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Ecotoxicological methodological guide for environmental monitoring: problematics, laboratory techniques and health risk investigation

BSB27-MONITOX

Editors:

Elena Zubcov
Antoaneta Ene

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Chisinau, 2021

CZU 504.06:[574+614]
E 15

The book is recommended for publication by the Scientific Council of the Institute of Zoology, Ministry of Education and Research, on 26th of July 2021.

The ecotoxicological guide is elaborated in the frame of the project with eMS code **BSB 27**, entitled *Black Sea Basin interdisciplinary cooperation network for sustainable joint monitoring of environmental toxicants migration, improved evaluation of ecological state and human health impact of harmful substances, and public exposure prevention* – MONITOX (Joint Operational Programme Black Sea Basin 2014-2020, financed by the European Union). There are used, also, some of the research results of the finalized international and national projects MIS ETC 1676 (2013-2015), AQUASYS (2015-2019) and of ongoing ones – BSB 165 HydroEcoNex (2018-2021), SOFT1/2/47 (2020-2021), AQUABIO (2020-2023).

The ecotoxicological guide is addressed to researchers, environmentalists, young students (PhD, master undergraduate students) and all those who want to become familiar with the theoretical and applied aspects of ecotoxicological investigations, which include monitoring, deciphering the migration processes of toxic substances in the environment and assessing the ecological status and functioning of aquatic ecosystems.

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MD-2068, Chișinău, str. Florilor, 1
Tel. 022-49-31-46

Descrierea CIP a Camerei Naționale a Cărții

Ecotoxicological methodological guide for environmental monitoring: problematics, laboratory techniques and health risk investigation / BSB27-MONITOX, Joint Operational Programme Black Sea Basin, Institute of Zoology; Center of Research of Hydrobiocenoses and Ecotoxicology; editors: Elena Zubcov, Antoaneta Ene. – Chișinău: S. n., 2021 (Î.S. F.E.-P. “Tipografia Centrală”). – 112 p.: fig., tab. – (Common borders. Common solutions, ISBN 978-9975-128-28-5).

Referințe bibliogr. la sfârșitul cap. – Financed by the European Union. – 200 ex.

ISBN 978-9975-157-99-5

SUMMARY

INTRODUCTION

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At the end of the twentieth century, emissions of anthropogenic pollutants have become proportional to the magnitude of the natural processes of migration and accumulation of various compounds. Problems directly related to chemical pollution of the biosphere, which often lead to acute toxicological and ecological situations, have suddenly worsened. This has led to the expansion and intensification of various studies on the extent and rate of environmental pollution, the search for effective methods of protecting the atmospheric air, natural waters, soil and vegetation, which are based on reducing the flow of chemical pollutants entering the biosphere with emissions from industry, waste, as well as limiting or eliminating the total toxic effects of various substances and compounds of anthropogenic origin on the plant and animal world, preventing their negative impact on human health.

According to the World Health Organization (WHO) (Promotion of Chemical Safety Unit & International Programme on Chemical Safety, 1992), out of over 6 million known chemical compounds, up to 500 thousand compounds are practically used; about 40 thousand of them are harmful to humans, and 12 thousand are toxic.

According to REACH data, there are currently over 140 thousand chemicals that are produced in quantities of over 1 ton/ year, of the more than 142 million substances registered in the Sci Finder Chemical Abstracts Service (August 2018). Most of these compounds could end up, in one case or another, in the environment.

Secondly, it is obvious that chemicals are not found alone in the environment, but as complex mixtures. Over time, a chemical substance may exist in environment in a low concentration or below the limit of detection, however the mixture of several substances can produce adverse effects.

Moreover, the products of the transformation of micropollutants, formed in the environment or through biological metabolism, are not always known or recorded and may be more toxic and persistent than the initial basic compounds. In fact, about 30 thousand types of new xenobiotic chemical compounds are produced annually.

Thus, there is a need to assess them in terms of the danger to living organisms, populations of organisms and biocenosis.

Ecotoxicology – a new environmental science – appeared at the stage of man's awareness of the need to form knowledge about fluctuations and changes in the state of his environment under the influence of a huge number of foreign and natural substances in the living environment.

Ecotoxicology – a new field of environmental science – has emerged as an interdisciplinary direction, developing in the border areas of toxicology, ecology, biology, geochemistry, soil science and other sciences.

Ecotoxicology is one of the fundamental disciplines of the general professional training of undergraduate, master or doctoral students, enrolled in the study programs of ecology, biology and environmental science, and which allows the future specialist to freely understand and comprehensively evaluate and analyze the effects of xenobiotics and ecotoxicants on the environment and the biological systems regarding these effects.

The theoretical part of ecotoxicology presents modern ideas about the transformation and circuit of toxic substances in ecological systems, the mechanisms for their inclusion in natural cycles, as well as the consequences of changes in the natural flows of substances in the biosphere – the violation of ecological balance and the transformation of elements of the biosphere, the reduction of biodiversity, the risk to human health.

Ecotoxicology lays the foundations for scientific approaches to the problem of adapting different-ranking communities of bodies and specifies the principles of assessing the toxicity of a substance from the point of view of toxicology and ecotoxicology, the characteristics of standardisation and the technicalitiesof risk estimation.

The practical part consists in forming the knowledge of the methods of environmental monitoring, necessary for the collection of environmental information, and the ability to interpret this knowledge in order to assess the state, sustainability and prognosis of the development of natural complexes.

The main purpose of ecotoxicology is to provide the fundamental knowledge about the sources of harmful substances in the environment, their distribution and effect on living organisms and communities of living things of different organizational ranks, ecosystems and the biosphere.

Thus, ecotoxicology is one of the components of the general professional training of the environmental specialists, regarding the understanding of the problems of evaluation and the comprehensive analysis of the effects on environmental objects and the reactions of certain natural environments to these effects.

Ecotoxicological researches aim to study the migration of ecotoxicants (natural substances) and xenobiotics (compounds obtained in the process of human activities, which are foreign to the metabolism of living organisms) in ecological systems, the mechanisms of their incorporation into biogeochemical circuit, as well as the consequences of changes in natural flows, the balance and transformation of the elements of the biosphere, the reduction of the biodiversity, the imbalance of ecosystems and the risk to human health.

The history of toxicology is lost in the depths of the centuries. Man faced the poisonous effects of various substances of plant and animal origin in the Stone Age. As the natural environment developed, more and more new toxic substances appeared, including those caused by man that is, as they were produced by man himself, either as intermediaries or as final products of his work activity.

In connection with the development of industry, chemistry and chemical technology, these toxic substances or „poisons" demanded special attention. As a matter of fact, there are no non-toxic substances in the environment. Under certain conditions, in certain doses, nutrients can also become dangerous to living things/organisms, causing damage or impairment of their functions and, ultimately, death. Thus, the toxicity of substances that are completely inert with regard to biological objects can be quantitatively designated as a tendency (but not equal) to zero. In connection with the above mentioned, it is possible to define ecotoxicology as a science that studies a property inherent to almost all the substances around us, both natural and anthropogenic, their circuit and transformation into the living environment and their toxic effects on the communities of aquatic and terrestrial living things, including man.

1

Chapter

ECOTOXICOLOGY AS A BRANCH OF SCIENCE

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1.1. GENERAL CONSIDERATIONS

The beginning of the development of a new branch of science – ecotoxicology – was the book *Silent Spring,* written by Rachel Carson and published in 1962, in which the author describes the cases of mass death of birds and fish due to the uncontrolled use of pesticides. R. Carson concluded that the harmful effects of pollutants on wildlife foreshadow an impending disaster for humans (Carson, 1962). This book has attracted the widespread attention of the public and the environmental protection societies, governments, which has intensified the drafting of several governmental laws regulating the xenobiotic emissions in the living environment.

The term „ecotoxicology" as a branch of science was designated by Renne Truhaut in 1961 – ***„Ecotoxicology is a branch of science that studies the interactions that determine the distribution and abundance of organisms" while toxicology is „the science that deals with the study of the adverse effects of substances on living organisms"***. The author brought together two completely different topics: ecology and toxicology. In fact, ecotoxicology, in addition to ecology and toxicology, also includes elements of other sciences (Truhaut, 1977).

For ecotoxicological research thorough knowledge is required of organic and inorganic chemistry, biochemistry, analytical chemistry, physical chemistry, toxicology, ecology, biology, medicine. It is also necessary to know some methods of statistical calculation.

The definition of ecotoxicology has been changed by several researchers.

Francois Ramade published in 1977 a textbook in which he emphasizes that: ***„Ecotoxicology studies the ways of contamination of the environment by natural and artificial pollutants produced by human activity, the mechanisms of action and the effects on the living creatures that populate the biosphere"*** (Ramade,1977). This definition was later clarified at the SCOPE (International Scientific Committee on Environmental Issues) conference in 1978. Here, Butler G.C. presented and then published the main objectives of ecotoxicology – ***„Ecotoxicology – is the branch of toxicology that studies the toxic effects of natural or artificial substances on living organisms that constitute the biosphere (animal, plant, terrestrial, aquatic), including the interaction of toxic substances with the physical environment in which living organisms exist"*** (Butler, 1984).

Valery E. Forbes & Thomas L. Forbes in 1984, then in the book *Ecotoxicology in theory and practice* edited in 1995, asserted that ***„Ecotoxicology is the field of study that integrates the ecological and toxicological effects of chemical pollutants on populations, ecosystem communities with their „fate" in the environment (transport, disintegration)"*** (Forbes & Forbes, 1984,1995).

P. Calow, in 1998, by conducting several investigations and editing textbooks on the ecotoxicology and protection of ecosystems, began to unite these two directions – ***„Ecotoxicology is the branch of science that deals with the protection of ecological systems against the adverse effects of synthetic chemicals”*** (Calow, 1998).

C.H. Walker, R.M. Sibly, S.P. Hopkin & D.B. Peakall, in their 1998 *Principles of Ecotoxicology,* wrote that: ***„Ecotoxicology is the science that studies the harmful effects of chemicals on ecosystems”*** (Walker et al.,1998).

With the development of the concept of *„ecotoxicology”*, it has undergone a certain evolution. Walker et al. (2001) again found that ***„Ecotoxicology is a science that studies the toxic effects of chemical agents on living organisms, especially in populations and communities, in certain ecosystems”***.

The topic of ecotoxicology is represented by organisms and biological systems of different levels (community, population, ecosystem, etc.), subject to anthropogenic pollution. Therefore, the fundamental principles of the functioning and structure of the natural population systems and biocenotic rank, actively developed by modern theoretical ecology, serve as a theoretical basis for ecotoxicology.

Interdisciplinary research areas related to ecotoxicology

The expansion and deepening of the concept of „ecology” led to the emergence of new independent scientific fields. These directions are in contact with each other: they have a common methodology, a conceptual apparatus, research objects and they use the same scientific results to solve their own specific problems.

As an example, *the chemical ecology* (Bogdanovsk, 1994; Egorov, 2009) studies the chemical changes under the influence of environmental factors. The central task is to study the behavior of anthropogenic substances: concentration in the environment, accumulation processes in the environment. The sphere of interest includes chemical processes in the environment due to changes caused by human activity. The close connection between ecological chemistry and ecotoxicologists is expressed in the fact that both fields use methods and techniques of chemicaland physico-chemical analysis (Korte, 1997).

The subject of ecotoxicological research is the establishment of the link between the dynamics of chemicals and the development of communities of living organisms in ecosystems. Unlike ecological chemistry, the objects of ecotoxicological research refer not only to anthropogenic substances, but also to natural substances, through which their interaction on living communities takes place, and to deciphering the role and importance of organisms in transport processes, circuitry and reducing or increasing the toxicity of chemicals.

A common feature of toxicologyi and ecotoxicology is that both fields of research use the methodology, the toxicological conceptual apparatus and the fundamental bases regarding the toxicity (Ecological Bulletins No. 36, 1984).

Toxicology is mostly a compartment of medicine that studies the physical, chemical properties of poisons (harmful and toxic substances), the mechanisms of their action on the human body and develops methods of diagnosis, treatment and prevention of poisoning (Golikov, 1972).

Toxicology is the study of toxicity and the toxic process. Toxicity and the toxic process are two basic concepts of modern toxicology. Toxicity is a property (capacity) of chemicals, which act on biological systems and cause damage (dysfunction) or death. Thus, toxicity is the ability to cause a disruption, illness or death. Chemicals that, under certain conditions, may present this property, are called

toxics (toxic substances) (Kutsenko, 2006; Lysenko & Dogadina, 2015; Oprea, 2007).

The action of a substance, which leads to disruption of the functions of biological systems, is called a ***toxic effect***. The basis of toxic effects is the interaction of a substance with a biological object at the molecular level.

The chemistry of the interaction of a toxic agent and a biological object (at the molecular level) is called the ***mechanism of toxic action***. ***Toxicant*** = poison = poisonous, harmful or dangerous substance (Kutsenko, 2006).

Ecotoxicology and toxicology, when toxicity and toxic processes are taken into account, use a common apparatus, models and conceptual methodology; these are their common features. However, toxicology and ecotoxicology study the toxicity of substances and the manifestation of the toxic process at different levels of organization of a biological object (Bazerman, Charles & René Agustin De los Santos, 2006).

Ecotoxicology treats a toxic material as a toxic component in the ecological processes of a living ecosystem or community, and toxicology – to an organ or organism. This is the fundamental difference between ecotoxicology and toxicology (Relyea & Hoverman, 2006).

In toxicology, the intake, metabolism and elimination of the toxic substance from a biological object and the toxic process are studied without an ecological perspective or ecological context (without taking into account the behavior in the ecosystem and the impact on populations and communities) (Lysenko & Dogadina, 2015; Oprea, 2007).

Ecotoxicology studies toxic effects above different communities of organisms up to the ecosystem level and thus the value of the environment becomes an active factor in development; in addition, a specific feature of ecotoxicology is the evaluation of migration processes and the circuit of toxic substances and the environmental consequences of the combined action of anthropogenic and natural factors on biological systems.

Ecotoxicology has the most interferences with the general toxicology and the toxicology of the environment. Drawing a parallel between these disciplines and ecotoxicology, it is necessary to highlight the following:

Toxicology deals with the takeover, absorption, diffusion, excretion of toxic substances by individual organisms. Ecotoxicology deals with the kinetics of pollutants in the abiotic environment (air, water, soil/sediment) and the biotic environment (trophic chain), the study of the transformations and biotransformations of chemicals at the level of communities of organisms and the ecosystem.

Toxicology aims to protect human/animals (viewed as an individual) from exposure to toxic substances. Ecotoxicology aims to protect the populations and the communities of different species, as well as ecosystems, from the harmful effects of exposure to toxic substances (Kutsenko, 2006).

A major importance for both toxicology and ecotoxicology is the relationship between the amount of chemical substance which the body is exposed to and the nature, degree of subsequent harmful effects (dose/response relationship, which would be the basis for estimating the hazard and risk posed by chemicals in the environment).

Ecotoxicology (Straalen, 2002) was for a while assimilated with environmental toxicology. Environmental toxicology addresses pollution problems in a limited way, namely:

- the way in which it exerts its physiopathological action on target organisms, in particular invertebrates, mammals (humans);
- the mechanism of action on the cellular and molecular level.

In the process of studying the effects of chemicals present in the environment on humans and human communities, environmental toxicology acts with established categories and concepts of classical toxicology and, as a rule, applies the traditional experimental, clinical and epidemiological methodology. The objective of the research in this case are the mechanisms, the dynamics of development, the manifestation of the adverse effects of the action of toxic substances and the products of their transformation in the environment on humans.

Ecotoxicology, different from Environmental Toxicology (Robinson & Thorn, 2005; Frumin, 2013), mostly addresses the effects of pollution directly on populations and ecosystems as a whole, in order to protect them completely and not on isolated components.

The basic concept of ecotoxicology, is also ***toxicity***, which in general terms can be defined as a property (capacity) of chemicals, which act on biological systems causing their damage or death, or the ability to cause degradation of biocenosis or the entire ecosystem.

The consequence of the toxic effect of substances on biological systems and the reaction of a biosystem to the action of a toxic agent that leads to the deterioration (*i.e.* impairment of its functions, viability) or death is called a ***toxic process***. External signs, recorded by toxic processes (toxic action), represent its manifestation (toxic effect) (Forbes & Calow, 1999).

The toxic process at the population level is manifested by (Bezel et al.,1994)***:***

- death of the population;
- increased morbidity, mortality, number of birth defects;
- decrease of the multiplication potential;
- violation of structural features (gender, age, space, size, mass structure, etc.);
- decrease of the average living possibilities and the degradation of the population composition.

The toxic process at the level of biocenoses is manifested by:

- changes in the population spectrum of cenosis, up to the disappearance of certain species and the emergence of new ones, which are not typical of this biocenosis (this can change the dominant species and, as a rule, diminish biodiversity);
- violation of interspecific relationships, reduced productivity of biocenoses as a whole system;
- possible degradation and disappearance of this ecosystem.

Methodological differences between ecotoxicology and environmental toxicology are completely erased when a researcher is asked to assess the indirect effect of pollutants on human populations (e.g. due to toxic changes in biota) or, on the contrary, to find out the mechanisms of action of chemicals in the environment on representatives of the one or other individual (Bezel et al.,1994).

In this sense, from a theoretical point of view, the „toxicology of the environment" as a science is only a special problem of „ecotoxicology", while the methodology, conceptual apparatus and structure of the sciences are the same (Robinson & Thorn, 2005).

The difficulties of establishing the ecotoxicology as an independent direction are today related to the absence of a sufficiently strict theoretical basis for unifying the accumulated experimental material and explain it.

The ambiguity of the initial data obtained under different natural conditions and under various influences, the lack of an explicit relationship between the field observations and experiments, the isolation of theoretical positions from their specific application to the solving of practical problems, are all signs of a novelty and the first steps of the new scientific direction.

BASIC OBJECTIVES OF ECOTOXICOLOGY

The content of the discipline „Ecotoxicology" is the study of the ecotoxicity and the main characteristics of the profile of the xenobiotic habitat, ecotoxicokinetics, ecotoxicodynamics and ecotoxicometry of ecotoxicants and xenobionts, the evaluation of biological communities of different rank subject to pollution of the living environment.

The most important tasks of ecotoxicology include: the study on fluctuations in species composition as well as in the relations of species at a trophic level. As far as ecotoxicology is concerned, the factor of reducing species diversity is particularly important (diversification), since a decrease in the number of species may lead to the disappearance in the first place of their previously existing indicator organisms. The changes of growth parameters and metabolism of organisms lead to the structural modification of the communities of species and cause pertrubations in the functioning of the entire ecosystem (Forbes & Calow, 1999; Panin & Bezel, 2008).

If epidemiological studies are carried out, the loss or reduction of the number of main species of organisms shall require an analysis of trends in the development of biological communities. Such studies are another task of ecotoxicology, which can help identify the degree of risk and prevent dangerous phenomena (Kaplin, 2006).

Thus, ecotoxicology presents a deep study of ecosystems, and the most important parts of it should be the problems of conservation (for undisturbed ecosystems) and restoration (for already affected systems). Both goals can only be achieved in functional modeling mode. However, due to the fact that there is no universal model, the development of such models, as well as the creation of laboratory and mathematical models or similar studies in natural conditions, are carried out on the basis ofthe objective set for solving a particular problem (Walker et al., 2002; Robinson & Thorn, 2005).

The nature and extent of the influence of chemical pollutants on the general ecological situation, biogeocenoses and biosphere components vary in different natural areas and even in relation to some species of animals and plants, and as a result of causing dangerous toxic-ecological situations local environmental disturbances often occur (Katrin Franke Bader, 2012).

Natural ecosystems have the ability to withstand both fluctuations in ordinary natural factors and changes in living conditions under anthropogenic influence. Therefore, the rational use and conservation of nature, as well as the properties that can detect negative or potentially dangerous environmental changes in the early stages become essential. Consequently, effective methods for early detection of certain changes are also needed (Israel, 1984).

Any transformation of the environment as a result of human activity can be called anthropogenic (or human factor). An anthropogenic impact that leads to a change in the chemical composition of one or more natural components of the environment is geochemical, as it inevitably involves this change in natural systems that interact with each other at different speeds and intensities (Panin & Bezel VS, 2008).

The works of several researchers (Truhaut, 1977; Forbes & Forbes, 1995; Schueuermann & Markert,1997; Connell et al., 1999; Forbes & Calow, 1999; Walker et al., 1998, 2001) have become classics in the development of ecotoxicology as a *scientific branch* in which the main notions, objectives, tasks, ecotoxicological research directions are stipulated and argued, and which refers to all the compartments of this chapter, as follows.

THE MAJOR OBJECTIVES OF ECOTOXICOLOGY ARE:

1. Obtaining the ecological and toxic data for risk assessment and environmental management;
2. Knowledge and establishment of chemicals dispersion laws in the environment;
3. Elaboration of the bases (empirical or theoretical) to enrich and improve knowledge about the behavior regarding the toxic effects of chemicals in the living system.

The following main issues are addressed in the above objectives:

- distribution of pollutants in the environment by identifying the disposal of chemical substances in the different compartments of the environment (air, water, soils, food);
- kinetics of pollutants (penetration modalities, storage in the environment, diffusion, their transformations into the environment);
- the effects of pollutants on living organisms, respectively the definition of the picture of harmful effects at the *individual* level *(disruptions of the biochemical, molecular and physiological structure and* function), as well as at the *population level (change in the number of individuals, change in the frequency of genes, modification of the entire ecosystem function);*
- recording the individual toxicity of toxic substances: dose, concentrations with standard tests or through complex systems;
- hazard or risk assessment for a particular toxic (based on toxicity data);
- assessment of the overall potential for disorders (changes) in the ecosystem;
- elaboration of „therapy" measures *(recovery);* taking preventive measures regarding the risk and analyzing them.

XENOBIOTIC PROFILE

The foreign substances in the environment (water, soil, air and living organisms) in the form (state of aggregation) allowing them to enter into physico-chemical and chemical interactions with biological objects of ecosystems constitute the xenobiotic profile of its biocenosis. In this list of substances, radioactive materials are also included.

The xenobiotic profile should be considered as one of the most important environmental factors (along with temperature, light, humidity, trophicity, etc., qualitative and quantitative characteristics) (Katrin Franke Bader, 2012).

The important elements of the xenobiotic profile are also substances that are xenobionts in the organs and tissues of living organisms, because all of them are consumed by others, sooner or later.

In contrast, chemicals that are fixed in solid, non-dispersed in objects in air and water, insoluble (stone, various solids, glass, solid plastic, etc.), cannot be considered as components of the xenobiotic profile.

Only bioavailable substances, which interact non-mechanically with the living organisms, are of interest. These are in the gaseous or liquid state, in the form of aqueous solutions, adsorbed on soil particles, solids, and in the form of finely dispersed dust (< 50 microns).

THE MOST COMMON COMPONENTS OF THE XENOBIOTIC PROFILE AND THEIR ROLE

- ***Oxygen*** is practically not a pollutant in the true sense of the word, but it can influence health by decreasing its concentration in the air and by decreasing the atmospheric pressure, the effect being determined by the decrease of the partial pressure at the level of the pulmonary alveoli and the alteration of the gas exchange. ***Oxygen and carbon dioxide*** (O_2 and CO_2) are determinants of the process of blood oxygenation. The specific phenomena that occur are phenomena of hypoxia or anoxia, their severity being dependent on the degree of decrease of the partial pressure.

- ***Ozone*** is naturally found in the composition of the atmosphere forming, due to the increased concentration, a real layer in the Earth's stratosphere. This layer or „***ozone shield***" has an important ecological role at a global level due to its ability to shield radiation from the ultraviolet sector of the spectrum of sunlight.

The concentration of ozone in the breathable atmosphere is normally quite low and is variable depending on the geographical conditions that determine a different incidence and intensity of ultraviolet radiation. Thus, in arid areas, the average concentration of ozone is about 15 parts per million (ppm), while in areas at higher altitudes it can reach values above 100 ppm.

Recent researches have shown that exposure of the human body to increased ozone concentrations leads to damage to the eyes, respiratory diseases and the overall breathing process. The effects are more strongly felt by young organisms.

- ***Carbon dioxide*** does not cause disturbances of the human body, only in situations where the passage of gas from the venous blood into the pulmonary alveole and its elimination through the exhaled air is prevented. In fact, toxic phenomena occur when the partial pressure of CO_2 in the air increases so much that they prevent it from being eliminated.

Initially, there is an increase in CO_2 in the blood less due to its penetration from the outside air, but because of the body's autointoxication. As the concentration in atmospheric air increases, its solubilization in the blood plasma also intervenes due to the increased partial pressure.

The first disorders occur around the concentration of 3%, being manifested by respiratory disorders (acceleration of breathing, cyanosis occuring, followed by respiratory and circulatory disorders).

- ***Sulphur dioxide*** **(SO_2)** causes mucosal irritation and bronchioles dilation. In contact with the blood forms sulfhemoglobin which imprints a reddish-brown color on the blood. Also, sulfur dioxide can disturb the synthesis activity of nucleic acids resulting in chromosomal aberrations, decrease in the growth rate. The harmful effects that occur on humans in connection with the concentration of SO_2 in atmospheric air are well known, regarding the impairment of the respiratory process.

Sulphur dioxide is naturally found in the atmosphere in the extremely low concentrations up to 0.2 ppm, the volcanic activity being the main source of sulphur dioxide. The main sources of sulphur dioxide pollution are dependent on human activity and are mainly represented by activities involving the combustion of fossil fuels. Estimates show that SO_2 emissions in the atmosphere can reach the figure of 145 million tonnes annually, which includes quantities due to the combustion of coal (70%) and other fuels (16%), the remaining percentages being provided by metallurgical industrial activities (processing of 1000 t of copper ore is equivalent to the release of 600 t of sulphur dioxide).

The concentrations of SO_2 in the atmosphere of industrialised cities vary from case to case, with an average value of 0.17 to 2 ppm, which must become of concern, as long-term exposure of homeothermic organisms, including humans, to concentrations higher than 0.1 ppm of sulphur dioxide in the air can induce serious damage.

- ***Hydrogen sulphide*** ***(H_2S)*** normally enters the composition of the atmosphere as a result of fermentation processes produced in the absence of oxygen (anaerobiosis) in which microorganisms specific to the terrestrial and aquatic environment are involved.

The quantity of hydrogen sulphide produced in this way by the bacteria is estimated annually at values of 68 million tons for the terrestrial environment and 30 million tons in the aquatic environmentc. Also, naturally, but as a result of volcanic activity, a significant amount of H_2S is permanently eliminated in the atmosphere.

It has been estimated that as a result of the industrial activities involved in the production of sulphur derivatives or the processing of ores containing sulphur, about 3 million tonnes of sulphur equivalent are released into the air annually.

It is known that in the geographical areas considered unpolluted the concentration considered normal of this gas in the atmosphere is about 2 ppm. The elimination of this highly toxic pollutant from the atmosphere occurs naturally through complicated chemical processes that determine the oxidation of hydrogen sulfide by producing sulfur dioxide.

This gas enters the body through the the respiratory tract and its effects are felt both at the short-term exposure and at medium- or long-term exposure, being linked to impaired breathing.

There is a great variability of the sensitivity of human individuals to the action of hydrogen sulfide but, in principle, depending on the amount inhaled, the effects are represented by constriction of the bronchi, the appearance of bronchitis and even of the bronchial spasm. The effect is more severe in young organisms.

➢ ***Nitrates and nitrites*** can cause harmful effects on humans through intoxication, a phenomenon that occurs through food and drinking water. According to the hygienic norms of the Republic of Moldova, the drinking water can have a nitrates' content of no more than 45 mg/liter and the presence of nitrites is allowed only exceptionally in the amount of 0.3 mg/liter in groundwater up to 60 m depth. Water becomes suspicious at concentrations of 50 mg nitrates/litre and toxic at 1 g/litre. The most common route of intoxication of man is the digestive one. In the digestive tract, nitrates undergo a series of successive transformations up to ammonia. These transformations are performed under the influence of bacterial flora and some enzymes.

The toxicity of nitrates and compounds resulting from their metabolism is initially manifested in the digestive tract through irritating and congestive effects on the digestive mucosa. Then, the irritating action is manifested on the kidney through congestion and hemorrhage.

After entering the bloodstream, nitrates and nitrites act paralyzingly on the vasomotor centers and veins, especially on small ones, causing vasodilation and hypotension.

These nitrogen compounds have negative influences on the endocrine glands, on the pituitary gland, but especially on the thyroid. Its nitrates and metabolites induce a state of hypothyroidism.

➢ ***Phosphates*** can cause polluting effects that manifest directly on humans through the appearance of intoxications. Some „impurities" contained in superphosphates, the most important of which is fluorine, also contribute to the increase of the toxicity. Phosphorus along with fluorine have an irritating effect on the mucous membrane of the digestive tract.

➢ ***Pesticides*** – are the largest group of xenobiotic substances (Rathore & Nollet, 2012; http://npic.orst.edu/factsheets/ ecotox.pdf) which, according to the way they penetrate the body and the character of the harmful action, are divided into:

- *ingestion products* (which penetrate the body with food),

- *contact products* (which act by touch with the skin),
- *breathing* products (which act through the respiratory apparatus).

➢ ***Organophosphorus insecticides*** have generally action by contact and ingestion and less often through breathing. It has a strong toxic action on the *nervous system, by altering the transmission of the nervous influx, causing spasms, convulsions and death*.

➢ ***Nematocides and molluscicides*** are pesticides with high toxicity that are applied to the soil. They act by ingestion and contact. The chronic effects of pesticide poisoning are most often observed on the liver and nervous system. Since the liver is the most important organ that metabolizes pesticides, it is the most injured (traumatized, affected).

➢ ***Metals-macroelements and trace metals*** are the most conservative and persistent ecotoxicants, the role of which is multilateral from those essential for life to those extremely toxic. The influence of macro- and micro-metals is dependent on their physico-chemical properties, quantity, form of migration, environmental factors (temperature, humidity, pH, oxidation-reduction processes, etc.) and, last but not least, the state and metabolic processes in the impacted living organisms.

CHANGING THE XENOBIOTIC PROFILE

In recent years, human activities have often significantly altered the natural xenobiotic profile in many regions (especially urban and industrial).

Chemicals that accumulate in the environment, in unusual amounts, contaminate the xenobiotic profile in which the so-called ecopollutants and xenibiotics are concentrated. (Katrin Franke Bader, 2012).

Changes in the xenobiotic profile may occur as a result of excessive accumulation of one or more pollutants in the environment, but this does not always lead to disastrous consequences for life.

Only ecopollutants accumulated in an amount sufficient to initiate a toxic process in the biocenosis (at any level of organization of living matter) can be designated as ecotoxicants (Harris et al., 2014).

One of the biggest practical problems of ecotoxicology is the determination of quantitative parameters for which the ecopollutant turns into an ecotoxicant.

THE BASIC COMPONENTS OF THE XENOBIOTIC PROFILE

Ecotoxicants – naturally occurring toxic substances that accumulate in organisms up to toxic amounts or are harmful to life (petroleum products, heavy metals, microbial biotoxins, etc.).

Xenobionts **-** substances that do not exist in nature and are synthesized by humans, which cause inhibition of vital processes or may become the cause of intoxication of living organisms and, ultimately, of man (pesticides, pharmaceuticals, detergents, dyes and other synthetic substances, including medicines obtained from plants and organisms).

Main pollutants

There are many classifications of pollutants, depending on toxicity, availability, chemical composition, etc. (Ciubotaru, 2003; Yufit, 2002).

Biogeochemical cycles now include a large list of synthetic compounds unknown to the virgin natural environment. These include, in particular, a large group of substances united by the general term „pesticides”, „phenols and their derivatives”, „freons”, „dioxins”, „benzopyrenes”, etc.

From classical geochemical positions, the notion of ***pollution*** means a change in the chemical properties of the environment, which are not associated with natural processes. When using this term, the biomedical meaning is most often introduced when ***pollution*** is taken into account from the point of view of the impairment of human health (Hoffman et al., 2003).

In this case, pollution means any change in the air, water, soil and food that has undesirable effects on human health, survival or activity.

The severity (degree) of exposure to pollutants is determined by three factors:

The first factor is the nature of the toxic pollutants, *i.e.* how active and harmful the substances tested for humans, plants and animals are;

A second factor is the concentration of pollutants, *i.e.* the content of air, water or soil per volume or mass unit;

The third factor is the durability/persistence of the substance, or the duration of its existence in air, water and soil.

The scale of pollution is divided by location:

- according to *the source of pollution* (around industrial enterprises, livestock farms, oil deposits, etc.);
- corresponding to *the geographical and state regions* (in the region, the basin of a region, republic, state);
- in compliance with *space* (in outer space – for example, the level of aircraft debris, etc.).

Pollution sources are divided according to the availability of pollutants. All sources of industrial emissions and wastewater discharge are *focal points* (point sources) of pollution, while *diffuse sources* are associated with agriculture, chemicalization, surface leakage from polluted areas, etc.

When assessing the impact of pollution on the natural environment, it is necessary to distinguish between *primary, secondary* and *indirect* effects (*e.g.* the direct impact of emissions from a metallurgical or chemical plant causes the primary effect of the vegetation loss in the immediate vicinity, up to the complete destruction of the soil (indirect effect)).

By the nature of the formation, the sources of pollution are divided into *natural* and *products formed by human activity.* Natural pollution is caused by the existence of the pollutant in unpolluted rocks and soils (e.g. fluorine, selenium in groundwater, or mercury, molybdenum in the rocks of Central Asia). Endemic areas of increased concentrations of some or other ecotoxicants in the environment are formed here.

In the process of natural pollution, in a narrower concept, natural catastrophic processes are also included – a strong volcanic eruption, landslides, etc., as well as geochemical pollution, which appeared in the process of formation and development of our planet. These processes can be both positive and negative. In the first case, there is an excess of element in a certain locality, and in the second, there is a deficiency (as an example – the excess of fluor, selenium and the deficiency of iodine in the groundwater of Moldova).

Pollution caused by human activities, called anthropogenic

Human actions are divided into ***industrial*** (metallurgy, coxochemistry, chemical, pharmaceutical, food, etc., caused by a single enterprise or the whole industry), ***agricultural*** (resulting from the application of fertilizers, pesticides, herbicides and other toxic substances, waste of animals and agricultural production) and ***military*** (chemical, radioactive).

By their nature, all types of pollution are reflected by physical (including mechanical, radioactive, magnetic), physico-chemical, chemical and biological effects (Panin & Bezel, 2008; Kaplin, 2006).

Physical pollution is associated with changes in physical environmental factors, such as: temperature – thermal pollution; wave parameters – electromagnetic pollution; noise – noise pollution; radiation parameters – radioactive contamination.

The only form of **physico-chemical** pollution is aerosol pollution, that is, air pollution by fine and solid liquid substances; an example of this form of pollution is the industrial smog or just the smoke.

The penetration of chemicals into the environment, which are initially lacking in this media or which are altering the natural concentration to a level above the normal rate, is called **chemical pollution** (heavy metals, pesticides, salts, detergents, polychlorinated biphenyls, dioxins, dyes, pharmaceuticals, etc.).

Biological pollution is associated with the introduction into and reproduction in the environment of allogeneic organisms, as well as with substances eliminated by microorganisms (they are the most toxic, known thousands of years ago), plants (during flowering – allergenic substances), products of decomposition of living organisms, various viruses and pathogenic microorganisms.

Natural sources of bioavailable xenobiotics, according to the WHO, include: windborne dust particles, sea salt, aerosols, volcanic activity, forest fires, biogenic particles of volatile nutrients (WHO. Promotion of Chemical Safety Unit & International Programme on Chemical Safety, 1992).

Most of the waste is generated in cities, where most of the world's population lives, and most of the various industrial sectors are concentrated. The anthropogenic flows of matter formed during the production activities of the urban population are extremely diverse, containing high concentrations of a wide range of chemical elements, including toxics (Ballschmiter & Zell, 1980).

By engaging in cycles of natural migration, anthropogenic flows lead to the rapid spread of pollutants in the components of the urban landscape, where their interaction with humans is inevitable (Izrael,1984).

The military actions have a significant impact on the environmental pollution process. As a result of the Second World War, thousands of tons of metals were stored on the battlefields. In1999,following a NATO military operation (ECETOC, 2021; https://moldova.europalibera.org/a/dou%C4%83-decenii-de-la-decizia-nato-de-a-interveni-militar-%C3%AEn-serbia/29508829.html).

Thus, on a relatively small scale in Yugoslavia, it has been observed that air, water and soil pollution has increased in Serbia both through the consumption of these toxic elements – lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) – but also due to the inadequate quality of food products imported or received for humanitarian purposes. According to the overall effect of forces on the environment in peacetime, this is comparable to the effect of a medium-sized industry (about 4% of all wastewater discharges, and 1.2% of emissions).

Ecotoxicology is targeted in three compartments:

- **Ecotoxicokinetics** investigates the fate of ecotoxicants and xenobiotic substances in the environment around (source/origin, distribution of abiotic and biotic elements of the environment, transformation and elimination from the environment around the world);
- **Ecotoxicodynamics** examines the specific mechanisms of development and forms of manifestation of the toxic process caused by the action of ecotoxicants and xenobiotic substances on biocenoses and/or the population, as well as on its components;
- **Ecotoxicometry** analyzes the methodological techniques for evaluating the ecotoxicity of pollutants.

1.2. ECOTOXICOKINETICS

Ecotoxicokinetics is a basic direction of ecotoxicology that investigates the processes that determine the diffusion of xenobiotics and ecopollutants in the ambient environment, namely:

- sources of their occurrence;
- distribution in the abiotic and biotic environmental elements;
- conversion/transformation of xenobiotics into medium;
- their elimination from the environment.

Among the ***sources of bioavailable ecotoxicants***, according to WHO (WHO, 1992) are: *dust particles, sea salts brought by the wind, volcanic activity, forest fires, biogenic particles, biogenic volatile substances*. However, the most *dangerous* source of ***xenobiotics in the environment***, the magnitude of which is constantly increasing, is human activity.

Among the substances persistent in the environment are also included the ecotoxicants, of which heavy metals and trace elements – in concentrations at the level of parts per million (ppm) – form one of the most numerous groups of ecotoxicants (lead, copper, zinc, nickel, cadmium, cobalt, antimony, mercury, arsenic, chromium, vanadium, molybdenum, selenium, strontium, boron, bismuth, beryllium, etc.), and xenobiotic substances – polycyclic hydrocarbons, dioxines and benzofurans, polychlorinated biphenyls, pesticides (in particular organochlorine pesticides – DDT, HCH, aldrin, lindane, etc.), pharmaceutical products, detergents, dyes, and many other substances.

Substances obtained by chemical synthesis, which as a rule are quite resistant to destruction, being persistent in the environment, are the most dangerous substances (Table 1.1).

The release of persistent pollutants into the environment leads to their accumulation, being the most vulnerable (sensitive) part of the ecosystems. After the cessation of their persistent toxic release, these remain in the environment for a long time. Thus, in the waters of Lake Ontario, high concentrations of the pesticide Mirex were determined in the 1990s, whose use was stopped in the late 1970s.

Table 1.1. The half-life of some xenobiotics (Kutsenko, 2002)

Xenobionts	Half-life	Substrate
DDT	10 years	Sol
TCDD	9 years	Sol
Atrazine	25 months	Apă (pH 7.0)
Phenantrene	138 days	Sol
Carbofuran	45 days	Apă (pH 7.0)
Phosphorous thiazoline	21 days	Sol (t + 15^0)
Yperite	7 days	Sol (t + 15^0)
Sarin gas	4 hours	Sol (t + 15^0)

In aquatic test ecosystems in Florida, in the period 1962-1964 it was sprayed for research purposes the Agent Orange, which for 10 years was observed in amounts of 10-35 ng/kg (CLA in the USA being of 0.1 pg/kg) (Lysenko & Dogadina, 2015).

