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The role of bioindicators and the modern instrumental methodology of ^{1}H NMR applied in ecological forensic expertise

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Abstract. Bioindicators and NMR performance offer new possibilities for forensic examination, directly in nature, at the site of the incident/ecological impact aimed at quickly finding out the truth about the crime and the criminal. The advantage of using biotic parameters (bioindication) lies in their greater objectivity and validity. The state of the boita is determined by the general state of the environment and promptly reacts to negative actions from various sources.

Keywords: pollution, bioindicators, Nuclear Magnetic Resonance (NMR), ecological forensic expertise.

Rolul bioindicatorilor și metodei instrumentale moderne ^{1}H RMN aplicate în cadrul expertizelor judiciare ecologice

Rezumat. Bioindicatorii și performanța RMN oferă noi posibilități pentru examinarea criminalistică, direct în natură, la locul incidentului/impactului ecologic menite să asigure aflarea rapidă a adevărului cu privire la infracțiune şi infractor. Avantajul utilizării parametrilor biotici (bioindicaţia) constă în obiectivitatea și temeinicia lor mai mare. Starea boitei este determinată de starea generală a mediului și reacționează prompt la acțiunile negative de diverse proveniențe.

Cuvinte-cheie: poluare, bioindicatori, Rezonanță Magnetică Nucleară (RMN), expertiza judiciară ecologică.

1. Introduction

The disturbance of environmental factors has a direct effect on the evolution of living beings, initially on their ability to adapt and later on their ability to survive, constituting, in extreme cases, factors for the elimination of certain species from food webs with drastic consequences on the evolution of biodiversity at the local level and with impact at a general level. This desideratum determined the need to evaluate the water quality of an aquatic basin and anthropogenic changes in aquatic ecosystems by means of bioindicators and modern technologies [1].

The large number of chemical compounds found in environmental factors requires the use of specific methods of analysis with high selectivity, specificity and sensitivity. Due to the continuous increase in the number of chemical compounds released into environmental factors that can contribute to the alteration of its quality, the development of new analysis protocols for their identification and quantification, even at the ultra trace level, has become a priority. Thus, in this article we highlight the role of NMR analysis in the assessment of environmental quality and the results of research carried out in this regard [2].

Numerous species of organisms manifest themselves as natural bioindicators of ecosystems, responding to changes in the environment to the presence of pollutants through changes in vital functions, or which accumulate pollutants in their bodies. Lake pollution indicators can be differentiated into sensitive species, which indicate the presence of a pollutant by the appearance of lesions or malformations, and accumulating species, which concentrate the pollutant in their body [1].

Diatoms are of particular importance in environmental monitoring, as they are good indicators of organic pollution in the aquatic environment. The dynamic nature of an environment leads to the incorporation of natural and anthropogenic materials of diverse provenance, characterizing a site to such an extent that it becomes highly distinctive and useful for forensic comparisons [1,3].

Diatoms have been used in forensic pathology for the diagnosis of death by drowning and to reconstruct or estimate the submergence of the post mortem interval (PMSI) of a body recovered from water. The use of diatoms as a diagnostic test for vital drowning is based on the assumption that, as long as the blood circulation is functioning (suggesting that the person was alive), these microorganisms will be transported and deposited in organs some distance from the lungs. In the absence of blood circulation (the individual was dead when placed in the water) they will stop at the walls of the lung alveoli. The blood circulation generated post-mortem by the autolysis process does not have the power to transport the diatoms and fix them at the level of the organs [4].

The consensus opinion currently accepted by specialists is that, provided adequate precautions are taken to prevent contamination, the demonstration of the presence of diatoms in organs such as bone marrow, brain, kidney, liver, spleen is strong evidence confirming death by drowning. If the degradative processes in the corpse are very advanced, the error in the assessment of the diagnosis of drowning is very possible, since the contamination - so not the destruction of the diatoms already fixed by inhaling the water – is obvious. In order to establish the place of immersion (where the vital drowning practically occurred), it must be confirmed, by histological examination, if the species

of diatoms found in the organs of the deceased are of the same kind as those present in the water from which the body was removed. Demonstrating the presence of diatoms and/or other physical particles or chemical pollutants in certain organs of the human corpse removed from the water is of real use both in the investigation of homicide and the investigative version, by establishing the exact cause of death and by locating the submersion area [1,5].

