Genotoxicants in marine and freshwater fish of the Barents Sea Basin

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Abstract. A comparative genotoxicity of marine and freshwater fish as indicators of the ecological hazard of the aquatic environment of the Barents Sea basin was analyzed. We used the Ames Salmonella/microsomes test to study mutagenicity of various tissues and liver fat of polar cod, sea bass, common whitefish and river bass with subsequent chromatography-mass spectrometry analysis of liver fat samples for the content of genotoxicants (organic pollutants). No significant excess of mutagenic activity was found in all fish samples except polar cod liver fat and sea bass liver fat. Promutagenic action caused gene mutations of both frameshifting and base pair substitution. Analysis of fat samples using the method of chromatography-mass spectrometry confirmed the presence of persistent organic pollutants in marine fish fat. The findings indicate that at present the ecology of the Barents Sea basin rivers is in more favorable conditions than the ecology of this sea.

1. Introduction

The aquatic environment comprises various ecosystems, such as rivers, lakes, oceans and seas. Impacts of industrial effluents on the aquatic environment are inevitable [1]. These impacts have occurred and still occur in both marine and freshwater environments, and, depending on the proportions of pollutants, may remain, causing ecological disturbances for long periods.

The Barents Sea remains highly natural and one of the last large and relatively intact marine ecosystems. At the same time, it is the most accessible region of the Arctic, and human activities are expanding rapidly. The basin contains one of the largest fish stocks in Europe.

Once pollutants are dumped into the aquatic environment, they are distributed and interact with the biota according to its features and to the conditions of the receiving environment, being subject to chemical, physical and biological transformations. Before the pollution reaches a destructive level, the organisms inhabiting the contaminated environments will initially respond with alterations at biochemical, cellular, physiological, and morphological levels. Fish as bioindicators are excellent experimental models for aquatic toxicology research and environmental biomonitoring because they are sensitive to chemicals and can inform about the possibility of human exposure to genotoxic substances in water.

Fish accumulate pollutants directly from contaminated water or indirectly through ingestion of contaminated food; fish also react sensitively to environmental changes or stressors in the form of low
concentrations of genotoxic substances present in the environment. In addition, these organisms respond to toxic agents in a similar way to higher vertebrates, allowing a better assessment of substances that are potentially teratogenic, mutagenic and carcinogenic to humans.

In aquatic ecosystems, pollutants originating from industrial activities, transport, and other human activities enter the sea through river inflow. Modern programs of environmental monitoring pay much attention to obtaining data using biotesting systems, including genetic ones. The mutagenic potential and genotoxicity of compounds are usually determined with the help of the Ames test [2] or its modifications. The test is considered an effective integral bioindicator of objects contamination by carcinogens and mutagens entering them from various sources [3]. It is equally important, in case of positive results of the conducted genetic test, to try to identify specific sources (carriers) of mutagenicity, which can be done using modern methods of chemical analysis [4].

The study was aimed at comparison of mutagenicity levels in sea and freshwater fish from the Barents Sea basin with subsequent analysis of samples of these fish for the content of ecotoxicants (genotoxicants, organic pollutants, xenobiotics) using chromatography-mass spectrometry.

2. Materials and methods

Marine fish: Polar cod (Boreogadus saida) and Sea bass (Sebastes glaucus) were caught in the fishing areas of the Barents Sea in summer 2019. Freshwater fish of the Barents Sea Basin: common whitefish (Coregonus lavaretus) and river bass (Perca fluviatilis) were caught in the Indiga and Pechora rivers in the same period of time.

Sampling and preparation of the samples for analysis were performed in accordance with the methodological guidelines of the FAO, ICES (International Council for the Exploration of the Sea) and other applicable regulative documents.

Analysis of mutagenic activity. The study samples were liver and muscle tissues of marine fish and fat isolated from fish liver. Fat was isolated using the traditional heat method at 90 °C. The mutagenicity of fish tissue extracts was determined using a modified semi-quantitative Ames Salmonella/microsomes test with a metabolic activation system based on the S9 microsomal fraction from rat liver induced by a solution of Aroclor 1254 [5].

To obtain extracts, 5 g of each sample was extracted three times with fivefold volume of methyl chloride. The extracts were evaporated dry in a film evaporator, then dissolved in 5 ml of dimethyl sulfoxide (DMSO). To each Petri dish, 100 μl of the test sample extract was added along with 100 μl of DMSO. As a total control, 100 μl DMSO per plate was used without sample extract. As a positive control, the effect of 2-aminoanthracene promutagen (0.5 μg per dish) was evaluated. The general control was tested on both strains in pentaplicate, the remaining samples were tested in triplicate.

