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ORIGINAL PAPERS

Analysis of the Mutual Influence of the Microalgae *Heterosigma akashiwo* (Raphidophyceae) and *Thalassiosira pseudonana* (Bacillariophyta)

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Abstract—The mutual influence of the rafidophyte microalgae *Heterosigma akashiwo*, which causes algal blooms, and the diatom *Thalassiosira pseudonana*, which is regularly present in sea waters, was studied under experimental conditions. Experiments were carried out with monocultures, monocultural filtrates, and mixed cultures of different initial concentrations. The growth and physiological state of the microalgae were assessed by flow cytometry. Both algae displayed inhibited growth in co-culture and on filtrates, but growth was suppressed to a greater extent in *T. pseudonana*. The fluorescence of chlorophyll *a* and the contents of reactive oxygen species and neutral lipids decreased in *H. akashiwo* cultures grown on *T. pseudonana* filtrate. Similar changes were noted in a *T. pseudonana* culture grown on *H. akashiwo* filtrate. It is concluded that these algae exhibit allelopathic activity in relation to each other.

Keywords: *Heterosigma akashiwo*, *Thalassiosira pseudonana*, co-culture, allelopathy, algal interaction, chlorophyll a fluorescence, reactive oxygen species, neutral lipids

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INTRODUCTION

The rafidophyte alga *Heterosigma akashiwo* causes "blooms" in different parts of the World Ocean [3, 27] that result in massive fish deaths [7, 30] caused by excretion of reactive oxygen species by the alga [20]. The concentration of its cells during "blooms" may reach 400000 cells/mL [2]. Therefore, it is important to study the ecology of *Heterosigma akashiwo* and the impact of both biotic and abiotic factors that influence its environment. Currently, there are few studies on the influence of biotic factors on *H. akashiwo*, including its interaction with diatoms [18, 32–34].

The direct mutual interaction of specific types of microalgae can be assessed under controlled conditions in a laboratory experiment. One of the methods to study biotic interactions is to experiment with monoculture filtrates, by which the "environment–population" relationships can be determined. Co-cultures make it possible to evaluate the "population–population" relationship, as well as to study the conditions under which dominance and species elimination occurs [21]. Mixed cultures are not just a mixture of monocultures, since their growth, biochemical composition and other indicators in a particular species can differ significantly from those of the same species recorded in monoculture [12].

The following indicators have been used in this study to assess the allelopathic influence on the physiological state of algae: chlorophyll *a* fluorescence, as an indicator of the state of the photosynthetic apparatus [14, 15]; the content of reactive oxygen species (ROS), which increases under adverse effects on a living organism [8], as well as the content of neutral lipids to assess the stress impact of unfavorable conditions on algae [4].

The diatom *Thalassiosira pseudonana* undergoes the phytoplankton succession cycle and is of great ecological importance as a species that influences the formation of "blooms" [16], including those in temperate and polar waters [10]. In the Far Eastern seas during the fall this alga may occur simultaneously with *H. akashiwo* [25, 26, 29]. Representatives of these genera in the form of cysts were found together in bottom sediments [24].

The purpose of this work was to study the mutual influence of microalgae *H. akashiwo* (Raphidophiceae) and *T. pseudonana* (Bacillariophyceae).

MATERIALS AND METHODS

This study is based on cultures of unicellular algae *Heterosigma akashiwo* MBRU_HAK-SR11 (Y. Hada) Y. Hada ex Y. Hara, *M. chihara* (Raphidophyceae)

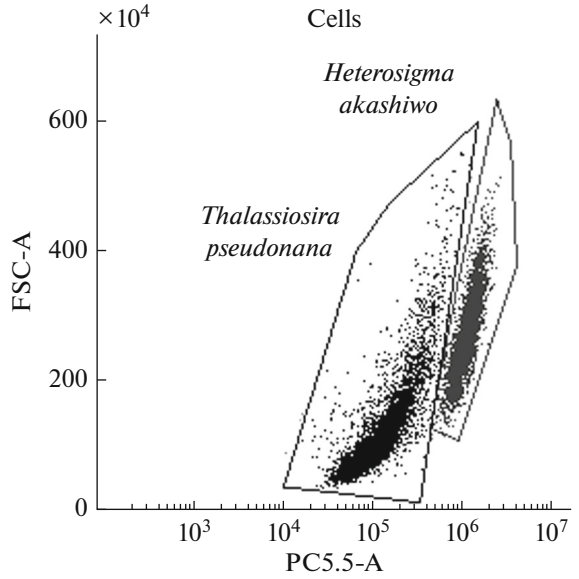

Fig. 1. The cell separation diagram of *Heterosigma akashiwo* and *Thalassiosira pseudonana* populations on a flow cytometer according to the size and chlorophyll *a* fluorescence. The abscissa shows the chlorophyll *a* fluorescence of cells in the PC5.5 channel; along the y-axis is direct light scattering, the FSC parameter, which displays indirectly the size of the cells.