NMR is a versatile technique that is capable of analyzing any analyte that has magnetically active nuclei such as ^{1}H or ^{13}C, as well as many heteronuclei (e.g. ^{19}F, ^{15}N, ^{31}P), a requirement that is met by almost all relevant compounds from an ecological point of view. NMR is not only useful for structure determination and compound identification, it can also be used to examine non-covalent interactions between wastewater components, such as pollutant-protein or pollutant-humic binding – which is very difficult to achieve using other techniques. The authors aimed at examining the use and potential of NMR application as a tool for understanding polluted water and wastewater treatment processes and effects. They mention that a deeper exploration of the various NMR techniques that have been and can be applied to the study of polluted waters is warranted. The mentioned authors aim at showing, that even a short case study can be convincing, that when different NMR characterization approaches are combined with NMR-based toxicity assessment, the result is a comprehensive understanding of both wastewater and its impacts, which cannot be matched by any other modern analytical approach [2,9].

NMR spectroscopy is undoubtedly the most powerful tool for the study of molecular structures and interactions and is increasingly applied to environmental research such as the study of wastewater. With over 97% of the planet's water being saltwater and two-thirds of fresh water frozen in ice sheets and glaciers, there is a significant need to maintain and reuse the remaining 1%, which is a precious resource critical to the sustainability of the most life on Earth. The sanitation and reuse of wastewater is an important method of water conservation, especially in arid regions, making the understanding of wastewater itself and its treatment processes a highly relevant area of environmental research. Here, the benefits, challenges, and subtleties of using NMR spectroscopy for wastewater analysis are considerable [5, 7].

The study demonstrates that, when applied comprehensively, NMR can provide unique insights into not only the structure, but also the potential impacts of wastewater and wastewater treatment processes. Finally, low-field NMR, which holds considerable future potential for on-site wastewater monitoring, is briefly discussed [7].

NMR spectroscopy is one of the most versatile tools in modern science, with abilities to study all phases (gases, liquids, gels, and solids), chemical structures, interactions,

interfaces, toxicity, and more. The authors hope that this analysis will inspire more scientists to adopt NMR, given its huge potential for both wastewater analysis in particular and environmental research in general [2].

Experimental research studies are capitalized with the results which determine the presence and abundance of a range of environmental indicators and guide subsequent strategy for the collection and analysis of a more in-depth forensic sample. Evidence from the aquatic environment is recognized as having the potential to contribute valuable circumstantial information regarding a particular crime: ecological (anthropogenic impact), death by drowning (accidental, suicidal, criminal) or a murder by other means, later concealed by drowning. This paper presents current applications of limnology, particularly algae and diatom analysis to introduce new and ongoing research into ecological forensics and forensics itself [3].

2. Metods and Materials

The water samples were collected from Valea Morilor Lake in Chisinau municipality seasonally during the years 2021-2023 according to the unified methods of collection and processing of field and experimental hydrobiological samples. Algae species were identified with the Optica B-510 POL microscope equipped with a digital camera, species determination according to the determinants in force and the literature in the field. The saprobiological analysis was based on the list of water quality indicator algae species [4], consulting specialists from the academic environment. In forensic expertise, chemists apply a set of approved methodologies, procedures and instructions, using calibrated measuring equipment and analytical techniques subject to strict quality control [4,8,9].

During the research, various methods were applied to highlight the degree of pollution of Valea Morilor lake in Chisinau municipality: chemical, biological, ecological and forensic. The versatility of NMR spectroscopy has led to the development and implementation of various types of NMR techniques, intended for the examination of the structure of various types of environmental samples, living and non-living, as well as the study of critical processes in the aquatic environment. Fixing, lifting, transporting and preserving traces of a biological/chemical nature according to methodical and forensic recommendations meet the conditions to be admitted as evidence in solving environmental crimes. From an applied point of view, forensics is inextricably linked to different fields, such as chemistry, biology, physics, anatomy, etc. Due to crimes, forensics requires a series of modern technical-scientific means and methods specially adapted and perfected to meet the needs of the criminal investigation process. Chemistry makes forensics available, some of which allow specialists and experts in this field to go beyond the classical sphere

by continuously processing new discoveries and adapting them to the methods and means specific to forensic chemistry.

