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BIOMONITORING OF ENVIRONMENTAL POLLUTANTS IN MARINE ECOSYSTEMS BY BIOLUMINESCENT BACTERIA

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Abstract

Bioluminescence is the process of creating biological light within the cell as a result of a chemical reaction in living organisms. In addition to being a visual feast in nature, the change in the amount of biological light created against various agents also leads to the formation of many application areas in terms of biotechnology. One of the areas where the application is carried out is marine ecosystems. Because the negative consequences of the increasing human population are the increase in pollutants from both industrial and anthropogenic sources. For this reason, in such habitats, it is necessary to detect pollutants with environmentally friendly, easy-to- apply, repeatable and sensitive bioassays and to take both pollution prevention and pollution removal measures. Considering the increasing environmental pollution, it is necessary to regularly perform biomonitoring in all ecosystems with innovative technologies, with the awareness that the earth can be without us but we cannot be without the earth.

Keywords: *bioluminescence; biomonitoring; marine ecosystem; pollutant*

What is bioluminescence?

Bioluminescence, the production and emission of light by living organisms as a result of a chemical reaction, is observed in many organisms, including fish, insects, medusae, dinoflagellates, fungi, squids, and bacteria [1]. While higher organisms such as insects and medusae produce light only in the form of intermittent flashes or flashes, the light produced continuously at a wavelength of 490 nm in bioluminescent bacteria demonstrates the uniqueness of the light produced by bacteria [2]. Bioluminescent bacteria are widespread in marine environments and are rarely found in freshwater, brackish water, and soil environments [3]. Bioluminescent bacteria have a wide distribution in a wide range of ecological niches (fish light organs, mammalian intestines and nematode intestines) and habitats (sea, freshwater, terrestrial areas and symbiotic relationships with the host) and are found in high abundance [4]. When we look at the sources of bioluminescent bacteria in marine environments, seawater and sediments are the first to be encountered [5]. Of course, in marine areas, they are found not only in shallow coastal areas but also in deep pelagic regions [6]. They can live freely in marine habitats, as well as with various living and non-living marine organisms, and can colonize marine animals as

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saprophytes, commensals and parasites [7]. When they live freely in seawater, their numbers are very low (0.01-40 cells/mL). When they colonize specialized organs of some living beings, their numbers reach quite high numbers (10^6 -10^9 cfu/g) [6]. In particular, certain bioluminescent species enter into species-specific symbiotic relationships with various marine fish and squids and reside in highly specialized light organs [8]. Due to this bacterial bioluminescence, many marine fish species acquire the bioluminescent property [1]. Recently, 4 naturally luminescent bacterial genera have been identified: *Vibrio, Photobacterium, Shewanella* and *Photorhabdus*. All identified bioluminescent bacteria belong to the class Gammaproteobacteria and 15 bioluminescent marine bacteria have been identified so far. Of these, 9 are *Vibrio* (*V. cholerae*, *V. fischeri*, *V. harveyi*, *V. logei*, *V. mediterranei*, *V. orientalis*, *V. splendidus* (biotype I), *V. vulnificus* and *V. salmonicida*) species and 4 are *Photobacterium* (*P. angustum, P. leiognathi*, *P. phosphoreum* and *P. mandapamensis*) species and these species belong to the Vibrionaceae family. The remaining 2 bioluminescent species belong to the *Shewanella* (*S. hanedai* and *S. woodyi*) species which belong to the Alteromo- nadaceae family. All of these species are Gram-negative, rod-shaped chemoorganotrophic bacteria. They do not form spores and can move with their polar flagella. All species of Vibrionaceae family are facultative anaerobic, but 2 *Shewanella* species are strictly aerobic bacteria [6]. In the studies conducted in 2007 and 2009, 5 new species were added to the 15 bioluminescent marine bacterial species. In the study conducted by Ast et al. in 2007, bioluminescent bacteria that had a symbiotic relationship with deep-sea fish were isolated and as a result of phylogenetic, genomic and taxonomic analyses, these isolates were found to be included in *Photobacterium* but were a new species and were named *P. kishitanii* [9]. Again, a new *Photobacterium* species was isolated and identified from the Sagami Bay water in Japan by another study group and named *P. aquimaris* [10]. Again, as a result of various studies conducted by Yoshizawa et al. in 2009 and 2010, 2 new bioluminescent *Vibrio* species were identified. Bioluminescent bacteria were isolated from various seawater samples at different times by this group, and many phylogenetic analyses showed that these strains were new *Vibrio* species as a result of DNA-DNA similarities and were named as *V. azureus* and *V. sagamiensis* [11, 12].