and *Thalassiosira pseudonana* MBRU_TSP-02 Hasle & Heimdal (Bacillariophyta). Algae were grown on medium *f* [9] prepared on the basis of filtered and sterilized sea water with a salinity of 32‰, in 250-mL Erlenmeyer flasks with a culture medium volume of 100 mL, at a temperature of 18°C, an illumination intensity of 70 μmol/m^2/s in areas of visible light and a light-dark period: 14 h of light and 10 h of dark. Cultures at their exponential growth stage were used as inoculum. The initial cell concentrations (ICCs) for *H. akashiwo* were 1.5×10^4 and 3.0×10^4 cells/mL and for *T. pseudonana* were 10×10^4 and 30×10^4 cells/mL.

The experiment was carried out in three stages. At the first stage, the dynamics of microalgae abundance and their physiological parameters (chlorophyll *a* fluorescence, ROS content, and neutral lipids) were studied in monocultures at different initial concentrations. At the second stage, growth and physiological parameters were studied in a culture of *H. akashiwo* grown on *T. pseudonana* filtrates, as well as in a culture of *T. pseudonana* grown on *H. akashiwo* filtrates. At the third stage, the growth of microalgae in co-cultures of *H. akashiwo* and *T. pseudonana* was studied without assessing physiological parameters.

Filtrates of *H. akashiwo* and *T. pseudonana* microalgae were obtained by filtering cultures through MFAS-OS-2 membranes (Vladipor, Russia); the culture of the *T. pseudonana* was preliminarily centrifuged for 10 min at 7000 rpm. To obtain filtrates, cultures of both algae were taken on the 10th day when they were in the same growth phase and the culture of *H. akashiwo* has the highest toxicity [33]. The filtrates were checked under a microscope to exclude the presence of algal cells.

The duration of the experiments was 14 days. Samples for cell counting were taken after 3, 7, 10, and 14 days. To assess the fluorescence of chlorophyll *a*, to determine the content of ROS and neutral lipids, samples were taken after 7 and 14 days of the experiment. The parameters were measured on a CytoFLEX flow cytometer (Beckman Coulter, United States). For further analysis, 10000 events (particles detected in a sample) were recorded during each measurement. The selection of algae cells from the total number of events detected by the cytometer was done on the basis of chlorophyll *a* fluorescence [14, 19].

The size of the cells is indirectly determined on the cytometer on the basis of the indicator of forward scattering (FSC); the smaller this indicator is, the smaller the cells are. *H. akashiwo* cells have more intense chlorophyll *a* fluorescence than *T. pseudonana* cells. Due to these features, the detected cells can be assigned to a specific species of the studied algae during their cocultivation (Fig. 1).

The chlorophyll *a* fluorescence intensity was recorded at a wavelength of 690 nm; the excitation wavelength was 488 nm on the PC 5.5 channel [14]. ROS production was assessed using a fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate; staining was carried out for 1 h at room temperature in the dark. The fluorescence index of its oxidized and diacetylated product was determined at a wavelength of 525 nm and excitation at a wavelength of 488 nm on the FITC channel [8]. The content of neutral lipids was determined by the fluorescence of Nile Red fluorochrome at a concentration of 1 μg/mL; staining was carried out for 15 min at room temperature in the dark; the excitation wavelength was 488 nm and the emission wavelength was 580 nm in the PE channel [1].

The experiments were carried out in triplicate. Statistical processing was performed using the Excel software. The figures show the mean values and standard deviations.

RESULTS

Growth and Physiological Parameters of Heterosigma akashiwo and Thalassiosira pseudonana in Monoculture

In the population of *Heterosigma akashiwo* with NCC 1.5×10^4 cells/mL, a pronounced lag phase was noted (Fig. 2a); the number of cells doubled only on the seventh day of the experiment, then their number increased more rapidly. An increase in the population of microalgae with an NCC of 3×10^4 cells/mL was noted at the beginning of the experiment; the number of cells increased monotonically and by the end of the experiment was 21.6×10^4 cells/mL. The fluorescence

