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HYDROCARBON-DEGRADING BACTERIA ASSOCIATED WITH INTESTINAL TRACT OF FISH FROM THE BALTIC SEA

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Abstract. The hydrocarbon-degrading bacterial diversity of the intestinal tract content of fish – the Baltic cod (*Gadus morhua*), plaice (*Platichthys flesus*) and the Baltic herring (*Clupea harengus*) – from the Baltic Sea has been investigated by molecular methods: DNA extraction, amplification polymerase chain reaction product and sequencing of partial 16S rRNA genes. The results of this study show that dense total heterotrophic bacterial populations occur in the intestinal tract of investigated fish. The data obtained showed that the abundance of hydrocarbon-degrading bacteria in the intestinal tract of fish varied from 2.40×10^4 to 1.08×10^5 cfu g^{-1} between fish species and was still high. Phenotypic examination of the recorded hydrocarbon-degrading bacteria from the intestinal tract of the Baltic cod, plaice and the Baltic herring revealed that they belong to *Aeromonas, Pseudomonas/Shewanella*. Molecular species of hydrocarbon-degrading bacteria found in the digestive tract of fish from the Baltic Sea were: *Aeromonas veronii, Aeromonas sobria, Shewanella spp. and Acinetobacter spp.* We argue that hydrocarbon-degrading bacteria in intestinal tract of fish take part in purification processes, as well as, bacteria in water and play a role in adaptation and survival of fish chronically exposed to pollution with hydrocarbons.

Keywords: pollution, fish, intestinal bacteria, abundance of hydrocarbon-degrading bacteria, 16S rRNA gene sequencing, species composition.

1. Introduction

Studies on the microflora of various ecological groups of fish are necessary for analysis of digestion mechanisms and feeding efficiency of fish from natural ichthyocenoses, control and correction of feeding efficiency of fish of pond populations, prevention and treatment of diseases, and scientifically grounded control of the quality and safety of the fish stock and products (Abramova 2004).

Extensive papers were published on various aspects of the microbial flora associated with fish eggs, skin, gills and intestine, and on the relationship of the intestinal microbiota to that of the aquatic habitat. The microbial populations within the digestive tract of fish are rather dense with numbers of microorganisms much higher than those in the surrounding water indicating that the digestive tract provides favourable ecological niches for these organisms (Cahill 1990; Austin 2002; Verner-Jeffreys *et al.* 2003; Hagi *et al.* 2004; Sugita *et al.* 2005; Ringø *et al.* 2006; Skrodenytė-Arbačiauskienė *et al.* 2006; McIntosh *et al.* 2008). The total number of bacteria isolated from the intestines of nine fish species by Cahill (1990) ranged from 10^5 to 10^8 cells per gram. The same author has shown that the density of microbial population in the fish intestine depends on the density of microorganisms in the ambient water distinguishing between the microflora of intestinal contents and the microflora closely connected with the intestinal wall. Although the relative abundance and diversity of bacteria inhabiting healthy fish are of undoubted interest, the role of these bacteria seems to be more important.

Fish harbour the communities of bacteria that fulfil necessary functions also (Sugita *et al.* 1991, 1997, 2002; Romirez, Dixon 2003). According to the data published, the bacteria of fish intestinal tract are related to numerous functions including the degradation of complex molecules such as starch (production of amylase by intestinal bacteria), cellulose, phospholipids, chitin and collagen; production of vitamins, etc.