In addition to these *Vibrio* species, *V. chagasii*, which was not reported to be bioluminescent when first identified but was determined to contain *lux* genes and to be bioluminescent as a result of the study conducted by Urbanczyk et al. in 2008, is also included in the new bioluminescent species [13]. In addition, bacteria that is not bioluminescent but is new *Vibrio* species have been identified and named. At the same time, species that were previously classified under non-bioluminescent species have also started to be reported as a result of the studies conducted. In bioluminescent bacteria, the question of their evolutionary origin has gained a new focus recently. Because *V. fischeri* members have been reclassified under a new genus (*Aliivibrio*). The taxonomy of luminescent bacteria, their evolutionary relationships and origins are frequently reviewed to better define them. The distribution of bioluminescent species among bacteria has still not been fully clarified. All species of the terrestrial genus *Photorhabdus* are bioluminescent, but marine genera that include bioluminescent species (*Aliivibrio*, *Photobacterium*, *Shewanella* and *Vibrio*) also include many non-bioluminescent and closely related species [14]. As is known, the sea is a habitat that preserves its mystery and about which very little is known. In addition, when it is considered that it is a habitat where many living creatures live, its mystery increases even more. Such reasons direct researchers to this habitat. As a result of research conducted in various seas, marine bioluminescent bacteria have been isolated and identified [15]. Bioluminescent bacteria use approximately 20% of their cellular energy to carry out this process. When we start from this point, it becomes clear that bioluminescence cannot be just a visual event. Researchers who think like this have started to investigate the biological role of bioluminescence in recent years. In addition, how this phenomenon can be brought to life or can be brought to life has also begun to be examined. When we consider it from an evolutionary perspective, since these organisms have acquired this quality and this event exists in nature, there are definitely areas where this process can be reflected [15]. Bioluminescence is a visual feast that exists in nature, as well as having the potential to be used in industrial and biotechnological areas. Research conducted in this direction has shown that this process can be used in many areas such as medicine and the food sector. Cancer, which was once considered one of the worst diseases, can now be eliminated with bioluminescence without side effects. Again, through this process, it is possible to determine the microbial load in various areas and to get rid of various toxic wastes that are harmful to the environment. Along with having such various application areas, this process is also capable of explaining the origin of microorganisms, which is a subject that all researchers are curious about. Because the detoxification feature of bioluminescent bacteria from toxic O_2 derivatives has enabled these creatures to continue their lives while living in an anaerobic environment

and their living conditions are aerobic. In this context, bioluminescent bacteria appear to be representative prokaryotes [15]. Bioluminescence is of course not a process that can only be applied in industrial and biotechnological fields. It also has areas of use in artistic terms. If bioluminescent bacteria are cultivated to form the desired shapes or letters, the resulting writings or shapes can be exhibited in dark exhibition halls [15].