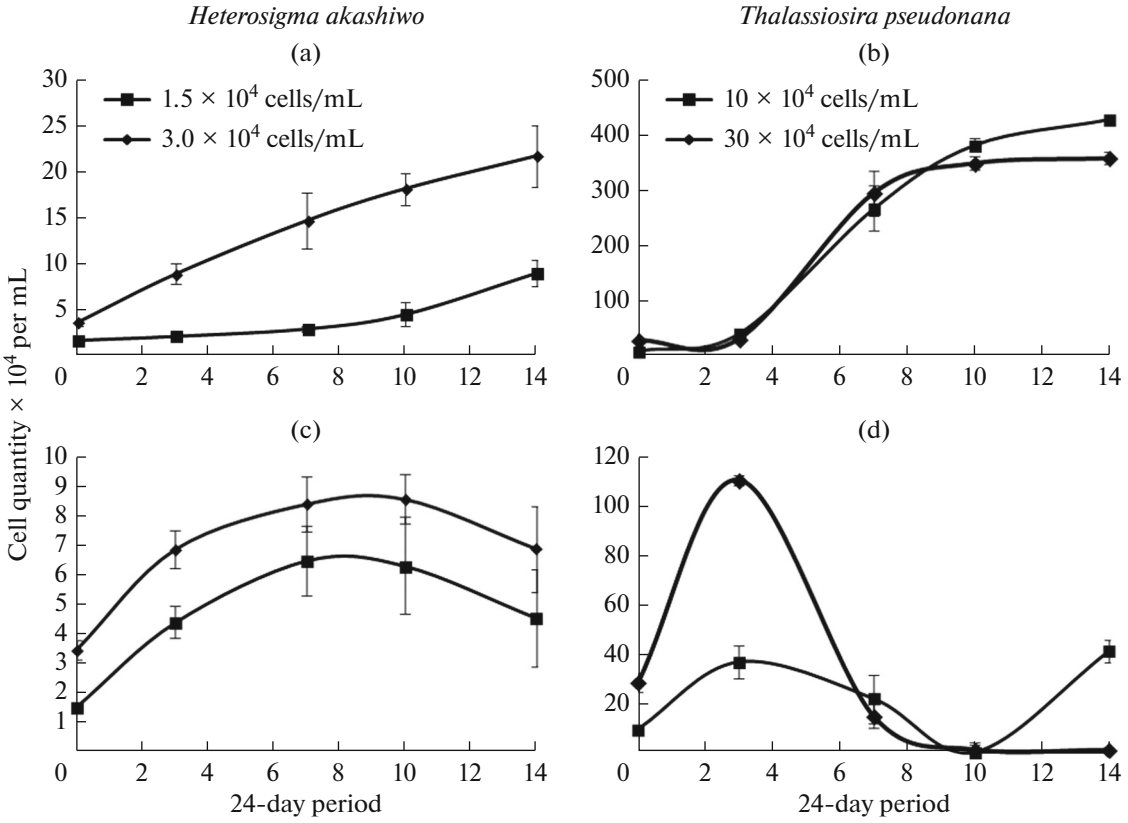

Fig. 2. The dynamics of the number of algae cells with different initial concentrations in monocultures. (a) *Heterosigma akashiwo*, (b) *Thalassiosira pseudonana,* and cultures grown on each other's filtrates: (c) *H. akashiwo* cultivated on *T. pseudonana* filtrate, (d) *T. pseudonana* cultivated on *H. akashiwo* filtrate.

of chlorophyll *a* in *H. akashiwo* in monocultures with different FCC values on the 7th and 14th days of exposure did not differ significantly (Fig. 3a), but slightly decreased by the end of the experiment. The content of ROS in the alga on day 14 was higher than on day 7 (Fig. 4a). In general, a higher content of ROS was noted in the culture with an NCC of 1.5 × 10^4 cells/mL. Regardless of the initial concentration of alga, the content of neutral lipids in *H. akashiwo* on the 14th day was higher than on the 7th (Fig. 5a).

At both initial concentrations in the monoculture of *Thalassiosira pseudonana*, the lag phase lasted up to 3 days, an exponential phase was observed from days 3 to 7, and a stationary phase was observed from days 7 to 14 (Fig. 2b). At NCC 10 × 10^4 and 30 × 10^4 cells/mL, the maximum abundance was 430 × 10^4 and 356 × 10^4 cells/mL, respectively. The fluorescence of chlorophyll *a* at NCC 10 × 10^4 cells/mL increased by the end of the experiment, while at higher NCC it decreased (Fig. 3b). The ROS content in *T. pseudonana* at the beginning of the stationary phase increased at both initial cell concentrations (Fig. 4b). The content of neutral lipids in the culture with NCC 10 × 10^4 cells/ml throughout the experiment was lower than in the culture with a higher initial concentration (Fig. 5b).