PROCESSES OF POLLUTANT ELIMINATION BY NON-DESTRUCTIVE PROCESSES

Evaporation and dilution processes. Some processes occurring in the environment contribute to the elimination of xenobiotics in the region, changing their distribution in environmental components. A pollutant with a high vapour pressure can easily evaporate from water and soil, and then migrate in other regions under the action of air currents. This phenomenon highlights the ubiquitous distribution of relatively volatile organochlorinated insecticides such as lindane and hexachlorobenzene (Katrin Franke Bader, 2012; Panin & Bezel, 2008).

Elimination under the influence of wind and atmospheric currents. The entrainment by wind and atmospheric currents of the toxic or soil particles on which the substances are adsorbed is also an important way of redistributing the pollutants into the environment.

In this regard, the example of polycyclic aromatic hydrocarbons (benzo[a]pyrene, dibenzopyrene, benz[a]anthracene, dibenz[a,h]anthracene, etc.) is characteristic. Benzo[a]pyrene and related compounds, both of natural (mostly volcanic) and anthropogenic origin (emissions from the metallurgical industry, the oil refining industry, thermal power plants, etc.), are actively involved in the biosphere circulation of substances, moving from one environment to another. In this case, as a rule, they are associated with solid particles of atmospheric dust.

Dust (1-10 μm) is preserved in the air for the long term and larger dust particles settle quickly enough on the soil and in the water at the site of formation. Moreover, the more intense the emission, the more dispersed the pollutants are.

The absorption of substances on particulate matter in water, followed by precipitation, leads to their elimination from the layers of water but increases their accumulation or level in sediments.

The redistribution of water-soluble substances depends on the nature of the rains and the circulation of groundwater. For example, herbicide atrazine, used to protect forest plants in rural farms and parks in the United States, is practically detected permanently in the surface waters. According to some investigations, up to 92% of the water bodies studied in the USA contain this pesticide. Since the substance is sufficiently stable and easily soluble in water, it migrates into groundwater and accumulates there.

ABIOTIC TRANSFORMATION AND DESTRUCTION

A large number of processes affect the persistence of some substances in the environment. The main processes are photolysis (destruction under the influence of light), hydrolysis (destruction under the influence of water) and oxidation (destruction under the influence of oxidation-reduction processes) (Vergeichik, 2009).

Photolysis. Light (especially ultraviolet rays) destroys chemical bonds and thus chemicals are decomposed. Photolysis occurs mainly in the atmosphere and on the surface of the soil and water. The rate of photolysis depends on the intensity of the light and the ability of the substance to absorb it. Unsaturated aromatic compounds, such as polycyclic aromatic hydrocarbons (PAHs), are increasingly sensitive to photolysis because they absorb light energy. Light accelerates other processes of substance degradation – hydrolysis and oxidation. In turn, the presence of photooxidants in the environment, such as ozone, nitrogen oxides, formaldehyde, acrolein, organic peroxides, significantly accelerates the process of photolysis of other pollutants.

Hydrolysis. Water, especially when heated, quickly destroys many substances. Essential bonds, for example in molecules of organophosphorus compounds, are very sensitive to the action of water, which determines the moderate resistance of these compounds in the environment.

The hydrolysis rate is largely dependent on pH values. As a result of the transformation of chemicals in the environment, new substances are formed. However, their toxicity may sometimes be higher than that of the original agent. Photochemical transformations in the environment of 2,4,5-trichlorophenoxiacetic acid, a well-known herbicide, can lead to the formation of an even more dangerous pollutant - 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Another well-known example is ***the formation of nitroso compounds*** (Vergeichik, 2009). Thus, according to scientists, in the soil (in an acidic environment), a number of pesticides are easily associated with nitrites. Among them are dialkyl thiocarbamates, thiocarbamoyl disulphides, phenoxyacetic acid salts, etc. The resulting nitroso compounds are currently considered to be potential carcinogenic substances.

Oxidation. The presence of photooxidants in environment, such as ozone, nitrogen oxides, formaldehyde, acrolein, organic peroxides, significantly accelerates the photolysis process. The decomposition of some xenobiotics in the process of oxidation diminishes their toxicity in aquatic ecosystems.

BIOTIC TRANSFORMATION

Abiotic destruction of chemicals usually occurs at low speed. Xenobiotics degrade significantly faster with the participation of biota, especially microorganisms (mainly bacteria and fungi), which use them as nutrients.

The process of biotic destruction occurs with the participation of enzymes. Biological transformations of substances are based on the processes of oxidation, hydrolysis, dehalogenation, cleaving of the cyclic structures of the molecule, cleaving of alkyl radicals (dealkylation), etc.

The degradation of a compound can be complete by the complete destruction, that is, mineralization (the formation of water, carbon dioxide and other simple compounds). It is also possible to form intermediate products of biotransformation of substances with a toxicity sometimes higher than the original agent (Perminova et al., 2020).

Thus, the transformation of inorganic mercury compounds can lead to the formation of more toxic organo-mercury compounds, in particular methylmercury. A similar phenomenon occurred between 1950 and 1960 in Japan, on the shores of the Minamata Bay, considered as one of the worst environmental catastrophes having human causes.

BIOACCUMULATION

Many toxic substances accumulate in the body of animals and plants, especially in aquatic organisms. The process by which organisms accumulate toxins, eliminating them from the abiotic phase (water, soil, air) and from nutrition (through the foodchain), is called bioaccumulation. Bioaccumulation has negative consequences both for the organism itself (reaching a harmful concentration in critical tissues) and for organisms that use this species as food.

The aquatic environment provides the best conditions for the bioaccumulation of compounds. Here there are myriads of aquatic organisms, which filter the water and accumulate a huge amount of substances diluted in the water up to the toxic level. Hydrobionts accumulate substances in concentrations sometimes thousands of times higher than those normally contained in water (Zubcov et al., 2012, 2013 a, b).

An example of an aquatic food chain, in the direction of increasing body size, is the following: dissolved substances – phytoplankton – planktonic invertebrates – benthic invertebrates – fish – birds of prey – poikilothermic animals that feed on fish.

If foreign substances are consumed, and if these substances cannot be „digested” or simply eliminated from the body, they begin to accumulate along the foodchain, especially if the substance has a long biological half-life.

The rate of accumulation of poisons that in most cases do not decompose, is about 10 for each stage of the food chain. In addition, the accumulation of toxic substances in trophic chains is often increased due to the slower response and limited mobility of animals accumulating these substances, since more poisonous individuals become more easily prey for predators than all other components of the food chain.

As a result, in the food chain of the aquatic ecosystem, the highest content of toxic substances is often observed in detritophagous and predatory fish. Poisonous substances in fish get into the body of predatory birds, pinnipeds, as well as, last but not least, through fish products in the human body.

Factors affecting bioaccumulation

The bioaccumulative tendency of ecotoxicants depends on a number of factors. The first is the persistence of xenobiotics in the environment. The degree of accumulation of a substance in the body is ultimately determined by its content in the environment. Substances which are quickly eliminated, generally are insufficiently accumulated in the body. An exception is the conditions in which the pollutant is constantly introduced into the environment (regions near industries, etc.). For example, cyanuric acid, which is a highly toxic compound due to its high volatility, is not a potentially dangerous substance, in the opinion of many experts. However, to date it has not been possible to completely exclude the fact that certain types of diseases, pregnancy disorders in women living near gold mining enterprises, where cyanides are eliminated in large quantities, are not associated with the chronic action of this substance. After the substances enter the body, their fate is determined by ***toxicokinetic*** processes. Soluble (lipophilic) substances, slowly metabolized in the body, have the highest bioaccumulation capacity.

The fatty tissue, as a rule, is the main place of long-term deposition of the xenobiotics. Thus, many years after exposure, high levels of Orange Agent were found in the adipose tissue and blood plasma of veterans of the U.S. Army and participants in the Vietnam War.

Many lipophilic substances are prone to sorption on the surfaces of various precipitated particles in water and air, which reduces their bioavailability. For example, benzopyrene sorption with humic acids reduces the ability to bioaccumulate in fish tissues and diminishes the toxicity three times. Fish in ponds with a low content of particles suspended in water accumulate more DDT than fish in eutrophic ponds with a high content of substances in suspension.

Substances that are metabolized in the body accumulate in smaller quantities than might be expected, based on their physicochemical properties. Inter-specific differences in the values of bioaccumulation factors of xenobiotics are largely determined by the particularity of their metabolism.

The importance of bioaccumulation. Bioaccumulation can be the basis not only of chronic acute toxic effects, but also of delayed ones. Thus, the rapid loss of fat, in which a large amount of the substance accumulates, leads to the release of a toxic product into the blood stream. Mobilization of adipose tissue in animals is often observed during the breeding season. In environmentally unfavorable regions, this can be accompanied by the mass death of animals when they reach the sexual maturity.

Persistent accumulated pollutants can also be transmitted to baby birds – with the contents of the yolk sac, to fish – by the content of roe, and in mammals – through breast milk. In this case, the offspring may develop effects that do not manifest in the parents (Katagi, 2010).

BIOAMPLIFICATION

Chemicals can circulate through chains and from victim organisms to consumer organisms. For substances with a high degree of lipophilia, this migration may be accompanied by an increase in the toxic concentration in the tissues of each subsequent organism of the trophic chain. This phenomenon is called **biomagnification** or **bioamplification** (Gobas et al. 1999; Kutsenko, 2002).

It is known that DDT was used to kill mosquitoes on one of the lakes in California. After processing, the pesticide content in the water was 0.02 ppm. After a certain period of time in plankton, DDT was determined at a concentration of 10 ppm, in the tissues of fish that consume plankton – 900 ppm, in predatory fish – 2700 ppm, and the birds that consumed fish accumulated 21000 ppm. The DDT content in the bird tissues that were not directly affected by pesticides was 1,000,000 times higher than in water and 20 times higher than in the first step of the food chain.

In the book „Silent Spring", Rachel Carson (1962) gives such an example. To combat the carrier of the „Dutch disease" affecting the elms – the European elm bark beetle *Scolytes multistriatus* – the trees were treated with DDT. Part of the pesticide ended up in the soil, where it was absorbed by earthworms and accumulated in the tissues. Migratory birds, which eat mostly earthworms, have accumulated this pesticide. One of them died, and at the other ones the reproductive function was affected and they laid sterile eggs. As a result, the fight against tree diseases led to the almost complete disappearance of the birds in numerous regions of the USA.

1.3. ECOTOXOCODYNAMICS

Ecotoxicodynamics is a compartment of ecotoxicology focused on deciphering specific mechanisms and toxic processes caused by the action of toxins on the communities of organisms and/or on biocenosis. The mechanisms by which substances can cause adverse effects in biogeocenosis are numerous but unique in each case. However, they can be classified. Thus, we can highlight the direct, indirect and mixed action of ecotoxicants.

- **Direct action – is the direct loss of the organisms of one or more populations due to some or more ecotoxicants or xenobionts of a xenobiotic profile.**

The mechanism of the direct action of the toxic substances leads to the death of representatives of sensitive species. The use of effective pesticides leads to the mass death of pests: insects (insecticides) or weeds (herbicides). This ecotoxic effect creates a strategy for the use of chemicals. However, in some cases there are associated negative phenomena. For example, in Sweden in the 1950s and 1960s, the methylmercriducyanamide was widely used to treat crop seeds. The mercury concentration in the cereals was higher than 10 mg/kg. The periodic storage of the treated seeds by the birds led to the fact that, after a few years, it was observed the massive death of pheasants, pigeons, partridges and other birds that had eaten cereal seeds and had been chronically intoxicated with mercury.

When assessing the environmental situation, it is necessary to take into account the fundamental law of ecotoxicology: ***the sensitivity of different types of living organisms to chemicals is always different.*** Therefore, the appearance of the pollutant in the environment, even in small quantities, can be detrimental to representatives of the most sensitive species. As an example, lead chloride kills daphnia during the day when it is contained in water in a concentration of about 0.01 mg/l, but these amounts pose a low risk to other species (Kutsenko, 2002).

- **The indirect action – represents the situation in which the xenobiotic profile or some abiotic components of the environment are no longer optimal for the existence of the population and, as a result, it no longer develops.**

The indirect action of the xenobiotics leads to the development of allobiotic states and special forms of the toxic process. In the late 1980s, about 18 thousand seals died in the Baltic Sea, North Sea and Ireland as a result of viral infections. In the tissues of dead animals, a raised content of polychlorinated biphenyls (PCBs) was found. It is known that PCBs, like other chlorine-containing compounds such as DDT, hexachlorobenzene, dieldrin, have an immunosuppressive effect on mammals. Their accumulation in the organisms led to a decrease in the resistance of seals to infections. Thus, without having direct action, such as the death of animals, the pollutant significantly increased their sensitivity to the action of other unfavorable environmental factors (Kutsenko, 2002; Panin & Bezel, 2008).

A classic example of this form of ecotoxic effect is the increase in the number of neoplasms and the decrease in reproductive abilities in the populations of people living in regions polluted by toxicants (the effect of dioxins in South Vietnam).

➢ Mixed action

Several toxic substances especially xenobionts have an effect both direct and indirect, that is, their influence is reflected by ***mixed action***. An example is that of the herbicides 2,4,5-T and 2,4-D which contain as an impurity a small amount of 2,3,7,8-tetrachloro-p-dioxin. The widespread use of these substances by the U.S. military in Vietnam caused direct damage to the country's flora and fauna and, indirectly, also affected human health (Panin & Bezel, 2008).

➢ *The action of chemicals depends on their toxic capacity and persistence*

Ecotoxicity represents the ability of a xenobiotic profile of the environment to cause adverse effects in the corresponding biocenose. In cases where the natural xenobiotic profile is associated with the excessive accumulation of a single pollutant in the environment, it is possible to conditionally speak of the ecotoxicity of only this substance.

According to Bezel et al. (1994), the adverse ecotoxic effects can be evaluated at different levels:

- ***at the level of the body*** – it is manifested by a decrease in resistance to other active environmental factors, a decrease in activity, the incidence of diseases, the death of the body, carcinogenesis, impaired reproductive functions, etc.
- ***at the level of the population*** – we observe the obvious loss of the population, the increase of morbidity, mortality, the decrease in the birth rate, an increase in the number of congenital defects, the violation of the demographic characteristics (age, sex, etc.), changes in life expectancy, cultural degradation;
- ***at the level of biogeocenosis*** – we observe obvious changes in the composition of the population until the disappearance of certain species and the appearance of new ones, which are not characteristic of this biocenosis, by destroying the interspecific relations.

In the case of the ecotoxicity assessment for only one substance in relation to representatives of only one species of living communities, the full qualitative and quantitative characteristics are considered, as well as in the classical toxicology (acute and subacute value, chronic toxicity, dose and the concentration causing mutagenic, carcinogenic, teratogenic effects, etc.).

However, in more complex systems, ecotoxicity is not measured by numbers (*quantitative*) and is characterized by a number of *qualitative* or semi-quantitative indicators, by the notions of „*danger*" or „*environmental risk*" (Hoffman et al., 2003).

Depending on the duration of the action of ecotoxicants ***on the ecosystem***, we can speak of acute and chronic ecotoxicity.

➢ Acute ecotoxicity

The acute toxic effects of substances on biocenoses can be the result of accidents and catastrophes, accompanied by the release into the environment of a large number of relatively unstable toxic substances or improper use of chemicals.

They are already well known different events. Thus, in 1984, in the city of Bhopal

(India), there was an accident at the factory of the American company Union Carbide of chemicals for the production of pesticides. As a result, a large amount of pulmonotropic metil isocyanate ended up in the atmosphere. Being a volatile liquid, the substance formed an unstable source of infection. However, about 200 thousand people were poisoned and 3 thousand of them died. The main cause of death was acutely developed pulmonary edema. Experts point out that in Bhopal there were unusually many children born with defects or who have developmental deficiencies, as well as various forms of cancer, diabetes and other chronical diseases (Kutsenko, 2002).

Another known case of acute toxicological and ecological disaster occurred in Iraq. The government of this state bought a large batch of grainseeds. Cereals sown for the purpose of pest control were treated with a fungicide (methyl mercury). However, this batch of cereals accidentally went on sale and was used for baking bread. As a result of this ecological disaster, more than 6.5 thousand people were poisoned, of which about 500 died. In 2000, in Romania, in one of the companies for the extraction of precious metals, as a result of the accident, there was a leakage of hydrocyanic acid and cyanide-containing products. Toxicants in large quantities entered the waters of the Danube, poisoning all living organisms hundreds of kilometers downstream (Kutsenko, 2002; Oprea, 2007).

The biggest environmental disaster was the use of highly toxic chemicals for military purposes. During the First World War, the conflicting countries used about 120 thousand tons of poisonous substances on the battlefields. As a result, more than 1.3 million people were poisoned, which can be considered one of the greatest environmental disasters in the history of mankind.

Acute ecotoxic effects do not always lead to the death or acute diseases of humans or other affected species. Thus, among the poisonous substances used in the First World War, there were also carcinogenic substances, which caused late death due to the formed neoplasms (Kutsenko, 2002; Lysenko & Dogadina, 2015).

➢ Chronic ecotoxicity

Chronic damage occurs with prolonged exposure to low concentrations. Sublethal effects are usually associated with chronic toxicity of substances. Often this implies a violation of reproductive functions, changes in the immune system, endocrine pathology, malformations, allergies, etc. However, chronic exposure to a toxic agent can also lead to deaths among individuals depending on the level of individual resistance.

The effect of long-term exposure on sulfur dioxide can be very noticeable. Vegetation studies in the area of metallurgical furnaces in Ontario (Canada) showed that at a distance of 16 km from them, 25 species of plants grew in a normal state and, as they approached the furnaces, their number decreased. At a distance of less than 1.6 km, no plant grew (Lysenko & Dogadina, 2015).

➢ Embryotoxic effect

It is well established that DDT, which accumulates in the tissues of birds such as wild duck, angler eagle, bald eagle, etc., leads to a thinning of the eggshell and, consequently, to the hitting and/or death of chickens. This is accompanied by a decrease in the number of birds (Frumin, 2013).

Examples of the toxic effects of various xenobiotics (including drugs) on human and mammalian embryos are well known.

➢ *Direct action of the product of biotransformation of pollutants with an unusual effect*

Field observations of fish in the state of Florida made it possible to identify populations with a large number of females with obvious signs of masculinization (strange behavior, alteration of the fin, etc.). These populations were found in a river downstream of an effluent and it was assumed that the effluent contained masculinizing agents.

However, studies have shown that in emissions there are no such substances: wastewater did not cause masculinization. It was also found that phytosterone was contained in wastewater (formed during the processing of raw materials), which, in the river water, was exposed to the influence of bacteria that converted the given substance into androgen, which caused an adverse effect (Kutsenko, 2002).

Thus, the interaction between xenobiotics and the biotic component of the environment (microorganisms) can cause significant effects on the population in the biocenosis.

➢ *Action mediated by reducing the food resources of habitats*

In one of the Canadian regions, an organophosphate pesticide with rapidly degradation in the environment has been used to combat pests (spruce worms) in the forestry industry. As a result of a sharp decrease in the number of caterpillars, about 12 million birds died from starvation.

The explosion of population size can occur due to the destruction of a competing species. In the United States, after the start of the use of synthetic pesticides to control certain types of plant pests, a small number of the mat butterfly caterpillar began to multiply rapidly, their number increasing from 6 to 16. This phenomenon is explained by the fact that in the world of insects, there is a complex system of relationships, and the number of species in a population of herbivorous insects is often controlled by other species, which are predators of these insects. The effects of pesticides may be more pronounced on predatory species. It is easy to see that the mechanisms of the ecotoxic action of substances on animals, under other conditions, can be used to estimate the impact on humans (Kutsenko, 2002).

➢ *The usual mechanisms and diseases of toxics entering the body*

The absorption of toxics represents their passage from the external environment into the blood or circulating lymph. The absorption of toxic substances, with all their structural variety, is based on common laws that involve transport through the membrane. The absorption of toxic substances is differentiated, depending on the segment of the digestive tract. Substances that are absorbed by this route (*e.g. cocaine, nicotine, potassium cyanide)* avoid the liver barrier and their detoxification is limited. Absorption is done by simple diffusion (passive transport) and active transport (Robinson &Thorn, 2005).

The active transport is carried out against the concentration gradient, with energy consumption. In clinical toxicology, active transport is present only for a limited number of toxics absorbable by digestive route and exceptionally, for those that penetrate transcutaneously or by lung route. By active transport are absorbed: *fluorides, chlorine, nitrites, nitrates, antimony, lead, copper, chromium.*

Simple diffusion is based on the selective permeability of the membrane and is generally produced without energy consumption, representing the most frequent mechanism of transport through biological membranes (Katrin Franke Bader, 2012).

Among the toxics that are absorbed by the mechanism of simple diffusion we can mention: *halogens, gaseous derivatives of sulfur, nitrogen, phosphorus, arsenic, carbon sulfide, ethyl alcohol, ethyl ether, phenol, carbon tetrachloride, etc.*

- ***The main ways of penetration of toxics into the body are:***
  - ***DIGESTIVE ROUTE:*** consumption of toxic plants, water containing toxic substances, fodder contaminated with toxics or toxins;
  - ***RESPIRATORY ROUTE:*** it is characteristic for volatile toxics, gaseous toxics or toxics in the form of aerosols (Katagi, 2010).

The respiratory route is a severe route of intoxication, because the absorption of toxics is done quickly, detoxification is diminished, toxics bypassing the liver barrier. Exceptions are liver-activated toxics (*e.g.* parathion). The respiratory route absorbsn the **volatile toxics** *(chloroform, ether)*, **gaseous toxics** *(carbon oxide, hydrocyanic acid, sulfur dioxide, hydrogen sulfide, ammonia)*, sublimating products, fine aerosol particles (less than 5 μ), electrically charged metal powders. **The mucous membranes** of the anterior airways (nasal, tracheal, bronchial) are of little importance for absorption. In general, toxics are expelled by coughing, sneezing. However, repeated contact can lead to intoxication (for example, alkaline salts such as sodium iodide, sodium salicylate can be absorbed). **The pulmonary system** has a high absorption capacity due to its large area, structure and vascularization.

- ***SKIN PATHWAY*** and through apparent mucous membranes: skin treatment of potentially toxic substances, accidental contact (Gobas et al., 1999).

The absorption of toxics through intact skin is difficult due to its structure and the hydrolipidic layer. The skin is considered a physiological barrier to foreign particles and chemical molecules. There are, however, two possibilities for the penetration of toxics into the capillaries in the dermis and from here into the general circulation, namely **transepidermic** (for fat-soluble substances with high coefficient of sharing) and **transfolicular** (from sebum into the sebaceous glands or hair follicle and from here to the dermis). ***Transcutaneous*** can be absorbed the gaseous and volatile substances (*hydrogen sulfide, carbon oxide, carbon dioxide, formic aldehyde, hydrocyanic acid), a* series of organic substances (liquid alyphatic hydrocarbons from C_6 to $C_{10;}$ *cyclic, terpene, aromatic hydrocarbons; alcohols, esters: acetates, butyrations; phenols; chlorinated solvents; organophosphorus insecticides, organochlorinated insecticides; liquid alkaloids: nicotine,* etc.), mineral substances (directly or after transformation in contact with the fatty acids in sebum – *thallium salts, some mercury salts, lead and bismuth salts, alkaline iodines).* In general, substances with high molecular weight hardly cross the skin, while toxics with low molecular weight, fat-soluble, non-ionic and non-pollar, penetrate the skin more easily. ***The transcutaneous absorption*** is favored by massage (by compressing the follicles), by sweating (by dissolving the toxics on the skin), by erosions (by discovering the lymphatic circulation of the chorith), by organic solvents, keratolytic substances, detergents.

- ***PARENTERAL PATHS*** are rarer ways of toxics penetration. Poisoning can occur in these ways by overdosing medicines, administration in unusual ways or fraudulent injection (Hoffman et al., 2003). Most of the poisoning is due to penetration in **multiple ways.**

The intravenous route allows the rapid absorption of toxics, the substances avoiding gastric and intestinal barriers.

The subcutanate path. The substance being introduced into the hypodermic tissue, by diffusion, reaches the circulation and exerts its toxic effect.

The intramuscular route ensures faster absorption of substances than the subcutaneous one. From the muscles, due to the rich vascularity, the substances reach the circulation by diffusion.

Water-soluble substances are most rapidly absorbed. The amount of toxic bound depends on the concentration of the toxic, the affinity for the places of fixation, the available coupling capacity of the plasma proteins. **There was a competitiveness** for the places of coupling between the toxics from the same group: the weak acids between them, the weak bases between them. Basic toxics have more coupling places than acidic ones.

1.4. ECOTOXICOMETRY

Ecotoxicometry is a compartment of ecotoxicology, within which the methodological techniques that make it possible to evaluate the ecotoxicity of toxic substances prospectively or retrospectively are taken into account. All the types of classical quantitative toxicological studies are fully used to determine ecotoxicity.

The acute toxicity of ecotoxicants is determined experimentally on several species representing different levels of trophic organization in an ecosystem (algae, plants, invertebrates, fish, birds, mammals).

The US Environmental Protection Agency (2011) calls for toxicity to be determined in at least 8 different freshwater types and marine organisms (16 tests) in determining criteria for the quality of water containing a toxic agent (EPA U.S., Clean Water Act Policy and Guidance Documents, 2018).

Repeated attempts have been made to classify the species of living beings by their sensitivity to toxic substances. However, for various toxic substances, the ratio of the sensitivity of living organisms to them is different. In addition, the ecotoxicological use of the ‘standard species’ of representatives of certain levels of ecological organisation to determine ecotoxicity from a scientific point of view is incorrect because the sensitivity of animals, even of species in these families, sometimes differs very significantly.

TYPES OF POISONINGS

Accidental poisoning can occur under **natural** conditions or in **man-made** conditions.

Poisoning in natural conditions occurs with toxic substances that are commonly found in nature, such as toxic minerals in drinking water (*fluorides, nitrates, iron)* or accumulated in plants *(molybdenum, selenium, cadmium)*, from toxic plants (especially *in drought conditions, affecting young animals at the beginning of grazing)*, venomous insects (*bumblebees, wasps, spiders)* and venomous snakes.

Poisoning in conditions created by human activity are the most common. The causes are: industrial pollutants, pesticides, chemical fertilizers (nitrate, phosphate, potassium), medicines, feed additives, mycotoxins. The poisonings caused are those made for criminal or euthanasic purposes.

Factors influencing toxicity. Toxicity factors are the totality of the conditions on which the toxicity of a substance or its degree of harmfulness depends (climatic conditions, water and soil temperature values, oxidation-reduction processes, etc.).

Toxicity depends on the physico-chemical properties of the toxic factor and the organisms, populations or ecosystems subjected to their influence (species, age, body weight, sex, degree of fullness of the digestive tract, diet, state of maintenance, state of health, predisposition, individual sensitivity, ratio between different species, genera).

PHYSICO-CHEMICAL PROPERTIES IN THE CHEMICAL SIDE, AGGREGATION SALT AND PARTICLE SIZE

Liquid salt promotes absorption and increases toxicity. Gaseous substances are rapidly absorbed and have a high degree of toxicity. The amorphous forms of solid toxics, being more soluble, are more easily absorbable and, as a result, more toxic, having an increased degree of toxicity.

Vegetable toxics. Their toxcity is influenced by the pedoclimateric and meteorological factors (in drought conditions, in the steppe regions, in some plants are concentrated cyanogenetic glycosides), the plant part (toxics are concentrated in the roots, seeds, pollen), the vegetation stage (toxics are found in larger quantities in certain phases of vegetation, for example glycosides and cyanogenetics – in maize, in the milk phase); in some plants the most frequent cases are found in pollen and flowers.

Combinations of substances may alter their activity in the sense of increasing or reducing toxicity. The associations may be synergistic (increase toxicity) or antagonistic (reduce toxicity).

COMBINATION OF TOXIC EFFECTS

Additing (1+1=2) (e.g. cocaine and adrenaline): the result represents a sum of the toxic effects of several toxicants;

Synergism (1+1>2): enhancing the toxic effects of a mixture of substances more than the sum of their toxic effects (Liess et al., 2016);

Antagonism (1+1<2): it treats toxicity through physical, chemical or pharmacodynamic effects. Antagonism can be reversible or irreversible. A particular case is antidotism. For example: tannins precipitate alkaloids; copper poisoning can be prevented by molybdenum and vice versa; potassium cyanide in combination with glucose is converted into cyanhydrin, a non-toxic product.

Clasification of the pollutants after their action

- ✓ **IRRITATING POLLUTANTS** cause irritative effects on the ocular mucosa and especially on the respiratory system. This group includes non-toxic powders, as well as a sum of gases and vapors such as *sulfur dioxide, nitrogen dioxide, ozone and oxidizing substances, chlorine, ammonia, etc*. (Katrin Franke Bader, 2012).

Irritating pollution is the most widespread of the types of pollution, resulting primarily from the processes of fuel combustion, but also from other sources.

- ✓ **FIBROSING POLLUTANTS** produce fibrous changes in the respiratory system. Among the most common are *silicon dioxide, asbestos and iron oxides, cobalt compounds, barium, etc*. They are much more aggressive in the industrial environment, determining specific illnesses. Intense dust pollution can lead to fibrous pulmonary changes (Katagi, 2010). **Powders** resulting especially from the building materials industry (*cement, asbestos, gypsum, magnesite)* can cause different symptoms to the human body, generally having ***irritating, toxic, allergic and carcinogenic action***. The mode of action of powders on the body depends on their physical and chemical structure. *The irritating action* is the consequence of the sharp shape or of a certain hardness of the particles that trigger a defense reaction from the exposed tissues or organs. Powders cause discomfort and specific diseases called coniosis. In the affected organ or tissue we observe different ***dermo-, ophthalmo-, entero-*** or ***pneumopathologies***.
- ✓ **ASPHYXIATING POLLUTANTS** are those that prevent the supply of oxygen to the body's tissues. Among the most important are *carbon oxide, nitrates, nitrites,* which form with hemoglobin a relatively stable compound *(carboxyhemoglobin)* and derivatives incapable of transporting oxygen and thus preventing blood oxygenation and the transport of oxygen to tissues (Gobas et al., 1999).

Depending on the concentration in the air and the exposure time, a certain proportion of carboxyhemoglobin is achieved, which can exceed 60% of the total hemoglobin, blockage of cellular respiratory enzymes *(cyanides)*, damage to hematoforming organs *(radioactive substances)*.

Acute *carbon oxide* poisoning is relatively rare, appearing practically only in enclosed spaces in the presence of important sources of CO (in rooms where the heating systems are malfunctioning, garages, underground passages for motor vehicles, in closed vehicles, etc.)

- ✓ **ALLERGENIC POLLUTANTS** in the atmosphere have been known for a long time. We can mention here the case **of natural pollutants *(pollen)*** as well as ***dust in the house*,** responsible for a very large number of respiratory or skin allergies (Hoffman et al., 2003).

Besides these, there are added pollutants from artificial sources – especially industrial – which can emit into the atmosphere a sum of complete or incomplete allergens. On the first place from this point of view, there is the*chemical industry* (plastics industry, pharmaceutical industry, insecticide factories, etc.). There are also reported situations with the occurrence of mass allergic phenomena, an example may be the one produced in New Orleans in 1958 in which the allergen was *identified in dust from industrial waste* (Kutsenko, 2002).

Allergens are substances that activate the immune system. Some allergens act directly as antigens, being recognized as foreign substances by white blood cells and stimulate the production of specific antibodies (proteins that recognize and bind to cells or chemicals foreign to the body). Other allergens act indirectly by binding and chemically modifying foreign substances so that they become antigens and cause an immune response.

- ✓ **TERATOGENIC SUBSTANCES** are chemical substances or factors that produce abnormalities during embryonic development and growth. Some compounds, which are otherwise not dangerous, can cause tragic problems at this sensitive stage of life. It is considered that the most widespread teratogenic in the world is alcohol. The consumption of alcohol during pregnancy can lead to a syndrome that involves the appearance of several symptoms: craniofacial abnormalities, mental retardation, behavioral problems.
- ✓ **CORROSIVE SUBSTANCES** act on the contact areas and on the excretory organs *(mineral toxics);*
- ✓ **PROTOPLASMIC AND PARENCHYMATIC TOXIC SUBSTANCES** cause fat degeneration of the liver and a tendency to hemorrhage *(carbon tetrachloride, phosphorus).*
- ✓ **CARCINOGENIC SUBSTANCES** are substances that cause cancer – invasive cells with uncontrolled growth that lead to the appearance of malignant tumors. The rate of cancer increased in most industrialized countries in the twentieth century, in the USA being now the second leading cause of death (Chapman, 2002). In the year 2000, for example, more than half a million people died. However, the increase in the frequency of cancer, especially in urban areas, has required the consideration of air pollutants as possible causative agents, all the more so as carcinogenic substances have been identified in the air in polluted areas.

We can classify the present substances in *organic* substances and *inorganic* substances. Among the inorganic carcinogenic pollutants we mention *asbestos, arsenic, chromium, cobalt, berylium, nickel and selenium.* Among the more widespread organic ones are polycyclic aromatic hydrocarbons such as *benzopyrene, benzoanthracene, benzofluoranthene,* etc. The last carcinogenic substance has been known for a long time, and the presence in the air indicates an increased risk of lung cancer.

Carcinogenic effects are also attributed to *organochlorine insecticides*, as well as to some *monomers* used in the manufacture of plastic masses.

✓ **NEUROTOXIC SUBSTANCES.** Neurotoxins are a special class of substances that specifically attack nerve cells – neurons. The nervous system is so important in coordinating and regulating the activity of organs, that the interruption of these activities causes numerous damages (Katrin Franke Bader, 2012).

The mode of action of neurotoxins may differ. Substances such as **lead** and **mercury** kill nerve cells and cause permanent neurological damage.

Anesthetics *(ether, chloroform)* and chlorinated pesticides *(DDT, dieldrin, aldrin)* break the nerve cell membrane necessary for nerve activity. Organophosphorus substances *(malathion, paration)* and carbamates *(carbaryl, zineb, maneb)* inhibit the enzyme acetylcholinesterase which regulates the transmission of the signal between the nevous cells and tissues or organs.

Most neurotoxic substances have acute and very high toxicity. Currently more than 850 compounds are considered neurotoxic.

The number of potential neurotoxics in the environment is constantly increasing and poses a great risk to humans and the environment. The neurotoxicity assessment is currently carried out to the greatest extent to predict and prevent the disease of human populations.

However, knowledge of the neurotoxic potential of environmental contaminants in ecosystems is very limited, as the neurotoxicity assessment is currently mostly focused on human exposure to individual chemicals.

Well-known human neurotoxic or neuroactive compounds, such as pesticides, pharmaceuticals and heavy metals, occur in the environment along with thousands of chemicals with neurotoxic potential unknown to different species and life stages. It has been estimated that up to 30% of all commercially used chemicals (~30,000 chemicals) may have neurotoxic potential.

In addition, in most aquatic ecosystems are recorded organic contaminants such as pesticides *(DDT, dieldrin, aldrin)*, heavy metals (Hg, Pb, Cd, As), etc. that are neurotoxic ones, but which can be determined by bioanalytical equipment and instruments capable of identifying these substances.

Unfortunately, we are not aware of any regulatory guidance available to identify neurotoxic risks in vertebrates or invertebrate animals. In addition, to date there is no European regulatory framework for the assessment of eco-neurotoxicity.

In the risk assessment and risk management of eco-neurotoxic substances, pesticides are a category of substances of particular interest. Some pesticides kill pests through neurotoxic mechanisms.

The European Food Safety Agency (EFSA) is responsible for the registration of pesticides and all other substances that may come into contact with or occur in food and are not evaluated in accordance with the REACH legislation – Regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals No. 1907/2006.

Water Framework Directive (WFD) (Directive 2000/60/EC of the European Parliament and Council Establishing a Framework for Community Action in the Field of Water Policy; OJ:L327, Dec. 22, 2000), aims at integrating biological and chemical information to obtain an overview of the quality of individual water bodies. According to the WFD, the chemical status of a body of water is determined by analyzing the concentrations of 45 priority substances.

A good chemical status is defined by the concentrations of all these substances below the average and maximum permissible environmental quality standards, which are defined to protect the environment and human health. As a result, regular chemical monitoring of water quality is carried out almost exclusively through targeted chemical analysis of a limited set of compounds (indicators).

However, there are some serious limitations related to the use of target chemical analyses of high volume samples to monitor the overall chemical status of a water body. First of all, since only a limited number of target substances are analyzed, the risk of other unknown substances present in the aquatic environment remains uninvestigated. Moreover, products of the transformation of micropollutants formed in the environment or through biological metabolism are not always known or recorded and may be more toxic and persistent than basic compounds (Katagi, 2010; Katrin Franke Bader, 2012; Vergeichik, 2009).

These limitations can thus lead to an incomplete assessment of chemical hazards and risks, requiring the exploration of alternative approaches. There is an urgent necessity to provide rapid evaluations of substances with unknown toxicological potential in order to prevent possible negative effects on consumers by the water suppliers and public health departments supervising the process.

THE ELIMINATION OF TOXICS FROM THE ORGANISM

The metabolized products and the untransformed fractions are eliminated from the body. In general, toxic substances have selectiveness for a route of elimination, but they can also be eliminated simultaneously in several ways.

A link between the route of penetration of the toxic and the organ of elimination cannot be established (for example, the atropine administered subcutaneously can be found in the stomach; mercury applied to the skin is found in the intestine).

The rate of elimination of toxics depends on the route of administration, the physicochemical properties, the fixation on plasma and tissue proteins, the metabolizations suffered, the route of elimination and the functional state of the route of administration (Chapman, 2002).

Elimination is the removal of the toxics and/or polar and ionized metabolites from the blood, lymph, interstitial fluid, cells or tissues. The route, speed, duration and degree of elimination vary in dependence on a number of physico-chemical and biological factors.

The harmfulness of a toxic is as greater as its elimination is slower.

In general, elimination is achieved through the metabolic processes of several organs, by renal, digestive, pulmonary and transcutaneous pathway.

The digestive tract is of little importance. A distinction should be made between the expulsion by vomit and faeces of insoluble or less soluble substances or those that have not been absorbed, and the elimination of substances that have entered the general circulation.

The elimination can be done by: saliva (alkaloids: quinine, strychnine; metals: mercury, lead, bismuth; iodine), gastric juice (morphine, halogens), bile (only products with molecular weight over 400 kDa, salts of heavy metals: lead, mercury, chromium, nickel; arsenic; alkaloids).

The kidney pathway is the major route for most toxics. This way eliminates toxic substances with a molecular weight lower than 150 kDa. The elimination is achieved by glomerular filtration, tubular resorption or tubular secretion.

Glomerular filtration is a process of passive ultrafiltration of a quantity of water and solvit substances with low molecular weight, polar, untethered to proteins. It depends on the hydrostatic pressure of the blood and the pressure of the blood proteins.

Tubular resorption represents the return to the renal tubules from circulation, by passive process, of large quantities of water, ionized inorganic substances, some fat-soluble non-ionized substances, undissociated acids and weak bases.

Tubular secretion consists of the active transport from blood capillaries to tubules against the concentration gradient. It has specificity and is made with energy consumption.

The kidneys eliminate most of the metal salts, iodides, nitrates, nitrites, alkaloids, pesticides. The determination of concentrations in the urine has diagnostic value for some toxic ones.

The pulmonary pathway is the fastest way of elimination. The elimination is made through the alveolar epithelium, by bronchial or nasal secretion. The speed of elimination depends on the degree of volatility. In this way, gaseous substances (hydrogen sulfide, carbon oxide), gaseous metabolites, volatile substances (alcohol, ether, chloroform, vegetal essences) are eliminated.