Petroleum hydrocarbon pollution in marine and estuarine environments is a global problem (Atlas, Bartha 1998; Baltrėnas, Vaišis 2007). Biodegradation by natural populations of microorganisms is the basic and the most reliable mechanism by which thousands of xenobiotic pollutants, including crude oil, are eliminated from the environment (Atlas, Bartha1998). Oil-degrading marine bacteria are of great significance in marine environments because it is well evidenced that a number of bacteria utilize a variety of hydrocarbons in nature and that bacterial oxidation rate may be as much as ten times the autoxidation rate. More than 100 species representing 30 microbial genera have been shown to be capable of utilizing hydrocarbons. In general, the population level of hydrocarbon utilizes and their proportion within the microbial community appear a sensitive index of environmental exposure to hydrocarbons. In unpolluted

ecosystems, the hydrocarbon utilizes generally constitute 0.1% of the microbial population; in oil-polluted systems they can rise to much higher levels (Leahy, Colwell 1990). The effects of environmental conditions on the microbial degradation of hydrocarbons and the effects of hydrocarbon contamination on microbial communities are areas of great interest (Delille, Delille 2000; Pucci *et al.* 2000; El-Tarabily 2002). In general, microbial communities from contaminated ecosystems can adapt to the presence of pollutants, producing shifts in the metabolic and generic diversity of the community (Macnaughton *et al.* 1999). In this context, the knowledge of the taxonomic and physiological characteristic of the autochthonous biocenosis belonging to a certain natural ecosystem can provide insights into the ecological function of these communities.

The information regarding the intestinal microbial flora in fish is abundant, however there is little information in the field of crude oil impact on intestinal microflora and hydrocarbon-degrading bacteria in the intestinal tract of aquatic animals (Šyvokienė, Mickėnienė 2000, 2004; King *et al.* 2005). Some data concerning crude oil impact on the intestinal bacterioflora in animals are available (George *et al.* 2001).

Almost all natural aquatic ecosystems contain populations of bacteria that can metabolize some oil components and related compounds even if those systems have not ever been exposed to oil or oil products (Leahy, Colwell 1990).

Fish are continuously exposed to a wide range of microorganisms present in their environment. Microorganisms that inhabit the digestive tract of fish are specialized to survive and multiply there (Cahill 1990).

The current study was initiated to investigate the abundance of hydrocarbon-degrading bacteria in the intestinal tract of fish from the Baltic Sea and to determine them to species level using the 16S rRNA gen sequencing technique**.**

2. Material and methods

The fish for microbiological investigations, i.e. the Baltic cod (*Gadus morhua*), plaice (*Platichthys flesus*) and the Baltic herring (*Clupea harengus*) are widespread in the Baltic Sea and were sampled near Būtingė once in July of 2006. All fish were caught before midday according to guidelines given by Thoresson (1996). Five specimens of cod, three of plaice and three of the Baltic herring were used for microbiological investigation of intestinal tract. All fish were kept on ice and examined within shortest time possible.

Populations of aerobic and facultative anaerobic heterotrophic bacteria occurring in the intestinal tract of the investigated fish were estimated using a dilution plate technique. The fish were killed by physical destruction of the brain, and the skin was then washed with 70% ethanol before opening the ventral surface with sterile scissors. From each fish intestinal tract, 1 g of intestinal contents was removed and suspended in 10 ml of sterile saline (0.85% (w/v) NaCl). The suspension was serially diluted to 10^{-7} and 0.1 ml of the solution was spread in triplicate on to agar media.

The media chosen were: tryptone soya agar (TSA, Oxoid, Hampshire, UK) with added 5% of glucose and 1% NaCl (for isolation of total heterotrophic bacteria) and oil agar (for hydrocarbon-degrading bacteria): 1 *l* distilled water, 4.0 g NH_4Cl, 1.8 g K_2HPO_4, 1.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4x7H_2O$, 20.0 g agar, 2 ml crude oil as hydrocarbon source, pH 7.4 (Ijah, Antai 2003). The same medium without the hydrocarbon source was used as a control. The inoculated plates were cultured 5–10 days at 20 °C and the number of colonies was counted. Bacterial numbers are reported as cfu (colony forming unit) g^{-1} of intestinal content.

