol ethoxysulphate (AES, C12, CAS NO. 68891-38-3), were tested for their effects on microalgae and biological contaminants.

Chlorella strain and test for its growth under surfactants exposure

Chlorella pyrenoidosa XQ-20044 was used in the present study. It was provided by the Algae Culture Collection of Wuhan Botanical Garden, Chinese Academy of Sciences. The algal seed were grown

autotrophically in shaking flasks under light intensity of $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ for 14 h per day and constant temperature (25 °C).

To study the effects of the SDBS surfactant on *Chlorella* growth, the seed cultures were inoculated into a bubbled column photobioreactor (PBR) at an initial density of $\text{OD}_{540} = 0.3$ (approximately 0.05 g L^{-1} dry weight), Light intensity at the surface of the column PBR was set at $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ with a light/dark cycle of 14 h/10 h. A thermostatic water bath was used to maintain constant culture temperature of 30 °C. The culture suspension was agitated with 200 mL min^{-1} air enriched with 1% CO_2 (v/v). The basal growth medium was BG-11. Concentrated SDBS solutions were added to the culture columns immediately after inoculation to reach final SDBS concentrations of 0, 2, 6, 10, 20, and 40 mg L^{-1} . Each cultivation was run in triplicate and proceeded for 3 days. The biomass dry weight (DW) and photosynthesis activity were monitored every day during cultivation. Fluorescein diacetate (FDA) dye was used to indicate the cell viability of *Chlorella pyrenoidosa* XQ-20044. Effects of the other four surfactants were evaluated by comparing DWs of the culture under the same conditions.

Grazer isolation and successive transfer cultures

Grazer samples were obtained from a microalgal mass culture test station at Yunnan province, China (26°29'29.6" N; 100°40'56.12" E). The grazers, namely *Poterioochromonas* sp. and *Hemiurosomoida* sp., were frequently observed and caused biomass loss during test cultivations of *C. pyrenoidosa* XQ-20044 in open raceway ponds from 2011 to 2019. We isolated *Poterioochromonas* sp. and *Hemiurosomoida* sp. cells and fed them *C. pyrenoidosa* XQ-20044 cells. After repeatedly re-isolation and feeding, steady cultures of the two grazers were established. In these steady cultures, *Poterioochromonas* sp. and *Hemiurosomoida* sp. grew heterotrophically by grazing on the *Chlorella* cells. For maintenance, the cultures were kept at room temperature (25 °C) with low light and occasional shaking. Diluted *C. pyrenoidosa* XQ-20044 suspension (approximately $\text{OD}_{540} = 0.1$) was fed to the *Poterioochromonas* sp. or *Hemiurosomoida* sp. cultures in a mixed ratio of 1:1 (v/v) every 2–3 days. By this way, successive transfer cultures of *Poterioochromonas* sp. and *Hemiurosomoida* sp. were established, which greatly facilitated the experiments.

Control of *Poterioochromonas* sp. and *Hemiurosomoida* sp. using surfactants

Based on the successive transfer cultures of the two grazers, the effects of SDBS, CDEA, SDS, AEO-7, and AES on *Poterioochromonas* sp. and *Hemiurosomoida* sp. were studied. Immediately after feeding with *Chlorella*, the cell densities of *Poterioochromonas* sp. and *Hemiurosomoida* sp. were counted and recorded. Generally, by feeding 1 L of the grazer culture with 1 L diluted *Chlorella* suspension every 2 days, the cell densities of *Poterioochromonas* sp. and *Hemiurosomoida* sp. reached at least 10^4 cells mL^{-1} and 8×10^2 cells mL^{-1} , respectively, which ensured fast growth during the following days if no surfactant was added. These grazer cultures were transferred into small flasks (80 mL working volume) and different volumes of the concentrated surfactant solutions were added to reach the desired concentrations. The flasks were kept at room temperature (25 °C) with low light and occasional shaking.

The experiments were performed in triplicate. A control culture that without surfactant addition was included in each experiment to show grazer viability and effectiveness of using the successive transfer culture. Morphological changes occurring during the first hour were observed using an optical microscope and recorded with a digital camera. After 24 h of surfactant exposure, the cell densities of the live *Poteriochromonas* sp. and *Hemiurosomoida* sp. were counted.

