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THE EFFECT OF CRUDE OIL ON THE SYMBIOTIC ASSOCIATION OF THE GREEN ALGA *ACROSIPHONIA ARCTA* (DILLWYN) GAIN AND EPIPHYTIC BACTERIA

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It was experimentally shown that the green alga *Acrosiphonia arcta*, inhabiting the Barents Sea littoral zone, remains viable for 10 days in case of exposure to crude oil introduced into seawater at a concentration of 5 mg·L^{-1}. This concentration corresponds to a weak oil spill in the marine environment. Morphological and functional changes in the symbiotic association of *A. arcta* and epiphytic bacteria on its surface were traced by the techniques of microbiology, light and electron microscopy, and physiology. During the experiment, most algal cells maintained a high level of photosynthesis, and their ultrastructure was preserved. Interestingly, by the end of the exposure, under the effect of crude oil, the proportion of chloroplasts decreased in algal cells, and the pyrenoid and starch granules disappeared. The dynamics of the number of epiphytic bacteria in the experiment and the proportion of hydrocarbon-oxidizing bacteria in the total number of cultivated heterotrophs were traced. The capability of *A. arcta* to absorb and transform oil products was shown. This algal species is capable of developing in oil-contaminated water areas on any substrate, preparing it for colonization by larger perennial macrophyte algae, and this determines the significant role of *A. arcta* in the restoration of coastal phytocoenoses.

Keywords: *Acrosiphonia arcta*, oil, seawater bioremediation, symbiotic association, epiphytic bacteria, photosynthesis, ultrastructure

As found earlier, macrophyte algae, inhabiting the Barents Sea and representing various systematic groups, are capable of absorbing diesel fuel [Pilatti et al., 2016; Voskoboinikov et al., 2018, 2020a]. In experiments on seawater purification from diesel fuel using algae, it was shown that a decrease in the content of oil products (hereinafter OP) in water occurred in parallel with their accumulation in plants. At the same time, on algae surface, degradation of OP occurred due to epiphytic hydrocarbon-oxidizing bacteria (hereinafter HOB), and this ensured their absorption and diesel fuel neutralization by plant cells. As known, HOB, that are in a mutualistic relationship with macrophyte algae, are able to oxidize almost all OP, with the degradation rate depending on the ratio of their constituent hydrocarbons [Atlas, 1978; Heitkamp, Cerniglia, 1987; Pirnik, 1977; Pugovkin, 2017]. Scant data on this issue have been obtained on algae representatives with lamellar thallus [Pugovkin, 2017; Ryzhik et al., 2019; Voskoboinikov et al., 2018, 2020b]. Interestingly, *Acrosiphonia arcta* (Dillwyn) Gain, 1912 belongs to pioneer species that prepare the substrate for its colonization by perennial dominant species [Malavenda et al., 2017].

The aim of this study is to identify morpho-functional changes, caused by the effect of crude oil, in the green alga *A. arcta* (a species with a siphonaceous thallus; an inhabitant of the littoral zone), to analyze the toxicant transformation by the symbiotic association of this alga and bacteria, and to determine the potential role of the species in seawater purification.

MATERIAL AND METHODS

A. arcta vegetative thalli, approximately equal in size and weight, were sampled on the coast of the Zelenetskaya Bay of the Barents Sea (69°07′09″N, 36°05′35″E) in summer. Foulers were removed, and thalli were placed in glass containers with 1.3 L of seawater. Seawater (salinity of 33‰) sampled in the alga habitat was pre-filtered through a cotton-gauze filter to remove large (visible) suspended matter. Then, oil from the field on Kolguev Island (Peschanoozerskoye oil and gas condensate field) was introduced into seawater at a concentration of 5 mg·L^{-1}, which corresponds to a weak oil spill in the marine environment and is 100 MPC (maximum permissible concentration) for water in terms of the total content of OP. According to regulatory documents, MPC for the total content of OP corresponds to 0.05 mg·L^{-1} [Normativy, 2020]. The experiment was carried out in a thermostatic box at a temperature of +7…+8 °C, irradiance of 16–18 W·m^{-2}, with a photoperiod 24L : 0D (it corresponds to the natural habitat of the alga in summer), and constant water aeration with air. Control (in containers with seawater with no oil) and experimental (in containers with introduced oil, 5 mg·L^{-1}) samples of the alga and water were taken for investigation at the beginning of the exposure (initial samples) and in 5 and 10 days. Changes in cell morphology were analyzed under a Mikmed-6 light microscope (LOMO, Russia) and a JEM-100C transmission electron microscope (Jeol, Japan). Under a light microscope, samples were examined *in vivo*. Samples for examining under an electron microscope (20–30-nm thick sections stained with toluidine blue) were prepared according to the standard technique [Voskoboinikov, Titlyanov, 1978]. The intensity of visible photosynthesis of the alga during the experiment was determined by the change in the oxygen content in water before and after thalli incubation using a HI 9141 oximeter (Hanna Instruments, Germany) and by the Winkler titration. The calculation was carried out in μg O_2 *per* 1 g wet weight of thallus *per* hour [Salakhov et al., 2020]. The total content of OP and concentrations of alkanes in water and the alga were determined by gas chromatography–mass spectrometry. Sample preparation and instrumental analysis were carried out according to EPA method 8270 (Semivolatile Organic Compounds by GC/MS) described in detail earlier [Voskoboinikov et al., 2018]. The mass fraction of crude oil components was calculated by the internal standard calibration. For water, the results are given in μg·L^{-1}; for the alga, in μg·g^{-1} dry weight.

