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ИнБЮМ – IBSS

Морской биологический журнал
Marine Biological Journal
2024, vol. 9, no. 1, pp. 86–97
https://marine-biology.ru

UDC 582.263-11:[57.04:665.7]

ANALYSIS OF PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF *ACROSIPHONIA ARCTA* (DILLWYN) GAIN CELLS AT THE EARLY STAGE OF STRESS REACTION FORMATION UNDER THE EFFECT OF DIESEL FUEL EMULSION

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Received by the Editor 29.07.2022; after reviewing 24.03.2023;
accepted for publication 09.10.2023; published online 22.03.2024.

Features of stress reaction formation were studied in cells of the green alga *Acrosiphonia arcta* under the effect of diesel fuel emulsion. Changes in indicators of oxidative stress (concentration of hydrogen peroxide and accumulation of products of lipid peroxidation) were analyzed; activity of antioxidant enzymes, intensity of photosynthesis, and condition of cells were investigated. As shown, during the first day of exposure to the toxicant, plasmolysis and disruption of the chloroplast structure occur in cells. The stress reaction develops in stages. At the first stage, the amount of hydrogen peroxide increases, the concentration of products of lipid peroxidation changes, and the activity of superoxide dismutase rises. At the second stage, catalase activity increases. By the end of the first day of exposure, against the backdrop of a drop in catalase activity, peroxidase activity rises (the third stage). The intensity of photosynthesis decreases by the end of the experiment. As suggested, under the effect of diesel fuel emulsion, the daily dynamics of the biological cycles of a number of enzymes may be disrupted.

Keywords: *Acrosiphonia arcta*, diesel fuel, catalase, superoxide dismutase, peroxidase, lipid peroxidation, hydrogen peroxide, photosynthesis intensity

Acrosiphonia arcta (Dillwyn) Gain, 1912 is a species of green algae widespread in the littoral zone of the Barents Sea [Malavenda, 2018], where it can form quite large thickets. It belongs to the first settlers preparing the substrate for colonization by perennial species of algae, for example, *Fucus* representatives. *A. arcta* has high adaptive capabilities: it can withstand a wide range of fluctuations in environmental factors, such as temperature, light, *etc.* [Sussmann, Scrosati, 2011].

With intensive industrial development, the anthropogenic load inevitably increases which includes the release of petroleum hydrocarbons into the environment [Patin, 2008]. In coastal cities, the most vulnerable zone is the near-shore area affected from both land and sea. The flora of such coastal sites is poor in terms of species composition; vegetation surviving here has mechanisms to neutralize toxicants and/or adapt to their occurrence [Malavenda, 2018; Milchakova, Shakhmatova, 2007; Shakhmatova, Milchakova, 2014]. Petroleum products slow down vegetation growth, as shown for *Ascophyllum nodosum* and *Laminaria digitata* [Bokn, 1985], and disrupt zygote formation and fucoid development [Thélin, 1981]. On the example of *Fucus* representatives, researchers also noted the lack

of significant changes in the intensity of photosynthesis and concentration of pigments under exposure to petroleum products, both long-term [Voskoboinikov et al., 2004] and short-term [Stepanyan, 2014]. However, the biochemical composition and activity of enzymes changed noticeably under their effect [Shakhmatova, Ryzhik, 2020; Voskoboinikov et al., 2004]. At the same time, when green algae are exposed to petroleum products, a decrease in photosynthesis intensity was recorded; also, significant damage and alteration in the biochemical composition of cells were registered [El Maghraby, Hassan, 2021; Klindukh et al., 2021; Pilatti et al., 2016; Ryzhik, Makarov, 2019; Voskoboinikov et al., 2018].

When an organism encounters a toxicant, several defense systems are gradually activated [Apel, Hirt, 2004; Kolupaev, 2007]. First of all, the formation of reactive oxygen species is intensified [Pokora, Tukaj, 2010; Vega-López et al., 2013] which activate the antioxidant defense system (catalase, superoxide dismutase, glutathione peroxidase, and so on) [Alscher et al., 2002]. Changes in superoxide dismutase activity were revealed for *Chlorella vulgaris* [Calderón-Delgado et al., 2019], *Phaeodactylum tricornutum* [Wang et al., 2008], and *Ulvaria obscura* [Salakhov et al., 2020]. Changes in catalase activity were recorded for *Palmaria palmata* [Voskoboinikov et al., 2020] and *Ulva* algae [Pilatti et al., 2016; Ryzhik, Makarov, 2019]. The nature of variations in activity of enzymes depends on the value and duration of the stress factor effect. Chronic exposure triggers profound changes in cycles of synthesis of proteins / amino acids, lipid metabolism (alterations in the composition of fatty acids and lipids), *etc.* [Nechev et al., 2002; Ramadass et al., 2015].

