



## Aquatic Organisms and Petroleum Hydrocarbon Degrading Bacteria Associated with Their Digestive System

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Laboratory investigation was carried out on the abundance and composition of bacteria in the digestive system of a total of 35 specimens, including bivalve molluscs, i.e. swan mussel *Anodonta cygnea* (Linnaeus, 1758) from Lake Spėra (Širvintos district) and swollen river mussel *Unio tumidus* (Philipson, 1788) from the Curonian Lagoon near Juodkrantė, zebra mussel (*Dreissena polymorpha*) from the Curonian Lagoon near Juodkrantė and an anostracan – *Chirocephalus josephinae* (Grube, 1853) from a pond in Ilčiukai village, Utena district, and in the water of related water bodies. Studies on bivalve molluscs (swan mussel and swollen river mussel), zebra mussel and an anostracan – *Chirocephalus josephinae*, as well as microbiological investigation of water demonstrated that the number of microorganisms in the digestive system of mollusc and anostracan species fluctuated and varied between different species and water bodies. The greatest percentage of HDB among total heterotrophic bacteria was found in the digestive system of swollen river mussels (21.53%) and in zebra mussels (19.99%) caught in the Curonian Lagoon and in the water of the lagoon (24%). A considerably smaller percentage of HDB was detected in the digestive system of swan mussels from Lake Spėra (17.6%) and in the water of the lake (16.66%). The smallest percentage of HDB was found in the digestive system of *Chirocephalus josephinae* (6.63%) and in the water of the Ilčiukai pond (2.72%). According to the values of abundance of petroleum hydrocarbon-degrading bacteria (HDB) and total coliform bacteria (TCFB) in the digestive system of aquatic organisms we can state that the water ecosystem of Ilčiukai pond was the least contaminated with petroleum, its products and sewage water, and the Curonian Lagoon water ecosystem was the most contaminated. Abundance of petroleum hydrocarbons degrading bacteria could be used as a bioindicator reflecting the level of ecosystem pollution petroleum and its products.

*Keywords: mollusks, crustaceans, digestive system, petroleum, bacteria*

### 1. Introduction

Increasing contamination of aquatic systems by different pollutants makes topical the problem of water self-purification. The concept of self-purification covers all natural processes influencing the degradation, transformation and utilization of pollutants.

Earlier investigations demonstrate an important ecological role of the normal microbiota of the digestive tract of aquatic organisms in their nutrition. Bacteria as active participants in the production of enzymes, amino acids and vitamins are necessary for the normal metabolism of an organism. However, the

stability of the balance of microbiota of the digestive tract of aquatic organisms and the importance of the functional activity of bacteria for homeostasis in an organism are conditioned by the trophic level, productivity and toxicity as well as many physical and chemical characters of a water body (Kuzmina et al, 2002; Gomez et. al, 2008; Navarrete, 2009).

The addition of petroleum induced no clear changes in total heterotrophic, amylolytic, or total coliform bacterial community counts in the digestive tract of molluscs. After a 10-days period of contamination, the total heterotrophic, amylolytic, or

total coliform bacterial community counts were on the same order of magnitude as the initial numbers. Before contamination with crude oil, the number of hydrocarbon degrading bacteria in the digestive system of the investigated mollusks was  $2.43 \log \text{CFU g}^{-1}$ . It was the first time that hydrocarbon degrading bacteria detected in the digestive system of mollusks were studied. Microbiological changes in the digestive tract of mollusks seem to serve as a criterion for the assessment of toxicity. In contrast to its effects on the total heterotrophic, amylolytic and total coliform bacterial counts, oil contamination induced obvious increases in the number of hydrocarbon degrading bacteria in the digestive system of mollusks during the initial 10 days of experiments (*in vitro*) (Šyvokienė, Mickienė 2004).

