Monitoring of oil hydrocarbons pollution in the Sea of Japan, based on detection of marker genes in microbial communities

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Abstract. By means of molecular biology techniques, metabolic potential of microbial communities within the regions of inshore water areas in the Sea of Japan with various anthropogenic load was explored. Presence of functional genes, responsible for oil hydrocarbons destruction, for microbial communities within the regions of inshore water areas in the Sea of Japan was first researched. In total microbial DNA from water mass in the regions with chronic anthropogenic pollution, the genes, responsible for oxidation of broad range of n-alkanes and polycyclic aromatic hydrocarbons, were found. Detection of marker genes in the background water area (in the Vostok Bay) was ever indicating ecological deterioration within this territory. Thereby, it was demonstrated, that molecular genetic methods, aimed at marker gene detection in total bacterial DNA from environment objects, proved themselves to be more effective technique for identification of oil hydrocarbons water pollution, in comparison with trivial culturable methods.

1. Introduction
A unique diversity of locally inhabiting organisms belong to the ecosystem of the Sea of Japan, due to its distinctive regional physiographic features. Likewise, a strong anthropogenic impact, caused by population growth, coastal urbanization, industrialization, as well as tourist development in the Russian Far East, are therein observed [1]. Sources of pollution of Far Eastern seas are enterprises of pulp-and-paper, power, and oil-gas industry, housing and communal services, ship-building and ship-repairing enterprises, merchant marine and navy. Traditional higher contents of oil itself and oil products are noticed, as a result of untreated domestic waste in bays, gulfs, port areas, including lay-up bases for vessels in all regions of the Far East, also inside inshore water areas in the Sea of Japan close to Vladivostok [2].

Ecological importance of bacteria acting as petroleum hydrocarbons destructors is very great, because it is known, that a complete destruction of them can not be done by higher organisms. In a coastal zone with permanent pollution with oil and its products, specific communities of heterotrophic microorganisms emerge, able to oxidize wide range of hydrocarbons and products of their transformation [3].

Earlier within the inshore water areas in the Sea of Japan the research of water mass microbial community was via culturing and microscopy techniques conducted, data on enzymatic activity were received, total microorganisms count was performed [4]. Taking into account the need to develop an
effective and modern monitoring system for marine environment pollution, by oil hydrocarbons polluted. At first, the research objective was set to carry out the molecular genetic analysis of water mass samples, taken from water areas with various anthropogenic load in the Sea of Japan, as well as to recognize marker genes in microbial communities, responsible for petroleum hydrocarbons destruction.

2. Material and methods

2.1. Researched regions
As the regions for the research water areas are undergoing different anthropogenic stress in the Sea of Japan, these regions were chosen: Zolotoi Rog Bay, the Nakhodka Bay, the Kievka Bay, the Stark Strait, and the Vostok Bay (figure1).

![Figure 1](image_url)

**Figure 1.** Place of sampling. 1 - Zolotoi Rog Bay; 2 - Stark strait; 3 - Vostok Bay; 4 - Nakhodka Bay; 5 - Kievka Bay.

Zolotoi Rog Bay, with its large industrial and port enterprises present, is considered to be one of the most troublesome districts. The heaviest pollution of chemical, petrolic, and thermal kind is traced in the bay, since treatment systems are not exploited [5].

The seaport, big in terms of cargo turnover in the Russian Far East, is situated along the Nakhodka Bay. The Partizanskaya river in southern Primorye, the second largest by river flow volume after the Razdolnaya river, falls into the head of the bay. Industrial and town public utilities, discharging sewage waters into the bay, vessels and port facilities, also river flows are the main sources of inshore waters pollution in the bay [6].

The Vostok Bay is located in the eastern part of the Peter the Great Gulf, near Nakhodka town. There are the Volchanka river flowing into the Vostok Bay in the northwest, and the Litovka river flowing into the Vostok Bay in the northeast. In 1989 the State Complex Marine Sanctuary was established, the Srednaya Bay is located under its authority [7]. Residents from all over the Far East spend their holiday time on the coast of the bay since July until the early September. There are no enterprises polluting the environment on the coast there.

The Kievka Bay is situated in the eastern part of Primorye coastline, it has latitudinal extension, open from the southern side, and classified as a type of unrestricted water exchange and substantial Riverside flow. On the riverside one can find none of housing facilities, and little recreational load happens in summer time [7].
The Stark Strait divides The Russian and Popov islands, strait depth is about 3 till 4.5 meters near the shore. Depth in the northern part of the strait range from 15 till 21 meters, and in the south it is 7.7—14.6 meters. Heavy north-directed current is observed [7]. The area is characterized by minimal anthropogenic load [8].

2.2. Sampling and plating

Samples were taken out of water mass in 5 various spots of each researched area in July 2016. For the purpose of obtaining biomaterial, water samples of at least 5 liter volume were infiltrated through Millipore filters of 25 mm in diameter ( pore diameter – 0.22 mcm).