The skin is a route of elimination for gaseous, volatile toxics and some solids. Thus, in this way are eliminated the alkaloids, arsenic compounds, heavy metals, iodides, bromides, camphor.

The mammary gland is a route of elimination for volatile compounds (alcohol, ether), organic bases (alkaloids: caffeine, morphine, quinine, antipyrine), acids (acetylsalicylic acid, barbiturate acid), pesticides, aflatoxins, mineral ions (lead, mercury, arsenic, iodine, bromine), chloralhydrate.

The activity of the human society causes multilateral changes in the environment. Before the development of industrial civilization, before the beginning of the XIX century, pollution was extremely limited in nature, quantity and distribution. Nowadays new problems have been added to the old problems – the presence in the environment of non-physical chemical compounds, to which biological systems cannot adapt.

Some terminological notions concerning the examination and assessment of toxicity

Toxics (poisons) are organic or inorganic substances with strong harmful activity on the body, capable of producing disorders even in small quantities. ***The poison can also be defined as a chemical substance that, introduced or formed into the body, produces disturbances or leads to death.***

Toxic – is a word with controversial etymology; it derives from the Greek „*toxon*" (bow with arrows with *poisoned tips*), or from the Egyptian word „*tako*" *(destruction, precipice, death)*. The toxic word derives from the Latin „*toxicus*" (*venomous, poisonous*) which derives by alteration from „taxicus" which, in turn, derives from the name of the plant *Taxus bacata* (yew), which contains a very toxic alkaloid. ***Poison*** – derives from the Slavonic verb „*otrăviti*" (to grieve deeply), a word that refers to the psychic background. Both terms can be used. In other languages, two terms are used for the same notion – so the French use *toxique* and *poison;* the Germans, *toxin* and *gift;* the English, *toxic* and *poison*.

Intoxication presents a morbid state produced under the action of an organic or inorganic substance of vegetal, mineral or synthetic origin, which causes profound disorders of cellular metabolism and which results in the disease of the body.

Toxins are organic substances with protein structure, produced by plant or animal organisms, which cause poisoning phenomena.

Toxicosis is a morbid state produced by toxins.

The dose is one of the most important indicators of toxicity:

- **maximum tolerated dose (DMT)** - the highest amount of substance that is borne by the body free of toxic phenomena;
- **lethal dose (DL)** - the minimum amount of substance causing the death of an adult animal;
- **zero lethal dose (DL0)** - the amount of substance that causes serious, but not fatal, toxic phenomena;
- **lethal dose 50 (LD50)** - the amount of substance that produces lethal effects in 50 out of 100 experienced animals within 24-48 hours;
- **lethal dose 100 (LD100)** - the amount of substance that produces lethal effects in all animals used in the acute toxicity experiment;
- **minimum lethal dose (DML)** - the minimum amount of substance that kills only one animal in the batch;
- **certain lethal dose (DLC)** - the amount of substance that causes death to animals in any situation.

Toxic substances that penetrate the respiratory route are characterized by:

- lethal concentration of toxic in the atmosphere (CL) - corresponds approximately to the lethal dose and is expressed in ppm (mg/m^3);
- lethal concentration 50 (CL50) - the amount of toxic (mg/m^3 air) that causes the death of 50% of the animals experienced within 4 hours;
- lethal concentration 100 (CL100) - the minimum amount of toxic (mg/m3 air) in the air breathed by experimental animals causing 100% mortality.

In the case of medicinal products, the **lethal dose DL50** is reported to the **effective therapeutic dose DE50** and the **therapeutic index (IT)** is obtained. If the therapeutic index is greater than or equal to **10**, the respective medicine does not present a danger at usual doses, and if it is less than **10**, it should be used with caution.

Due to the large differences in toxic doses, Hodge & Sterner (2005) have developed a classification whereby all substances are identified in 6 categories according to the DL50 value (expressed in mg/kg), a classification which is also accepted at the moment:

- extremely toxic (less than 1 mg/kg);
- very toxic (1-50 mg/kg);
- moderately toxic (50-100 mg/kg);
- slightly toxic (500-5000 mg/kg)
- practically non-toxic (5000-15000 mg/kg);
- relatively non-toxic (over 15000 mg/kg).

In toxicology, the spectra of the toxic process are based on the properties of the toxic product; toxic substances, biological substances, radiation and other harmful factors are considered harmful agents (ECETOC, 2021). Since the effects can be considered on the basis of a variety of signs, the conceptual dose (the affectable dose) is used to indicate the amount of substance acting on a biological object. The dose of effect can be measured directly using technical means and expressed in appropriate units (mg/kg, mg/m^3, Gray, cells/kg, etc.).

The dose-effect relationship in most cases will be recorded by a general pattern: with increasing the dose increases the degree of damage to the system. In ecotoxicology, in determining the dose-effect relationship, the main aspect arises from the intraspecific variety of communities, organisms and ecosystems. In this regard, the dosage of a particular substance that causes damage often differs significantly (Hughes, 1996).

Accordingly, the dose-effect relationship reflects the properties not only of the toxic substance, but also of the organism on which it acts. In practice, this means that the quantitative assessment of toxicity, based on the study of the dose-effect relationship, must be carried out by different modelling with various biological objects and by correctitudingthe non-useof methods for processing the data obtained. In general, the graph is an S-shaped curve of the normal log-distribution, symmetrical to the central point, used in the experimental ia toxicologist. The question of estimating the range of effective doses for biological systems is complex and inextricably linked to the concept of the norm. The theory of the norm on biological systems is not sufficiently developed at present. The most characteristic indicator of the norm of biological systems is the ability to thus change its functional parameters in changing the conditions of existence in order to keep the system in optimal conditions. The population as a system of interconnected individuals, due totheinitial heterogeneity of its individual ecological-functional groups, is characterized by the diversity of their response to any external influence (Ostroumov, 2004).

For ecotoxicological regulation it is necessary to optimize the interaction by limiting the anthropic impact on environmental capacities and this aims at optimizing the use of resources or renewables. The main criterion for determining the permissible environmental load is the absence of a decrease in productivity, stability and ecosystem diversity. The death of some organisms in this case is not estimated as critical.

For each ecosystem, it should be identified its own environmental quality criteria, depending on the ecological potential of the ecosystem and the ecological capacities of the region. The basis of the development of environmental standards is the theoretical concept of a systematic approach to regulating the quality of the natural environment.

Yurie Israel (Israel, 1984) has developed ideas about the maximum allowable capacity for the environment (CMA) on this ecosystem. The significance of the CMA is based on the concept of the sustainability of ecosystems, since their normal functioning under anthropogenic pressure is possible only if the CMA is not exceeded, which is derived taking into account the combined and complex action of all the impact factors on a particular ecosystem. To date, the general principles for justifying environmental regulations are implemented by developing the concept of assimilative capacity of ecosystems.

A common approach in defining their critical points is based on the assumption that the criterion for the normal state of an ecosystem is the normal distribution of its variables. In this case, the maximum and minimum critical values are found in the functional link of normal statistical distribution.

An achievement of this approach for the evaluation of biogeochemical CAM of the chemical elements (especially their metals), which are microcomponents of waters of origin of both natural and anthropogenic origin. Each of these components of the environment must be within the limits of those biologically tolerant of aquatic organisms, communities and populations with an ability to achieve its optimal physiological functions.

For stability of the ecosystem or aquatic functioning, the main criterion is the self-protection capacity and the balance between the quality of the aquatic environment and the diversity of hydrobionts. Indeed, between the biological structure of the ecosystem and its capacity for self-purification, there is an interdependent functional relationship.

Therefore, the regulation of water quality should be done by determining the size of the self-treatment capacity, the tolerance of hydrobiocenoses, the values of which should serve as water quality standards (Zubcov et al., 2015; Moiseenko, 2008). Self-purification is an integrated process of the operation of three components – water column, underwater sediments, aquatic organisms – organically related to the ecosystems of waterbodies, so that it is an indicator not only of chemical pollution but also of the physico-geographical changes (hydrological, climatic).

1.5. ENVIRONMENTAL RISK ASSESSMENT

Risk is a probabilistic feature of the threat that arises for the environment in terms of possible anthropogenic influences or other phenomena. In a system of environmental risk assessment, any impact (be it a chemical factor or an energy field) that causes changes in biological systems (both positive and negative) is called a *stressor* (Hoffman et al., 2003). In this sense, any ecotoxic agent is undoubtedly a stress factor (Liess et al., 2016).

The concept of risk assessment includes two elements: risk assessment and risk management (Negrei & Hat, 2013; Oprea, 2007).

Risk assessment is a scientific analysis of its origin, including its identification, and the determination of the degree of danger in a given situation. In ecotoxicology, the concept of risk is associated with sources of danger to the ecological systems and processes that occur in them (Calow, 1998). Environmental indicators of damage (environmental risk) in this case include: destruction of the biota, harmful, sometimes irreversible effects on ecosystems, deterioration of the quality of the environment due to pollution, increased likelihood of specific diseases, land erosion, loss of forests and water bodies (*e.g.* Aral Sea), etc.

Risk management is an analysis of the risk situation itself, the development and justification of a management decision, usually in the form of a regulatory act aimed at reducing the risk, finding ways to reduce the risk.

The development of risk theory has led to the consistent formation of principles characterizing the attitude of society to ensuring the smooth operation of anthropogenic objects - sources of danger to the environment:

- the *zero* risk principle, which reflects the confidence that the risk will not be applied; is the principle of the successive approximation of absolute security, *i.e.* at zero risk, involving the study of certain combinations of structures, alternative technologies, etc.;
- the principle of *minimum* risk, according to which the level of danger is set at as low a level as possible, based on the justification of any costs for the protection of a person;
- the principle of *balanced* risk, according to which the different natural risks and anthropogenic impact are taken into account; the degree of risk of each event and the conditions under which people are at risk are studied;
- the *principle of acceptable* risk, based on the analysis of the ratio „cost-risk", „benefit-risk", „cost-benefit".

The concept of acceptable risk implies that the complete elimination of the risk is practically impossible or not economically viable. In line with this, a rational safety limit shall be set which optimises the costs of risk prevention and the extent of the damage in the event of an emergency.

*The first step (phase) of risk assessment is to **identify hazards*** - defining a real danger to a person or the environment. Here an important role is assigned to scientific research. Tests to identify the hazard are reduced to the search for danger signals and the selection of such a signal on the existing background.

Methods of approval, selection (*e.g.* various medicines), modelling the behavior of different substances in the environment, monitoring and diagnosis (symptom assessment, effects of exposure) are important for identifying hazards. Note that all evaluation, diagnostic and forecasting problems should be attributed to the monitoring system.

Diagnosis begins with the observation of deviations - for these deviations it is necessary to correctly identify the „disease". Almost all the data obtained through monitoring require evaluations, especially diagnostic ones.

When a hazard is identified, the first question is about what exactly constitutes a hazard and when calculating a risk, what is its extent. It is necessary to determine the likelihood of occurrence of this danger and the likelihood of adverse effects. Perspective, intuition and extrapolation can be used to determine the calculation of risk (Calow, 1998).

At the stage of the review of the risk assessment procedure, the analysis is carried out at a qualitative level.

The second stage – ***the exposure assessment*** – is an assessment of how and the environment in which, at what quantitative level, at what time and for what period of exposure the actual and expected exposure occurs; it is also an estimate of the doses received, if available, as well as an estimate of the number of people who are exposed to such exposure and for whom it appears to be probable.

The size of the exposed population is one of the most important factors in deciding on the priority of protective measures that arise when using the results of the risk assessment for the purpose of 'risk management'.

Ideally, the exposure assessment is based on the actual pollution monitoring data of different environmental components (ambient air, indoor air, soil, surface water, drinking water, food).

However, this approach is often impossible due to high costs. In addition, it is not always possible to assess the relationship between pollution and its source, and it is insufficient to predict future exposure. Therefore, in many cases, different mathematical models are used to assess the dispersion and dispersion of atmospheric emissions, their sedimentation on the soil, the diffusion and dilution of pollutants in groundwater and / or open water bodies.

The third stage – ***the evaluation of the dose-effect relationship*** – is the search for quantitative models that associate the received dose of a substance with the prevalence of an adverse effect (for health), that is, with the probability of its development. Similar patterns, as a rule, are revealed in toxicological experiments. However, their extrapolation from a group of animals to a human population is associated with too many uncertainties.

As the risk assessment procedure is complex and suffers greatly from the known uncertainty, the United States Environmental Protection Agency (U.S.EPA) developed and approved a plan for such a reseach standardization activity (Measurement Methods Standardization Strategy Document; Science Models and Research Tools (SMaRT) Search). This contains a description of the sequence of problem solving, taking into account the uncertainties and assumptions in order to obtain, to some extent, unified, approximate information about the likelihood of adverse effects on the environment.

According to this plan, the environmental risk assessment includes the following steps:

1. Formulating the problem and drawing up a plan for analyzing the situation.
2. Analysis of the environmental situation.
3. Data processing, formation of findings and presentation of materials to the customer.

As a rule, the environmental risk assessment is carried out in the form of an ordered research, performed in order to obtain information of a promising or retrospective nature and necessary for the client (legislation, management structures, etc.) to make administrative decisions.

Therefore, unlike scientific ecotoxicological studies, in which the objective rules of biocenosis reactions to the action of a stress factor are taken into account, when defining the ecotoxic risk, the environmental characteristics to be studied and „protected" may be the characteristics of a biosystem that has a value in public opinion as being very significant (Bezel et al., 1994).

The methodology for environmental risk assessment is not fully developed. In most cases, its conclusions are qualitative, descriptive. Attempts to introduce quantitative assessment methods face serious difficulties.

This is due to the complexity of ecosystems, the complex impact of stressors on the environment (not only chemically, but also physically and biologically), insufficient knowledge of the characteristics of the ecotoxic danger of a huge amount of xenobiotics used by humans, etc. In this sense, in the opinion of the environmentalists themselves, the assessment of environmental risks is largely an art that requires a permanent development.

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2

Chapter

ECOTOXICOLOGICAL INVESTIGATIONS OF AQUATIC ECOSYSTEMS

(study of ecotoxicants)

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2.1. INTRODUCTION

Aquatic ecotoxicology involves the solution of a wide range of problems related to the study of biodiversity, with the condition of the sustainable functioning of hydrobiocenoses in order to determine the support capacity of aquatic ecosystems with different anthropization rank, to quantify the migration of ecotoxicants and to elaborate the bioremediation processes of aquatic ecosystems. The deciphering of the processes of migration, bioaccumulation, bioamplification and of the role of ecotoxicants, especially metals, in the functioning of aquatic ecosystems is a very current problem of world importance, a special role having the quantification of the contribution of hydrobionts communities in the processes of self-purification and secondary pollution of inland waters.

Quantification of the functional role and weight of the main groups of hydrobionts in the biogenic migration of metals, in the processes of self-purification and secondary pollution of inland waters, as well as determining the influence of chemicals on fish development in early ontogenesis, metabolism and development of aquatic organisms, the development of production-destructive processes, were the basis for the formation of the concept of of the assessment of the buffer or resistance capacity of the aquatic ecosystems in Moldova, the determination of the limit of the tolerance of some aquatic organisms to chemicals (Zubcov, 1997,2000).

These complex multi-annual ecotoxicological investigations were carried out within several national (AQUSYS, 2014-2019; AQUABIO 2020-2023) and international (MIS-ETC 1150, 2012-2015; MIS ETC 1676 – INPOLDE, 2013-2015; BSB27 MONITOX and BSB165 HydroEcoNex, 2018-2021) projects.

The ecotoxicological investigations of the flowing aquatic and lake ecosystems, as well as the assessment of the quality of water, fishery products, risks assessment and the elaboration of the scientific bases for the sustainable valorization of aquatic resources can be targeted in two compartments – those with a purely ecological aspect, and the ecotoxicological ones (Figure 2.1).

The basic sources of metals in inland waters are bedrocks, soils, as well as the technogenic pollution. Metals are one of the most numerous persistent ecotoxic substances which, penetrating into surface waters, play a very important role and, depending on concentrations and forms of migration, become

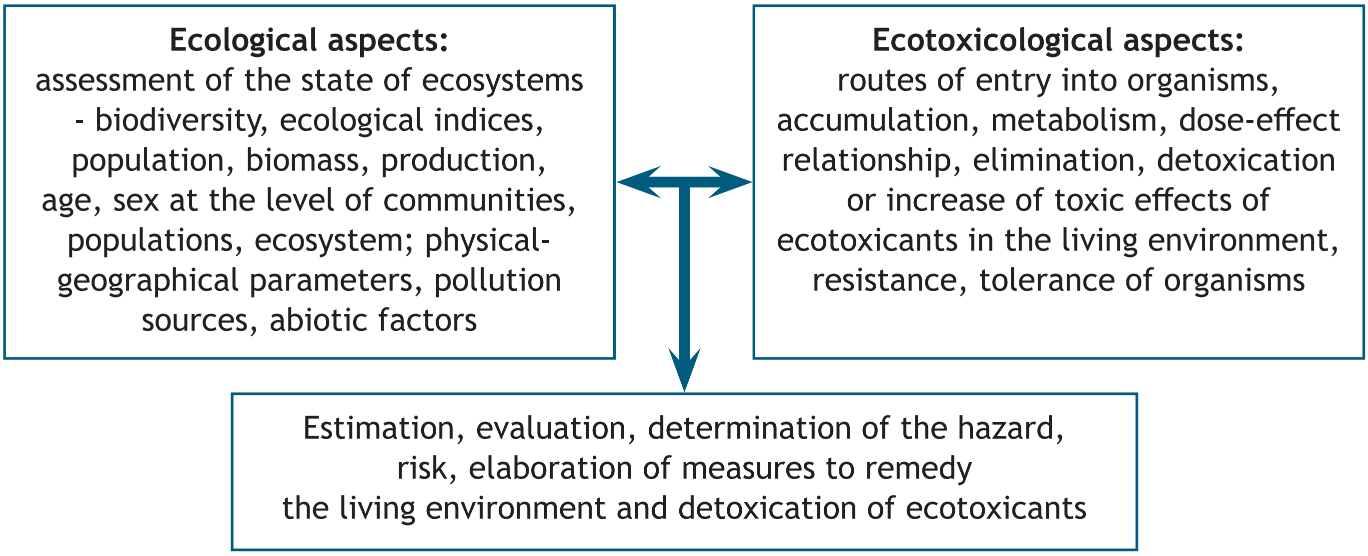

Figure 2.1. The main compartments of ecotoxicological investigations

essential elements biocatalyzing the biochemical processes or, conversely, toxic and dangerous elements.

The large number of metals, the varieties of their migration forms, and the wide range of concentrations are the basis of several classifications of this group of ecotoxic substances, from „elements of life”, „biometals”, to „toxicants” and „heavy metals”, according to their influence on living organisms. Nevertheless, there are also classifications based on the chemical properties, according to the distribution in the environment, etc. (Strakhov, 1979; Linnik & Nabivanets,1986; Szyczewski et al., 2009).

Strakhov (1979) introduced for the first time the notion of the ***form of migration***, and established that a considerable part of the metals migrates to the river waters in the form of suspensions, emphasizing the importance of research of the ratio between soluble and suspended forms of migration, as an indicator of the geological and physico-geographical processes of the regions.

The migration capacity of metals in surface waters and their forms of migration are conditioned both by the properties of metals themselves and by the physico-chemical peculiarities of the environment, *i.e.* by the value of the pH, temperature, oxidation-reduction conditions, hydrological regime, the presence of complexing agents, suspended substances, the vital activity of the hydrobionts (Strakhov, 1979; Linnik & Nabivanets,1986).

Human activity introduces substantial corrections in the distribution and migration of metals in the aquatic environment. The state of the largest rivers, lakes and reservoirs in the world demonstrates that the dynamics of metals, their forms of migration, depend to a great extent on the complex of anthropogenic factors (industrial wastewater, surface runoff from agricultural land, urbanized territories and highways).

The pollution of surface water gave impetus to a new research direction – the toxicological one, and thus a series of synthesis works on environmental pollution, including surface water, were published. Penetrating the aquatic ecosystems, metals may become the cause of hydrobiontes poisoning and, ultimately, of humans (Moore, Ramamoorthy, 1984).

The difficulty of determining the resistance limits lies in the fact that, unlike organic compounds including the persistent ones, metals do not decompose, but pass only from one state to another, which complicates in a large extent the evaluation of the influence of metals on aquatic animals and plants.

In this context, the investigation of the migration of metals in surface waters represents today one of the current scientific directions of research in the field of ecotoxicology. The ecotoxicological researches, aimed at clarifying the biological importance of metals and their toxic action on hydrobionts and water quality, correspond to the requirements of several Directives and Regulations in the field of conservation of the living environment (Directive 2000/60/EC; SETAC Europe 29th Annual Meeting 26–30 May 2019; REACH — Regulation No 1907/2006; WHO Promotion of Chemical Safety Unit & International Programme on Chemical Safety, 1992; Philip et al., 2018).

All the above-mentioned directions of research are current for the Republic of Moldova, where the shortage of good quality water is acutely felt, and the anthropogenic action on the environment is constantly increasing.

2.2. METHODOLOGICAL GENERALIZATIONS

The ecotoxicological investigations of the metals were carried out on the Dniester River and the Prut River - the main aquatic arteries of the Republic of Moldova, which are transboundary water bodies, because they originate on the territory of Ukraine in the Carpathian Mountains and carry their waters through the regions with dense populations of Ukraine, Moldova and Romania (Figure 2.2).

The Cuciurgan refrigerent reservoir and the Costești-Stânca Dam lake are also border ecosystems with a complex destination. On both rivers were built dam lakes with hydropower purposes. In the river basins of both ecosystems are concentrated large main industrial centers of the nominated countries, therefore, the quality of their waters largely depends on the anthropogenic influence in these regions and on the implementation of nature protection measures, based on scientific elaborations and knowledge of the legalities of intrabasin processes of distribution and migration of chemical elements.

In order to establish the regularities of the distribution and migration of metals in the system „water-suspended substances-underwater deposits-hydrobionts” and to determine the role of the main natural and anthropogenic factors that influence their direction and forms of migration, the material was systematically collected from 10-12 points of the river ecosystems (on the Dniester river from Naslavcea to Palanca, on the Prut river from Costești-Stânca to Giurgiulesti; in recent years also from Criva - to Costeşti), monthly or quarterly, in accordance with standardized regulations and reflected in the recently edited guide (Hydrochemical and hydrobiological sampling guidance, 2015). The analysis and complex monitoring of the state of aquatic ecosystems, including the determination of metals, were carried out by classical methods and according to ISO standards (Guidance on the Monitoring of Water Quality and Assessment of the Ecological Status of Aquatic Ecosystems, 2020).

Figure 2.2. Hydrographic basin of cross-border rivers of the Republic of Moldova

In parallel, a hydrological database was created (flow rate of flowing water, speed of water, amount of suspensions, transparency) containing hydrochemical data (mineralization, oxidation-reduction processes, pH value, gaseous regime, content of organic substances in water, suspensions, silts); climate data (temperature, amount of atmospheric precipitation); materials about the volume of wastewater and the amount of use of fertilizers and pesticides containing metals; geological data (chemical composition of soils and rocks, hydrogeological parameters) from the hydrographic basins.

In addition, the amount of metals was determined in the wastewater overflowing in the investigated aquatic ecosystems, in atmospheric precipitation (rains and snow), in the leakages from the industrial territories (thermal power plant, municipality of Chishinau) or from the agrarian squares (vines, orchards).

The water samples, subjected to the analysis of the metal content, were immediately filtered, *in situ,* through membrane filters with a pore diameter of 45 microns, in order to separate the dissolved metals from the suspended forms. The samples of the silts and the biological samples were processed no later than 12-24 h after collection. In silts it was determined the total content of metals, in 5 particle size fractions, in sludgeous solutions obtained by centrifugation for 30-45 minutes with a speed of 2500-3000 rotations/min.

In the suspended substances and the underwater deposits, besides the determination of the total microelement content and the total amount of organic substances, the mobile forms of the metals were also determined: Me_1 - the easily soluble and easily interchangeable adsorbed carbonates, Me_2 – the organomineral form and Me_3 – the metals that are in association with the amorphous iron and manganese hydroxides. The indicated forms were separated by the method of gradual extraction, according to a scheme previously described in detail (Zubkova, 1996).

In order to establish the level and regularities of metals bioaccumulation in aquatic plants and animals and to evaluate the contribution of the main groups of hydrobionts in the biogenic migration of chemical elements in order to elucidate the role of metals in the functioning of aquatic ecosystems, samples of superior algae (macrophytes) planktonic and benthic invertebrates were collected quarterly. Hydrobiont samples were divided by body size, weighed and subjected to the analysis of metals and humidity. In paralel, hydrobiological research was carried out: diversity, herd, biomass, production.

In order to evaluate the influence and establish the level of accumulation of the metals in the early stages of fish ontogeny, the content of microelements in roe and larvae at different stages of their development (morula, gastrula, organogenesis, after hatching of larvae, larvae of 3, 6 and 12 days) was investigated. Numerous experimental works and modelings were carried out in the conditions of enterprises of fishing households with larvae, brood, cargo fish and breeding fish (Zubkova N., 2011).

To determine the level of accumulation of metals in fish, the muscles of the body, skin, gills, liver, gonads were analyzed.

In order to establish the influence of the metals on the size of the primary production of phytoplankton and the destruction of the organic substance, experimental works were carried out directly *in situ* on the Dniester and Prut rivers, and Dubasari, Cuciurgan and Costești-Stanca accumulation lakes. For experiments, a quantity of water was collected, part of which was taken for the analysis of metals, and in others solutions of metals were added in concentrations ranging from the minimum to the maximum possible for the given ecosystem (from 5 to 10 concentrations).

Bottles (transparent) were filled with the water samples, obtained with the addition of metals together with the reference water (without addition), in order to determine the primary production and the destruction of organic matter (opaque bottles), which were subjected to the exposure for 24 hours, at a depth of 30 cm from the surface of the water; then, using the method of determining the dissolved oxygen, the production and the destruction values were calculated by the classical method (Guidance on the Monitoring of Water Quality and Assessment of the Ecological Status of Aquatic Ecosystems, 2020).

In order to assess the buffering capacity of the aquatic ecosystems in Moldova and the quality of the water according to the content of the metals, the above-mentioned investigations were made *in situ* modelings and several laboratory experiments. Thus, the investigations were carried out according to the scheme presented in Figure 2.3.

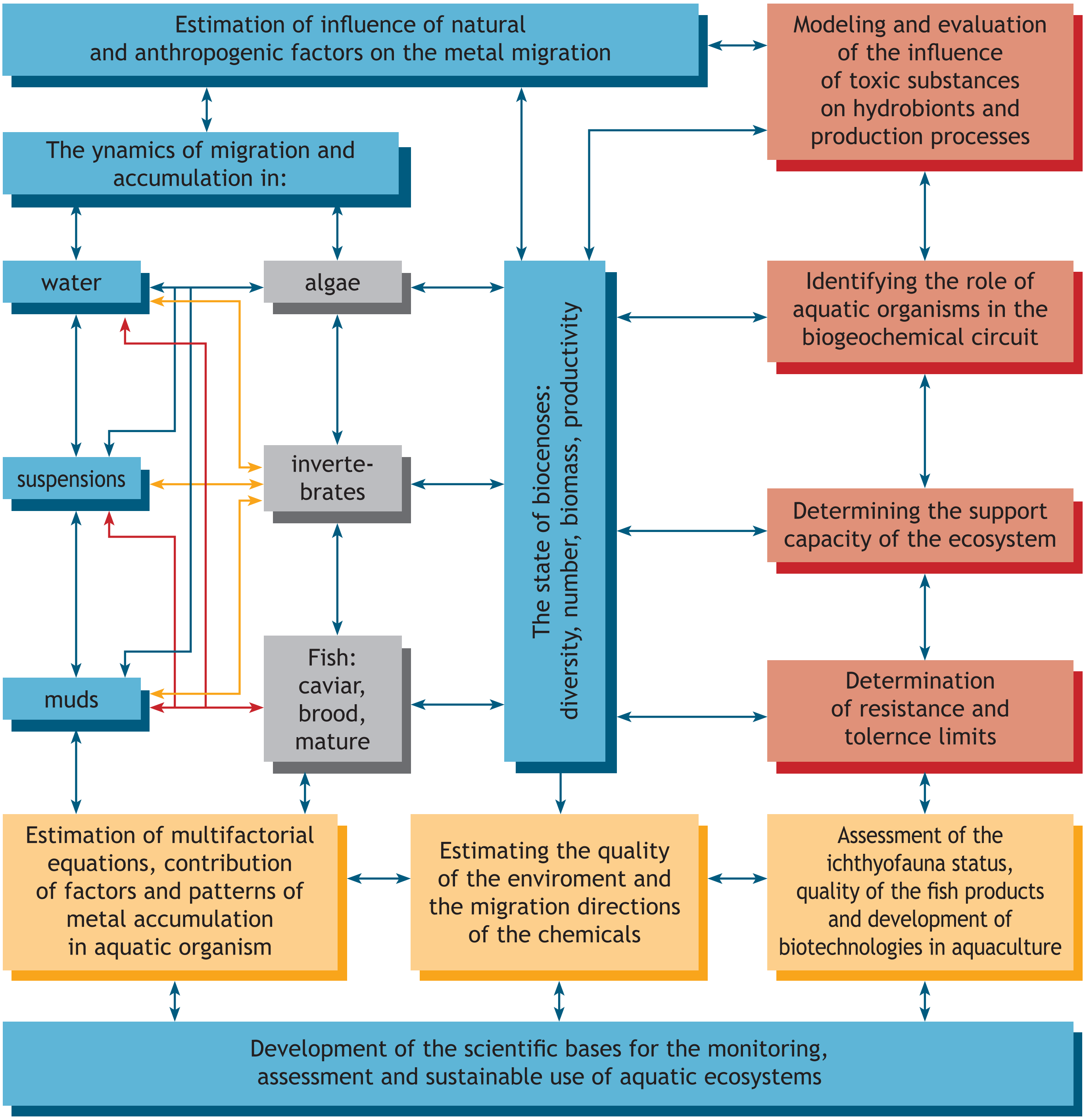

Figure 2.3. Scheme of ecotoxicological investigations of metals in aquatic ecosystems

2.3. RESULTS OF ECOTOXICOLOGICAL INVESTIGATIONS

The ecotoxicology and biomonitoring of metals in aquatic ecosystems, with the purpose of establishing tolerance limits and assessing the resistance of aquatic plants and animals in the conditions of instability of the physico-chemical properties of the aquatic environment, is a major problem for several reasons.

First of all, these investigations contribute significantly to the solution of the fundamental problems – establishing the evolution of the specific diversity of the hydrofauna, deciphering the mechanisms of regulating their numerical herds, the processes of bioproductivity, the trophic structure of the communities, the circuit and the flow of the chemical elements in the trophic chains of the ecosystem. The applicative spect consists in the protection of the aquatic fauna and flora genofond, and the elaboration of recommendations on restoration and sustainable valorization of aquatic resources (Zubcov et al., 2016).

The importance of the multi-annual complex investigations carried out allowed for the first time, by performing the polyfactorial analysis, to determine the quantitative parameters of the share of the main factors (the water flow, the quantity of suspensions, the volume of the discharged wastewater, the quantity used by agrochemicals and fertilizers) in the dynamics of the migration of metals in water, suspensions, silts of the Dniester and the Prut River.

The given regularities are described by equations such as:

$$y = a_0 + a_1x_1 + a_2x_2 + \dots + a_nx_n$$

For example, in the case of aluminum and titanium, the natural factors such as water flow (Q, m^3/s) and the amount of suspended substances in water (S, mg/l), are determinants, and for Răut river (a tributary of Dniester river) these metals fall into the following equations:

$$Al = 0.330 \cdot Q + 0.012 \cdot S + 1.2; \quad R = 0.82$$
$$Ti = 0.159 \cdot Q + 0.011 \cdot S - 0.7; \quad R = 0.86$$

The main sources of aluminium in aquatic ecosystems are the mountain rocks and soils of the region. The weight of the natural factors in the dynamics of migration of these metals exceeds 80% both in the Dniester river and in the Prut river and their tributaries. It has not been determined an obvious influence of anthropogenic factors on the dynamics of aluminum and titanium.

In the 80s and 90’s of the last century, the dynamics of copper and zinc in the investigated waters was in a pronounced dependence on the amount of agrochemicals with the high content of these metals (blue vitriol and zeneb), used against diseases of the vines and orchards, the weight of these agrochemicals in the migration processes of copper and zinc in the hydrographic basins of the aquatic ecosystems oscillating within the limits of 40-70%.

The use of chemical preparations and fertilizers on agricultural fields during the development of intensive agriculture strongly influences the dynamics of migration Cu, Zn and Mn in the aquatic ecosystems of the Republic of Moldova (r=0.70-0.92), to which contributes the abundant character of atmospheric precipitations, the heavily dismembered relief and the intense processes of erosion (Zubcov et al., 2013 a).

The wastewater of cities and industrial enterprises pollutes the aquatic ecosystems with Ni, Zn, Cu, Ag, Cd (r>0,73), modifies the ratio of dissolved and suspended forms of metal migration, which is especially felt downstream of the Chisinau municipality, the cities of Soroca, Ribnita, Tighina, Tiraspol, Iasi, Cahul, especially during the etiage period.

Some aspects of the migration of metals in technogenic aquatic ecosystems have been elucidated, as for example, the storage-cooling lake of the Cuciurgan thermal power plant. For this ecosystem, the multiannual trend of the dynamics of metals was determined – the indicators of the thermal power plants activity (Mo, V, Cd, Pb, etc.). A strong dependence was established on the time factor (A, year), the amount of burnt coal (C, t/year) and fuel oil (P, t/year) which, for the concentrations of molibden in water (Mo, µg/l) and vanadium in suspensions ((V_s, µg/l), is described by the following equations:

$$Mo = 0.91 \cdot A + 4.33 \cdot C + 3.5 \cdot P - 1809; \quad R = 0.81$$
$$V_s = 1.04 \cdot A + 6.97 \cdot C + 0.1 \cdot P - 2068; \quad R = 0.82.$$

The operation of the thermal power plant started the pollution of the water of the refrigerant basin with V, Mo, Ni, Cd, Mn, and of the underwater deposits – also with Pb, Zn, Cu, the concentrations of which are in a direct correlational dependence on the amount of fuel burnt at the station (r=0.76-0.97). The increased temperatures are reflected on the oxidation-reduction processes that change the migration of metals in the „water-suspension-silts” system. In this ecosystem, secondary pollution processes are recorded, when metals from the silts enter the water layer. In the area of the power plant and **in the atmospheric precipitations**, the concentration of metals is 3-15 times higher compared to that outside the area.

The underwater depositions of fl. The Dniester and the Prut river refer to the sandy muds of the Dubasari dam lake, to the clay muds and sludges of the Cuciurgan basin. Suspended substances and underwater deposits of reservoirs and rivers are powerful accumulators; for several metals, their content in the silts is visibly higher than in the parental rocks and regional soils.

It has been established that in the adsorbed complex, together with the easily soluble carbonates and in the associations of the amorphous iron and manganese hydroxides of the underwater deposits are concentrated 56-78% of metals in mobile forms of migration and accessible for hydrobionts. It was determined the dependence of the content of different forms of metal migration and their distribution in the particle size fractions of underwater deposits on the amount of clay particles (G, %) finely dispersed and that of organic substances in them (B, %), which are described by regression equations, such as for example, for the accumulation of the content of some metals (Cu, Mn, Ni) in the silt of the Dubasari accumulation:

$$Cu = 0.29 \cdot G + 42.23 \cdot B - 49.5; \quad R = 0.94$$
$$Mn = 0.90 \cdot G + 0.08 \cdot B + 68.5; \quad R = 0.99$$
$$Ni = 0.75 \cdot G + 1.56 \cdot B + 117.7; \quad R = 0.99.$$

According to one of the main concepts of biogeochemistry and toxicology (Filenko, 1986), organisms and biocenoses not only adapt to the chemical factors of the environment, but, in turn, they actually modify the composition of the environment in accordance with the needs of the living in the process of development and reproduction. In connection with this, one of the criteria for establishing the permissible level of metal content in biological objects is the determination of dependencies between their concentration and the intensity of bioproduction of hydrobionts in the ecosystem. If the polyfactorial influence of natural conditions on intrabasinic processes and also the physiologic-chemical characteristics of hydrobionts are not taken into account, a non-objective picture of the situation can be obtained, because metals represent simultaneously both vitally necessary elements and toxic elements (Zubcov et al., 2016).

We considered it necessary to determine the limits of the concentrations of metals in water of Dniester and Prut rivers, and of the Dubasari, Costești-Stanca and Cuciurgan reservoirs, to elucidate the optimal concentrations and those which can have an inhibitory action on the production-destructive processes (Zubcov, 1997,2000). The analysis of the obtained results proves that for all the investigated aquatic ecosystems, common regularities of the influence of some or other metals on the production of phytoplankton and the destruction of organic substances are pursued, most of them being described by polynomial equations of 2-4 order (Figure 2.4).

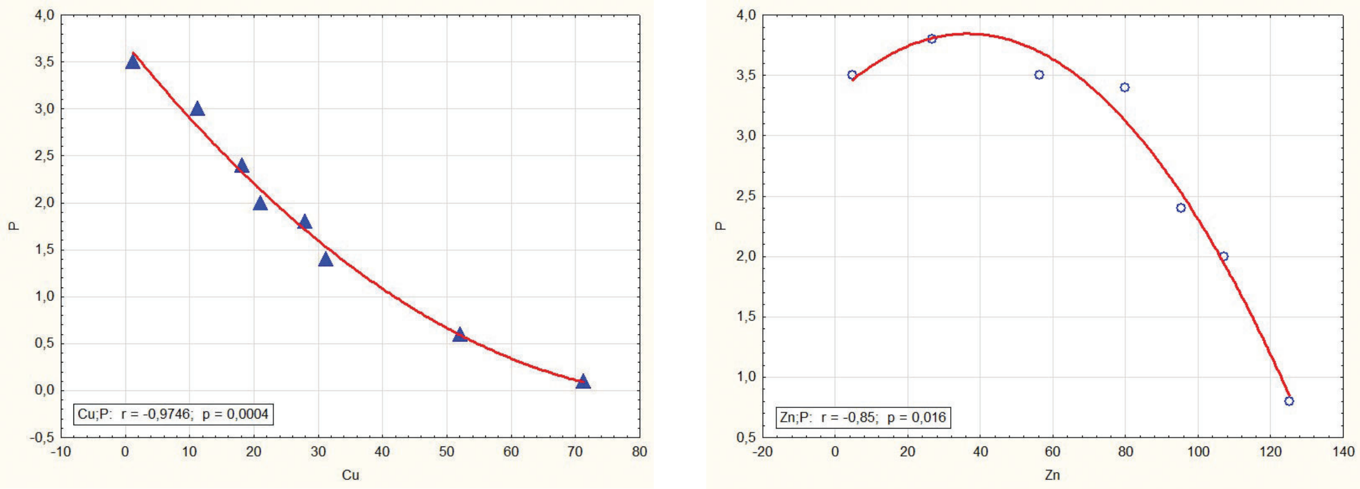

Figure 2.4. Dependence of the values of the phytoplankton primary production (P, mg O2 /l) on the concentrations of some metals (Cu, Zn)

Thus, there were established the optimal or favorable concentrations for the functioning of the ecosystems *(which do not influence the production-destructive processes)*, the admissible concentrations (*gradually decreases the primary production)* and the extreme or critical concentrations for the aquatic ecosystems (*the size of the primary production decreases sharply and reaches the zero value)* that allow to attribute the aquatic ecosystems in the category of unpolluted, polluted and heavily polluted or dirty (Zubcov, 1997, 2000).