The environment is under constant pressure of different substance destruction processes intensively performed by different microorganisms. The course of such processes depends on the two principal factors, namely the microorganism-biodegrader and the substrate, in which the former is functioning. Easier consumption by a microorganism of substances contained in the substrate conditions more intensive destruction processes. Besides, some microorganisms easily consume metabolites isolated by other microorganism species and therefore are intensively developing, whereas other microorganisms use for their development mineral and organic contaminants (Kalėdienė et al, 2003). Microorganisms need a particular adaptation period during which changes of different biochemical associations with the environment take place. The synthesis of some metabolites is being induced, of others suppressed and genetic mechanisms can sometimes be widened. Some substances are accumulated into the cells and degraded into minor compounds for the organism to recognise. Microorganisms immediately consume such compounds as an additional co-metabolite substrate. A possibility for a microorganism to metabolise one or another substrate appears only when the environment contains at least a small amount of certain organic compounds which stimulate and regulate the growth of a cell (George et al, 2001; Piehler et al, 2002).

Microbiological degradation of contaminants otherwise than chemical degradation occurs under rather moderate conditions and does not require great monetary investments (Hagi et al, 2004).

The deficit of food forces different microorganism species to use the same substrates and transform them into different compounds. Different environmental conditions change the development of local microorganisms. It is important to evaluate the contribution of separate microorganisms and of their associations in general in degrading petroleum and its products (Nayak et al, 2010).

Aquatic organisms are affected by a great variety of ever-changing environmental factors, including xenobiotics, and microbiota of their digestive system are under continuous pressure of different toxins. Microbiota of the digestive system of aquatic organisms and the scope of possibilities of adaptation

of bacteria communities to xenobiotics are insufficiently investigated as yet. One of essential impacts of contamination is deterioration of bacterial digestion of organisms, which causes further changes in the organism. The ability to degrade and utilise hydrocarbons is characteristic of many bacteria and fungal species. The most active petroleum degraders are bacteria, in particular of the following genera: *Vibrio*, *Aeromonas*, *Pseudomonas*, *Flavobacterium*, *Micrococcus* and *Sarcina*. Researchers have described 25 genera of hydrocarbon-degrading bacteria and 27 species of fungi isolated from sea water and sediments. The least investigated and hardly controllable hydrocarbons are those that are naturally released into the sea, ocean or atmosphere (Leahy et al, 1990).

The effect of microorganisms on substances depends on their systematic grouping, number of cells, ability to synthesise different enzymes and isolate organic acids and other metabolites into environment. Nearly 400 microorganism enzymes participating in catabolism processes of different substances are known (Barclay et al, 1998; Tünde et al, 2000). They cause changes in the chemical and physical characteristics of substances. The ability of microorganisms to develop on different substances and destruction of substances can be described by correlative relationship (Ellis et al, 2001).

Microorganisms use hydrocarbons of petroleum and its products as the only source of carbon and perform biodegradation of contaminants thus contributing to self-purification of water. The concept of self-purification covers the entirety of natural processes conditioning degradation, transformation and utilisation of contaminants, which leads to the recovery to the original state of the water ecosystem. Many components polluting the environment accumulate in different trophic levels and disturb vital functions of organisms. Of particular importance are early contamination impacts, namely effects on microbiota of the digestive tract, which cause further changes in the organism. Due to the settled way of life, water filtration and ability to accumulate pollutants, different molluscs are a good reflection of the quality of the environment (Šyvokienė et al, 2004). The effects of petroleum hydrocarbons on one of the links of the food chain, namely bacteria of the digestive system of aquatic organisms, are insufficiently investigated.

The purpose of the work was to investigate the greatest percentage of HDB among total heterotrophic bacteria in water and digestive systems of aquatic organisms sampled in different habitats.

## **2. Material and methods**

Microbiological investigations were carried out at the Laboratory of Genotoxicology of the Institute of Ecology of the Nature Research Centre in 2009-2010.

We carried out a laboratory investigation on abundance and composition of bacteria in the

digestive system of a total of 35 specimens, including bivalve molluscs, i.e. swan mussel *Anodonta cygnea* (Linnaeus 1758) from Lake Spėra (Širvintos district) and swollen river mussel *Unio tumidus* (Philipson 1788) from the Curonian Lagoon near Juodkrantė, zebra mussel (*Dreissena polymorpha*) from the Curonian Lagoon near Juodkrantė and an anostracan – *Chirocephalus josephinae* (Grube 1853) from a pond in Ilčiukai village, Utena district, and in the water of related water bodies. All aquatic organisms were kept on ice and examined within shortest time possible.

For each microbiological analysis 5-10 aquatic organisms specimens were used. Populations of aerobic and facultative anaerobic heterotrophic bacteria occurring in the aquatic organisms were counted using the dilution plate technique (Hansen et al., 1999).

