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Bioassay standardization issues in freshwater ecosystem assessment: test cultures and test conditions

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Abstract – The use of bioassays for environment contamination assessment, in particular for aquatic ecosystems, has gained topicality and found extensive applications over the past decades. The methodologies are well established, but the standardization of bioassay conditions needs to be better defined in order to assure applicability as an efficient analytic tool and that results are ecologically relevant. This paper addresses the analysis of the current international situation and the specifics of Russian practice in culturing and use of test organisms for freshwater bioassays. Standardization of bioassays calls for the utilisation of pertinent sources of test cultures and the provision of appropriate cultivation conditions. The paper discusses and reviews recommendations for the selection of test species and their culturing in the context of established ecotoxicological targets and the assessment of sensitivity to various reference toxicants. The significance of the quality of water utilized for test cultures and sample dilution is highlighted. Strict water quality requirements and synthetic media options are analyzed and due consideration is given to temperature and illumination conditions. Toxkit microbiotests are discussed as an alternative to toxicity bioassays alleviating the need for continuous stock test culturing and maintenance.

Keywords: Toxicity tests / standardization / reference toxicants / culture and dilution water / test conditions / toxkit microbiotests

Résumé – Questions de normalisation des essais biologiques dans l'évaluation des écosystèmes d'eau douce : cultures expérimentales et conditions des tests. L'utilisation des essais biologiques pour l'évaluation de la contamination de l'environnement, en particulier pour les écosystèmes aquatiques, est devenue d'actualité et a trouvé de nombreuses applications au cours des dernières décennies. Les méthodologies sont bien établies, mais la normalisation des conditions d'essais biologiques doit être mieux définie afin d'assurer, et l'applicabilité en tant qu'outil d'analyse efficace, et que les résultats sont écologiquement pertinents. Le présent document traite de l'analyse de la situation internationale actuelle et des particularités de la pratique russe en matière de culture et d'utilisation d'organismes de test pour les essais biologiques en eau douce. La normalisation des essais biologiques exige l'utilisation de sources pertinentes de cultures expérimentales et la mise à disposition de conditions de culture appropriées. Le document discute et examine les recommandations pour la sélection des espèces de test et leur culture dans le contexte des cibles écotoxicologiques traditionnelles et l'évaluation de la sensibilité à divers produits toxiques de référence. L'importance de la qualité de l'eau utilisée pour les cultures expérimentales et la dilution des échantillons est soulignée. Les exigences strictes en matière de qualité de l'eau et les options en matière de fluides synthétiques sont analysées et les conditions de température et d'éclairage sont dÛment prises en compte. Les tests microbiologiques Toxkit sont considérés comme une alternative aux essais biologiques de toxicité, ce qui réduit la nécessité de cultiver et d'entretenir les animaux en continu.

Mots-clés : Tests de toxicité / normalisation / substances toxiques de référence / eau de culture et de dilution / conditions d'essai / tests microbiologiques toxkit

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1 Introduction: demand for test cultures for environmental testing

Biotesting for environmental samples is integral to environmental regulations in many countries, including Russia. Lab testing, involving application of 'short-term bioassays' gives results that complement bioindication research data and improve credibility of biodiagnostics of the ecological quality of natural and man-made environments. The drive for extensive application of bioassays in Russian environmental practice was propelled by *Criteria for Categorizing Hazardous Wastes by Classes of Environmental Hazard* (approved by the RF Ministry for Natural Resources (Order No. 511) enforced in 2001 (FR, 2001). Pursuant to this document, experimental toxicity bioassays based on response of hydrobionts, along with calculations of the concentration of certain toxic components determined by chemical methods, have to be performed for the waste hazard characterization.

A sufficiently broad range of freshwater toxicity bioassays have been devised and are to date used in global practice. The applicability of toxicity bioassays as efficient analytical tools should be supported by their standardization and validation (Keddy *et al.*, 1995; Slabbert and Venter, 1999; Wadhia and Thompson, 2007; Kokkali and van Delft, 2014). According to OECD guidelines (2004, 2006, etc.), principal bioassay aspects to be standardized are methods of preparation of stock and test solutions, details or composition of cultivation and dilution water, incubation conditions (temperature, light intensity and periodicity, dissolved oxygen, pH, etc.). Biological, technological and financial challenges primarily associated with the continuous cultivation and maintenance of test cultures with the desired sensitivity preclude extensive implementing of routine toxicity testing in practice. Well-developed research infrastructures are the key components both for the advancement of basic research and generation of state-of-the-art technologies and innovations. Russia does not however have a well-functioning system of supplying users with established and defined test cultures supported with reference or authentication data. Ongoing are vigorous discussions around the creation of national biobanks which over the past decades have been increasingly gaining significance for the standardization of biological sample treatment.