Field test of sodium dodecyl benzene sulfonate (SDBS) as a pesticide to control flagellates and ciliates contaminations

SDBS was used as a pesticide to control *Poteriochromonas* sp. and *Hemiurosomoida* sp. in *Chlorella* mass cultivation. The field test was conducted at the microalgal mass culture test station at Yunnan province, China (26°29'29.6" N; 100°40'56.12" E). Detailed information about the raceway ponds and general cultivation parameters can be seen in our previous study [37]. *Chlorella pyrenoidosa* XQ-20044 was firstly cultivated in greenhouse-covered raceway ponds (5 m², 1000 L) using BG-11 medium and solar irradiation. Then, approximately 2 L of the *Poteriochromonas* sp./*Hemiurosomoida* sp. culture suspension was added empirically into each pond on the 2nd or 3rd day. The grazer cultures acted as seeds to bring about *Poteriochromonas* sp./*Hemiurosomoida* sp. contamination, which was validated later by microbial community analysis using metagenomics data. 1 – 2 days after the addition, the grazers increased in density and could be easily observed under the microscope and counted using a counting chamber. Such development was very similar to the natural occurrence of *Poteriochromonas* sp. or *Hemiurosomoida* sp. contamination in *Chlorella* mass cultivation. After several days of cultivation and development when marked increases in the grazer density were observed, 10 mg L⁻¹ SDBS was added to the ponds. For the control experiments, the development of the two grazers was not interfered with by any extra operation. The experiments were conducted in parallel. Chl *a* content and grazer density were monitored every day to indicate *Chlorella* growth and grazer development, respectively.

The SDBS pesticide was also applied in a 20 to 200 m² cascade culture of *Chlorella* in October 2019. The cultivations were performed according to our previous study [37] and continued for 20 days. For the first 10 days, the cultivation was conducted in a greenhouse-covered 20 m² raceway pond (4000 L cultural volume) and then the culture suspension was transferred to an 200 m² open raceway pond (40000 L cultural volume) to inoculate a new cultivation. Solar irradiations during the culture period is given in Additional file 4. The cascade culture was microscopically monitored twice a day and two rounds of naturally occurring bio-contamination were observed. The SDBS pesticide (10 mg L⁻¹) was used to control these contaminations.

Measurements

Biomass dry weight (DW) and Chlorophyll *a* (Chl *a*) content were measured to evaluate *Chlorella* growth. About 10 mL of algal suspension was filtered through a pre-dried filter paper (0.45 μm). The filter paper holding the cells was washed with 10 mL ddH₂O, dried at 105 °C for 4 h and weighed to calculate the microalgal DW (g L⁻¹). Chl *a* was extracted from live cells with hot DMSO (70 °C) and was quantified

spectrophotometrically [38]. An equation ($DW \text{ g L}^{-1} = 38.14 \times \text{Chl } a \text{ mg mL}^{-1}$, $R^2 = 0.9979$) was estimated from an uncontaminated *Chlorella* mass culture and used to calculate *Chlorella* DW for those samples that had contamination during the field test.

The photosynthetic status of *Chlorella* was evaluated by measuring the chlorophyll fluorescence parameter (F_V/F_M) using a PAM 2500 fluorometer according to Xie et al.'s method [39]. Fluorescein diacetate (FDA) staining was used to determine *Chlorella* viability (cell membrane integrity) according to the methodology described by Serra-Maia et al [40]. Briefly, *Chlorella* cells were centrifugally collected from 1 mL of culture suspension (approximately $OD_{540} = 0.5$) and washed twice with PBS (pH 7.4). The cells were re-suspended in 200 μL of PBS added with 4 μL of 2% FDA (dissolved in acetone) and kept in the dark for 10 min at room temperature. The fluorescence intensity was measured on a plate reader (Tecan Infinite M200 PRO) with wavelengths of excitation and emission light of 480 and 530 nm, respectively. Pictures were also taken on a Leica DMI8 C microsystem.

