Chapter 7

Biotests in Ecotoxicology: Current Practice and Problems

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Additional information is available at the end of the chapter

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Abstract

Nowadays ecotoxicology plays the role of a theoretician – methodical unifying centre for the optimization of man – biosphere relations and sustainable existence of life on the Earth. The main basis for its development is the classical toxicology—studies of chemical compounds' effects on man, but ecotoxicology is the original part, following it. According to the modern concept, the ecotoxicology is a science for migration, transformation and utilization of different toxic ingredients (with organic, inorganic or organic-mineral chemical nature; with natural biotic or abiotic origin and artificial, mainly anthropogenic origin) in the environment and their impact on Macro- biological systems with different levels of integration as groups of individuals, population, community, ecosystem, etc. studied in ecology. In this chapter, the types of ecotoxicological tests are discussed in detail with a set of examples about used species, advantages and disadvantages of different types of toxicity testing. The application of exposed natural ecosystems or man-made analogue systems is also commented as the environmentally more realistic approach for ecotoxicological testing. These test systems are increasingly becoming in aquatic ecotoxicology practice, but they are contemporary challenge in terrestrial testing. The development of test systems for realistic assessment of contaminant toxicity is essential for the efficient control of human influence on the environment.

Keywords: ecotoxicology, bio tests, acute, chronic, mono-species, multispecies, biomarkers, kits

1. Introduction: the contemporary meaning of ecotoxicology as a complex science

Ecotoxicology is a scientific discipline, which of the modern stage of man-biosphere relations, is developed as the theoretician – methodical unifying centre for the optimization of these relations for the sustainability of life existence on Earth. The main basis for the development of ecotoxicology is classical toxicology—the research of drugs and chemical compounds effects on man. The modern concept of ecotoxicology is that it is the original part following the classical toxicology, which studies migration, transformation, degradation and utilization of toxic ingredients (with organic, inorganic or organic-mineral chemical nature; with natural biotic or abiotic origin and artificial, mainly anthropogenic origin) in the environment and their impact on Macro- biological systems with different levels of integration as groups of individuals, population, community, ecosystem, etc. studied in ecology (MBS) [1, 2] and others.

The main objects of the ecotoxicological studies are the both: (1) toxic ingredients and their "behaviour" in the five main environments such as air, soil, terrestrial, water (incl. sediments) and biotic and (2) the responses of MBS in nature. The studied toxicants can be: (1) by the chemical nature: organic, inorganic or organic-mineral; (2) by the origin: natural (biotic or abiotic) and artificial (mainly anthropogenic origin); (3) by the toxicity: toxicants in the Black list, toxicants in the Grey list, etc. and (4) by the main environment of circulation: air, water, soil or different bio toxicants. The migration, transformation and degradation of toxic ingredients depend on the internal (endogenous) factors, that are, chemical properties of the toxicant and external (exogenous) factors or features of the environment. The studied MBS at different levels of integration can be: diverse individuals as elements of the population; homogeneous and heterogeneous populations as elements of the communities; heterogeneous communities as elements of the bio cenosis; ecosystem as a functional unity between biotope and bio cenosis; landscape, biome and biosphere formed by a corresponding set of ecosystems and their environment. The responses of MBS also depend on the endogenous factors (level of integration and features of the MBS) and exogenous factors (the characteristics of toxicants and habitats). Therefore, according to the used objects, ecotoxicology is an interdisciplinary complex science, developing on the border of chemical, biological, medical, ecological, environmental, economic, social and legal sciences. It can also be considered as applied environmental science studying the biological effects of anthropogenic ingredients. According to the main objects concerned, the main sections of ecotoxicology are *Toxicant dynamics and Bio toxicology.*

Toxicant dynamics considers the migration, transformation and utilization of toxicants according to the characteristics of different environmental areas. There is a great difference in the evaluation of contaminants effects in the laboratory and in the environment. The physical and chemical changes of compounds in the environmental migration lead to the changes of their impact dose that vary in different environments. Therefore, the toxicants in nature often have an indirect effect on biosystems, changing the physical and chemical environment to act upon an indirect effect on the survival of organisms. For the terrestrial environment of great importance to toxicant migration is the temperature-precipitation Dynamics of habitat. In climatology, biogeography and ecology, this dynamics is well characterized by "climatic diagram" ("ombro-thermal" diagram) by Lyubenova [3]. The toxicants emission during the period of drought and semi-drought (calculated on the chart curves) poses an extra risk to the environment, because of the bio systems stress state, limited by temperature – precipitation patterns. The characteristics of different environments and the particular environment are

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important for the sensitivity of bio systems to toxicant. The toxicity and behavior of contaminant in the environment are also dependent on the concentration, but also to a large extent, on the predominant form (molecules, ions, complexes, etc.) of migration in biotope and of taken by bio systems.

The development of analytical methods is very important in this division for solving the series of toxicological problems. For example, with the appearance of inductively coupled plasma mass spectrometry (ICP/MS) as a method of measurement, it is possible to separate and determinate the toxicity of various forms of each toxicant. Therefore, the chemical and physical measurements for assessing the toxicity of the substances and their forms are important for determining the valid toxic concentration in the bioassay and complementary test system reply.

Bio toxicology examines the effects of toxicants on sensitive test systems and further on MBS in nature. For the manifestation of this effects is necessary to consider four main phases in passing of toxicants in bio systems, namely biological absorption and assimilation, metabolism, transport and excretion from the system. At the MBS level, biological uptake and accumulation from the environment (bioaccumulation) in the food chains (biomagnification) and accumulation in different organs or elements of bio system (bioconcentration) can be characterized by calculating several geochemical coefficients [4]: biological absorption coefficient (BAC); relative absorption coefficient, (RAC); acropetal coefficient (AC); temporal absorption coefficient (TAC); litter-mulch coefficient, A; and relative rate of transformation of the organic matter (RRTOM).

There are few main differences between classical toxicology and ecotoxicology: (a) the usage of bio-test, including selected for this purpose; (b) the main objects for acute toxicity measurement are different – *Daphnia spp*., or laboratory rat (c) the reference of induced toxicological effects on sensitive/representative test objects to MBS in nature; (d) the usage of standardized methods and indicators (good laboratory practice) guaranteed the results recognition everywhere, etc. The requirements for the test bio systems are: susceptibility of cultivation and maintenance in laboratory, low-cost, highly responsiveness to the toxicant, mass usage and vast database available, representativeness of the exposed species to MBS, low correlation with other assessments in the same trophic level and strong correlation with a series of changes in ecosystems [1]. Today, in laboratory tests with test objects, most often are reported mortality, reproductive capacity, changes in growth, development, behaviour, biochemical, genetic changes and other.

The MBS are characterized by a complex structural and functional organization and the specificity of the set of internal regulatory mechanisms that support the system in equilibrium, which should be considered in the toxicological effect extrapolation, as well as for assessment of ecosystem health and predicting the risk. Therefore, the models, adequately reflecting the responses of MBS in nature, require the knowledge of structure, function and the mechanisms for ensuring the existence and integrity of MBS and the behaviour of toxicants in the current climate. The main functions and features of MBS have been deeply commented by Lyubenova [5].

