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Impact of hydrocarbon oxidizing strains *Serratia grimesii* and *Bacillus* sp.3 on microcenosis of molluscs and ecosystem components under oil pollution impact

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Abstract. Bivalved molluscs are natural filter feeders of the aquatic environment due to their physiological characteristics. Passing sea water containing emulsified oil through them, they bind its droplets in pseudofeces, thereby accelerating water self-purification. Mussels prove to be resistant to oil pollution. Experiments have shown that in aquariums with mussels the content of petroleum hydrocarbons decreases faster than in the tanks without mussels. The interaction of mussels with oil has been studied in detail. It has been established that when passing through the body of mussels the group composition of oil products changes. Oil resignification is noted, which is also observed during its natural weathering. Currently, for the post-purification of natural ecosystems from oil pollution, the method of introducing active hydrocarbon-oxidizing microorganisms is widely used, often with the simultaneous incoming of additional biogenic elements, the so-called bioaugmentation. In this regard, the study of the interaction of oil-oxidizing microorganisms and molluscs is of practical and scientific interest. The aim of the work was to study the interaction of hydrocarbon-oxidizing microorganisms on molluscs Unio and other components of the microecosystem when modeling the process of oil pollution. A study was made of river water, soil and molluscs Unio from model microecosystems for contamination by saprophytic and hydrocarbon-oxidizing microflora with the additional introduction of hydrocarbon-oxidizing microorganisms – *Serratia grimesii* and *Bacillus* sp.3 under conditions of Caspian oil pollution.The values of the bacterial composition of saprophytic and hydrocarbon-oxidizing microflora obtained in the experiment suggest that the processes of restoration and reclamation of a water body take place due to the structural and functional organization of ecosystems and communities.

Keywords: model microsystems, pollution, microorganisms, oil, microflora

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Научная статья

Влияние углеводородокисляющих штаммов *Serratia grimesii* и *Bacillus* sp.3 на микроценоз моллюсков и компоненты экосистем под воздействием нефтяного загрязнения

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Аннотация. Двустворчатые моллюски по своим физиологическим особенностям являются естественными фильтраторами водной среды. Пропуская через себя морскую воду, содержащую эмульгированную нефть, они связывают ее капельки в псевдофекалии, тем самым ускоряя самоочищение воды. При этом отмечено, что мидии устойчивы к нефтяному загрязнению. В экспериментах показано, что в аквариумах с мидиями содержание нефтяных углеводородов уменьшается быстрее, чем в аналогичных емкостях без мидий. Взаимодействие мидий с нефтью подробно изучено. Установлено, что при прохождении через организм мидий изменяется групповой состав нефтепродуктов. Отмечается осмоление нефти, которое наблюдается и при ее естественном выветривании. В настоящее время для доочистки природных экосистем от нефтяного загрязнения широко используется метод интродукции активных углеводородокисляющих микроорганизмов, зачастую с одновременным внесением дополнительных биогенных элементов, так называемая биоаугментация. В связи с этим изу-

чение взаимодействия нефтеокисляющих микроорганизмов и моллюсков представляет практический и научный интерес. Целью работы являлось изучение взаимодействия углеводородокисляющих микроорганизмов на моллюсков рода *Unio* и других компонентов микроэкосистемы при моделировании процесса нефтяного загрязнения. Проведено исследование речной воды, грунта и моллюсков рода *Unio* из модельных микроэкосистем на обсемененность сапрофитной и углеводородокисляющей микрофлорой при дополнительном внесении углеводородокисляющих микроорганизмов – *Serratia grimesii* и *Bacillus* sp.3 в условиях загрязнения каспийской нефтью. Выявленные в ходе эксперимента значения бактериального состава сапрофитной и углеводородокисляющей микрофлоры позволяют говорить о процессах восстановления и рекультивации водного объекта, что обусловлено структурно-функциональной организацией экосистем и сообществ.

Ключевые слова: модельные микросистемы, загрязнение, микроорганизмы, нефть, микрофлора

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Introduction

Molluscs form an important link in the`food chains in aquatic and terrestrial ecosystems. By their physiological structure bivalves are natural water-filtering organisms. The role of bivalved molluscs is especially great as biofilter feeders that clean water bodies from organic pollution. When filtering seawater containing emulsified oil, they bind its drops in pseudofeces, thereby enhancing the self-purification of water [1-4]. In addition, they absorb and accumulate heavy metals in their body [5-8].