Cell densities of *Poteroochromonas* sp. and *Hemiurosomoida* sp. were counted with two types of plankton counting chamber (0.1 mL, 400 counting squares and 0.1 mL, 100 counting squares) after fixing with Lugol's solution. Only 1 μL of Lugol's solution (10%) was used for each 10 mL of sample to inhibit grazer swimming but avoid cell disruption. At least three independent countings were conducted for each sample. To study the morphological changes of grazer cells exposure to surfactant, the cells were continuously monitored under microscope, and a small device was used to assist video recording. Description of the device and the recorded videos can be seen in Additional file 3. The outdoor samples were also subjected to metagenomic sequencing to evaluate whether the microbial community was consistent with that expected.

All the above analytical experiments were performed in triplicate and the results were analyzed for variance using SAS 9.13 at a significance level of $\alpha = 0.05$. Tukey's multiple comparison tests were done where applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XW performed indoor and outdoor cultivations and examinations, analyzed and interpreted the data, and draft the manuscript. AZ, YD, XZ participated in the outdoor cultivation and examination. LL, YH, KW, and YY participated in the laboratory investigation and data visualization. YG and YL designed the experiment and critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

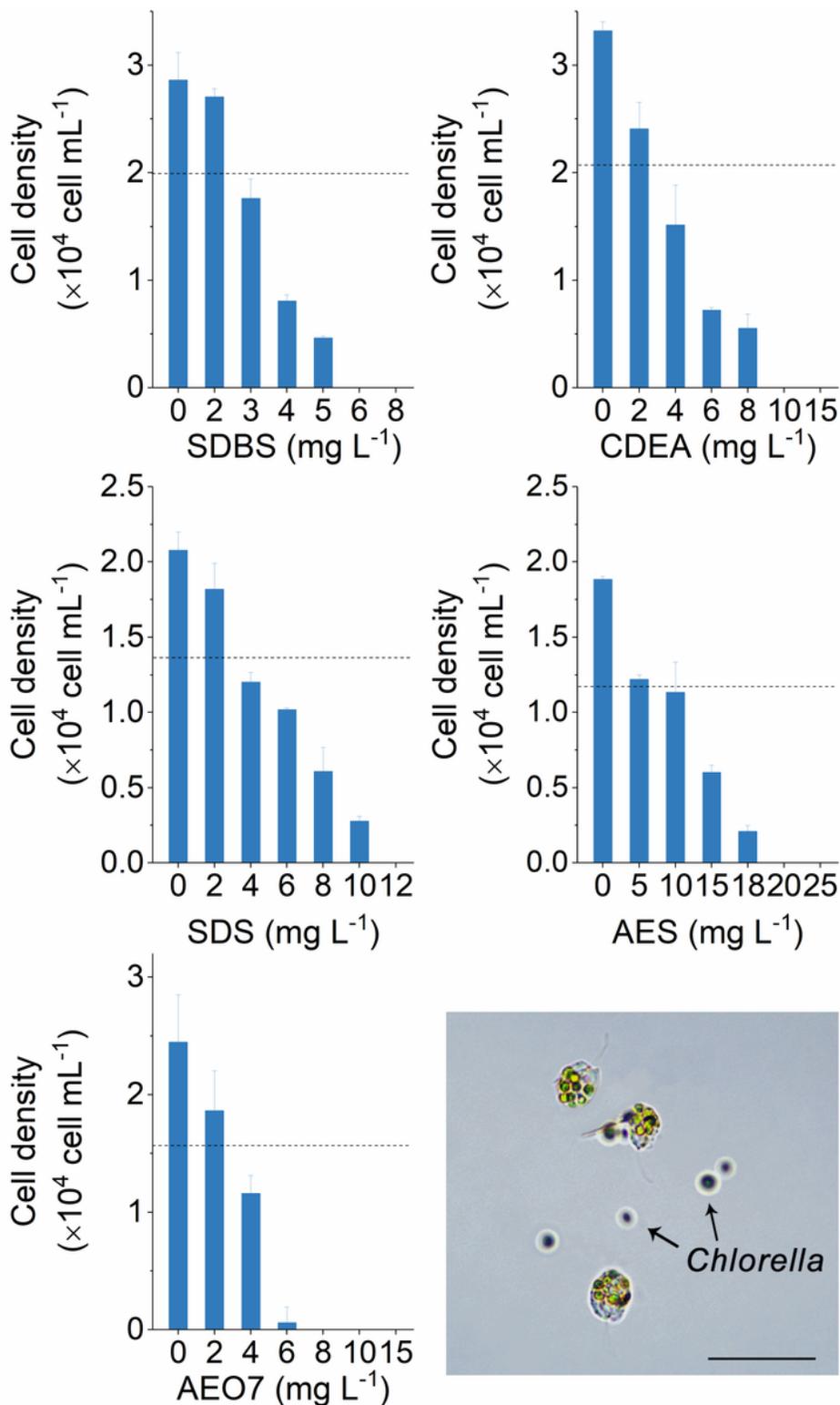


Figure 1

Toxic effects of five surfactants on *Poterioochromonas* sp. growth. SDBS, Sodium dodecyl benzene sulfonate; CDEA, coconut diethanolamide, SDS, sodium dodecyl sulfate; AEO-7, fatty alcohol polyoxyethylene ether; AES, alcohol ethoxysulphate. Dash lines represent initial cell densities at the beginning of the experiment. Microscopic image of *Poterioochromonas* sp. with *Chlorella* cells inside their body is shown at the bottom-right corner. Scale bar = 30 μm .