Indicator strains were histidine-auxotrophic Salmonella typhimurium TA-98 and TA-100. Strain TA-98 records frameshifting mutations, and TA-100 - base pair substitution mutations. Mutagenicity was judged by the frequency of reversions to histidine (His+) prototrophy, detected on plates with minimal medium. The experiments without metabolic activation (-MA) were conducted to evaluate the direct mutagenic effect of the studied extract. The experiments with metabolic activation (+MA) were conducted to identify the mutagenicity of the products (promutagenic effect) formed during the biotransformation of the extracts.

The results of the experiments are presented as effects of solvent (DMSO), 2-aminoanthracene, tissue extracts and fat. An increase in the number of colonies of his+ Salmonella revertants compared with the control indicates the presence of a mutagenic effect. The final result (table 1) are the values expressed as the ratio of the number of his+ revertants of Salmonella colonies grown in the presence of the test sample to those in the control tests. If the ratio value is close to 2 (1,7-2,0), then the mutagenic effect is usually considered slightly positive. If the test-to-control ratio is exceeded 5 or more times, the mutagenic effect is average.

The data were statistically processed using software package Statistica 10.0 (StatSoft Inc., USA). The reliability of the deviation of the number of colonies in the experiment with a two-fold or higher mutagenic index compared with the control corresponded to the probability p <0.01.
Identification of organic compounds in marine fish fat was made by gas chromatography-mass spectrometry. Sample preparation: 2.5 g of liver fat was dissolved in 5 ml of methylene chloride and applied to a column filled with silica gel, eluted with 40 ml of hexane, then with 30 ml of methylene chloride. The eluates were combined, and concentrated to a volume of 1 ml. The analysis of organic compounds was carried out using a time-of-flight mass spectrometry with a Pegasus 4D electron ionization spectrometer (LECO, USA) [6]. The ionization energy was 70 eV, the scanned masses were 29–500 Da. For gas chromatography, a 0.25 μm thick and 30 m long capillary silicone column SPB-5 was used; the applied temperature mode was as follows: 40 °C (4 min.) – 10 °C (1 min.) – 300 °C (10 min). 1 μl of each sample was introduced into the splitless chromatograph injector at a temperature of 270 °C. Perdeuterized naphthalene, phenanthrene and chrysene were used as internal standards. Identification of the substances to be determined was carried out by comparing the obtained mass spectra with the reference mass spectra of the NIST14 library. Their evaluation was performed by the method of peak areas normalization using an automatic data processing system.

3. Results and discussion
The results of the mutagenic activity analysis of the liver and muscle tissues of marine fish and fish fat are presented in table 1.

Table 1. The Ames test of fish tissues and liver fat.

| Sample                        | Salmonella strains |
|-------------------------------|--------------------|
|                               | TA-98 (+ MA) | TA-98 (- MA) | TA-100 (+ MA) | TA-100 (- MA) |
| DMSO                          | 1.0           | 1.0           | 1.0           | 1.0           |
| 2-aminoanthracene             | 13.0          | 2.1           | 7.1           | 2.8           |
| Polar cod liver               | 1.8           | 1.3           | 1.6           | 1.2           |
| Polar cod muscle tissue       | 1.1           | 0.9           | 1.2           | 1.1           |
| Sea bass liver                | 1.9           | 1.4           | 1.6           | 1.3           |
| Sea bass muscle tissue        | 1.2           | 1.0           | 1.2           | 1.2           |
| Polar cod liver fat           | 6.7           | 1.1           | 4.4           | 1.9           |
| Sea bass liver fat            | 3.9           | 0.9           | 4.1           | 1.3           |
| Common whitefish liver fat    | 2.1           | 1.9           | 2.2           | 1.2           |
| River bass liver fat          | 1.1           | 1.3           | 1.9           | 0.9           |

*The table shows the average data of three experiments in the form of the ratio of the number of colonies per plate in the experiment to control (DMSO). +MA - presence, -MA - absence of metabolic activation. A significant excess of the number of revertant colonies over control p<0.01 is underlined.

No significant excess of mutagenic activity over control was found in all fish samples except polar cod liver fat and sea bass liver fat. As table 1 presents, all extracts of the marine liver fat showed genotoxicity. In this case, the mutagenic effect was manifested both on the TA 100 strain and on the TA 98 strain, which indicates the presence of mutagens causing mutations such as frameshifting and pair base substitution. The mutagenic effect was manifested only in the presence of the S9 fraction (Aroclor 1254-induced liver metabolic activation system).

Thus, the samples of sea fish fat contained promutagenic compounds - genotoxicants. the mutagenic effect of which manifests itself after metabolic activation in the cytochrome P-450 system. Possibly, these are polycyclic aromatic hydrocarbons (PAHs), as well as some other organochlorine compounds [7].