Mussels are known to be resistant to oil pollution. Experiments have shown that in aquariums with mussels, the content of petroleum hydrocarbons decreases faster than in the aquariums without mussels. The interaction of mussels with oil has been studied in detail. There was found a change in the group composition of petroleum hydrocarbons during the passage through the body of the mussel. Oil gumming is observed, which is typical for natural weathering processes [9-11].

At present, the introduction of active hydrocarbon-oxidizing microorganisms is widely used for the remediation of natural ecosystems often accompanied by the simultaneous introduction of additional biogenic elements, which is also called bioaugmentation [12-14].

In this regard, the study of the interaction of oil-oxidizing microorganisms and molluscs is of practical and scientific interest.

In addition, *Unio* bivalved molluscs are a valuable food resource for the inhabitants of the Caspian Sea. Almost all of them, with the exception of large forms with a strong, thick shell, serve as a favorite food for demersal fish – benthophages (i.e., feeding on bottom animals), including many commercial fish – Bream (*Abramis brama*), Bleak (*Alburnus alburnus*), River eel (*Anguilla anguilla*), Asp (*Aspius aspius*), South European satin (*Atherina boyeri*), Blue bream (*Ballerus ballerus*), White bream (*Blicca bjoerkna*), Common loach (*Cobitis taenia*). Sturgeons are also included: Siberian sturgeon (*Acipenser baerii*), Russian sturgeon (*Acipenser gueldenstaedtii*), Spike (*Acipenser nudiventris*), Persian sturgeon (*Acipenser persicus*), Sterlet (*Acipenser ruthenus*).

Some fish, due to the predominance of small molluscs in their food, were called “mollusc-eaters”, such as, for example, the Caspian roach (*Rutilus caspicus*). Areas where, along with other bottom animals (polychaete worms, brittle stars, etc.), mass development of small bivalved molluscs is observed, serve as feeding grounds for various bottom commercial fish.

Accordingly, being part of the fish-human food chain, mussels are transport agents of oil products and heavy metals in the human food. This points to the special importance of the studies of the above processes.

Materials and methods

The research was focused on studying *Unio* river molluscs, water, soil, and strains of hydrocarbon-oxidizing microorganisms *Serratia grimesii* and *Bacillus* sp.3. The samples of molluscs, water and soil were collected in the Krivoy Buzan River, the Krasnoyarskiy district of the Astrakhan Region. The hydrocarbon oxidizing strains for the experiment were kindly provided by the Department of Hydrobiology and General Ecology of Astrakhan State Technical University.

To identify the impact of hydrocarbon oxidizing microorganisms on *Unio* molluscs and other test objects the study of 4 microecosystems was performed by 3 replicate samplings. Molluscs were placed in aquariums with stocking density of 15 individuals per 20 litres of river water with permanent aeration and soil (sand) from the same water body.

Once a week different concentrations of oil (0.5, 1.0 and 1.5 ml) were added to the experimental microecosystems. In the beginning of the experiment a consortium of microorganisms consisting of *Serratia grimesii* and *Bacillus* sp.3 strains was introduced into the microecosystems. To obtain strain suspensions each of the strains was inoculated in an Erlenmeyer flask containing 200 ml of M9 minimal medium, with exposure to Caspian oil to the finite concentration of 1.5%, by introduction of the inoculation dose of microorganisms in an amount of 1 ml of the suspension ($5.4 \cdot 10^8$ CFU/ml) per 1 litre of the microecosystem water. These strains possess oil-oxidizing properties and are proposed as a tool of aquatic systems remediation from oil pollution [15]. Model microecosystems sets:

1. Reference set: river water, river soil, molluscs, suspension of strains *Serratia grimesii* and *Bacillus* sp.

2. Experimental set 1 – river water, river soil, molluscs, suspension of strains *Serratia grimesii* and *Ba-*

cillus sp. 0.5 ml of oil was added every week (4 times during the experiment).

3. Experimental set 2 – river water, river soil, molluscs, suspension of strains *Serratia grimesii* and *Bacillus* sp. 1.0 ml of oil was added every week (4 times during the experiment).

4. Experimental set 3 – river water, river soil, molluscs, suspension of strains *Serratia grimesii* and *Bacillus* sp. 1.5 ml of oil was added every week (4 times during the experiment).

The experiment on the study of the overall impact of oil and oil oxidizing microorganisms on molluscs and microflora in the model system lasted for 30 days. The samples of the model ecosystems were taken 3 times: at the starting point (at the launch of the experiment), in 15 days and in 30 days (at the end point of the experiment).

