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Modern trends in the development of bioassay methodology of aquatic environments

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Bioassay is now an integral part of the "Effect-directed analysis" (EDA). We analyzed current research in the field of biodiagnostics and environmental monitoring programs, which used bioassay methods. The modern bioassay methodology is developing in the following areas: the development and implementation of new bioassay methods, the development of special bioassay devices, the detection of new informative test-functions based on the accounting of sublethal effects in laboratory organisms, the evaluation and interpretation of the results of toxicological analysis of environmental components. We propose three directions for evaluation and optimizing bioassay approaches and methods. First, we propose an algorithm for selecting protocols of bioassay. This algorithm is based on the ranking of sensitivity of bioassay methods to the most important pollutants in the territory of research. This approach will allow using only the most informative and sensitive bioassay protocols in the further researches. The second direction in optimization of bioassay methods is strict standardization of maintenance conditions of test-organisms. We recommend verification of the influence of abiotic and biotic factors on the test culture during the entire life cycle of individuals of a biological species. Life expectancy and ability of individuals to reproduce are universal criteria of health for many animals. The third part of our work is the development of a system of test-functions for laboratory animals consistently evaluated during a toxicological experiment. This approach allows taking into account the different effects (lethal, sublethal, chronic and delayed) in the process of testing various substances or aquatic environments. We tested this system of bioassay using *Daphnia magna*. The system of test-functions includes 14 response effects, which we took into account in three generations of crustaceans. The earliest responses of *D. magna* are estimated from changes in motor activity and trophic activity of crustaceans. Delayed effects are diagnosed by changes in fertility in the F_2 and F_3 generations, as well as the emergence of abortive eggs. Implementation of the proposed directions of bioassay optimization will allow taking into account the multiplicity of obtaining objective results of ecotoxicological analyses. Researchers can consistently use three parts of evaluation and optimizing of bioassay approaches at the planning stage of environmental studies and continue to implement them in the research process.

Keywords: bioassay, bioassay methodology, methods of bioassay, test-function, laboratory test-organism.

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Современные тенденции развития методологии биотестирования водных сред

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При анализе современных исследований в области биотестирования и работ, представленных в международных наукометрических базах данных, выделены направления развития методологии биотестирования. Наиболее актуальны работы по созданию новых методик биотестирования, разработка специализированного оборудования для биотестирования, повышающего точность и объективность исследований, поиск новых тест-функций «классических» тест-организмов, из числа которых наиболее востребованы оценки предлетальных эффектов. Предложены три блока оценки и оптимизации подходов и методов биотестирования. В первую очередь, при планировании экологических исследований необходимо обоснованно выбирать перечень биотестов. Для этого разработан алгоритм выбора биотестов, основанный на ранжировании чувствительности набора методик биотестирования к приоритетным загрязняющим веществам района исследования. Этот подход позволит использовать только наиболее информативные и чувствительные биотесты. Вторым блоком оптимизации подходов и методов биотестирования должна стать строгая стандартизация условий культивирования выбранных тест-организмов. На примере *Daphnia magna* сформулированы научно обоснованные рекомендации по их содержанию и периодическому контролю здоровья тест-культуры. Завершающим направлением оптимизации подходов биотестирования и развития методологии группы методов является предложенная система последовательного учета спектра тест-функций базового тест-организма

D. magna. Оценка 14 доступных для учёта тест-функций *D. magna* приводит к диагностике летальных, нелетальных, хронических и отсроченных эффектов. Реализация предложенных направлений оптимизации биотестирования позволит учесть многофакторность получения объективных результатов экотоксикологических анализов.

Ключевые слова: биотестирование, методология биотестирования, методы биотестирования, тест-функции, тест-организмы.

The principle of environmental effect-directed analysis includes justification of all stages of monitoring: from sampling and preparation of samples to a combination of chemical research methods with bioassay of the environmental components [1]. At the stage of bioassay, a "battery of bioassays" is formed, which guarantees the assessment of responses of organisms belonging to different systematic groups [2–4]. The choice of bioassays should take into account "the contradiction between the need for express toxicity assessment and the environmental reliability of these assessments" [5]. This problem is solved by tests for accounting of pre-lethal effects: FET-test (fish embryo toxicity) on embryotoxicity [6, 7], bioluminescent tests, for example, "bioluminescent nanopaper" [8], as well as various methods of genotoxicity and mutagenicity of aquatic environments assessment [9, 10].