2 Diversity and quality of test cultures for bioassays

2.1 Diversity of test cultures

Test cultures and detailed procedures for their use are described in international standards or guidelines. The most widely applied standards have been produced by the Organization for Economic Cooperation and Development (OECD) and the International Organization for Standardization (ISO). Standard methods have also been produced by the American Standards for Testing and Materials (ASTM). In Canada, biological test methods are developed by the Environmental Science and Technology Centre and approved by the Environment and Climate Change, and marked as EC (environmental Canada). In the UK, standards are issued by the British Standards Institution (BSI). In Germany, the German Institute for Standardization (DIN - Deutsches Institut für Normung) is in charge of developing standards. The French Association for Standardization (AFNOR) is the French national organization for standardization, which is responsible for the development of standards and technical solutions for evaluation of environmental quality. Russia has a member body in ISO. The national member body that can represent Russia in ISO is the federal agency on technical regulating and metrology (GOST R). GOST R is the federal executive body, implementing inter-industry coordination and functional regulating in the fields of standardization, metrology and conformity assessment (https://www.iso.org/). In Russia, biotesting procedures are defined in state standards and methodologies and presented on two registers. The first is the federal register (FR) that is approved by the federal agency on technical regulation and metrology. The second is the register of environmental regulatory documents (ERD) that is approved by other of national standards body – the environmental protection committee. Table 1 lists the test species for freshwater bioassays covered by standards and guidelines including ISO, OECD and ASTM.

Russian standards have progressed to incorporate the utilisation of freshwater toxicity bioassays employing cells/organisms such as:

- germinal cells of mammals (cattle) (ERD, 2009);
- crustaceans – *Daphnia magna* and *Ceriodaphnia dubia* (ERD, 2007, 2014b; FR, 2007b);
- ciliate holotrichs – *Paramecium caudatum* (ERD, 2007, 2015);
- microalgae – *Pseudokirchneriella subcapatata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*) and *Chlorella vulgaris* (ERD, 2011, 2014a, 2014c; FR, 2007a);
- lyophilized bacteria culture – a genetically modified strain of *Escherichia coli* (ERD, 2010).

An important condition for using bioassays in toxicity assessment pertaining to natural environment is accurate identification of the test species. Many standards, especially those dealing with the use of macrophytes, freshwater algae and cyanobacteria, list "strains shown to be suitable for the test" and specify their sources and collections available for their purchase (OECD, 2006, 2011a; EC, 2007). In particular, algae strains usable for bioassays are ATCC 22662 or CCAP 278/4. Additionally, the standards set requirements for the characteristics of recommended species (appearance, size, cell volume, cell dry weight and growth rate). The description of a strain, source and method of collection, and culture conditions used is an integral part of the test report.

The need for biotesting and the value of the results for inference of quality assessment of natural and man-made environments are regulated by corresponding international and regional legal acts. In the UK, bioassays have been used in a number of statutory roles (Wadhia and Thompson, 2007): (a) in direct toxicity assessment (DTA) for effluent control; (b) in control of emissions under the water resources act and approval for the integrated pollution prevention and control (IPPC); (c) in identification of "special wastes" under the special waste regulations (SWR) under Section 2 of the environmental protection act 1990; and (d) in the UK national

Table 1. Test species for freshwater toxicity tests.

ISO	OECD	ASTM
Vertebrates: amphibians, fishes		
Danio rerio (formerly known as *Brachydanio rerio)* Hamilton-Buchanan (Teleostei, Cyprinidae)	*Oncorhynchus mykiss*; *Pimephales promelas; Danio rerio* (formerly known as *Brachydanio rerio); Oryzias latipes*	*Morone saxatilis;* Salmon, Trout, and Char; Northern pike; Fathead minnow; White sucker; Channel catfish; Bluegill; Gulf toadfish; Sheepshead minnow; Silversides
ISO 7346-1(2,3):1996; ISO 12890:1999; ISO 15088:2007	OECD 210; OECD 212 (+*C. carpio*); OECD 215 (- *P. promelas*)	E1241-05(2013); E1768-95(2013); E 1711–95 (2008)
		Fishes, macroinvertebrates and amphibians E 729–96 (2007)
Invertebrates: rotifers, worms, mollusks, crustaceans, insects		
Cladoceran crustaceans *Daphnia magna* Straus *Ceriodaphnia dubia*	*Daphnia magna Straus*	*Daphnia magna Straus*
ISO 6341:2012 ISO 10706:2000 *Ceriodaphnia dubia*	OECD 202; 211	E1295-01 (2013) E1193-97 (2012) *Ceriodaphnia dubia*
ISO 20665:2008 Rotifer *Brachionus calyciflorus*		E1768-95(2013) Rotifer *Brachionus calyciflorus*
ISO 20666:2008		E1440-91(2012)
Anostracan crustacean *Thamnocephalus platyurus*	Sediment-dwelling larvae of dipteran Chironomus sp.: *C. riparius*; *C. tentans*; *C. yoshimatsui*	Two test organisms, amphipod crustacean Hyalella azteca and midge Chironomus dilutes (formerly known as C. tentans; Shobanov *et al.*, 1999)
ISO 14380:2011 Ostracod crustacean *Heterocypris incongruens*	OECD 219	E1706-05(2010) Benthic invertebrates (*e.g.* Diporeia spp. and *Lumbriculus variegates*)
ISO 14371:2012 Nematode *Caenorhabditis elegans*		E1688-10 Bivalve freshwater mollusks belonging to the family Unionidae or Margaritiferidae
ISO 10872:2010		E2455-06(2013)
Plants: algae, higher plants		
Pseudokirchneriella subcapitata (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*); Scenedesmus subspicatus Chodat.	*Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*); *Desmodesmus subspicatus*; *Navicula pelliculosa*; anabaena flos-aquae *Synechococcus leopoliensis*	*Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*)
ISO 8692:2012 *Lemna minor*	OECD 201 Plants of the genus *Lemna* (duckweed): *L. gibba; L. minor; L. aequinoctialis; L. major; L. paucicostata; L. perpusilla; L. trisulca; L. valdiviana*	D3978-04(2012) *Lemna gibba G3*

