The influence of diesel fuel on morpho-functional state of
*Ulvaria obscura* (Chlorophyta)

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**Abstract.** There was researched the influence of diesel fuel (DF) on morpho-functional state of *Ulvaria obscura* (Chlorophyta) of the Barents sea. The reaction of algae on oil products depends on concentration and duration of toxicity. Under the concentration up to 5 mg/l (100 MAC - maximum allowable concentration) there were no visible changes in cell structure during 10 days of experiment; by its end the photosynthesis of algae decreased, probably due to growth of bacterial film on the surface of thalli, preventing photosynthetic apparatus from the light. On the 7th day the number of heterotrophic bacteria increased twice, staying on this level till the end of experiment, the number of hydrocarbon oxidizing bacteria also increased. Under 1000 MAC the chloroplasts lost green colouring, the intensity of photosynthesis significally decreased, but plants maintained vitality. On the 3rd day under 2000-3000 MAC the vacuolization of cells, the destruction of cell structures occured, parallel with the cessation of photosynthesis and the death of macrophytes. The activity of antioxidant system (AAS) in experiments with DF was high, appeared by increasing of formation of hydrogen peroxide and intensity of superoxide dismutase and catalase ferments synthesis. *U. obscura* adapts to concentrations of DF up to 2000 MAC.

1. **Introduction**

In the coastal zone of the Barents sea the most anthropogenic burden, especially the influence of oil and oil products (OP) lays on the algae macrophytes [1] of the littoral zone. There was revealed that fucus algae [2], [3] showed high stability to the pollution of such toxicant. During the experiments with *Ulvaria obscura* there was noted that this type of algae have a stability to the influence of low concentrations of oil products (60 PMC) and an ability to sorb them [4]. There was shown the stability of *Ulva lacuca* an *U. obscura* to a short-time influence of a diesel fuel (DF) film [5].

In the literature, there is no information on the range of tolerance to DF of *U. obscura*, morphological and functional changes in this species under prolonged influence of a hydrocarbon pollution. Obtaining these data for *U. obscura*, the dominant species of algae macrophyte of the littoral of the northern seas, will help to understand the possible changes under the influence of OP of littoral phytocenoses.

The goal of research - To determine the effect of various concentrations of diesel fuel on the morphological and functional state of green algae *U. obscura*.

The object of research - green algae *Ulvaria obscura* (Kützing) Gayralex Bliding (Chlorophyta) widespread on the Murmansk coast, growing in the zone of intertidal and upper sublittoral [6].

2. **Materials and methods**
Algae were selected for the experiment in August - September 2019 on the east coast of the Barents Sea in the Zelenetskaya Bay, in the region where a seasonal biostation of the MMBI KSC RAS is located. The experiment was carried out in a thermostatically controlled chamber at a temperature of 8-10 °C and a constant lighting of 100 W / m². Before a start of experiment, *U. obscura* collected on the littoral were acclimated to laboratory conditions. For this, they were placed in a glass vessels with pure marine water for 7 days. The thalli were immersed in glass containers containing 1.5 liters of marine water with a salinity of 35 ‰. DF (summer) was added to the container with algae under a concentration of 0.86, 4.3, 43, 86, 129 mg / l, which corresponded to 20, 100, 1000, 2000 and 3000 MAC (maximum allowable concentration). According to “The Judicial and regulatory acts of the Russian Federation” (2016), the MAC of oil products in water for fishery reservoirs is 0.05 mg / l [7]. Algae in marine water with no addition of oil product was used as a control sample. The duration of the experiment was 10 days. Every 3 days, the photosynthetic activity of algae and the morphological state of cells were analyzed.

Analysis of morphological changes in algae cells was carried out visually using a LOMO Mikmed - 6 microscope.

The fresh mass of plants was determined using weights (VLT-310, accuracy class II) after removing droplet moisture from the surface using filter paper according to the generally accepted method [8].

The photosynthesis rate was calculated from the change in the oxygen content in water during the incubation of thalli (μg O2 per 1 g of wet weight of thallus per hour) using a HANNA HI 9141 oximeter (Germany). Algae in containers without oil products served as control. The content of hydrogen peroxide was determined by a modified spectrophotometric method [9].

Catalase activity (CAT) was measured using a spectrophotometric method [10]. The calculation of the activity of enzymes (CAT and SOD) was carried out on a dry weight.

Superoxide dismutase (SOD) activity was determined by Hyannopolitis [11]. The enzyme activity of the antioxidant system was evaluated at the end of the experiment in options from control up to 1000 MAC.

To account for the number of cultured heterotrophic bacteria, the limiting dilution method [12] using liquid media was used: for heterotrophic bacteria - modified Zobell 2216E medium [13] for hydrocarbon-oxidizing bacteria (BOB) - MMS medium [14] with the addition of summer grade DT as the sole source of carbon and energy [15]. The most probable number of bacteria was determined using the McCreary tables [16]. The count of microorganisms was carried out in all studied concentrations, changes were similar. There was obtained data presented changes of number of microorganisms under the concentration of 100 MAC, as the most informative.

Statistical processing of the obtained results was carried out using the Excel software package.