In the current year it was proved that the elements Cd and Bi, already at concentrations of 1.5-2.0 µg/l, inhibit the production processes of phytoplankton. Co enhances these processes up to concentrations of 4.5-5.0 µg/l and Se – up to 46-50 µg/l. The support capacity at increased concentrations in the Prut river is higher than in Dniester river, which is directly caused by the imbalance of the Dniester river hydrological regime.

The establishment of flows and the revealing of the metal accumulation legalities in aquatic plants and animals, along with those of the production-destruction processes, were the basis for the formation of the concept of the evaluation of the buffer capacity of the aquatic ecosystems in Moldova depending on the dynamics of the metals content. Thus, a new methodology of assessing the state of aquatic ecosystems is theoretically substantiated and proposed, according to which the waters of the Dniester, Prut, Dubasari and Costești-Stanca accumulation lakes were favorable in 85-94% of cases and only in 2-6% of cases were critical for the production-destruction processes, while in the Cuciurgan reservoir, in 68-84% and 8-17% of cases, respectively (Zubcov et al., 2013 a, b).

The range of oscillations of the concentration of metals in the investigated aquatic plants is quite large and is conditioned by the taxonomic peculiarities of the plants, the content of metals in water and the muddy deposits, the chemical properties and the biological importance of the microelements and, also, of the season (Zubcov et al., 2013a, b). However,

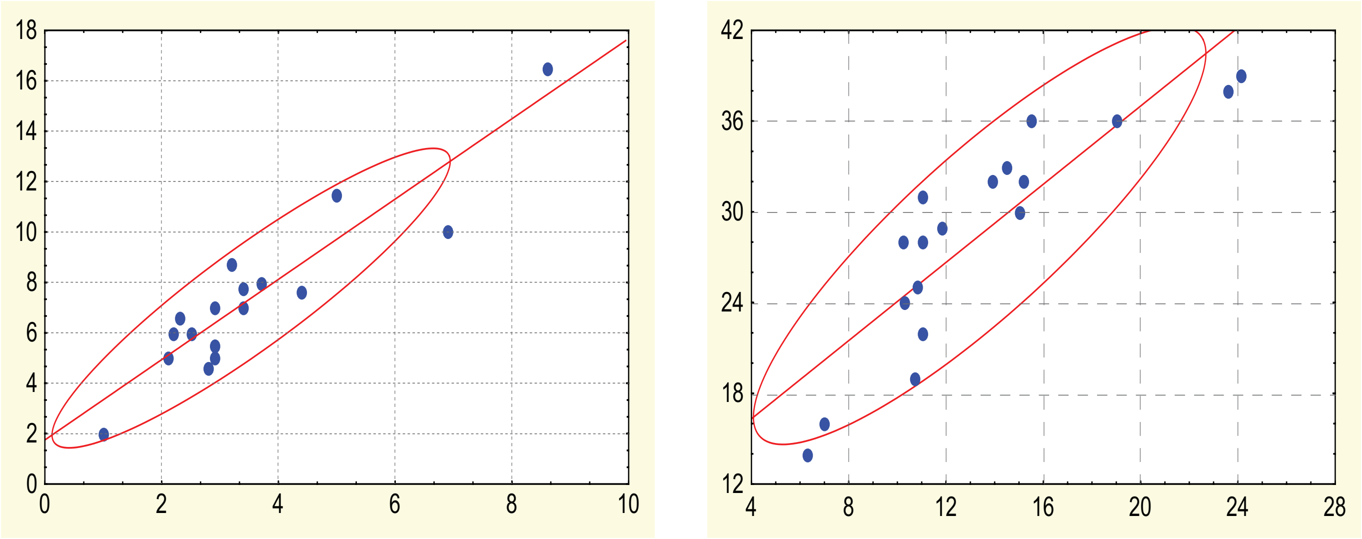

Figure 2.5. Dependence of metal concentrations (Pb, Mo) in aquatic plants
on those in the water of Cuciurgan reservoir

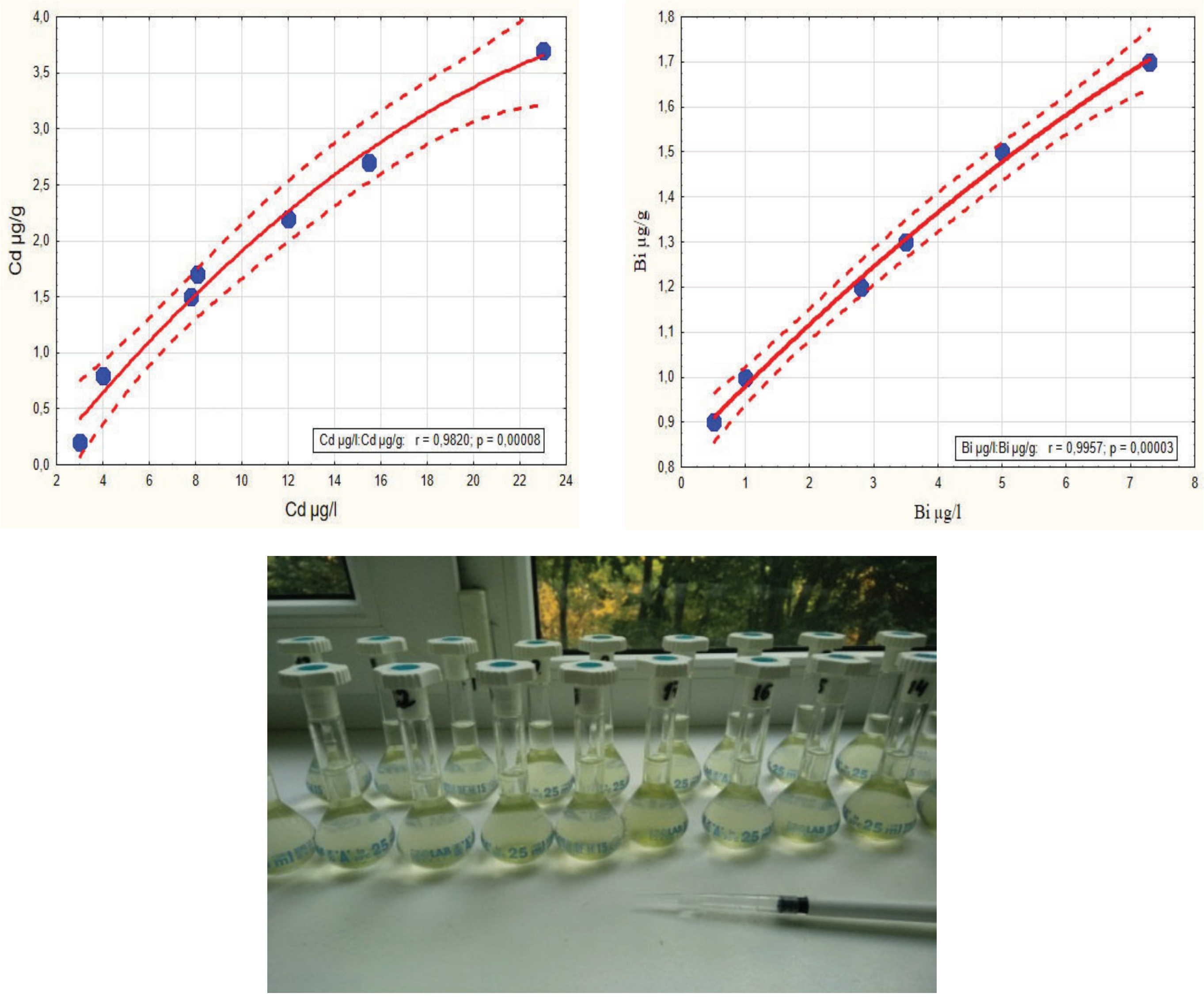

Figure 2.6. Dependence of metal concentrations (Bi, Co) in *Chlorella vulgaris*
on those in water in laboratory experiences

the multiannual investigations have allowed us to establish the dependence of the content of a series of microelements in aquatic plants on their concentrations in water, which fall within the linear one (Figure 2.5).

By laboratory modeling it was established the accumulation of metals in phytoplankton (Figure 2.6).

The range of oscillations of metal concentrations in benthic invertebrates in the aquatic ecosystems of Moldova is very high and is conditioned both by the variation of the natural conditions of the environment, the biological importance of metals, as well as by the taxonomic and age peculiarities of the hydrobionts.

For all groups of benthic animals, the functional dependence of the accumulation of metals on their body mass was established (Toderaș et al.,1999). This dependence is described quite truthfully by the equation of linear dependence of the type:

$$lg\ y = lg\ a + b\ lg\ W,$$

where:

y – concentration of the micro-nutrient in µg/exp.,

W – the mass of the individual in mg,

a – the coefficient showing the size of the concentration of the chemical element in µg/exp., when the mass of the body is equal to 1 mg,

b – the angular coefficient that determines the change of the concentration of the microelement at the increase of the body mass.

Examples of linear dependencies are the following:

Gammarus balcanicus (n = 27):

$$lg\ Mn = lg(-1.361 \pm 0.040) + (1.134 \pm 0.064)\ lg\ W,\ r = 0.98,$$

Limnomysis benedeni (n = 15):

$$lg\ Ni = lg(-1.692 \pm 0{,}020) + (1.013 \pm 0.044)\ lg\ W,\ r = 0.99,$$

Dreissena polymorpha (n = 39):

$$lg\ Pb = lg(-2.291 \pm 0{,}032) + (0.783 \pm 0.015)\ lg\ W,\ r = 0.99,$$

The obtained research results of the allow to find that the mass of the body of benthic invertebrates, along with the environmental conditions, represents one of the main factors that determine the level of accumulation of metals.

It is very important to take this into account when comparing the level of accumulation of metals in hydrobionts in different aquatic ecosystems because, otherwise, non-objective conclusions may be drawn. For example, in *Dreissena polymorpha* from Dniester river, with body mass within 2-800 mg, the average content of some microelements seems to be higher than that of *Dreissena polymorpha* in the Cuciurgan reservoir, with a body mass of 2-4000 mg. At the same time, in individuals with close mass values, the content of microelements in the molluscs of the river is considerably lower compared to that of the refrigerent lake.

It was determined that, among the benthic invertebrates, the molluscs have the highest weight in the process of forming metals in the circuit. In Figure 2.7. we give several examples of the dependence and degree of accumulation of metals (27) in two species of molluscs *(Crassiana crassa, Unio tumidus)* on their concentrations in water (laboratory modelings).

Thus, the functional role was quantified and, for the first time, quantitative indices

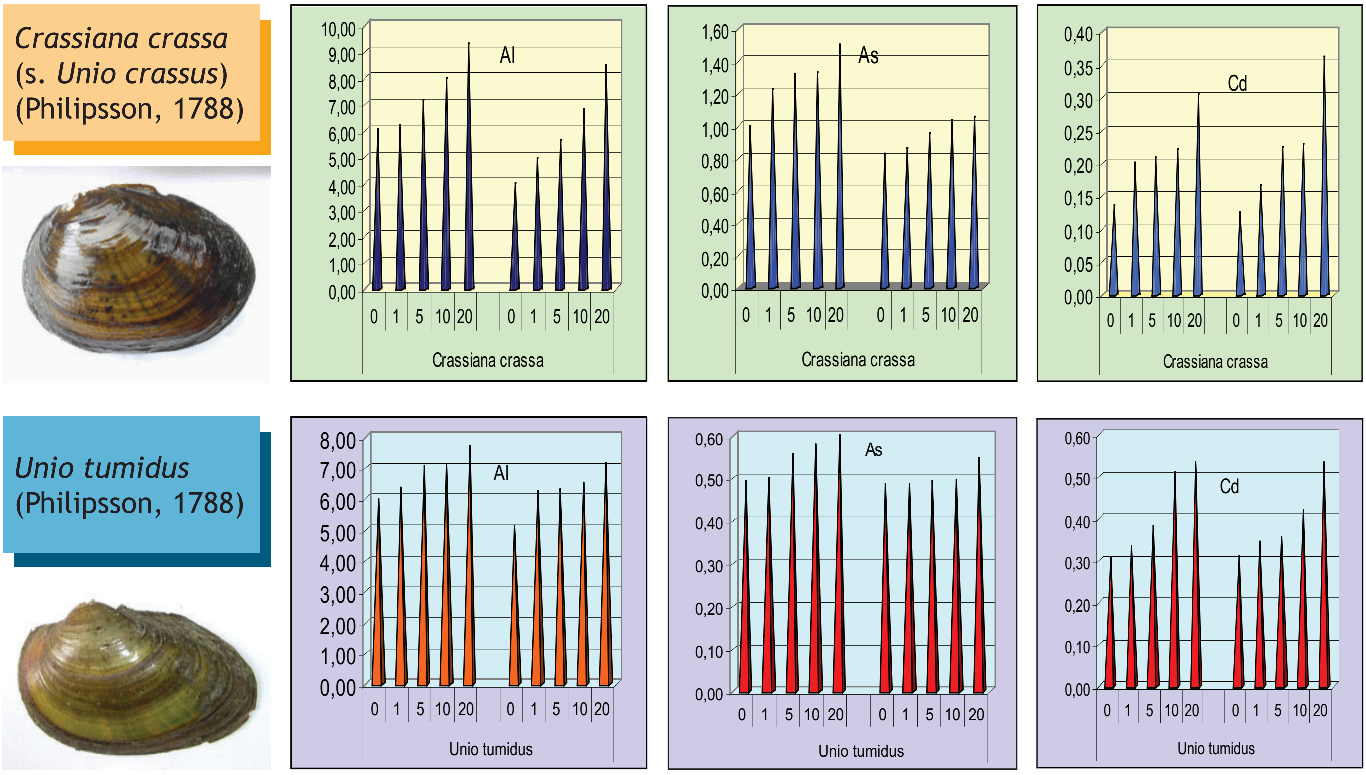

Figure 2.7. Dependence and degree of accumulation of metals in two species of molluscs *(Crassiana crassa, Unio tumidus)* on their concentrations in water (laboratory modeling with 27 metals)

of the contribution of the main groups of hydrobionts to the biogenic migration of microelements were obtained. It was evaluated the possibility of using aquatic plants, of the mass species of hydrobionts, especially of daphnia, chironomids, myzids, molluscs and fish eggs and brood as monitors and bioindicators in the implementation of integrated ecological monitoring in aquatic ecosystems.

The content of metals in fish is one of the links of biological monitoring of metals and evaluation of the direction of the substance and energy circuit in aquatic ecosystems. The research of the phenomenon of accumulation of metals in fish has both an ecological and hygienic importance. In the conditions of increasing anthropogenic influence on aquatic ecosystems, the problems of accumulation, in particular of metals, gain a major importance (Zubcov et al., 2019a,b). This information is particularly valuable, because the trace elements, in optimal quantities, are always necessary for humans, but in the case of abnormal concentrations can become the cause of imbalance, endemic diseases and even intoxication.

Some metals accumulate in the food chain, which can eventually affect the health of humans who feed on fish products. There are well known cases of intoxication of people with cadmium and mercury as a result of eating polluted fish (Zubcov et al., 2012).

It was determined that in the case of eggs and larvae of fish, their microelementary composition constitutes an accurate image of the dynamics of the microelement content in water (r=0.90-0.98) and has distinctive peculiarities depending on the species (Figure 2.8.).

Fish eggs and larvae can, therefore, serve as a safe test when assessing water quality by metal content.

It is established the synergism and antagonism of metals in the process of acculating metals in roe, larvae and brood of fish. Here we must mention that the presence of zinc in water reduces the toxicity of cadmium and, in turn, zinc and manganese suddenly decrease

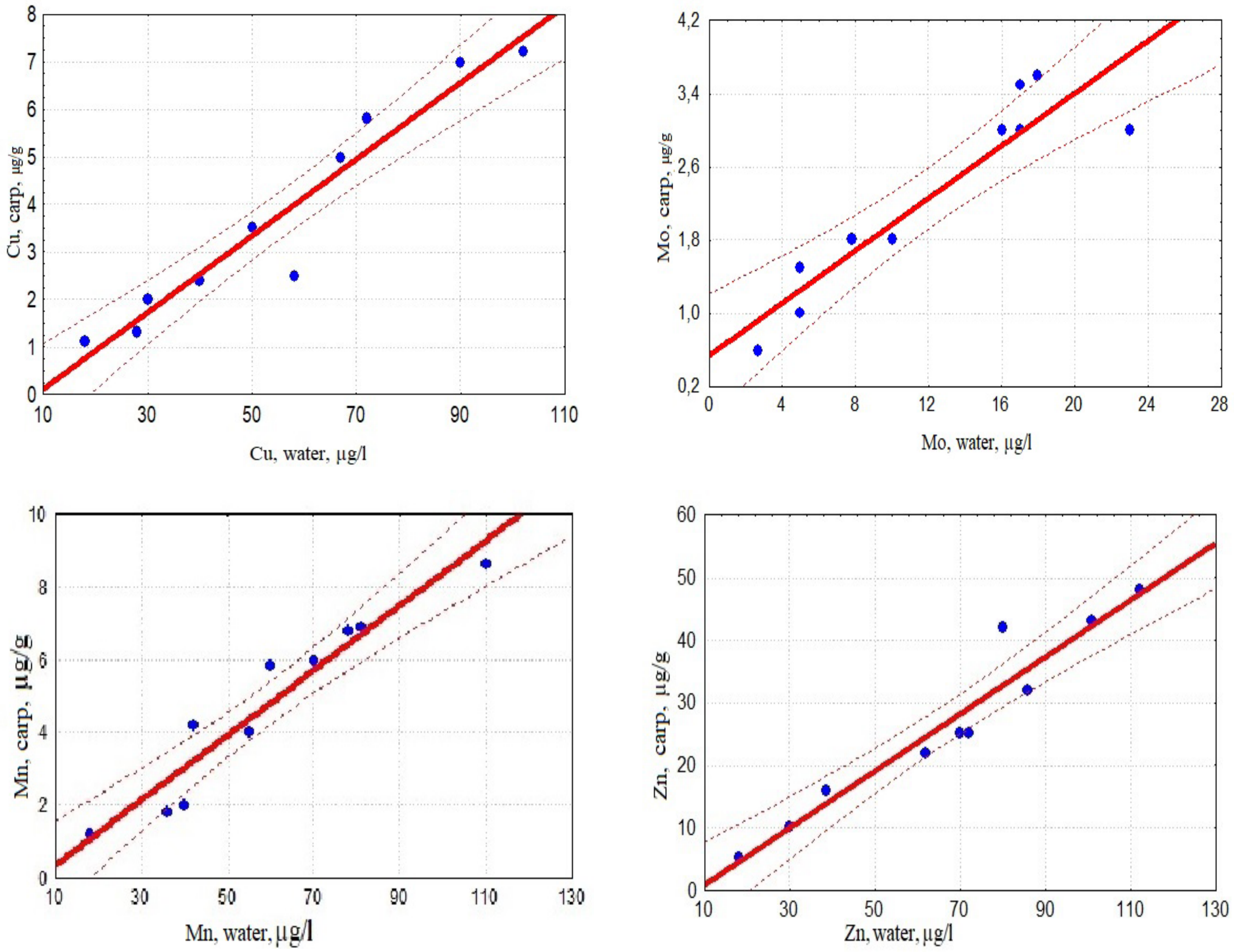

Figure 2.8. Dependence of the accumulation of metals in the larvae of *Ciprinus carpio* (r=0.88-0.98) *(modelling in production conditions)*.

the toxicity of copper and nickel. Even more so, when copper, zinc, manganese was added to the Dniester water, each in concentrations of 40-50 µg/l, a reverse effect was obtained. It was established that a stimulating action on the development of the eggs had the complex of these microelements and, as a result, the share-part of the viable hatched larvae increased (Zubcov et al., 2016).

The dynamics of the accumulation of microelements in fish fry depends more on the nutritional base and less on the concentration of microelements in water, being conditioned by the taxonomic and age peculiarities of the fish.

Fish have a fairly developed homeostasis mechanism, that regulates the processes of accumulation and redistribution of microelements between different organs depending on the plastic and generative metabolism of fish and their needs for one microelement or another. In the period before spawning, the intense processes of accumulation of biologically important microelements in gonads predominate, accompanied by the decrease of their concentrations in skeletal muscles, and in the growth period - vice versa. However, the microelemental composition of organs and tissues is a function of the environment composition.

The processes of accumulation of metals in the organs and tissues of sexually mature fish have a rather complicated and diverse character, conditioned by the complex of environmental factors and the physiologic-biochemical status of the organisms. Therefore, the link between the level of accumulation of metals in the organs and tissues of the investigated fish and the environment is not questioned, although in fish the mechanism of homeostasis is quite developed, which regulates the processes of accumulation of chemical elements (Zubcov et al., 2012).

When the metals enter the body of fish, their distribution through different organs takes place – a process dependent on biological necessity. In most cases, the maximum concentrations of Pb, Mo, V, Cu, Ni, Cd are detected in the liver, of Mn, Al, Zn – in gonads, and the minimum concentrations of Mn, Al, Ni, Mo, Cu, Zn – in skeletal muscles and of Pb, Cd, V, Ti – in gonads. It was established the permanent change of the content of microelements throughout the vital cycle of fish, for example, the accumulation in the liver of Fe, Cu, Zn and Mn during the period of intense nutrition, the increase in concentrations of Fe, Zn, Mn, Co, Cu, Mo in gonads directly before spawning – a process accompanied by a decrease in their content in muscles (Zubkova N., 2011; Zubcov et al., 2016).

Heavy metals, according to the decision of numerous international commissions on environmental protection and health care issues, are reported as a priority pollutants (WHO. Promotion of Chemical Safety Unit & International Programme on Chemical Safety, 1992; Philip et al., 2018). It is known that, in not so high concentrations, practically all metals participate in biochemical processes arising in living organisms and are an indispensable part of many vitamins, ferments, metalloproteins and other compounds, without which metabolic processes are impossible. However, with the intensification of anthropogenic action on aquatic ecosystems, most of the time we encounter the problem of the toxic influence of metals on hydrobionts, including on the growth and development of fish.

We have conducted experiments to assess the impact of different concentrations of metals in water on the development of roe, the growth of larvae and brood of fish. The experiences with the individuals of one year, two years and those who are sexually matured, in order to elucidate the action of metals – microelements contained in water and fodder – were carried out in fish tanks, plastic pools and experimental ponds. In experiments, simultaneously with the assessment of the state of fish, their biometric parameters, the concentration of accumulated microelements and a whole complex of indices were also recorded, such as the values of temperature, pH, hardness of water, concentrations of the basic components of the ionic composition of water, biogenic elements, organic substances, etc. (Zubkova N., 2011; Zubcov et al., 2017).

The results of the experimental works showed that the eggs of the species investigated by the fish are more sensitive to the action of the increased concentrations of metals at the gastrula stage and at the beginning of the organogenesis stage. The increase of the concentrations of copper, nickel, lead, manganese, zinc, cobalt in water (even insignificant – within the limits of 5-15 µg/l) in the embryo hatching stage and in the first 48 hours can lead to the destruction of up to 50-70% of carp, silver carp, bighead carp, grass carp and roach larvae. It turned out that when using the water from Dniester river, the effect of depression, in the case of the investigated fish, is already manifested when adding to the water 8-15 µg/l of lead and cadmium, 20-30 µg/l of copper and nickel, 30-40 µg/l of cobalt and over 80-100 µg/l of zinc and manganese.

It was established that the incubation of the eggs in the water containing 20-30 µg/l of lead and cadmium already at the gastrula stage, a pathological development of the eggs is observed (voids of an indeterminate form are formed in the cells) and, finally, at the hatching, the monstrous forms of the larvae (over 75-88%) prevail over those with a normal development. The introduction of these metals into the water at the initial stage of gastrulation in concentrations above 30 µg/l causes the loss of a considerable number of eggs, their development is stopped and a mass destruction of embryos (up to 85%) is observed already at the stage of organogenesis (Zubkova N., 2011).

In connection with the fact that the larvae, in the first 48 hours until the filling of the swim bladder and the transition to the mixed type of nutrition, are very sensitive in the case of a mixture from the outside, in order to carry out experiments for evaluation of the influence of new microelements, most of the time we have used three days larvae.

As a criterion for assessing the influence of metals on the larvae, their biometric indices and the number of perished larvae were taken. In all aquariums the same density of larvae was maintained and, if in one of the three variants their perishing was recorded, then the same number of live larvae was extracted from the other aquariums.

From each category of 3, 6, 12 and 24 days, 40-50 individuals were chosen, they were weighed, their total length was measured, and at the end of the experience (over 12 or 24 days), their total protein and metal content was also determined.

The results of the research have shown that lead, cadmium, nickel and copper in concentrations of 20-40 µg/l hinder the growth and development of carp and phytophagous fish larvae (Figure 2.9.).

It is also necessary to note that lead and cadmium from each other are synergists, because when they are used simultaneously, their negative action on the growth of larvae is doubled.

After an exposure of 12 days, the share of the larvae perished in the experience with the addition of copper was 22-30%, of cadmium – 23-32%, of lead – 21-24%, of nickel – 20-25%, and in the control group – 16-17%. We must also note that in aquariums with the addition of cadmium, lead, nickel and copper, the larvae perished mainly in the first 6 days, and in the reference ones their perishing had a uniform character throughout the entire period (Figure 2.10.).

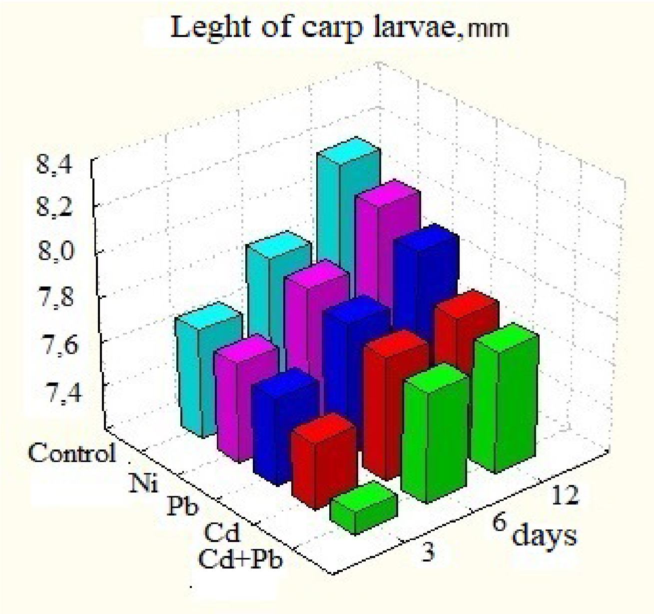

Figure 2.9. Change in the length of carp larvae (in mm) under the influence of metals after filling the swim bladder

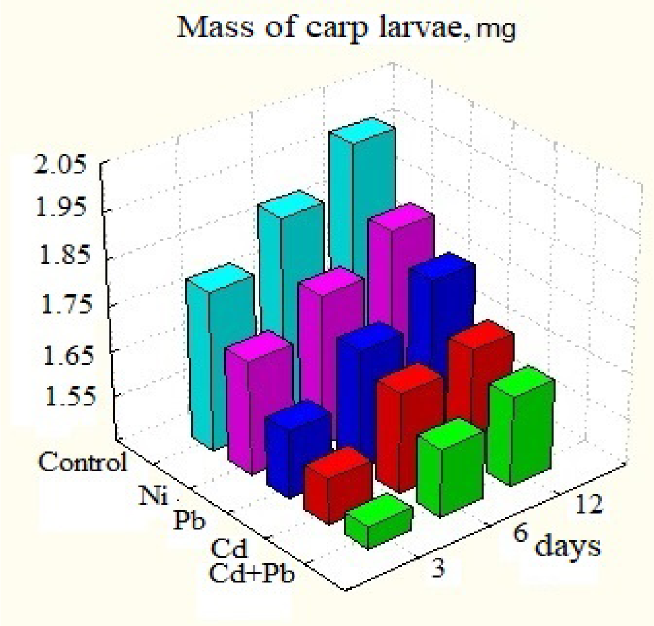

Figure 2.10. Change in the mass of carp larvae (in mg) under the influence of metals-microelements after filling the swim bladder

Therefore, according to the results of experimental research, we can conclude that cadmium in concentrations of 20-25 µg/l, nickel and lead in concentrations of 25-30 µg/l and copper starting with 30-40 µg/l, inhibit the growth and development of larvae aged 3-6 days of carp, silver carp and bighead carp.

Experiments with one-month old fry have shown their higher resistance to the investigated metals, compared to roe and larvae. These experiments were also carried out in aquariums, the fry were fed 2 times in 24 hours with ordinary compound feed and clorel suspension.

Experimental investigations were also carried out with sexually mature individuals, namely with carp and silver carp, with individuals of the same age, with a close body weight and similar degree of maturity of the gonads (Zubkova N., 2011).

In the experience with a duration of 15 days, increased concentrations of a whole complex of metals (copper zinc lead nickel cadmium) were used, which periodically, up to 5% cases, are found in the surface waters of Moldova. The fish were fed with compound fodder and clorel suspension once in 24 hours, but the food was consumed slowly, so in the last 7 days the feeding of the fish was interrupted.

The obtained results allow us to conclude that the increased concentrations of metals, especially the extremely high ones, even in the case of a not too long time of action in the last stages, directly before the spawning, can be essentially reflected on the effectiveness of the eggs laying; if the eggs get into the water with such a metal content, then they also perish for the most part.

2.4. CONCLUSIONS

- Quantitative estimation of the share of natural (hydrological, biological parameters) and anthropogenic (pollution, daming of rivers, etc.) factors in the processes of migration and forecasting of metal flows in the investigated ecosystems;
- Determination of the coefficients of migration and biogeochemical mobility of metals in aquatic ecosystems and their hydrological basins, which are converted by human activity;
- Establishing the regularities of accumulation of chemical substances in aquatic organisms, quantifying the functional role and the share of the main groups of hydrobionts in biogeochemical migration, determining the influence of chemical substances on the development of hydrobionts and production-destructive processes.
- Determination of the dangerous level of certain substances toxic to the functioning of aquatic ecosystems.
- Deciphering the accumulation processes and the role of metals and other chemical substances in the development of fish at various stages of development (eggs, larvae, brood, unmatched individuals and breeders), redistribution and change of the ratio between different metals in fish organs depending on plastic and generative metabolism, as well as the argumentation of the importance of different metals in assessing the quality of fishery products are of major importance; some of them being pioneering, are of importance for the development of ecological and ecotoxicological investigations.

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3

Chapter

EVALUATION OF EMERGING MICROBIAL CONTAMINANTS IN WATER

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3.1. GENERAL CONSIDERATIONS

Microbial communities are a fundamental part of aquatic ecosystems and are of great importance for the circuit of matter and energy. Heterotrophic bacteria play a decisive role in river ecosystems, in the degradation of organic matter that comes primarily from allohtone sources and less from native sources. Their contribution to river self-purification processes is of great interest in water quality assessment. Thus, bacteria are ideal sensors due to the rapid response to changing environmental conditions (Kavka et al., 1996; Findlay, 1991).

Aquatic environments are heavily contaminated with dangerous microorganisms as a result of pollution. Thus, discharges of wastewater into freshwater and coastal sea waters, are the main source of fecal microorganisms, including pathogens.

Any microorganisms are considered to be emerging contaminants (EC) because they have the potential to cause known or suspected negative effects on ecosystems or on the quality of human and animal life (Figure 3.1) (Rosenfeld & Feng, 2011; Gomes et al., 2020).

These microorganisms are usually spred throughout the environment, especially through feces, urine or wastewater. In this

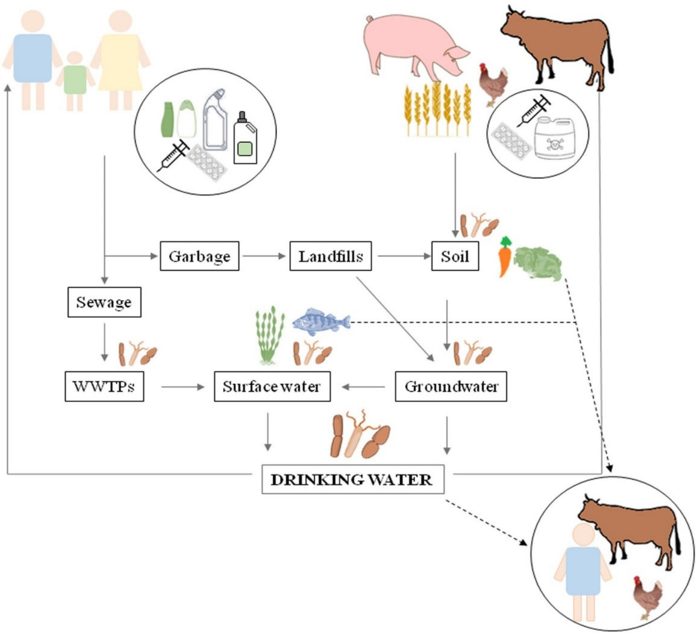


Figure 3.1. Dispersion of microbial EC into different ecosystems and organisms continuously exposed to them (WWTPs wastewater treatment plants) (*after* Gomes et al., 2020)

category of microorganisms are usually included some viruses that are found in natural or wastewater, such as: human polyomaviruses, hepatitis E virus and human adenoviruses. Antibiotic resistant bacteria (ARB) and their genes (ARGs) occur in nature and are also considered contaminants of emerging concern (Gomes et al., 2020). The presence of ARGs and ARB in different ecosystems worldwide is in correlation with many factors with anthropogenic influence on living organisms (humans, animals, and plants) (Figure 3.2) (Wang et al., 2020).

Although the presence of ARB and ARGs in various ecosystems around the world is particularly worrying, water being recognized as the main reservoir.

The spread of ARB and ARGs by fecal and human contamination is determined by three different ways (Figure 3.3): (1) man can be

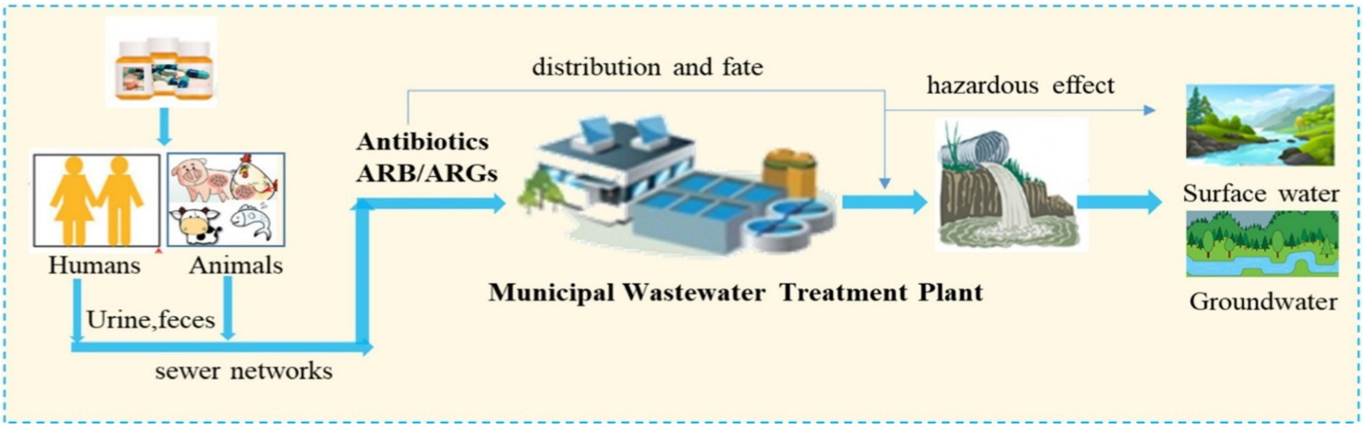


Figure 3.2. The route of ARB and ARGs in correlation with antibiotics dispersion in aquatic environments (*after* Wang et al., 2020)

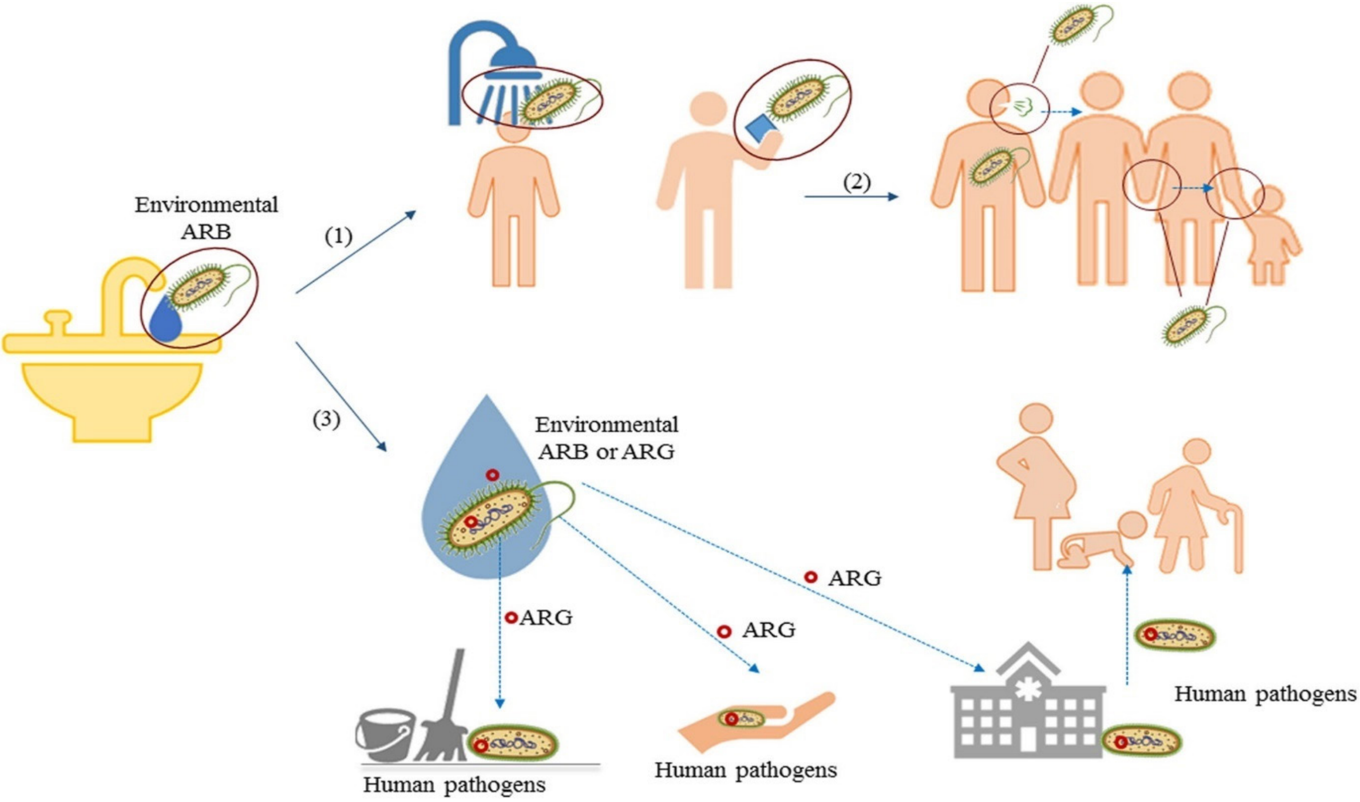


Figure 3.3. Spread of ARB and ARGs via fecal and human transmission (*after* Wang et al., 2020)

contaminated with an antibiotic-resistant pathogen by direct ingestion of drinking water, although no human-to-human transmission occurs; (2) human-to-human transmission occurs after direct infection through the consumption of contaminated water; and (3) horizontal transfer of ARGs to human pathogens (Wang et al., 2020).

Most microbiological indicators used to assess the microbiological quality of water are the faecal indicator bacteria (FIB), including *E. coli* and enterococci, which have been accepted to indicate fecal pollution and to assess microbial risks associated with direct targeting of real human pathogens (Saingam & Yan, 2020).