To determine the target parameters in the model ecosystems the total abundance of microorganisms and the abundance of hydrocarbon oxidizing organisms were determined during each sampling for the 4 study targets:

1. Water.
2. Soil.
3. Scraping from the mollusc shells.
4. Washes from the mollusc gills.

The total number of heterotrophic microorganisms was determined by the method of decimal dilution and germination on the solid medium. The cultures were incubated for 4 days at the temperature of 25-27°C [16].

The mean amount of CFU (N) in 1 ml was calculated by the formula

$$N = \frac{c}{(n_1 + 0,1x\,n_2)\,xd},$$

where c – sum of colonies counts on all the plates; n_1 – number of the first dilution plates; n_2 – number of the second dilution plates; d – is the first dilution coefficient; 0.1 – coefficient accounting for the factors of the first and the second dilutions; x – multiplication [17-19].

The oil oxidizing microflora was determined by the method of decimal dilutions with the postgermination on solid mineral medium M9 with addition of the Caspian oil in amount of 1% of the volume. The cultures were incubated at the temperature of 25-27°C [20-21].

Results

In the model experiment the variations of microorganism abundance in the water of the microsystems under study were analyzed. The total microbial count and the abundance of hydrocarbon oxidizing microorganisms were taken into account.

The results of the microbial studies showed that at the starting point of the experiment the average abundance of microorganisms didn't change (about $0.50 \cdot 10^4 – 0.70 \cdot 10^5$ CFU/ml) (Fig. 1).

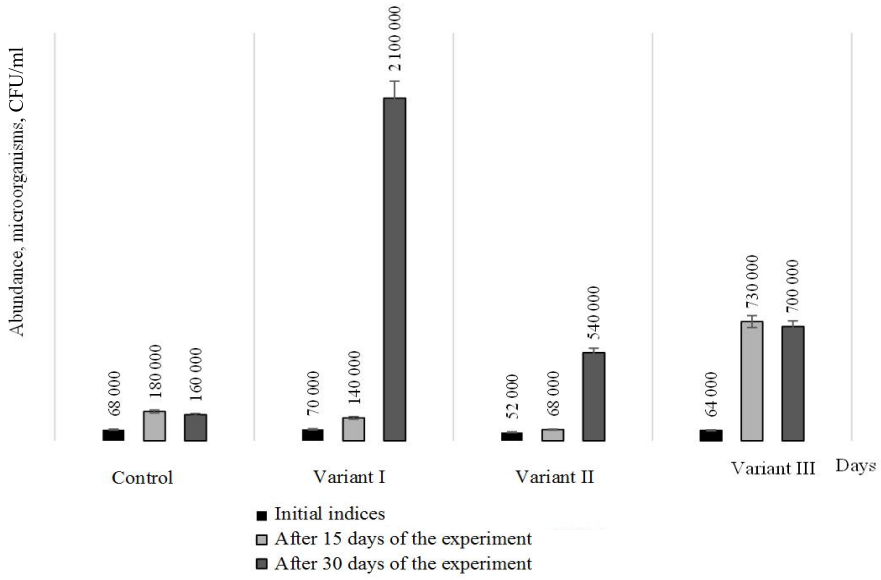

Fig. 1. The abundance of saprophytes in the water of the model systems

The further observations showed that the abundance of microorganisms in the reference set slightly increased – up to $1.6\text{-}1.8 \cdot 10^5$ CFU/ml. A different pattern is observed in the microecosystems, to which different concentrations of oil were injected. On the 15th day of the experiment, set 1 showed insignificant growth of microflora up to $1.4 \cdot 10^5$ CFU/ml. However, on the 30th day the count of microorganisms rose to $21 \cdot 10^5$ CFU/ml. In this set, 0.5 ml of oil was introduced into each of the aquariums weekly, which was an additional source of carbon. This oil concentration inhibited the sharp growth of microorganism. In addition, the suspensions of the studied strains were introduced in sets 1, 2 and 3 at the beginning of the experiment, which could also affect the growth of microorganisms.

A similar pattern with an increased number of microorganisms was observed on the 30th day of the experiment in sets 2 and 3. However, the increase ranged from $0.54 \cdot 10^5$ CFU/ml to $7.0 \cdot 10^5$ CFU/ml.

The abundance of oil oxidizing microorganisms was determined before the introduction of oil and suspension of strains *Serratia grimesii* and *Bacillus* sp.3 CFU/ml (Fig. 2).