Colonies larger and different from those on the substrate-free control plates were selected for further investigations. Bacterial colonies on oil agar were divided into different types according to colonial characteristics i.e. shape, size, elevation, surface, colour, edge and opacity. Three to five representatives of each colony type were streaked and re-streaked on fresh media to obtain pure cultures. Total of 100 hydrocarbon-degrading bacteria isolates from the contents of intestinal tract of investigated fish were identified to genus by phenotypic properties. Each isolate was classified to the genus level using a modified version of the scheme (Sugita *et al.* 1981, 2002) and utilized gram-staining, morphological observation, pigmentation, motility, the OF test, KOH test, oxidase test, catalase test, spore observation and the O/129 sensitivity test.

5 pure hydrocarbon-degrading bacteria grown on nutrient agar were used for PCR amplification.

A suspension of 0.1–0.3 g of each bacterial isolate in 0.5 ml of TE buffer was distributed in 3 Eppendorf microtubes. Bacterial chromosomal DNA was isolated by using Genomic DNA Purification Kit #K0512 (Fermentas, Lithuania) (www.fermentas.com).

DNA extracted from bacterial isolates was PCR amplified with 16S bacterial primers w001 F8 (5′-AGA GTT TGA TCM TGG CTC-3′), w002 R 1492 (5′-GGT ACC TTG TTA CGA CTT-3′), w007 R 1100 (5′-CTC GTT GCG GGA CTT AAC-3′), w012 R 700 (5′-TAC GCA TTT CAC CAT ACA-3′) and Taq DNA polymerase (recombinant) (Fermentas, Lithuania).

The PCR temperature profile was 95 °C for 4 min. followed by 30 cycles 95 °C for 1 min., 50 °C for 1 min., 72 °C for 2 min. All PCR amplifications were performed with Gene Amp PCR system in a termocycler (Mastercycler, Eppendorf). The PCR products, which had an expected sizes of about 1500, 1100 and 700 base pairs (bp) were examined for purity and size in 0.8% agarose gels, visualised by staining with ethidium bromide and photographed under UV light. Reactions products were purified and ligated into pUC57/T vector according to the supplier's instructions and transformed into high efficiency XL1-Blue *Escherichia coli* cells using a commercial InsT/Aclone^{TM} PCR Product Cloning Kit #K1213 (Fermentas, Lithuania). Transformants were selected using blue-white screening and multiplied by culture in Luria-Barton medium containing ampicillin. Nucleotide se-

quencing reactions were conducted using the plasmid templates with inserts. A selected number of plasmids with 16S rDNA fragments were sequenced on an ABI Prism 377 DNA sequence using BigDye^R Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and primers: M13/pUC sequencing primer (–46), 22-mer and M13/pUC reverse sequencing primer (–46), 24-mer according to the manufacture's guidelines. Search of nucleotide sequence homology of 16S rDNA gene was done using the Blast algorithm and the sequences were aligned using the CLUSTALW software program (Thompson *et al.* 1994; Altschul *et al.* 1997).

Data are presented as the mean and standard error (SEM) of three determinations.

3. Results and discussion

The physiology of the fish gut differs in many respects from that of the homeothermal animals (Buddington *et al.* 1997) and this can be expected to affect the bacterial numbers and the species' composition. The relevant findings of the published studies on fish intestinal microbiota can be summarized as follows: the total cultivable bacterial numbers are seldom higher than 10^6cfu/g^{-1} (Ringø 1993; Ringø, Olsen 1999), the many typical genera of homeothermal animals such as *Bifidobacteria*, *Bacteroides*, *Eubacterium* (Isolauri *et al.* 2004), are either absent or only occasionally present. Lactic acid bacteria (LAB) are relatively common, but their numbers are low (Ringø *et al.* 2000). Typical fish specific species and genera include, among others *Pseudomonas*, *Aeromonas* and *Vibrio* (Sugita *et al.* 1996; Ringø, Olsen 1999; Hagi *et al.* 2004). The significance of the intestinal microbial community to the health and well-being of fish is poorly known, and also the knowledge of the dietary effects on the composition of microbiota is limited also.