Such microorganisms are sometimes referred to as „conditional" or „opportunistic" pathogens. Usually these non-pathogenic or „saprophytic" microorganisms are common in the aquatic environment and other environments, but in some biological conditions they are able to infect, colonize and cause diseases in humans or animals.

The opportunistic pathogenic strains *Escherichia coli*, *Mycobacterium* spp., *Clostridium perfringens* and strains of the *Bacillus cereus* group were detected as potential hosts of ARGs. Based on multivariate statistics, it has been shown that the factors that influence the antibiotic-resistant gene in the waters, were multiple and interactive.

Water environments were found to be reservoirs of ARGs, which may be hosted by some opportunistic bacterial pathogens. There were established correlations between ARGs, mobile genetic elements (MGEs), bacterial communities, antibiotics, and opportunistic bacterial pathogens (Schets et al., 2015).

The monitoring of the microbiological emerging contaminants in water may have a range of aims, including:

(1) *Source water monitoring*: to establish the quality characteristics to be able to determine the health risks.
(2) *Operational monitoring*: to assess the performance of the processes of water treatments.
(3) *Validation* to establish the performance of a control assays.
(4) *Compliance monitoring*: to confirm whether the set targets for water quality, e.g., the standards for fecal indicator organisms.
(5) *Surveillance*: to continuously assess the safety and acceptability of the water quality.
(6) *Outbreak investigation*: to investigate the source of the outbreak and the effectiveness of measures taken to control the outbreak by testing for reference or specific pathogens.

In evaluation of water microbiological quality, the target microorganisms may be pathogenic, or they may be harmless indicator microorganisms. Indicators (model microorganisms) are being used in water quality management and health risk assessments because their detection is usually more rapid and less costly compared to the detection of pathogens. In this context, three principal indicators are taking in account for water microbiological analysis, as follows:

(1) *Contamination indicators*: microorganisms that demonstrate the type of the contamination such as the heterotrophic plate count to indicate level of organic contamination including also fecal contamination.
(2) *Fecal indicators*: microorganisms that indicate the presence of fecal contamination, such as *Escherichia coli* to indicate fecal pollution and consequently that a vast array of potential pathogens might be present.
(3) *Index and model organisms*: microorganisms indicative of pathogen presence, such as F-specific RNA bacteriophages as models of human enteric

viruses, and *E. coli* as an index for *Salmonella* spp.

Sometimes, additional indicators need to be assessed depending on the purpose of the examination and the type of water under study. These include the intestinal enterococci and *C. perfringens* (whose spores withstand disinfection much better than bacterial vegetative cells). Furthermore, fecal indicator bacteria do not reflect the presence of pathogens that are not of fecal origin and may be part of the natural aquatic microbiota, such as *Legionella* spp., *Pseudomonas aeruginosa*, and *Vibrio* spp. (Schets et al., 2015).

The most relevant microbiological indicators are those that appear in large numbers in human or animal feces, since feces are the most important source of pathogens in water. Thus, the indicators have the advantage that they can be detected and enumerated using simple, inexpensive methods that give results in a relatively short period of time.

In water samples can be detected a wide range of pathogenic microorganisms. Most commonly, quantitative tests are carried out that require several stages: pre-enrichment (1/4 reactivation), selective enrichment, isolation by plate culture methods, identification by biochemical, serological and / or molecular tests and, possibly, epidemiological patterns.

For pathogenic microorganisms, that are of fecal origin and that can actively multiply in the water environment (e.g. *Legionella* spp, *Pseudomonas aeruginosa*, *Vibrio* spp.), direct detection is required because there are no suitable model organisms (Schets et al., 2015).

Generally, the microbiological analysis of water refers to a wide variety of microorganisms, including viruses, bacteria and protozoa, but this chapter only reviews the detection of bacteria.

3.2. METHODS FOR THE QUALITATIVE AND QUANTITATIVE DETECTION OF BACTERIA IN WATER SAMPLES

Several methods are available for detecting bacteria in water, such as: cultural methods, microscopic methods, molecular methods and immunological methods.

Sampling and storage of samples

An appropriate specific sampling strategy of the sampling site is essential to design the monitoring plan in conjunction with the parameters to be tested and the methods used for testing. Selecting a suitable design for sampling, its frequency is also taken into account in a control strategy (Schets et al., 2015).

From natural environments, water samples are collected asepticly, in sterilized borosilicate glass bottles of 250 ml, from a water depth of 20-30 cm. The samples are then processed immediately in the laboratory or kept at 4 °C for maximum of 24 hours.

Cultural methods

Microorganisms from water microbiota are cultured in the laboratory conditions when adequate nutrients are supplied, and optimal growth conditions are provided. It will be taking in account that not all microorganisms present in a water sample microbiota can be cultured, and that an obtained count does not reflect the true number in a sample, because only the microorganisms that was viable, i.e., capable of growth on the culture medium used and under the culture conditions applied are counted.

Usually, culture methods for bacteria count are divided into two categories: (1) plate count method and (2) liquid enrichment method.

In the **plate count method**, a test sample is inoculated onto the surface of a growth medium that has been solidified by addition of agar (spread-plate method). Each individual cell of the target organism will multiply into colonies that are visible to the naked eye. The results are therefore expressed as „colonies-forming units" (CFU). CFU represent one or more cells of the target organism.

In **liquid enrichment method**, a test sample is inoculated into a growth medium that has been formulated to stimulate growth of the target organisms and to suppress growth of all other organisms („background microbiota"). For cultivatin an appropriate incubation temperature and time enhances the selective nature of the growth medium are used. If the target organism is present in the test sample, this will result in a positive signal. In simplest assay, liquid enrichment methods therefore give qualitative (presence/absence) information. To obtain semi-quantitative information, it is common to examine a series of different volumes (e.g., 100, 10, 1, and 0.1 mL). If a series of different volumes is examined in replicate, i.e., three - or fivefold, it is possible to use a statistical method known as the „most probable number" (MPN) technique to estimate the original concentration of the target organism. The precision of this estimate is low (e.g., the 95% confidence interval of a fivefold MPN estimate is roughly between one-third and three times the analytical result). The precision of this method is basically determined by the distribution of the target microorganisms in the sample (Schets et.al., 2015).

Heterotrophic plate count bacteria

The heterotrophic bacteria are microorganisms that indicate a correlation with the pollution of water by easily degradable organic matter. These are aerobic and facultative anaerobic bacteria which can growt on solidified Plate Count Agar medium (PCA), by incubation at 22°C within 48-72 hours (Kohl, 1975).

The heterotrophic plate count, formerly known as the *standard plate count*, is a procedure for estimating the number of live, culturable heterotrophic bacteria in water. Pour plate method is a comparatively simple enumeration procedure applicable to samples where densities of target microorganisms are sufficient to yield appropriate numbers of culturable elements for counting purposes (Standard Methods, 9215:2017).

In this method, a small sample volume (approximately 0.1-1 mL) containing suspended or decimal diluted bacteria cells suspension of the sample is homogenized with fluidified agar (Plate Count Agar) and tempered (about 15 mL of PCA fluidized at a temperature of 100 °C and tempered to 45°C) in a sterile Petri dish.

Homogenization is carefully carried out to disperse the sample evenly in the culture medium and then allow to solidify. Inoculated and solidified Petri boxes shall be reversed as quickly as possible in order to avoid condensation on the lid. Incubation of plates is carried out at 22°C for 48-72 hours. Colonies that will grow both on and inside the culture medium are carefully counted to obtain as accurate a number of cells as possible per unit of sample volume. Each colony represents one „colony-forming unit" (CFU) per milliliter of water sample. Isolated cells form single colonies that can be used to obtain pure cultures (Figure 3.4.a. Indirect counting technique) (Reasoner & Geldreich, 1985; Allen et al., 2004; Brown & Smith, 2015; Standard Methods, 9215:2017).

The number of micro-organisms present in the sample is determined using the formula:

$$CFU/mL = \frac{\text{colonies counted}}{\text{actual volume of sample plated, mL}} \quad (1)$$

For an accurate counting of microorganisms, the optimal colony count should be within the range of 30 to 300 colonies/petri plate (Figure 3.4.b. Colony counting by indirect method of counting microorganisms) (Cappuccino & Sherman, 2008).

In order to facilitate the interpretation of data on water quality from the point of view of pollution by organic matter, microbiological results have been classified by a classification system for water pollution by organic matter, presented in Table 3.1 (ICPDR, 2001). Depending on the number of heterotrophic bacteria per millilter of water analyzed, water pollution by organic matter is classified into five classes of pollution: low, moderate, critical, strong and excessive.

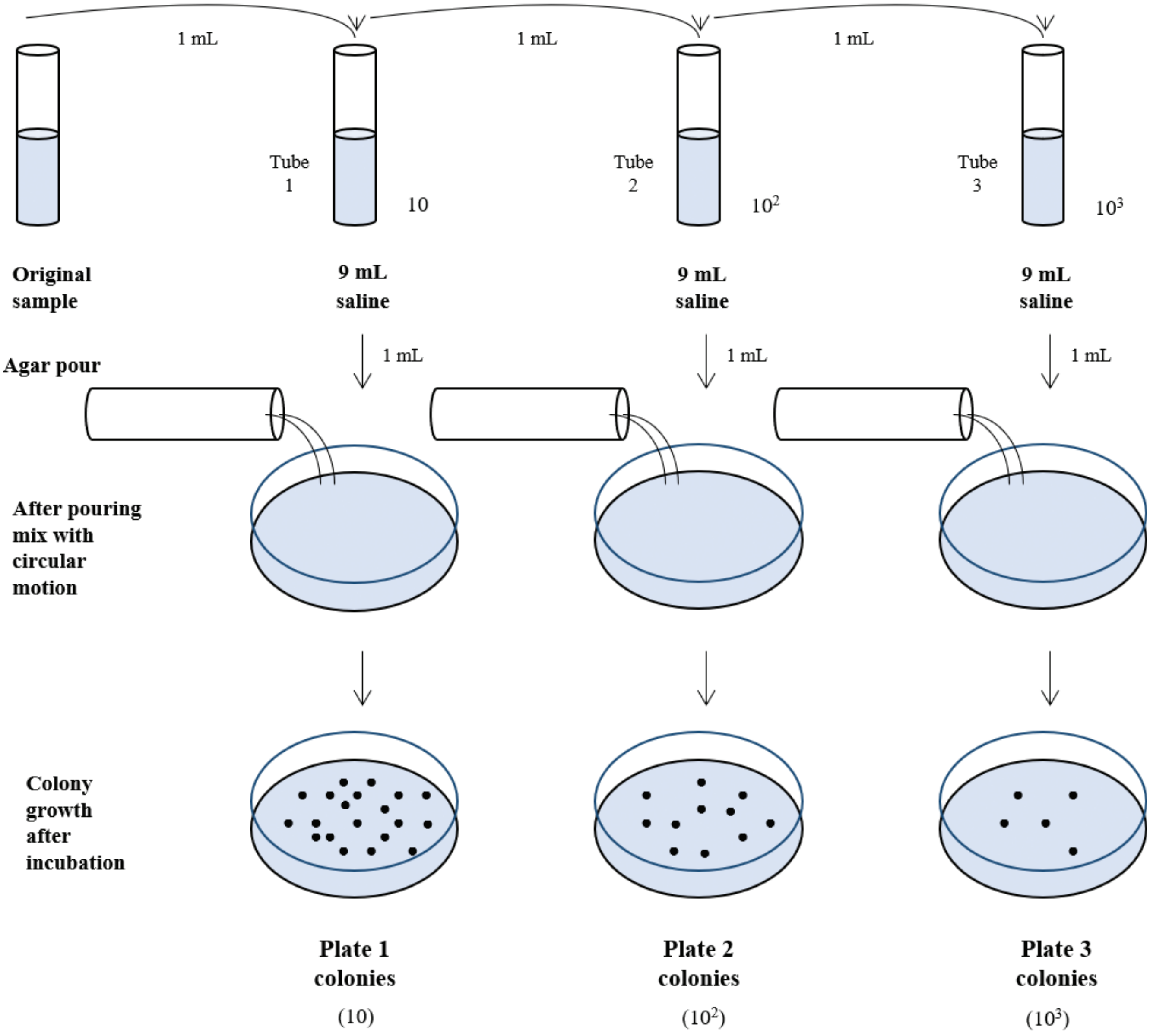
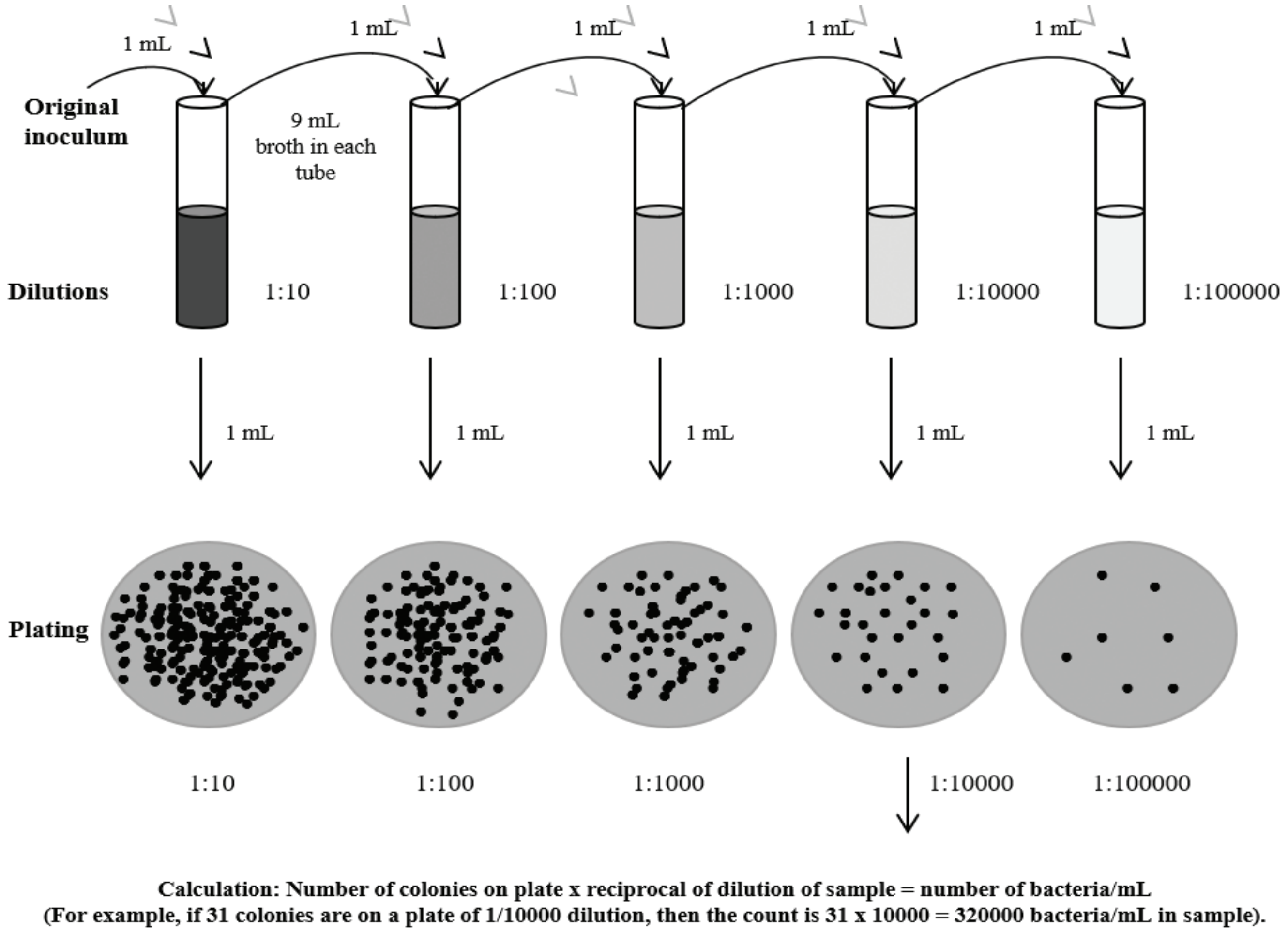


Figure 3.4. Heterotrophic bacteria plate count assay
a. Technique of indirect counting of micro-organisms



Calculation: Number of colonies on plate x reciprocal of dilution of sample = number of bacteria/mL
(For example, if 31 colonies are on a plate of 1/10000 dilution, then the count is 31 x 10000 = 320000 bacteria/mL in sample).

Figure 3.4. (continued)
b. Colony counting by indirect method of counting microorganisms

Table 3.1. System for classification of microbiological water quality according to pollution by organic matter

Microbiological evaluation of water quality		Clasa				
		I	II	III	IV	V
Indicator	Level of organic pollution	Low	Moderate	Critical	Strong	Excessive
Heterotrophic bacteria (colony count at 22°C)	CFU/mL water	< 500	> 500 - 10 000	> 10 000 - 100 000	> 100 000 - 750 000	> 750 000

Detection of coliforms in water

Microbiological water safety is achieved by monitoring organic contamination using indicator organisms such as total coliforms and *Escherichia coli*. Total coliforms are a group of bacteria commonly found in aquatic environments, soil and vegetation, as well as in the intestines of mammals, including humans.

Total coliform bacteria are commonly used to assess the overall sanitary quality of water, but also in situations where fecal contamination is present, since total coliforms are more numerous than *E. coli*, thus representing a more sensitive microbiological indicator (Feng et al., 2020).

The term „coliform" describes a group of enteric bacteria. This term is not a taxonomic classification, but is a definition used to describe a group of gram-negative anaerobic bacteria, optional, in the form of sticks, which ferment lactose with acid and gas production in 48 hours at 35 °C.

The presence of coliforms and *Escherichia coli*, particularly, in water microbiota is accepted as prediction of a recent fecal contamination, it is the possible to be accompaigned by pathogens. Thus, the concept of using of total coliforms as an indirect indicator of health risk it is complicated in practice, due to the presence of other enteric bacteria like *Citrobacter* spp., *Klebsiella* spp. and *Enterobacter* spp. with similar metabolic behaviour and phenotypic characteristics like *E. coli*, so that they are not easily distinguished from it (Figure 3.5.) (USEPA, 2002; Duncan & Horan, 2003; WHO, 2017; Standard methods, 9221:2017).

Most strains of *E. coli* are not considered pathogens, but they can also be pathogens causing infections in hosts with low immunity. *E. coli* belongs to the *enterobacteriaceae*family, which incidentally includes many genera, including known pathogens such as *Salmonella* spp., *Shigella* spp. and *Yersinia* spp.

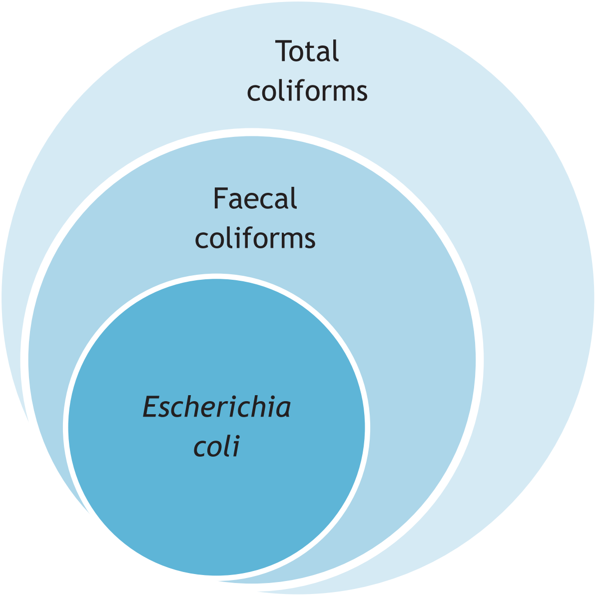


Figure 3.5. Schematic representation of the group of coliform bacteria

However, there are pathogenic strains of *E. coli* that cause gastrointestinal disease in healthy people when accidentally ingested (Feng et al., 2020).

The fecal coliform group consists mainly of *E. coli*. Some enteric bacteria, such as *Klebsiella* spp. can also ferment lactose, and therefore can be considered as a fecal coliform bacterium. The inclusion of the bacteria *Klebsiella* spp. in the group of fecal coliforms decreased the correlation factor of this group with fecal contamination. Thus, the implementation of methods for the rapid detection of *E. coli* is important in the microbiological control of water (Directive 7/EC, 2006; Wade et al., 2003).

Principle of the method for determining the most likely number of coliforms

The most probable number (MPN) is an important culture method used to estimate of total and fecal coliforms in drinking and surface waters, soils and sediment. Garthright (1998) showed that the MPN test is a statistical estimate of culturable units and assumes such units are randomly distributed within a sample. Functionally, the MPN test is based on dilution of sample until a dilution range which permit to counting of the targeted microorganisms. Actual dilutions required are based on experience and MPN tables are commonly based on inoculation of media with 3, 5, 10 or 12 sample portions at each dilution. The MPN is useful when cells are present at low densities in analysed samples. Coliform concentration is reported as the number of colonies forming units (CFU) per 100 mL of water (APHA, 2012; WHO, 2017).

The NMP test is performed in the following steps (Feng et al., 2020):

- Presumptive test for coliforms, fecal coliforms and *E. coli*
- Confirmed test for coliforms
- Confirmed test for fecal coliforms and *E. coli*
- Completed test for *E. coli*.

MPN - Presumptive test for coliforms, fecal coliforms and E. coli

The presumptive test is a screening test of sample waters analysis to predict the presence of total coliforms. The working plan is as follow. Preparing decimal dilutions with sterile Butterfield's phosphate diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform concentration. Shake all suspensions 25 times in vortex mix for 7 s. Using at least 3 consecutive dilutions, inoculate 1 mL aliquots from each dilution into 3 tubes with Lauryl tryptose (LST) broth for a 3 tube MPN analysis (other analysis may require the use of 5 tubes for each dilution). Lactose Broth (LB) may also be used. Incubate LST tubes at 35°C± 0.5°C . Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. If the presumptive test is negative, no further testing is performed. If, however, any tube in the series shows acid and gas, the water is considered with microbiological risk and the confirmed tests are necessary to be performed. Perform confirmed test on all presumptive positive (gas) tubes (Figure 3.6).

MPN - Confirmation test for coliforms

From each gassing LST or lactose broth tube, transfer a loopful of suspension to a tube of Brilliant green lactose bile (BGLB) broth. Incubate BGLB tubes at 35°C ± 0.5°C and examine for gas production at 48 ± 3 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions. In order to confirm the presence of coliforms the working plan presented in Figure 3.7 is recommended to be applied.

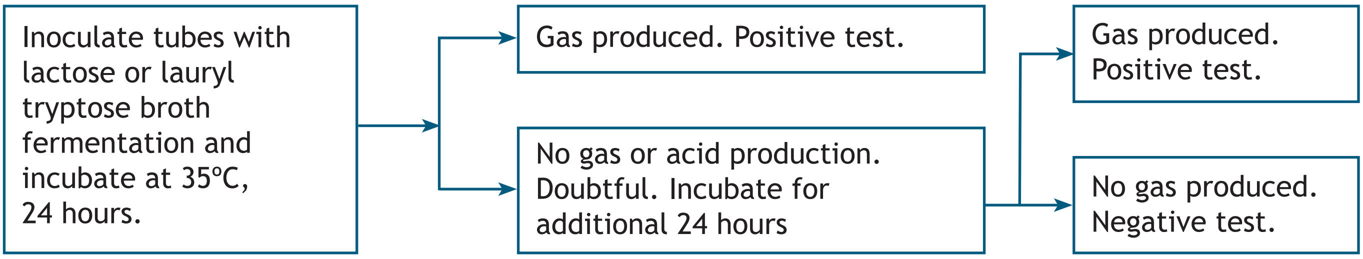


Figure 3.6. Schematic presentation of the presumptive test

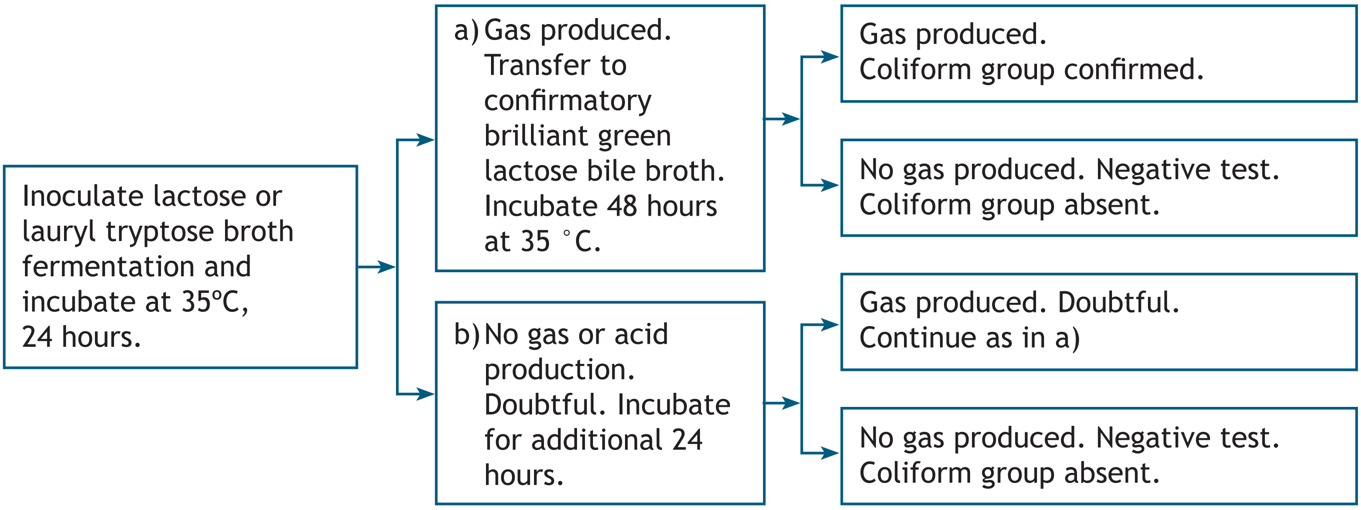


Figure 3.7. Schematic presentation of the confirmation test

MPN - Confirmatory test for coliforms and E. coli bacteria

From each gassing LST or Lactose broth tube from the presumptive test, transfer a loopful of each suspension to a tube of EC broth. Incubate EC tubes 24 ± 2 h at 44.5°C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN.

MPN - Completed test for E. coli

To perform the completed test for *E. coli*, gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a Levine's eosin-methylene blue (L-EMB) agar plate and incubate for 18-24 h at 35°C ± 0.5°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to Plate Count Agar (PCA) slants, incubate them for 18-24 h at 35°C ± 0.5°C and use for further testing (Figure 3.8) (Standard Methods, 9221:2017).

The confirmatory tests are as follows (Feng et al., 2020):

1) Perform Gram stain - All cultures appearing as Gram-negative, short rods should be tested for the IMViC (see below points 2÷5) reactions below and also re-inoculated back into LST to confirm gas production.
2) Indole production - Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°C ± 0.5°C . Test for indole by adding 0.2-0.3 mL of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test.
3) Voges-Proskauer (VP)-reactive compounds - Inoculate tube of Methyl-red VOGES-PROSKAUER broth (MR-VP broth) and incubate 48 ± 2 h at 35°C± 0.5°C. Transfer 1 mL to 13 × 100 mm tube. Add 0.6 mL α-naphthol solution and 0.2 mL 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops.
4) Methyl red-reactive compounds - After VP test, incubate MR-VP tube additional 48 ± 2 h at 35°C± 0.5°C. Add 5 drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.
5) Citrate metabolism - Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 h at 35°C ± 0.5°C . Development of distinct turbidity is positive reaction.

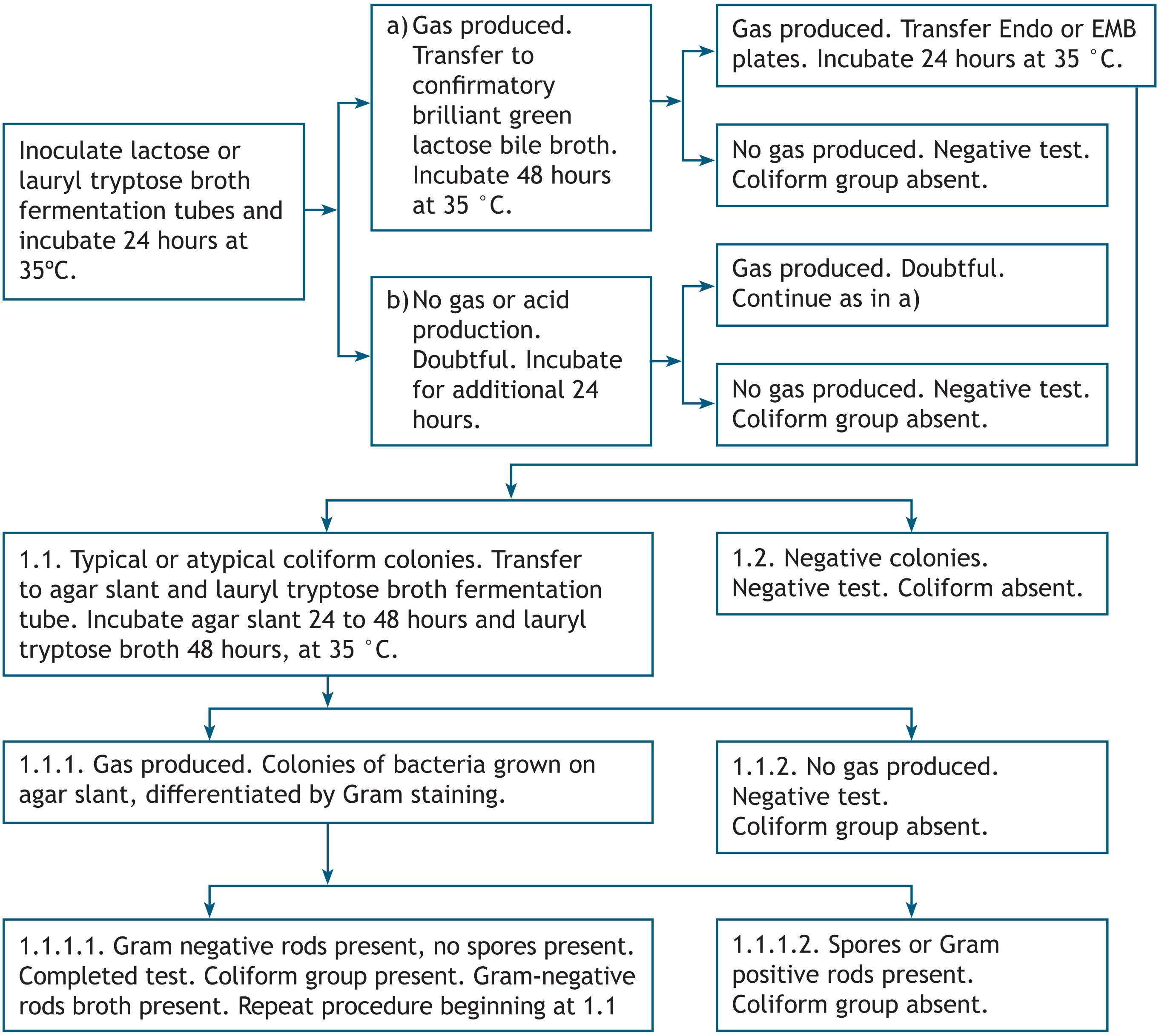


Figure 3.8. Schematic outline of completed test for *Escherichia coli* detection

6) Gas production from lactose – Inoculate a tube of Lauryl tryptose broth (LSB) and incubate 48 ± 2 h at 35°C ± 0.5°C. Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction.

All cultures that (a) ferment lactose with gas production within 48 h at 35°C, (b) appear as Gram-negative nonsporeforming rods and (c) give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN of *E. coli* based on samples of EC tubes in 3 successive dilutions that contain *E. coli* is then performed.

Calculation of MPN

Based of the number of positive tubes resulting from the incubation on selective media of the three sets of dilutions in correlation with the Table 3.2 data, the the MPN can be established and then CFU/mL taking in account the lowest dilution factor of the three dillutions used for calculation.

Example: A water sample was diluted by making 3 successive decimal dilutions. From each dilution, 5 test tubes with selective medium were inoculated, resulting in a total of 15 inoculated tubes. After incubation, the *first group of test tubes inoculated from the first dilution showed 4 positive tubes. The second group of test tubes inoculated from the second dilution showed 2 positive tubes, and the third group of tubes inoculated with dilution 3 showed 1 positive tube. The MPN index according to Table 2 data, for 4, 2 and 1 positive tubes, is 26. The CFU/mL for the analyzed water sample is: 26 × 10 = 260* (Feng & Hartman, 1982).

Table 3.2. Table for establish MPN index

Number of positive tubes			MPN Index	Number of positive tubes			MPN Index
Dilution group 1	Dilution group 2	Dilution group 3		Dilution group 1	Dilution group 2	Dilution group 3	
0	0	0	< 2	**4**	**2**	**1**	**26**
0	0	1	2	4	3	0	27
0	1	0	2	4	3	1	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	30
1	1	0	4	5	0	2	40
1	1	1	6	5	1	0	30
1	2	0	6	5	1	1	50
2	0	0	4	5	1	2	60
2	0	1	7	5	2	0	50
2	1	0	7	5	2	1	70
2	1	1	9	5	2	2	90
2	2	0	9	5	3	0	80
2	3	0	12	5	3	1	110
3	0	0	8	5	3	2	140
3	0	1	11	5	3	3	170
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
4	0	0	13	5	4	4	350
4	0	1	17	5	5	0	240
4	1	0	17	5	5	1	300
4	1	1	21	5	5	2	500
4	1	1	26	5	5	3	900
4	2	0	22	5	5	4	1600
-	-	-	-	5	5	5	>1600

To transform fecal indicator concentrations into levels of faecal microbial pollution, a five-tier water quality classification system is recommended to be use that integrates bathing water quality guidelines with the European Water Framework Directive (EU-WFD). The system defines five classes of faecal pollution, classes I and II are lower, and quality classes III, IV and V exceed the faecal pollution limit values for good bathing water quality (Table 3.3) (Directive 60/EC, 2000; Kavka et al., 2006; Kirschner et al., 2015, 2017).

Table 3.3. Microbiological classification system of water quality according to fecal pollution

Microbiological evaluation of water quality		**Clasa**				
		I	**II**	**III**	**IV**	**V**
Parameters	**Fecal pollution**	**Low**	**Moderate**	**Critical**	**Strong**	**Excessive**
Total coliforms	**CFU/100 mL water**	**< 500**	**> 500 - 10 000**	**> 10 000 - 100 000**	**> 100 000 - 1000 000**	**> 1000 000**
Fecal coliforms (thermotolerant coliforms)		**< 100**	**> 100 - 1 000**	**> 1 000 - 10 000**	**> 10 000 - 100 000**	**> 100 000**
Fecal streptococci (*Enterococci*)		**≤ 40**	**> 40 - 400**	**> 400 - 4 000**	**> 4 000 - 40 000**	**> 40 000**
Escherichia coli		**≤ 100**	**> 100 - 1 000**	**> 1 000 - 10 000**	**> 10 000 - 100 000**	**> 100 000**

Microscopic Methods

Microscopic methods for direct enumeration of bacteria in water has only limited applications in health-related water microbiology because the detection limit is relatively high and because the microscopic image does not clearly provide information to the identify of the bacteria. It is considered that the microscopic methods do not commonly differentiate between living and dead cells, which makes interpretation of analytical results in terms of health risks impossible. Some advanced methods asspecific immunofluorescence methods using selective antibody preparations are available (Schets et al., 2015). Several methods have been developed to assess the viability of single cells by microscopic methods, such as exclusion of certain dyes (indicating integrity of the cell wall), reduction of tetrazolium salts (indicating active respiratory metabolism), and cell elongation in the presence of nalidixic acid (indicating active biosynthesis). These methods are time consuming and require the expertise of a research laboratory. The limitations are that in a bacterial population under stress the detectability by culture methods is lost more readily than the viability using microscopic methods. On resuscitation from this ‘viable but nonculturable’ (VBNC) state, the cells regain culturability and the renewed ability to cause infection.

Molecular methods

The detection of a specific sequence within the DNA or RNA of the target organism is principle of molecular methods. Compared to culture and microscopic methods, molecular methods generally provide results more rapidly, although sometimes less quantitatively (Schets et al., 2015). These methods do not have the limits for detection of nonculturable organisms.

Detection of mRNA, viability polymerase chain reaction (PCR) methods allow differentiation between live and dead organisms.

In recent years, molecular methods for the detection of bacteria have been explored in many research laboratories. Fluorescence *in situ* hybridization involves direct detection of the DNA or RNA of specific bacteria after concentration on membrane filters with sequence-specific oligonucleotide probes labeled with fluorescent dyes, without the need for pre-culture.

PCR can be used for direct detection of bacteria in water samples and be applied to identify bacterial isolates. Although PCR is a sensitive method, concentration of water samples is nevertheless required. PCR detects nonculturable bacteria but may also detect dead organisms or past contamination because DNA is very stable in the environment. mRNA molecules, on the contrary, are very labile and have a rapid turnover. PCR cycle consists of three steps: denaturation of the DNA at high temperature, annealing of sequence-specific primers to the target sequence at a lower temperature, and elongation of the primers at a temperature that is optimal for the thermostable polymerase enzyme.

Reverse transcriptase-PCR (RT-PCR) assay can amplify mRNA, which indicates the presence of living or recently dead bacteria in a sample.

The principle of RT-PCR is that the PCR step is preceded by a reverse transcriptase step in which the mRNA is transcribed into a copy DNA strand, which is used as target in the PCR reaction. Differentiation between live and dead cells can also be accomplished by viability-PCR strategies in which the DNA of damaged cells is excluded from PCR amplification by the covalent binding of an intercalator to the DNA of the damaged cells. An alternative for the discrimination between live and dead cells is preceding the PCR by a short enrichment step allowing culturable bacteria to multiply.

PCR is a presence/absence technique, but real-time quantitative PCR (qPCR) systems have been developed that allow direct molecular detection and quantification of microorganisms in water samples. In qPCR methods, the amplified DNA is detected during the PCR reaction in 'real time'. For typing of bacterial isolates, DNA-sequencing is often used.

Next-generation sequencing enables the rapid production of thousands or millions of sequences, allowing for detection of whole genomes.

Experimental techniques used in the frame of the project BSB27-MONITOX

Detailed knowledge of faecal pollution in aquatic environments is crucial for river basin management activities in order to maintain safe waters for recreational and economic purposes. The evaluation of the level of microbiological contamination of the Danube River and Black Sea water in the target areas of the BSB27 MONITOX project in the SE part of Romania was carried out by counting the heterotrophic bacteria and total coliforms.

The collected water samples were tested for different populations of microorganisms in the laboratories of the BioAliment Platform of the Dunarea de Jos University of Galati, Romania, by incubating the diluted water samples on specific media for the growth of coliform bacteria and mesophilic aerobic bacteria (Figures 3.9-3.16).