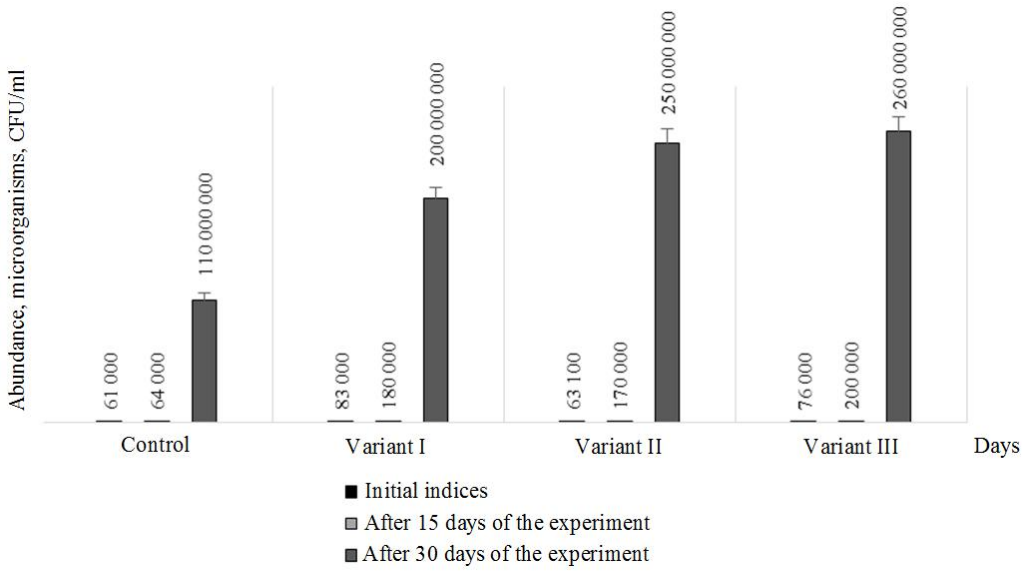

Fig. 2. Abundance of hydrocarbon oxidizing organisms in the water of the model systems

The count of hydrocarbon oxidizing microorganisms in the reference sample did not vary significantly in the course of the experiment and remained within $0.12\text{-}0.17 \cdot 10^5$ CFU/ml. The abundance of hydrocarbon oxidizing microorganisms grew slightly in sets 1-3. Thus, the introduction of oil and suspension of oil oxidizing microorganisms into aquariums stimulated the steady growth of bacteria, though no sharp changes in abundance were recorded.

The abundance of microorganisms in the soil of the model systems. Bacteria play an important role in destruction of hydrocarbons as a main source of carbon and energy. Bacteria decompose petroleum products both in the bottom sediments and in the pallial cavity of the molluscs, which hydrocarbons enter in the course of molluscs' life. Therefore the study of microorganism abundance in the soil is as important as it is in the water.

The experiment results showed that the abundance of microorganisms in the reference sample remained almost unchanged. Concentration of bacteria in the experimental samples (1-3) slightly increased: from $1.9 \cdot 10^5$ CFU/ml up to $2.5 \cdot 10^5$ CFU/ml (Fig. 3, 4).