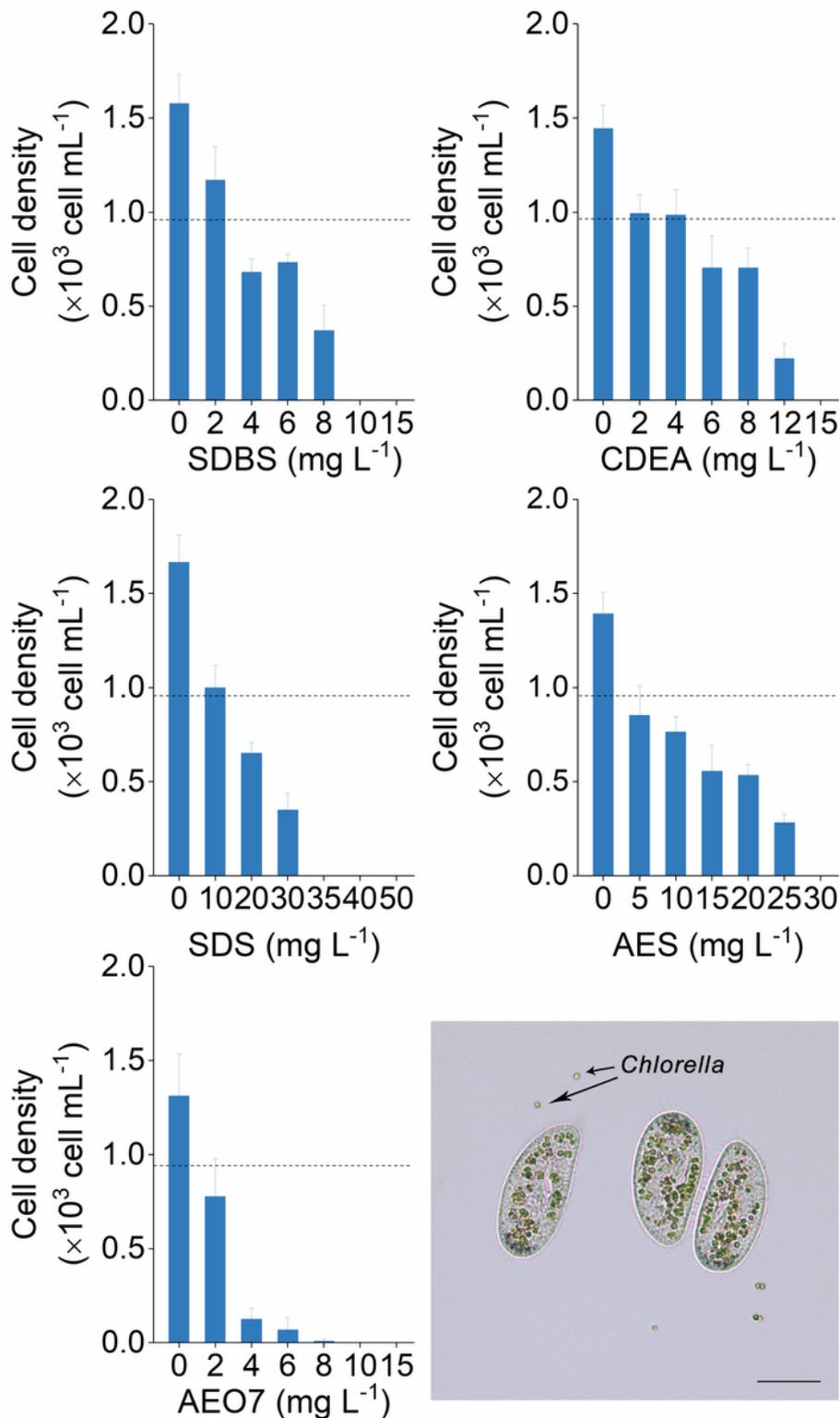


Figure 2

Toxic effects of five surfactants on *Hemiurosomoida* sp. growth. SDBS, Sodium dodecyl benzene sulfonate; CDEA, coconut diethanolamide, SDS, sodium dodecyl sulfate; AEO-7, fatty alcohol polyoxyethylene ether; AES, alcohol ethoxysulphate. Dash lines represent cell densities at the beginning of the experiment. Microscopic image of *Hemiurosomoida* sp. with *Chlorella* cells inside their body is shown at the bottom-right corner. Scale bar = 60 μm .