The surface of aquatic organisms specimens was sterilised with 95% ethanol, and aquatic organisms were dissected to remove their digestive system. The content of the digestive system was removed to sterile Petri dishes. Each set of experiment involved five or ten specimens of aquatic organisms. All digestive system samples were weighed and placed into a test tube, and then nine volumes of diluents were added. The tenfold dilution was further done serially. The volume of 0.1 ml of least dilutions (expected to give 30 to 300 colony-forming units, CFUs), was plated in triplicate on solid media. Incubation was carried out aerobically at 20 °C for seven days, except for MacConkey agar plates, which were incubated at 37 °C for 7 days. The number of CFU in the digestive tract of *O. mykiss* was established on four media: tryptone soya agar (OXOID) was chosen for isolation of total heterotrophic bacteria (THB), milk agar for proteolytic bacteria (PB) as a separate group of total heterotrophic bacteria, PB were identified according to the zones of protein (casein) hydrolysis on milk agar, MacConkey agar (OXOID) was used for total coliform bacteria (TCB), the Voroshilova-Dianova agar with crude oil for petroleum hydrocarbon-degrading bacteria (HDB) as a separate group of heterotrophic bacteria, and the same agar without crude oil was used as a control. Bacterial colonies appearing on each plate were counted, and a CFU per 1 g (wet weight) of the digestive system content was obtained (Кузнецов, Дубинина, 1989).

The average values and mean square deviations were calculated (Sakalauskas, 2003).

### 3. Results and Discussion

The gastrointestinal bacterial flora of the studied aquatic organisms was dominated by heterotrophic Gram-negative bacteria. The greatest abundance of autochthonous THB ( $2.8 \times 10^6$  per  $g^{-1}$  of digestive system content) and PB ( $1.75 \times 10^6$ ) was detected in the digestive system of swan mussels from Lake Spėra. Bacteria get into the digestive system with food from the surrounding environment. Bacteria are assumed to play an important role in nutrition when their abundance is  $10^4$ – $10^5$   $g^{-1}$  of the content of the digestive system (Kuzmina et al., 2002; Navarrete, 2009).

Although THB predominated in the microbiota of the studied aquatic organisms, PB, TCFB and petroleum HDB were isolated, too. Petroleum hydrocarbon-degrading bacteria were detected in high numbers of  $13 \times 10^3$ – $1.18 \times 10^6$  per g of the content of the digestive system of aquatic organisms and  $221$ – $18 \times 10^3$  per ml of water. Petroleum products that aquatic organisms consume with food can be partly degraded by enzymes of bacteria present in intestines. A comparison of swollen river mussels with zebra mussel from the Curonian Lagoon showed that the greatest numbers of all studied functional groups of bacteria bacterial communities were in microbiota of the digestive system of swollen river mussels he smallest abundance of the studied bacterial communities was found in the digestive system of *Chirocephalus josephinae*, ranging from  $13 \times 10^3$  to  $2 \times 10^5$  per  $g^{-1}$  of microbiota of the digestive system. High numbers of petroleum HDB were detected in the microbiota of the digestive system of swan mussels from the Curonian Lagoon and in the water of the lagoon. The smallest abundance of petroleum HDB was found in *Chirocephalus josephinae* from the Ilčiukai pond and in the water of this pond (Figures 1–3). Russian researchers (Ильинский et al., 1986) determined that the abundance of hydrocarbon-degrading bacteria in the Mozhaisk reservoir (one of the main potable water reservoirs in Moscow) was 25-250 per ml water.

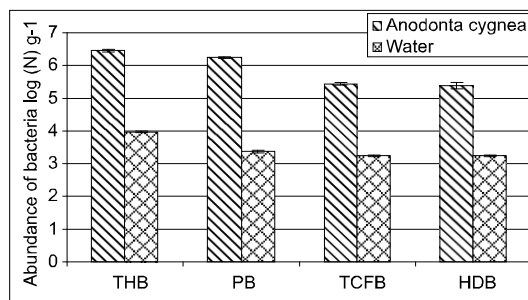


Fig. 1. Abundance of bacteria in the digestive system of *A. cygnea* and water from the Lake Spėra: THB – total heterotrophic bacteria, PB – proteolytic bacteria, TCFB – total coliform bacteria, HDB – hydrocarbon degrading bacteria