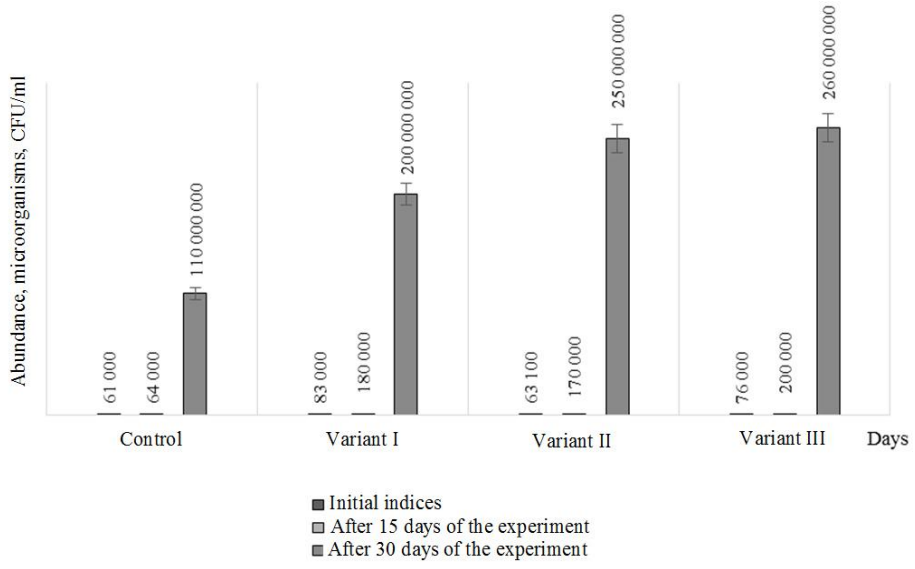

Fig. 3. Total abundance of saprophytes in the soil of the model systems

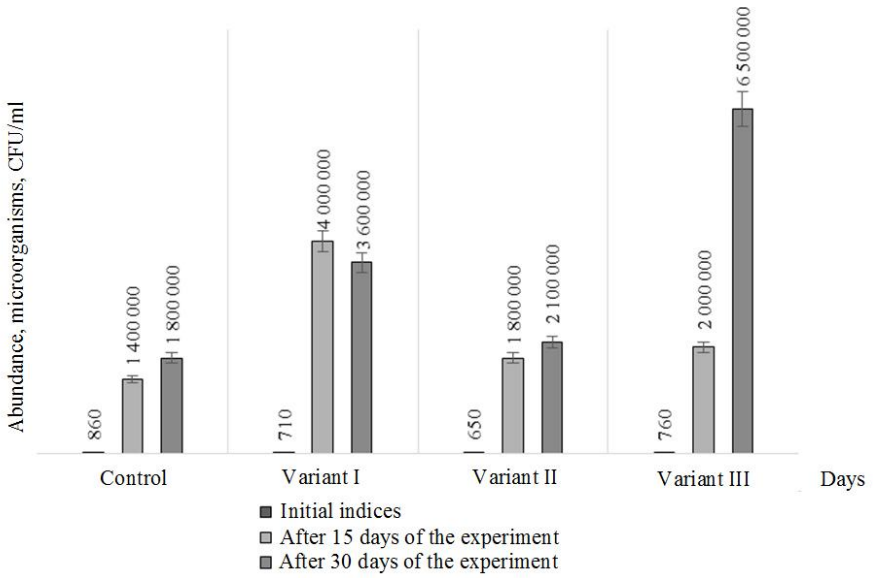

Fig. 4. The abundance of hydrocarbon oxidizing organisms in the soil of the model systems

The analysis of the results shows that the abundance of hydrocarbon-oxidizing microorganisms in the reference sample varied slightly in the course of the experiment, ranging from $18 \cdot 10^5$ CFU/ml to $86 \cdot 10^5$ CFU/ml. The abundance of oil destructors in set 2 trebled. On the 15th day the abundance of hydrocarbon oxidizing microorgansims in set 1 amounted to $40 \cdot 10^5$ CFU/ml, and on the 30th day it remained almost the same. A step-by-step increase was recorded for set 3: the abundance on the 30th day of the experiment reached $65 \cdot 10^5$ CFU/ml.

The abundance of microorganisms on the molluscs' surface in the model ecosystems. The count of microorganisms on the surface of molluscs' shells is extremely important while assessing the impact of oil and suspension of strains *Serratia grimesii* and *Bacillus* sp.3 on microecosystems. The results showing the distribution of total microorganisms count are presented in Fig. 5.

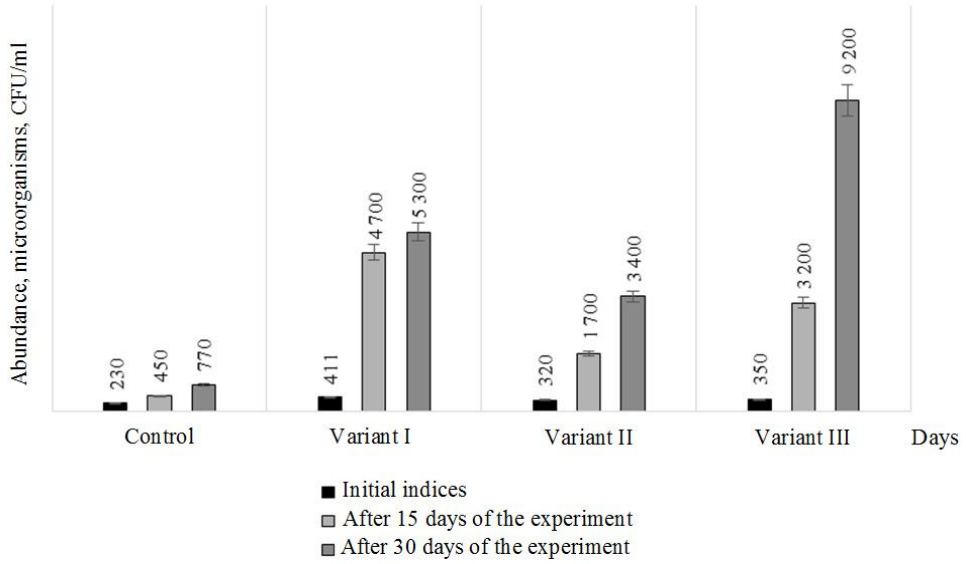

Fig. 5. Total abundance of saprophytes on the molluscs' shells in the model ecosystems

