

Influence of Vladivostok coastal waters pollution on a microflora of mussel *Crenomytilus grayanus*

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Abstract. Taxonomic structure of bacterial community for mussel *Crenomytilus grayanus* digestive system from coastal waters of Vladivostok city, which is characterized by considerable anthropogenic impact was studied. Specimens of *Micrococcaceae* family predominated in the mollusc microscopic flora by the quantity of selected strains (62%). The order of *Actinomycetales* (namely genera of *Actinomyces* and *Pimelobacter*) were large (11%). In addition, there were numerous representatives of Enterobacteriaceae family (9%), *Vibrio* genus (9%), and *Paracoccus* genus (9%) selected. There were found such pathogenic and opportunistic microorganisms, as *Escherichia coli*, *Listeria monocytogenes*, and *Klebsiella spp* in microbial communities of mussels. Even though pathogenic microflora does not predominate in the biotic community, it nevertheless shifts the balance to increase extrinsic microflora within the hydrobiont. Detection of such potentially harmful bacteria indicates the insufficient sanitary and epidemiological state of the water area researched, the bay persistently polluted with municipal domestic drain waters.

1. Introduction

The Sea of Japan is a favourable site for fishery and cultivation of various hydrobionts aggregations due to its environmental and geographical factors [1]. However, increasing pollution of water areas considerably affects natural and cultivated populations of marine animals [2, 3].

Vladivostok is a seaport with over 600000 people, and it is situated on the Muraviev-Amursky peninsula and the islands in the Peter the Great Gulf of the Sea of Japan. Vladivostok inshore waters are rich with marine animals. Species like herrings, smelts, navagas, flatfish, greenlings, rudds, so-iuy mullets, mussels, trepangs, scallops, octopuses, crabs inhabit them [4]. Despite natural original conditions, the water areas, which are adjacent to the city, have been tremendously influenced by anthropogenic stress since the mid-nineties of the last century. The main sources of seawater pollution close to the city are industrial wastes and domestic sewage from Vladivostok city, and other residential areas and settlements, as well as polluted waters of rivers running into the sea nearby. During the period of preparation for the APEC Vladivostok Summit 2012, there were three complexes of treatment facilities constructed, and the fourth complex was redeveloped, that was set to improve ecological situation in this region [5].

Crenomytilus grayanus is prevalent in the waters of southern Primorye, including many bays of Vladivostok islands. This surf type is popular among local people, however, systematic monitoring of microbiological safety for these objects is lacking. While mussels let water through mantle cavity, nutritious matter, as well as various organic and inorganic pollutants, also microorganisms which are



agents of infectious diseases, can get into animal bodies with seawater and be accumulated inside [2]. Thus, industrial waste and sewage waters in the big city can produce a negative effect on natural microbial communities in the marine environment, thereby degrading the sanitary-epidemiological state of water areas and opening the risk of human population infections through contaminating seafoods [2, 6]. Therefore, the research objective was to estimate the influence of the anthropogenic pollution on the microflora composition of *Crenomytilus grayanus* and its habitat in Vladivostok coastal water areas.

2. Material and methods

2.1. Sampling

The Ajax bay was chosen as the research area, the bay is situated in northeastern part of the Russian Island within the Vladivostok city (Primorsky krai of the Russian Federation). The seafloor of the bay is substantially stony, and sand-stony. Previously, the technogenic pollution of the bay with oil hydrocarbons, phenols, and heavy metals was observed [7].

Adults of *Crenomytilus grayanus* of the same size, water and sea ground samples from the species microhabitat for experiments. Samples were taken at a depth of 5 - 6 metres in the Ajax Bay during the late summer-early autumn periods during 2014-2015. In the laboratory environment, the mussel gastrointestinal tracts were extracted with a scalpel, and they were homogenized. After serial dilution the tissue homogenate, water and sediment were plated on solid modified marine broth (MMB) medium, thereafter they were cultivated at the temperature of 25°C inside the thermostat during two days [8]. The obtained single colonies were separated and replated for isolated bacterial strains could further be taxonomically determined.

2.2. Identification of obtained strains of cultivated heterotrophic bacteria

Identification of obtained bacteria strains was carried out on a basis of the analyzed nucleotide sequences in the 16S rRNA gene fragment. DNA extraction was made using the improved Echt method [9-11]. Two primers were chosen for the operation: 926F 5'-AACTCAAAGGAATTGACGG-3' and 1522R 5'-AAGGAGGTGATCCARCCGCA-3'[12]. A positive control (earlier separated bacterial DNA) was employed for the PCR efficiency evaluation. A negative control (no DNA adding) was used to prove unavailable contamination of foreign DNA reagents.