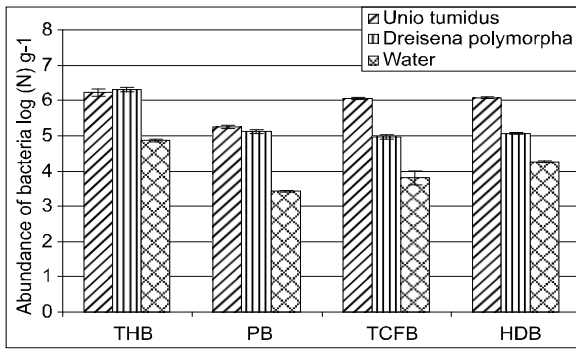


Fig. 2. Abundance of bacteria in the digestive system of *U. tumidus*, *D. polymorpha* and water from the Curonian Lagoon

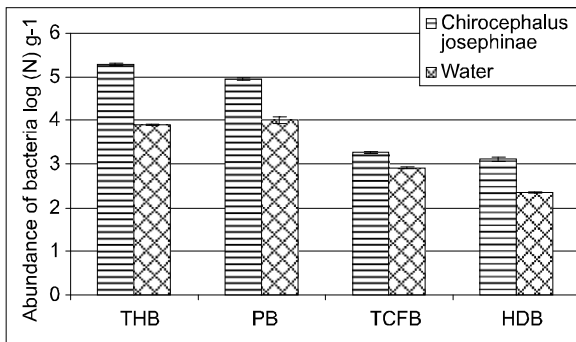


Fig. 3. Abundance of bacteria in the digestive system of *Chirocephalus josephinae* and water from a pond in Ilčiukai Village

The greatest percentage of HDB among total heterotrophic bacteria was found in the digestive system of swollen river mussels (21.53%) and in zebra mussels (19.99%) caught in the Curonian Lagoon and in the water of the lagoon (24%). A considerably smaller percentage of HDB was detected in the digestive system of swan mussels from the Lake Spėra (17.6%) and in the water of the lake (16.66%). The smallest percentage of HDB was found in the digestive system of *Chirocephalus josephinae* (6.63%) and in the water of the Ilčiukai pond (2.72%). Petroleum HDB may constitute from 0.13% to 50% in soil microbiota, from 0.003% to 100% in marine ecosystems (Leahy et. al, 1990), from 0.05% to 66.67% in the Curonian Lagoon.

The greatest abundance of the total coliform bacteria was found in the microbiota of the digestive system of swollen river mussels ( $1.14 \times 10^6 \text{ g}^{-1}$ ) and the smallest ( $2 \times 10^3 \text{ g}^{-1}$ ) in anostracan – *Chirocephalus josephinae* from the Ilčiukai pond. The abundance of TCFB in the water of the pond constituted 822 cells per 1 ml.

A normal intestinal microbiota of fish serves as accumulator of externally ingested or internally produced toxic substances. It is the first to engage in the degradation of natural or alien, useful or hazardous substances and in the synthesis of needed elements (amino acids, vitamins, enzymes and others). Xenobiotics, however, may suppress bacterial activity and produce a negative impact on the efficiency of assimilation and adaptation capabilities of the host organism. Investigations have revealed that vital functions of the intestinal microbiota of fish

and other aquatic organisms depend on the ecological status of the environment. The impact of anthropogenic pollutants causes changes in both water composition and the composition and enzymatic activities of predominant bacteria in the digestive tract of fishes (Шивокене, 1989; Skrodenytė-Arbačiauskienė, 2007).