The water samples from the Valea Morilor lake in Chisinau municipality were taken at the end of October 2021 from three distinct sectors of the lake, with different accumulation of green algae, as the given research is part of a larger prospective study, also aiming involvement of algae in the ecobiological indication of water quality [3]. Valuing evidence by applying logic, scientific and forensic methods is the basic requirement in conducting ecological forensic expertise. Since the evidence collected in the case of a crime has an inhomogeneous character or a different chemical composition, they require various high-performance technical means and new examination methods. The veracity of the conclusions depends on the appropriate research methods and techniques, and modern high-performance technologies ensure the examination of the small volume of the presented material with high certainty. The amount of evidence in forensic chemical analysis is often at the level of microtraces/microparticles. In some perishable cases, for this reason, it requires fast (express) chemical methods of investigation and automated processing of the obtained data.

To perform the ^{1}H NMR experiments, the water samples were diluted with 10% D2O (v/v) (99.9%, Sigma-Aldrich), being subsequently transferred directly into the NMR vial (5 mm). The measurements were made at 25°C on Bruker AVANCE III 400 NMR spectrometer, 400 MHz operating frequency, equipped with 5 mm PABBO BB-1H/D Z-GRD Z108618/0071 sample head.

To suppress the water resonance, the pulse sequence "Water suppression using 3-9-19 pulse sequence with gradients" (eng.) was used. Direct ^{1}H NMR analysis of DOM took one hour per sample. This acquisition time of the ^{1}H spectrum, which involved the recording of 1024 scans, allowed the qualitative functional analysis study of DOM to be performed. After recording, the NMR spectra were subjected to Fourier transformation, using LB 0.3 Hz (LB- line broadening, eng) and the usual procedures of automatic spectrum post-processing (including baseline and phase adjustment). The ^{1}H NMR spectra of DOM in all analyzed samples demonstrated the most numerous resonances in the typical region for unsubstituted aliphatic hydrocarbons (0.5-2.05 ppm). Signals characteristic of protons adjacent to functional groups, such as carbonyl, ester or amide in the 2.1-3.10 ppm region of the ^{1}H spectra are missing, but instead, resonances are present in the 3.1-3.90 ppm region, which identify C-H bonded to the oxygen atom, i.e. hydrogen from carbohydrates [2,8,9].

3. Obtained Results and Discussion

Currently, a large amount of waste, plastic, vegetable remnants has accumulated in Valea Morilor lake, among which fish die daily. Dozens of dead fish can be seen floating on the surface of the lake. Given the high temperature that is favourable for the development of bacteria and algae, this fact has led to the so-called "blooming" of the water [5,6]. Phosphorus and nitrogen concentrations also contributed to this. Phosphorus and nitrogen reach the lake water through the small streams, including the Durlești stream. The big danger now appears as a consequence of last year blue-green algae death because when they decompose, they absorb the oxygen that is in the water and release toxins that are harmful to other aquatic life, also causing fish to die [6].

According to the specialists from the Environment Agency, the exact cause of the death of the fish is not known yet. Over the course of several years at the same time, in their opinion, this phenomenon occurs because the water is not sufficiently oxygenated. The O_2 share in the water varies from 85% to 140%, compared to the saturation norm, being maximum in the summer period, together with the massive development of algal vegetation.

Water mineralization oscillates between 700 and 1070 mg/l. The water of the lake belongs to the hydrocarbonate-sulfate-chlorine and hydrocarbonate-chlorine-sulfate category from the Mg+K and Mg+Na groups; the pH is slightly alkaline. To solve the problem, the municipal authorities proposed to procure 25 additional aerators, to activate in addition to the 13 already existing. Later, the specialists took samples that proved that there is insufficient oxygen in the water, which leads to the death of the fish.

Eutrophication contributes to the overgrowth of various species of algae in water bodies. The presence of an increased amount of nutrients in the water contributes to the intensification of the process of photosynthesis and cell division, which ensures the exaggerated reproduction of algae. Water pollution occurs due to a large concentration of chemical or biological waste that reaches the lake. They are dangerous, posing a great threat to human health. Some of the most common problems faced by fish species are: insufficient oxygen and organic pollution, thermal pollution, pollution with persistent synthetic compounds, etc.

In order to obtain a more complete picture regarding the state of water quality, the assessment was also extended to the biological components that can store information at a structural and functional level, in time and space, etc. The ecobiological indication of water quality allows for the express assessment of the level of pollution of the aquatic environment, presenting an advantage for forensics in the framework of ecological forensic

expertise that requires a quick assessment. The rapid assessment of the quality of the aquatic environment is becoming more and more effective and current.

The dominant composition of algae species differs in different research periods. The development of the phytoplankton of the Valea Morilor lake reaches maximum levels in spring and summer. In the winter period, the phytoplankton is very poor and contains mostly diatom algae. The diversity of phytoplankton species varies under the influence of the hydrological regime, climatic conditions and the degree of significant water pollution.