The Growth and Physiological Parameters of Heterosigma akashiwo and Thalassiosira pseudonana Cultures on Filtrates

When *H. akashiwo* was cultivated on *T. pseudonana* filtrates, an increase in the number of cells was noted in the first 3 days (Fig. 2c); at NCC 1.5 × 10^4 cells/mL the growth of *H. akashiwo* was more intense than in the monoculture. However, at a higher FCC the number of cells throughout the experiment was lower than in the monoculture. After 10 days, the number of cells decreased in both cases. Regardless of the initial concentration, the fluorescence of chlorophyll *a* in *H. akashiwo* during the entire experiment was lower than in the culture grown on pure medium (Fig. 3c). The ROS content was higher in the culture with NCC 3 × 10^4 cells/mL (Fig. 4c); this indicator remained at the same level on the 7th and 14th days of the experiment. In the culture grown on *T. pseudonana* filtrates, the content of neutral lipids was lower than in the monoculture, but it also increased by the end of the experiment (Fig. 5c).

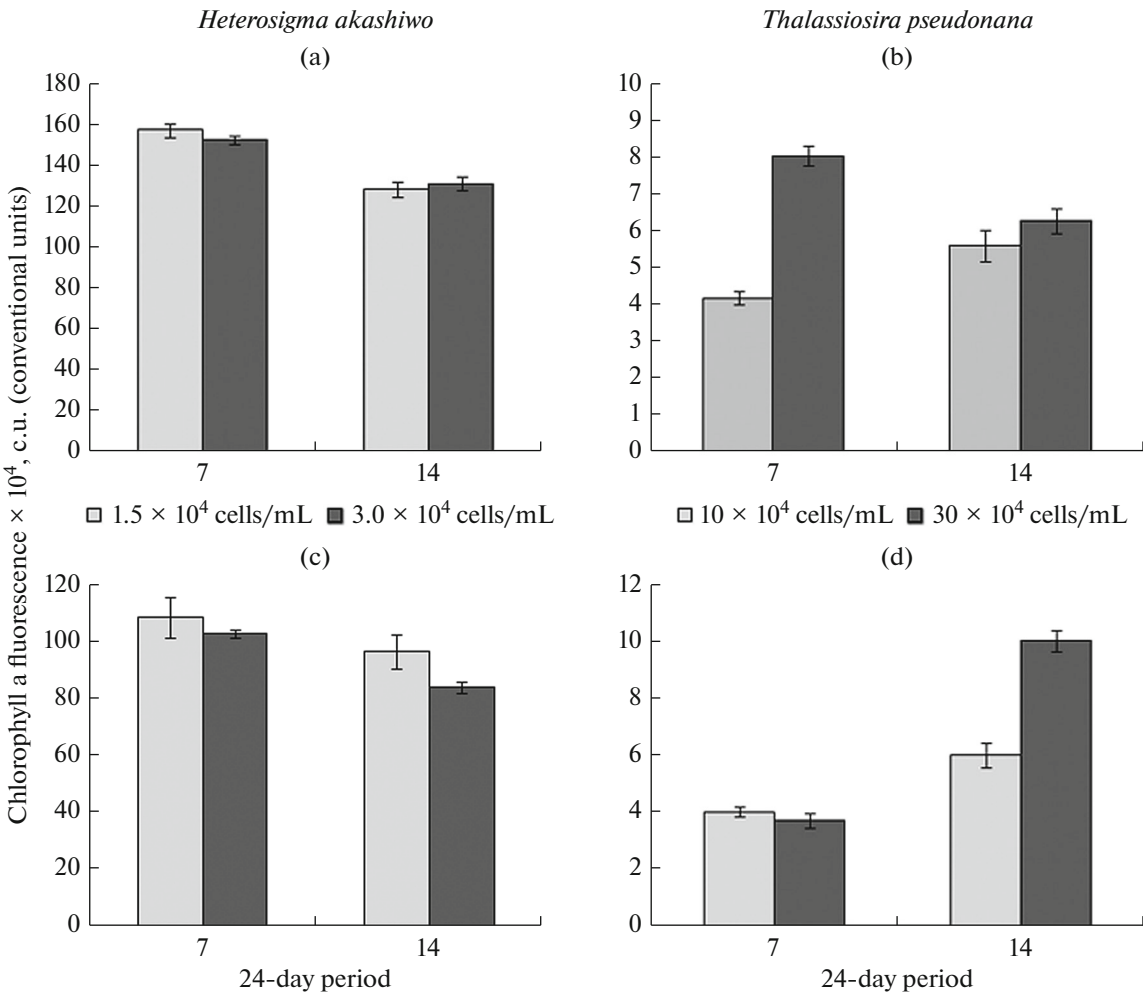

Fig. 3. Chlorophyll *a* fluorescence detected in channel PC 5.5 in algae with different initial concentrations in monocultures. (a) *Heterosigma akashiwo*, (b) *Thalassiosira pseudonana*, and in cultures grown on each other's filtrates: (c) *H. akashiwo* cultivated on *T. pseudonana* filtrate,(d) *T. pseudonana* cultivated on *H. akashiwo* filtrate.