The analysis of scientific works for the period of 2013–2017 on the basis of "Web of Science Core Collection" database demonstrates the relevance of the issues of the development and application of bioassay methods in the practice of scientific research (Fig. 1).

The total number of responses to the query "bioassay" was 55805. It should be noted that researches from other natural sciences using methods of studying the organisms' reactions to a variety of environmental factors are entered this collection, in addition to results of environmental bioassays.

Thanks to such analysis of bases of scientific publications, methodical works, materials of specialized conferences the following modern directions of formation of bioassay methodology were defined.

1. Accumulation of the Bank of bioassay methods. Although scientists know hundreds of bioassay methods, this positive trend has its drawbacks. The variety of methods raises for researchers a question of their choice in the context of specific practical tasks. At the same time many developers propose bioassays using organisms that are not always available to a wide range of researchers and users of monitoring systems [10, 11].

2. Development of available specialized bioassay equipment for test automation. At the same time, you should not neglect reliable visual methods as new devices are expensive and have to undergo a complicated procedure of inclusion

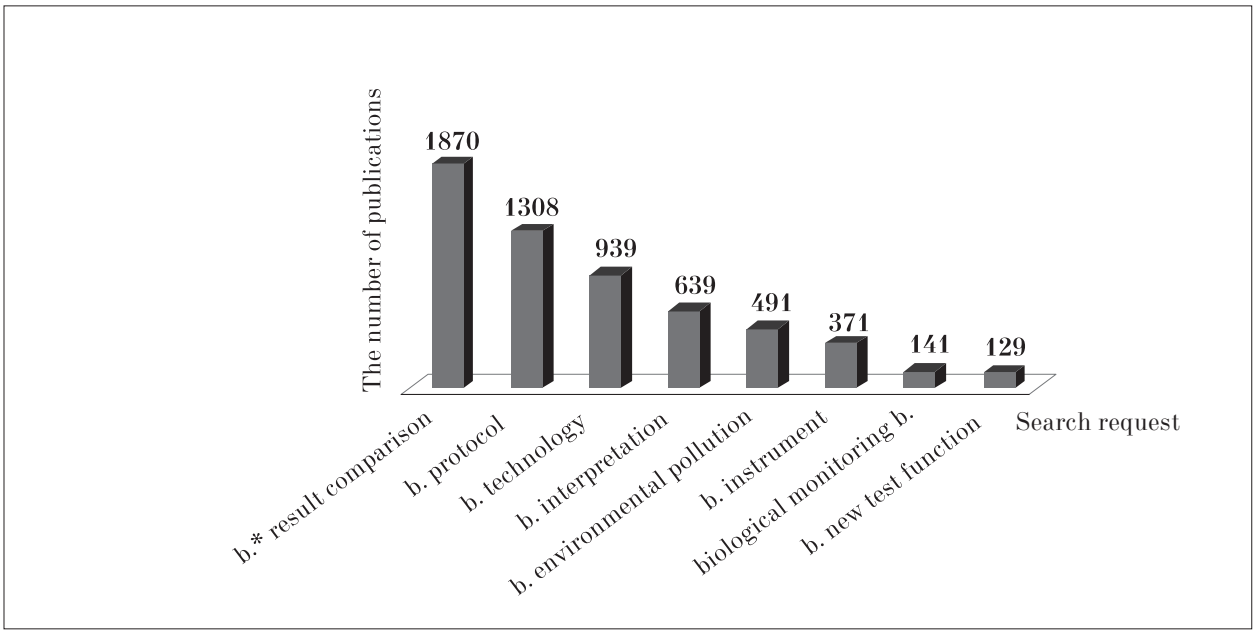

Fig. 1. The ratio of the number of publications on the topic "bioassay"*
Note: b. – bioassay*