In order to receive pure bacteria cultures, which are able to biodegrade hydrocarbons, water samples were plated on Voroshilova-Dianova agar medium with addition of crude oil at 1% concentration, and incubated at room temperature during 7 days (24 hours each) [9]. Further work was performed on collection of obtained isolates to reveal functional genes, responsible for oil hydrocarbons degradation.

2.3. Research molecular methods

For extraction of total DNA each filter was minced and supplemented with 1 ml of TE buffer, 0.05 g of polyvinylpyrrolidone, and lysozyme (final concentration at 15 mg/ml), after that the mixture was incubated at 37°C within 1 h, stirring it every 10 minutes. Lysate and sodium dodecyl sulfate (SDS) at 10% concentration were combined to get final solution concentration of 1 %, next incubed during 15 munites at 37 °C temperature. Thereafter 3 successive freezing cycles in liquid nitrogen were started, and defrosting cycles at 56˚С (10 min) were continued. The next stage was centrifugation at 13.4 thousand rpm within 15 minutes. Further total DNA separation was made using reagent kit of Lytech (Russia) according manufacturer’s inscription from clause No.2.

For DNA separation out of pure culture strains a phenol-chloroform extraction method was applied [10, 11].

While conducting PCR-amplification, specific primers were operating on functional bacterial genes, which are responsible ones for decomposition of broad range of alkanes (alk) and polycyclic aromatic hydrocarbons (nar/nah) (table 1). PCR of total DNA and pure cultures was performed in 10 mcL amplification kit “AmpliSens” (Russia), containing 5 mcL of Redmix, 0.2 mcL of dNTP, proper primers 0.2 mcL each, and 3.4 mcl of H₂O. Amplifiers BIS (Russia) and «MyCycler» (Bio-Rad Laboratories Inc., USA) were enabled into amplification process.

| Table 1. Oligonucleotide primers. |
|-----------------------------------|
| Primer | Sequence 5' – 3' | Reference |
|--------|------------------|-----------|
| ALK 2F | GAGACAAATCGTCTAAAACGTAA | [12] |
| ALK 2R | TTGTATTATTCAAACATATGCTC | |
| ALK 3F | TCGAGCACATCCGCGCCCACCA | |
| ALK 3R | CCGTAGTGCTCGACGTAGTT | |
| Ps.putidaF Alk-B | TGGCCGGCTAATCCGATGATCGGAATCTGG | [13] |
| Ps.putidaR Alk-B | CGCGTGGTGTAGTCGAGCCTGAAGGTG | |
| NarAa-1010F | TACCTCGGGCCGACCTGAAGTTCTA | |
| NarAa-1611R | AGTGTTCACGCCGAGCTAGTCTTG | [14] |
| NarAb-2392R | GATGGTGTCTGTAGTCTAGCAGCA | |
| NarAb-2031F | GCACTCCTACCCGAGGATCTC | |
| NahAc-Ac1014r | CTCRGGCATGTCTTTTTC | |
| NahAc-Ac149f | CCCYGGCGACTATGT | [15] |
For optimal DNA amplification with primers for \( alk \) genes (350-550 bp) the selection of conditions for amplification was made. The following programmes were chosen: 94 °C – 3 min (1 cycle), 94 °C – 60 sec, 44 °C / 54 °C – 60 sec (for pure cultures DNA/total DNA), 72 °C – 30 sec (30 cycles); 72°C – 3 min.

For optimal DNA amplification with primers for \( nar/nah \) genes (404-625 bp) also the selection of conditions for amplification was made. The following programmes were chosen: 95°C – 3 min (1 cycle), 94 °C – 40 sec, 55 ° C – 60 sec, 72 °C – 60 sec (30 cycles); 72°C – 3 min.

Amplification products were divided through electrophoresis in 1 % agarose gel, coloured by ethidium bromide, and visualized in ultraviolet light. PCR-products purification was made through cutting fragments of expected length and gel extraction.

Sanger sequencing was by capillary instrument ABI 3130 x1 Genetic Analyser conducted. Primary structure of obtained fragments was in the Center for collective use “Genomics” in Novosibirsk city identified.

3. Results and discussion

In the research process, the total DNA, separated from bacterial filters, was tested for functional genes, responsible for destruction of \( n \)-alkanes and polycyclic aromatic hydrocarbons (PAHs). Similar genes were detected in 3 water areas out of 5 (table 2).

| Area                  | Marker genes |
|-----------------------|--------------|
|                       | ALKB | NarAa | NarAb | NahAc |
| Zolotoi Rog Bay       | +    | -     | +     | -     |
| Nakhodka Bay          | +    | -     | -     | -     |
| Stark Strait          | -    | -     | -     | -     |
| Vostok Bay            | +    | +     | -     | +     |
| Kievka Bay            | -    | -     | -     | -     |

Genes, determining alkane hydroxylase (\( alk \)) synthesis, providing degradation of short-chain and long-chain alkanes, were in DNA of microorganisms in the Zolotoi Rog Bay, the Nakhodka Bay, and the Vostok Bay detected. Genes, responsible for PAHs destruction, were in total DNA, isolated from water mass of the Zolotoi Rog Bay and the Vostok Bay, identified. In other samples genes, investigated with the help of chosen primers, were not found.