Antioxidant enzymes which are biomarkers can be used to detect metabolic disorders caused by xenobiotics [Díaz-Báez et al., 2004; Geret et al., 2003; Inupakutika et al., 2016; Mallick, 2004; Shakhmatova, 2004]. The speed of activation of defense systems is important for further adaptation of an organism to a toxicant.

However, issues of the rate of the stress response formation and features of the involvement of various antioxidant system components in the cell protection from oxidative stress remain poorly studied, especially for macrophytic algae inhabiting the Arctic zone. We assume as follows. Based on the intensity of the response development and changes in enzyme activity, it will be possible to conclude on the fate of vegetation: whether it will be able to adapt to effects of the toxicant or die. As noted earlier, the fate of cells will depend on the changes occurring at the moment of contact with the toxicant [Shiu et al., 2020]. Thus, analysis of the indicators of the antioxidant system and photosynthetic activity on the first day of cell contact with petroleum products is significant for understanding the mechanisms of adaptation formation.

The aim of this study is to determine the rate of activation of *Acrosiphonia arcta* antioxidant system in response to the contact of this alga with a diesel fuel emulsion. The nature of the effect will be assessed by markers of oxidative stress (concentrations of hydrogen peroxide and lipid peroxidation products) and the state of enzymes of the antioxidant system (superoxide dismutase, catalase, and peroxidase).

MATERIAL AND METHODS

Experimental work was carried out in July 2020 at the seasonal biological station of the Murmansk Marine Biological Institute (Dalnie Zelentsy village, the Barents Sea eastern coast). This area belongs to ecologically clean spots of algae growth.

The alga thalli were sampled from the littoral zone of the Dalnezelenetskaya Bay and placed in laboratory conditions: a thermostatically controlled room with irradiance of 150 $W \cdot m^{-2}$ (24 h light : 0 h dark), water temperature of +8…+10 °C, and constant aeration of vegetation in vessels. The mode of irradiance

was chosen in accordance with features of the photoperiod (polar day) at the time of the experiment. The alga was acclimated to laboratory conditions for three days. Then, one part of the alga was placed in control vessels [pure seawater with salinity of 33‰], and another part was placed in experimental vessels [seawater with salinity of 33‰ with summer diesel fuel (state standard GOST 305-82) added at a concentration of 43 mg·L^{-1}]. In each variant of the experiment, 8 alga thalli were used (with a total mass of < 50 g). The concentration of petroleum products corresponded to the maximum noted for the Kola Bay coastal waters in 2014–2016.

The experiment lasted for one day. Indicators were measured during the day in 1, 3, 7, 10, and 24 h, in triplicate. In total, 70 samples were processed. Physiological and biochemical indicators were determined with a PE-5300VI spectrophotometer (Ecroskhim, Russia).

The content of hydrogen peroxide was established in accordance with a modified spectrophotometric method [Bellincampi et al., 2000]. It is based on the oxidation of iron Fe^{2+} with hydrogen peroxide to Fe^{3+} forming stained compounds with xylenol orange. Optical density was measured at a wavelength of 560 nm.

The level of lipid peroxidation (hereinafter LPO) was assessed by the accumulation of active products of thiobarbituric acid [Esterbauer, Cheeseman, 1990]. Measurements were carried out at 540 nm.

The supernatant for determining the activity of catalase and superoxide dismutase (hereinafter CAT and SOD, respectively) was obtained in the following way. The alga with the weight of 150–200 mg was ground on ice in a mortar, with 2,000 μL of extraction buffer added (K/Na-phosphate buffer). The homogenate was centrifuged for 5 min at 12,000 g; then, the supernatant was sampled.