Overview of application areas of bioluminescence

Bioluminescent bacteria have been used frequently in biotechnological applications in recent years due to the advantages of their radiation being easily, rapidly and quantitatively measurable. Their use as detectors for the search for carcinogenic compounds or for monitoring general toxic activities constitutes a suitable example for these features [16]. Luciferase enzyme isolated from various bioluminescent bacteria has been used in many different types of bioassays. FMN and long-chain aldehydes are important compounds for the specific search and quantification of luciferase reaction components [16]. Depending on the developments in genetic engineering and molecular biology, genes encoding luciferase enzyme in bacteria have been cloned and with the development of these cloned lux systems, it has been possible to act as "reporters" for specific promoters in measuring the activity of other genes [16]. Bioluminescence has a wide range of applications in medicine and the food sector. In medicine, lipopolysaccharide assay has areas of use such as determination of albumin binding capacity, imaging of 70 psychopharmacological substances, diagnosis of dental diseases, diagnosis and treatment of cancer, effectiveness of drugs and as a biosensor [17]. Bioluminescence also has a wide range of applications in the food sector. It is used for determination of microbial load, quality control of products, raw materials and water, determination of contamination, hygienic monitoring, sanitation control, determination of shelf life and determination of compliance with HACCP standards [17]. The microbial quality of raw milk can be determined by using the ATP bioluminescence method [18]. Again, microbial contamination in dead poultry can be detected by using rapid microbial ATP bioluminescence assays [19]. Another study that can be done with ATP bioluminescence technology is to determine the total living biomass. All living organisms contain and use ATP, their primary source of energy. When cells die, they lose most of their ATP. The amount of ATP in a living cell is directly proportional to its volume. There is a correlation between the amount of cellular ATP and the mass and number of cells. Bioluminescence also plays an important role in ocean ecology. It has been used to locate shoals of commercial fish near the surface in many parts of the world with video cameras, and the nighttime movements of ships, torpedoes, and submarines during wartime have also been recorded using bioluminescence. It has many other uses beyond the ocean: biomedical assays, pollution imaging, and neuro- muscular and developmental physiology. Bioluminescent systems extracted from marine organisms are now widely used as intracellular markers that signal the presence of potentially damaging radicals such as active oxygen or a specific biochemical event. Photoproteins extracted from jellyfish provide a wealth of information about the role of intracellular calcium. Green fluorescent protein (GFP) extracted from jellyfish has a wide range of use as an intracellular marker. The *gfp* gene and the *luc* gene encoding firefly luciferase are used as biomarkers to monitor the effects of microbial 71 inoculants in bioremediation. The *luc* gene is used for imaging in the bioremediation of gasoline or chlorophenol. The *gfp* gene is used to monitor bacteria in soil chlorophenol degradation based on the fluorescence of the GFP protein. These systems are being genetically cloned and modified to demonstrate their biomedical utility. Genes controlling bioluminescence of marine bacteria have also been identified and cloned. These and jellyfish genes can be added to other organisms as reporter genes. They report on the activation of other genes to which they are attached and do this by causing the emission of light that can be easily visualized. Changes in the light emission of bioluminescent marine bacteria or dinoflagellate cultures have also been used to image a wide range of toxic pollutants. Bioluminescence, which plays an important role in ocean ecology, is now increasingly being used in non- marine areas. Christopher Contag is a bioluminescence researcher at Stanford University. He became very interested in the idea of glowing in the dark and developed a method that could use the process that occurs in fireflies to study living tissue. The reason for this interest is that fireflies produce light through a chemical reaction that depends on the presence of ATP. If ATP is present in a sample, it is a good indicator that life is present in that sample. If firefly compounds are mixed with the sample and a light-emitting reaction is produced, this is likely to indicate that ATP is present in the environment. Biologists also use this approach to combat tuberculosis. If the enzyme luciferase is added to a cultured sample of tuberculosis and an antibiotic is added, the strength of the antibiotic can be tested. If the drug fails, the bacteria will continue to grow and emit light. This

method allows researchers and doctors to reduce the time required for drug treatment from three months to three days. This research has also been used in examples of gene activation therapy. Here, genes treated with luciferase were imaged with U.V. cameras. If light is visible, this means that the luciferase gene is active. This method is a powerful approach to studying many biological phenomena. Because this way, it is possible to work on live animal surfaces or deep tissues for a longer period of time. In recent years, researchers have found that the bioluminescent effects of fireflies will destroy cancer cells within themselves. In other words, a firefly is in the position of a powerful weapon producer against cancer. In a new study, researchers in London have added a firefly gene that activates bioluminescent light to modified cancer cells. In this way, a new method has been found to fight this disease. A light source known as luciferin causes the modified cancer cells to glow as if they were a firefly. When a light-sensitizing agent is added, this combination is lethal. The cells produce enough light to initiate their own death. This firefly technique (Cancer Destruction Activating Bioluminescence or BLADe) may provide a new and comprehensive approach to photodynamic therapy. This therapy is an effective treatment method that uses bursts of light to attack tumors located on the surfaces of internal organs or near the skin surface. As part of the therapy, cancer cells are treated with a photosensitizer and then exposed to laser or other external beams. The light stimulates the production of active oxygen species that can destroy the cancer cells. The cancer cells are modified to express the firefly luciferase gene and then incubated with luciferin in the laboratory. The cells become miniature lamps that emit light that triggers their own destruction. After a photosensitizer is added, the cells produce toxic substances that force them to commit suicide. Here, the light is produced within the tumor cell and therefore does not need to be penetrated from outside. This method has been used successfully in cells that cause prostate cancer. Luciferase can be transferred to primary tumors and migrate from there to disseminated cancer cells. While animals were used in toxicity screening in the water industry, bacterial assays are now used. In order to protect society and the environment from pollution and pests, the toxicity of both potable and wastewater must be monitored. Traditionally, the necessary tests have been performed using fish. However, the use of animals in toxicity tests is a general concern and the need for alternative methods arises. Bacterial assays provide results in a short time and therefore can be used for many toxicity tests and water controls. At the same time, the toxicity of pollutants and metals found in high concentrations in environmental wastes can be measured successfully and easily using the bioluminescent species *V. fischeri* (*A. fischeri* in the new classification) [20].