The number of cultivated heterotrophic bacteria was determined by the limiting dilution analysis [Rukovodstvo, 1980] using liquid Zobell media for common heterotrophs [Prakticheskaya gidrobiologiya. Presnovodnye ekosistemy, 2006] and MMS medium for HOB [Koronelli, Iljinsky, 1984; Mills et al., 1978]. Obtained number of cultivated bacteria was recalculated *per* 1 g wet weight of the alga thallus.

RESULTS

Changes in the oil product content in water and the alga during the experiment. The fraction of Kolguev crude oil dissolved in dichloromethane, was characterized by the predominance of n-alkanes in the C_8–C_{30} range with maximums in the C_{14}–C_{16} range. The content of isoprenoids (pristane and phytane) was no more than 7% of the total sum of n-alkanes. Initial seawater contained 495 μg·L^{-1} of OP,

which is 10 MPC (Table 1). During the experiment (10 days), the content of OP in initial seawater with no oil increased to 1,527 μg·L^{-1} ("water + *A. arcta*"). The value of the indicator reflecting the degree of hydrocarbon transformation (∑n-alkanes / ∑OP) increased as well – from 0.06 to 0.10–0.12.

Table 1. Content and proportion of alkanes and oil products (OP) in water samples during the experiment, μg·L^{-1}

	Water	Water + *A. arcta*	Water + OP			Water + *A. arcta* + OP		
	0 days	10 days	0 days	5 days	10 days	0 days	5 days	10 days
Sum of n-alkanes	28.6	185	1,569	247	185	1,569	54	95
∑n-alkanes / ∑OP	0.06	0.12	0.28	0.13	0.16	0.28	0.09	0.08
Total content of OP	495	1,527	5,552	1,954	1,158	5,552	628	1,166

When oil was introduced, the content of OP (on the 1st day) was 5,552 μg·L^{-1}. By the 10th day of the experiment, the total content of OP in water samples decreased by 79%. With the presence of *A. arcta*, the total content of OP in water decreased by 88% on the 5th day and increased to 1,166 μg·g^{-1} on the 10th day of the exposure.

The total content of oil hydrocarbons (hereinafter OH) in *A. arcta* control sample (0 days) was 2,686 μg·g^{-1} (Table 2). By the 10th day, the value decreased by almost 30%.

With the presence of *A. arcta* in the medium with OP, the content of OH in the alga thallus increased significantly on the 5th day of the experiment but decreased on the 10th day.

Table 2. Content and proportion of alkanes and oil products (OP) in *Acrosiphonia arcta* samples during the experiment, μg·g^{-1} dry weight

	Control		Experiment		
	0 days	10 days	0 days	5 days	10 days
Sum of n-alkanes	102	156	102	1,482	867
∑n-alkanes / ∑OP	0.04	0.08	0.04	0.19	0.16
Total content of OP	2,686	1,929	2,686	7,930	5,395

Changes in the alga viability, morphology, and physiology during the experiment. Both control (with no oil) and experimental algae (with introduced oil) remained viable until the end of the exposure. In the control sample, thalli had an intense green color. In the experiment sample, the color intensity in some thalli decreased by the 10th day.