CAT activity was measured by a modified spectrophotometric method [Korolyuk et al., 1988]: 2 mL of 0.03% hydrogen peroxide solution was added to 0.1 mL of the supernatant. To a blank sample, instead of the supernatant, 0.1 mL of distilled water was added. The reaction was stopped after 10 min by adding 1 mL of 4% ammonium molybdate. The intensity of the developing color was measured at a wavelength of 410 nm against a control sample to which 2 mL of water was added instead of hydrogen peroxide.

SOD activity was determined according to [Giannopolitis, Ries, 1977]. Optical density of the content of vessels was assessed at 560 nm. Activity of enzymes (CAT and SOD) was calculated on a dry weight basis.

Peroxidase activity was analyzed by the Boyarkin method [Metody, 1987]. It is based on determining the rate of benzidine oxidation in the presence of hydrogen peroxide and peroxidase. Optical density was measured at a wavelength of 590 nm every second for 120 s. The difference between initial and final optical density was taken into account. Enzyme activity was calculated on a dry weight basis.

The rate of photosynthesis was measured by the Winkler titration. The change in oxygen content in water during incubation of thalli was calculated (μg O_2 *per* 1 g of thallus wet weight *per* h). The alga in vessels without petroleum products served as the control.

The content of dry matter was determined according to a generally accepted method [Metody, 1987]. The alga thalli, after removing droplet moisture from the surface with filter paper, were weighed on a VLTE-310 scale (Gosmetr, Russia) (accuracy of 0.001 mg), dried in a desiccator for 24 h to a constant weight at +105 °C and re-weighed. Dry matter content was assessed as the proportion of dry weight to wet weight.

The state of the alga cells was analyzed by light microscopy under a Mikmed-6 microscope (LOMO, Russia) at a magnification of ×400.

The significance of differences between options was calculated for initial data by the Student's *t*-test, with a probability of 95% ($p \leq 0.05$). To assess the significance of the effect of the pollution factor, the one-way analysis of variance (ANOVA) was applied. The data obtained were processed and analyzed in MS Office Excel 2010 statistical package.

RESULTS AND DISCUSSION

State of the alga cells. Control samples remained intact until the end of the study (Fig. 1A). In experimental samples, after 3 h of exposure, perforated chloroplast structures expanded. By the end of the first day, the chloroplast decreased; in a number of cells, it acquired a granular structure. The development of plasmolysis was noted (Fig. 1B, C).

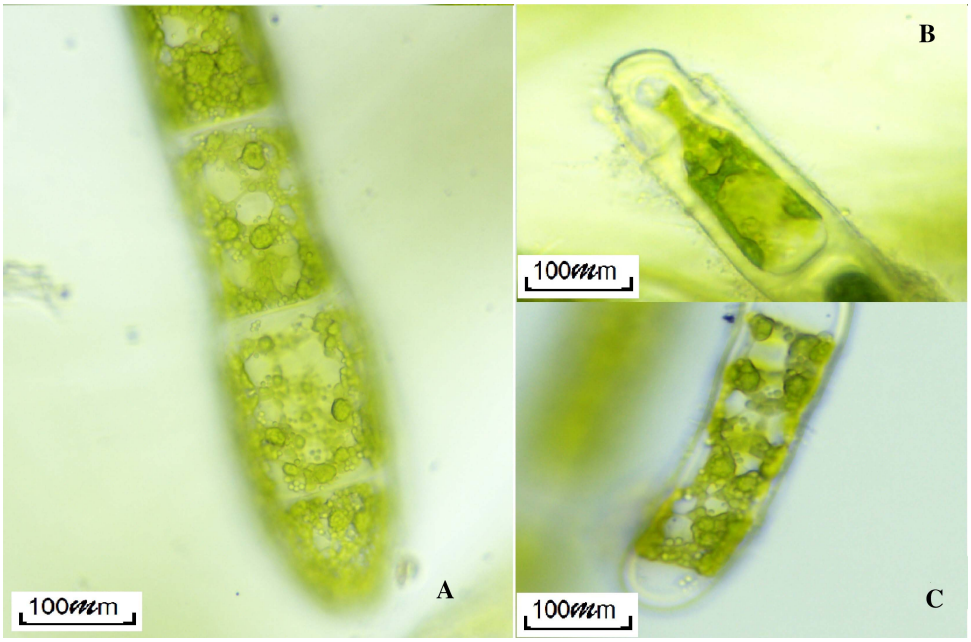

Fig. 1. *Acrosiphonia arcta* cells at the end of the experiment (24 h): A, the control; B, C, cells after exposure to water containing diesel fuel