The main aim of chapter is to comment the contemporary knowledge and established practice in the usage of bioassays to study the environmental toxicity of ingredients. The acute, chronic, mono-species and multi-species tests are discussed. Moreover, the analytical and biochemical methods for determining the initial damage at the molecular level on acute and chronic exposure are commented, too. The molecular markers (biomarkers) or indicators are very important for the early diagnosis of damages and the interests for new developments are growing steadily.

2. Ecotoxicological testing: contemporary knowledge and gaps

The ecotoxicological effects of contaminants on bio systems and MBS are developed as sub lethal and lethal responses. The earliest toxicological responses (change in biological systems) are detected at the cellular level. Some of the most important effects are changes in the structural components of the cell membrane (e.g. breach links between proteins and lipids); suppression of certain enzymes (e.g. microsomal enzymes); damage to the whole or partial metabolic changes (e.g. the synthesis of carbohydrates) [6]; changes in DNA correlation, respectively mutations and modification of cell growth [7], etc. At the macro-bio system level, effects related with their structure and functioning can be observed: efficiency of energy utilization and transmission through the food chains [8]; bio-depressant effects (inhibition of growth and reproduction) [9] and bio-stimulant effects (e.g. eutrophication) on population or community [10]; changes in the nature of biological cycle—capacity (the amount of chemical elements involved in biomass per year), intensity (of productive processes, energy transformation and destructive processes), chemistry (determined by the leading elements in the cycle), openness (e.g. balance of import and export); bioaccumulation of toxicants (higher concentration in the biomass than in the surrounding medium) [11]; bio magnification (increasing the concentrations in each higher-trophic level) [12]; bio concentration (accumulation in separate organs or elements of bio-system), etc. In most cases, plants and animals are more exposed to the combined effects of many pollutants simultaneously [13]. The interaction between them may increase or decrease the toxicity of the mixture and hence alter the response of the biological system. The effects on biosystem exposure on two or more toxicants may result in the following combining toxicological responses: supplementary response (e.g. in simultaneous action of two organophosphate compounds) [14]; synergistic (reinforced) response (e.g. response of rat to concomitant ingestion of hepatic toxins – ethanol and carbon tetrachloride) and depressed (antagonistic, reduced) response, when the antagonistic reaction between toxicants exists: for example, chemical (e.g. the toxic effect of Se and Hg) [15], competitive (e.g. the toxic effect of CO), uncompetitive (e.g. the toxic effect of atropine and organophosphorus insecticides), functional (e.g. the toxic effect of barbiturate decreased vascular pressure) and predisposing (e.g. the reduction of organophosphorus insecticides toxicity with piperonyl bioksid by blocking the activity of cytochrome P450, responsible for the metabolism of organophosphates) antagonism. The interactions between toxicants and natural chemicals in the environment result in formation of new molecules or complexes, changing their expected utilization. When toxicity is unknown, the conducting tests use a wide range of concentrations and report of "all or none" response. The dose–response relationship is the tested biological effects to 4–5 toxicant concentrations that cause from 20 to 80% mortality. This value is only representative of acute exposure, not chronic one. The LD_{50} and ED_{50} variables are influenced by many factors: behaviour of animals [16], age [17], sex [18], temperature [19], water quality (hardness) [20], pH [21], etc. Nevertheless, the 50% response rate is used, because it is the most reproducible response and can be calculated with high reliability. There are three main types of systems for the contaminants exposure of aquatic organisms: *Flowing-through, Static and Renewal* [1 and others]. Flowing-through systems are preferred for the study of acute toxicity. They are recommended for toxicants with high volatility and pollutants that are unsustainable in water [22 and others]. The static systems are applied mainly in short-term tests (≤96 h with fixed or slowly degradable materials and at a low load (biomass/water volume) of the test organisms. Static systems are used at limited availability of studied pollutant critical load with residual water or receiving toxic effects at critical levels of the components of the test. The renewal systems are applicable in work with small organisms that may be lost in watercourse systems or that are very sensitive to streams. They are also used in the case of limited test material. A *recirculation test* is similar to a static test except that the test solutions and control water are pumped through an apparatus, such as filter, to maintain water quality but not reduce the concentration of test material. The water is returned to the test chamber. This type of test is not routinely used because of uncertainty about the effect of the apparatus (aerator, filter and sterilizer) on the test material [1].

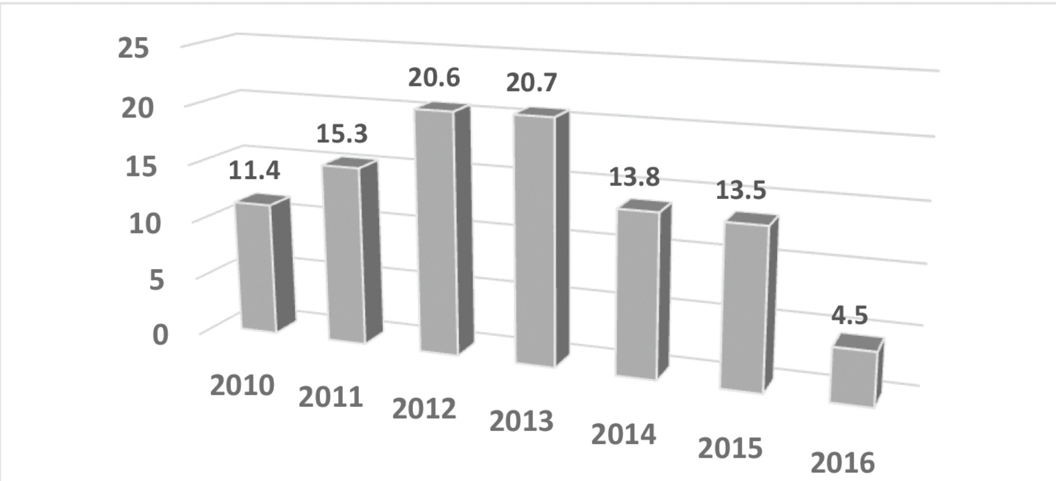

Figure 1. Percentage distribution of ecotoxicological studies published based on random sampling.

In the random sample of 384 published studies, 535-conducted bio-tests were considered. For the studied period (2010–2016), an exponential increase of published ecotoxicological studies to 2012 can be observed—**Figure 1**. The level is kept in 2013 and the percentage sharp fell in 2014, while in 2015 the published ecotoxicological studies are closed to that in 2014. We do not have compete data for 2016, but it seems that this trend will likely keep. Furthermore, the scientific community is concerned about finding new environmentally acceptable agents and technologies in industry, agriculture and households, which is gradually becoming a priority in the new solutions. No less is the role of environmental legislation, the timely testing of new toxicants and the introduction of regulations and restrictions.

Among the reviewed studies, the tests for toxicity of aquatic environment prevail – 67%, of which these for the toxicity of freshwater are 35%, the terrestrial ones are about 20% and those concerning three environments – 13% (**Figure 2**).