PCR was performed with 25 mcL reaction mixture, containing 1×TaqMan buffer B, 2.5 mM of MgCl₂, 250 mcM of dNTP, 2 activity unit of Taq DNA Polymerase (Sileks, Russia), 15 ng of DNA, and 0.25 mcM of each primer (Syntol, Russia). Initial denaturation was performed at 95 °C (2 minutes) with 37 following denaturing cycles at 95°C during 15 seconds, annealing at 56°C during 10 seconds, and elongation at 72°C during 40 seconds, with final elongation at 72°C during 1 minute.

All PCR-products were visualized through horizontal electrophoresis in 1.5% agarose gel with the Tris/Borate/EDTA buffer (TBE buffer), containing ethidium bromide (10 mg l⁻¹). Sequencing reaction was carried out with 10 mcL reaction mixture under the following protocol: 6 mcL of distilled water, 2 mcL of quintuply sequencing buffer, 0.6 mcL of Big Dye Terminator Cycle Sequencing kit v.3.1 (Thermo Fisher Scientific, the USA), 0.4 mcL of primers, and 1 mcL of PCR-products obtained. Sequencing reaction with forward and reverse primers was performed in different test tubes. Reaction was processed in the Bio-Rad T 100 amplifier (the USA) under the following protocol: initial denaturation at 96°C (1 minute) with 30 further denaturing cycles at 95°C during 10 seconds, annealing at 56°C during 9 seconds, and elongation at 60 °C during 50 seconds, with final elongation at 60°C during 60 seconds. Reaction products were purified with the use of ethanol. 8 mcL of distilled water and 32 mcL of 96% ethyl alcohol were added to a sample, which was thereafter centrifuged at maximum rpm within 15 minutes. After that, the previous alcohol was decanted, and 120 mcL of 70% ethyl alcohol was infused to centrifuge the sample for within 10 minutes, then the liquid was promptly discharged. Dried sample with addition of formamide (18 mcL) was denatured at 95°C during 2 minutes and sequenced in ABI 3130 Genetic Analyser (Perking-Elmer Biosystems, the USA).

The assembling and alignment of sequences were done with Pregap 4 v.1.4 software (England), and the analysis of DNA sequences was fulfilled via BLAST resource (Basic Local Alignment Search Tool) (NCBI, the USA).

3. Results and discussion

While making the research 181 strains of cultivated heterotrophic bacteria were isolated, there were 67 bacteria strains segregated out of water. 61 ones out of sediment, 53 ones out of mussels (table 1).

Table 1. Taxonomical diversity of bacterial communities from Ajax bay.

Taxon	Quantity of strains		
	water	sediment	mussel
order Actinomycetales:	10	5	6
genus <i>Actinomyces</i>	4	5	3
genus <i>Corynebacterium</i>	3	0	0
genus <i>Pimelobacter</i>	0	0	3
genus <i>Rhodococcus</i>	3	0	0
family Micrococcaceae:	8	15	14
genus <i>Arthrobacter</i>	2	8	6
genus <i>Micrococcus</i>	6	7	4
genus <i>Kocuria</i>	0	0	4
family Enterobacteriaceae:	8	7	5
genus <i>Escherichia</i>	6	6	3
genus <i>Citrobacter</i>	1	0	0
genus <i>Klebsiella</i>	0	1	1
genus <i>Proteus</i>	0	0	1
genus <i>Yersinia</i>	1	0	0
family Clostridiaceae:	2	5	4
genus <i>Clostridium</i>	0	3	2
genus <i>Sarcina</i>	2	2	2
family Flavobacteriaceae:	4	1	0
genus <i>Flavobacterium</i>	2	1	0
genus <i>Olleya</i>	2	0	0
family Halomonadaceae:	5	2	0
genus <i>Cobetia</i>	3	1	0
genus <i>Deleya</i>	2	1	0
family Moraxellaceae:	9	5	2
genus <i>Acinetobacter</i>	4	3	0
genus <i>Moraxella</i>	5	2	2
Other families:	19	17	19
genus <i>Acetobacter</i>	0	1	0
genus <i>Aerococcus</i>	1	0	4
genus <i>Bacillus</i>	2	4	3
genus <i>Cytophaga</i>	0	1	0
genus <i>Enterococcus</i>	1	4	0
genus <i>Listeria</i>	1	1	1
genus <i>Microbacterium</i>	2	0	0
genus <i>Paracoccus</i>	1	2	5
genus <i>Phyllobacterium</i>	1	0	0
genus <i>Pseudomonas</i>	4	5	1
genus <i>Pseudoalteromonas</i>	0	0	1
genus <i>Staphylococcus</i>	3	2	2
genus <i>Streptococcus</i>	1	0	0
genus <i>Vibrio</i>	4	1	5
Total:	67	61	53

All investigated microorganism strains were identified and assigned to the 34 genera of bacteria. Representatives of the family *Micrococcaceae* predominated in the mollusc microflora as 26% of the segregated strains. Bacteria of the *Actinomycetales* order, namely genera *Actinomyces* and *Pimelobacter*, appeared also numerous and made up 11%. In addition, many specimens of *Enterobacteriaceae* family (9%), *Vibrio* genus (9%), and *Paracoccus* genus (9%) were selected.