Possible effect of contamination was assessed based on the abundance and qualitative parameters of bacteria of different aquatic organisms from different water bodies, namely swan mussel, swollen river mussel, zebra mussel and an anostracan – *Chirocephalus josephinae*. It should be noted that contaminants could be removed with the help of living organisms or their enzymes systems. The abundance of total heterotrophic and proteolytic bacteria in microbiota of the digestive system of the studied bivalve molluscs, zebra molluscs and *Chirocephalus josephinae* was found to be greater than the abundance of the total coliform bacteria and petroleum hydrocarbon-degrading bacteria (Figures 1–3). However, the highest value of abundance of TCFB was registered in swollen river mussels caught in the Curonian Lagoon near by Juodkrantė (Figure 2). Their numbers were higher in the water of the Curonian Lagoon than in the Lake Spėra (Figure 1), and the smallest TCFB numbers were found in the pond in Ilčiukai village (Figure 3). It is difficult to objectively evaluate low contamination with sewage water of the latter habitat; however, bacteriological investigations of three habitats and comparative data obtained showed that Ilčiukai habitat was the least contaminated with sewage water. It should also be noted that the numbers of TCFB in the digestive system of *Chirocephalus josephinae* caught in the Ilčiukai pond were considerably lower (Figure 3).

Petroleum hydrocarbon-degrading bacteria constitute a great portion of the local population of microorganisms in a petroleum contaminated environment. In the areas where accidental oil spills occur, the local microbiota affected by large amounts of petroleum hydrocarbons because of chemotaxis is replaced by the nearby existing petroleum-oxidising species. Usually, the diversity of microorganisms in contaminated areas is lower than that in uncontaminated areas. Such ground is most frequently dominated by gram-negative bacteria (Korda et. al, 1997; Kanaly, Harayama, 2000). Chemotaxis is a selective preference for degrading bacteria to occupy contaminated areas (Pandey, Jain, 2002).

The greatest abundance of petroleum HDB was registered in microbiota of the digestive system of swollen river mussels and zebra mussels from the Curonian Lagoon. It should be noted that petroleum HDB dominated in the water of the Curonian Lagoon compared to THB and TCFB (Figure 3). A little smaller numbers of petroleum HDB were found in the digestive system of swan mussels. A comparison of these two bivalves shows that the digestive system of the swollen river mussel accumulates a greater amount of petroleum HDB (Figures 1, 2).

The abundance of the studied functional groups of bacteria in the digestive system of *Chirocephalus*

*josephinae* from Ilčiukai pond was found to be considerably lower compared to respective bacteriological data in molluscs from Lake Spėra and Curonian Lagoon nearby Juodkrantė. The abundance of petroleum HDB in the water of Lake Spėra was slightly lower than in the water of the Curonian Lagoon (Figures 1–3).

A comparative analysis of water bodies showed that the lowest numbers of TCFB and petroleum HDB were in the microbiota of Ilčiukai pond (Figure 3). The greatest numbers of petroleum HDB were detected in the water of the Curonian Lagoon, followed by the Lake Spėra (Figures 1, 2).

The environment itself constantly suffers from biodegradation processes intensively induced by different microorganisms. Such statement can be supported by numerous data. It was found that biodegradation processes in water and sediments are influenced by the amount of dissolved oxygen, temperature, medium pH, biogenic elements, salinity etc, which change in time. In addition, change of the chemical composition of hydrocarbons, change of the composition and structure of microbiota, new substances appearing as a result of vital activities of microorganisms influence the speed and quality of biodegradation of petroleum. Petroleum and its products get into the digestive system of aquatic organisms from the environment. The functional activity of petroleum HDB is related to the existence of petroleum hydrocarbons as a nutritional substrate and the only source of carbon and to participation of bacteria associated with hydrobionts in the process of self-purification (Šyvokienė et al, 2004; Bairagi, Balcazar, 2008).

A comparison of the percentage of petroleum HDB in THB of microbiota of the digestive system of swollen river mussels, zebra mussels, swan mussels and *Chirocephalus josephinae* showed that the percentage of petroleum HDB was the greatest (21.53%) in the microbiota of the digestive system of swollen river mussels from the Curonian Lagoon compared to respective percentages in the digestive systems of other investigated aquatic organisms.

According to the values of abundance of petroleum HDB and TCFB we can state that the water ecosystem of Ilčiukai pond is the least contaminated with petroleum, its products and sewage water, and the Curonian Lagoon water ecosystem is the most contaminated.

Results of the current study demonstrate that analysis of fish digestive tract bacteria disturbances could be used in environmental monitoring programmes for the evaluation of distribution of toxic compounds in water ecosystems and for ecotoxicological risk assessment in the petroleum and its products pollution zones.

Based on literature data and results of the present study we can state that the abundance of petroleum hydrocarbon-degrading bacteria could be used as a bioindicator reflecting the level of water pollution of an ecosystem.