Effects of microorganisms and pollutants on marine ecosystems

When we look from space, it becomes clear why we should call our planet an ocean instead of a land mass. Because more than 70% of the earth's surface is covered with interconnected aquatic habitats. Life in the oceans began approximately 3.5 billion years ago, and for two-thirds of our planet's existence, there were only microorganisms as life forms. Accordingly, we can say that the development and continuity of all other marine life forms depend on the past and current activities of microorganisms [21]. These tiny microorganisms, which have great importance in the marine ecosystem, reach enormous numbers. When we look at the studies on the density of microorganisms in different samples; it is seen that Whitman et al. predicted that the number of bacterial and archaeal cells in marine environments is between 10^{28} and 1^{029}, including the first 10 cm of the sediment. The number of viruses in the oceans is thought to be approximately 10^{30}. These numbers are unimaginably high. If we include the subsurface sediments, this number would be 10 times larger. If we were to place all the marine virus particles end to end, we could say that it would be about 10 million light years. This is 100 times the distance in our own galaxy [21]. When evaluated from these perspectives, determining the microorganisms in marine ecosystems and how these species are affected by various aquatic pollutants is of great ecological importance. Water is by nature a universal solvent, a natural reservoir, and a carrier of both biogenic and xenogeneic chemicals. The magnitude of this problem is partially expressed in the U.S. Chemical Industry's Statistical Handbook (1998), which states that the industry produces 70,000 chemical products in 12,000 facilities annually. The broad ecological impact of chemicals on the health and well-being of aquatic com- munities poses a hazard and risk assessment problem for both ecotoxicologists and resource managers. Analytical chemists have made advances in collecting, separating, and identifying waterborne chemicals at nano and picogram concentrations. Ecotoxicologists have made similar strides in the identification and characterization of environmental toxicants [22].

A chemical in the environment can be a pollutant at one concentration and toxic at another. Bioassay testing based on dose-response experimental designs has become a critical element in defining the nature

of environmental toxicants over the last fifty years. Today, toxicological bioassays are based on a five-element experimental design: sample, biota, time, endpoint, and dose response [22].

The microtox toxicity test was first described by Bulich (1979) and uses the marine bacterium *Photobacterium phosphorum*, now renamed *Aliivibrio fischeri*, to assess the toxicity of aquatic pollutants. *Aliivibrio* sp. emits light as a natural byproduct of respiration, and this luminescence response can be easily measured with a sensitive photometer. The change in the amount of light emitted by bacteria when exposed to a toxicant is proportional to the ability of the toxicant to inhibit metabolism and is an indicator of its toxicity [23].

The luminescent bacterial strains evaluated in Bulich's water toxicity test were *Photobacterium mandapamensis* (ATCC 27561), *Lucibacterium harveyi* (ATCC 14126) (currently *Vibrio harveyi*), *Photobacterium phosphoreum* (ATCC 11040), and *Photobacterium fischeri* (currently *Aliivibrio fischeri*) (NRRL B- 11177). These bioluminescent bacterial strains were evaluated for toxicity testing by stimulation with sodium lauryl sulfate. All bioluminescent bacteria showed a decrease in light output when challenged. Only strain NRRL B-1117 was used to develop the test because it provided consistently stable light production and sensitivity to a wide range of toxicants [24]. One of the first microbioassays to be commercialized was the Microtox system of Beckman Instruments for the petroleum industry. This test was developed as an acute aquatic toxicity bioassay to replace traditional fish and invertebrate tests for monitoring potentially toxic wastewater from drilling operations [22]. The Microtox test is based on the measurement of changes in light emitted by a nonpathogenic, naturally luminescent marine bacterium when exposed to a toxic substance or a sample containing toxic substances. The Microtox test is a short-term acute toxicity bioassay that combines the advantages of a biological test with the speed and ease of use of a laboratory instrument. The Microtox test has been effectively used in the toxicity monitoring and assessment of industrial wastewater, municipal wastewater, sewage and sludge, lake and river water, agricultural and stormwater runoff, aqueous extracts of contaminated soils and sediments, groundwater, drilling muds, various industrial inorganic and organic chemicals, herbicides, pesticides, mycotoxins, pollutants and chemical mixtures.