The results of this study show that dense bacterial populations occur in the intestinal tract of investigated fish (Fig. 1). These results are in accordance with those found for other fish species (Ringø, Birbeck 1999; Šyvokienė, Mickėnienė 2000; Al-Harbi, Uddin 2004; Sugita *et al.* 2005; Ringø *et al.* 2006).

Generally bacteria are abundant in the environment in which fish live and it is impossible to avoid them being component of their diet. The bacteria ingested by the fish along with their diet may adapt themselves to the environment of the gastrointestinal tract and form a symbiotic association (Ringø, Birkbeck 1999). The abundance of total heterotrophic bacteria in the intestinal tract of fish varied from 1.56×10^5 to 6.00×10^5 cfu g^{-1} depending on fish species. The differences in results may be due to differences in feeding of fish. Heterotrophic counts are representative of a small group of active bacteria that react immediately to changes in nutrient supply (Delille, Delille 2000).

Earlier reviews (Cahill 1990; Ringø *et al.* 1998; Ringo, Birkbeck 1999) suggested that the gastrointestinal tract microbiota of fish are simpler than those of endothermic animals. However, recent studies on Arctic charr (Ringø *et al.* 2006), Atlantic salmon (Bakke-McKellep *et al.* 2007) and Atlantic cod (Ringø *et al.*2006) demonstrate that this statement may need revision, as several new isolated bacterial species have not been previously reported as a part of the intestinal microbiota in fish.

In the present investigation, a considerable population of hydrocarbon-degrading bacteria has been obtained in the intestinal tract of investigated fish (Fig. 2).

The ubiquitous distribution of oil degrading bacteria has already been reported in a wide variety of niches (Leahy, Colwell 1990; Delille, Delille 2000). Almost all natural aquatic ecosystems contain populations of bacteria that can metabolize some oil components and related compounds even if those systems have not ever been exposed to oil or oil products (Leahy, Colwell 1990). Hydrocarbon-degrading bacteria are present in low numbers in unpolluted environments. These populations increase in number when petroleum hydrocarbons enter natural habitats (Pucci *et al.* 2000; El-Tarabily 2002).

There are several possible sources for the establishment of intestinal gut flora and it is generally believed that the processes of bacterial colonization in fish are complex and depend upon the bacterial flora of live feed and water (Ringø, Birkbeck 1999).

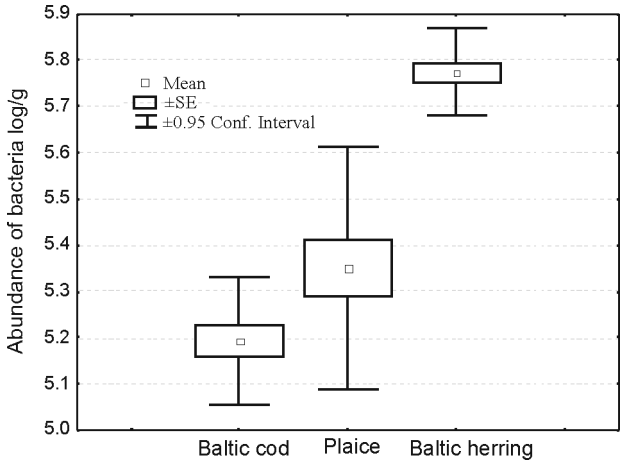

Fig. 1. Abundance of total heterotrophic bacteria in the intestinal tract of fish from the Baltic Sea