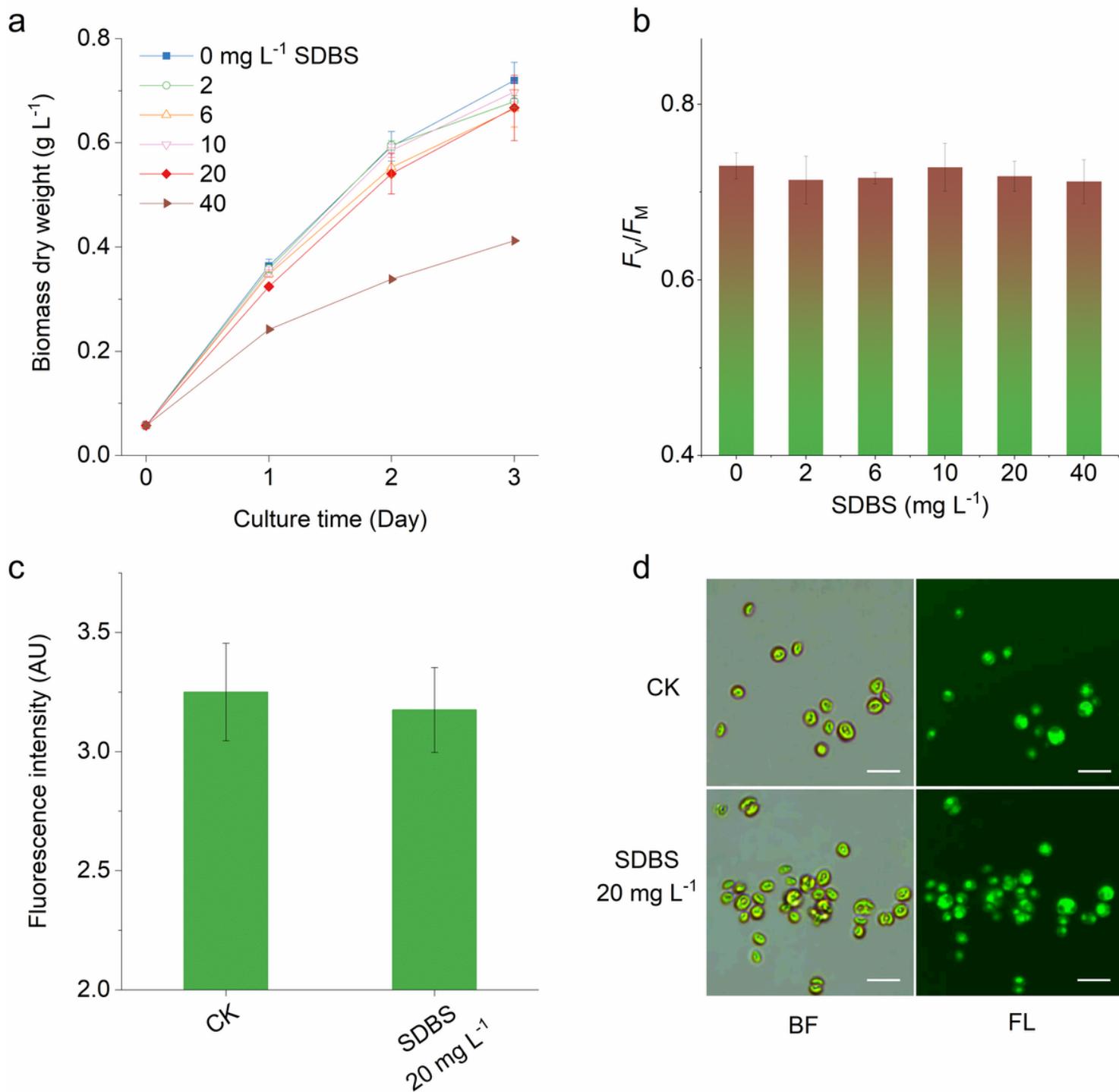


Figure 3

Effects of sodium dodecyl benzene sulfonate (SDBS) on *Chlorella pyrenoidosa* XQ-20044. (a) Cell growth, (b) Photosynthetic activity, (c) FDA staining fluorescence intensities, and (d) Bright field (BF) and fluorescence (FL) image of the cells. Scale bars = 10 μm.

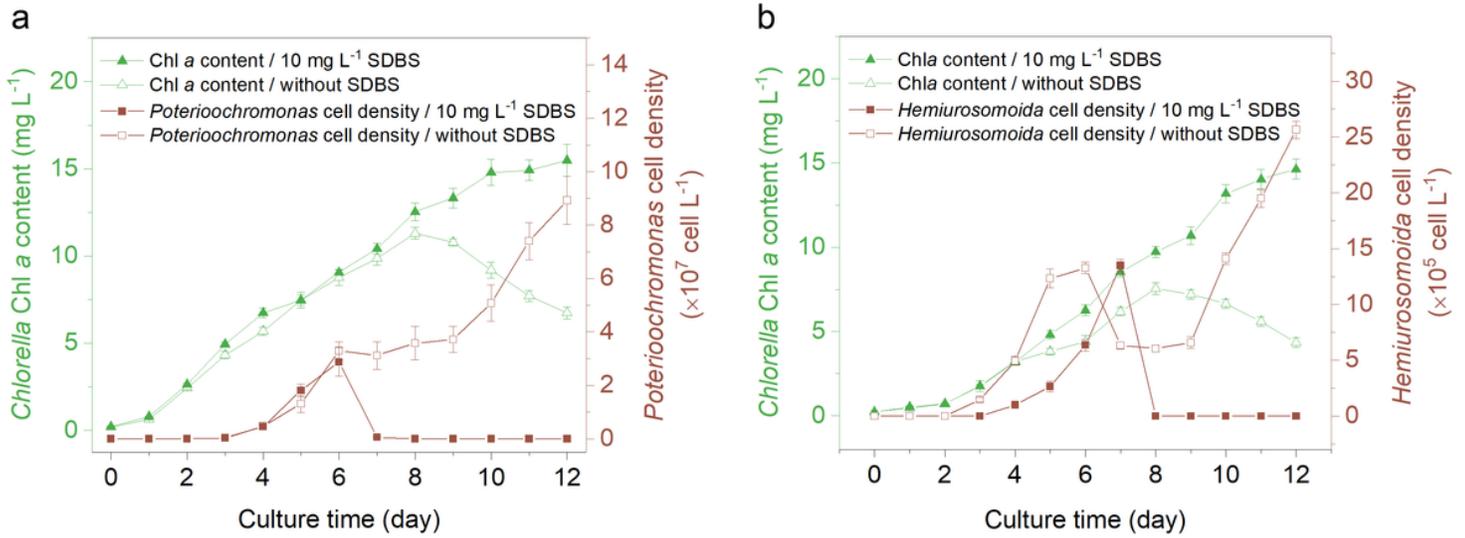


Figure 4

Changes in cell density of Chlorella and inoculated contaminating organisms in 5 m² raceway ponds. The contaminating organisms were *Poterioochromonas* sp. (a) and *Hemiurosomoida* sp. (b), with open symbols indicating the cultivation without SDBS treatment, and closed symbols indicating the cultivation treated with 10 mg L⁻¹ SDBS on the 6th day (a) and the 7th day (b).