Basic principle of microtox bioanalysis system

The toxicity of pollutants such as heavy metals and organic compounds is usually evaluated using chemical analytical methods such as liquid chromatography, gas chromatography, and mass spectrometry to report the concentration of certain toxic substances. However, these methods have some limitations such as expensive equipment, longer analysis time, and the need for skilled experimental operators. Alternatively, researchers have begun to use living organisms such as fish, algae, and bacteria as biosensors to monitor pollutants. Among these bioassays, bacterial bioluminescence test has the characteristics of high sensitivity, short-term detection, and easy operation. Its basic principle is that the luminescence intensity of living bacteria is directly related to their metabolic activities [25].

$$\mathbf{FMNH_2 + RCHO + O_2 \rightarrow 0.1\ hv + FMN + H_2O + RCOOH}$$

Light output is determined before and after exposure to a bioluminescent sample. When toxic compounds present in the sample interfere with the mechanism responsible for light emission, the intensity of light output decreases. The decrease in light intensity is proportional to the degree of toxicity present in the sample. Thus, the EC_{50} value can be measured using different concentrations of the sample [26].

Microtox toxicity test system

The Microtox system is a simple standardized toxicity test system that uses a suspension of the marine luminescent bacterium *Aliivibrio fischeri* as the bioassay organism. The Microtox bioassay is a measure of the acute toxicity of aqueous solutions. The Microtox Test System measures the increase or decrease in light output of test organisms relative to a reference sample, while subjecting

A. fischeri to osmotically adjusted, serially diluted samples. The Microtox TM analyzer, a temperature-controlled photometric instrument, quantitatively measures the light output of each suspension before and after sample addition [22].

The protocols are simple, well-defined, and easy to implement. The test organism is stored freeze-dried, eliminating the cost of continuous culture [27]. A selected strain of *A. fischeri* NRRL B-11177 is

included in the bioassay as the Microtox Reagent bacteria [22].

The Microtox Toxicity Test System includes four toxicity tests:

a. Microtox Acute Toxicity Test

b. Microtox Solid-Phase Toxicity Test

c. Microtox Chronic Toxicity Test

d. Mutatox Genotoxicity Test

a) ***Microtox Acute Toxicity Test:*** determines the acute toxicity of samples from surface water, groundwater, wastewater, leachate, and organic or aqueous sediment extracts.

b) ***Microtox Solid-Phase Toxicity Test:*** exposes bioluminescent bacteria directly to sediment-bound chemical contaminants in an aqueous suspension of a test sample.

c) ***Microtox Chronic Toxicity Test*****:** the chronic test is designed to mea- sure the adverse biological effects that occur when an organism is exposed to toxic stress for a prolonged period of time. Test samples are; from surface water, groundwater, wastewater, leachate and organic or aqueous extracts in liquid phase.

d) ***Mutatox Genotoxicity Test:*** mutatox determines the genotoxicity of liquid phase samples from surface water, groundwater, wastewater, leachate and organic or aqueous sediment extracts by measuring the light changes produced by bioluminescent bacteria. The test is used as a screening tool to detect the presence of genotoxins, which are DNA-damaging substances, in these mixtures and their metabolites. Non-luminescent bioluminescent bacteria (dark mutant strain) in a growth cycle are exposed to a test substance at sublethal concentrations. The toxicological endpoint of Mutatox is qualitative and provides a yes-no assessment of DNA-damaging substances]27].