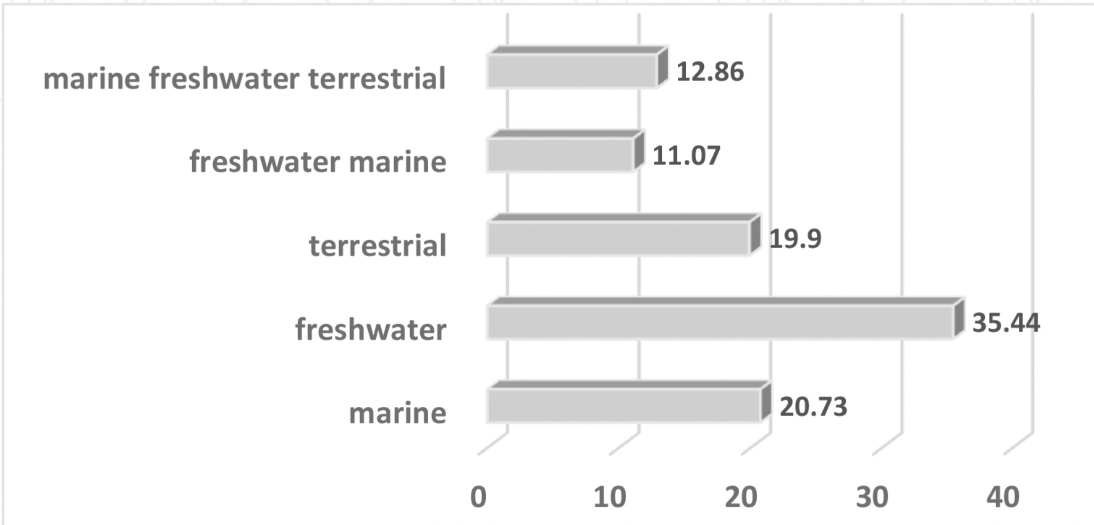

Figure 2. Percentage distribution of ecotoxicological studies by media published in random sample.

During the years, the focus of researches has been on the toxicity of different environments, for example, in 2011, prevailed these for the aquatic environment; in 2012, for the terrestrial; in 2013, again for the aquatic; and in 2015, again for the terrestrial (**Figure 3**).

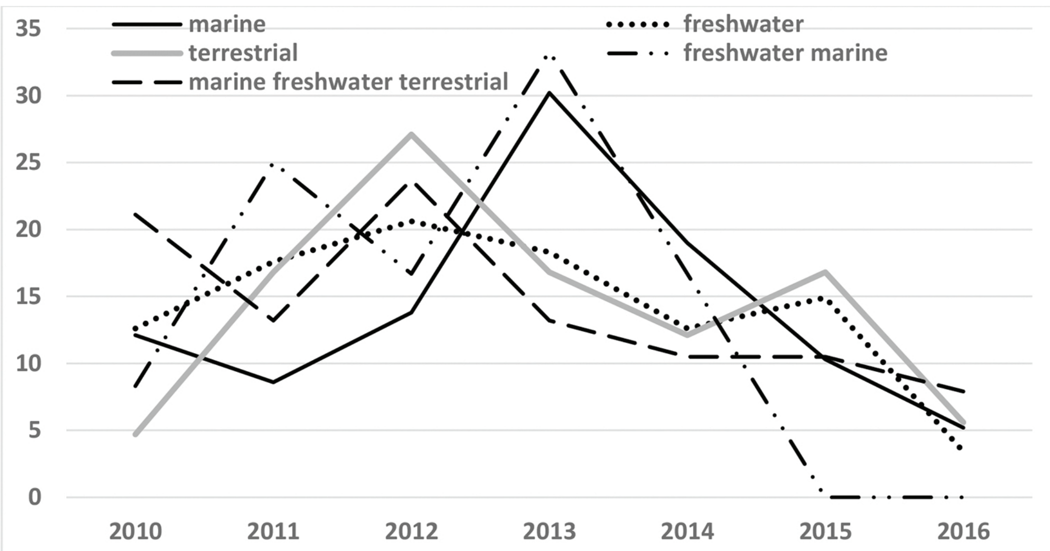

Figure 3. Percentage distribution of ecotoxicological studies by media and years published in random sample.

For the research period in the toxicological testing, 25 groups of biological systems have been used as most tests have been performed with crustaceans and fishes, 22% and 20%, respectively (**Figure 4**). Common test objects are also insects (9%), molluscs (9%), algae (8%) and the plants (6%). In the ecotoxicological studies, 61 crustacean species, 51 fish species, 27–17 insect species, molluscs, algae and higher plants have been used (**Figure 5**).

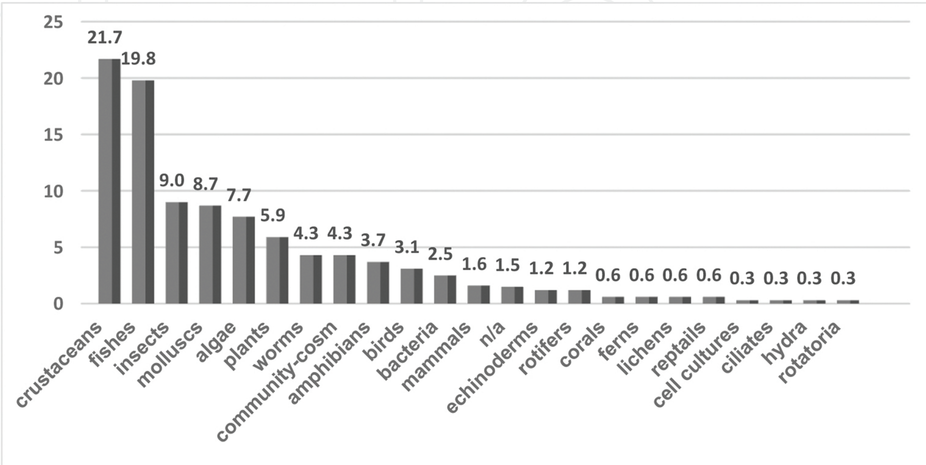

Figure 4. Percentage participation of biological groups for ecotoxicological testing in random sample (2010–2016).

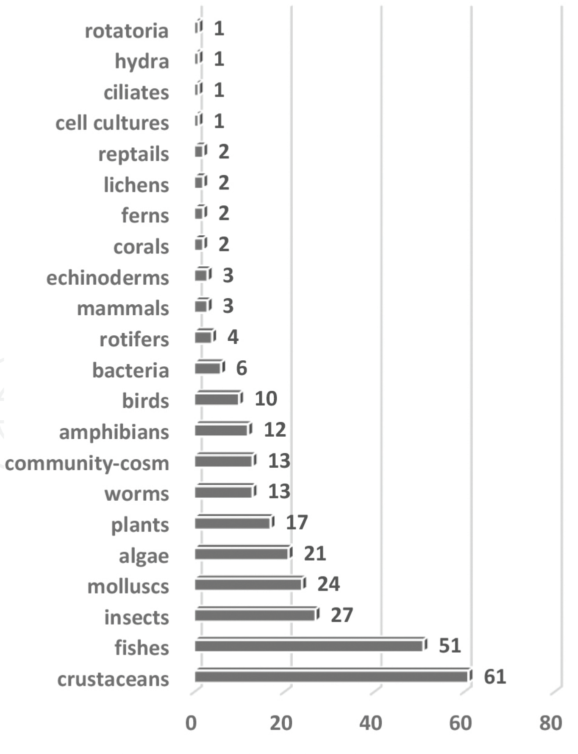

Figure 5. Participation of biological groups (number of species) for ecotoxicological testing in random sample (2010–2016).