Light-optical and electron microscopic observations in 5 and 10 days of the experiment did not reveal any morphological changes in the alga cells with no oil (the control sample) compared to the initial variant.

The cell cytoplasm adhered tightly to the inner side of the plasma membrane. On cell sections, oval-shaped chloroplasts were detected (1 in Fig. 1a), located close to the plasmalemma.

In most cells, chloroplasts were combined into the photosynthetic reticulum. Chloroplasts contained thylakoids running parallel to each other along the long axis and a submerged pyrenoid with starch granules (2 in Fig. 1a) forming a sheath. Starch granules were abundant outside the pyrenoid as well – in the stroma of chloroplasts (3 in Fig. 1a). In the cytoplasm, there were up to three mitochondria

per cell section, 1–2 μm in size, with single cristae; moreover, several electron-dense granules were recorded, mostly rounded, 1.5–3 μm in diameter. On the outer side of the shell of *A. arcta* thallus, there were single bacteria in the initial variant.

In the experiment, 10 days after oil introduction, plasmolysis was observed in single cells of *A. arcta* thallus in *in vivo* samples. The plasmalemma separation from the inner surface of the thallus cell membrane was recorded not only under a light microscope, but also on sections under electron microscope. At this stage of the experiment, a degrading pyrenoid was observed in chloroplasts of single cells; it was not revealed in most cells. There were no starch granules. At the same time, there were no signs of damage to the lamellar system of chloroplasts (1 in Fig. 1b). The chloroplast stroma was quite dense, with abundant thylakoids. In the cytoplasm, compared to that of the control sample, on cell sections, there was an increase in number (up to 4–7) and size (up to 2.5 μm) of mitochondria, as well as in size of electron-dense globules (up to 4 μm) (4 in Fig. 1b). On the outer side of *A. arcta* shell, there were abundant microorganisms of various shape and density forming a continuous layer in some areas (7 in Fig. 1c).