Markers of oxidative stress. Normally, in cells, hydrogen peroxide is constantly present (its level ranges within 0.004–0.005 z^{-1} dry weight), as well as LPO products (their concentration ranges within 0.003–0.005 c. u.·g^{-1} dry weight). Most likely, a decrease and increase in the content of these substances in a cell are caused by the occurrence of daily rhythms of changes in the activity of physiological processes (Fig. 2A, B).

Under the effect of diesel fuel, the concentration of hydrogen peroxide in experimental samples increases by 1.5 times during the 1st hour. This rise is followed by a gradual decrease (by 2 times). The level of LPO in the experimental alga decreases during the 1st hour; by the 7th hour of exposure, it rises almost 2 times; and by the 10th hour of observation, it drops. By the end of the experiment, LPO increases.

It is worth noting as follows: changes in the level of LPO in the control and experiment are in antiphase. In 1 and 10 hours of the study, in the control, accumulation of LPO products was observed, while in the experiment, their concentration decreased significantly (Fig. 2A, B).

Activity of enzymes of the antioxidant system. In the control, SOD activity remained unchanged from the 1st to 7th hour of the study, increased by 1.3 times by the 10th hour, and did not change until the end of the experiment. Under the effect of diesel fuel, SOD activity rose by 1.5 times in the 1st hour, decreased by 2 times by the 7th hour, and became equal with the indicator in the control by the first day (Fig. 2C).

CAT activity in the control did not change during the 1st hour. By the 3rd hour, a 3.6-fold rise in activity was recorded; by the 7th hour, a 3-fold drop; and by the 10th hour, a 1.5-fold increase. Until the end of measurements, the values of CAT activity remained high. In experimental samples, there was a gradual rise in CAT activity (by 3 times) from the 1st to 7th hour. By the 10th hour, enzyme activity decreased by 2.5 times; until the end of exposure, it did not change (Fig. 2D).