2.1. Ecotoxicological tests for short–term (acute) and continuous (long–term,6 chronic) toxicity

The accepted types of toxicants impact on exposed biosystem are: acute (high doses and for a short time, typically 24 – 96 h); sub-acute (repeated exposure for one month or less at lower doses than those in the acute exposure); sub chronic (multiple exposure, for 1–3 months) and chronic (exposure for more than 3 months at doses representing about 1/100 to 1/1000 of the acute dose). The exposure intervals definition varies for different biological systems, media and toxicants.

For the period concerned, the studies of acute toxicity clearly prevailed over the chronic tests. In a number of studies for clarifying the actual toxicity, a series of both tests have been performed. The practical application of subacute and sub-chronic tests is low or negligible (**Figure 6**), but the trend of increasing the interest for these tests in 2014 and 2015 is noticeable.

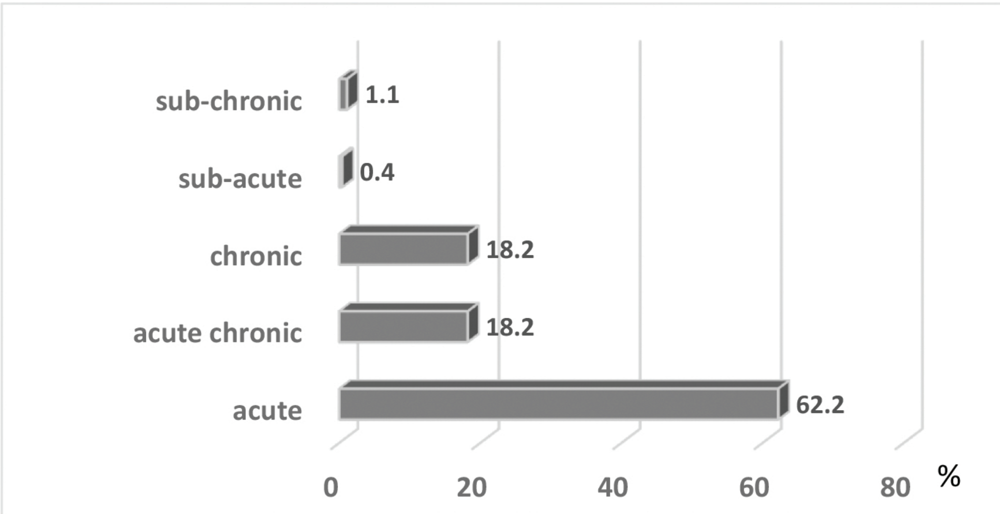

Figure 6. Percentage participation of acute and chronic tests in random sample (2010–2016).

Acute ecotoxicological testing has two main applications in environmental risk assessment. One of the applications is in conducting the screen test, for example, to determine whether the toxicant is biologically active at test doses for used indicators. The second type of application is the determination of acute toxicity—measuring the dose-response function and determination of LC_{50}/LE_{50} for a predetermined period. The acute toxicity tests is the first step for detecting the total toxic effects caused by toxicant. Many studies have been dedicated for searching to the most sensitive species to conduct acute tests. The practice shows that there are no universal species, that are the selection of species depends on the type of toxicant and the affected ecosystems. Virtually every hydrobiont is suitable for conducting the acute tests, but one of the most used are *Daphnia magna* [23–26], *Pseudokirchneriella subcapitata* [27, 28]; many mussel species like *Dreissena polymorpha* [7] and *Mytilus edulis* [29]. Fish species are also very often used as test system: *Danio rerio* [30, 31], *Gambusia holbrooki* [32], *Cyprinus carpio* [33, 34], *Oreochromis niloticus*s [35, 36]. The terrestrial test objects include bees [37, 38], *Mus musculus* [39], *Megascops asio* [40], *Podarcis sicula* [41] *Aquila adalberti*, soil invertebrates [42] and other.

The bacterial species, especially *Vibrio fischeri*, are also used in many ecotoxicological tests [27, 28, 43] and others. Today the most used plant test objects are different species of algae [44] and duckweed (*Lemna* spp.) [45]. They have a high reproductive capacity and therefore the study includes several generations in a relatively short period. Intensity of photosynthesis [46] and growth [47] are measured at algae or growth [48] at duckweed. Reporting indicators for the photosynthesis intensity are: concentration and ratio of pigments in photosynthetic biomass [49], amount of released oxygen [50], assimilated $14CO_2$ [51], ATP production [52] and number of cells [53]. Short-term sublethal tests are used to evaluate the toxicity of effluents to aquatic organisms [54], but some authors use terrestrial organisms [38]. These methods are developed by the EPA, and only focus on the most sensitive life stages. The endpoints for these tests include changes in growth, reproduction and survival as NOECs, LOECs, LC50s and EC50s [25] and others. Acute toxicity resulted in abortion rate of eggs and embryonic stages [25], reduced offspring and egg production [55], reduction in hatching success [56], decrease in fecundity [57], decrease in fertility [57], failure of metamorphosis [58], delayed development [59] and abnormalities and deformities in fish larvae [60]. Despite the results cited above, [61] reported no significant effects of the insecticide indoxacarb on the eggs, young and old larval stages and the pupal stage of two species of Trichogramma [62] and observed no negative effects on *Daphnia magna* embryonic development or hatching rate to insecticide Carbaryl up to concentrations almost 1000 times of the median effect concentration (EC_{50}) of neonate survival in acute tests. Furthermore, [63] suggested that adaptation to tolerate PCBs has altered the sensitivity of *Fundulus heteroclitus* to oxidative stress during embryonic development, demonstrating a cost of the PCB resistance adaptation and [64] reported resistance of *Fundulus heteroclitus* from the Atlantic Wood Superfund site on the Elizabeth River to the acute toxicity and teratogenesis caused by polycyclic aromatic hydrocarbons (PAHs) and others.