On *H. akashiwo* filtrates, the number of *T. pseudonana* cells (lag phase absent) on the third day increased to 370 × 10^4 and 111 × 10^4 cells/mL at the ICC of 10 × 10^4 and 30 × 10^4 cells/mL, respectively (Fig. 2d). By the end of the exponential growth phase of the culture, the number of cells decreased and continued to decrease until the tenth day. On day 14, in the culture with low FCC, the number of cells increased to 41.5 × 10^4 cells/ml, at the same time in the culture with FCC 30 × 10^4 cells/mL it remained at the level of day 10, 1.5 × 10^4 cells/mL. The fluorescence of chlorophyll *a* in the culture with NCC 10 × 10^4 cells/mL did not differ from that in the algae in monoculture (Fig. 3d). In a culture with a higher FCC the fluorescence was initially lower than in monoculture, but by the end of exposure it was higher than in the monoculture. The ROS content increased by the end of the exposure and was higher in the culture with NCC 30 × 10^4 cells/mL than in the culture with NCC 10 × 10^4 cells/mL (Fig. 4d). The content of neutral lipids during the cultivation of *T. pseudonana* on *H. akashiwo* filtrates significantly decreased by the end of the experiment (Fig. 5d).

The growth of Heterosigma akashiwo and Thalassiosira pseudonana in Co-Cultivation

When *H. akashiwo* and *T. pseudonana* were co-cultivated with an NCC of 15 × 10^4 cells/mL, the number of *H. akashiwo* increased from the beginning of the experiment and did not differ from this indicator in monoculture for 10 days (Fig. 6a). By the end of the exposure, the growth of the algae slowed and its abundance was lower than in the monoculture. The culture with NCC 3 × 10^4 cells/mL had a 3-day lag followed by intensive growth. However, the number of cells in the culture was less than in the control sample. As in the monoculture, the number of cells increased more intensively at higher NCC. The growth of *H. akashiwo*