Table 1. (continued).

ISO	OECD	ASTM
ISO 20079:2005 *Myriophyllum aquaticum* ISO 16191:2013 *Spirodela polyrhiza* ISO 20227: 2017	OECD 221	E1415-91(2012) Certain species of freshwater and saltwater microalgae E1218-04(2012) Macrophytes E1841-04(2012)
Bacteria *Pseudomonas putida* ISO 10712:1995 *Aliivibrio fischeri* ISO 11348-1:2007 *Salmonella typhimurium* (TA 100 and TA 98; TA1535/ pSK1002) ISO 11350:2012 ISO 11350:2012 ISO 16240:2005		A multi-trophic level freshwater community E1366-11

marine monitoring programme (NMMP). In Canada, in compliance with the Canadian environmental protection act, 1999, all substances on Canada's domestic substances list (DSL) must be categorized. Categorization involves evaluation of the substances on the basis of their persistence, bioaccumulation, and inherent toxicity. Categorization of inherent toxicity is based on a criterion of 1 mg/L for acute LC50 (EC50) values. This numerical cut-off is in agreement with some well-recognized international initiatives such as the organization for economic cooperation (EC, 2003). In Russia, bioassay results are used for waste hazard assessment that is statutory by the RF Ministry for Natural Resources Order No. 536 dated December 4, 2014 (FR, 2014). On approval of the criteria for categorizing wastes by classes of hazard I–V on the basis of the adverse environmental impact level. To characterize hazardous waste an experimental toxicity bioassay based on response of hydrobionts is performed, along with calculations of certain toxic component concentrations determined by chemical methods.

Given such diversity of bioassays test cultures it is necessary to select one that is fit-for-purpose depending on the objectives of the toxicity testing and nature of the test material. The most pertinent and prudent choice of tests should be determined by the relevance of the factors associated with the investigative operation (Wadhia and Thompson, 2007). Each ISO standard precisely defines the application scenarios it applies to and those it does not apply to, or is applicable under certain conditions. For example, "ISO 14380:2011 is not applicable to the testing of unstable chemicals (hydrolyzing, absorbing, etc.) in water unless exposure concentration is measured...", ASTM E 1850–04 describes a standard guide for selection of resident species as test organisms for aquatic and sediment toxicity tests. This standard is as the title suggests designed for guidance in selection of resident species to be used as test organisms in aquatic and sediment toxicity tests and is not exclusively for testing particular chemical types. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA, 2013) provides a list of mandatory toxicity tests for effect assessment of various pesticides having different modes of action. For acute effect assessment of pesticides with an insecticidal mode of action, with a herbicidal mode of action and for other pesticides, it is necessary to carry out toxicity tests with *Daphnia* sp. (*D. magna* preferred) and *Oncorhynchus mykiss*. In the case of pesticides with an insecticidal mode of action, additional toxicity tests with another arthropod (*e.g*. *Chironomus* sp. or *Americamysis bahia*) are advisable. For chronic effect assessment of different pesticides, a broader set of toxicity tests has to be performed: *Daphnia* sp., *Chironomus* sp. or *Lumbriculus* sp., green alga (*e.g. P. subcapitata*) and additional non-green alga (*e.g*. diatom *Navicula pelliculosa*), *Lemna* sp. or *Myriophyllum* sp. or *Glyceria maxima* (for pesticides with a herbicidal mode of action).