Chronic ecotoxicological testing is subjected to determine whether the long-term toxicant exposure that is supposed to be present in the environment, can have a significant detrimental impact on ecosystems. The number of selected species that inhabit the ecosystem is tested to toxicant exposure. The threshold of chronic concentration complies with the reactions of the most sensitive species. In the practice, the following assumptions are made to the chronic toxicity determination: selected individuals are with respective sensitivity to toxicant corresponding to the representative natural groups; chronic threshold concentration set for the most sensitive species, is the starting chronic toxicity for the ecosystem; studied species are the most sensitive to the toxicant in the ecosystem. The chronic tests provide information allowing extrapolation of the effects at the community and ecosystem level [1]. In the chronic tests, the bio-reactions upon exposure to toxicants for a long time are examined [65], often as long as the entire life cycle. After running the assay, the established initial concentration causing chronic sensitivity of the ecosystem is compared with the expected concentration of toxicant in the environment. Effect of severe chronic toxicity can be expected at concentrations exceeding the established in the environment initial or threshold concentrations. For the predicting of toxicants chronic effects, commonly three categories of tests are used: including life cycle [66], including the most sensitive life stages [67] and functional. By the lifecycle tests, the contaminant impact of chronic exposure on reproduction, growth, survival and other indicators of several generations of test organisms are examined. The tests begin with eggs, larvae or juveniles and continue until the reproduction of test organisms. The used toxicant concentrations range from causing strong negative impact to at least one that has no influence on studied indicators (compared to controls). Most commonly aquatic organisms that can complete their life cycle in laboratory are used—algae, invertebrates and others. In these tests, the calculation of survival and fertility by age is conducted according to equations of Lotka or Yuler [1]. The isolated generations in the period of youth and the period of maturity may be also used for these tests. The toxicity tests on the life cycle require considerable time and costs, especially for vertebrates. The tests conducted on the most sensitive life stages are used for studying the contaminants impact of chronic exposure to the survival and growth of eggs [68] and larvae [69] of fish. The indicative tests have been developed with the early – eggs floating on the water surface or eggs laid on the bottom of rivers or estuaries, embryos or larvae. In surface water micro-layer are concentrated heavy metals, detergents, chlorinated oil hydrocarbons, etc. The sediments also accumulate a number of toxicants. The tests start with the exposure of groups of fertilized eggs or embryos through the system for supply of serial concentrations of tested toxicant. The range of concentrations should include substantial effects and lack of impact. The species inhabiting cold water, for example *Salmo gairdneri*, are usually exposed for a period of 330–570 days [1] while inhabitants of warm water, for example zebrafish, are exposed from 30 to 250 days [70]. The parameters of the measurement include survival, growth and teratogenesis. The benefits of testing embryos and larvae are: saving time and money; creating opportunities for the study of larger number of species compared with the life cycle tests; calculated thresholds for chronic toxicity can be extrapolated to many more species for a wide range of areas and trophic levels than the potential in the implementation of testing lifecycle; the needs to conduct these tests due to the insufficient data on the fish toxicity. The concentration of toxicant that causes chronic toxicity effects on eggs, embryos and larvae vary among the same species and among different species. It depends on the duration of the conducted test—stage of the life cycle or the whole life cycle. For example, the studies with small *Salmo gairdneri* fish has the highest degree of sensitivity to six toxicants, while the eggs are relatively resistant, because of the bio–absorption alteration. The early life-staged tests are not considered as valid, if mortality in the control sample is greater than 30% [1]. Some authors published results of conducting tests with eggs [71], fish embryos [28], larvae [72] and early stages of development [24], as through them the potential toxic effect is reflected.

By the *functional tests*, the effects of toxicants on various physiological functions of test objects are studied. The fishes and other aquatic organisms react physiologically and with behavioural changes on toxic exposures. For example, changes are observed in blood chemistry [73], enzymatic activity [74], histology [75], swimming behaviour [76], sensory perception and disease resistance. This testing has some disadvantages: the effect of adaptation to toxicant is absent and the reported effects differ from the real ones; the inability to capture all variation in functional parameter for MBS; the inability to extrapolate the results to MBS. In general, the information is about the relationship between functional individual bio-effects to toxicants and the survival, growth and reproductive capacity of the populations in community. The data for the discussed test categories are used to determine the threshold concentration of the toxicant causing chronic toxicity.

Bioaccumulation tests are toxicity tests that can be used for hydrophobic chemicals that may accumulate in the fatty tissue of organisms. The toxicants with low water solubility generally can be stored in the fatty tissue due to the high lipid content in this tissue. The storage of these toxicants within the organism may lead to cumulative toxicity. Some authors report results from bioaccumulation in different tissues and organs like liver, kidney, gills, embryo tissue and accessory glands [60, 77]. Bioaccumulation tests use bio concentration factors (BCF) to predict concentrations of hydrophobic contaminants in organisms [78] and others. The BCF is the ratio of the average concentration of test chemical accumulated in the tissue of the test organism (under steady state conditions) to the average measured concentration in the water.

Tests with sediments. At some point, most chemicals and elements originating from both anthropogenic [79] and natural sources [80] accumulate in sediments. For this reason, sediment toxicity can play a major role in the adverse biological effects seen in aquatic organisms, especially those inhabiting benthic habitats [81]. A recommended approach for sediment testing is to apply the Sediment Quality Triad (SQT), which involves simultaneously examining sediment chemistry, toxicity, and field alterations so that more complete information can be gathered [82]. Collection, handling and storage of sediment can have an effect on bioavailability and for this reason standard methods have been developed to suit this purpose [83]. Some ecotoxicological tests for assessing sediments quality are published [84]. The worms [42], clams [85], fish [86] and phytoplankton [87] are mostly used as test objects.

2.2. Mono–species tests and multi–species tests

For the period concerned, the studies of acute toxicity clearly prevailed over the chronic tests. In a number of studies to clarify the actual toxicity the series of both tests have also been performed. The practical application of subacute and sub-chronic tests is low or negligible (**Figure 6**), but the noticeable trend of increasing the interest for these tests in 2014 and 2015 is observed (**Figure 7**). The analyses carried show that in 2012, 2014 and 2015, the tests with two species as test objects and multi-species ones were applied in most published studies. In 2013, the focus is mainly on tests with communities and multi-species ones.

Mono-species tests are appropriate in determining the toxicological effects on individual characteristics of species such as mortality [88], growth [89], reproductive capacity [41], behaviour [38] and other but have limited significance for the consequences on the entire ecosystem from the pollutants impact [36]. The disadvantages of mono-species testing are: the responses of individuals are often not sufficient to extrapolate responses of other (even very close) species and determine the real toxical effects in nature; the identifying of sensitive species or groups to the toxicant is expensive; the influence of indirect effects from intra-population and inter-population relationships on toxicity cannot be observed; the standardized laboratory conditions in conducting mono-species tests are different from the conditions in the biotope [90]. The influence of many additional abiotic and biotic factors is always present in the field effects changing significantly the eco–toxicological response. The mono–species tests have been used for years for the simulation of multi-species effects in ecosystem although the existing inadequacy. Therefore, the results obtained in mono-species tests are more often used in practice and have the same rank of importance to those of multispecies tests enable to assess indirect effects, such as the changes of structure and the functioning of ecosystem (changing in competition, predation, energy flow and circulation of substances, etc.).