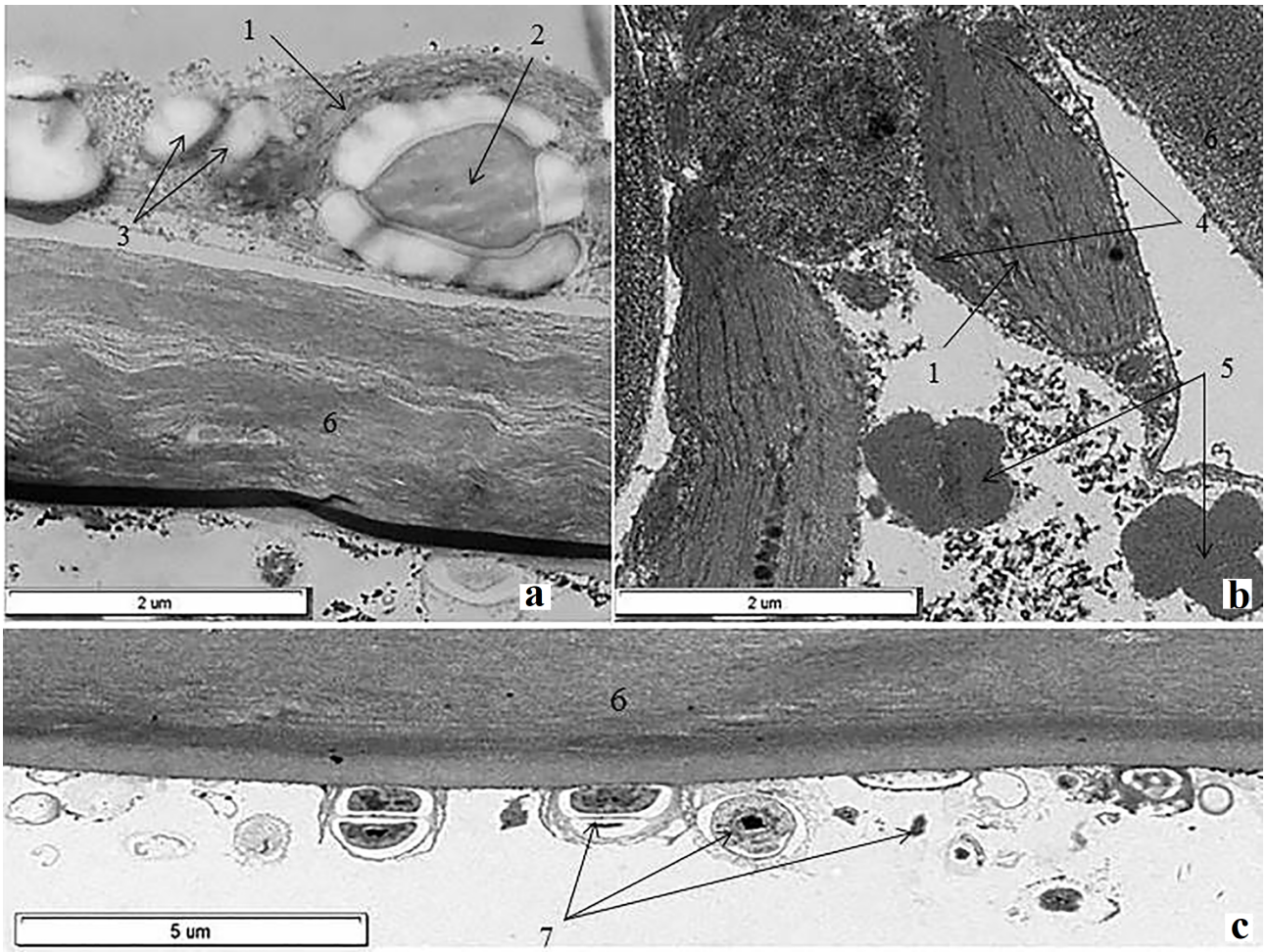

Fig. 1. Cell structure of *Acrosiphonia arcta* under oil contamination: a, control; b, c, experimental sample after 10 days of exposure. Legend: 1, chloroplast; 2, pyrenoid with starch granules; 3, starch granules in the chloroplast stroma; 4, mitochondria; 5, electron-dense globules; 6, algal shell; 7, epiphytic microorganisms on the surface of the alga

The intensity of photosynthesis in the initial samples of *A. arcta* was 0.42 μg O_2 *per* 1 g wet weight *per* hour (Fig. 2).

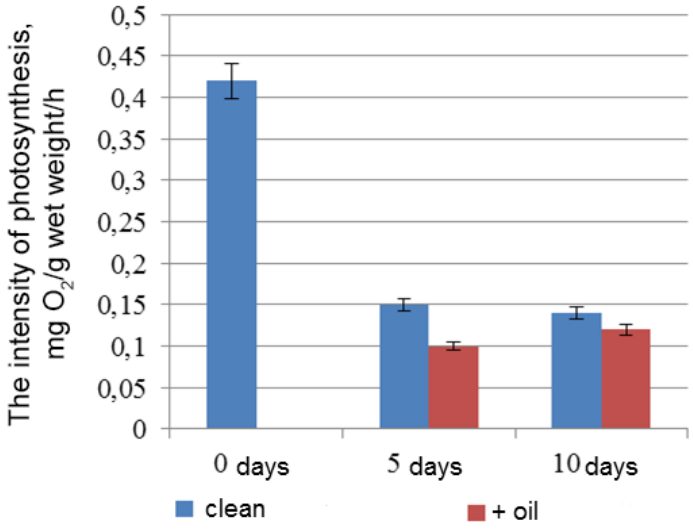

Fig. 2. Intensity of *Acrosiphonia arcta* photosynthesis during the experiment

In 5 days, in the alga of the control sample, the intensity of photosynthesis decreased by 2.8 times and did not change until the end of the exposure. In experimental samples of *A. arcta*, on the 5th day, there was a decrease in the intensity of photosynthesis by 1.5 times compared to the control variant. By the end of the exposure, the value almost did not differ from the control one.

Change in the number of epiphytic bacteria. During the experiment, a significant change in the number of cultivated heterotrophic bacteria was recorded – within several orders of magnitude. *Prior* to being placed under experimental conditions, *A. arcta* had a rather high (up to 9 orders of magnitude) number of cultivated epiphytic bacteria – more than 20 million cells·g^{-1} for heterotrophs and 4.3 thousand cells·g^{-1} for HOB (Fig. 3).