Evidence of the successful use of a battery of bioassays with different species pertaining to different trophic levels to understand the toxicological profile and to evaluate toxicity of environmental samples and toxicants has been provided in many studies (Doherty *et al.*, 1999; Castillo *et al.*, 2000; Wadhia and Thompson, 2007; Khangarot and Das, 2009). "No single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection." (EC, 2007). Back in 1998, a battery of six standard ecotoxicity tests was proposed by the French ministry of environment in order to assess hazardous wastes (Slabbert and Venter, 1999). In Russia, it is recommended to use a combination of two bioassays based on responses of species belonging to different taxonomic groups and trophic levels.

2.2 Quality of test cultures

Standardization of toxicity tests, apart from other conditions, demands the evaluation of the sensitivity of test species to reference toxicants (García *et al.*, 2010). Toxicity tests using reference toxicants improve the understanding of certain toxicants' impact on aquatic organisms (Jorge and Moreira, 2005), which can be of help in the selection of the most suitable test organisms (test systems) for analyzing toxicity of particular substances. The use of the most sensitive test organisms (test systems) shows promise for elucidating better water quality information that is more protective of native species (Struewing *et al.*, 2015). In the standards (or guidelines) toxicity tests with reference toxicants are compulsory to assure that the test conditions are reliable, to assess the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision of results obtained by the laboratory for that chemical (OECD, 2004a, 2012a; EC, 2007).

The US EPA document (1994) proposes to use the following compounds as reference toxicants: sodium chloride NaCl, potassium chloride (KCl), cadmium chloride ($CdCl_3$), copper sulfate ($CuSO_4$), sodium dodecyl sulfate (SDS), and potassium dichromate ($K_2Cr_2O_7$). This list is however incomplete and, alongside with these, toxicologists also employ endosulfan, lindane, malathion, pentachlorophenol, ammonium chloride, chloroform, sodium lauryl sulfate, phenol, xylene, carbon tetrachloride, sodium fluoride, and ethyl alcohol (Snell *et al.*, 1991; Khangarot and Das, 2009). A wide range of reference toxicants contributes to a deeper insight into the test organism sensitivity to substances of various nature and action mechanisms. Meanwhile, it is desirable that a reference toxicant meets certain requirements: is easy to measure analytically, poses a minimal hazard to the use and the toxicity of these chemicals is not affected significantly by changes in water quality (EC, 2007). Among the most widely applicable are phenol, $ZnSO_4$ or $CuSO_4$, and $K_2Cr_2O_7$ (Tab. 2). Phenol as a component of dyes, polymers, drugs and other organic substances and a ubiquitous environmental pollutant is a widely used reference toxicant for many bioassays (Ricco *et al.*, 2004; EC, 2007; Michałowicz and Duda, 2007; Park *et al.*, 2012). Some tests require the use of an individual model toxicant. For the freshwater harpacticoid copepod *Attheyella crassa*, the authors (Turesson *et al.*, 2007) used the fungicide tebuconazole, which is classified as a reproduction toxic substance, as a reference substance.

The toxicity test procedure with reference toxicants suggests testing of the toxicant's sensitivity to serial dilution and evaluation of EC50 or LC50. Russian methodologies (FR, 2007a, 2007b; ERD, 2011, 2014a etc.) indicate a tolerance range for the LC50 (EC50) of a reference toxicant. If the experimentally determined LC50 (EC50) of the test culture falls within this range, it is supposed to be of desired quality and applicable for the bioassay. Other standards set forth a somewhat different procedure that includes a comparison of results for a reference toxicant with historical test results to identify whether they fall within an acceptable range of variability and plotting of a warning chart (EC, 1990, 2005, 2007). Successive ICps (IC50) are plotted on a warning chart and examined to determine whether the results are within ±2 standard deviations (= warning limits) or ±3 standard deviations (= control limits) of values obtained in previous tests using the same reference toxicant and test procedure. The results that do not fall within the acceptable range indicate a change in the test organism health or genetic sensitivity, a procedural inconsistency, or a combination of these factors.

3 Standardization of test conditions

It is impossible to obtain unified and high quality results of ecotoxicological studies without stringent standardization of test conditions. This review will further elaborate on cultivation and dilution water as well as incubation conditions.

3.1 Water for cultivation and dilution

The utmost importance of the quality of water used for test culture cultivation and sample dilution has been highlighted in many specialty documents and publications (US EPA, 1994; OECD, 2004a, 2011a, etc.) A few water types can be used for test culture cultivation and dilution: natural water (surface or groundwater), dechlorinated tap water or synthetic water (reconstituted water, artificial media, reagent water) (US EPA, 1994; ISO, 1996; OECD, 2004b, 2011a). The same water is advised for cultivation and dilution. The EC document (2007) recommends control/dilution water for various types of aqueous samples. For effluents, elutriates, leachates: reagent water or receiving water; for receiving water: reagent water or upstream water; for reference toxicants: reagent water; for chemicals: reagent water or receiving water.