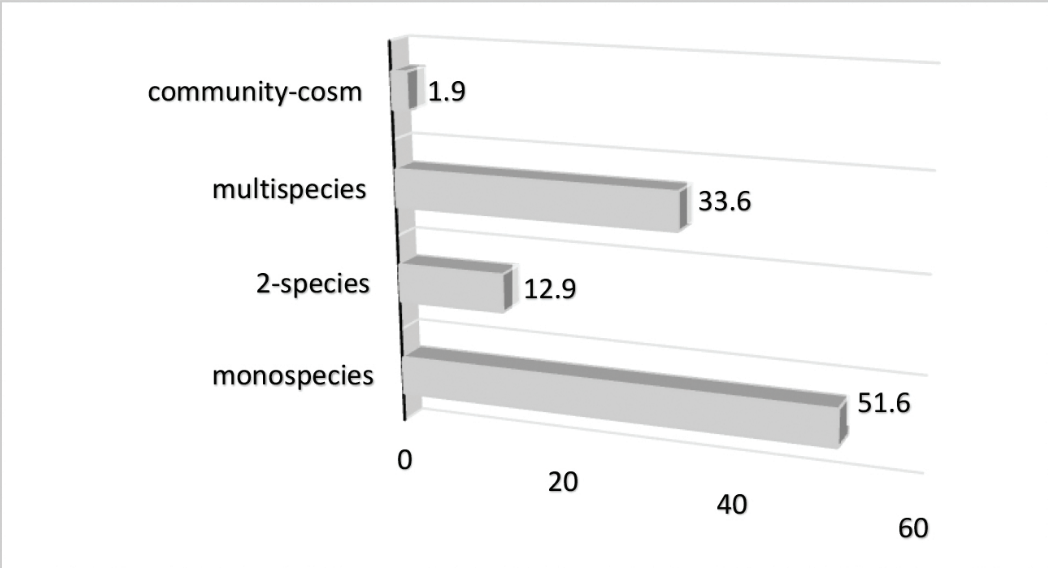

Figure 7. Percentage participation of tests with different number of test objects used in random sample (2010–2016).

Multi-species tests. At every level of biological organization appear new features that could not be recognized even by the most intense and careful studies of low levels. The complexity of MBS makes unacceptable the assumption that the responses received in toxicity tests of various species are applicable for predicting the behaviour of system of integrated species [91]. Therefore, the biological effects within the natural system differ considerably from those in laboratory tests [1]. Only acute toxic effects can be accounted with available methods. Another way to resolve the testing problems is by the identification of sensitive or/and key species or groups of species for MBS, but it is very expensive approach. We also need to bear in mind that in multispecies tests many answers are skipped due to unification of indicators, individuality of responses and statistical unreliability of additional indicators. So, we are faced with the inability to reproduce the experimental results to MBS level due to the variability and specifics of relationships, self-organization and self-regulation of different MBS. In other words, the uniqueness of each MBS leads to the inability for the toxicant assessment unification. The toxic effects extrapolation to ecosystem level requires good description of the biotope conditions and availability of data on the structure and functional processes. The lack of classification and characterization of ecosystem types in relation to specific environmental factors and bio-system features hampers the ecotoxicological research interpretation. For example, the population generally shows a lower sensitivity to pollutants than the individuals do. It seems that environmentally more realistic approach includes monitoring the effects of exposure to toxic impact in natural ecosystems or man-made systems specially designed to resemble fully the characteristics of natural systems (multi-species tests with the key representatives of studied ecosystem – model ecosystems, microcosm, mesocosm, lake-coral systems and others). The most optimal combination of test objects for aquatic ecosystems

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includes species of algae, crustaceans and fish, reflecting their functional structure. The micro-ecosystem is not an absolute analogue of the natural ecosystem, but a small system with sufficient complexity that could allow realistic ecological study of the typical characteristics of ecosystem in nature. Their application to environmental toxicology has been of interest, where fate and behaviour of contaminants markedly modify the exposure of biota to them and hence the environmental hazard [1]. In the aquatic micro-cosmology, there are two major achievements even in the last century: the ecosystem model and the food chain model. The ecosystem model is used to study the inherent ecosystem properties and functions in the level of integration—cycling of substances, energy flow and homeostasis. The food chains model is applied in studying the relationships predator-prey, environmental efficiency in the transmission of energy in trophic levels, etc. The experiments with food chains model is simpler and easier to manage compared to experiments with ecosystem model. The main problem is in selecting the appropriate microcosm types for different studies. Many species tests include a set of standard reproductive tests on daphnia, where the dose–response relationship is determined depending on the supply of nutrients [1]; large – (CEPEX, 1300 m^3); medium – (10–150 m^3) and small-sized (1.4 m^3) bags or tanks is used for the isolation of communities living in the open sea. Usually the larger the system is, the longer it will operate and the environmental conditions, community structure and functioning will look more like natural. The artificial macro-bio-systems differ by the nature of communities; by the period of submission of the matter—long and short duration, also by using materially closed systems. The classical example is Taub test [1]. He performed to some degree a standardized toxicity test with 24 identical 3.6 1 containers that are "infected" with a total of 10 algal species, five animal species and unknown set of bacteria. The duration of the experiment was 60 days due to deterioration of the community in the absence of normal biological cycle. In Kersting classical test [1], the lack of mineral circle was overcome. He has developed a microcosm with 151 Compartments, in which primary production, secondary production and decomposition are separated to prevent overconsumption. The balance between production and decomposition in this type of microcosm proves its sustainability for months or even years in relatively stable conditions, which corresponds to the "ecological temporal periods". These types of models that are designed to resemble certain types of natural systems (isomorphic models) usually include sedimentary part and benthic invertebrates and operate under watercourse conditions. In most cases, it is expected the water, sediments and biota to be modelled from the site. There are designed different tanks in size (560 1 or 13 m^3) with a variable depth to external structures that are best described as model flows, model channels and model lakes of various sizes. These test systems can combine the evaluation of biological effects with studies on transformation of pollutants in the environment. Test systems modelling the changes of interactions between populations under the chemical stress were also developed in the last century. The tests of Cairns and Lundgren [1], studying the interactions of algae and daphnia populations under chemical stress, are well known [1]. The usage of a battery of bioassays involving different species at different trophic levels is an efficient and essential tool for predicting environmental hazards to the aquatic ecosystem. For example [85], the adverse effects of wastewater treatment plants (WWTPs) on sediment quality at the Bay of Cádiz (SW, Spain) were investigated using a battery of acute bioassays and chemical contamination. The author concluded that the test may provide complementary information for diagnose of environmental factors that can impair aquatic communities. In other study [92], a battery of marine and freshwater species representing different trophic levels was used, and compared the bioassay of sensitivity levels to pharmaceutical residues of three antidepressants (fluoxetine, sertraline and clomipramine). The bioassays like the algal growth inhibition test (*Skeletonema marinoi* and *Pseudokirchneriella subcapitata*), the micro-crustacean immobilization test (*Artemia salina* and *Daphnia magna*), development and adult survival tests on *Hydra attenuata*, embryotoxicity and metamorphosis tests on *Crassostrea gigas*, and in vitro assays on primary cultures of *Haliotis tuberculata* hemocytes were selected. The importance of test battery usage showing the difference in sensitivity between bioassays hence high interspecies variability in EC50–values was underlined. The battery of bioassays and representative aquatic organisms (*Vibrio fischeri,* microalgae *Pseudokirchneriella subcapitata* and invertebrate *Daphnia magna*) for assessing the acute toxicity of water-extractable fractions of biochar-amended soil was published [93]. Based on the obtained results, the authors suggested that the battery of rapid and cost-effective aquatic bioassays that account for ecological representation can complement analytical characterization of biochar-amended soils and risk assessment approaches for surface and groundwater protection. The battery of bioassays was used [94] to assess the impact along a river due to a leak of effluent from an Installation of Cleansing and Uranium Recovery (Tricastin, France) and provided an estimation of exposure conditions that occurred along the river. The acute toxicity of the effluent was evaluated and compared to the toxicity of uranium nitrate in bioassays using *Chlamydomonas reinhardtii*, *Daphnia magna*, *Chironomus riparius* and *Danio rerio*.