Figure 3.9. Preparing culture media, a – weighing and dosing of culture media, b - sterilization of culture media and pipette tips

a

b

Figure 3.10. Sample processing – a, b – realization of dilutions in sterile physiological serum and their distribution in sterile Petri dishes

Figure 3.11. Omogenization of the dillution samples with culture media in sterile Petri dishes

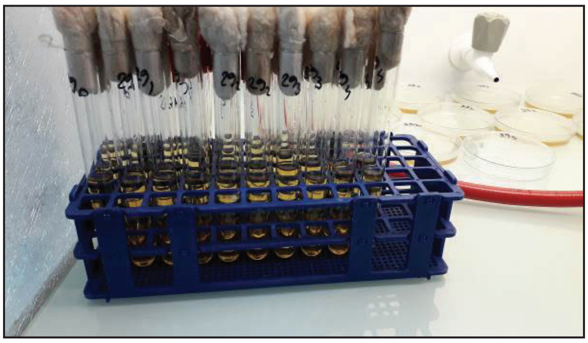


Figure 3.12. The method of multiple tubes: Inoculation of the sample dilutions in specific medium for presumptive test

Figure 3.13. The incubation of the Petri dishes and tubes with the sample dilutions at 37 °C for 48 hours

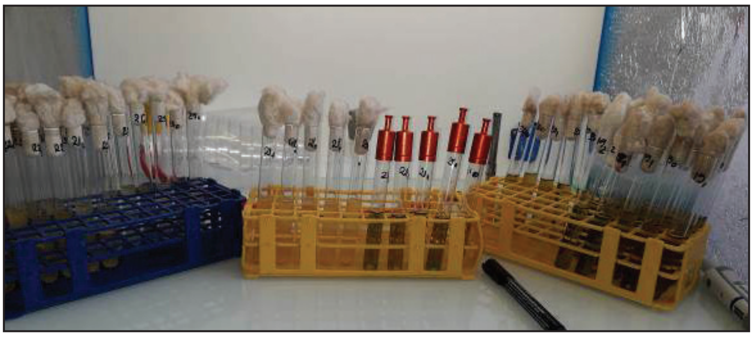
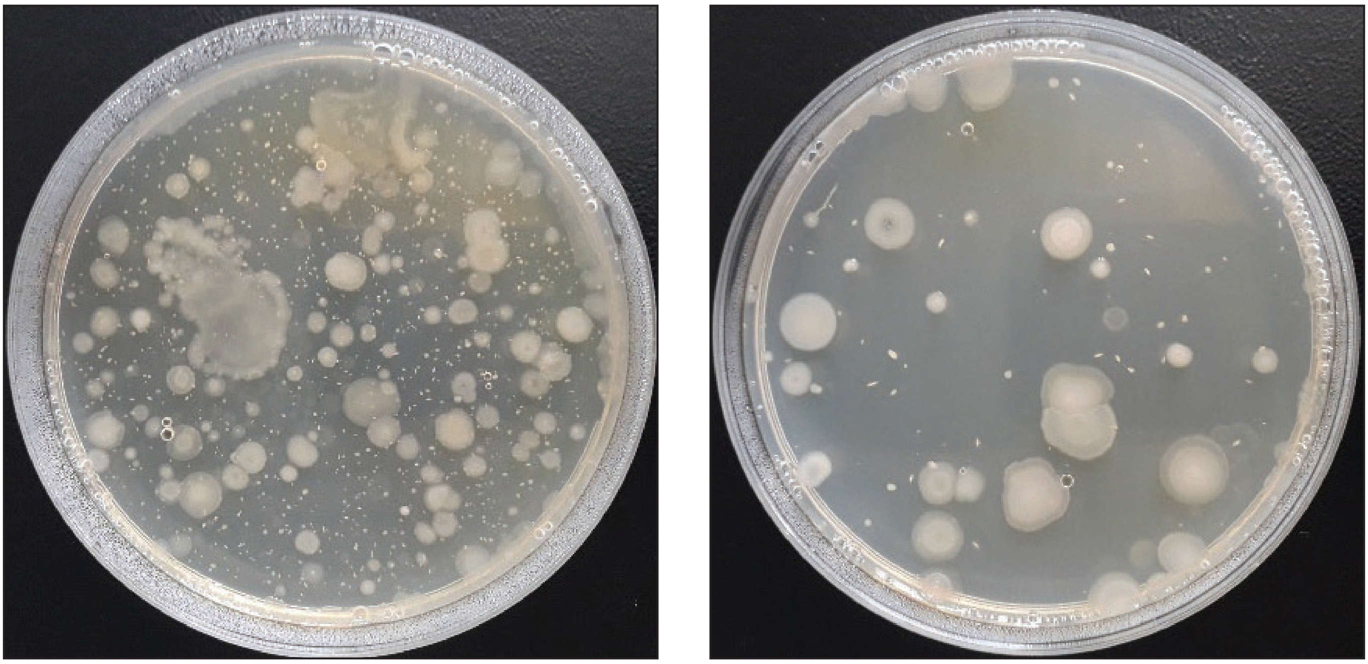


Figure 3.14. Multiple tubes method: inoculation from positive test specific medium for confirmatory test

Figure 3.15. The method of multiple tubes: Positive tubes of the confirmatory test after incubation at 37 °C for 48 hours



a b

Figure 3.16. Colony of heterotrophic bacteria on PCA medium (a – first and b – second dilution of the sample)

The determinations of total mesophilic aerobic bacteria were carried out in pour plate using plate count agar followed by incubation at 37°C for 48 h, method provided by the Romanian Standard STAS 3001-91. The medium used for cultivation of microorganisms were Plate Count Agar (Sigma Aldrich, Sweden). The number of heterotrophic bacteria was expressed as colony forming units (CFU) per mL of sample.

Coliforms at 37°C were determined through the most probable number (MPN), with three sets of three tubes according to STAS ISO 4831-92. This method involved two tests, the presumptive test and the confirmatory test. For presumptive test was used broth of meat with lactose and Durham tubes. The confirmatory test was performed with Brilliant Green Bile 2% broth (Scharlau, Spain) with Durham tubes. After incubation, the presence of the gas in Durham tubes and the change color of the medium from green in yellow indicated the growth of coliform bacteria.

Coliforms were present in all water samples, and their number varied between 600 and 250 000 CFU/100 mL (Vasile et al., 2019). The number of total mesophilic bacteria is closely correlated with the amount of organic matter in water. The number of bacteria in the water samples analyzed ranged from 1.55×10^2 to 6.08×10^3 CFU/mL. These values were higher than the values recorded by Ungureanu et. al. (2014) within the project with code MIS ETC 1676 (INPOLDE) funded by the EU through the Joint Operational Programme Romania-Ukraine-Republic of Moldova: the total mesophilic aerobic bacteria were in the range of 1.8 x 10 CFU/mL and 4.6 x 10 CFU/mL, and the MPN of coliforms, between 60 CFU/ mL and 600 CFU/ mL.

The results obtained by our team in the comparative study of the microbiological contamination of the surface water from the Danube and Black Sea basins (Ene et al., 2020) demonstrate a decrease in microbiological contamination in 2020 during the COVID-19 pandemic, in all the samples collected from the Black Sea coast, the Danube arms, the Danube-Black Sea confluence and the Danube River downstream of the city of Galati.

Overall, microbial water quality assessment is needed to assess the significance of the growing anthropogenic impact on the Danube River, the Danube Delta and the Black Sea. According to the international classifications for bathing waters, the water samples were characterized mainly by a moderate number of coliform bacteria. The exception was made by some sites located in several sectors of the Lower Danube River, upstream of the city of Galati, for which critical values were met.

3.3. CONCLUSIONS AND PERSPECTIVES

Quality assurance and traceability of the microbiological assays of water is the principal criteria for the relevance of the results. The analysis plan must be establiseh for each sample taking in account numerous factors related to level and type of contamination, physico-chemical ctahateristics of analysed samples, the techniques used, detection limits etc. Thus, if water is highly contaminated, some methods may require multiple dilutions for accurate results, whereas water with a very low contamination levels may require the analysis of multiple replicate samples to obtain reliable results. In microbiological examinations, the nature and age of the water sample, as well as the growth medium and the conditions of incubation can have a strong influence on the species isolated and the count obtained. Consequently, different microbiological methods have variable accuracies. This means that the standardization of methods and laboratory procedures is of great importance if criteria for the microbial quality of water are to be uniform across different laboratories both within country and internationally.

Establishing the standard methods to be used for routine examinations is necessary for the relevance of the results. A relevant microbiological investigation plan should take into account: (1) the use of Standard Operating Procedures; (2) proper documentation to warrant traceability of samples and used protocols; (3) the use of relevant control samples, which provide information about the relevance and the reproductibility of the results; (4) the participation in external quality assessment schemes (proficiency testing) to evaluate the entire testing process and reporting of results.

The microbiological controls of water could rely on the analysis of faecal pollution indicators. The classical indicators together with the improvements on the treatment and disinfection to control waterborne outbreaks offer predicted information about microbiological quality of water. However, bacterial indicators do not predict parasite and virus's contamination, more resistant to disinfection. Furthermore, the investigation of classical indicators may be substituted by the direct detection of pathogenic microorganisms, but in this case the methods are much more expensive and involve special resources (infrastructure, highly qualified operators and financial investion).

The relevance of the control, data collection and safety assurance of water at global level, motivated the authorities in the development and implementation of more preventive approaches, like the *Water Safety Plans* proposed by World Health Organization (WHO).

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4

Chapter

EMERGING CONTAMINANTS IN THE ENVIRONMENT - ECOTOXICOLOGICAL EFFECTS

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4.1. GENERAL CONSIDERATIONS

The emerging contaminants or pollutants (ECs or EPs) comprise, in a broad sense, any synthetic or naturally-occurring type of substance or chemical or any microorganism that is not usually monitored by any monitoring agency or regulated in the environment, with potentially known or presumed ecological and human health side effects (European Environment Bureau, 2018).

These types of emergent contaminants include chemicals found in pharmaceuticals, personal care products, pesticides, industrial and household products, surfactants, industrial additives and solvents. Many of these substances are ceaselessly used and released back into the environment at very small concentrations, which may, in the long run, cause chronic toxicity, endocrine disruption in humans and aquatic wildlife and the development of pathogen bacterial resistance (European Environmental Bureau, 2018).

According to the European Environment Bureau and UNESCO, all of these factors are converging to address the urgent need to strengthen the scientific knowledge and to adopt the most appropriate approaches to monitor different emerging pollutants in water and wastewater resources, to assess their potential human health and environmental risks, and to prevent and control their disposal into water resources.

In the last years, the availability of a solid and sensitive instrumental method for chemical analysis has enabled different studies on the emergence of many contaminants in the environmental systems at very low concentrations (Farré et al., 2012; Carvalho & Santos, 2016; Enachi et al., 2019).

These types of emerging contaminants include a broad variety of chemicals (Figure 4.1) characterized by a wide distribution and persistence in the environment due to their intensive use for different purposes. Pharmaceutical and personal care products, plasticizers, flame retardants and pesticides are the ECs main categories (Sauvé & Desrosiers, 2014; Enachi et al., 2019).

The fate and the potential effects and risks of these compounds on ecosystems and human health at such concentrations have been a real concern for the scientific world. This notorious interest justifies the reasonably large number of reviews and scientific articles regarding the analytical determination (Albero et al., 2015), the occurrence, distribution and risks of emerging contami-

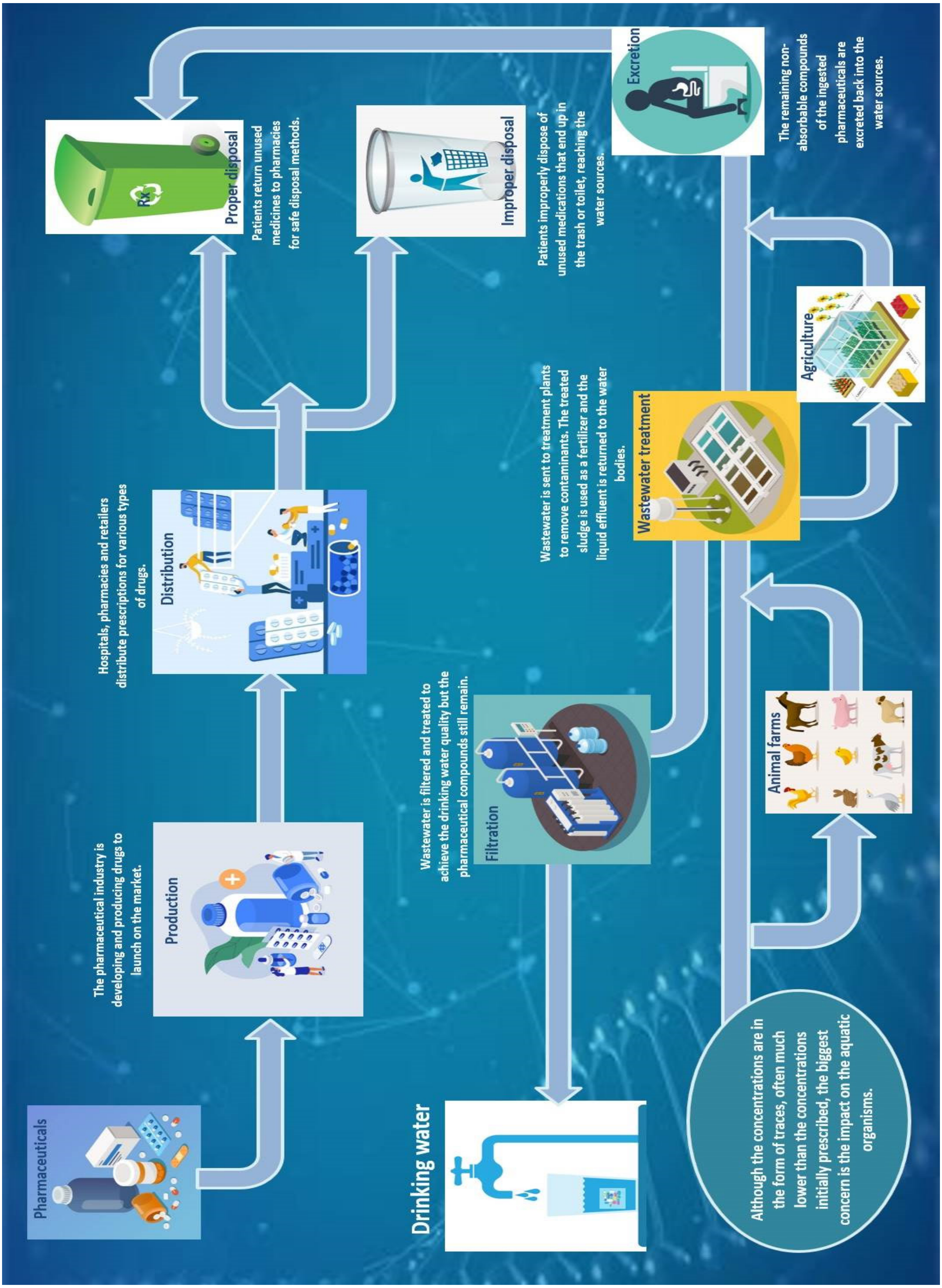
Figure 4.1. The most representative emerging contaminants

nants in the environment, especially in the aquatic compartments (Pal et al., 2014; Carvalho & Santos, 2016; Enachi et al., 2019). Nonetheless, little is currently known about the potential toxicological and ecological impact of these types of substances on the aquatic ecosystems (Bellenger & Cabana, 2014; Carvalho & Santos, 2016). In term, due to the high incidence rate of these compounds in the environment, the EU, EMEA and CDC developed a series of water quality regulations (EC 2000, 2001, 2008, 2013; US EPA, 2014; CE, 2015a).

Consequently, further scientific research that concerns their effects on the environmental and human health is required in order to provide the necessary means to the competent authorities that will have to establish effective and adequate monitoring strategies and appropriate regulation for the targeted substances (Murphy et al., 2012; Enachi et al., 2019). In recent years, the manufacture and widespread application of synthetic chemical compounds have become crucial in the industrial sectors. However, the low biodegradability of these compounds is the serious disadvantage associated to their extensive use.

Therefore, a significant interest continues to grow for the treatment and remediation of these compounds, here including antibiotics, contraceptives, endocrine disruptors, pesticides, hormones, pharmaceuticals and dyes synthetic pollutants primarily due to the fact that these substances can cause nervous, hormonal and reproductive system disorders with serious health outcomes (Bilal et al., 2018a; Hernandez-Vargas et al., 2018; Ullah et al., 2018).

These synthetic chemicals exhibit a cytotoxic activity and can cause obesity, diabetes, cardiovascular and reproductive disorders or even lead to other disorders like cancer. By discharging the effluents from the communal wastewater that contain these emergent contaminants (pharmaceuticals, dyes from the paper or textile, agriculture or household industries), this action leads to a serious environmental pollution (Bilal et al., 2018b; Bilal et al., 2019; Enachi et al., 2019).



The pharmaceutical industry is developing and producing drugs to launch on the market.

Hospitals, pharmacies and retailers distribute prescriptions for various types of drugs.

Patients return unused medicines to pharmacies for safe disposal methods.

Patients improperly dispose of unused medications that end up in the trash or toilet, reaching the water sources.

The remaining non-absorbable compounds of the ingested pharmaceuticals are excreted back into the water sources.

Wastewater is sent to treatment plants to remove contaminants. The treated sludge is used as a fertilizer and the liquid effluent is returned to the water bodies.

Wastewater is filtered and treated to achieve the drinking water quality but the pharmaceutical compounds still remain.

Although the concentrations are in the form of traces, often much lower than the concentrations initially prescribed, the biggest concern is the impact on the aquatic organisms.

Figure 4.2. Environmental pollution with pharmaceutical compounds

Numerous scientific reports have shown a variable number of sources and routes for the emergence of emerging contaminants, and in recent years this intensive research has been conducted in order to recognize their effects upon the environment (Rasheed et al., 2018a).

As each area has its own specific topography, climate, and environmental interactions, so they have a specific type of contaminants in that region. The production of contaminants and their impact on the environment depends entirely on their use and disposal.

Predominantly, these contaminants are produced through an on-field route, i.e. wastewater treatment plant, soil, wastes of a medical center, poorly treated raw materials, animal and livestock and industries (Fi-gure 4.2.). The wastewater treatment plants are also a source of pharmaceutical contamination as they are designed to eliminate the easily biodegradable nitrogenous, carbon and phosphorous compounds present in the wastewater (Bilal et al., 2019; Enachi et al., 2019).

4.2. EU REGULATION AND LEGISLATION REGARDING THE EMERGING CONTAMINANTS (PHARMACEUTICALS, ENDOCRINE DISRUPTORS)

Among these emerging contaminants, many pharmaceutical compounds such as antibiotics and endocrine disruptors can be found in the water ecosystems. Although the presence of these substances in the environment has been extensively studied, the long-term effects on humans and especially on ecosystems are still unknown.

These compounds are not yet included in the list of priority substances, but they were added in the first observation list (which became available in September 2014), to obtain the needed information on the pollutants that due to their toxicity might be suitable for prioritization, but for which there is a lack information about their appearance in Europe. The monitoring should be carried out once a year whilst a requirement of the EU Commission has also to develop a strategic approach for the water pollution by pharmaceuticals (Directive 2013/39/UE).

The EU Directive 2013/39/EU sets the environmental quality standards (EQS) for a list of 45 substances of priority concern (antibiotics excluded) in surface water and biota across EU due to their widespread use and their high concentrations in rivers, lakes, transitional, and coastal waters (EC, 2013).

In addition, from the perspective of the European Environment Agency (EEA), more monitoring data are needed to further define pharmaceuticals as emergent pollutants, which presently, they are not included in the routine monitoring programs at the European Union level (Chițescu et al., 2015).

During the last years, European Commission has increasingly promoted and supported several directives, projects, actions, and initiatives to mitigate against the widespread antimicrobial resistance, including here the surveillance of antibiotic consumption and research on the environmental contamination by these drugs in Europe (EC, 2011; CDC, 2015; ECDC, 2015a; EFSA, 2015; EMA, 2015a; EC, 2015 b,c).

In particular, the monitoring of priority substances listed in Annex X of the Water Framework Directive is of major concern (EC, 2000, 2008). Many of these substances may cause endocrine-related malfunctions both in wildlife and humans and are known as endocrine-disrupting compounds (Annex VIII-group 4) (EC, 2000).

In particular, the monitoring of priority substances listed in Annex X of the Water Framework Directive (WFD, 2000/60/EC) is of major concern (EC, 2000, 2008). Many of these substances may cause endocrine-related malfunctions both in wildlife and humans and are known as endocrine-disrupting compounds (Annex VIII-group 4) (EC, 2000).

The European Commission considers the water quality as one of the main priorities to achieve the environmental sustainability, ecological balance, and human health and well-being.

Consequently, regulations to minimize the adverse impacts of their production and consumption in the aquatic environment have also been implemented. Nonetheless, the current EU legislation for good-quality water in Europe does not cover a wide range of emerging contaminants, including here antibiotics, due to the lack of knowledge and understanding about their toxicity and environmental occurrence.

The recent Decision 2015/495 established a watch list of substances that may pose a significant risk for the aquatic environment in the EU, but available data are insufficient to conclude on the actual risk.

Thus, in all the countries, including Romania, where this type of monitoring programs does not exist, new programs, directives and regulations must be introduced at the institutional level to oversee the presence of pharmaceuticals and other emergent contaminants in the water environment.

4.3. ANTIBIOTICS

Antibiotics in the aquatic environment

The antibiotics' discovery is considered one of the most significant scientific achievements of the 20th century, revolutionizing both the human and veterinary medicine.

Antibiotics are natural, synthetic and semi-synthetic compounds which possess antimicrobial activities (Catteau et al., 2018; Szymańska et al., 2019; Kumar et al., 2019).

Antibiotics represent probably the most successful family (Figure 4.3) of drugs that

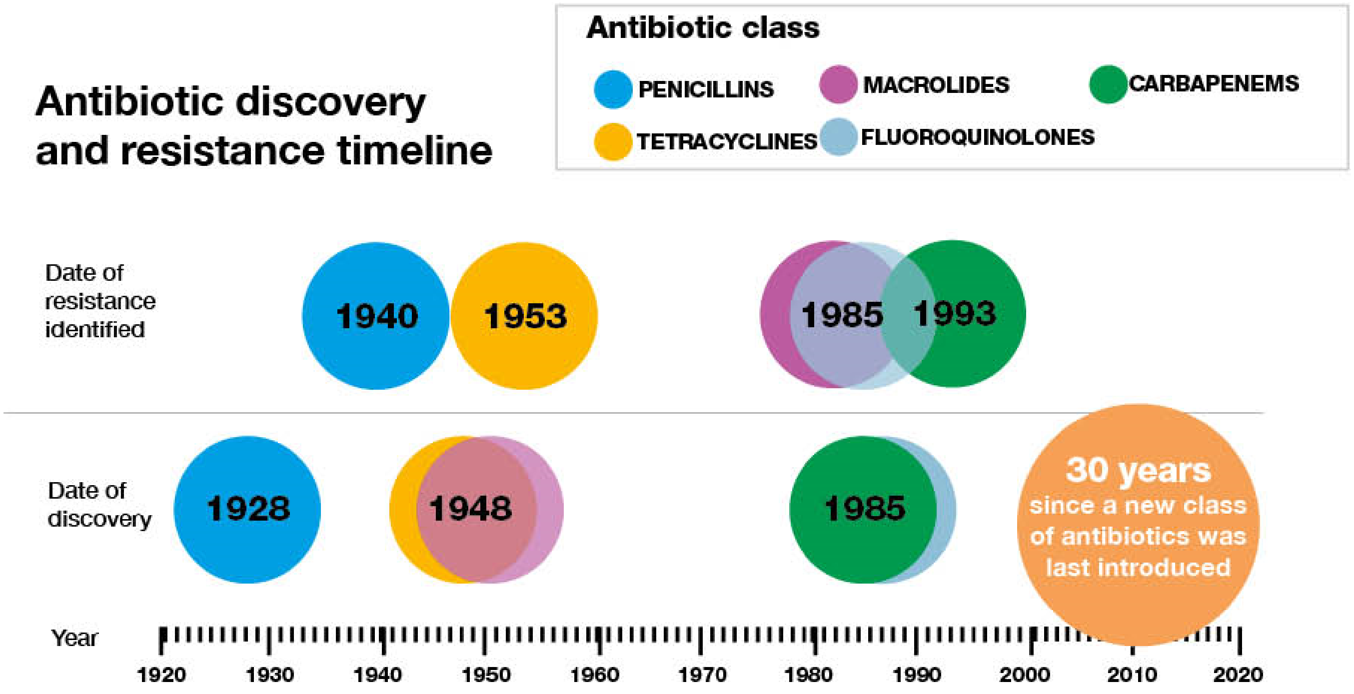


Figure 4.3. Antibiotic discovery and antibiotic resistance (Carvalho & Santos, 2016)

are used to treat the microbial infections in humans and animals with specific action.

The era of antibiotics began with Alexander Fleming in 1928 that accidentally discovered penicillin. Since this scientific discovery, many antibiotics have been synthesized for human, plants and animal health (Carvalho & Santos, 2016; Enachi et al., 2019).

The major entrance sources in the environment and pathways of these emerging contaminants and their by-products (metabolites and transformation products) are the municipal, agricultural, and industrial wastewater.

Also, the rate of consumption has reached substantial amounts (30% to up 90%) of antibiotics administered to humans and animals, so that these compounds are excreted into the waste stream via urine and feces (mostly unmetabolized).

Another problem is that the conventional wastewater and recycled water treatments are only partially effective in the removal or degradation of antibiotics.

The accelerated and continuous use of all known antimicrobials' compounds for the benefit of humans, animals, and agriculture led slowly but surely to their regular and repeated release into the environment and natural aquatic ecosystem.

Antibiotics as chemical compounds not only affect the targeted population but also influence the non-targeted population with high toxicity impact. The main problem of all antibiotics and other antimicrobial pharmaceutical compounds lies in the development of antibiotic resistance in all organisms.

The antibiotic resistance in the bacterial population represents one of the most serious health risks and concerns of humans (Szymańska et al., 2019; Kumar et al., 2019; Enachi et al., 2019).

The antibiotics emergence potential in the surface water can disturb the key bacterial cycles, mechanisms or processes that are critical to maintain the aquatic balance or the agricultural balance of the soil and the animal production.

The wastewater treatment pilot plants effluents, leakage of sewage and agricultural waste are some of the secondary contributors as these compounds are not entirely metabolized and may escape degradation.

Nevertheless, depending on their mobility and persistence in the soil-water environment, antibiotics and their by-products may reach the surface waters and ground waters, and, potentially, the drinking waters.

According to the aqueous environment matrices, the studies showed that the detected concentrations generally range from ng L^{-1} to µg L^{-1}.

The assessment of the antibiotics environmental contamination levels is fundamental so that the current state of knowledge on their pathways, transport, fate, and effects in the environment would be determined and improved (Szymańska et al., 2019; Kumar et al., 2019).

In the scientific literature there are several review articles on this type of compounds' sources, occurrence, fate, and risks.

Furthermore, some publications highlighted a critical review regarding the removal efficiency of antibiotics from wastewater through different treatment processes.

Also, the scientists assessed the role that wastewater treatment processes may play in the emergence, transport, and dissemination of the antibiotic resistance in the environment (Ebert et al., 2011; Pleiter et al., 2013; Rico et al., 2014; Szymańska et al., 2019; Kumar et al., 2019).

The environmental analysis of the antibiotics state of the art focuses on the samples' preparation, analyte stability, degradation and matrix effects.

Antibiotics and their metabolites (Figure 4.4) are continually discharged into the natural environment so that many studies considered the fact that some antibiotics

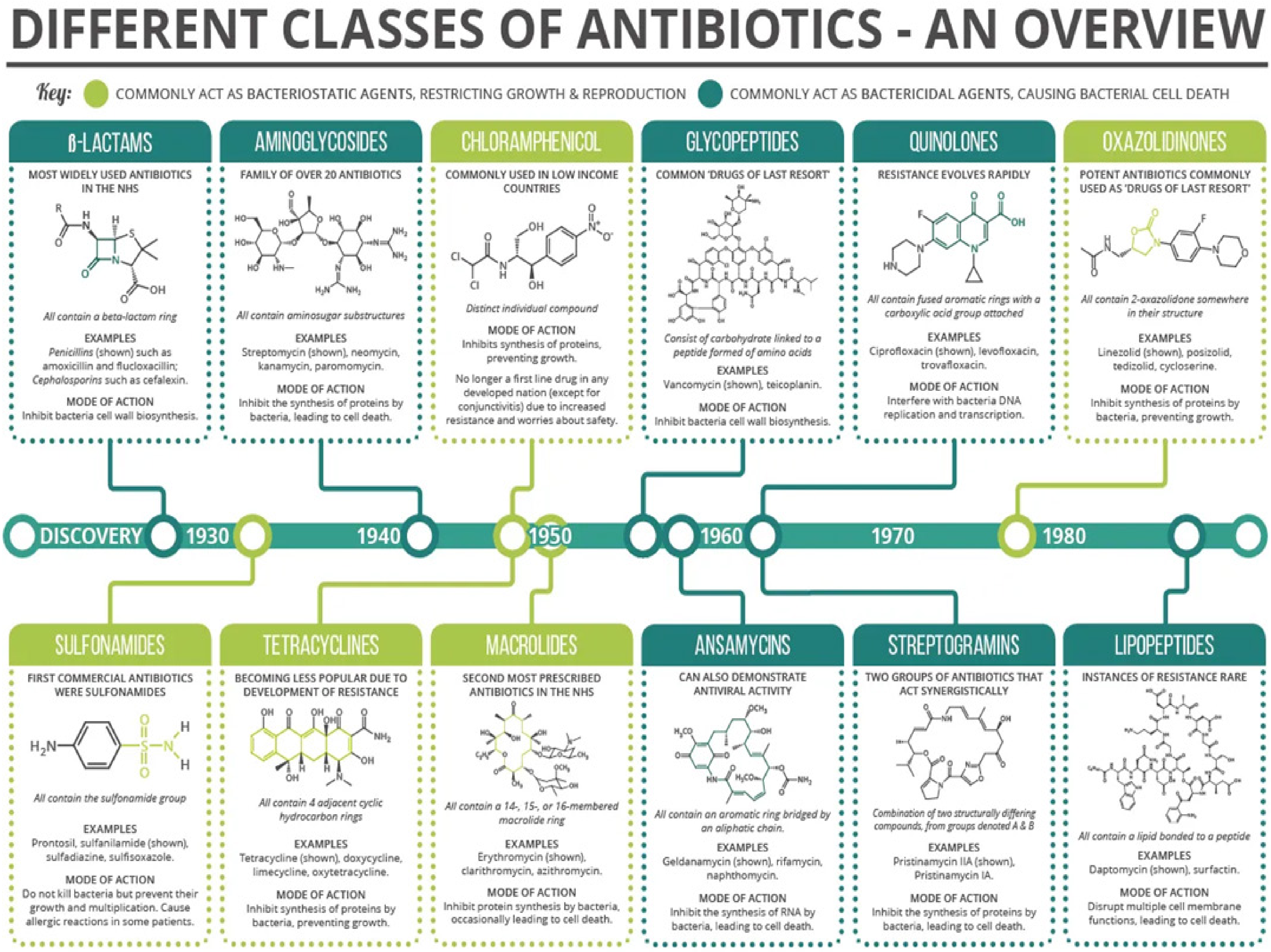


Figure 4.4. Different clases of antibiotics

Source: https://i0.wp.com/www.compoundchem.com/wp-content/uploads/2014/09/A-Guide-to-Different-Classes-of-Antibiotics-Aug-15.png?fit=1323%2C935 with low molecular weight (< 1000 D) quickly dissolved in the water bodies and hence resulting the persistence and recalcitrance of antibiotics (i.e., beta-lactams, aminoglycosides, lincosamides, macrolides, nitrofurans, amphenicols, phosphonates, quinolones and fluoroquinolones, rifamycins, sulfonamides, and tetracyclines) and their isomers (Krzeminski et al., 2018).

Moreover, the discharge of the hospital waste, veterinaries, pharmaceuticals pilot plants, dairies, animal excreta, domestics, animal husbandry, municipal waste and poultry do also shape and highlight the antibiotic pollution threat (Carvalho & Santos, 2016; Kumar et al., 2019; Enachi et al., 2019).

Concerning the antibiotics use in agriculture, there has been shown that it enhances the livestock growth, bee-keeping, and fish farming but as an adverse reaction it also contaminates the environment with the excretion of unused antibiotic and metabolites from the feces of the poultry animals (Kumar et al., 2019). Hence, the antibiotic residues indirectly contaminate the soil and water resources.

Compared to animals, in plants, the antibiotics consumption is very low. Antibiotics are frequently detected as emerging compounds in the waterways of the agricultural land points (Toolaram et al., 2016; Carvalho & Santos, 2016; Kumar et al., 2019). The pharmaceutical industry contributes significantly to the total antibiotics concentration added to the influent of the sewage treatment plant, besides the domestic and industrial effluents.

The improper disposal of unused or expired drugs, which usually are directly discharged in the sewage network or deposited in the landfills, waste effluents from manufacture or accidental spills during manufacturing or distribution can also be taken into consideration as significant routes of contamination (Akici et al., 2018).

Antibiotics - Ecotoxicological effects

The high ecological risk of antibiotics in the aquatic environment has been regarded as a growing concern all over the world. Antibiotics, as active molecules, are specially used to fight against pathogenic bacteria. However, in the environment non-targeted organisms are unavoidably exposed to these residues and their metabolites.

The primary producers and decomposers, which are essential for a sustainable ecosystem, are vulnerable to the presence of this emergent contaminants. Consequently, antibiotics can disrupt in the aquatic environment many vital ecosystem processes. These potential ecotoxicological effects are difficult to predict, especially in complex matrices.

The antibiotics' acute or chronic ecotoxicity has been assessed by several standard ecotoxicity assays that were done on different trophic levels organisms, such as bacteria, algae, invertebrates, and fish.

Grinten et al. (2010) studied and compared the sensitivity of the algal class, cyanobacterial and bacterial bioassays against different antibiotics classes. For three antibiotics, namely trimethoprim, sulfamethoxazole and streptomycin, the cyanobacteria presented a higher vulnerability rate compared to the the green algae.

Li et al. (2014) found that two quinolones, difloxacin and oxifloxacin, exhibited a notable higher toxic effect on *Vibrio fischeri* among the 21 tested antibiotics, with a 20% inhibitory concentrations of 19 and 23 μmol L^{-1}.

Ebert et al. (2011) studied the toxicity of enrofloxacin and ciprofloxacin of various photoautotrophic aquatic organisms and discovered that while enrofloxacin presented a higher toxicity to green algae, ciprofloxacin was more toxic to cyanobacteria.

Rico et al. (2014) also suggested that the environmentally relevant enrofloxacin concentrations were not likely to result in direct or indirect toxic effects on the tropical eutrophic ecology of freshwater microecosystems.

Generally speaking, the antibiotics toxicity studies in the aqueous environment only take into account each individual pharmaceutical compound. Since antibiotics emerge into the environment as a mixture with other anthropogenic contaminants, additive or synergistic effects should also be considered in the environmental risk assessments (Carvalho & Santos, 2016).

Pleiter et al. (2013) assessed the toxicological effects of different types of antibiotics on green algae, both in isolation and in different combinations.

The obtained results suggested that erythromycin in particular was highly toxic to green algae. Mostly, the antibiotics' toxic effects were enhanced when they were combined to other antibiotics. The antibiotic by-products can also possess a bioactivity and a much higher toxicity, by being stable and mobile in the environment than their parent compounds. Additionally, these compounds can revert back to their parent antibiotic, representing a reservoir of contaminants (Evgenidou et al., 2015).

Despite the recent scientific advances on this topic, only small pieces of information are available to understand the extent of the environmental contamination and their importance in terms of the overall risk (Toolaram et al., 2016; Carvalho & Santos, 2016; Kumar et al., 2019). Overall, the undertaken studies demonstrated that any type of antibiotic can be toxic to non-targeted organisms, or to pose a potential ecological risk for the aquatic organisms considering the nowadays usage patterns. However, very few publications regarding the ecotoxicity of antibiotics and their by-products are available to assess and understand the real scenario (Szymańska et al., 2019).

Therefore, further studies are needed in this area, considering the high rate of emergence of contaminants in the environment.

Another route for the antibiotics to enter the aquatic ecosystems is by mixing the sewage waste, that usually is incompletely degraded and the wastewater treatment plants least eliminated effluents.

Also, the high rate of consumption also influences the emergence of antibiotics from veterinary and poultry, aquaculture and manure applications or by the improper disposal of unused antibiotics. From that moment on, it can be predicted how will the microorganisms from a certain ecosystem will behave which in term will lead to antagonistic effect on other microorganisms.

One of the most important issues of the pharmaceutical and medical industries is the antibiotic resistance (Figure 4.5).

This phenomenon spreads at the speed of light globally through the high consumption rate. Fluoroquinolones detected in the hospital effluents (up to 87,000 ng/L) displayed a high genotoxicity and human health risks on long-term use. Fluoroquinolones are frequently found in the hospital waste and they are more toxic to prokaryotes than eukaryotes, here including freshwater algae, fishes and zooplankton and finally through the process bioaccumulation they reach the human bodies.

Internationally, the antibiotic consumption data is insufficient because of variation in their prescription and administration.

All the international data available on antibiotic usage is estimated and approximated, since many countries do not declare their monitoring data, and hence the actual data remains unrevealed (Carvalho & Santos, 2016; Kumar et al., 2019; Szymańska et al., 2019; Enachi et al., 2019).

The antibiotics that are used in the veterinary and poultry industry increase the reluctant mechanism of bacterial strains to survive under the antibiotic stress thus resulting in the evolution of multidrug-resistant bacteria.

These resistant strains of bacteria are also reported both in the hospital environment

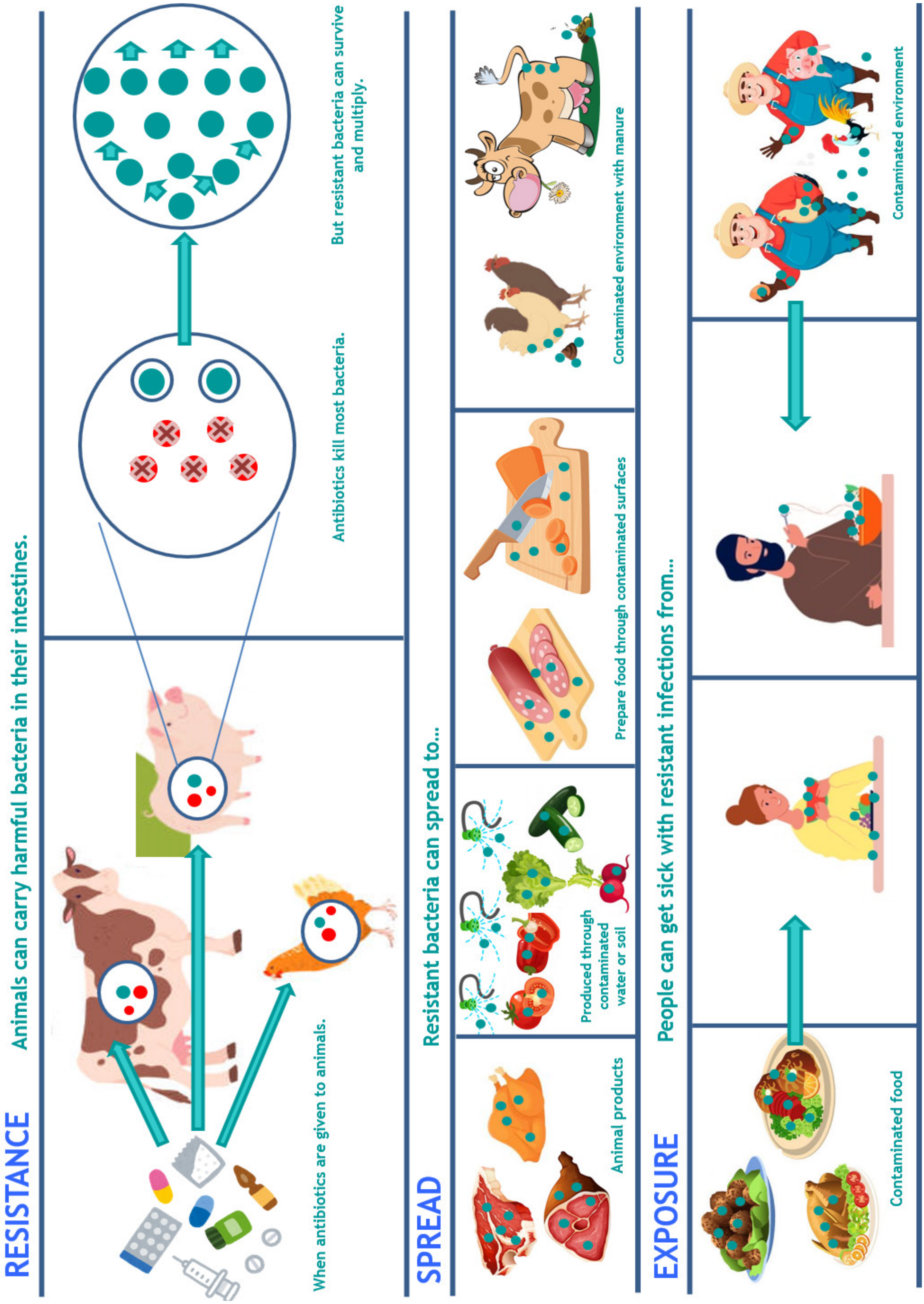


Figure 4.5. Antibiotcs resistance from farm to table

but also in the aquatic ecosystems as well as the soil environment. Studies on *Bifidobacterium, Clostridium, Escherichia coli, Enterococcus* and *Lactobacillus* clearly showed that their structural composition and metabolic activities are severely affected by antibiotics as these compounds play a crucial role in the ecology change particularly on the niche where the genetic exchanges take place.