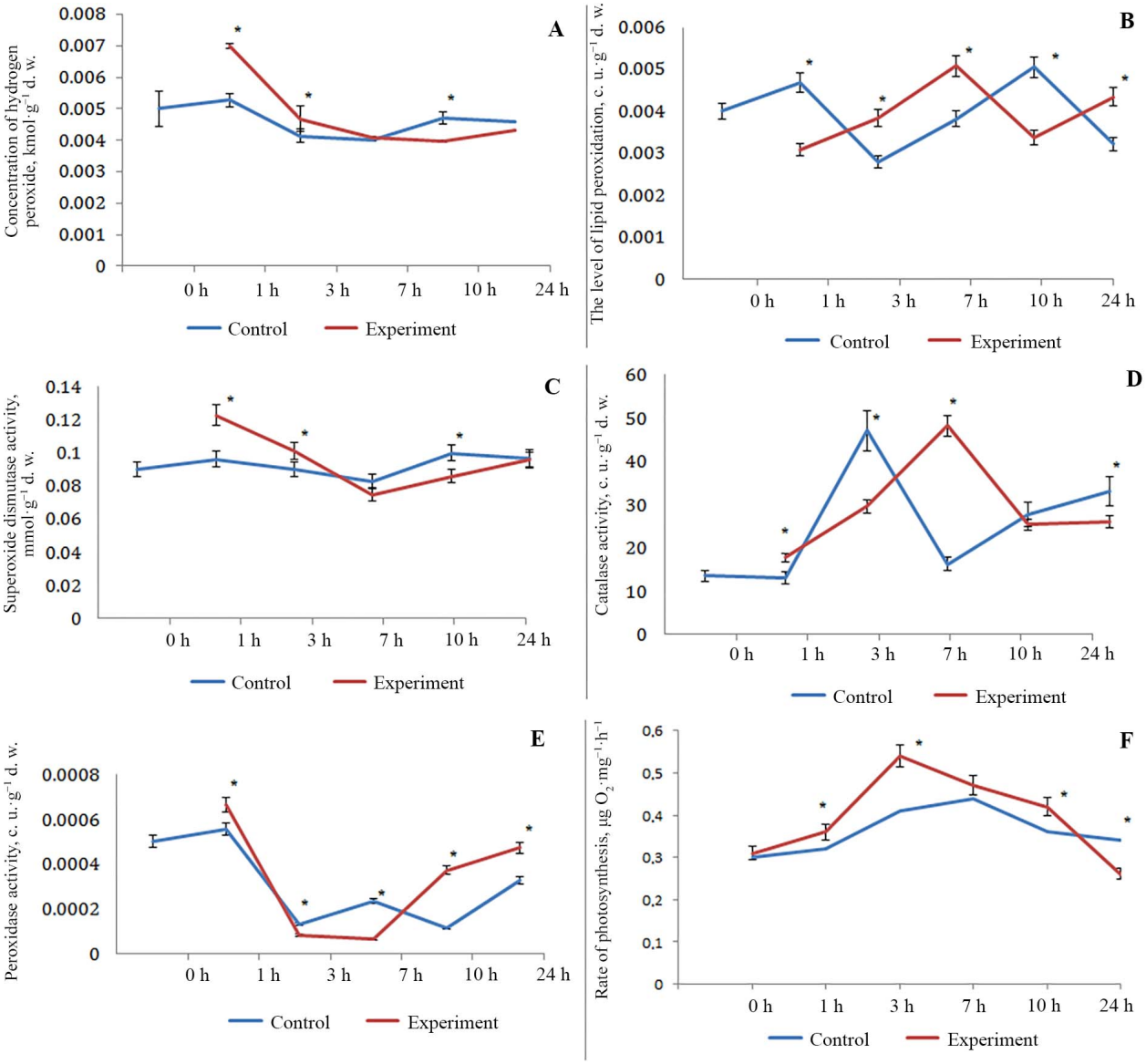

Fig. 2. Changes in the main physiological and biochemical parameters of *Acrosiphonia arcta* during the experiment: A, hydrogen peroxide concentration; B, the level of lipid peroxidation; C, superoxide dismutase activity; D, catalase activity; E, peroxidase activity; F, rate of photosynthesis. Data in the graphs are presented as arithmetic means; bars indicate standard deviation [* marks significant differences with the control ($p \leq 0.05$)]

During the experiment, peroxidase activity varied significantly in control samples and in those under exposure.

In the control, there was a wave-like change in enzyme activity. During the 1st hour, a slight increase in peroxidase activity was noted, and by the 3rd hour, there was a 6-fold drop. In the 3rd to 7th hour, activity rose by 2 times. Then, it decreased by 2 times. By the end of the experiment (the 24th hour), the value increased by 3 times compared to the previous values (Fig. 2E). In experimental samples, there were also a rise in peroxidase activity by the 1st hour (by 1.3 times) and a drop by the 3rd hour (by 7 times). At the same time, from the 7th hour of exposure, there was an increase in peroxidase activity. By the 10th hour, it rose by 5 times, and by the 24th hour, by 1.5 times compared to the previous values; it was significantly higher than peroxidase activity in the control (Fig. 2E).

Rate of photosynthesis. During the experiment, the rate of photosynthesis was measured as well (Fig. 2E). In the first hours, experimental samples were characterized by an increase in the rate of photosynthesis compared to the control. The most significant differences were observed by the 3rd hour of measurements: the values were 1.3 times higher than control ones. By the end of exposure, the rate of photosynthesis of experimental samples became 1.3 times lower than that of the control.

DISCUSSION

A change in the activity of antioxidant enzymes in response to stressors of various nature is a universal reaction of any organism [Milchakova, Shakhmatova, 2007; Regoli et al., 2002; Ryzhik et al., 2019; Sardi et al., 2016; Shakhmatova, 2004; Zhang et al., 2004]. Literature sources mainly discuss long-term effects of petroleum products and corresponding alterations in an organism. Specifically, on the example of *Hypnea musciformis*, a complex pattern of changes in various biochemical parameters was shown: a decrease in the content of chlorophyll *a*, a decline in the concentration of phenolic compounds, and an increase in the content of carotenoids. A change in cell morphology was noted as well, in particular in the structure of a cell wall surface [Ramlov et al., 2014, 2019]. As established for the microalga *Pseudokirchneriella subcapitata*, fresh and, to a greater extent, used motor oil caused a rise in the activity of antioxidant enzymes (first, SOD activity increased; then, peroxidase and CAT activity rose); thus, oxidative damage to biomolecules decreased [Ramadass et al., 2015]. In cells of green algae, with prolonged exposure to a toxicant, researchers recorded the development of plasmolysis, destruction of chloroplasts, *etc.* [Salakhov et al., 2021; Voskoboinikov et al., 2018].