The dysfunction occurring in plant communities and its effects on the plant populations structure and functioning is very well studied, especially for systems with poor species composition and simple structure. The pioneer studies have been published [1]. The imbalance is defined as a sudden change of the resource base that led to a clearly visible response. The hierarchical series of responses to these impacts can be predicted. The responses of the plant may influence the plant populations—reproduction, density spatial structure, rate of growth, mortality, body age, genetic variations, interspecies connections, etc. The different responses of plant populations can lead to major change of plant community as species composition, species richness, distribution of included genera, succession processes, etc. At the ecosystem level, these changes affect the primary productivity, the intensity of respiration, the intensity of mineralization and other functional processes. These responses depend on the type, frequency, intensity, duration and heterogeneity of dysfunctions. In some cases, we can evaluate the different obtained effects to the intensity and combination of impacts in models of vegetation structure and dynamics changes. By the comparison between the responses of exposed plant communities and the responses of untreated ones that grow on compatible soil types under similar topography and climate, the imbalance can be evaluated. Further, it would be possible to find a correlation of the results from laboratory tests, such as root growth, the growth of algae in soils or soil extracts, with actual plant data. There are some attempts to create models and study the complex toxicity on terrestrial ecosystems, but they are mostly with cultural ecosystems. For example, a model toxicological investigation of cultural plant-soil complex treated with wastewater have been published [95, 96]. Today, the conducting ecotoxicological studies with model ecosystems are common practice in the aquatic toxicology.

While it can be considered that to some degree the problems associated with the study of the toxicological effects in the aquatic toxicology are resolved, this is not the case in terrestrial toxicology.

Tests with nanoparticles Nanoparticles represented by a group of toxicants as TiO_2 [97], ZnO [44], carbon nanomaterials (CNMs) [98], core-shell copper oxide [99], silver (AgNPs) [49] and others are tested in ecotoxicology. Mainly, tests with aquatic organisms are conducted like algae [97–99], plants and invertebrates [97].

Biomarkers The most used biomarkers is the activity of antioxidant enzymes like catalase (CAT) [100], superoxide dismutase (SOD) [101], glutathione S-transferase (GST) [102], glutathione peroxidase (GPX) [101] and glutathione reductase (GR) [101] or their gene expression. Many authors use Hsp70 (heat shock proteins) and their expression for determining the toxicity of a pollutant [102, 103]. When using fish as test object, the haematological parameters are often applied as biomarkers [104]. Behavioural biomarkers are applied for frog tadpoles [105], clams [106]; fish [31], cladocerans [107]. When using plants as test object, cell viability (mitochondrial activity) and plant physiology (chlorophyll) are used as biomarkers [108].

3. About the studies of ecosystem health (ecosystem diagnosis)

The evaluation methods for ecosystems health assessment are usually based either on *risk assessment* or on *bio assessment* [1]. Most studies attach importance of *risk assessment*, but it is appropriate when the effect caused by known toxicant from one or more known sources with relatively high emissions and expected acute effect. The risk assessment focuses on the chemical composition, the impact of environmental toxicity and laboratory data. The acute tests with test object definitions as well as the available kits usually are used. The data of plants and soil invertebrates can be used to study the response of the short exposure, especially when impacts were made at regular intervals. The upper layer of the soil (5 cm) and plants (root, leaf and stem) are collected for the acute toxicity testing. It is essential also to measure physiological parameters—respiration, photosynthesis, pigments as well as microbial communities indexes. The short response of soil microbial community is also suitable indicator for this review—for example, the intensity of soil respiration of exposed and unexposed soil. The studies about the ecosystem's risk assessment using GIS, aerospace technologies and calculation of State Vector also were published [109–113 and others].

Bio assessment is applied for the complex effects of mixed toxicants or for low non-specific toxicity similar to chronic effects. The assessment focuses on ecosystem characteristics, factors causing stress and their importance and usage of measurements and models for chronic effects assessment. Bio assessment includes micro- and macro-research to perform controlled tests for ecosystem under impacts. It is necessary to know the characteristics of studied ecosystems and principles of their self-management and self-control. The bio-assessment of ecosystem state is based on the results of tests series with "critical" ecosystem components for chemical, physical and biological effects on ecosystems. There have been published 44 different characteristics important for the bio-assessment of ecosystem state and the eight of them are identified as critical ones of varying importance for different ecosystems. These ecosystem characteristics are as follows: (1) The features of the habitat to maintain biodiversity and reproduction of organisms; (2) The phenotypic and genotypic diversity of organisms; (3) The length of food chain, supported by biotope; (4) The determining biological production level of active and stored nutrients in biotope; (5) The features of biological turn-over for maintaining the ecosystem existence; (6) The energy flux to maintain the trophic structure; (7) The set of feedbacks for self-regulation and (8) The capacity to regulate the toxic effects, including capacity for biological absorption, metabolism and decomposition of toxicant, linking with the anthropogenic influences buffering. The importance of every critical characteristic depends on the ecosystem geographical location (i.e. eco region) and whether the system is aquatic or terrestrial. Some authors published bio assessments for different ecosystems, by selection of a set of indexes for the noticed critical characteristics [114–117]. The complex of 44 characteristics, even the complex of eight of them, the variation in the relative importance of each of them for the different ecosystems and the lack of standardized indices and methods for each of them, makes the representative assessment unlikely at each case. The representative assessment of ecosystem can only be done with a few "standard" test systems types. Today the scientific community makes efforts to resolve these methodological problems, not only in relation to ecosystem diagnosis, but also to assess the ecosystem capacity, assets and services [118].

The need of test systems classification leads to the publication of a set of investigations in the last century. For example, the classification [1] is based on the following criteria: environment (air, water and soil); time of exposure (long, medium, occasionally, etc.); concentration of toxicant (mg/l, mg/m^3); used organisms (bacteria, fish, mammals, plants, etc.); type of exposure (through food, air, dermal, etc.); the effects on test objects (genetic, toxic, bio-accumulation, etc.); measuring methods; test type (common, standard, experienced, screening); requirements for variability, accuracy and precision of values and technical requirements for personnel and laboratory equipment. The developed protocols, however, require significant modifications depending on the type of ecosystem and environmental factors, the objectives of the study and more mentioned in the specific dynamic action analyses (SDA). To assess the possible effects on ecosystem level, the responses of dominant species are usually investigated on a set of sample plots. The mostly used indicators are grouped as indicators of plant community, plant chemistry (major cations, nitrogen, phosphorus, iron, zinc, etc.), aboveground and water insects (some populations of *Homeoptera* are extremely sensitive to changes in the chemical and species composition of communities), soil invertebrates, soil chemistry (indicators for mineralization potential of organic nitrogen and phosphorus, variable cations, pH, inorganic nitrogen, total nitrogen, organic and inorganic phosphorus), water chemistry, organic matter decomposition (the activity of heterotrophic microorganisms and species of class Arthropoda, etc. [119, 120 and others]. A lot of plant community indicators and indices are applied at ecosystem level as plant species composition and density ($number/m^2$); plant biomass, separated into herbaceous and woody, above- and underground, live and dead; the average height of the stems; specific leaf mass (SLM); stalk weight; total dry weight (W); total leaf area (Le), etc. The relative indexes are also calculated as: the relative growth [$Rw = (1/W)\cdot(dW/dT$]; relative leaf area [$Re = (1/La)\cdot(dLa/dT)$]; leaf ratio [$F = La/W$]; full leaf evaluation [$E = (1/La)\cdot(dW/dT)$] and others. Soil invertebrates as earthworms, spiders and nematodes are sufficiently