From all the antibiotic classes, sulfonamides and fluroquinolones are the most persistent in environment followed by macrolides whereas aminoglycosides and ß-lactams are the least persistent. Tetracyclines can also persist for a relatively long period in the absence of sunlight, at the expense of their mobility (Szymańska et al., 2019; Kumar et al., 2019).

The existing fundamental data on the fate and effects of antibiotics and antibiotic resistance in the environment is crucial in order to perform proper risk assessment studies.

The future undertaken research should be designed to contribute to the improvement of the European legislation to stop the antibiotic resistance, as well as to highlight the development of rational water and wastewater management practices for the protection of humans and ecosystems.

4.4. CONTRACEPTIVES

Contraceptives in the aquatic environment

Oral contraceptives are a class of endocrine disruptors that usually are commercialized on the market as a combination of different steroidal hormones.

Mainly, contraception is achieved by inhibiting the ovulation through the combined activity of two main components: estrogen and progestin.

In order to ensure a much wider adherence to the contraception methods, recent advances in oral contraceptives focused on the development of new formulations that present fewer side effects and additional benefits, besides the much-desired contraception (Lecomte et al., 2017; Enachi et al., 2019).

Recently, the presence of this type of chemicals with hormonal activities in the environment has become a major concern worldwide.

In several well-documented studies, the aquatic wildlife reproduction has been adversely affected by these emergent contaminants.

Steroid hormones, a type of emergent contaminants, are a major part of the commercialized oral contraceptives that have the potential to exert estrogenic effects at the low ng L^{-1} level.

The most used form of hormonal contraception is the combination of steroid hormones.

As a result of the high rate of consumption among women (Figure 4.6.), the steroid hormones and their metabolites are constantly excreted in the sewerage environment and continue to bioaccumulate in the environment.

Over the past 50 years, the use of this type of chemical pollutants and their release into the environment has increased considerably, leading to major concerns about their impact on wildlife and humans (Jarošová et al., 2015).

These environmental concerns have been recognized unanimously by many institutions and authorities, although the industrial development, agrochemistry and human chemical consumption produce an increasing amount of chemical pollutants in the environment, especially in surface waters.

Due to the low overall biodegradability rate of several of these molecules, there is an accumulation of oral contraceptives chemicals in the aquatic environment (Lecomte et al., 2017).

Therefore, the presence of these molecules is a risk to both to human life and to wild animals.

THE CHEMISTRY OF ORAL CONTRACEPTIVES

The first oral contraceptive, norethindrone, was synthesised by Carl Djerassi in 1951. This graphic looks at the range of compounds used and how they work.

THE NATURAL HORMONES

Oral contraceptives contain synthetic versions of two hormones produced naturally by the body: estrogens and progestogens. Both hormones have roles in the female menstrual cycle.

PROGESTOGENS

Can be used in combination with estrogens, but also on their own in progestogen-only pills. These pills must be taken continuously and within 3 hours of a specific time every day. Recommended for breast-feeding women, as it doesn't affect milk production.

99.9% EFFECTIVE

(WHEN TAKEN CORRECTLY)

HOW ORAL CONTRACEPTIVES WORK

Steady levels of estrogens and/or progestogens in the body as a result of oral contraceptives trick the pituitary gland into thinking a woman is already pregnant, stopping it from releasing hormones that stimulate ovulation, and preventing pregnancy. Progestogens promote formation of a thicker layer of cervical mucus, which makes it difficult for sperm to reach the uterus, and also affect the uterine lining and make it harder for an egg to attach.

ESTROGENS

Combined oral contraceptive pills include an estrogen as well as a progestrogen. Most are taken over a 28 day cycle, with 21 pills taken, followed by a week of no pills. They must be taken within 12 hours of a specific time every day to maximise protection.

Figure 4.6. Oral contraceptives and their effects on the human health
Source: https://www.compoundchem.com/2015/02/03/oral-contraceptives/

Of these hundreds of these human-created and synthesized molecules, those with endocrine disruption activities attract particular attention due to their potential disruptive activities that can conduct even at very low doses several endocrine disruptive processes in humans and in the aquatic wildlife, linking them to diseases.

In addition to the current natural hormones, including here 1,7-estradiol, estrone and estriol, the presence of various pharmaceuticals products such as ethinyl estradiol, drospirenone and levonorgestrel contraceptives was also detected, these emergent contaminants being in concentrations up to ng L^{-1} in the surface water (Adeel et al., 2017).

Nowadays, more and more concerns are raised due to the increasing scientific evidence of disruptive activity that affects the endocrines.

The lack of a legal definition and regulations are also incriminated for their significant presence in the environment (Lecomte et al., 2017; Ohore at al., 2019).

Contraceptives - Ecotoxicological effects

The presence of estrogens like 1,7-estradiol, estrone, estriol, the ethinyl estradiol, drospirenone and levonorgestrel has been identified in the aquatic environment more and more often in the past few years.

The main sources of this contamination are the human population, which discharges around 30,000 kg/year, and livestock, which discharges 83,000 kg/year (Adeel et al., 2017; Lecomte et al., 2017). Maybe the most important concern regarding these contraceptive compounds is the very high density of animals which generate large amounts of manure, a significant source of hormone, into the soil and surface water.

In addition to the endogenous sexual steroids found in the manure of livestock (natural physiological excretion process), over 90% of the cattle from all over the world receive steroid hormone treatment (by implant or via feeding) in order to accelerate their growth (CEAH USA, 2013). The majority of the contaminated manure is used as a fertilizer, without any other additional decontamination treatment, and the surface runoff is likely to transport the hormones from the cropland to the aquatic ecosystems (Gall et al., 2014).

The presence of 1,7-estradiol, estrone, androstenedione, testosterone, and progesterone (ranging from 5 to 250 ng L^{-1}) in was detected in the runoff from the feedlot's surfaces during a rainfall simulation after the animals were removed from the pens (Mansell et al., 2011).

Similarly, numerous studies highlighted the presence of these natural and synthetic estrogens (estrone, 1,7-estradiol, and estriol), androgens (trenbolone, and androgens receptor agonist up to 50 times more potent than testosterone), and progestagens (progesterone and melengestrol) in the rivers that are situated nearby animal farms (Lecomte et al., 2017; Enachi et al., 2019).

Nonetheless, it should be noted that a recent study linked the urinary estrogen levels, including their metabolites, to a higher breast cancer risk in postmenopausal women, and the risk doubled between the lowest and highest percentile (Plotan et al., 2014; Lecomte et al., 2017).

Surprisingly, even if the overexposure to the endogenous estrogens was suggested to be a significant risk factor, a definitive link between the environmentally present natural estrogens and breast cancer or other types of cancer incidence has not been demonstrated.

In the majority of contraceptive pills, the major synthetic hormones are progestins, with a release estimated to reach 700 kg/year. This environmental release may conduct to the estrogen overexposure of the human population and of the aquatic animals. At the same time, the environmental rates of progestins are not very well documented (Gall et al., 2014; Mansell et al., 2011; Plotan et al., 2014; Jarošová et al., 2015).

In addition to the vertebrate origin of the anthropogenic and natural estrogens, the emerging natural compounds found in water are phytoestrogens and mycoestrogens.

All the results obtained through scientific research emphasize the importance of high-flow events in the hormone export, and thus to better understand their metabolic and ecotoxicological pathways.

4.5. ENDOCRINE DISRUPTORS

Endocrine disruptors in the environment

Endocrine disruptors represent one the main classes of emergent pharmaceutical contaminants, the most important being the bisphenols. These substances are phenolic, organic synthetic compounds that are used as an additive or monomers in the production of polycarbonate plastics and epoxy resins.

Bisphenol A is a reproductive, developmental, and systemic toxicant, often classified as an endocrine-disrupting compound. The bisphenols absorption into the human body can result in the development of metabolic disorders such as low sex-specific neurodevelopment, immune toxicity, neurotoxicity and interference of cellular pathway (Sabanayagam et al., 2013; Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

Therefore, the presence of these compounds and their analogues in the environment has recently attracted global attention. As a representative, Bisphenol A is an organic synthetic compound belonging to diphenylmethane derivatives and bisphenols, being usually used as an additive in the production of polycarbonate plastics and epoxy resins. Regarding its predominance, bisphenol A is the most predominant bisphenol (Sabanayagam et al., 2013; Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

The assessment of human exposure to bisphenol A (Figure 4.7) showed that humans are directly or indirectly exposed to several concentrations of BPA (0.27–10.6 ng/mL), with a globally estimated intake of 30.76 ng/kg per body weight per day by dust inhalation, dermal exposure and ingestion.

Furthermore, it was reported that the major route for the bisphenol A exposure is via ingestion, accounting about 90% of the bisphenol A exposures.

In addition, the large amount of bisphenol A and its analogue are used in the production of plastics and hence the improper recycling methods will result in an enormous endocrine disrupting compounds amount especially in the aquatic system (Rezg et al., 2014; Enachi et al., 2019).

This product would be biodegraded and acted upon by the sunlight into micropieces such as microplastics, and subsequently, the bisphenol A in the plastics can easily be ingested and bioaccumulated along the food chain by diffusion.

The biomonitoring data reveals that tons of industrial bisphenol containing products are ubiquitously present in the environment and consequently these substances become even more harmful (Sabanayagam et al., 2013; Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

The use of bisphenol A in the industrial production of plastic bottles, lining of cans, thermal papers etc., and their presence in the environment has raised important questions regarding its potential environmental impacts, particularly on children's health and what is the exact concentration at which this compound can be considered as environmentally harmful.

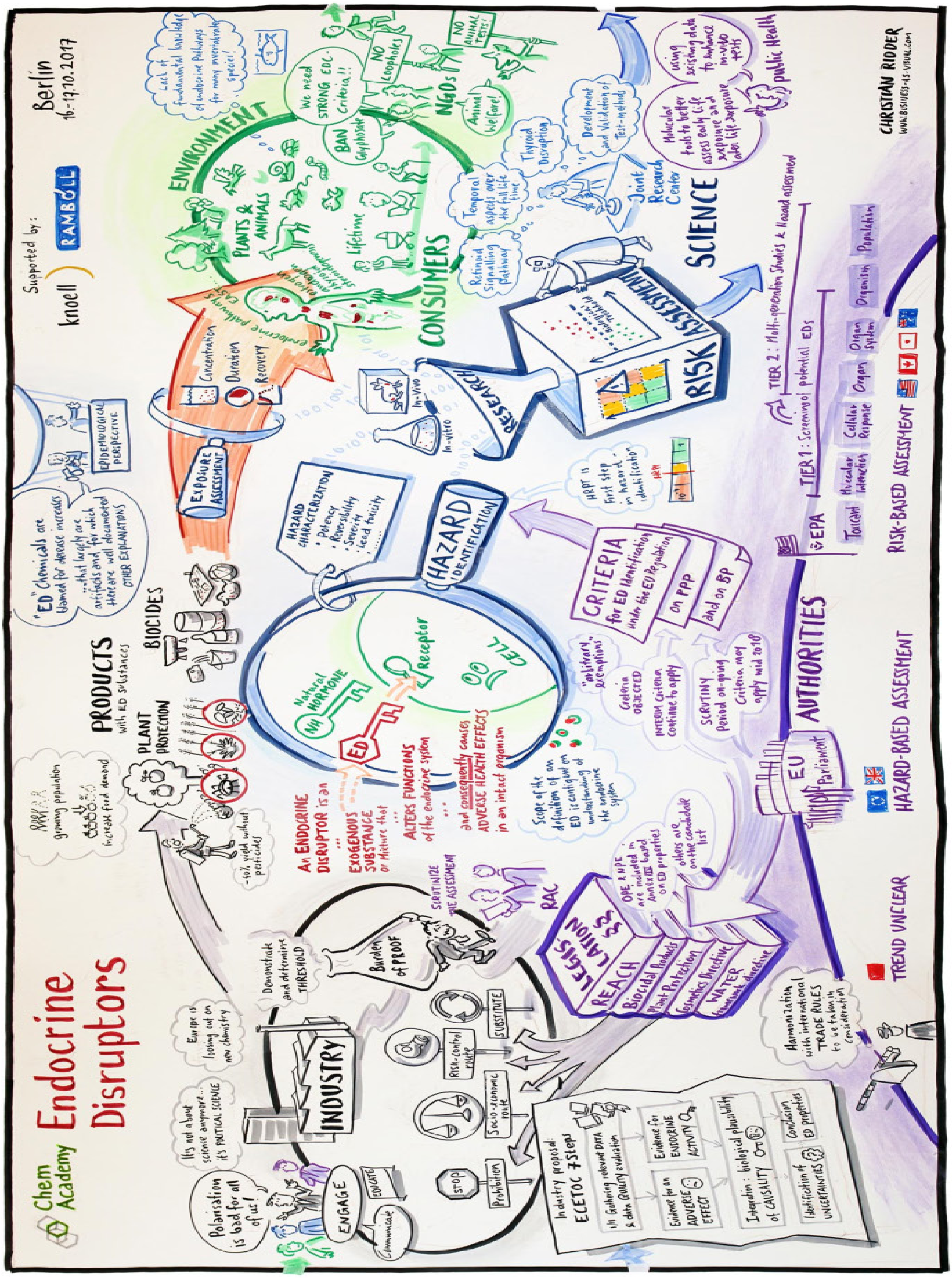


Figure 4.7. Endocrine disruptors and their environmental route
Source: https://www.chem-academy.com/sites/chem-academy/files/bilder/gr/END4764_Graphic_Recording_thumb.png

Several studies and surveys reported the assessment of low dose all over the world which led to the conclusion that there is insufficient information to assess the human health risks due to the inconsistency of the results (Ohore & Zhang, 2019).

Endocrine disruptors - Ecotoxicological effects

Several reports have been published in regards to the human and animal experiments, studies that describe the effects of bisphenols exposure. Generally, bisphenols act on the hormonal level by distorting the hormonal balance and inducing several estrogenic effects through the binding to estrogen-related receptors (Ohore at al., 2019). The resultant effects are several of which numerous hormonal related abnormalities have been reported (Sabanayagam et al., 2013; Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

The bisphenol A was also reported to trigger an early onset of development of sexual characteristics and obesity. In males, the exposure to a high serum concentration of bisphenol A (between 1.53-2.22 μg/L) especially during the developmental stage resulted in feminization of the male fetuses, testicular and epididymis atrophy, alteration of sperm parameters, and the reduction of testosterone levels. The most possible mechanism is that the bisphenols induce a phosphorylation which contributes to the activation of the P450 genes cytochrome (CYP gene) expression in both mRNA and protein levels, which finally results in the alteration of the normal sex hormone ratio (Toner et al., 2018; Ohore & Zhang, 2019). Many studies have shown that the overall effects of bisphenols, especially the A type, on the male reproductive system have shown to be more pronounced in the fetal period. In females, the high serum concentration of bisphenol A (1.53-2.22 μg/L) induces changes in the estradiol serum concentration, which results in a hormonal balance distortion and in metabolic abnormalities such as early puberty, menstrual irregularities, increased likelihood of the endometriosis, higher implantation failure, and ineffective gonadotropin fertility treatment.

Finally, the effects of the bisphenol A paradigm upon estrogen hormone possesses a much stronger signaling than the equimolar concentration of estrogen. The binding of the bisphenol A to the estrogen-related receptors has resulted in multiple negative outcomes due to its distortion of the hormonal balance (Hines et al., 2017; Ohore & Zhang, 2019).

Several reports have also shown that bisphenol A displayed the alteration of the immune system through a multi-targeted approach. This compound caused the alteration of the transcription of genes and thus exhibiting sex differences during neonatal periods, which in the end resulted in decrease of the sex genes expression. This impact had an immunomodulatory role and resulted in a compromised auto-inflammatory response and the deregulation of the immunoglobulin inhibition of Tregs (Sabanayagam et al., 2013; Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

It was suggested that this emergent compound binds to estrogen-related receptor γ, which is expressed strongly in the mammalian brain development and promotes the differentiation and maturation of the fetal brains disorders. In a research conducted lately to determine the effect of the bisphenol A analogue on the adrenocortical function, H295R which is an angiotensin-II-responsive steroid-producing adrenocortical cell, was used as a model of toxicity comparison. The results showed several inhibitory effects on the hormonal adrenocortical function (Ohore & Zhang, 2019). Additionally, the bisphenol A exposure affects the development of the thyroid and hypothalamic pituitary testicular axis by modulating the hormone synthesis and by induction related to the endocrine disruption of the thalamus and spleen. This process is also determining a conformational change in the tertiary ability to recruit coactivator proteins and in the inhibition of the transcriptional activity induced by triiodothyronine (T3) (Sabanayagam et al., 2013; Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

Recent findings have indicated that bisphenol A induced an increased tumor necrosis factor (TNF α) and conformational changes in the estrogen-related receptors. The DNA methylation and histone changes might be the most important key events in the mechanism of the bisphenols' toxicity. Moreover, the pathological outcomes concerning the exposure to bisphenols vary with the routes, concentration and dose. The effects of the exposure on the glucose regulation have been extensively studied. The studies indicated that the exposure to high level of bisphenols whether A or F, (10–400 mg/kg) caused oxidative stress and the disruption of pancreatic β-cell function which in term resulted in a glucose regulation complication (Rezg et al., 2014; Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

Furthermore, the bisphenols exposure was tested on mice families to better understand their effect on the insulin signaling in the brain. As a result, it was discovered that, in fetal mice (8 months of age) offspring, the insulin signaling including the insulin phosphorylated extracellular signal regulated the protein kinase and the glucose transporter, factors that were significantly decreased. Also, the hyper-insulinemia, glucose intolerance and insulin resistance were reported to be associated to the bisphenols' exposures (Ohore & Zhang, 2019). This showed that the bisphenols analogues determine a negative impact on the glucose metabolism. The bisphenols are conjugated primarily to the mono-glucuronide from rat liver where a deconjugated condition occurred at a high dose exposure and the resultant metabolites were excreted into the bile.

The exposure to bisphenol A and its effects on the cardiovascular system were studied and the reports suggested a possible relationship between the exposure and the hypertension condition that severely affected the cardiovascular system during a hypoxic event. Furthermore, the exposure affected the angiogenesis by stimulating the production of vascular endothelial growth factor, which resulted in an uncontrolled neovascularization and in the increase of the interventricular septal thickness (Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019).

Nonetheless, it was also reported that the exposure to bisphenols within a varying concentration of 1–250 μg/mL, caused an increase of the calcium levels in incubated erythrocytes with the strongest effect noted for bisphenol A and bisphenol F which particularly resulted in the increase of the phosphatidylserine translocation in red blood cells and arrhythmias (Hines et al., 2017; Toner et al., 2018; Ohore & Zhang, 2019). The presence of these emergent substances that disrupt the endocrine activities in the environment (Figure 4.8) has attracted considerable attention.

In addition to the chemical analysis, several studies reported and quantified the endocrine activities of the bisphenols. The results suggested that the bisphenols' paradigm and the alternative signaling of estrogens are the dominant contributors to the high estrogenic activity, thus affecting the hormonal regulations. The use of bisphenols in the production of polycarbonate plastics and epoxy resin has been identified as an important contributor to the bisphenols' pollution of the aquatic ecosystems.

BISPHENOL-A

BPA

$C_{15}H_{16}O_2$

WHAT IS BISPHENOL-A (BPA) ?

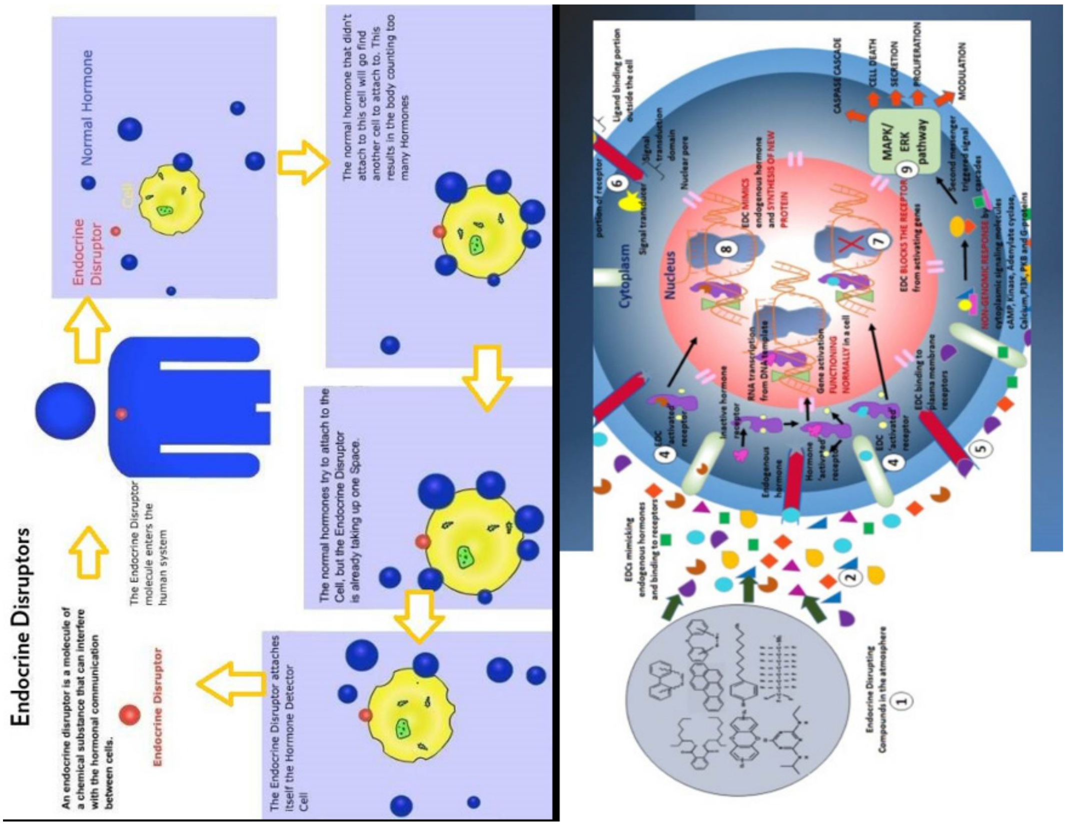
- Bisphenol-A (BPA) is a synthetic form of estrogen.
- It is a chemical generally used to harden plastic and coating the inside of food and drink cans.
- It can easily dissolve into the food and hot liquids that are kept in the plastic or plastic coated containers.
- It can easily enter into our body after eating or drinking from such containers.

FROM WHERE CAN BISPHENOL-A (BPA) ENTER INTO OUR BODY?

Baby bottles (Bottle feeding)
Canned food packages
Soda or juice cans
Plastic food containers
Plastic water bottles

SOURCES OF BPA (BISPHENOL-A)

Plastic toys for children
Plastic utensils for microwave
Blenders and food processors
Plastic food plates
Plastic cups for drinking tea/ coffee



Endocrine Disruptors

An endocrine disruptor is a molecule or a chemical substance that can interfere with the hormonal communication between cells.

The Endocrine Disruptor molecule enters the human system

The normal hormone that didn't attach to this cell will go find another cell to attach to. This results in the body counting too many Hormones

The normal hormones try to attach to the Cell, but the Endocrine Disruptor is already taking up one Space.

The Endocrine Disruptor attaches itself the Hormone Detector Cell

Figure 4.8. Endocrine disruptors and they interfere with the hormonal communication between cells
Adapted after: https://www.sproutsanfrancisco.com/get-educated/endocrine-system-endocrine-disruptors-harms

4.6. THE ASSESSMENT OF THE EXPOSURE DEGREE TO PHARMACEUTICAL COMPOUNDS IN THE AQUATIC ENVIRONMENT

Based on the experimental assessment of the environmental risk represented by pharmaceuticals, the guides of the European Medicines Agency (EMA) on the environmental risk assessment (ERA) of pharmaceutical products for human and veterinary were adopted in 2005 and 2006. The guidelines EMA EMA/CVMP/ERA/418282/2005 and EMEA/CHMP/SWP/4447/00 apply to all new applications for the release on the market authorization of a pharmaceutical product. Human pharmaceuticals enter the environment predominantly through the surface water. Therefore, in the EMA guidelines, the limit of action of pharmaceuticals is the surface water. In the phase I of the risk assessment procedure, the estimation is based only on the medicinal substance, regardless of the mode of administration, pharmaceutical form, metabolism and excretion.

Pharmaceuticals are now being classified by their Predicted No Effect Concentration (PNEC) values and Critical Environmental Concentration (CEC) values to assess their environmental risks. PNEC is defined as the pharmaceutical concentration at which no pharmacological effect is expected to occur for a specific organism. A PNEC value is estimated using ecotoxicity testing data.

- Ecological risk assessment based on the PEC/PNEC ratio (PEC) and predicted no-effect concentrations (PNEC)
- PEC (predicted environmental concentrations) – is a function of the maximum daily dose and is calculated according to EMEA guidelines (Guideline on environmental impact assessment for veterinary medicinal products EMA/CVMP/ERA/418282/2005; Guideline on the environmental risk assessment of medicinal products for human use, Doc. Ref. EMEA/ CHMP/SWP/4447/00)

$$\text{PEC surface water (mg/l)} = \frac{\text{DOS Eai x Fpen}}{\text{WASTEWinhab x DILUTION}}$$

where:

PEC Surface Water = Predicted environmental concentration r surface water
DOSEai= Maximum daily dose consumed per inhabitant
Fpen= Market penetration factor of active ingredient
WASTEWinhab= Volume of wastewater generated per inhabitant
DILUTION= Dilution of effluent

The volume of wastewater generated per inhabitant per day (WASTEWinhab) is set to 200 L/(inh x d), and the dilution factor is set to 10. Action limit for phase II (environmental fate and effects analysis) is set on 0.01mg/L. If the PEC value is equal to or greater than 0.01 µg/L, the evaluation in the second phase is mandatory. The pharmaceutical compounds for which logKow> 4.5 are particularly studied in terms of persistence, bioaccumulation capacity and toxicity.

In addition, a comparison can be made between the measured environmental concentrations (MECs) derived from the detection and quantification of specific pharmaceutical residues in the water and sediment and the PEC values calculated to verify the accuracy of the used models. The Phase II risk assessment is based on the physico-chemical properties of the targeted substance, its effects, the toxicological studies carried out to date which are being reviewed and also the degradation of it in the environment is taken into account:

- Adsorption – Desorption Using a Batch Equilibrium Method (OECD 106/OECD 121/OPPTS)

- Ready Biodegradability Test OECD 301
- Aerobic and Anaerobic Transformation in Aquatic Sediment System (OECD 308)
- Algae, Growth Inhibition Test (OECD 201)
- Daphnia sp. Reproduction Test (OECD 211)
- Fish, Early Life Stage Toxicity Test (OECD 210)
- Activated Sludge, Respiration Inhibition Test

Based on them, the Predicted No Effect Concentration (PNEC) for water, groundwater and microorganisms is calculated.

The PNEC calculation is based on modeling and data on persistence in the environment (ability to be degraded), transport (ability to penetrate biological membranes and accumulate within flora and fauna) and biotransformation (ability of organisms in the environment to metabolize and detoxify the pharmaceutical product, eliminating any harmful effects resulting from the bioaccumulation of the drug). PNEC is based on LC50 values (lethal concentration 50), divided by arbitrarily defined constants (10–100–1000).

The PEC/PNEC ratio is called the risk coefficient (RQ). A PEC/PNEC ratio of <1 suggests that there is no significant risk associated with the presence of those pharmaceuticals in the environment, while a ratio ≥ 1 suggests that a further level B environmental risk characterization is needed in phase II.

Another way to assess the risk for the environment is to determine the critical environmental concentration (CEC) (Peake, 2016). The concentration of a pharmaceutical product that, according to the available clinical data, may cause a pharmacological effect in fish (positive or negative), is used instead of PEC, together with logP (the partition coefficient is closely related to the bioaccumulation capacity) to obtain a value for the fish (fish steady-state plasma concentration FssPC). The use of the CEC parameter, rather than the PEC, means that the assessment is independent of the exposure and can be used to evaluate the potentially harmful pharmaceuticals when their use or sales data are not available in a particular country.

Finally, the evaluation method by QSAR modeling (quantitative relationship – activity) can also be used to classify the hazard posed by pharmaceutical substances in the environment (Peake, 2016). By this method, the physico-chemical properties of a molecule are mathematically correlated with the minimum concentration (C) required to induce a pharmacological effect in a given organism.

4.7. DETERMINATION OF THE RISK OF EXPOSURE TO HUMAN HEALTH

To calculate the risk of a chemical to the human health, a presumed no-effect concentration is compared to the concentration measured or predicted in the environment. This no-effect concentration for individual pharmaceuticals or their metabolites that can be ingested daily without any expected health consequences is called acceptable daily exposure (ADE) or acceptable daily intake (ADI).

ADI is based on the ratio between the experimentally established minimum concentration at which an adverse effect is observed (NOAEL) and the safety factor (SaF), as follows:

$$\text{ADI (for humans)} = \frac{\text{NOAEL (experimental dosis)}}{\text{SaF}}$$

SaF is usually a multiple of 10 and represents a specific area of uncertainty inherent to the validity of the data considering possible differences in response between humans and animals in long target studies.

In the case of missing NOAEL (or NOEL) data, the ADI may be determined by referring to a lowest-observed-adverse-effect level (LOAEL), and applying an additional SaF.

Toxicological risk index (HQ) for each substance could be expressed as the ratio of the chronic daily intake (CDI) to the reference value:

$$HQ = CDI/ADI$$

The RfD derives from the ratio of the NOAEL data to various safety factors and is defined as an estimate of the daily exposure of the human population (including sensitive subgroups) deemed to be without risk of health adverse effects even when exposure persists throughout life.

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5
Chapter

IONIZING RADIATION IN THE ENVIRONMENT – RADIOLOGICAL RISK

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5.1. GENERAL CONSIDERATIONS

Ionizing radiation. Environmental radioactivity

Radiation is the emission, propagation and transfer of energy in any medium in the form of **electromagnetic waves** or **particles**.

Living beings coexist with radiation from their origins. Without radiation from the sun there would have been no life on earth and without infrared radiations we would not be able to heat ourselves. In addition to these natural sources of radiation, humans have been able to develop various devices that are based on radiation or use radiation. We use radiation when we listen to the radio, talk on our cell phones, heat breakfast in the microwave, toast our bread or undergo a radiological investigation . The higher the frequency of electromagnetic radiation, the higher its energy. *Electromagnetic* waves or *radiations* can be classified according to their energy into *ionizing radiations*, which have enough energy to produce the ionization of the atoms of the matter they pass through (*e.g.* X-rays) or *non-ionizing radiations*, which do not have enough energy to break the bonds of the atoms and to produce ionization (*e.g.* microwaves) (Nat, 2005).

Radiations of a *corpuscular* nature, which are present in the form of particles with mass (for example, alpha radiations), are included within ionizing radiation, since in all cases they are capable of producing ionization.

There are several types of ionizing radiations, which interact differently with the substance they cross, depending on their nature and energy and the type of material crossed (Figure 5.1):

- **Alpha radiation** (α) is the nucleus of helium 4 that is emitted in certain nuclear decays and consists of two neutrons and two protons. It has a high mass, but is not very penetrating: a sheet of paper or the human skin itself is enough to protect us from its effects.
- **Beta radiations** are beams of electrons (beta negative – β^-) or positrons (beta positive – β^+) released in certain nuclear decays. They have a smaller mass than alpha particles, although they are somewhat more penetrating: they can penetrate a sheet of paper and between one and two centimeters of living tissue, but they cannot penetrate an aluminum sheet.
- **The X and gamma rays** are electromagnetic radiations without electric charge or mass. These types of radiation have different origins; gamma-rays (γ) come from the disintegration of the unstable nuclei of some radioactive elements, and the X-rays come from the outer layers of the atom, where the electrons are found. This type of radiation is quite penetrating; it passes through the sheet of paper and aluminum foil and, to stop them, a layer of lead with sufficient thickness is required.
- **Neutrons** (n) released in nuclear reactions are a highly penetrating type of radiation.

Having no electrical charge, neutrons easily penetrate the structure of certain atoms and cause their division. They can be absorbed with certain chemical elements such as cadmium or boron. A layer of water or concrete stops the neutron beams.

If a parallel (collimated) beam of monochromatic nuclear radiation of initial intensity I_0 passes through a layer of material of thickness x, the intensity I of the beam decreases after an exponential **attenuation law** (Figure 5.2):

$$I(x) = I_0 e^{-\mu x} \qquad (1)$$

where μ is called *the attenuation coefficient* which depends on the nature of the substance crossed by the beam and the energy of the incident radiation (Ene & Pantelica, 2011a).

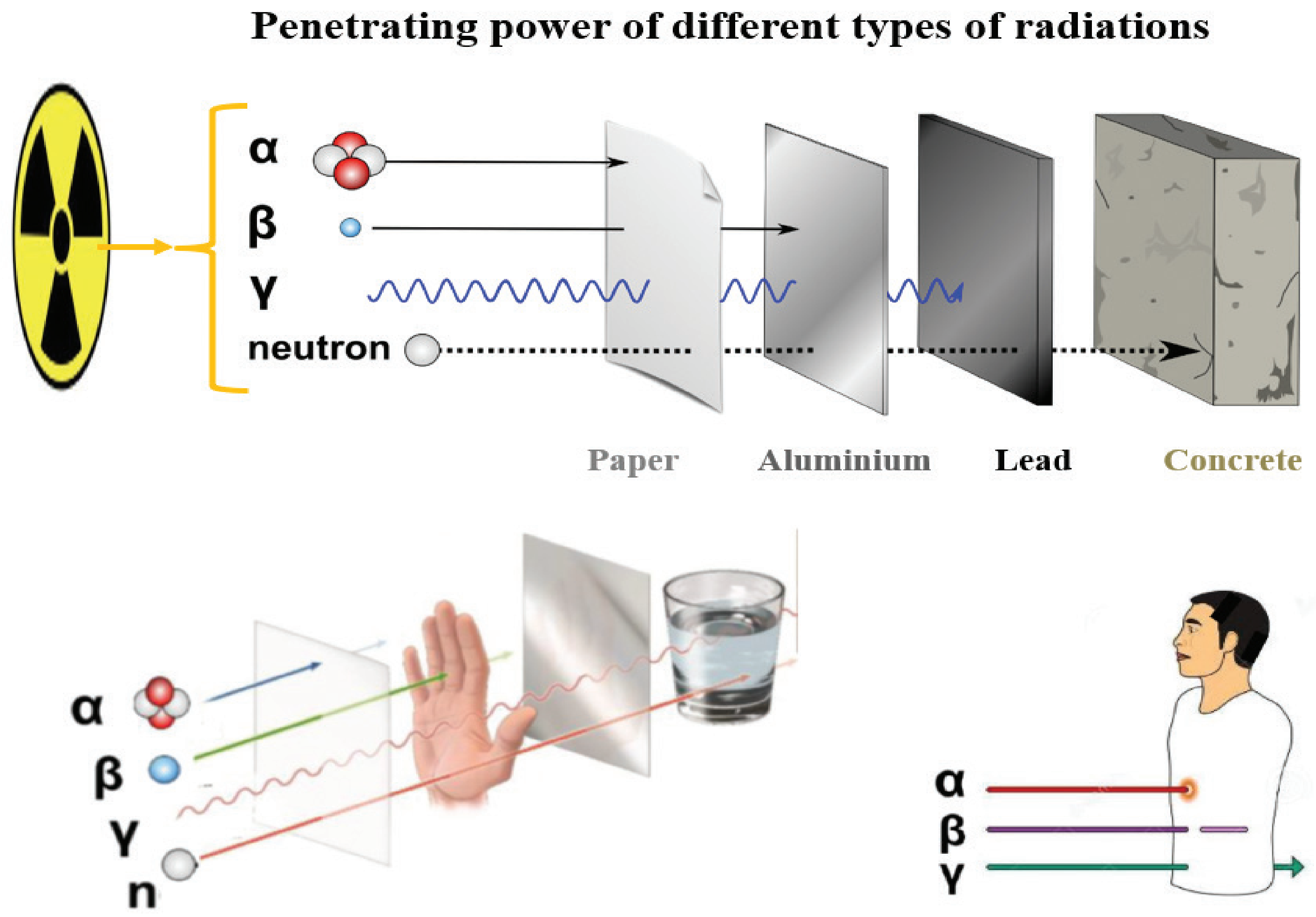

Figure 5.1. Modes of interaction of nuclear radiation beams (α, β, γ, n) with matter

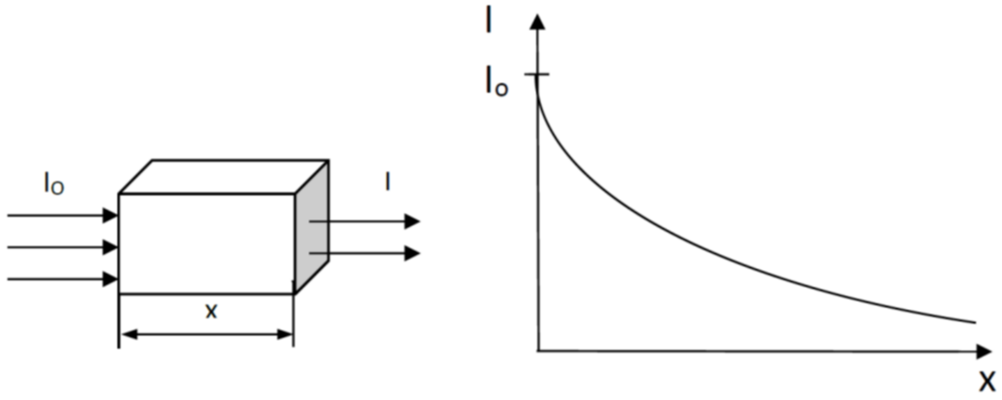

Figure 5.2. Exemplification of the attenuation law of a beam of radiation in the substance

The structure of matter is made up of molecules which, in turn, are formed by atoms. For centuries, scientists believed that the atom is the smallest part of matter, hence its name, atom, which means „indivisible". However, further research has allowed the representation the atom as being formed by a nucleus and electrons that rotate around it.

The concrete, quantitative representations regarding the atomic structure of chemical elements had as a first point of approach the experiments of scattering of α particles (He ions) on very thin metal sheets, carried out by the „father of atomic theory" – Ernest Rutherford. The conclusion that has been reached from these experiments is that the nucleus is the central part of the atom, in which practically the entire mass of the atom is concentrated and, because the atom is electrically neutral, it has a positive electric charge. After Chadwick's discovery of the neutron in 1932, it was determined that the nucleus consists of two types of nucleons: *protons* and *neutrons*. Protons have a positive electric charge and electrons negative charge, while neutrons are called so because they are not electrically charged. Protons and electrons attract each other by coulombian forces.