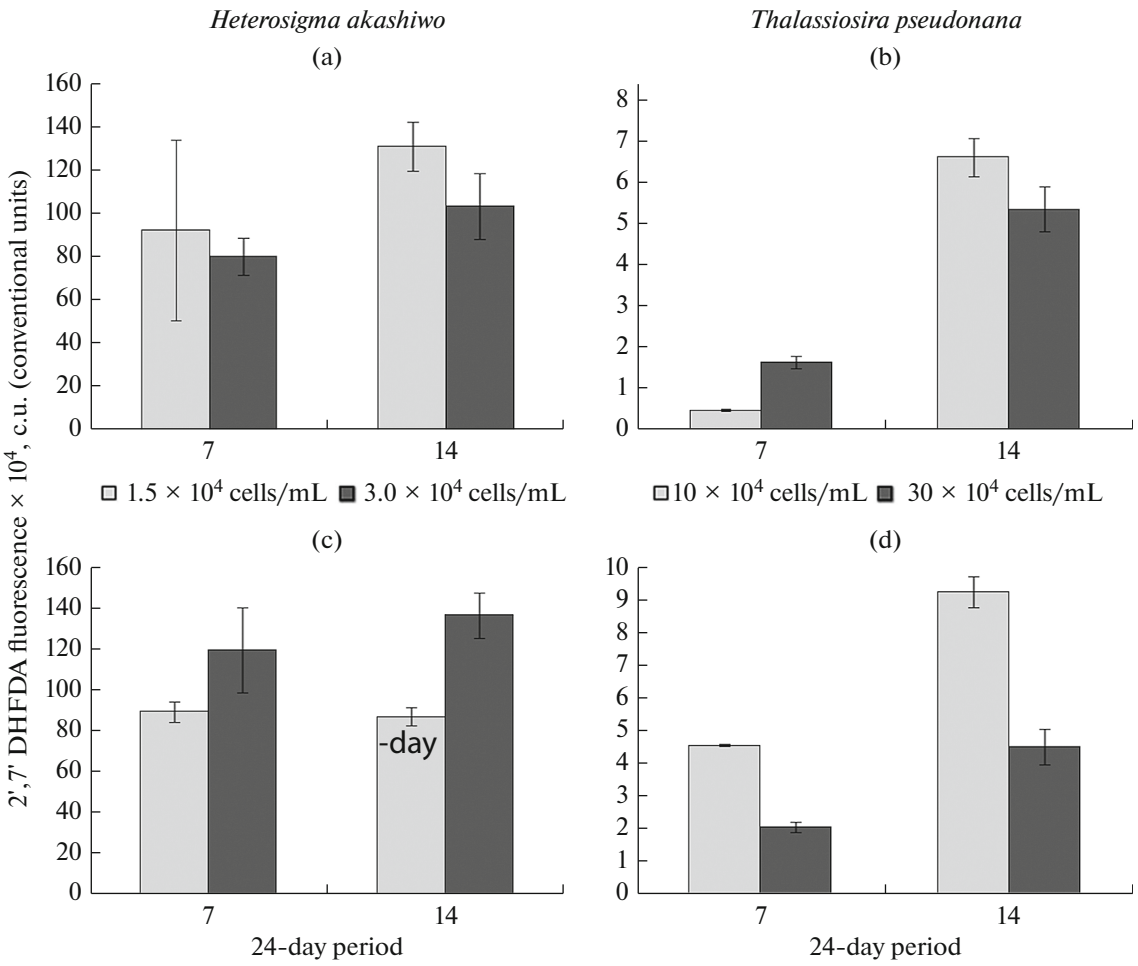

Fig. 4. The fluorescence of cells marked with the indicator of presence of the active oxygen 2',7'-dihydrofluorescein diacetate in algae with different initial concentrations in monocultures (a) *Heterosigma akashiwo*, (b) *Thalassiosira pseudonana*, and in cultures grown on each other's filtrates: (c) *H. akashiwo* cultivated on *T. pseudonana* filtrate,(d) *T. pseudonana* cultivated on *H. akashiwo* filtrate.

when co-cultivated with *T. pseudonana* did not depend on the concentration of *T. pseudonana*.

The growth of *T. pseudonana* with NCC 10 × 10^4 cells/mL in a mixed culture was inhibited throughout the experiment and remained at a constant level (Fig. 6b). In the culture with NCC 30 × 10^4 cells/mL the number of cells decreased slightly for 7 days and increased to 60 × 10^4 cells/mL only by the tenth day.

DISCUSSION

This study demonstrated that the culture of *Heterosigma akashiwo* had a greater effect on *Thalassiosira pseudonana* than the culture of *T. pseudonana* had on *H. akashiwo*, when co-cultivated and grown on filtrates. Yamasaki et al. [33] found that the co-cultivation of *H. akashiwo* and *Skeletonema costatum* changed the growth of only *S. costatum*, which decreased by 13% compared to its growth in monoculture. Simultaneously, when growing *H. akashiwo* on extracts of *Phaeodactylum tricornutum*, the death of most of the *H. akashiwo* cells as a result of lysis was noted. In surviving cells, membrane damage was observed, as well as a decrease in esterase activity and chlorophyll *a* fluorescence [32].

The culture of the alga *T. pseudonana* with NCC 30 × 10^4 cells/mL co-cultivated with *H. akashiwo* grew more intensively than with NCC 10 × 10^4 cells/mL; however, the opposite effect was noted when grown on filtrates. Yamasaki et al. [33, 34] noted that the growth of *S. costatum*, at low *S. costatum* FCC, was inhibited when exposed to *H. akashiwo* filtrates, but this inhibition did not occur in a culture with high *S. costatum* FCC. One possible explanation of this fact is that the growth inhibition noted in the experiments is associated, among other things, with smaller sizes of *T. pseudonana* than those of *H. akashiwo*. Small species with a fast metabolism are more sensitive to substances with allelopathic activity [4]. It should be noted that when growing *T. pseudonana* on filtrates,