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sensitive to the quantity and quality of plant roots and plant detritus and can influence or be influenced by microbial populations. This group of consumers is identified as the potential regulator of the decomposition and production processes. The macro arthropods and earthworms are associated with the fragmentation of detritus, while the micro arthropods and nematodes are more important for the micro-organisms populations. Earthworms are a standard system for the bio assessment. The soil nematodes and the species of *Scarabaeidae* family are linked to net primary production and are sensitive to changes in plant chemistry. The number and trophic composition of soil invertebrates are measured together with the biomass of plant roots and rhizomes and dead biomass. The plant carbon distribution changes in stress are also a good indicator for toxicity. The data for a living and dead biomass in relation with the number and composition of soil invertebrates provides possibility for ecosystem state assessment. Usually the number of earthworms and large arthropods is measured in spring and autumn using soil samples of 0.1 m^2 and 30 cm depth. The micro arthropods and nematodes are measured by mechanical separation of soil samples.

For the forest ecosystem health assessment, the widespread indicator is defoliation that can do possible to calculate the ratio of damage of ecosystem: $C = [\sum(n \cdot k)/NK] \cdot 100$, where n is number of trees with respective scores of defoliation (first to fifth score); N—the total number of trees; K – maximum score on the scale. The forest ecosystems are considered to be damaged at $C > 30\%$ [121] Percentage of defoliation is determined by sight with guidance, where the habitus of crowns with different rates of defoliation for each tree species is given.

Dendro-chronological analysis for ecosystem health assessment. By the dendrochronological analysis, the impact of external factors (including contaminants) on the radial growth of stems may be determined. Depending on the change of these variables, the characteristic pattern of the tree ring series was formed. The pattern includes: successively alternating narrow and wide rings of lighter or darker wood; changes in the density of the tree rings; change in the ratio between early and late wood, changes in the chemical, cytological and histological characteristics of tree rings, etc. The year with changes in growth or with special annual ring, is named *different special year*. The different special years are very important in cross-dating and in identifying the age of trees, as well as the time of stressed events by climatic factors, pollution, disease, pests, etc. The samples are taken with Presler's driller on 1–1.5 m height of the stem and placed in special templates. They are measured by LINTAB™. Through the statistical processing of obtained series of values, the influence of considered stressors or bio assessment can be performed [121]. The main indicator for dendro-chronogical, especially dendrochemical analysis, is the growth index (It). It is the ratio between measured and calculated value for tree ring (Wt/Gt) by the best reflected to the course of tree stem growth trend ($R^2 > 0.45$). Thus, the influence of age on the growth is eliminated and the environmental information in the rings is enhanced. The analysis of content of chemical elements in annual rings and its dynamics can provide valuable information about changes in the environmental toxicity. There is information for over 70 chemical elements that can be absorbed from the soil by root system, also through the bark or caught by the leaves and moved to the xylem. Many authors have found that the vertical transport of nutrients thousand times exceeds the horizontal one, that is, radial migration of elements in annual rings is minimal, because they form the insoluble complexes [1, 3, 111 and others]. Therefore, the content of chemical elements in the wood gives a general picture of the environmental factors influenced on tree species for their lifetime. The chemical memory of annual rings also can be used to estimate the changes in the environmental toxicity. Recently, the set of dendrochronological indicators have been developed for the forest state assessments: number of eustress periods; their duration, frequency and depth; eustress years (unfavourable climatic type of years), reactive tree functional types and eustress-climatic predictive patterns [122–124]. The authors perceive eustress as a repeating state of restricted radial growth rate of tree stems within a period of one or multiple years and caused by unfavourable environmental factors. This state encompasses the numerous other reactions of tree species. The level of radial stem growth (or tree ring width) is the main parameter that the developed holistic approach operates with, as well as the growth index, which is the main indicator for the statistical determination of low growth threshold (categorized as eustress). The study of the forest ecosystem state is based on the assessment of eustress depth (A) – $A = \frac{1}{s}\sum_{i=1}^{s}(1 - It_i)$, duration (D) – the number of adjacent eustress years, and frequency (F) – the number of stress years for a period of 100 years, and the creation of eustress nomenclature by five-graded scales. The performance evaluation of eustress in particular localities allows the expression of reactive functional type of tree species. For example, functional type F4D5A4 means that in particular locality the typical for trees of that species are frequent, very long and deep eustresses and that "forest behaviour" puts the existence of the forest under some risk. For the analysis of eustress based on the periods with limited growth, SP-PAM 2.0 software has been developed [125]. Thus, these analyses can be applied for the forest ecosystem state assessment.

Nowadays, *the kits* are widely used for short-, long-term and risk ecotoxicological assessment, as well as for ecosystem health assessment and for quality monitoring of water and wastewater, because they are rapid, sensitive and cost-effective way [126–128]; determining the impact of bio-toxins produced by blue-green algae [129–131], chemicals for mutagenicity [132], chemicals and wastes released in aquatic, terrestrial environments and sediments [127]. Different model organisms are used like algae, aquatic invertebrates, bacteria and plants.

4. Conclusion

New synthetic chemicals are recorded each year and the legislation in countries requires the immediate conduction of the both – toxicological and ecotoxicological testing. The scale of the potential ecological impacts on the environment and biota requires fast and accurate assessments of toxicological effects. The practical importance of ecotoxicology for the existence and functioning of the MBS is constantly growing. The toxicity may be different for different species in the ecosystem and for the same species in different ecosystems. Furthermore, toxicants do not only directly affect the biological system being evaluated, but may have an indirect negative effect on it, altering both abiotic and biotic parameters in the ecosystem. The various populations of the same species under different environmental conditions will respond differently to a given concentration of toxicant. In ecotoxicology practice, the number of species is used as

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test objects, and the results are extrapolated to all groups of organisms in the ecosystem. The variation in size, physiology, evolution, ontogeny and geographical distribution are some of the important parameters that usually do not fit exactly. However, some of the basic tests have demonstrated its great importance in the understanding of contaminants effects on the environment. The series of variables must be considered for the realistic assessment of environmental toxicity and MBS state. The reported sublethal effects often refer to changes in the structure of MBS that can lead to their degradation. A greater variation in the responses of individuals, populations and ecosystems observed in nature are compared with these reported under laboratory conditions, due to the mutual influence. This fact requires the more intensive usage of multi-testing systems—micro- and mesocosms and new developments. The analysis of situation and problems of ecotoxicological testing makes it possible to outline the directions in which to focus future efforts. They are related to the search of sensitive species for acute and risk testing, developing of new biomarkers and kits, especially for the study of terrestrial toxicity, formation of model systems (micro- and mesocosms) by key members of the ecosystem trophic network for multi-species testing and modelling the toxic effects at MBS level, which is especially true for the terrestrial ecotoxicology.

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