Nuclear physics, through its object of study on the constitution, properties and transformations of the atomic nucleus, has established its complexity and special physical properties. Some of these properties are of principled importance, such as those related to the stability and structure of the nucleus and mechanisms of the nuclear reactions, and others are of practical interest, such as obtaining and using nuclear reactions, radioactive isotopes, or nuclear energy (Nat, 2005).

The nuclei are defined by two numbers: *the atomic number* Z which represents the number of protons contained in the nucleus, that is, it indicates the charge of the nucleus that is equal to Ze (where e = the electron charge), and by the *mass number* A which is the number of nucleons in the nucleus (protons and neutrons), being close to the atomic mass of the chemical element. The number of neutrons contained in the nucleus is NE = A – Z. The atomic species defined by a certain composition of the nucleus, i.e. Z protons and (A-Z) neutrons is called ***nuclide***, symbolized by $^{A}_{Z}X$. Nuclides that have the same A number are called *isobars*, those that have the same Z are *isotopes*, those with the same NE = A – Z are *isotones* and those who have the same A and the same Z but differ in radioactive properties are called *isomers* (Nat, 1998; Ene, 2002).

Atomic nuclei can be stable and non-stable. Stable nuclei remain unchanged over time, and non-stable ones undergo spontaneous transformations. A nucleus with a certain number of nucleons, A, is stable when there is a well-determined ratio between the number Z of protons and the number NE=A-Z of neutrons, and the nuclear energy levels correspond to a well-established minimum energy configuration. If these conditions are not met, the nucleus is unstable and spontaneously undergoes various transitions to sooner or later reach a stable nucleus. These transitions are called *radioactive transitions* and their ensemble, supported by an unstable nucleus, represents the radioactive ***decay***. The *daughter* nuclide (resulted from decay) may not be stable and decomposes into a third party, which can continue the process, until finally a stable nuclide is reached. It is said that successive nuclides in a set of decays form a *radioactive series* or a *radioactive family* (Marcu & Marcu, 1996).

The effect of radioactive decay is the transition from an atom with an initial nucleus with higher energy to an atom with a lower energy derivative nucleus, achieved by one or more radioactive transitions, called *decay schemes*. The difference between the two energies is released during decays in the form of nuclear radiations: corpuscular (beams of elementary particles, atomic nuclei, ions: α particles, β^-/β^+ particles, protons p (H^+), deuterons $^2H^+$, tritons $^3H^+$, heavy ions Li^+, Na^+, etc.) or penetrating electromagnetic radiation (X and γ radiations) (Nat & Ene, 2003a; Ene, 2006).

If at time t there is a number N of nuclei of a particular atomic species, **the law of radioactive decay** is written as follows:

$$N = N_o\, e^{-\lambda t} \qquad (2)$$

where N_o is the number of nuclei existing at the initial moment (t = 0) and λ is the *radioactive constant*, which characterizes the respective radionuclide.

In practice, another quantity is used, called *the half-life* $T_{1/2}$, which represents the time interval during which half of the number of given nuclei disintegrates. The half-life is a specific quantity of each radionuclide and varies in a very wide range, from 10^{-7} s to 10^{15} years. The connection between the two quantities caracteristic to the decay processes ($T_{1/2}$, λ) is:

$$T_{1/2} = \frac{\ln 2}{\lambda} \qquad (3)$$

A radioactive source is characterized by its *activity,* Λ, which represents the number of disintegrated nuclei in the unit of time:

$$\Lambda = \left|\frac{dN}{dt}\right| \qquad (4)$$

If $\Lambda_0 = \lambda N_0$ is the initial activity, one can write for Λ a relationship similar to (2) :

$$\Lambda = \Lambda_0\, e^{-\lambda t} \qquad (5)$$

The unit of measurement of radioactive activity is the *Becquerel* (Bq), 1Bq=1 dez/s, or the derived unit, *the Curie* (Ci), 1 Ci= 3.7 · 10^{10} Bq.

Natural and artificial sources of radiations

Life on Earth evolved in the presence of cosmic radiation or that generated by the lithosphere. Human activities lead to a gradual and continuous increase in the amount of radiation received by organisms. In addition to controlled radiation sources in medicine, industry and research, there are also certain uncontrolled sources of radioactive pollution: radioactive falls as the products of nuclear explosions and weapons tests, and radioactive residues produced by economic and research activities. To the sources of artificial irradiation is added the natural radioactivity. Natural irradiation should be considered to be responsible for at least one third of all genetic infirmities.

Ionizing radiations of natural origin are present in the surrounding nature. In addition to cosmic radiation, ionizing radiation is produced as a consequence of the presence of radioactive materials existing in the Earth's crust. Three-quarters of radioactivity in the environment comes from natural elements. The natural radioactivity of terrestrial origin is mainly based on the radioactivity of K-40 radionuclides and those of three natural radioactive series, called uranium (U-238), actinium (U-235) and thorium (Th-232) series (Marcu & Marcu, 1996; Nat & Ene, 2003a; Ene & Pantelica, 2011b; Pintilie et al., 2016; Moghazy et al., 2021). Radioisotopes that are part of the three natural radioactive families (denoted by A,B,C in the Figure 5.3) are linked to each other by successive transformations α and β, the generator being the radionuclide with a high half-life.

Not all places on Earth have the same level of radioactivity. In some areas of India, for example, radioactivity is 10 times higher than the European average. The reason lies in the sands of India, which have a high content of ores rich in thorium. Mountainous areas also have a relatively high level of radioactivity, due to the composition of granitic rocks. In addition to this geographical variability, certain activities such as the manufacture of ceramics, the production of fertilisers or the extraction of gas and oil, may increase the

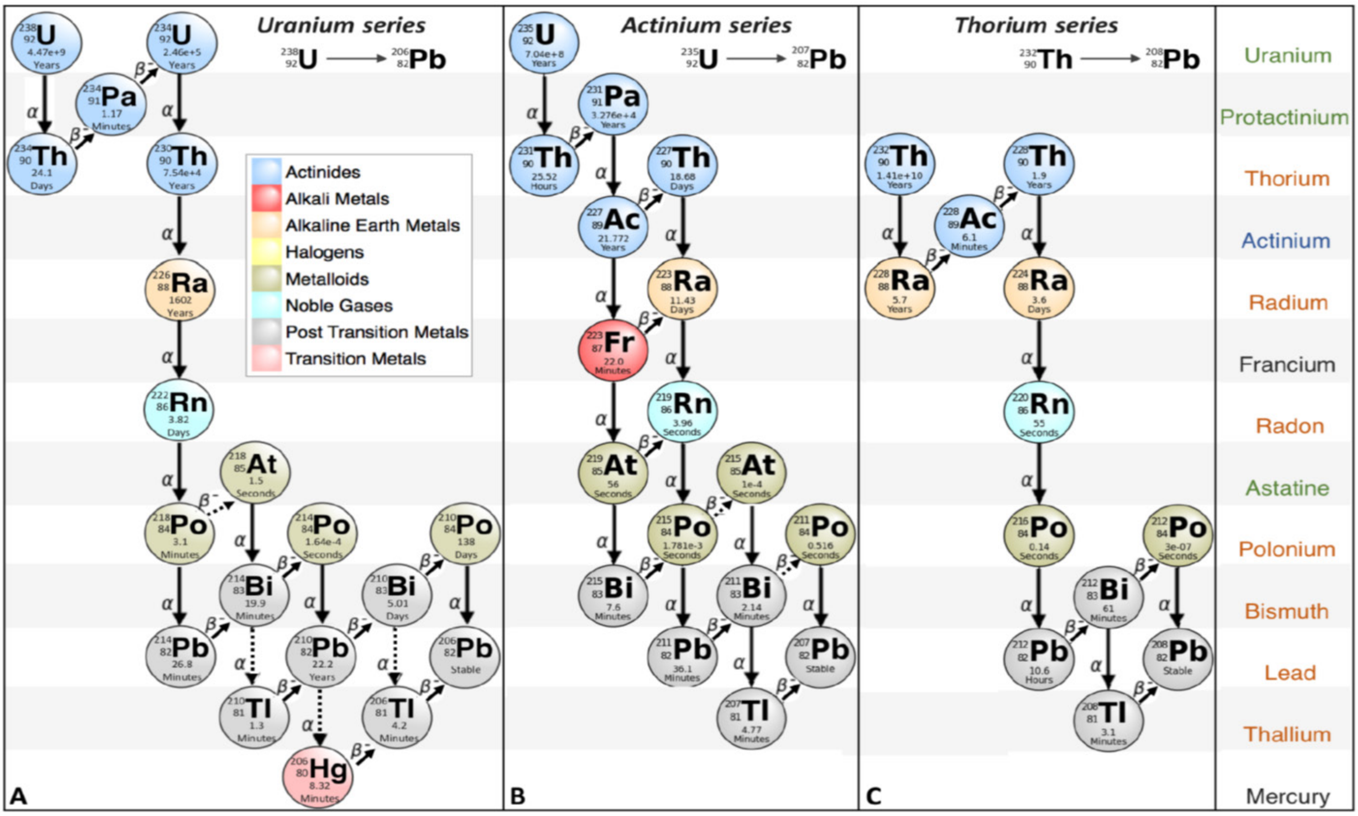

Figure 5.3. Natural radionuclides of the U^{238}, U^{235} and Th^{232} series
(*after*: Change Laura Tan, 2016)

doses due to these radionuclides of natural origin, not only for workers, but also for the rest of the population (UNSCEAR, 2000).

Radioactivity can also exist in our homes, mainly from *radon* gas (WHO, 2009). This gas is produced as a consequence of the decay of uranium contained in rocks. The amount of radon gas that accumulates in a house depends on its situation, the materials that have been used in its construction and our way of life. Radon emanates from rocks and is concentrated indoors, so it is highly recommended that homes, public spaces and workplaces be well ventilated (Ene & Vodarici, 2020).

On the other hand, cosmic radiation is generated in nuclear reactions that occur inside the sun and other stars. The atmosphere filters these rays and protects us from their dangerous effects, because outside the atmosphere, in space, the level of radioactivity is much higher. When we climb a mountain, that protection diminishes and the cosmic radiation is more intense. The same happens when we travel by plane, being more exposed to radiation.

Radioactive elements also exist in many foods (Pintilie et al., 2017, 2018 a,b) and in drinking water (Pintilie et al., 2016). Even our bodies are a source of radiation, since they store small amounts of radioactive potassium, a product necessary for the human body.

Radiation can also be produced artificially. In 1895, the physicist Roëntgen, conducting experiments with cathode rays, discovered the first type of artificial radiation that the humans used: X-rays. These are electromagnetic waves caused by the braking of beams of accelerated electrons on certain materials inside a vacuum tube.

The release of energy can occur as a result of nuclear fission reactions, that is, breaking the nucleus of the atom. Some elements are more suitable than others to produce these types of reactions. This is the case with uranium-235, with a tendency to absorb any neutron that collides with it. When this happens, uranium-235 gains weight, becomes more unstable and ends up breaking into various fragments, releasing other neutrons. If these neutrons are absorbed, in turn, by other uranium-235 atoms, a sequence of chain reactions occurs, generating significant amounts of radioactivity and energy.

Once the properties and potential of radiations began to be known, their applications were also developed, as well as the techniques for obtaining artificial radioactive materials. X-rays and gamma rays are used in medicine to diagnose multiple physical problems through imaging. Radiation is also used in the treatment of cancer and other diseases. The industry also benefits from radiation applications in X-ray techniques, industrial measurement, food sterilization, pest control, etc. In addition, fission reactions are caused in nuclear power plants that release a great deal of energy in the form of radiation, allowing the production of electricity.

Elements of dosimetry

In order to express the biological effects produced by the nuclear radiations, a radiological system of dosimetric quantities and units was defined (Gaspar, 1996; Nat & Ene, 2003b; Angelescu et al., 2005; Creangă, 2005; Nat, 2005), the most important being:

- *absorbed radiation dose* ***D***, refers to any type of ionizing radiation and represents the amount of energy dε absorbed by the unit of mass *dm* of living tissue:

$$D = \frac{d\varepsilon}{dm} \qquad (6)$$

The unit of measurement for the absorbed dose **D** is *Gray* (Gy; 1Gy=1J/1kg), or the tolerated unit, *rad* (1 rad = 10^{-2} Gy).

- *The equivalent* dose (biological dose) ***H***:

$$H = w_R\, D \qquad (7)$$

is the absorbed dose multiplied by a weighting adimensional factor for radiation, w_R, which expresses the biological efficiency of a type of radiation.

For most of the radiations used in industry or medicine (X, γ, β-) $w_R = 1$, so that: D=H. Exceptions are made for alpha particles ($w_R = 20$) and neutrons ($w_R = 2.5 - 21$) (Figure 5.4).

The unit of measurement of the equivalent dose is *Sievert* (Sv) and represents the dose of incident radiation that produces under identical conditions the same biological effects as the dose of 1 Gy from the standard X radiation. The tolerated unit of measurement, often used in practice, is *rem* (1 rem = 10^{-2} Sv).

In practice, *the rate of the equivalent dose* is measured, DR, which is the biological dose dH corresponding to the irradiation time unit dt,

$$DR = dH/dt$$

and is expressed in Sv/s (or submultiples μSv/h, nSv/h) or rem/s. Maximum permissible dose rate value of the ambient range is 0.250 μSv/h.

The applications of ionizing radiation are based on their interaction with matter and the behavior of radiation in it. Radioactive materials and ionizing radiation are widely used in medicine, industry, agriculture, teaching and research.

- *The effective dose* ***E*** is defined as the sum over the whole body of the equivalent doses of each organ or tissue, H_T, multiplied by a weighting factor per tissue, w_T:

$$E = \Sigma_T (w_T\, H_T) \qquad (8)$$

The unit of measurement of the effective dose is *Sievert* (Sv).

In Figure 5.4. is illustrated the difference between the equivalent and the effective dose, as well as between the effects of different types of radiations and the sensitivities of the various organs to their action.

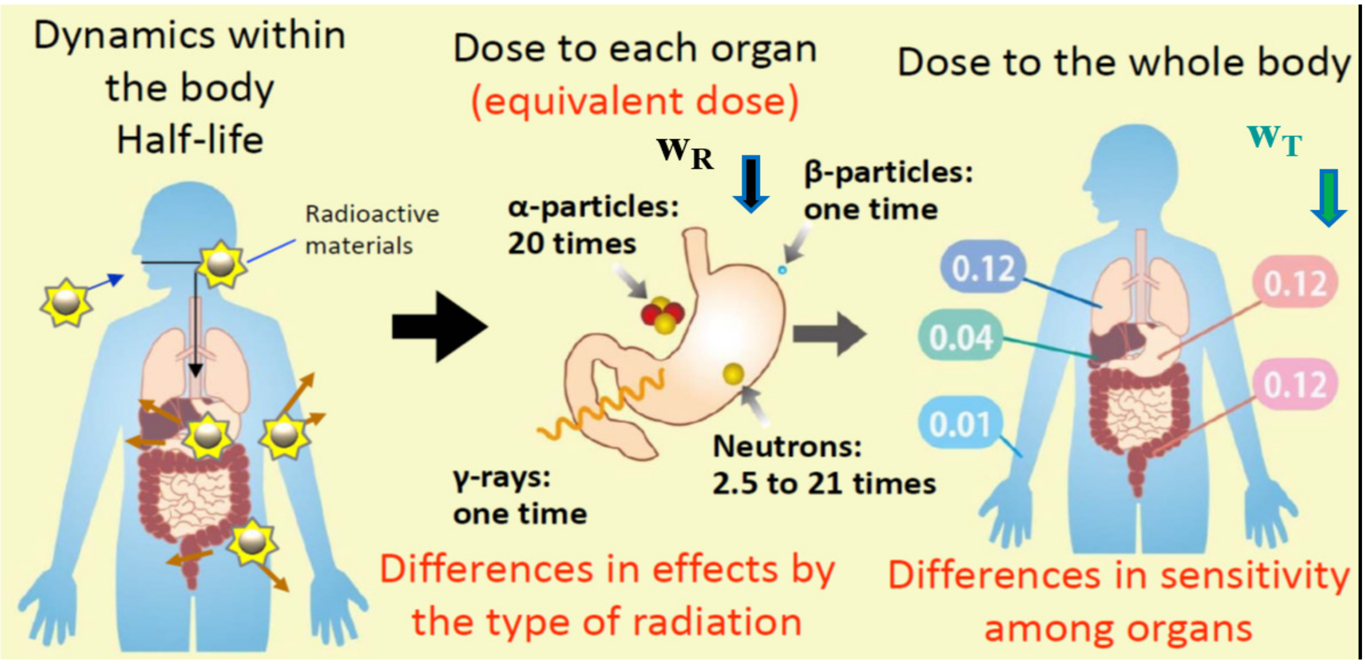

Figure 5.4. The difference between the equivalent and the effective dose, the effects of radiation and the sensitivity of different organs to their action
Adapted after: https://www.env.go.jp/en/chemi/rhm/basic-info/

Applications of ionising radiations

In medicine, the use of ionizing radiation is part of the application of diagnostic techniques, radiation therapy and nuclear medicine.

Radiodiagnosis comprises the set of procedures for visualizing and exploring human anatomy using images and maps. Some of these applications are: the obtaining of radiographies by means of X-rays for the identification of internal lesions and diseases, the use of radioisotopes in computed tomography to generate three-dimensional images of the human body, fluoroscopy and interventional radiology, which allows visual monitoring of certain surgical procedures.

Radiation therapy allows the destruction of tumor cells and tissues by applying high doses of radiation to them.

Nuclear medicine is a medical specialty that includes the use of radioactive material in uncapsulated form for diagnosis, treatment and research. An example is the radioimmunological test, a laboratory analytical technique used to measure the amount and concentration of numerous substances (hormones, drugs, etc.) in biological samples from the patient.

In the industrial field, the applications of ionizing radiations are many and varied. The industry takes advantage of the ability of radiation to pass through objects and materials and the fact that the negligible activities of their radionuclides can be measured quickly and accurately, providing accurate information about their spatial and temporal distribution (Ene, 2006).

Some of the most significant applications of ionizing radiation in industry are: the sterilization of materials; measurement of the thicknesses and densities or levels of filling of tanks or containers; measurement of the degree of humidity in bulk materials (sand, cement, etc.) in the production of glass and concrete; gammagraphy or industrial radiography, for example, checking welded joints in pipes; security detectors and X-ray surveillance at airports and official buildings; smoke detectors; pipes leak detectors and analysis of carbon-14 by radioactive dating to accurately determine the age of various materials.

There are also many applications of ionizing radiatiosn in agriculture and food, for example, to determine the efficiency of fertilizer absorption by plants, to determine the humidity of a plot of land, and thus to optimize the necessary water resources, to combat pests and to extend the shelf life of food by irradiating it with rays gama.

Apart from previous technological achievements, the use of radiation has been an incredible advance in all types of research activities, such as studies of cellular and molecular biology of cancer, molecular pathologies, genetic evolution, gene therapy, drug development, etc.

Radioactive waste

Radioactive waste is generated as a result of processes carried out in the different types of nuclear and radioactive installations distributed throughout the territory. Radioactive waste may occasionally be generated as a result of incidents involving radioactive material. The origin of the radioactive waste that is currently generated or has already been generated is as follows:

- Operation of nuclear power plants
- Operation of their factories of nuclear fuel elements
- Operation of radioactive installations for industrial, medical, agricultural and research purposes
- Operation of radioactive waste disposal facilities
- Reprocessing of spent fuel from nuclear power plants
- Dismantling and closure of nuclear power plants or installations
- Sterile resulted from uranium extraction and manufacture of uranium concentrates
- Incidents that occur occasionally.

This waste is classified into different categories, depending on the type of management currently planned or implemented in different countries.

The regulation on the protection of health against ionising radiation defines the activities and tasks that involve the generation, storage area or handling of waste containing natural radionuclides, better known as **NORM waste**.

The radiological characterization of waste consists in checking whether or not its radioactive content is lower than the legislated levels of declassification. If the radioactive content is less than or equal to the levels of declassification, the waste may be managed by the usual conventional means in accordance with the applicable legal regulations.

The declassification levels for norm waste management are those recommended by the European Union in its publication *Application of the concepts of exemption and disposal of natural radiation sources* and have been established taking into account the following considerations:

- The annual effective dose is 300 μSv/year.
- All routes of radiation exposure (inhalation, ingestion, external exposure) are taken into account.
- Comprehensive scenarios and specific parameters are considered.
- All routes of exposure are analyzed in each of the scenarios under consideration, selecting the most restrictive radionuclide for each case and determining the specific activity that would give rise to 300 μSv/year.

Ministerial orders issued at national level require that norm waste management be carried out with methods and procedures for the characterization, classification and management of this waste.

5.2. INTERACTION OF RADIATION WITH LIVING TISSUE AND RADIOLOGICAL RISK

Interaction of ionizing radiation with living matter

Radioactive contamination is the transfer of radioactive material from the environment to different structures, objects or organisms, being *external* (by deposition on the recipient's surface) or *internal* (by penetration inside the receiver).

The main exposure ways of the human body to ionizing radiations are: by inhalation, ingestion, diffusion and penetration of radioactive material (internal contamination), or by direct contact, cutaneously, with radioactive materials deposited on the skin, hair, nails (external contamination) (Bacalum, 2021) (Figure 5.5).

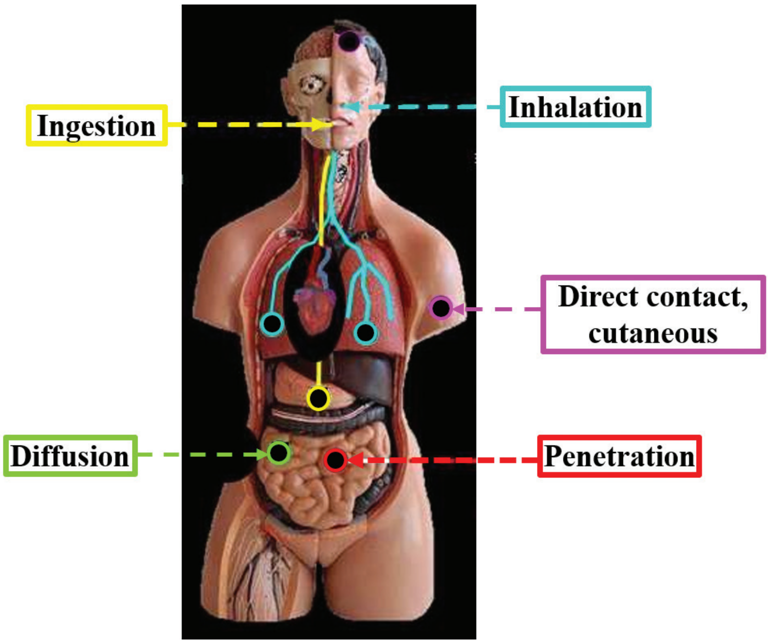

Figure 5.5. Exposure pathways of the human body to ionizing radiation
Adapted after Bacalum, 2021.

Figure 5.6. presents the internal and external exposure of the population to radionuclides and fission products resulting from nuclear accidents.

Radiation, when it penetrates into matter, and especially in the case of charged particles - alpha, protons, fission fragments and electrons - tends to break the atomic bonds that hold the molecules together in cells through the ionization process. These changes can be more or less serious, depending on the radiation dose received.

The essential elements in the study of the **interaction of radiation with the biological material** are related to the amount of energy and the type of energy transferred, as well as the type of energy into which it is transformed, so the effects of irradiation depend on the nature and energy of the radiation and on the characteristics of the body that can be highlighted in this way (UNSCEAR, 2000; Creangă, 2005; Nat, 2005).

The main effects are:

- excitations (electronic, nuclear, molecular);
- ionizations;
- chemical effects (photochemical reactions, acceleration of some biochemical reactions or triggering of others, breakages or bond formations, etc.);

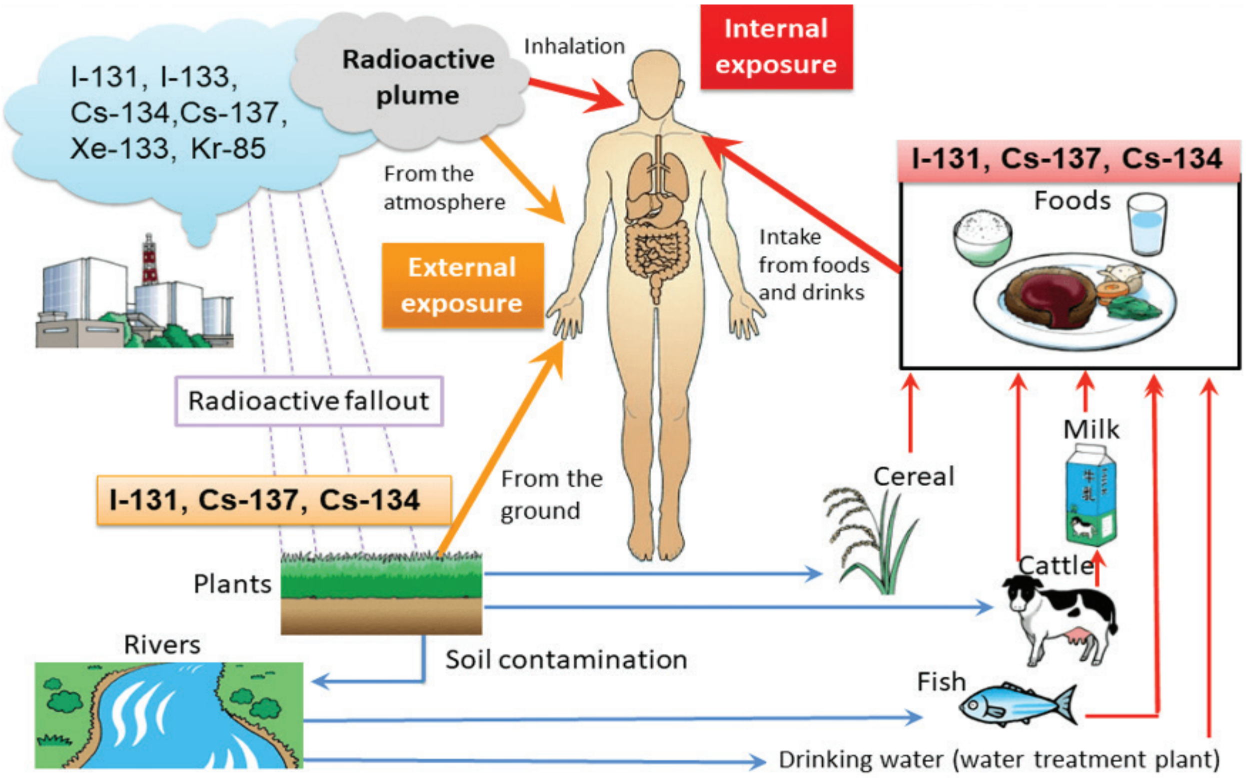

Figure 5.6. Internal and external exposure to radiation resulting from nuclear accidents
Adapted from: https://www.env.go.jp/en/chemi/rhm/basic-info/

- thermal effects (heating, protein denaturation, evaporation of water, carbonization).

The transferred energy to organism can disrupt, reversibly or irreversibly, physiological processes.

Penetrating radiation can act on the living substance in three ways:

- by direct action,
- by indirect action,
- through remote action.

The direct action occurs as a result of excitations and ionizations in the living cell and determines the breaking of chemical bonds, which leads to the partial or total destruction of the tissue functions in the body, and implicitly to functional disturbances that damage important macromolecules: proteins and aminoacids.

The indirect action leads to the appearance of other chemical elements as a result of radiochemical processes. Thus, the water in the tissues is ionized, and the decomposition products (ions, free radicals, etc.) become reducing or oxidizing agents for the molecules in the living substance.

Remote action causes the spread of radioactive substances also in unradiated areas, radioactively contaminating these areas.

There are two types of biological effects of ionizing radiation (Nat, 2005):

- somatic effects,
- genetic effects.

Somatic effects occur in the irradiated individual in the form of skin lesions, changes in blood composition, bone degradation, degradation of the nervous system, neoplasms, etc.

Genetic effects occur in the generations following the irradiated individual through changes in the genetic code such as: malformations, chromosomal aberrations, mental deficiency, etc.

The effect produced by penetrating radiations depends on their energy and intensity, on the exposure time, but also on the individual's sensitivity or radiosensitivity.

Radiosensitivity is the property of cells, tissues or organs to respond through morphological and functional changes, qualitatively and quantitatively differentiated according to the size of irradiation. The most radiosensitive are the cells in the multiplication phase (Nat, 2005).

Biological effectiveness is determined by the level of the value of the energy absorbed by irradiation processes, necessary to produce a certain biological effect. Thus, particles that produce high linear ionization or have a high *linear energy transfer* (LET) (the amount of radiation energy that is deposited per unit of length of its path), such as alpha particles or protons, lead to stronger biological effects than particles that produce lower linear ionization, such as electrons.

Depending on the response (effects) at the dose of radiation received, biological effects are divided into two groups (Figure 5.7) (Bacalum, 2021):

a) b)

Figure 5.7. Dose-effect relationship in the case of: a) deterministic effects; b) stochastic effects

- Deterministic effects
- Stochastic effects

Deterministic effects are characterized by a determined (direct) causal relationship between dose and effect and the response reactions of the irradiated organism appear from a certain minimum value of radiation dose (***threshold value)*** and that is why they are also called ***threshold effects***. They are evaluated in terms of ***severity:*** for any radiation dose value higher than the threshold, the severity of the effect becomes more pronounced as the dose increases (Figure 5.7a). The severity of the effects is dependent on the amount of energy of the radiation that is absorbed by the tissue, i.e. by LET; thus, at radiation with low LET (X, gamma, beta particles of high energy, the tissue absorbs a small fraction of the incident energy, while at radiation with high LET (alpha particles, neutrons), the tissue absorbs a larger fraction of the incident energy. The minimum level of the radiation dose at which the effect becomes manifest varies from one individual to another and in relation to the irradiation conditions, but it manifests itself only in the irradiated individual and does not spread to the offspring (it targets only the somatic cells, not the mature sexual cells, involved in the reproductive function) (Bacalum, 2021).

Stochastic effects are reactions of the body's response at any level of irradiation, the effects can occur from doses placed below the threshold doses (***no-threshold effects*** or ***zero-threshold effects)***. They concern the consequences of irradiation at the population level, whether it is a population of cells or a population of organisms obeying a *dose-effect relationship of probabilistic type*. Thesupport evaluated within *risk* terms, the risk of their occurrence in a population being proportional to the dose received, the chances of occurring increasing as the dose increases, without influencing their severity (Figure

5.7b). Stochastic effects are associated with long-term (chronic) and low-level irradiation exposures. At the body level, they include the consequences on tissues and organs when the cells are not destroyed but only transformed, when changes (mutations) occur in their genetic apparatus and the mutant cells will propagate an altered genetic information in the cells resulting from their division. If irradiation occurs at the level of somatic cells, then *bodily effects* occur (which manifest themselves only in the irradiated individual); after a longer and variable period most likely can lead to cancerization (neoplasia) / the appearance of radio-induced cancer. If irradiation occurs at the level of the sexual cells, then *hereditary effects* take place (which are transmitted to the descendants of the irradiated individual); after a longer and variable period – can most likely lead to cancerization (neoplasia) / the appearance of radioinduced cancer (Bacalum, 2021).

If matter is a biological tissue with a high water content, the ionization of water molecules can give rise to so-called *free radicals* that exhibit a high chemical reactivity, sufficient to modify the important molecules that are part of the tissues of living beings. These changes may include chemical changes in DNA, the basic organic molecule that is part of the cells that make up our body (Figure 5.8). In Figure 5. 9. are presented the main types of cellular modifications caused by ionizing radiation.

Health risk

The increase and diversification of the use of radioactive isotopes and nuclear energy also leads to an increase in the risk of radiocontamination of the environment and living organisms. Radionuclides that reach the air, water and soil affect the biosphere through trophic chains and are also transferred to the human

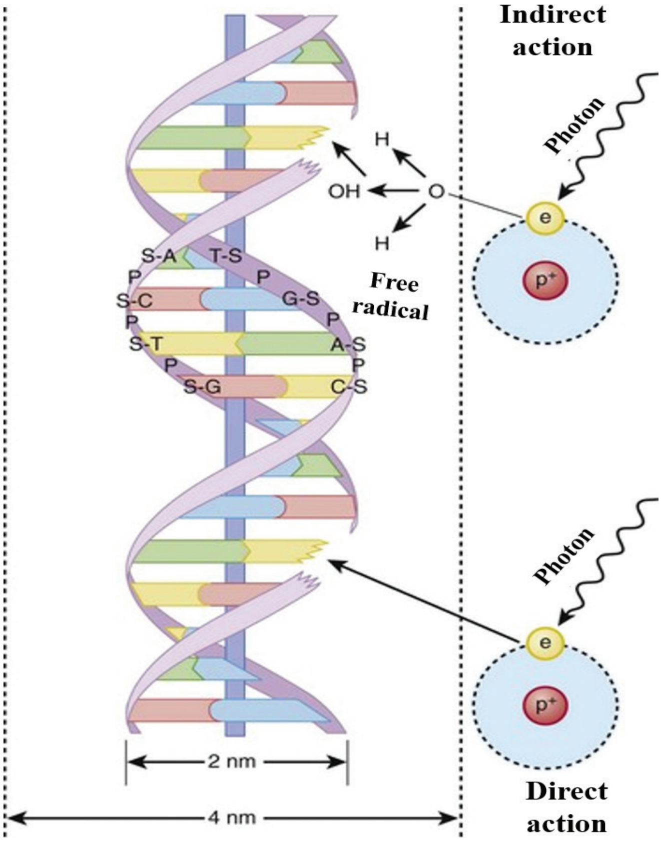

Figure 5.8. Modifications in DNA caused by the direct and indirect action of ionizing radiation

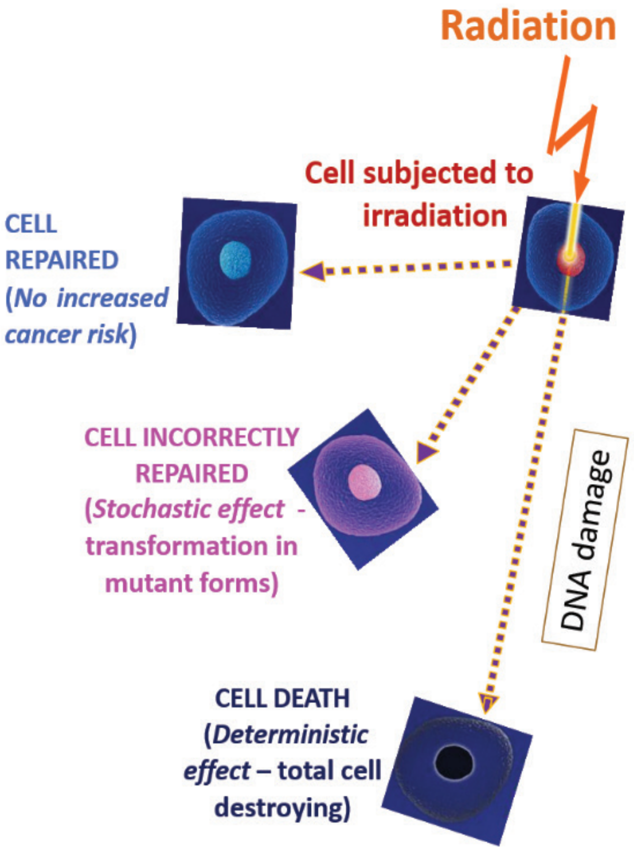

Figure 5.9. Cellular modifications caused by ionizing radiation

body (UNSCEAR, 2000). The investigation of the level of environmental radioactivity and the assessment of the health risk of radionuclides emitting different types of radiations are of great importance in the management of the environment and the assessment of radiological hazards to human health and ecosystems.

The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR, 2000) estimated at a global level that natural sources have a contribution to the annual effective dose of 2.4 mSv year^{-1}; of this, 1.2 mSv is due to radon, 0.5 mSv to terrestrial gamma radiation, 0.4 mSv to cosmic radiation and 0.3 mSv to food and water ingestion. The contribution of the artificial radiation background of 0.6 mSv year^{-1} is added to this dose.

The International Commission for Radiological Protection (ICRP) and World Health Organization (WHO) have started measures for decreasing the population exposure and prescribed limits of the concentrations of radionuclides in food and drinking water and risk coefficients for population (ICRP, 2007), by age group (Pintilie et al., 2016, 2017, 2018 a,b), as well as allowed concentrations of radon in housing and public spaces (300 Bq m^{-3}) (WHO, 2009).

Using the conversion factors published by the International Atomic Energy Agency (IAEA, 2014) and the annual rate of consumption of a food item, the **annual effective dose**, Def, can be estimated using the following formula (Pintilie et al., 2016, 2018b):

$$\text{Def} = \Sigma\ (A_X \times R \times CF)\ \text{Sv year}^{-1}$$

where: A_X is the concentration of radionuclide X (X = 210Po, 210Pb, 238U, 232Th, 40K, 226Ra, etc.) (Bq kg^{-1}), R is the annual consumption of food (kg year^{-1}) or water (L year^{-1}), and CF is the effective dose conversion factor for adults or children (Sv Bq^{-1}). For example, for calculations, the following dose conversion factors can be used for adults (in Sv Bq^{-1}) (IAEA, 2014): 1.2 × 10^{-6} for 210Po, 6.9 × 10^{-7} for 210Pb, 4.5 × 10^{-8} for 238U, 2.3 × 10^{-7} for 232Th, 6.9 × 10^{-9} for 40K and 2.8 × 10^{-7} for 226Ra.

The **Lifelong Cancer Risk** (LR) assessment is carried out using the formula:

$$LR = \text{Def} \times L \times RF$$

where:

LR represents the risk of cancer for life, Def is the effective annual dose (Sv year^{-1}), L is the life span (years) and RF is the risk factor for fatal cancers for the entire population (Sv^{-1}). The annual effective dose of ingestion of uranium and thorium radionuclides reported by UNSCEAR (2000) is 110 μSv year^{-1} (for adults). The value of the nominal risk coefficient RF adjusted for cancer and hereditary effects, published by the ICRP (ICRP, 2007), is 5.7 × 10-2 Sv^{-1}.

In the case of radionuclides existent in soils or building materials, based on the concentration activity values of natural radionuclides or radionuclides originating from natural series, A_X, the hazard risk should be calculated using the total outdoor absorbed dose rates at 1 m above the ground surface due to γ-rays emission in air by the terrestrial radionuclide X (X = ^{226}Ra, ^{232}Th, and ^{40}K), using the following equations (UNSCEAR, 2000; Ene & Pantelica, 2011b; Ene et al., 2020; Moghazy et al., 2021):

- **Radium equivalent activity ():**

$$Ra_{eq} = A_{Ra} + 1,43A_{Th} + 0,077A_K \leq 370$$

- **Air absorbed gamma dose rate (D_{air}), nGy/h:**

$$D = 0.462A_{Ra} + 0,621A_{Th} + 0,0417A_K < 57$$

- **External (γ radioactivity) level index (*Iy*):**

$$I_\gamma = \frac{A_{Ra}}{300} + \frac{A_{Th}}{200} + \frac{A_K}{3000} \leq 1$$

- **External hazard index (H_{ex})**

$$H_{ex} = \frac{A_{Ra}}{370} + \frac{A_{Th}}{259} + \frac{A_K}{4810} \leq 1$$

- **Internal hazard index (H_{in})**

$$H_{in} = \frac{A_{Ra}}{185} + \frac{A_{Th}}{259} + \frac{A_K}{4810} \leq 1$$

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Joint Operational Programme Black Sea Basin 2014-2020
Institute of Zoology
September 2021