Long-term experiments often fail to identify the true response to exposure to a toxicant. Specifically, studies on *Fucus vesiculosus* did not reveal a rise in CAT activity after prolonged (more than 10 days) contact with diesel fuel [Ryzhik et al., 2019]. However, under natural conditions, CAT activity was significantly higher in algae exposed to chronic pollution of a high level than in algae of ecologically clean spots [Shakhmatova, Ryzhik, 2020].

The current study provides data on changes in the activity of antioxidant enzymes in *A. arcta* cells in response to exposure to diesel fuel during the first day of the experiment. To date, it is established that the intensification of formation of reactive oxygen species results in an increase in the activity of antioxidant enzymes involved in the formation of long-term adaptations [Kolupaev, 2007; Kolupaev, Karpets, 2010; Migdal, Serres, 2011; Rogozhin, 2004].

According to the results of our study, stress reactions begin to form in the alga cells by the 1st hour of exposure. In particular, plasmolysis develops, and the structure of chloroplasts is disrupted.

During the first day, three blocks of rapid stress reactions can be distinguished; those are launched in stages. One of the first, implemented immediately after the beginning of exposure, is the intensification of LPO processes. Malondialdehyde and other LPO products are unique signals for enhancing the synthesis of antioxidant enzymes. After the 1st hour of the experiment, a decrease in LPO level and an increase in SOD activity were registered compared to the control. LPO processes develop at high speed; as shown on the example of higher plants (wheat sprouts), a significant accumulation of LPO products can occur already within the first 10–15 min of exposure [Rogozhin, 2004]. To neutralize them, the pool of SOD is used which was in a cell before the onset of exposure. Moreover, *de novo* synthesis begins, since SOD is an inducible enzyme. According to literature data, on the example of a study on ultraviolet radiation effect on wheat sprouts, it was revealed as follows: at the initial stages of exposure, the available supply of SOD is used to utilize reactive oxygen species; then, the synthesis of enzyme begins [Rogozhin, 2004]. In parallel, we can observe an increase in the concentration of hydrogen peroxide in *Acrosiphonia* cells. Also, during this period, CAT synthesis is activated, and its maximum activity occurs at the 7th hour of measurements (the second block of reactions).

Then, CAT concentration decreases in the studied alga, and the level of peroxidase increases (the third stage). Identified features may be a consequence of inhibition of CAT activity by high concentrations of hydrogen peroxide and/or products of the split of petroleum hydrocarbons, as well as the possible transition of this enzyme to another form allowing to perform an oxygenase function [Kolupaev, Karpets, 2010; Kolupaev et al., 2011]. At the same time, a decrease in CAT activity was accompanied by an increase in peroxidase activity. With the similarity of functions they perform, it indicates the compensatory nature of the changes. The publications of a number of researchers showed compensatory changes in some components of the antioxidant system under inhibition of the activity / reducing the content of its other components [Apel, Hirt, 2004; Miroshnichenko, 1992].

The study also revealed an increase in the rate of photosynthesis in the experimental alga in the first hours of measurements (the 1st, 3rd, 7th, and 10th) and a decrease by the end of exposure.

A rise in the rate of photosynthesis we observed within the first hours of the experiment may be due to features of the Winkler titration: to measure photosynthesis, plants are transferred to a medium without a toxicant for 30–60 min. We assume that transferring plants to clean water for measurements caused a temporary activation of photosynthesis, since toxic effect of diesel fuel was reduced. However, when plants remain in the experiment for a longer period, this effect is not observed, because irreversible changes are accumulated in cells. Apparently, a shift in prooxidant/antioxidant reactions towards LPO processes in cells of the experimental alga after a day of exposure led to a change in the structure of chloroplasts, plasmolysis, and, accordingly, a decrease in the rate of photosynthesis.