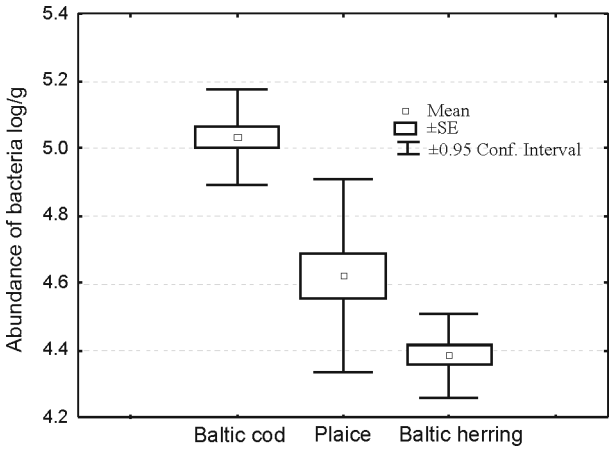

Fig. 2. Abundance of hydrocarbon-degrading bacteria in the intestinal tract of fish from the Baltic Sea

The obtained data showed that the abundance of hydrocarbon-degrading bacteria in the intestinal tract of fish varied from 2.40×10^4 to 1.08×10^5 cfu g^{-1} between fish species and was still high. Our previous investigations have shown that the addition of crude oil into an environment of molluscs resulted in an increase of two orders of magnitude in the number of hydrocarbon-degrading bacteria in the intestinal tract (Šyvokienė, Mickėnienė 2004).

Hydrocarbon-degrading bacteria were found in the liver and bile of fishes: gold-spotted trevally (*Carangoides fulvoguttatus*) and bar-cheeked coral trout (*Plectropomus maculatus*) also (King *et al.* 2005). The authors argue that these fish species have a potential as indicator species for assessing the effect from exposure to petroleum hydrocarbons.

Phenotypic examination of the recorded hydrocarbon-degrading bacteria from the intestinal tract of the Baltic cod, plaice and the Baltic herring revealed that they belong to *Aeromonas, Pseudomonas/Shewanella* (Table 1).

Table 1. Diagnostic features of hydrocarbon-degrading bacterial genera

Genus	Diagnostic features
Aeromonas	Gram-negative rods, motile, polar-flagella, facultative anaerobic, oxidase positive, catalase-positive
Acinetobacter	Gram-negative rods, aerobic, oxidase-negative, catalase-positive
Shewanella	Facultative anaerobic, Gram-negative, motile by polar flagella rods

The 16S rRNA gene sequences obtained from the hydrocarbon-degrading bacteria from fish was deposited in the EMBL data library under accession numbers: EU916707, EU916708, EU916709, EU916710, EU916711.

Phylogenetic analysis based on 16S ribosomal DNA (rDNA) sequences showed that isolates of hydrocarbon-degrading bacteria from the intestinal tract of fish were closely related to *Aeromonas veronii, Aeromonas sobria, Shewanella* sp. and *Acinetobacter* sp. (Table 2).

From the intestinal tract contents of the Baltic cod, the hydrocarbon-degrading bacteria belong to *Aeromonas veronii*, from plaice – to *Aeromonas sobria* and *Acinetobacter* sp., from the Baltic herring – to *Shewanella* sp. and *Acinetobacter* sp.

Table 2. Molecular species of hydrocarbon-degrading bacteria from the digestive tract of fish

Closest species	Closest database sequence	Similarity %
Aeromonas veronii	AF 099023	99
Shewanella sp.	CP000503	99
Acinetobacter sp	AM 184270	99
Aeromonas sobria	X74683	100
Acinetobacter sp.	AY 576723	99

Ringø *et al.* (2006) isolated and identified the following Gram-negative bacteria from Atlantic cod, which are not normally isolated from the gastrointestinal tract of fish: *Acinetobacter johnsoni, Chryseobacterium spp., Ochrobactrum spp., Psychrobacter cibarius, P. fozii, P. glacincola, P. luti, P. psychrophilus* and *Sejongia antarctica.*

Acinetobacter johansonii are Gram-negative, oxidase-negative, catalase-positive and rod shaped bacteria, which have been isolated from freshwater aquaculture habitats (Miranda, Zemelman 2002), Atlantic cod (Ringø *et al.* 2006) and Atlantic salmon (Bake-McKellep *et al.* 2007).

In the current study, we isolated and identified *Acinetobacter* spp. bacteria which are able to degrade oil hydrocarbons from the content of intestinal tract of plaice and the Baltic herring.