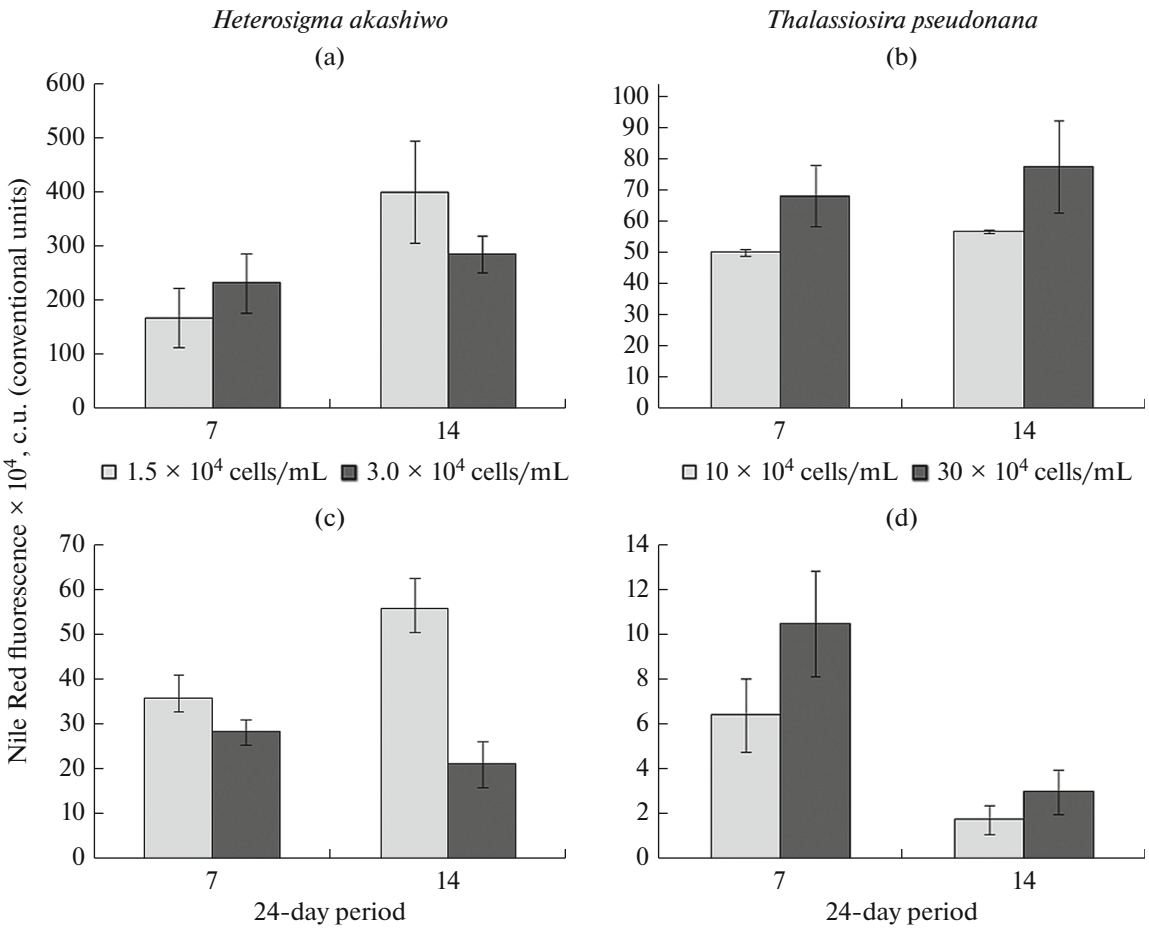

Fig. 5. The fluorescence of cells marked with Nile Red, an indicator of neutral lipid content, in algae with different initial concentrations in monocultures (a) *Heterosigma akashiwo*, (b) *Thalassiosira pseudonana*, and in cultures grown on each other's filtrates: (c) *H. akashiwo* cultivated on *T. pseudonana* filtrate, (d) *T. pseudonana* cultivated on *H. akashiwo* filtrate.

the number of cells first increased and then clearly decreased. The same trend was observed when the freshwater green alga *Chlorella vulgaris* was grown on filtrates of various cyanobacteria [17].

Despite the fact that the growth of *T. pseudonana* and *H. akashiwo* populations was suppressed they did not die by the end of the experiment, although it is known that substances secreted by microalgae, for example, representatives of the genus *Alexandrium*, can cause cell immobilization and lysis within several hours [6, 11, 18, 35].

A highly competitive effect in co-cultivation can be achieved not only due to the suppression of one culture by another with toxic exometabolites, but also due to the withdrawal of nutrients during the faster growth of one of the cultures or due to a greater need for nutrients in one species than in another. Small diatoms often have a high growth rate of the population, which can lead to faster removal of nutrients from the environment compared to competitive species [21]. This is thought to be the reason for inhibition growth in *H. akashiwo* when co-cultivated with *T. pseudonana*, although, as noted, it was weaker than when grown on *T. pseudonana* filtrates. Pichierri et al. [22] noted that filtrates of diatoms *Skeletonema marinoi* and *Thalassiosira* sp. inhibited the growth of *Ostreopsis* cf. *ovata*, respectively, to 56 and 78% of the growth in monoculture. These authors attributed this fact to high production of polyunsaturated aldehydes and fatty acids by diatoms, which impact other algal species. Diatom aldehydes act as signaling molecules, causing cascade reactions of targeted type in the cell, which leads to its death. Species of the genus *Thalassiosira* negatively affect the cell cycle and induce apoptosis in other microalgae species [5, 16].

In the present investigation it was noted that the growth of the *T. pseudonana* culture was suppressed to a greater extent at *H. akashiwo* FCC of 3×10^4 cells/mL than at FCC of 1.5×10^4 cells/mL. We believe that the concentration of both species is important and not just the more allelopathically aggressive species, because at 30×10^4 cells/mL *T. pseudonana* ICC, demonstrate a pronounced increase in the number of cells despite a 7-day lag

phase. This phenomenon was not observed at FCC 10×10^4 cells/mL. In the experiment on the co-cultivation of *Trichormus doliolum* and *Anabaena variabilis* it was found that an allelopathically active species must reach a certain concentration before it begins to dominate [13].