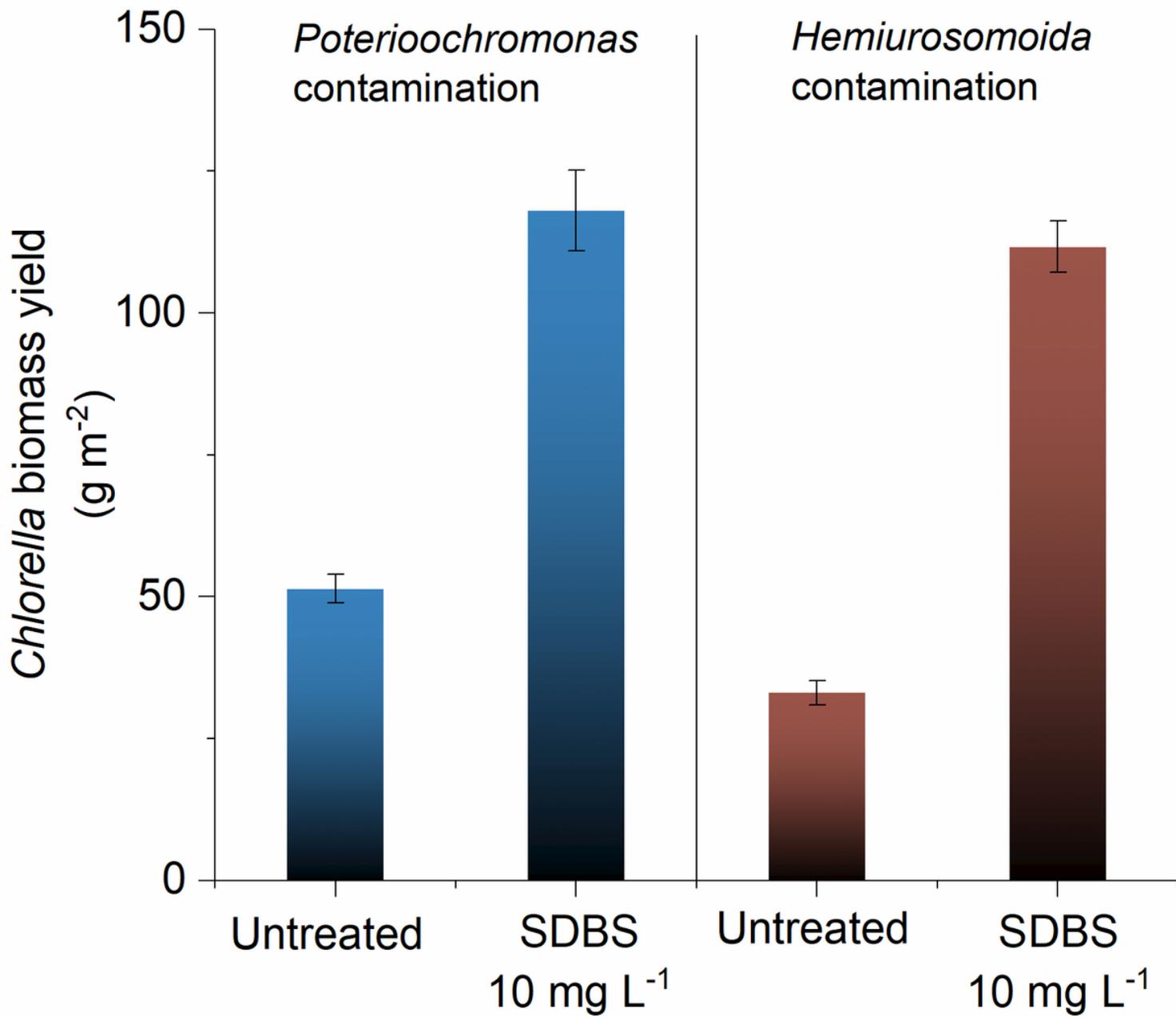


Figure 5

Comparison of biomass yields between the contaminated cultivations with and without SDBS treatment.