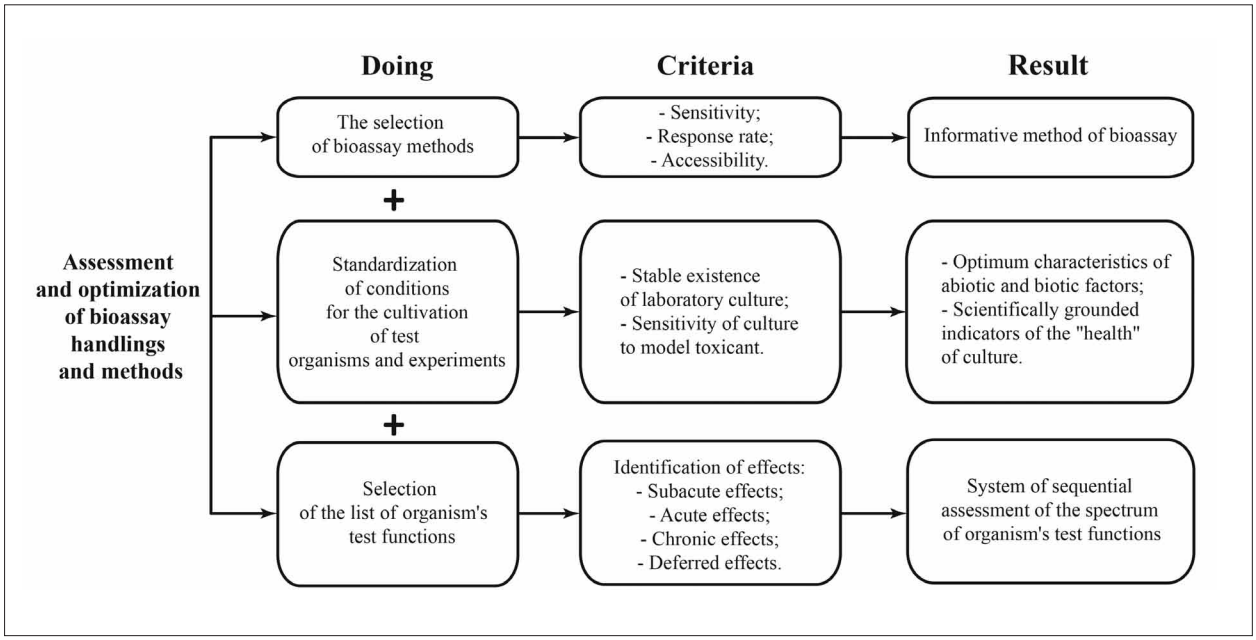

Fig. 2. The flowchart of directions of assessment and optimization of bioassay approaches and methods

into the Russian Federal register of equipment (recommended).

3. Searching for new test-functions of "classic" test-organisms. Now assessment of genetic disorders of test organisms is at the peak of popularity among developers of bioassays. However, it is difficult to introduce such bioassay methods in mass practice as they are expensive and also demand highly qualified specialists.

4. Assessment and interpretation of results of the toxicological analysis. A reference point in this area are works in which the role of organic matter in formation of stability of aquatic ecosystems at chemical influence is proved, the positive effect of bioaccumulation processes for water detoxification is shown, the effects of labile and non-labile forms of toxicants are described [12–14].

Assessment and optimization of bioassay approaches and methods for obtaining reliable and objective results in multi-factor conditions for the implementation of toxicological analysis methods became the purpose of our work.

Materials and methods

The article presents the generalized results of several our studies aimed at identifying the features and problems of bioassay methods, correction of conditions of toxicological experiments, obtaining reliable bioassay's results. Details of separate methods are disclosed in the "Results and discussion" section.

The database of toxicological analyses for selection of the directions of optimization of bioassay's approaches and methods was obtained on the basis of the accredited research eco-analytical laboratory of Vyatka State University. Approbation of the proposed approaches was carried out on the natural waters selected in the environment, as well as on natural waters with added simulated pollution.

Results and discussion

We propose some directions of assessment and optimization of bioassay approaches and methods reflected in the following flowchart (Fig. 2).

This scheme includes three blocks, each of which is represented by the directions, criteria and the result of optimization and assessment of selected research bioassay's approaches and methods.

The implementation of this flowchart is recommended to be started from **the selection of bioassay methods**. The following selection algorithm should be based on the ranging of the bioassays' sensitivity (i.e. certain bioassay methods) to priority pollutants.

1. Establishing non-lethal and lethal doses of the test substance on the basic bioassay. We propose to use the biotest on mortality of *Daphnia magna* Straus as one of the most common in the world bioassay practice. Definition of the average lethal concentration of toxicants at the

same time is not obligatory if it is not a research problem. Such approach considerably reduces the workload.