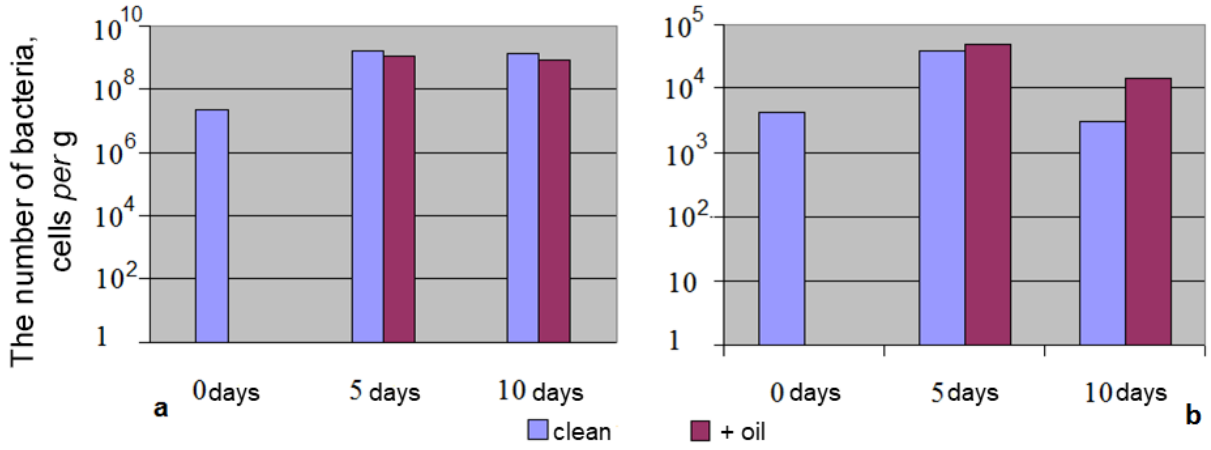

Fig. 3. Number of cultivated epiphytic heterotrophic (a) and hydrocarbon-oxidizing (b) bacteria in the experiment

By the 5th day of the exposure, in the control sample, the number of epiphytic bacteria of *A. arcta* was approximately 81.5 times higher (1.6×10^9 cells·g^{-1}) than in the initial variant (2.2×10^7 cells·g^{-1}). After oil introduction (100 MPC), their number increased by about 53.5 times – up to 1.1×10^9 cells·g^{-1} (the number of bacteria rose by more than 2 orders of magnitude). By the 10th day, there was a decrease in the number of epiphytic bacteria compared to previous values – by 1.2 times in the sample with oil and by 1.4 times in the control variant (up to 7.8×10^8 and 1.3×10^9 cells·g^{-1}, respectively).

The proportion of HOB in the alga before the experiment accounted for 0.02% of the total number of cultivated bacteria (4.3×10^3 cells·g^{-1}). By the 5th day, their proportion in the control sample decreased to 0.002% (3.02×10^4 cells·g^{-1}); in the experimental sample, the value was 0.005% (4.9×10^4 cells·g^{-1}). By the 10th day, with a decrease in the number of cultivated bacteria compared to that on the 5th day, the proportion of HOB decreased as well – to 0.0002% in the control and to 0.002% in the experiment (3.1×10^3 and 1.5×10^4 cells·g^{-1}, respectively).

DISCUSSION

The experiments have shown as follows: *A. arcta* remains viable for 10 days at such a content of OP in water, which is 10 times higher than the MPC and 100 times higher than 0.05 mg·L^{-1} (the value taken as 1 MPC for fishery reservoirs) [Normativy, 2020]. According to this study and previous ones, the level of seawater contamination by OP at the sampling site ranges from 2 MPC in winter and spring to 10 MPC in summer [Voskoboinikov et al., 2018, 2020b]. In the water area of the Zelenetskaya Bay, there is a diving center; therefore, an increase in contamination may result from the intensified navigation of small vessels with the onset of the season in July. Apparently, the development of the alga under low contamination by OP ensured its resistance to this factor.

The changes in the mass fraction of alkanes and the total content of OP in water in experiments with crude oil and the green alga, as well as without the alga, are shown in Tables 1 and 2. As already noted, in 10 days of the experiment, the content of OP in the initial water with no OP increased to 1,527 μg·L^{-1} ("water + *A. arcta*"). The value of the indicator reflecting the degree of hydrocarbon transformation (∑n-alkanes / ∑OP) increased as well – from 0.06 to 0.10–0.12. This indicates slight "introduced" oil contamination. The alga itself may initially contain a significant amount of OH on the surface.