Therefore, at the next stage of our research, samples with mutagenic activity and river bass liver fat (for control) were studied using chromatography-mass spectrometry to identify possible sources (carriers) of genotoxicity. Table 2 shows the average content of persistent organic pollutants in the studied samples.
Table 2. Persistent pollutants found in marine and freshwater fish fat.

| Compound                        | The content of detected compounds (µg/kg) in fish fat |  |
|---------------------------------|-----------------------------------------------------|---|
|                                 | Polar cod liver fat | River bass liver fat | Sea bass liver fat |
| DDT                             | 5.4                  | 4.8                  | 2.4               |
| DDE                             | 43.8                 | 9.7                  | 28.5              |
| DDD                             | 9.9                  | 8.1                  | 3.9               |
| Naphathalene (PAH)              | 91.3                 | 4.1                  | 72.3              |
| Dimethylnaphthalene (PAH)       | 36.9                 | 2.1                  | 31.5              |
| Methylnaphthalene (PAH)         | 13.4                 | 0.9                  | 15.6              |
| Dihydronaphthalenes (PAH)       | 957.0                | 18.9                 | 827.1             |
| Phenanthrene (PAH)              | 69.7                 | 11.2                 | 99.1              |
| Dibutyl phthalate               | 401.6                | 14.9                 | 299.1             |
| Naphthenes                      | 3112.2               | 31.5                 | 2415.2            |
| Alkylbenzenes                   | 3565.1               | 37.9                 | 4419.4            |
| Trichlorobiphenyl (3 isomers)   | 5.9                  | 2.0                  | 12.5              |
| Tetrachlorobiphenyl (8 isomers) | 21.1                 | 1.2                  | 18.4              |
| Pentachlorobiphenyl (7 isomers) | 23.8                 | 2.1                  | 19.6              |
| Hexachlorobiphenyl (7 isomers)  | 17.9                 | 1.4                  | 16.7              |

As follows from table 2, the organic contaminants were found in all samples of fat (isomers of tri-, tetra-, penta- and hexachlorobiphenyls; polycyclic aromatic hydrocarbons; alkylbenzenes; naphthenes), but their content was much lower in river fish than in sea fish. However, the pesticide DDT and its metabolites – DDE and DDD is found in freshwater fish at about the same level as in marine fish. DDT is a persistent biocide and it is toxic for fishes. Its bioaccumulation comes mainly from its content in water. Note that DDT and its metabolites. the genotoxicity of which has been proven by a number of researchers [8], may be responsible for the mutagenic effect in the Ames test. Thus, it indicates that at present the ecology of the Barents Sea basin rivers is in more favorable conditions than the ecology of the sea.

Fats of both types of marine fish are quite similar in content of persistent toxicants. It can be assumed that the metabolites of DDT. PAHs and, possibly, nitrotoluene are responsible for the recorded promutagenic effects. Apparently, due to the increasing pollution of marine ecosystems with organic toxicants and xenobiotics, it seems appropriate to expand their list in the documents of food legislation. Particular attention should be paid to compounds that are included in the list of priority eco-toxicants of the US Environmental Protection Agency [9]. They are anthracene, phenanthrene, nitrotoluene, dibutyl phthalate, and alkylbenzenes.

We may assume that PAHs enter aquatic ecosystems with petroleum products and phthalates. commonly used as plasticizers, are the result of industrial pollution of water systems [10]. In addition to river runoff, hydrocarbons also enter the region through the Atlantic Current, as well as from ship spills and drilling operations. Petroleum development poses a major threat to the natural resources of the Barents Sea region [11].

Oil tanker accidents and illegal discharges of oil into the sea are widespread practices resulting in the Barents Sea an absorber of persistent organic pollutants. The methylnaphtalene have long term negative effects on aquatic environment [10]. Biphenyl polychlorides are very persistent. liposoluble. but less soluble in water. Their transfer happens from sediments to fish via food. Nevertheless. the observed phenomenon requires a further examination.
4. Conclusion
The obtained data on the mutagenicity of marine fish fats indicate the environmentally hazardous biotransformation of organic pollutants of artificial origin. Such effects are currently recognized as the most dangerous in terms of the distribution scale and the degree of impact on living organisms [12].

It should be noted that mass spectrometry does not allow detecting all possible compounds with potential mutagenic activity, since the analysis carried out on the basis of existing libraries of standards includes only about 80% of possible contaminants. Nevertheless, on the basis of the data obtained, it can be assumed that the metabolites of DDT, PAH and, possibly, nitrotoluene are responsible for the identified mutagenic effect.

Despite the fact that the concentrations of detected regulated toxicants do not exceed permissible levels, and the content of detected non-regulated toxicants is relatively low, the total amount of pollutants was sufficient to show the promutagenic effect of fats in the Ames test, which indicates the potential danger of long-term use of such products for humans.

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