The results of published scientific works can be a reference point for the choice of a series of tested concentrations, if the toxicant was previously subject to studying by other bioassays, as well as the actual content of the interesting substance in the studied environment is known. Otherwise, it is necessary to be guided by operating doses of homologous substances (for organic compounds) or the most close substances on genesis (for inorganic). The planned testing doses should be added to surface or subterranean natural water, peculiar to the region of potential pollution; that will allow obtaining the data adequate to the natural waters' chemical background. The use of distilled water is not recommended.

2. Conducting bioassay by all available bioassay methods within the carried-out work. The responses to model aquatic environments with additives of non-lethal and lethal doses established for *D. magna* in the basic bioassay are estimated. If necessary for catching of differences in sensitivity of bioassay's examinees, effects of padding doses of priority substances are estimated. The control environment used for model operation remains the same, i. e. initially matched one.

3. Comparison of the received results to determination of bioassay sensitivity level and their distribution in a range of sensitivity. First of all it is recommended to be guided by weight percentages of the studied substance, but not by multiplicity of the regulatory values (MPC, UDC, etc.) if they are available. Standards should be used for further orientation in degree of pollution and a response of test-organisms to it. This condition will eliminate the difficulties and possible discrepancies caused by different values of MPC for the waters of particular appointment and soils different on a typology and properties.

4. Establishing of chronic toxic effects on *D. magna* and/or *Ceriodaphnia affinis*. The procedure is not mandatory and can be used to distinguish the close sensitivity of the two species of lower crustaceans or for immediate assessment of chronic toxic effect of the studied substance if it is included in research problems.

This algorithm is approved on several substances with obtaining sensitivity ranges that allows choosing the optimal bioassay methods. The use of such approach before large-scale monitoring researches will allow you to save funds without decrease in objectivity and reliability of the obtained decisions.

The second block of the scheme includes **standardization of cultivation conditions for the test-organisms**. Due to its specific features each test-culture requires the development of the reference habitat conditions allowing to support its health for a long time. The health of test-organisms' culture is understood as its ability to exist for a long time as model population with a stable life expectancy of individuals and preservation of self-reproduction ability, provided that the optimal abiotic and biotic factors of its existence are created. To consider the problem of standardization of cultivation conditions for test-organisms and their health monitoring synchronized groups of *D. magna* were selected from the entire list of laboratory cultures as an experimental model, with the help of which the basic bioassay was previously proposed.

Despite the centuries-old history of cultivation, the conditions of keeping of these crustaceans and carrying out experiments are not unified. In particular, the following recommendations on *D. magna* planting density occur in official bioassay protocols of different countries: 10 individuals per 1 liter of cultivated water [15], 20 individuals per 1.6 liter [16], 20–25 individuals per liter of water [17, 18]. At the same time, according to environmental laws, it is known [19] that mortality and fertility (two main test functions of *D. magna*) as well as biomass accumulation, food behavior and some other parameters depend on population density.

The study of the influence of *D. magna* model population density on fertility and natural mortality, as well as the influence of cultivation temperature on these characteristics were described in detail in our work [20]. We submit the main recommendations on standardization of cultivation conditions and health monitoring of *D. magna* as the basic test-organism.

1. Life expectancy and reproduction ability are universal health criteria for many animals. For a more operational assessment of the state of *D. magna* culture, used in chronic experiments, we propose to observe "the day of the appearance of the first juveniles" index in each new synchronized *D. magna* culture. The deviation of this index from the average value of more than 5 days will indicate reproduction problems with test culture and a decrease of the fertility test-function validity.

2. The suitability of *D. magna* culture to the bioassay needs to be determined not only by sensitivity to a model toxicant, as this test estimates adequacy of the acute effects. When carrying out chronic experiments, it is proposed

Table

List of test functions used for the comprehensive assessment of the toxicity of aquatic environments