As also mentioned earlier, when oil was introduced at a concentration of 5 mg·L^{-1}, the measured OP content (on the 1st day) was 5,552 μg·L^{-1}. During the experiment, the total content of OP in water samples without the macrophyte alga gradually decreased; on the 10th day, the value was 1,158 μg·L^{-1} (*i. e.*, it dropped by 79%). With the presence of *A. arcta*, a decrease in the total content of OP to 628 μg·L^{-1} (by 88%) on the 5th day was followed by an increase to 1,166 μg·L^{-1}. On the 10th day of the exposure, the content of OP in water with the macroalga was higher than in control samples (without the alga). This can be explained by the fact that the alga does not consistently absorb OP: there are periods of "returning" absorbed hydrocarbons to the environment until a certain equilibrium is established. Apparently, this is due to the life cycles of bacteria inhabiting surface of macrophyte algae. The concentrations of n-alkanes and isoprenoids (phytane and pristane) changed throughout the experiment, in general, in proportion to the total content of OP.

Initially, *A. arcta* thallus contained 2,686 μg·g^{-1} of OP. On the 10th day of the experiment, the content of hydrocarbons in *A. arcta* decreased to 1,929 μg·g^{-1}. It can be assumed that one part of OP was transformed in the thallus cells, and another part returned to the aquatic environment.

In the experiment with oil introduction, the absorption of OH by the alga surface was registered. The maximum content of OP in the alga cells was recorded on the 5th day of the exposure – 7,930 μg·g^{-1}. On the 10th day, the total content of OP in the alga decreased to 5,395 μg·g^{-1}. Given the data on water, we may assume that some of the hydrocarbons diffused back into water.

The processes of transformation of hydrocarbons in *A. arcta* can be traced by the changes in the values of the indicator ∑n-alkanes / ∑OP. Its decrease by the 10th day reflects active destruction of the main oil components – n-alkanes. However, by the 10th day, these processes were not fully completed. Changes in the total content of OH in water and simultaneously in the alga indicate that *A. arcta* purifies water by absorbing OH.

Visual and microscopic observations of the changes in *A. arcta* throughout the experiment showed as follows. Despite the fact that the alga remained viable under oil contamination corresponding to 100 MPC, destructive changes were registered in single thallus cells – enlightened protoplasm, reduction of the formed pyrenoid in chloroplasts and its starch sheath, and a decrease in the number and partial volume of starch granules on cell section followed by their disappearance. However, until the end of the exposure, chloroplasts in most thallus cells retained their integrity, and there were no signs of damage to the internal membrane structure. At all the stages of the experiment, electron-dense globules were registered in the cytoplasm. We cannot be sure in the nature of these formations, but we do not exclude that the globules are the product of the transformation of absorbed OP. Their presence in the alga cells, not only in the experiment with oil introduced into seawater, but also in the initial sample, may be due to the long-term habitation of the alga in an environment contaminated with OP (10 MPC) before our study. Moreover, the alga itself can synthesize hydrocarbons, *e. g.*, phytane. The intensity of photosynthesis decreased in the alga of both the control (with no OP) and experimental (with introduced OP) samples in 5 days by almost 3 times compared to that of the initial variant. However, in 10 days of the exposure, the values of photosynthesis in the sample with introduced oil did not differ from those registered in the initial variant. This fact may reflect the adaptive capacity of the alga photosynthetic apparatus to oil contamination. It is confirmed by minimal changes in the photosynthetic apparatus throughout the exposure. An increase in the intensity of photosynthesis in the presence of small doses of oil in the environment was revealed in experiments with other algae species, and this confirms the possibility of OH absorption by macrophyte algae and OH inclusion in the metabolism [Salakhov et al., 2020, 2021; Stepanyan, Voskoboinikov, 2006]. Possible changes in metabolism are evidenced by an increase in the number and size of mitochondria and the number of mitochondrial cristae on cell sections during the experiment.

The number of epiphytic bacteria correlated with changes in the concentrations of OH in the alga. By the 5th day, there was an increase in the number of heterotrophic bacteria, *inter alia* HOB. By the end of the exposure, their number decreased compared to the value on the 5th day. OH, which are accumulated in algae, can be a growth factor for microorganisms. Moreover, a sharp rise in the number of heterotrophic bacteria both in the experiment and in the control may be due to the fact as follows: during the vital activity of macrophytes, substances are released into the environment that contribute to the development of heterotrophic microorganisms.