Summer diesel fuel was chosen as a pollutant of marine water, as the main oil product used by large-tonnage and small-size fleet.

3. The results
In the control variant cells 4-5 of different forms are chaotically located. The chloroplasts are stained with saturated green color, distributed throughout the cell, however, most of them were located parietally. Until the end of the experiment, the structure of cells, their color remained unchanged. (Fig. 1A).
Figure 1. State of algae cells of Ulvaria obscura after 10 days under the influence of DF: A – a control sample of algae; B – algae cells under the influence of DF under the concentration of 1000 MAC, C – algae cell under the influence of DF under the concentration of 1000 MAC, D – under the influence of DF under the concentration of 3000 MAC.

Under the influence of DF at a concentration of 20 and 100 MAC in cells, no visible changes are observed in comparison with the control variant of the experiment. Under the action of oil products at a concentration of 1000 MAC, an increase in the intercellular space and a change in the color of cells from bright green to yellow-green (Fig. 1B) are noted, however, that changes were not lethal for the cells, algae remained viable until the end of experiment.

Significant changes in the morphology of algae occurred when the concentration of DF became higher than 2000 MAC. In part of the cells, plasmolysis is observed on the 3rd day of the experiment. On the 10th day of the experiment, the contents of cell adjoins one of the inner sides of the cell, acquires a brown color, and destruction of chloroplasts is observed (Fig. 1B). Under the influence of DF at a concentration of 3000 MAC on the 10th day of the experiment, the organelles in cells and the cells themselves seem swollen, have a rounded shape, transparent, and in some cells, dense rounded structures are shifted to the periphery, the cells lose their bright green color, and the components of the photosynthetic apparatus are destroyed.

An analysis of the enzymes of the plant's antioxidant system showed that they quickly respond to the introduction of a toxicant.

In the control, the content of hydrogen peroxide is about 0.0046 μmol / g dry weight (Fig. 2). With prolonged presence of algae in an environment with diesel fuel in cells, an increase in the concentration of hydrogen peroxide (as compared with the control) occurs, which is especially noticeable at low concentrations of OC.

Figure 2. Changes in the content of hydrogen peroxide and the activity of enzymes of the antioxidant system in algae cells during the experiment: a - hydrogen peroxide; b - superoxide dismutase; c – catalase.
SOD reacts on the adding of a toxicant by the increasing of the activity in all experimental variants in 1.5-1.8 times compared to the control. The activity of catalase in all variants of the experiment was approximately the same and 3 times higher than in the control.

The photosynthesis of ulvaria in the control variant on the first day of the experiment was 0.36 μg O₂ / g wet mass per hour (Fig. 3). After 3 days, photosynthesis in the control thalli of algae was close to the initial one and amounts to 0.34 μgO₂ / g of wet mass per hour. On the 6-10th day of experiment, the photosynthesis of ulvaria in the control variant is 2.5 times less than the initial value.

[Figure 3. The photosynthetic activity of Ulvaria obscura during the experiment]

The value of photosynthesis under the influence of DF under concentration of 20 MAC for 3 days does not change compared with the control sample. On the 6-10th day of the experiment, the photosynthetic activity of algae decreased by 2 times compared with the control sample. Exposure to diesel fuel at a concentration of 100 MAC reduces the intensity of photosynthesis in the first 3 days of the experiment by 1.5 times, and after 6 days - by 3 times. On the 10th day of the experiment, the level of photosynthesis in algae reduces by 13 times compared with the control sample, but at the level of 6 days of the DF at a concentration of 1000 MAC in the first 3 days of the experiment reduces the photosynthetic activity of algae by 4 times. On the 6-10th day of the experiment, the value of photosynthesis is aligned in comparison with the control variant.

DF at a concentration of 2000 and 3000 MAC after 3 days of the experiment reduces the photosynthetic activity of algae by 1.5 times, after 6 days of the experiment, the value of photosynthesis is aligned with the control values. After 10 days of experiment in algae under the influence of these concentrations of oil products, photosynthesis is absent.

Microbiological studies showed that on the third day of the experiment, a slight increase in cultured heterotrophic bacteria was observed on the surface of macrophyte thalli both in the control and in the presence of oil products at a concentration of 100 MAC (Fig. 4).
Figure 4. The total number of cultivable heterotrophic bacteria and the number of hydrocarbon-oxidizing bacteria (HOB) at 100 MAC of oil product (DF)

At the same time, the number of hydrocarbon-oxidizing bacteria increased to 100% of all cultivated heterotrophic bacteria. This suggests that by the third day of the experiment, almost all cultivated bacteria were able to use oil hydrocarbons as a nutrient substrate. In the control, the number of HCO bacteria did not change.

On the seventh day of the experiment, with 100 MAC of oil products, there was a visible increase of the number of heterotrophic bacteria, which remained at a high level in the future. Moreover, the number of hydrocarbon-oxidizing bacteria decreased by the end of the experiment. This is explained by the conditions of the experiment in which it was carried out: constant lighting, lack of water change, which leads to the accumulation of organic matter as a result of macrophyte activity. Most bacteria use a lighter digestible organics released by algae as a nutrient substrate and accumulate in the system during the experiment. In experimental containers with higher concentrations, a visible increase of the number of cultured bacteria was also noted.