#### 4. Conclusions

A regularity showing the dependence of the abundance of autochthonous and allochthonous bacterioflora in the digestive system of aquatic organisms upon the species of the organism and the environment was established. The abundance of total heterotrophic and proteolytic bacteria in microbiota of the digestive system of the studied bivalve molluscs (swan mussel and swollen river mussel), zebra mussel and an anostracan – *Chirocephalus josephinae* was greater than the abundance of coliform bacteria and hydrocarbon-degrading bacteria.

The greatest value of abundance of TCFB and petroleum HDB was detected in the digestive system of swollen river mussels and zebra mussels from the Curonian Lagoon and in the water of the lagoon, compared aquatic organisms with from Lake Spėra and the Ilčiukai pond and the water of these water bodies.

The greatest percentage of HDB between THB was registered in the digestive system of swollen river mussels (21.53%) and zebra mussels (19.99%) caught in the Curonian Lagoon and in the water of the lagoon (24%). A considerably lower percentage of HDB among THB was detected in the digestive system of swan mussels from Lake Spėra (17.6%) and in the water of the lake (16.66%). The lowest respective proportion was registered in the digestive system of *Chirocephalus josephinae* (6.63%) and in the water of Ilčiukai pond (2.72%).

According to the values of abundance of petroleum HDB and TCFB we can state that the water ecosystem of Ilčiukai pond was the least contaminated with petroleum, its products and sewage water, whereas the Curonian Lagoon water ecosystem was the most contaminated. The abundance of petroleum hydrocarbon-degrading bacteria could be used as a bioindicator reflecting the level of water pollution of the ecosystem.

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## Naftą skaidančios bakterijos, susietos su vandens gyvūnų virškinimo sistema

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*(gauta 2013 m. rugsėjo mėn.; atiduota spaudai 2013 m. gruodžio mėn.)*

Atliekant laboratorinius tyrimus iširta 35 dvigeldžių moliuskų: didžiausia geldenės *Anodonta cygnea* (Linnaeus, 1758), surinktos iš Spėros ežero (Širvintų rajonas), pleštakiautės geldutės *Unio tumidus* (Philipson), 1788, *Dreisena polymorpha*, surinktos iš Kuršių marių ties Juodkrante, *anostracan species Chirocephalus josephinae* (Grube, 1853) tvenkinyje (Utenos rajonas) virškinimo sistemos ir biotopo vandens mikrobiotos gausa, funkcinių bakterijų grupių sudėtis.

Nustatytas dėsningumas, rodantis autochtoninės ir alochtoninės mikrobiotos gausos vandens gyvūnų virškinimo sistemoje priklausomybę nuo gyvūno rūšies ir gyvenamosios aplinkos. Tirtųjų dvigeldžių moliuskų, dreisenų ir *anostracan species* virškinimo sistemose bendrų heterotrofinių ir proteolitinių bakterijų rasta daugiau, lyginant šias funkcines bakterijų grupes su tirtosiomis žarnyno grupės ir angliavandenilius skaidančiomis bakterijomis. Pleštakiautės geldutės ir dreisenos, sugautų Kuršių mariose, virškinimo sistemoje ir vandenyje rasta daugiau bendrų žarnyno grupės ir naftos angliavandenilius skaidančių bakterijų negu Spėros ežero ir tvenkinio gyvūnų virškinimo sistemoje ir vandenyje. Didžiausias HDB/THB gausos santykis – 21,53 proc. – nustatytas pleštakiaučių geldučių, dreisenų – 19,99 proc., sugautų Kuršių mariose virškinimo sistemoje ir vandenyje – 24 proc. Gerokai mažesnis HDB/THB gausos santykis buvo nustatytas didžiosios geldenės iš Spėros ežero virškinimo sistemoje, kuris sudarė 17,6 proc. Spėros ežero vandenyje šis rodiklis sudarė 16,66 proc., o mažiausias šis santykis – 6,63 proc. – nustatytas *anostracan species* virškinimo sistemoje ir 2,72 proc. tvenkinio vandenyje. Naftos angliavandenilius skaidančios bakterijos kaip bioindikatoriai atspindi tiriamojo vandens telkinio užterštumo nafta ir jos produktais lygį.