The presence of pathogenic and opportunistic microorganisms, such as *Escherichia coli*, *Listeria monocytogenes*, and *Klebsiella spp* in the microflora of mussels in the Ajax Bay turned out to be important feature of the microflora. Detection of similar potentially hazardous bacteria proves the insufficient sanitary and epidemiological state of the water area researched, which is caused by persistent pollution with municipal domestic drain waters, and also by significant recreational pressure in summer time. As a strong factor, which has an influence to the ecological conditions and the microbial variety in the Ajax bay waters, a heavy contamination with waste oil products from private small-size vessels can appear. It is interesting to note that, only in the Ajax bay waters 2 bacteria strains belonging to the *Olleya* genus were determined, some of the specimens can be found in sea waters while extensive oil spills occur [13], as literature data report, which play the role of decomposers for polycyclic oil hydrocarbons [14], including high-toxic benzo[a]pyrene [15].

When analyzing hydrobionts microflora composition, one cannot fail to take into account their environmental conditions, as well as the peculiar microbiota of this environment [16]. Though, water and sediment microbiota have a substantial impact on the researched animals microflora (table 1), the certain groups of microorganisms, detected in the environmental objects of a habitat, did not occur among mussel symbionts. Thus, for example, there were not any *Corynebacterium*, *Rhodococcus*, *Flavobacterium*, *Olleya*, *Cobetia*, *Deleya*, *Acinetobacter*, *Enterococcus*, and *Microbacterium* bacteria genera in mollusc associates that indicates, a definite existing selectivity during the development of *Crenomytilus grayanus* bacteria communities. It is worth mentioning that in order to exclude probable desultory data only the microorganism groups, selected in two or more strains from the habitat, were accounted.

In addition, our data demonstrated, that in the mussel microflora of the Ajax bay there were some bacteria found, which presence in the water and ground samples of this area was not discovered: the representatives of genera of *Kocuria* (4 strains), *Pimelobacter* (3 strains), and *Pseudoalteromonas* (1 strain).

Other authors received similar results at *Crenomytilus grayanus* microflora investigations [17]. Thus, *Streptomyces*, *Enterobacteria* and yeasts occur only as mollusc associates, and were not determined in the waters of mollusc natural habitat [17]. Mediterranean oysters example demonstrates that *Cytophaga*, *Flavobacterium*, *α -Proteobacteria*, *Bacillus*, *Shewanella* bacteria are not medium-selected, but only in mollusc microflora representative [18]. During the study on *Mytilus galloprovincialis* microflora Cavallo et al [19] pointed out that the exceptional luminescent bacteria for mussels became the following genera: *Hotobacterium* and *Lucibacterium*, as well as *Vibrio*, *Aeromonas*, *Pseudomonas*, *Acinetobacter/Moraxella*, *Flavobacterium*, *Micrococcus*, also bacteria of *Enterobacteriaceae* family. *Moraxella*, *Cytophaga*, *Flavobacterium*, *Bacteroides*, *Bacillus*, *Micrococcus* and *Streptomyces* were discovered only in mussels, and not in water, as indicated in the paper about *Mytilus trossulus* [6].

Such facts could be related with the rare specimens of inhabiting microorganisms accumulated inside mollusc body. Penetrating hydrobiont organism, they can colonize its gastrointestinal tract to possess thereby an advantage over free-living microorganisms. Therefore, also pathogenic bacteria, potentially hazardous for human beings, can take a preferential development under specific circumstances that indicates the necessity of a permanent microbiologic monitoring.

As the conducted research found, the anthropogenic contamination of Vladivostok water areas has a considerable impact on microbial communities residing in water, sediments and gastrointestinal tract of *Crenomytilus grayanus* in the Ajax bay. Although opportunistic microflora does not prevail in the biocenosis, it still upsets the balance toward the increasing quantity of the non-typical for hydrobiont

microflora. Meanwhile, the ability of mussels to accumulate certain microorganism groups exposes us to higher risks of contaminated products.

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