Based on these data we tended to highlight the role of modern technologies (NMR) and bioindicators in the assessment of anthropogenic impact in highly polluted areas with application of data in forensic expertise. As a result of the examination of the samples, the strongest signals in the obtained spectra are those of the solvent (waste water) in the region 4.40-5.40 ppm. In the spectrum, you'll notice that there are no signals in the range of 5.60-6.10 ppm, which is characteristic of derivatives containing alkenyl groups. However, in the region corresponding to aromatic protons at 8.30 ppm, you will observe the respective signals.

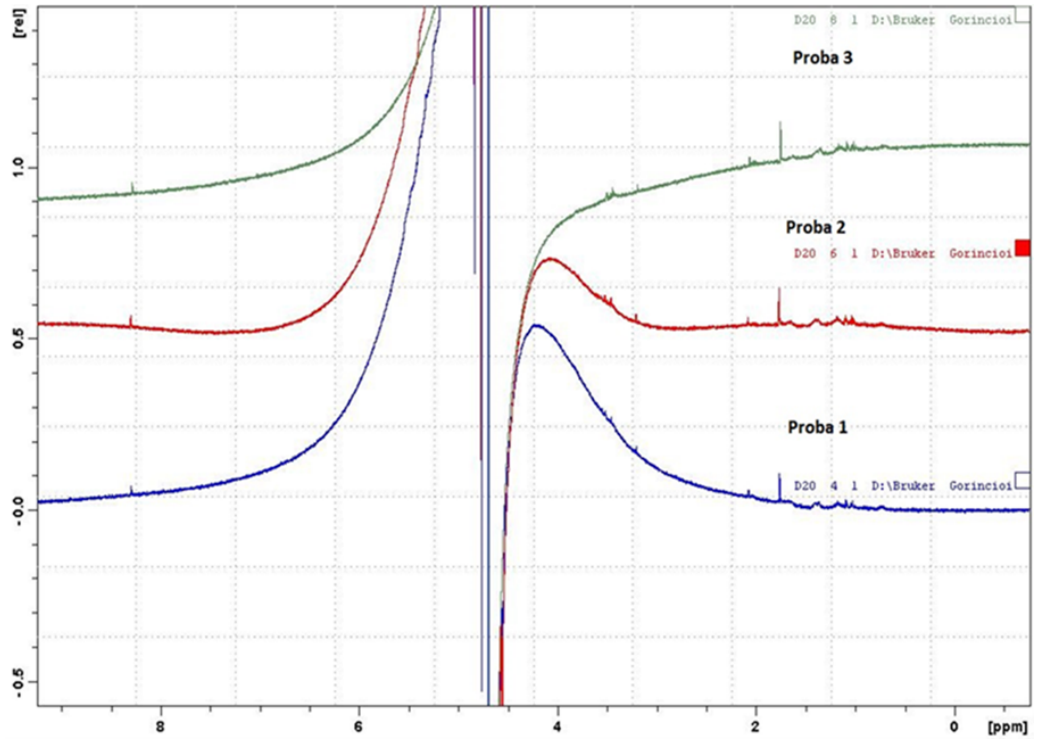

Figure 1. ^{1}H NMR spectra of DOM in water samples taken at the end of October, 2022 from Valea Morilor lake in Chisinau municipality.

Analyzing the water sample from Valea Morilor lake in Chisinau municipality, which was taken in June 2022 from the area with many rotting organisms (dead fish), in the ^{1}H NMR spectrum, the same groups of signals were found at a qualitative level, as it was mentioned above for the samples taken in autumn Fig. 1. The signals characterizing the spectrum area typical for unsubstituted aliphatic hydrocarbons (0.5-2.05 ppm) include the signal at 1.76 ppm apparently more intense, which would be favorable to a higher concentration of these compounds in this sample. Based on these data, it can be concluded about the composition of DOM in the warm period of the year, as the result of both water eutrophication due to algae (unsubstituted aliphatic hydrocarbons, carbohydrates) and, probably, of anthropogenic pollution (aromatic compounds). In the discussed sample, the signal was attested at 0.012 ppm, which can be attributed to organometallic compounds, probably water pollutants. Also, the typical area for aromatic compounds is richer in signals: signals at 6.45, 6.96, 7.10, 7.23 and 8.29 ppm are attested, which probably speaks of a more extensive pollution (although the signals are of low intensity).