№ п/п	Test-functions	Method of evaluation	Special devices	Exposition
1	Mortality	Visual	No	96 hours
2	Motor activity	Visual	No	3–96 hours
3	Trophic activity	Instrument method	Spectrophotometer	5 days
4	Delay / stimulation of formation of brood chambers	Visual + microscopic method	Microscope	5–10 days
5	Reducing linear dimensions	Microscopic method	Microscope with micrometer	10 and 25 days
6	Body pigmentation	Visual	No	25 days
7	Delay in the hatching of the first offspring	Visual	No	7–12 days
8	«Quality» of the juveniles	Visual + microscopic method	Microscope with micrometer	25 days
9	Fertility	Visual	No	25 days
10	The proportion of abortive eggs from the number of the juveniles			Whole life cycle
11	Average lifetime of individuals			Whole life cycle
12	Mortality in the generations of F_2 and F_3			25 days of life of individuals F_2 and F_3
13	Fertility in the generations of F_2 and F_3			
14	Abortive eggs in generations F_2 and F_3			

to monitor the daily increase of mortality index in a specially created group of crustaceans once a year. This parameter is stable and equal to 1.1 with a variation of less than 10%. Significant deviations from this parameter will indicate the health problems of culture.

3. The optimal density of *D. magna* in the habitat should be 25 individuals per 1 liter. Low planting density leads to high fertility, which deplete the culture. The effect is similar to cultivation in warm conditions (25 °C) which reduces the life expectancy of crustaceans. High planting density causes a developmental delay of individuals even in conditions of optimum temperature and sufficient nutrition.

The proposed standard parameters of cultivation conditions and the state of the test-organism *D. magna* can be adapted for other cultures, using the leading principle: regular monitoring of vital parameters throughout the life cycle of organisms.

An important section of the assessment and optimization of bioassay approaches and methods is the third block of the proposed scheme, including **the determination of the spectrum of test functions of an organism.** According to [5], "experiments of 2–3 days duration seldom adequately characterize biological and ecological threat of specific pollution". Therefore, it is important to propose for users of bioassay methods convenient bioassay complexes for assessment both the test-functions of early diagnosis and delayed effects.

To solve this problem, 14 test functions of *D. magna*, manifested at different times of the experiment, depending on the toxicity of active substances, were combined into a uniform system. First of all, relatively easily diagnosed reactions of *D. magna* were chosen, which begin to appear from the first days of the experiment on sublethal doses of toxicants, that is, meet the criteria of accessibility, expressiveness and sensitivity (Table). Test functions based on changes in biochemical parameters of crustaceans, on the contrary, were not used because of the high laboriousness and the need for higher qualification of performers, as well as additional costs for materials and reagents.

The proposed list from 14 test-functions is available to accounting for most of employees of bioassay laboratories. The algorithms for determining most of these test functions are well-known or understandable, based on their name, and are also described in certified or

research techniques. We proposed and tested the modification of visual accounting of motor activity in [21].

Effects are diagnosed by test functions, sequentially manifested during the experiment, the developed scale reflects the time of manifestation of test-functions (Fig. 3). Depending on the established effects, the toxicity of the sample can be estimated not only qualitatively, but also quantitatively on many indexes as well as ranked by the intensity of their manifestation.

Bioassay according to this scheme during the whole life cycle of the conditional *D. magna* first generation and 25 days of life of the crustaceans' second and third generations allows tracing three main effects depending on contact duration of the tested aquatic environment and an organism according to S.A. Kutsenko's classification [22]:

– lethal effects manifested in acute intoxication which develops as a result of the action of substances for a limited period of time (typically up to several days);

– sublethal effects, manifested in subacute intoxication, which develops as a result of continuous action of the toxicant(s) for up to several tens of days;

– chronic and delayed effects, manifested in chronic intoxication, which develops as a result of the prolonged exposure to the toxicant(s).

Such approach will allow characterizing in details the investigated aquatic environments, new substances and materials, to predict their ecotoxicological effects in entrance into environment.

The presented three blocks of the considered scheme at consecutive joint realization will allow estimating and optimizing approaches and methods chosen for environmental research bioassay, as well as developing the most rational plan of environmental research or regular monitoring using ecotoxicological methods.

Conclusion

The basic development tendencies of group of bioassay methods are analyzed in this work. The scheme of assessment and optimization of bioassay approaches and methods, including three blocks of actions which differ in their application availability is developed on this basis.

The proposed algorithm for setting the bioassay sensitivity allows comparing not only different test organisms but also specific bioassay methods. As a result, the researcher is not required to select test functions of sensitive species and to determine the exposure and other parameters of the experience in the further. This action is directed to assessment of bioassay methods in the context of specific tasks of the planned or carried out researches.