A decrease in the intensity of chlorophyll *a* fluorescence in *H. akashiwo* grown on *T. pseudonana* filtrate was observed in the present study. Previously, Long et al. [18] documented the same effect in *Alexandrium tamarense* filtrate linked to a simultaneous change in lipid composition in *Chaetoceros muelleri*. Based on the assessment of the esterase activity of *C. muelleri*, the authors concluded that these disorders are the result of damage to the outer membranes, including the production of ROS by algae. One of the reasons for the decrease in chlorophyll *a* fluorescence may be the destruction of chloroplast membranes, which was observed in *P. tricornutum* when co-cultivated with *A. tamarense* [35]. The same effect was observed in algae from different divisions during co-cultivation with *Sinechocystis* sp. [4, 28], as well as in *Ostreopsis* cf. *ovata* when co-cultivated with the diatom *Licmophora paradoxa* [31].

During our presently described experiment, a slight increase in ROS was registered when *T. pseudonana* was grown on *H. akashiwo* filtrate. Earlier, this phenomenon was observed by Long et al. [18] in *C. muelleri* when co-cultivated with *A. tamarense*. ROS can act as signaling molecules that affect the genetic processes in cells, which leads to both the appearance of nonviable cells and the emergence of organisms that are more adapted to the negative effects [5].

In the present experiment, during cultivation on filtrates in cultures of both algae, the content of neutral lipids decreased. Poulin et al. [23] noted that *Karenia brevis* metabolites destroy membrane lipids in *T. pseudonana* and *Asterionellopsis gracialis* simultaneously with growth inhibition.

Thus, according to the results we obtained, microalgae *H. akashiwo* and *T. pseudonana* not only affected their mutual growth, but also changed their physiological states. Both algae showed a decrease in chlorophyll *a* fluorescence and the content of neutral lipids. The content of ROS in *H. akashiwo* in the monoculture was higher than in the algae grown on the filtrate. This observation suggests that *H. akashiwo* is more toxic in monospecific blooms than in mixed phytoplankton communities. Since the growth and physiological state of algae were suppressed when they were grown on filtrates, we conclude that the studied microalgae exert more allelopathic than competitive influence on each other. At a higher FCC the number of cells in cultures of *H. akashiwo* and *T. pseudonana* reached higher values.

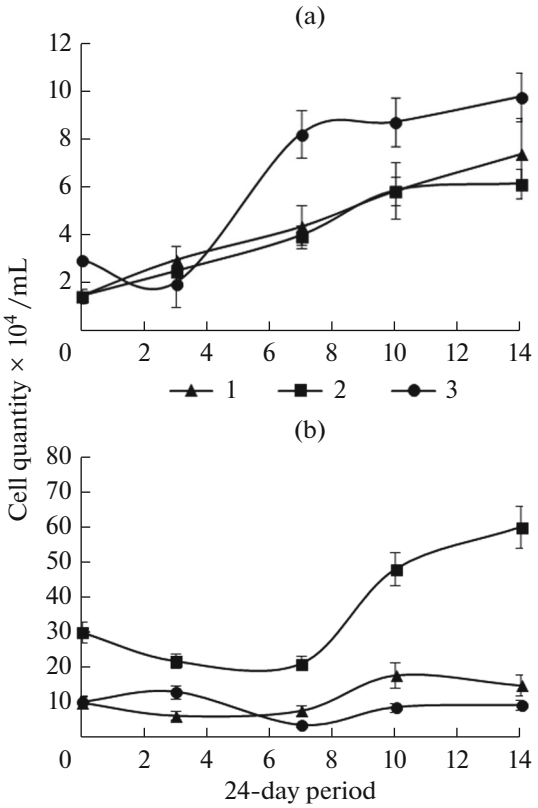

Fig. 6. The dynamics of the number of algal cells *Heterosigma akashiwo* (a) and *Thalassiosira pseudonana* (b) in a mixed culture. (1), Cultures of *H. akashiwo* with initial cell concentration (ICC) of 1.5×10^4 cells/mL and *T. pseudonana* with ICC of 10×10^4 cells/mL; (2), cultures of *H. akashiwo* with ICC of 1.5×10^4 cells/mL and *T. pseudonana* with ICC of 30×10^4 cells/mL; (3), cultures of *H. akashiwo* with ICC 3×10^4 cells/mL and *T. pseudonana* with ICC 10×10^4 cells/mL.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. This article does not contain a description of any research using humans and animals as subjects.

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