The abundance of microorganisms in the reference sample did not vary significantly throughout the experiment. The introduction of oil and strain suspension led to the increase in the count of microorganisms up to $0.092 \cdot 10^5$ CFU/ml. The highest values were recorded for the microecosystem in set 3, the lowest values – for set 2 (abundance increase up to $0.034 \cdot 10^5$ CFU/ml).

The variations in abundance of oil oxidizing microorganisms are shown in Fig. 6.

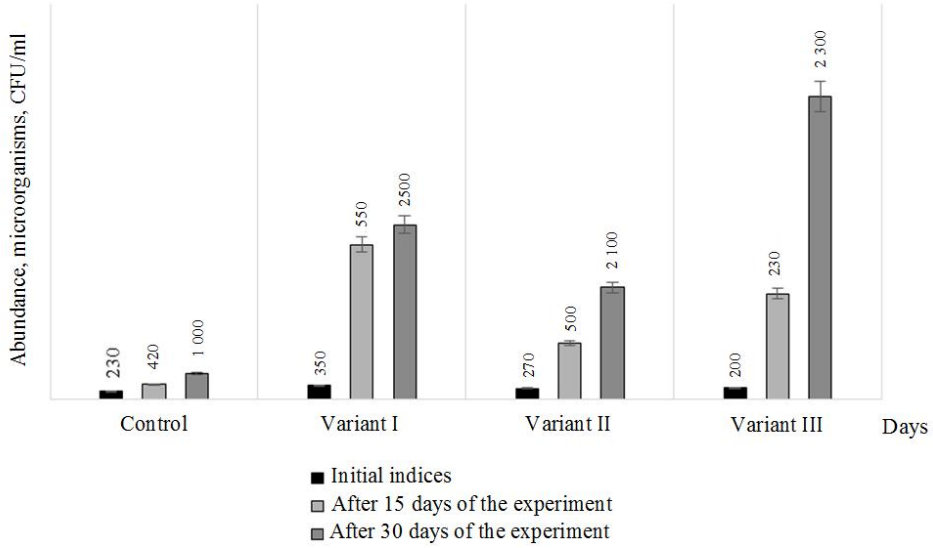

Fig. 6. Abundance of hydrocarbon oxidizing microorganisms on the molluscs' surface in the model ecosystems

The results analysis showed that the abundance of hydrocarbon-oxidizing microorganisms in the reference sample changed insignificantly throughout the whole experiment (by four times). The abundance of hydrocarbon-oxidizing microorganisms in sets containing oil and suspension of strains rose by two orders.

In the course of the experiment, the abundance of heterotrophic bacteria in the pallial cavity of molluscs varied from $0.010 \cdot 10^5$ CFU/ml to $0.062 \cdot 10^5$ CFU/ml (Fig. 7).