Our data obtained showed that hydrocarbon-degrading bacteria from the intestinal tract of the Baltic cod and plaice belong to species *A. veronii* and *Aeromonas sobria.*

A recent review by Sugita and Ito (2006) devoted to phylogenetic analysis based on 16S ribosomal DNA (rDNA) sequences of bacteria from the intestinal tract of flounder. The obtained data showed that 82 representative isolates were closely related to three major species of marine vibrios, *Vibrio scophthalmi-Vibrio ichthyoenteri* group, *Vibrio fischeri* and *Vibrio harvey* with similarities of 97.2–99.8%, 96.4–100% and 98.6–99.5% respectively. These findings indicate that intestinal bacteria from Japanese flounder were mainly composed of *Vibrio scophthalmi-Vibrio ichthyoenteri* group and *Vibrio fischeri* (Sugita, Ito 2006).

Aeromonas isolates were obtained from fish intestines, water and sediments from an urban river (Sugita *et al.* 1995). The results obtained by authors strongly suggest that aeromonads are indigenous in fish intestines and have the potential to be predominant in aquatic environments. In addition, it was reported that all of the *Aeromonas* isolates from the intestinal tracts of six species of freshwater-cultured fishes constituted five *Aeromonas* species: *Aeromonas caviae, Aeromonas hydrophila, Aeromonas jandaei, Aeromonas sobria* and *Aeromonas veronii* (Sugita *et al.* 1995).

However, according to Dügenci and Candan (2003) *Aeromonas* strains were isolated from the intestinal tract of Atlantic salmon from freshwater and the Black Sea. Five of motile *Aeromonas* strains isolated from freshwater and one motile *Aeromonas* strain isolated from the Black Sea salmons were identified as *A. caviae*. The rest were identified as *A. sobria*.

Shewanella putrefaciens is a Gram-negative facultatively anaerobic bacteria belonging to the family *Vibrionaceae.* It is assumed that *S. putrefaciens* is derived from the coastal marine environment. Possibly, the organism is the part of the normal microflora of marine fish (Austin, Austin 1999). Therefore it is surprising, but very interesting that the bacterium was isolated from freshwater fish (Kozinska, Pekala 2004). From the fish digestive

tract we isolated *Shewanella* spp., which was able to degrade oil hydrocarbons. According to Floodgate (1984), in marine environment *Shewanella* spp. and *Pseudomonas* spp. are often involved in the degradation of hydrocarbons.

Earlier we established that in the intestinal tract of fish from the Curonian Lagoon (the Baltic Sea basin) oil hydrocarbons are degraded by: *Aeromonas allosaccharophila, Aeromonas eucrenophila, Aeromonas media* and *Pseudomonas flavescens* (Voverienė *et al.* 2002).

We argue that hydrocarbon-degrading bacteria in an intestinal tract of fish take part in purification processes as well as bacteria in water and may play a role in adaptation and survival of fish chronically exposed to pollution with hydrocarbons. The controversial hypothesis that the fish gut microbiota might not be as simple as believed should stimulate bacteriologists to obtain more information on bacteria colonizing the digestive tract of fish and their potential beneficial role.

4. Conclusions

1. Hydrocarbon-degrading bacteria were obtained in the intestinal tract of all investigated fish and varied from 2.40×10^4 to 1.08×10^5 cfu g^{-1} between fish species and was still high.

2. Molecular species of hydrocarbon-degrading bacteria found in the digestive tract of fish from the Baltic Sea were: *Aeromonas veronii, Aeromonas sobria, Shewanella* sp. and *Acinetobacter* sp.

3. We argue that hydrocarbon-degrading bacteria in fish intestinal tract take part in purification processes, as well as, bacteria in water and may play a role in adaptation and survival of fish chronically exposed to pollution with hydrocarbons.