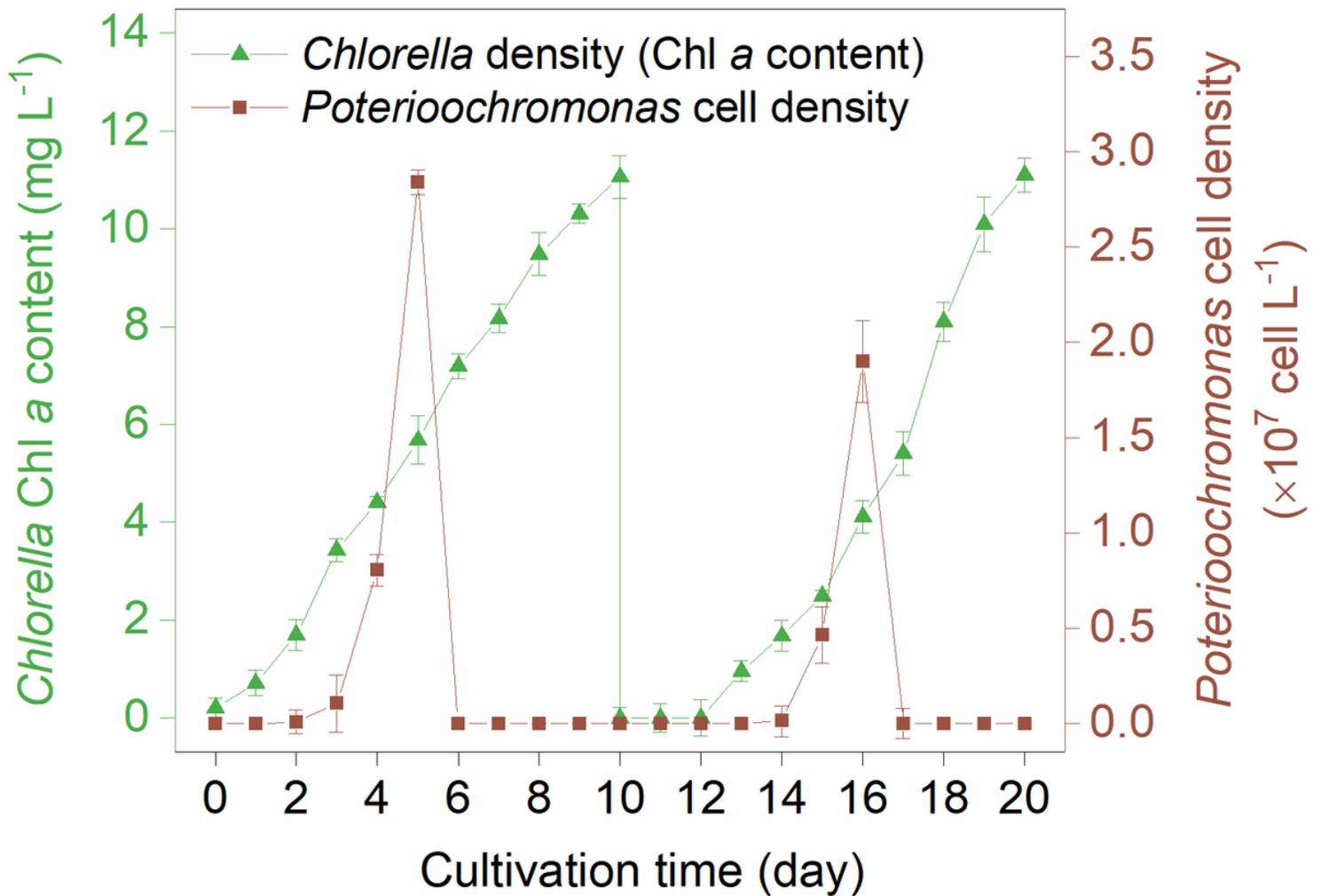


Figure 6

Cascade cultivations of *Chlorella* in raceway ponds and contamination control using SDBS. The first 10 days of cultivation was conducted in a 20 m² pond and the next 10 days was in a 200 m² raceway pond. Both observed contaminants were *Poterioochromonas* sp. and 10 mg L⁻¹ SDBS was added on the 5th and 16th day.

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