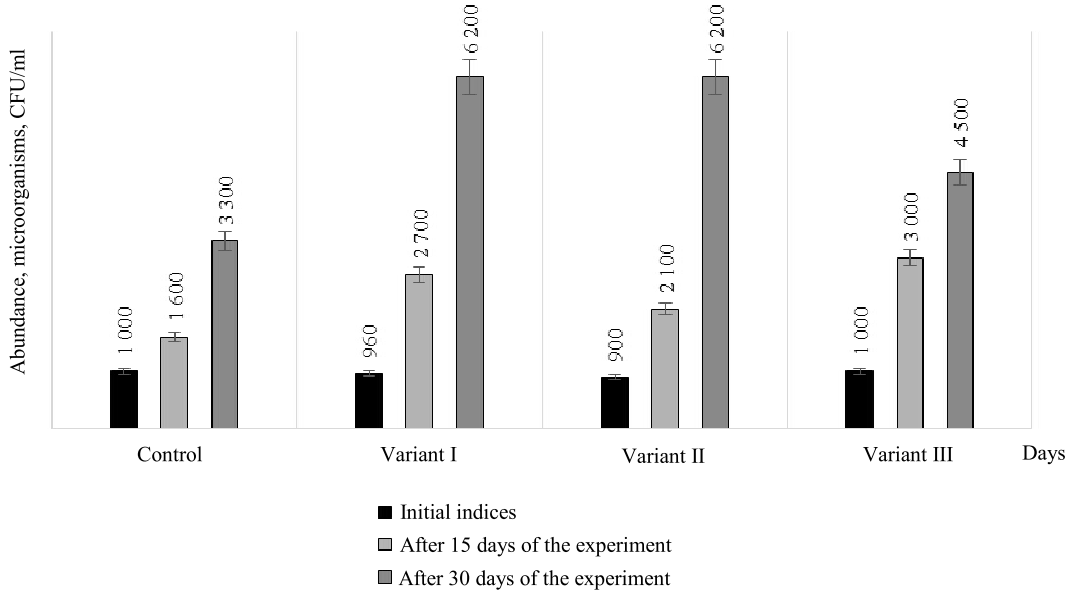

Fig. 7. Total abundance of microorganisms in the pallial cavity of molluscs in the model systems

The abundance of hydrocarbon oxidizing microorganisms in the reference sample did not change in the course of the experiment ranging within $0.018 \cdot 10^5$ – $0.025 \cdot 10^5$ CFU/ml (Fig. 8).

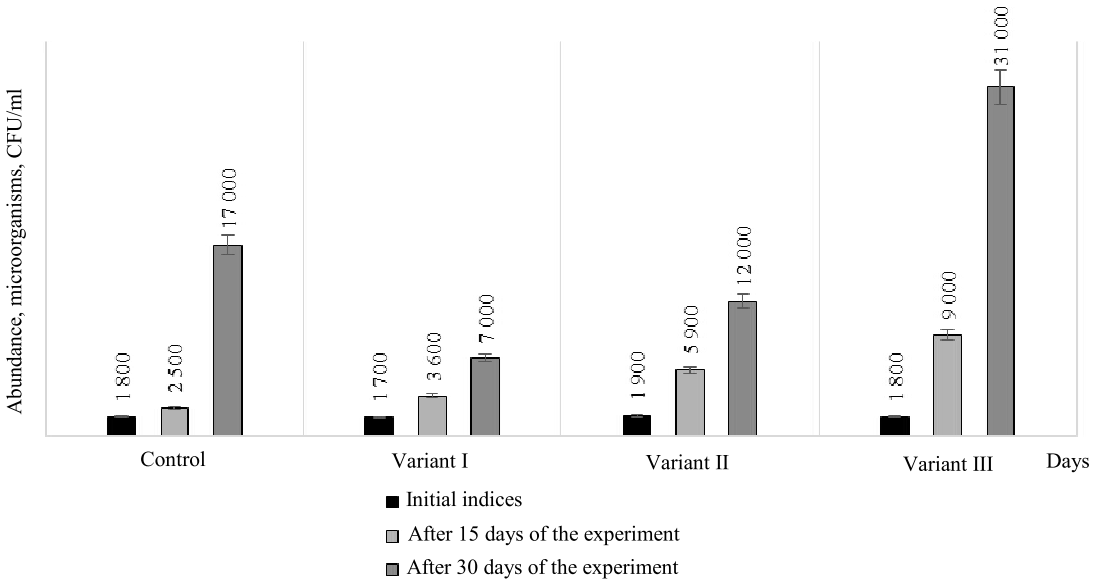

Fig. 8. Control abundance of hydrocarbon oxidizing microorganisms on the molluscs' surface in the model ecosystems

The abundance of hydrocarbon-oxidizing microorganisms in sets containing oil and suspension of strains rose by one order. The peak of abundance increase ($0.090 \cdot 10^5$ CFU/ml) was recorded in set 3, while the minimum was registered in set 2 ($0.059 \cdot 10^5$ CFU/ml) (Fig. 9).