Changes in physiological parameters during the first hours of exposure to stressors were registered for various groups of organisms, and those affected not only the state of the antioxidant system, but also the protein-synthesizing and energy apparatus of a cell. For example, for microalgae and microorganisms, significant changes were shown in ratios of protein/carbohydrate and cell growth rate / survival occurring upon contact with dissolved diesel fuel during the first day of exposure [Shiu et al., 2020]. According to the results of a research on the effect of petroleum product film, with a short-term exposure (one tidal cycle), green algae experience cell plasmolysis, a decrease in the rate of photosynthesis, and an increase in the intensity of respiration [Ryzhik, Makarov, 2019].

At the same time, we established a shift in the daily cycle of CAT and LPO level in experimental samples compared to control ones. This may indicate a disruption in daily rhythms of CAT activity

and LPO processes. A number of publications provided data on the existence of biological rhythms in algae in production of antioxidants, and those are important for cell functioning [Carvalho et al., 2004]. Studies on ultraviolet radiation effect on the state of the antioxidant complex in cereal plants showed a similar result [Rogozhin, 2004]. Disruption of rhythms can negatively affect the resistance of vegetation under changing environmental conditions, and this is confirmed by data of our research.

Thus, the results of the study allowed to establish as follows. An increase in the activity of antioxidant enzymes in *Acrosiphonia arcta* cells exposed to diesel fuel occurs during the 1st hour of the experiment and is an adaptive response of the alga to a rise in the concentration of hydrogen peroxide. During exposure, different time maximums of the activity of SOD, CAT, and peroxidase were established. This corresponds to modern ideas on the sequence of the antioxidant response: SOD → CAT/peroxidase.

Importantly, the negative effect of diesel fuel is not only due to disruption of physiological processes in cells, but probably also due to biorhythms that allow organisms to adapt to periodically changing factors.

This is especially true for littoral plants exposed to periodically changing environmental factors, in particular the tidal cycle when disruption of synchronization can cause the death of algae.

The study was carried out within the framework of the Russian Science Foundation grant No. 22-17-00243 "Radiation oceanology and geoecology of the coastal shelf of the Barents and White seas. Bioinert interactions in the system bottom sediments – water – macroalgae – microorganisms; their role in the remediation of the marine coastal ecosystem during radiation and chemical pollution in the Arctic."

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АНАЛИЗ ФИЗИОЛОГО-БИОХИМИЧЕСКИХ ПОКАЗАТЕЛЕЙ КЛЕТОК *ACROSIPHONIA ARCTA* (DILLWYN) GAIN НА РАННЕЙ СТАДИИ ФОРМИРОВАНИЯ СТРЕСС-РЕАКЦИИ ПОД ДЕЙСТВИЕМ ЭМУЛЬСИИ ДИЗЕЛЬНОГО ТОПЛИВА

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Проведено исследование особенностей формирования стрессовой реакции в клетках зелёной водоросли *Acrosiphonia arcta* на воздействие эмульсии дизельного топлива. Проанализированы изменения показателей окислительного стресса (концентрация перекиси водорода и накопление продуктов перекисного окисления липидов), активность ферментов антиоксидантной системы, интенсивность фотосинтеза и состояние клеток. Показано, что в течение первых суток воздействия токсиканта в клетках происходит развитие плазмолиза и нарушение структуры хлоропластов. Стрессовая реакция формируется поэтапно: на первом этапе увеличивается количество перекиси водорода, изменяется концентрация продуктов перекисного окисления липидов, повышается активность супероксиддисмутазы; на втором этапе происходит активизация каталазы; к концу первых суток воздействия на фоне снижения активности каталазы увеличивается активность пероксидазы (третий этап). Интенсивность фотосинтеза снижается к концу эксперимента. Выдвинуто предположение, что под воздействием эмульсии дизельного топлива может происходить нарушение суточной динамики биологических циклов ряда ферментов.

Ключевые слова: *Acrosiphonia arcta*, дизельное топливо, каталаза, супероксиддисмутаза, пероксидаза, перекисное окисление липидов, перекись водорода, интенсивность фотосинтеза