The water quality can be assessed against the key indicators such as the salinity, hardness, pH, and dissolved oxygen concentration. Their recommended values vary a little in the corresponding documents of different countries, though generally they fall within the following ranges: alkalinity – 60–70 mg/L; pH – 7.0–8.2; oxygen ≥3–6 mg/L (>60% of the air saturation value) (EPA guidance for *Ceriodaphnia* tests, OECD, 2004a, 2011b, 2012a, 2012b; FR, 2007b). The recommended hardness values as $CaCO_3$ indicated in the documents show a greater differentiation: <400 mg/L (OECD, 2011b), 140–250 mg/L (OECD, 2004b, 2012b), 250 ± 25 mg/L (ISO, 6341), 80–100 mg/L (EPA guidance for Ceriodaphnia tests), and 80–250 mg/L. In addition, a need for monitoring of some other parameters is pinpointed, *e.g.* amounts of metals, organic carbon, and chlorine and chlorinated derivatives. In accordance with specific standards (APHA, 1992; US EPA, 1994; OECD, 2012b, 2013a) the Al, As, Cr, Co, Fe, Pb, Ni, and Zn content expressed as total metal should not exceed 1 μg/L each; the Cd, Hg, and Ag content expressed as total metal should not exceed 100 ng/L each. Furthermore, total organochlorine pesticides plus PCBs should be less than 50 ng/L. If dilution water is from a natural source, conductivity and total organic carbon (TOC should be <2 mg/L) or chemical oxygen demand (COD should be <25 ng/L) ought to be measured (OECD, 2004b, 2011b). If dechlorinated tap water is used, chlorine analysis is desirable (residual chlorine should be <10 mμ/L) (OECD, 2004b, 2011b). For zebrafish cultivation, Jin *et al.* (2009) used charcoal filtered water (under semi-static conditions). Special requirements are set for the natural

Table 2. Test species sensitivity to the most widely used reference toxicants.

Test cultures	Reference toxicants and LC		
	$K_2Cr_2O_7$	$CuSO_4$	Phenol
Cladoceran crustacean *Daphnia magna* Straus	48 h LC50 = 0.2–0.8 mg/L (response range) ERD*, 2014b	48 h LC50 = 54.6 μg/L 4 d LC50 = 34.2 μg/L Struewing *et al.*, 2015	48 h EC50 = 9.13 mg/L Lilius *et al.*, 1995
Cladoceran crustacean *Ceriodaphnia*	*Ceriodaphnia affinis* 24 h LC50 = 0.9–2.0 mg/L (response range) ERD, 2007	*Ceriodaphnia dubia* 48 h LC50 = 27.6 μg/L 7 d LC50 = 52.0 μg/L Struewing *et al.*, 2015	
Anostracan crustacean *Thamnocephalus platyurus*	24 h LC50 = 0.100 mg/L with 95% confidence limit of 0.052–0.148 mg/L International Interlaboratory Comparison, Protocol Toxkit		
Benthic ostracod crustacean *Heterocypris incongruens*		6d LC50 = 5.79 mg/L The results of a reference test with this compound should be in the range 2.21–9.37 mg/L International Interlaboratory Comparison, Protocol Toxkit	
Amphipod crustacean *Hyalella azteca*	7 d LC50 = 137 μgCr/L with 95% CL 106–176 (Cr was added in tap water as anion salt) Borgmann *et al.*, 2005	7 d LC50 = 90 μgCu/L with 95% CL 82–99 (Cu was added in tap water as atomic absorption standards) Borgmann *et al.*, 2005	
Rotifer *Brachionus calyciflorus*		24 h LC50 = 0.026 mg/L with 95% CL 0.022–0.030 Snell *et al.*, 1991	24 h LC50>150 mg/L Snell *et al.*, 1991
Green micro-algae *Selenastrum capricornutum*	72 h EbC50 (reduction of biomass) = 0.52 ± 0.21 mg/L 72 h ErC50 (reduction of growth rate) = 0.84 ± 0.27 mg/L International Interlaboratory Comparison (2006), Protocol Toxkit		
Green micro-algae *Chlorella vulgaris* Beijer	22 h EC50 (optical density) = 0.4–1.6 mg/L (response range) ERD, 2014a 22 h EC50 (delayed fluorescence) = 0.2–0.8 mg/L (response range) ERD, 2014c		
Ciliate protozoan *Paramecium caudatum Ehrenberg*	24 d LC50 = 0.55–0.89 g/L ERD, 2007	30 min EC50 (chemotoxic reaction) = 0.01–1.0 mg/L ERD, 2015	

Table 2. (continued).