Applications of microtox bioanalysis

Since the development of the Microtox test in 1979 [24], microbiologists, ecologists, biologists, ecotoxicologists and other researchers worldwide have widely used this test for toxicity assessment of chemicals, wastewater, industrial effluents and a wide variety of environmental samples. The Microtox test system is also used in water quality monitoring, soil extract testing, contaminated sediment or field investigations and environmental impact and risk assessment studies [28, 29]. The main uses and applications of Microtox testing are:

1. Rapid toxicity screening of wastewater and receiving waters
2. Effect monitoring and biomass protection in water and wastewater treatment plants
3. Toxicity testing of sewage, sludge and contaminated soil and sediments
4. Toxicity monitoring and assessment of agricultural, storm-water and combined sewage flows
5. Toxicity assessment of groundwater, surface water and drinking water
6. Toxicity screening of inorganic and organic chemicals based on individual and quantitative structure-activity relationships (QSAR)
7. Prediction of other bioassay results and process changes
8. Determination of toxicity of potentially hazardous wastes
9. Screening and testing of various environmental samples
10. Toxicity screening of landfill leachates
11. Monitoring of efficiency and effectiveness of drinking water treatment operations and systems
12. Detection and prediction of irritation in substances and products designed for industrial, pharmaceutical and cosmetic uses
13. Monitoring of environmental impacts
14. Regulatory decision-making and compliance monitoring
15. Toxicity screening of drilling muds, additives and reservoir effluents
16. Detection and control of toxic commercial wastes and spills
17. Identification and prioritization of effluents and discharges for toxicity-based control
18. Comparison and correlation with other conventional and unconventional toxicity bioassays
19. Ecotoxicological monitoring and testing
20. Toxicity determination assessment (TIE) and toxicity reduction assessment (TRE) studies and investigations

21. Toxicity assessment of fossil fuel process waters and phenolic compounds
22. Toxicity testing of biocides, pesticides, herbicides and bactericides
23. Industrial waste streams and process control monitoring
24. Quality control monitoring of raw materials, new chemicals and formulations
25. Determination of toxicity of plasticizers, stabilizers, antioxidants and surfactants
26. Toxicity screening of mycotoxins and biological toxins
27. Marine toxicity data
28. Toxicity testing of wastewater from plastic, resin, wood, pulp, textile and leather industries.

Advantages of the microtox test system

This simple and rapid test provides an indication of test substance toxicity after only 5-30 minutes of exposure, whereas other acute toxicity tests of comparable sensitivity typically require exposure times of 24 to 96 hours [23]. This rapid response time meets the needs of the toxicologist to conduct routine toxicity experiments as well as to respond to emergency situations such as wastewater, chemical spills, and detection of toxic substances. The Microtox protocol and rapid toxicological determination (<30 minutes) allow for large sample collection capacity both in the laboratory and in the field [22]. The Microtox test is widely used because it is simple, rapid, inexpensive, and can be performed with minimal laboratory facilities [23].

Easy to use and convenient

- ✓ Simple, fast and practical
- ✓ Highly sensitive, reliable and repeatable
- ✓ Precise and accurate data/information
- ✓ High degree of standardization
- ✓ Excellent quality control
- ✓ Economical (low cost/testing, labor saving)
- ✓ Time/Cost-effective
- ✓ Fast turnaround time; results in two hours or less
- ✓ Instant testing capability (laboratory testing or field studies)
- ✓ Availability of test reagents and ease of storage
- ✓ Availability, consistency and stability of bacterial reagents (long-term use)
- ✓ Excellent correlation with common acute toxicity tests
- ✓ Applicable to testing solid and liquid samples
- ✓ Applicable to testing highly turbid and colored samples
- ✓ Statistical advantage of using a large number of test organisms
- ✓ Allows screening of large numbers of samples in a relatively short time
- ✓ Requires small sample volumes
- ✓ Requires little laboratory space
- ✓ Does not require elaborate laboratory facilities
- ✓ Specialized expertise/skilled manpower not required
- ✓ Suitable for compliance monitoring/new product testing [28].

Conclusion

The exponential increase in the world population and the resulting increase in environmental pollution are endangering all life forms. One of the polluted areas under threat is marine ecosystems. The fact that marine environments cover a very large area raises the view that various pollutants will be diluted by being released into these habitats. However, due to the increasing pollutants along with the increasing population, the threshold value of marine ecosystems in terms of pollutants is also exceeded. For this reason, both the prevention and elimination of polluting factors are necessary. In this context, biomonitoring should be done using bioassays that are sensitive to the environment and fast, and the abundance and diversity of all species, which are *insurance policy* of nature, should be protected.

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