Interestingly, with a significant increase in the number of HOB, their proportion relative to the total number of heterotrophic bacteria in the experiment remained quite low, in the presence of oil as well. The maximum proportion of HOB was observed in the alga *prior* to oil introduction into the medium; then, the value decreased. This was especially noticeable on the 10th day of the exposure: the difference was about an order of magnitude.

When estimating the number of cultivated heterotrophic bacteria in the control and experiment, some discrepancies arose with the results of electron microscopic analysis, in which significantly more bacteria were registered on the alga surface in the presence of oil than in the control (Fig. 1a, c). This can be explained by the fact that most of the bacterial community does not grow on nutrient media [Meyer-Reil, 1977; van Es, Meyer-Reil, 1982; ZoBell, 1946]. It is believed that about 10% of the entire community is capable of cultivating on media, with from 40% to more than 70% of the community being able to use OH as a nutrient substrate [Buckley et al., 1976; Panov, 1990]. However, this does not mean that other ("unaccounted") bacteria present in the environment are not capable of destructing OP. Cultivation on nutrient media allows to identify bacteria that can quickly adapt to conditions of contamination (within the framework of a laboratory experiment as well), to obtain quite reliable results, and to follow the trends occurring in bacterial communities under the effect of various contaminants (in our case, oil).

Conclusion. The obtained results showed the ability of the green alga *Acrosiphonia arcta* not only to withstand oil contamination of 100 MPC for 10 days (in terms of level, it is comparable to a weak oil spill), but also to participate in the purification of seawater from oil products. The first revealed fact is likely to be due to the formation of adaptive reactions during the development of the alga under low contamination by oil products, while the second, by analogy with characteristics of other studied representatives of phytobenthos, can be due to the formation of a symbiotic association of algae and hydrocarbon-oxidizing bacteria. These facts are supported both by the preservation of the structure and function of the photosynthetic apparatus at an oil contamination of 100 MPC and the presence of abundant microorganisms on the alga surface. With an extremely low biomass of *A. arcta*, its percentage contribution to the volume of neutralized oil products in the Barents Sea coast is much lower than that of *Laminaria* or *Fucus* algae [Voskoboinikov et al., 2020a]. However, *A. arcta* occurs in all latitudes of the World Ocean, is highly resistant to the environmental factors of the Barents Sea coast [Lüning, 1984; Wiencke et al., 1993], and is capable of developing in oil-contaminated water areas on any substrate, preparing it for colonization by larger perennial macrophyte algae and their passage through early developmental stages, at the same time participating in the bioremediation of the marine environment from oil. Thus, the role of *A. arcta* in the restoration of coastal phytocoenoses is quite significant.

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ВЛИЯНИЕ СЫРОЙ НЕФТИ НА СИМБИОТИЧЕСКУЮ АССОЦИАЦИЮ ЗЕЛЁНОЙ ВОДОРОСЛИ *ACROSIPHONIA ARCTA* (DILLWYN) GAIN И ЭПИФИТНЫХ БАКТЕРИЙ

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Экспериментально показано, что зелёная водоросль *Acrosiphonia arcta*, обитающая на литорали Баренцева моря, сохраняет жизнеспособность в течение 10 дней при воздействии сырой нефти, введённой в концентрации 5 мг·л$^{-1}$ в морскую воду. Данная концентрация соответствует слабому разливу нефти. Методами микробиологии, световой и электронной микроскопии,

а также физиологии проанализированы морфофункциональные изменения у симбиотической ассоциации акросифонии и эпифитных бактерий на её поверхности. Показано сохранение высокого уровня фотосинтеза и неповреждённой ультраструктуры у большинства клеток водорослей в течение всего эксперимента. Вместе с тем к концу опыта под воздействием нефти в клетках водорослей уменьшается доля хлоропластов, исчезают пиреноид и гранулы крахмала. Прослежена динамика численности эпифитных бактерий в эксперименте и доля углеводородокисляющих бактерий в общем количестве культивируемых гетеротрофов. Продемонстрирована способность акросифонии поглощать и трансформировать нефтепродукты. Способность данного вида водорослей развиваться в загрязнённых нефтью акваториях на любом субстрате, подготавливая его для заселения более крупными многолетними водорослями-макрофитами, определяет важную роль *A. arcta* в восстановлении прибрежных фитоценозов.

Ключевые слова: *Acrosiphonia arcta*, нефть, биоремедиация морской воды, симбиотическая ассоциация, эпифитные бактерии, фотосинтез, ультраструктура