Identification of genes, responsible for oil hydrocarbons decomposition, in total DNA samples was the evidence of oil contamination with respect to oil-contaminated areas. Marker genes, detected in the Zolotoi Rog Bay and the Nakhodka bay, proved data on chronic pollution by oil products in these bays. Meanwhile, absence of marker genes in samples from background regions (the Kievka Bay and the Stark Strait) indicated the insignificant anthropogenic load within those water areas. Detection of marker genes in the background Vostok Bay, however, showed the environmental deterioration on that territory. One of possible reasons of current situation can be geographical proximity of the Vostok bay and the Nakhodka Bay. Polluted waters from the Nakhodka Bay might be transferred by means of a current to the Vostok Bay, undergoing minimal anthropogenic load.

Besides carrying out the research of total bacterial DNA, we studied metabolic peculiarities for separate bacterial strains, selected from water in specified regions. As a result of the work conducted in agarose medium, containing crude oil, 7 strains of bacteria were obtained: 6 ones from the Nakhodka Bay, and 1 from the Golden Horn Bay. From water samples, taken in other water areas, none of bacteria cultures, able to provide oil oxidation, were separated. It is important to mention, though in total DNA marker genes were in samples from the Vostok Bay determined, we did not
succeeded in obtaining pure cultures of bacteria, which can grow in oil-containing medium. This can be testifying to both weak concentration of oil-oxidizing bacteria in territorial water, thus, impossible for us to isolate them as a separate culture, and possible ability for oil degradation in the Vostok Bay for uncultured bacteria.

Molecular genetic studies of obtained bacteria strains demonstrated, that all 7 strains contain 3B-group alk genes, responsible for degradation of broad range of n-alkanes, and do not contain 3B-group alk genes, responsible for PAHs decomposition (table 3). The similarity of bacteria comprising alk genes with gram-negative bacteria of Acinetobacter genus, which have decoded alk genes structure and participate inside various ecosystems in degradation of oil and oil derivatives, was via phylogenetic analysis of producted nucleotide sequences revealed, resembling bacteria of Micrococcus, Bacillus, and Corynebacterium genera too.

| Area          | Strain № | Length, bp | Similarity, % | Closest sequence of functional genes (NSBI) | Source of isolation of the closest relative |
|---------------|----------|------------|---------------|--------------------------------------------|------------------------------------------|
| Nakhodka Bay  | 1NN2a/1  | 170        | 99%           | CP007437 Micrococcus luteus                | Collection of strains, Germany            |
|               | 4N/3     | 205        | 95%           | CP001628 Micrococcus luteus                | Collection of strains, Germany            |
|               |          |            | 98%           | DQ288004 Bacterium alkW92 AJ009584 Acinetobacter calcoaceticus (alkM gene) | Soil bacterium                           |
|               | 23N/8    | 221        | 96%           | H530362 Acinetobacter guillouiae (alkB gene) | Strain cultivated on alkanes (C12, C16)  |
|               | NGIL3/7  | 220        | 99%           | CP0000227 Bacillus cereus                  | Strain from industrial production        |
|               | NG1a/10  | 164        | 93%           | CP007437 Micrococcus luteus                | Collection of strains, Germany            |
|               | 17N/11   | 222        | 82%           | EU853413 Corynebacterium variabile (alkB gene) | Oil-oxidizing bacteria from coastal surface waters of Xiamen Island |
| Zolotoi Rog Bay | ZR32/2  | 220        | 99%           | CP0000227 Bacillus cereus                  | Strain from industrial production        |

For the first time, the presence of microbial functional genes, responsible for oil hydrocarbons destruction, in the inshore water areas in the Sea of Japan was researched. In total, microbial DNA from water mass in the regions with chronic anthropogenic pollution to the genes, responsible for oxidation of broad range of n-alkanes and polycyclic aromatic hydrocarbons, were found. Meanwhile, absence of marker genes in samples from background regions (the Kievka Bay and the Stark Strait) indicated the insignificant anthropogenic load within those water areas. Detection of marker genes in the background water area (in the Vostok Bay) was ever indicating ecological deterioration within this territory. Analysis of the data received gave the opportunity to specify molecular genetic techniques, intended for marker genes detection in total bacterial DNA from environment objects, as more
effective technique for identification of petroleum hydrocarbons water pollution in comparison with trivial culturable methods.

Acknowledgments
The research was fulfilled with financial support of the Russian Scientific Fund (Agreement No. 14-50-00034)

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