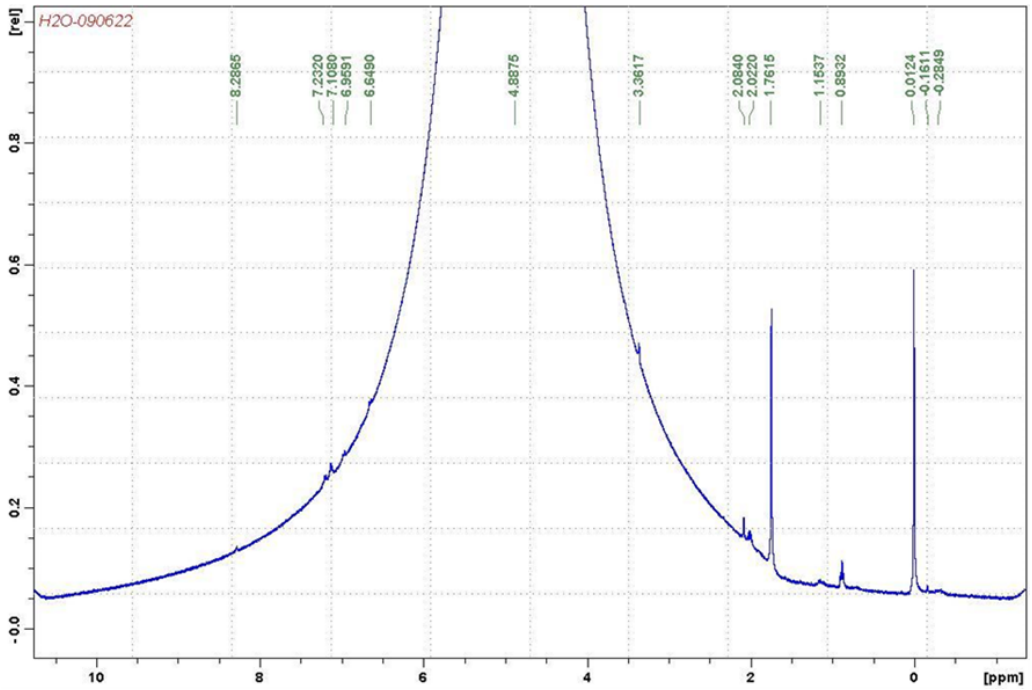

Figure 2. The ^{1}H NMR spectrum of DOM in the water sample taken on June 9, 2022, from the Valea Morilor lake in Chisinau municipality.

It is interesting to compare the ^{1}H NMR spectrum of DOM in the water sample from the Valea Morilor lake in Chisinau municipality, taken on June 9, 2022, with that of DOM

spectra in the water samples taken on March 27, 2023, i.e. during the cold period (Fig. 2). The spectra were recorded with the same NMR experimental parameters set, practically demonstrating the absence of DOM in the analyzed water, i.e. confirming the absence of eutrophication in the cold period of the year and the absence of pollution.

Ensuring the validation of the obtained results, we resorted to the use of scientific materials from the country and abroad, the creation and interpretation of diagrams according to data from the literature in the field [9].