Test cultures	Reference toxicants and LC		
	$K_2Cr_2O_7$	$CuSO_4$	Phenol
Macrophyte *Lemna paucicostata*			72 h EC50 (growth of fronds) = 2.70 μM 72 h EC50 (maximum quantum yield of PS II (F_v/F_m) = 1.91 μM Park *et al.*, 2012
Bacteria *Aliivibrio fischeri*			Bioluminescence inhibition 5 min EC50 = 16.8 mg/L 15 min EC50 = 18.3 mg/L Hernando *et al.*, 2006

* Environmental regulatory document.

Table 3. Synthetic water formulations.

Macro nutrients	Standard freshwater prepared with deionized water (according to US EPA, 1994) moderately hard water	Standard freshwater prepared with deionized or distilled water (ISO medium according to ISO 6341)	Medium M4 and M7 (according to OECD, 2004, 2011b)
	Concentration, mg/L		
$NaHCO_3$	96	67.75	64.8
$CaCl_2 \cdot 2H_2O$	120	294	293.8
$MgSO_4 \cdot 7H_2O$	123	123.25	123.3
KCl	4	5.75	5.8
$NaSiO_3 \cdot 9H_2O$	–	–	10.0
$NaNO_3$	–	–	0.274
KH_2PO_4	–	–	0.143
K_2HPO_4	–	–	0.184

water quality because the composition of natural freshwater may change with season and region (Klüttgen *et al.*, 1994). Therefore it is necessary to make sure that water matches the preset characteristics and has constant quality throughout the test. In the case of natural water, the water quality characteristics should be measured at least twice a year or when it is suspected that these characteristics may have changed significantly. The test culture should not express signs of stress and should reproduce well in the water used for cultivation. A dilution water type may depend on the objective of research (US EPA, 1994).

Any constant quality water in which the test species shows suitable long-term survival and growth (OECD, 2012b, 2013a) or reconstituted water in line with ISO 7346 for adult fishes or ISO 12890 for embryo-larval forms can be used for fish cultivation and sample dilution. The synthetic media presently applied that are most successful for culturing successive generations of various invertebrate organisms are listed in Table 3.

The composition of M4 and M7 media (formula according to OECD, 2004a) is rather complex. Apart from macro nutrient elements, these media comprise trace elements and vitamins. The Elendt M4 medium is harder and the amount of trace elements (except for $ZnCl_2$, $CaCl_2 \cdot 6H_2O$, KI, Na_2SeO_3, and NH_4VO_3) is 4-fold higher than in M7. The M4 medium was first time described by Elendt and Bias (1990).

It should be noted that, if an interaction is suspected between hardness ions and the test substance, lower hardness water should be used and, thus, Elendt Medium M4 must not be used in this situation (OECD, 2004b, 2011b). The US EPA document (1994) also caters for the preparation of synthetic water with different hardness: very soft, soft, moderately hard, hard, and very hard; however, the most used is moderately hard water. Where natural or dechlorinated water is applied and in order to ensure that the dilution water will not unduly influence the test result (for example by complexation of the test chemical), or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis (OECD, 2004a).

To ensure the required concentration of dissolved oxygen, all synthetic media (the standard freshwater and seawater) used in Toxkit microbiotests must be aerated for at least 15 min prior to use for the hatching of the cysts and for the preparation of the toxicant dilutions. The dissolved oxygen and pH are measured, as a minimum, in the controls and in the highest test substance

concentration at the beginning and end of the test. The dissolved oxygen concentration in the controls should be in compliance with the validity criterion. The oxygen level in control and test vessels should be reported as the air saturation value. The pH should not normally vary by more than 1.5 units over the course of the test (Weltje *et al.*, 2009).

Growth media prepared with deionized water according to the protocols of OECD 201, US EPA medium AAP, and ASTM, are used for freshwater phytoplankton tests. Growth medium, 20X AAP, and modified Swedish standard medium (SIS) for freshwater macrophytes are given in the OECD Guideline 221 "Lemna sp. Growth Inhibition Test" (Tab. 4). Steinberg medium is typically used for aquatic macrophyte culturing and testing (Park *et al.*, 2012).

In the test with the diatom *N. pelliculosa* the media (original medium from OECD TG 201 according to ISO 8692 and US EPA medium AAP according to ASTM) must be supplemented with $Na_2SiO_3 \cdot 9H_20$ to obtain a concentration of 1.4 mg Si/L (OECD, 2006). The paper by Rahman *et al.* (2014) clarifies that phytoplankton was cultured in OECD media (OECD, 2011a) at a reduced concentration of total phosphorus (0.10 mg/L) to more closely reflect natural waters (Dodds *et al.*, 1998). Moreover phosphate concentrations are known to influence As toxicity in phytoplankton (Duester *et al.*, 2011; Levy *et al.*, 2005).