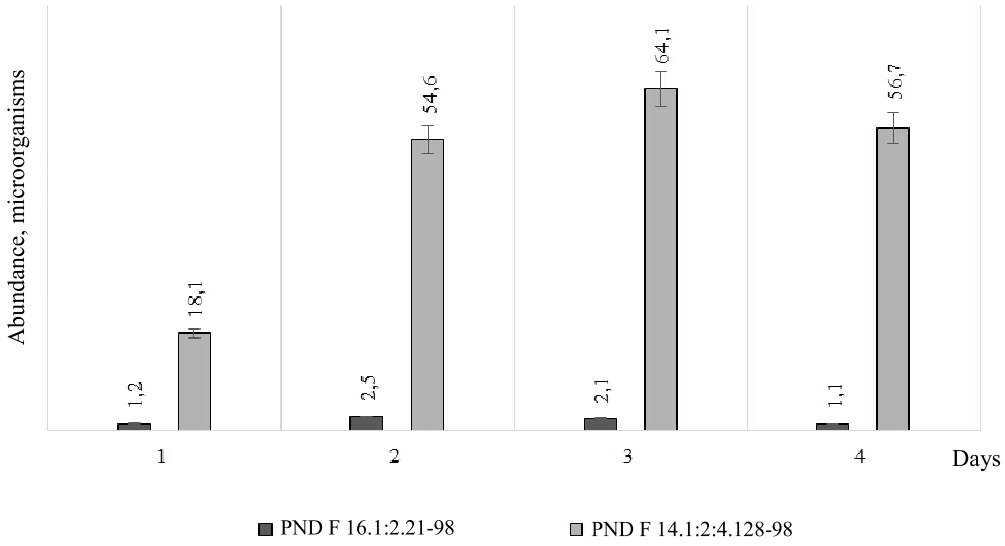

Fig. 9. Residual content of oil in the water (mg/dm^3) and soil (mg/kg) after introduction of microorganism strains: 1 – Reference sample; 2 – 0.5 mg/l of oil + microorganism strains; 3 – 1.0 mg/l of oil + microorganism strains; 4 – 1.5 mg/l of oil + microorganism strains; PND F 14.1:2:4.128-98 – Quantitative chemical analysis of waters. Method for measuring the mass concentration of oil products in samples of natural, drinking, waste water by the fluorimetric method on the liquid analyzer «Fluorat-02»; PND F 16.1:2.21-98 – Quantitative chemical analysis of soils. Method for measuring the mass fraction of oil products in soil and soil samples by the fluorimetric method using the «Fluorat-02» liquid analyzer

Discussion

The tests of the river water, soil, and *Unio* molluscs from the model micro-ecosystems for the presence of saprophyte and hydrocarbon-oxidizing microflora accompanied by introduction of hydrocarbon-oxidizing microorganisms *Serratiagrimesii* and *Bacillus* sp.3 under the conditions of Caspian oil pollution showed that the abundance of heterotrophic microflora rises as the habitat changes (oil and suspension of oil-oxidizing microorganisms). However, the recorded growth of bacterial

count was insignificant and ranged within one order. No variations of heterotrophic microflora concentration were recorded in the reference samples.

The change in abundance of oil oxidizing microorganisms during 30 days of the experiment was different in the experimental sets. The maximum concentration of oil destructors was recorded in the pallial cavity of molluscs: $3.0 \cdot 10^5$ CFU/ml. The maximum values in water ($0.043 \cdot 10^5$ CFU/ml) were recorded in experimental set 2. The highest abundance of oil oxidizing microorganisms ($65 \cdot 10^5$ CFU/ml) was recorded in set 3.

Concentration of oil destructors on the surface of the molluscs' shells rose slightly.

Identification of the residual oil content after introducing the microorganism strain increased by 2 and more times in sets 2 and 3. The residual oil content in the soil of all the experimental sets was by 3.02, 3.54 and 3.13 times higher than the reference values.

Conclusion

Oil and petroleum hydrocarbons are among the major pollutants of the hydrosphere. An efficient mechanism for the destruction of these substances was developed in the course of evolution.

The results of the implemented experiment showed that the abundance of heterotrophic microflora increases when the habitat conditions change (oil and the suspension of oil oxidizing microorganisms are added). However, the recorded growth of bacterial count was insignificant and ranged within one order. No variations of heterotrophic microflora concentration were recorded in the reference samples.

The change in abundance of oil oxidizing microorganisms within 30 days of the experiment was different in the experimental sets. The maximum concentration of oil destructors was recorded in the pallial cavity of molluscs: $3.0 \cdot 10^5$ CFU/ml. The maximum values in water ($0.043 \cdot 10^5$ CFU/ml) were recorded in experimental set 2. The highest abundance of oil oxidizing microorganisms ($65 \cdot 10^5$ CFU/ml) was recorded in set 3. The concentration of oil destructors on the surface of the mollusc shells rose slightly.

On the whole, the exposure to strain suspension and oil produces additional pressure on the filtration capacity of molluscs, as the bacterial content in the molluscs' internals rises, which affects significantly the self-purification capacity.

Thus, the values of bacterial content of saprophytic and hydrocarbon-oxidizing microflora identified in the course of the experiment point to the processes of restoration and remediation of the water body, which is conditioned by the structural and functional organization of the ecosystems and communities.

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