4. The discussion

Ulvaria obscura, being one of the main components of coastal biocenoses, responds to the introduction of DF into the environment by changing the morphological and functional characteristics.

Low concentrations of the toxicant (up to 100 MAC) did not affect the morphology of the cells, while an increase in the concentration damages the photosynthetic apparatus. The results of the experiment show that at concentrations of 1000 MAC and more, the pigment apparatus is destroyed first. Apparently, the destruction of chlorophyll, a change in the ratio of chlorophyll to carotenoids leads to a weakening of the green color of the cells in the first stages of the experiment, and the acquisition of brown color of the thalli by the end of the experiment. The change in the pigment apparatus, as the first reaction of chloroplasts, under the influence of altering factors, was repeatedly observed on unicellular algae and macroalgae, including under the influence of OP [17]. Studies on the effect of DF on the green alga Enteromorpha intestinales of the Barents Sea showed a twofold (compared to control sample) decrease in the photosynthetic activity of algae when exposed to petroleum products at a concentration of more than 200 MAC [2]. A decrease in photosynthesis in ulvaria is noted not only in experimental, but also in control samples. However, if in plants exposed to high doses of the toxicant, photosynthesis is completely stopped by the end of the experiment, then in the control variant and at low intensities of DF, photosynthesis is fixed. The decrease in the intensity of photosynthesis in ulvaria, including in the control, we explain the formation of a bacterial film on the surface of the thallus, which reduces the level of lighting necessary for the full operation of the
photosynthetic apparatus. A similar negative effect from the symbiosis of algae and microorganisms is confirmed by the obtained data on an increase in the number of bacteria during the experiment (Fig. 4) and is reflected in the literature [18], [19].

*Ulvaria obscura*, being one of the main components of coastal biocenoses, responds to the introduction of DF into the environment by changing the morphological and functional characteristics.

Morphological changes in ulvar cells were detected with a concentration of DF in water under 2000 and higher on the 3rd day of the experiment. Marked plasmolysis, and then vacuolization of cells, destruction of chloroplasts, end on the 10th day with the destruction of cytoplasmic structures. Apparently, these destructive changes are already irreversible, and the next stage of damage under high concentrations can be expected to violate the structure of the thalli.

Under the influence of oil products in algae cells, an increase in the content of hydrogen peroxide is noted in comparison with the control (Fig. 2). Hydrogen peroxide is formed during various physiological reactions and in the neutralization of a number of reactive oxygen species (ROS). According to the data of A. Garifzyanov and colleagues [20] under stress conditions, the amount of ROS increases, activating the defense system and triggering an additional synthesis of antioxidant system enzymes. An increase in the concentration of hydrogen peroxide in the cells may indicate an increase in oxidative processes and an increase in the number of powerful oxidizing agents such as superoxide radicals, which is manifested in the activation of the complex of enzymes SOD-catalase. SOD converts superoxide radical to a less active form (hydrogen peroxide), and catalase neutralizes hydrogen peroxide.

A slight decrease in the activity of AOS enzymes (Fig. 2) at the end of the experiment in experimental samples of ulvar at a concentration of DT 1000 MAC indicates a weakening of the metabolism in plants. The fraction of ROS formed as a result of exposure to a toxicant is higher than during synthetic processes, for example, during photosynthesis. This is also confirmed by a decrease in the intensity of photosynthesis at the end of the experiment (Fig. 3).

It is known that in the majority of the bays of the Murmansk coast of the Barents Sea, the background level of OP pollution ranges from 2 to 8 MAC. In the littoral zone, the level of pollution is usually much higher [21]. Thus, algae, the inhabitants of the littoral, undergo a certain stage of adaptation to the influence of OP during the development process. This can explain the ability of macrophyte species to exist at a pollution level of 100 MAC and higher, revealed in our and other studies.

Previously, in experiments with *U. obscura*, it was shown that this type of algae is not only resistant to relatively low concentrations of DF (60 MAC), but is also capable, like a number of other macrophytes, of accumulating and neutralizing DF [4]. A large role in this process is played by hydrocarbon-oxidizing bacteria, which carry out the destruction of OPs on the surface of the thalli, making them available for absorption by plant cells. Apparently, the detoxification of oil hydrocarbons also plays a role in maintaining the viability of ulvaria under conditions of OP contamination. However, under the high concentrations, ulvaria can not withstand high concentrations of the toxicant.

Thus, *U. obscura* green algae can survive for a long time in conditions of environmental pollution by diesel fuel up to 100 MAC, withstand short-term pollution up to 1000 MAC. In some cases, this is ensured by a transition to a lower level of metabolism, activation of the antioxidant system of the cell, characterized by a decrease in photosynthetic activity. In many respects, the preservation of viability in the littoral zone under conditions of moderate pollution with oil products is due to symbiosis with hydrocarbon-oxidizing bacteria. The higher level of pollution observed during the spill period, as well as in places of constant sources of pollution, leads to the death of this species of algae.

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