In Russia, the new system for preparing of laboratory water was proposed by researchers of the Faculty of biology of Lomonosov Moscow State University (Filenko *et al.*, 2015). The system consists of three-step purification and "biologization" of tap water. Tap water purified through a flowing filter is put in the first aquarium and dechlorinated two weeks by aeration with atmospheric air with a pump. The water is then transferred to the second aquarium containing quartz sand and higher aquatic plants, which are necessary for its "biologization". Numerous data attest to an active environmental role of the higher aquatic plants and water saturation of metabolites of macrophytes (Kirpenko and Usenko, 2012). Water in the second aquarium is continuously filtered by an external filter (*e.g.* Tetra EX 600 Plus), which is necessary for water purification from turbidity, mixing of the upper and lower layers of water, and gas exchange. The aquarium is illuminated 12 h a day. The water in the second aquarium is "biologized" within 14 days. The water is then transferred to the third aquarium with a pump. This water is then ready to be used for the cultivation of aquatic organisms and dilution of the samples in the ecotoxicological tests. The "biologized" water has the following hydrochemical parameters: pH 8.0–8.5; salinity – 0.2–0.3 g/L, the oxygen content 8–11.5 mg/L.

Uspensky, Tamiya and Prat's culture media (ERD, 2011, 2014a) are applied for cultivation and dilution in tests with microalgae (*Scenedesmus quadricauda* and *C. vulgaris)*. The media formulations are generally similar, varying in salt saturation and Calcium content. The Ca-containing Uspensky's medium, has a different composition from the other growth media.

3.2 Illumination and temperature

In addition to the cultivation and dilution water quality, optimal light and temperature conditions (for water and air temperature) have to be maintained. These parameters are of a great physiological value and differ for different groups of aquatic organisms (Snell *et al.*, 1991).

Crustaceans are less demanding in terms of light conditions. *Daphnias* have eyes capable of rolling to maintain the proper angle with respect to the light source. Light with 3000 lux intensity however inhibits to a certain extent respiration of cladocerans. Vessels with cladocerans should be optimally illuminated by fluorescent lamps in the 500–1000 lux intensity range during 16 hour light and 8 hour dark periods (OECD, 2004a, 2012a; FR, 2007b; ERD, 2014b). Similar requirements for the illumination regime are set for culturing of rotifers *Brachionus calyciflorus* (ISO, 2008b; ASTM, 2012a), anostraca (fairy shrimps) *Thamnocephalus platyurus* (ISO, 2011), ostracods *Heterocypris incongruens* (ISO, 2012a), and larvae of chironomids *Chironomus* sp. (OECD, 2011b).

Algae culturing requires steady fluorescent lighting, *e.g.* of the cool-white or daylight type. Strains of algae and cyanobacteria differ in their light demand, therefore light intensity should be tailored for each test culture. For green algae described in the OECD standard (2006) light intensity is recommended to be in the range of 4440–8880 lux, which is equivalent to 60–120 $\mu E\, m^{-2} s^{-1}$. By Russian standards, the range is somewhat lower, and for freshwater algae (*S. quadricauda* and *C. vulgaris)* illumination should be within the 3000–5000 lux range (FR, 2007a; ERD, 2011). Some species of cyanobacteria, in particular *Anabaena flos-aquae*, grow well at lower light intensities and may be damaged at high intensities. For such species average light intensity in the range of 2960–4440 lux (equivalent to 40–60 $\mu E\, m^{-2} s^{-1}$) should be selected (OECD, 2011a). Continuous warm or cool white fluorescent lighting should be used to provide light intensity in the range of 6500–10000 lux (85–135 $\mu E\, m^{-2} s^{-1}$) for cultivation of Lemna sp. (OECD, 2006).

It is important to understand that light, especially in UV-range, may also affect the toxicity of a contaminant. On one hand, light can also interact with certain contaminant compounds in a phenomenon known as photo-induced, photo-activated, or photo-toxicity (Roberts *et al.*, 2017). The researchers remark that toxicity of metals, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons is enhanced by light (Wernersson *et al.*, 1999; Roberts *et al.*, 2017). On other hand, light can add to a toxicity decrease in photo-degradable, substances and mixtures. This phenomenon broadly refers to an interaction between a chemical and light resulting in photo-inactivation. Such effect is fixed for a wide range of organic, inorganic, and microbial contaminants (Sousa *et al.*, 2013; Na *et al.*, 2018; Rubasinghege *et al.*, 2017).

Thus in all consideration a need for strict monitoring of the lighting conditions is paramount. The ASTM Standard Guide E1733-95(2014) contains information on types of artificial light sources that mimic spectral ranges of the sunlight, light components, calculations of the biologically effective radiation, and other relevant data. The lighting conditions described in the standard are applicable to tests with most organisms and use of most chemicals. Secondly, studying toxicity of difficult substances and mixtures such as photo-degradable substances calls for special biotesting conditions. In short-term acute fish and *Daphnia* tests breakdown of chemical structures by

Table 4. Culture medium formulations for macrophytes and phytoplankton.