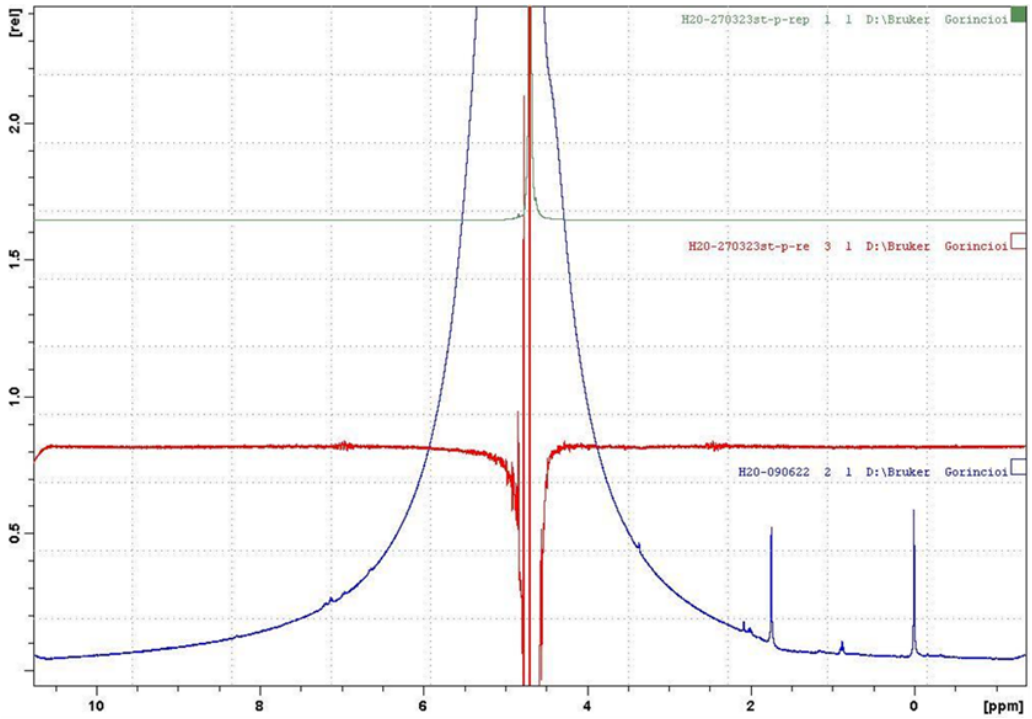

Figure 3. ^{1}H NMR spectra of DOM in water samples taken at the end of March, 2023 (red, green), June 9, 2022 (blue), from Valea Morilor lake in Chisinau municipality.

The samples were examined during the sampling period, using the NMR method for the detection and measurement of substances. In addition to these, the research activity was guided by the recommendations offered by international research with reference to modern technologies and chemical forensic expertise. The research results demonstrate that separation techniques and advanced NMR techniques are indispensable in environmental studies. According to the results obtained in the research carried out, the environmental factors are polluted/contaminated with a varied range of chemical compounds that create a permanent chemical stress on the environment.

The samples taken in different periods of the years of studies capable of determining the chemical compounds were examined, thus creating the premises for a more detailed assessment of the chemical stress to which the Valea Morilor lake hydroecosystem is subjected. The use of biomonitors and the application of specific methods to identify pollution sources together with NMR analysis made it possible to determine the sources of pollution of environmental factors and obtain useful information for the authorities responsible for water quality management.

The development of this method allows the introduction of a new direction that we want to apply in forensics in the case of pollution of watercourses based on persistent organic pollutants in water samples or sediments. We believe that this direction of research is welcome, considering the fact that, at the international level, it is widely applied in the ecological assessment of the environment.

Databases can be built with the NMR spectra of DOM, which can later be used in the authentication of unknown samples. NMR spectroscopy plays an essential role in understanding the nature of different types of environmental components and associated processes, including the different forms of organic matter found in soil, water and air, and is used to elucidate the state of water, organics, pollutants and metals in the environment.

4. Conclusion

Experimental research studies by evaluating algae as biological monitors capitalize through this work on the application of limnology, especially diatoms, to introduce new and ongoing research in ecological and forensic forensics.

DOM research in natural waters with the application of ^{1}H NMR spectroscopy is pioneering in the Republic of Moldova. The use of the ^{1}H NMR technique allowed the determination of the organic matter dissolved in the waters of Valea Morilor lake in the Chisinau municipality, which consists of a complex mixture of aliphatic and carbohydrate structures, with a minor contribution of aromatic compounds. NMR provides a very large amount of information on the chemical composition of water in a relatively short time and with minimal sample preparation.

The results of this initial qualitative functional analysis study are to be completed by quantitative analysis studies, based on the same method and by comparison with the currently applied and validated methods.

The methodological assurance of the actions of the ecological judicial expert in the examination of aquatic ecosystems is a good ground of conduct in the case of ecological damage, and the compensation of the damage from the polluter is the right of everyone not to be deprived of the normal state of the environment in which they live.

The theoretical-practical significance of the paper consists in the proposal and application of the modern instrumental method ^{1}H NMR in forensics. Nuclear Magnetic Resonance Spectroscopy (NMR) and the opportunities offered by this analytical method to apply measurement data at both molecular and macroscopic scales facilitate its rapid advancement in environmental science studies. The obtained results provide evidence of how the bioindicators respond to changes and react to pollutants in the aquatic environment. The ecological damage that affects the ecosystem, man and his goods, constitutes the first and most important stage of the process of perfecting the normative framework in the field.

The complex of analysis methods applied in carrying out chemical-forensic expertise by licensed forensic experts, with the use of advanced technologies and equipment, with the application of approved procedures and instructions, as well as with ensuring the quality control of the results, make this type of expertise a source of valid results, with high precision, scientifically substantiated and correlated with the factual data of the criminal prosecution.

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