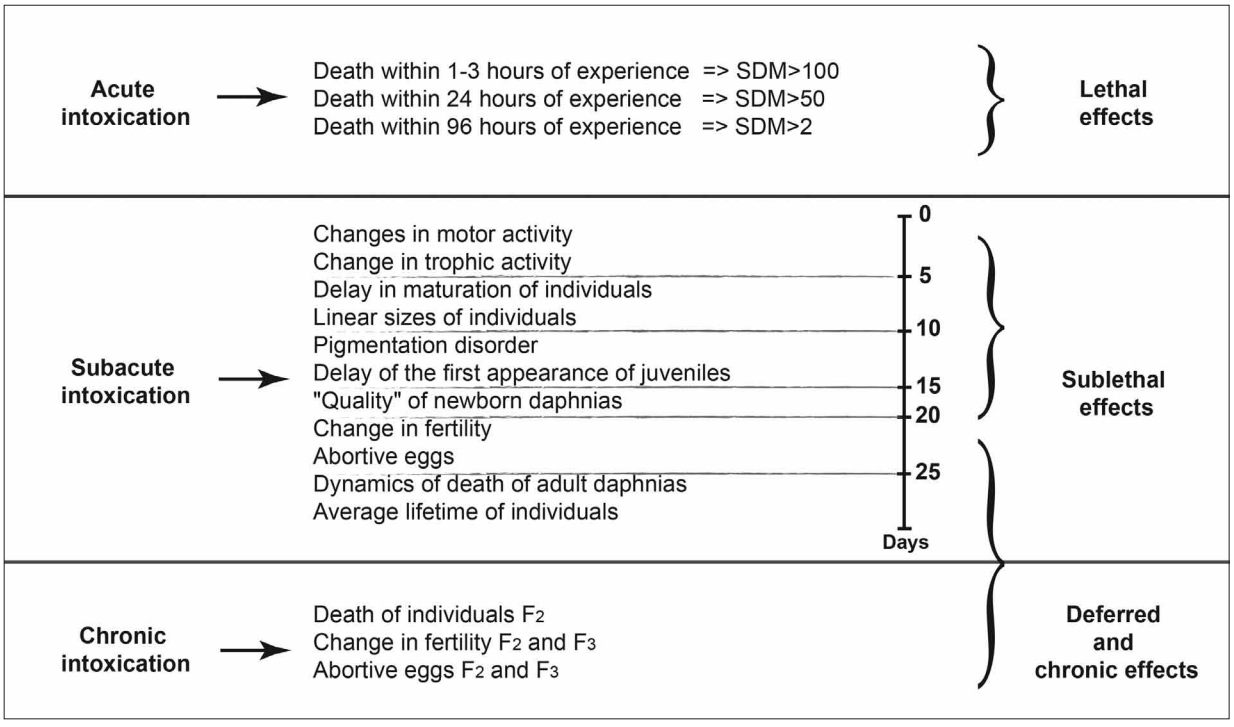

Fig. 3. System for sequential assessment of test-functions spectrum of *D. magna* (SDM – safe dilution multiplicity)

Further exact standardization of test organisms' cultivation conditions allows to set high quality criteria of test-cultures. The example of *D. magna* shows a list of regulated cultivation parameters and health monitoring of test-culture. The signs recommended for assessment of *D. magna* laboratory culture help to separate seasonal fluctuations of test organisms' condition from systematic deterioration in their health. The proposed standardization parameters of cultivation and health monitoring of test-culture can be adapted for the majority of other test-organisms.

As optimization of bioassay approaches profound studying of various responses of basic test-organism, but not increase in "the battery of bioassays" is proposed. At such approach both test-functions of early diagnostics, and delayed effects are estimated. The implementation of such approach is approved on the example of *D. magna* – a test-organism, basic in a number of parameters. The system of a sequential estimation of *D. magna* test-functions spectrum is developed. Its advantages include the possibility of widespread introduction into bioassay practice, ranging the degree of toxicity of the tested environments, assessment of the lethal, sublethal, chronic and delayed effects of the tested aquatic environments and substances.

Thus, the bioassay methodology continues to develop. Many modern developments complicate the algorithms of actions for the researchers. This is not always justified, and also it does not allow carrying out mass introduction of advanced designs. The proposed flowchart of assessment and optimization of bioassay approaches and methods is directed to the choice of the most informative methods for reduction of researcher's operating time, quality control of the used test-cultures for obtaining reliable results and is focused on the use of basic test-organisms with the most accessible test-functions.

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