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ANGLIAVANDENILIUS SKAIDANČIOS BAKTERIJOS BALTIJOS JŪROS ŽUVŲ VIRŠKINAMUOSIUOSE TRAKTUOSE

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Santrauka

Angliavandenilius skaidančių bakterijų įvairovė Baltijos jūros žuvų – menkių (*Gadus morhua*), plekšnių (*Platichthys flesus*) ir strimėlių (*Clupea harengus*) žarnynų turinyje tirta molekuliniais metodais: DNR išskyrimas, polimerazės grandininės reakcijos produkto amplifikacija ir 16S rRNR genų sekvenavimas. Tirtų žuvų žarnynuose nustatytos gausios heterotrofinių bakterijų populiacijos. Angliavandenilius skaidančios bakterijos išskirtos iš visų tirtų žuvų žarnynų turinio. Jų kiekis žuvų žarnynuose svyravo nuo $2{,}4\times10^4$ iki $1{,}08\times10^5$ kfv (koloniją formuojantis vienetas) ir buvo pakankamai didelis. Fenotipiniais bakterijų identifikavimo metodais nustatyta, kad angliavandenilius skaidančios bakterijos, išskirtos iš Baltijos jūros žuvų žarnynų turinio, priklausė *Aeromonas Pseudomonas/Shewanella* gentims. Molekuliniais metodais nustatyta, kad angliavandenilius skaidančios bakterijos tirtų žuvų žarnynuose yra *Aeromonas sobria, Aeromonas veronii*, *Shewanella* spp. ir *Acinetobacter* spp. Galima daryti prielaidą, kad žuvų žarnynuose gyvenančios angliavandenilius skaidančios bakterijos dalyvauja savivalos procesuose kaip ir vandenyje gyvenančios bei angliavandenilius skaidančios bakterijos ir padeda adaptuotis ir išgyventi žuvims vandenyse, pastoviai teršiamuose nafta ir jos produktais.

Reikšminiai žodžiai: tarša, žuvys, žarnyno bakterijos, angliavandenilius skaidančios bakterijos, 16S rRNR genų sekvenavimas, rūšinė sudėtis.

УГЛЕВОДОРОДРАСЩЕПЛЯЮЩИЕ БАКТЕРИИ В ПИЩЕВАРИТЕЛЬНОМ ТРАКТЕ РЫБ БАЛТИЙСКОГО МОРЯ

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Резюме

Разнообразие углеводородрасщепляющих бактерий в содержимом кишечника рыб: трески (*Gadus morhua*), камбалы (*Platichthys flesus*) и салаки (*Clupea harengus*) из Балтийского моря было исследовано молекулярными методами: выделение ДНК, амплификация ПЦР продукта, секвенация 16S рРНК генов. В кишечнике исследованных рыб найдены многочисленные популяции гетеротрофных бактерий. Углеводородрасщепляюшие бактерии выделены из содержимого кишечника всех исследованных рыб. Их численность колебалась в пределах $2{,}4\times10^4$ – $1{,}08\times10^5$ КФЕ (колонию формирующая единица) и была достаточно высокая. Фенотипическими методами идентификации бактерий установлено, что углеводородрасщепляюшие бактерии из пищеварительного тракта рыб принадлежали родам *Aeromonas* и *Pseudomonas/Shewanella.* Молекулярными методами определено, что углеводородрасщепляюшие бактерии из пищеварительного тракта рыб принадлежали видам *Aeromonas sobria, Aeromonas veronii, Shewanella* spp*., Acinetobacter* spp*.* Можно предположить, что углеводородрасщепляющие бактерии из пищеварительного тракта рыб участвуют в процессах самоочищения, как и бактерии, обитающие в водной среде, а также помогают адаптироваться и выжить рыбам в водах, постоянно загрязняемых нефтью и ее продуктами.

Ключевые слова: загрязнение, рыбы, бактерии кишечника, углеводородрасщепляющие бактерии, секвенация 16S рРНК генов, видовой состав.

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