For macrophytes			For phytoplankton (algae and cyanobacteria)			
Salts	Swedish Standard (SIS) Lemna growth medium according to OECD 221	20X AAP growth medium according to OECD 221	Salts	Original medium from OECD TG 201 according to ISO 8692	US EPA medium AAP according to ASTM	Uspensky's medium according to ERD, 2011
	Concentration, mg/L			Concentration, mg/L		
$NaNO_3$	85	510	$NaHCO_3$	50	15.0	–
Na_2CO_3	20	–	$NaNO_3$	–	25.5	–
$NaHCO_3$	–	300	NH_4Cl	15	–	–
KH_2PO_4	13.4	–	$MgCl_2{\cdot}6H_2O$	12	12.16	–
$K_2HPO_4 \cdot 3H_2O$	–	30	$CaCl_2{\cdot}2H_2O$	18	4.41	–
$MgSO_4 \cdot 7H_2O$	75	290	$MgSO_4{\cdot}7H_2O$	15	14.6	25
$CaCl_2 \cdot 2H_2O$	36	90	KH_2PO_4	1.6	–	–
H_3BO_3	1.0	3.7	K_2HPO_4	–	1.044	25
$MnCl_2{\cdot}4H_2O$	0.2	8.3	KNO_3	–	–	25
$MnCl_2{\cdot}6H_2O$	–	240	K_2CO_3	–	–	34.5
$Na_2MoO_4{\cdot}2H_2O$	0.01	145 μg/L	$FeCl_3{\cdot}6H_2O$	0.064	0.160	–
$ZnSO_4{\cdot}7H_2O$	0.05	–	H_2BO_3	0.185	0.186	2.86
$ZnCl_2$	–	66 μg/L	$MnCl_2$	0.415	0.415	1.81
$CuSO_4{\cdot}5H_2O$	0.005	–	$ZnCl_2$	0.003	0.00327	
$CuCl_2{\cdot}2H_2O$	–	0.24 μg/L	$ZnSO_4{\cdot}7H_2O$	–	–	0.222
$Co(NO_3)_2{\cdot}6H_2O$	0.01	–	$CoCl_2{\cdot}6H_2O$	0.0015	0.00143	–
$CoCl_2{\cdot}6H_2O$	–	29 μg/L	$CuCl_2{\cdot}2H_2O$	0.00001	0.000012	–
$FeCl_3{\cdot}6H_2O$	0.84	3.2	$Na_2MoO_4{\cdot}2H_2O$	0.007	0.00726	–
Na_2-EDTA$\cdot 2H_2O$	1.4	6.0	Na_2-EDTA$\cdot 2H_2O$	0.100	0.300	–
MOPS	490	–	MoO_3	–	–	0.01764
–	–	–	NH_4VO	–	–	0.02296

photolysis may be reduced or prevented by working in a darkened environment and using red light where necessary (ISO, 1997; OECD, 2000a). In algal tests, it may be possible to determine toxicity of the parent substance using one of two approaches: the first approach is based on selective removal from the illumination source of light wavelengths responsible for photolysis whilst retaining those wavelengths necessary for photosynthesis; the second approach involves carrying out the test using a dark exposure phase followed by an illuminated phase (OECD, 2000b).

A temperature range is specific for each test culture; however, generally, test organisms, for the most part, are mesophilic and need culturing and testing at temperatures in a range of 18–24 °C. Where thermophilic and cryophilic test cultures are used, the temperatures should be higher and lower, respectively. The Russian procedure (ERD, 2014a) provides for the use of a thermophilic strain of *C. vulgaris,* for which the optimal temperature range is 36.0 ± 0.5 °C. The choice of the temperature sometimes is influenced by the need to minimize the length of the test. In the above mentioned procedure (ERD, 2014a) test lasts 22 h, *i.e.* it is shorter than other tests with microalgae that last from 48 to 72 h (ERD, 2011). Temperature can influence the rate of hydrolysis of some substances that degrade in the test system. The adjustment of this parameter, within the range permitted for the test, may therefore be appropriate to optimize exposure concentrations of the parent substance (OECD, 2000a).

3.2.1 Toxkits

Biological, technological and financial challenges primarily associated with the continuous cultivation and maintenance of test cultures preclude from implementing toxicity bioassays in practice. Dr Guido Persoone and his research team at the Ghent University and the company MicroBioTests Inc. in Belgium, developed miniature biotests (Toxkit microbiotests) with selected test organisms that are independent from culturing or maintaining of living test cultures (Blaise, 2000; http://www.microbiotests.be/). The major advantage of Toxkit microbiotests, in comparison to conventional bioassays, is that the test organisms are incorporated in the kits in a "dormant" or "immobilized" form, from which they can be activated "on demand", prior to performance of the toxicity test. Data from comparative research of various laboratories in different countries and own interlaboratory exercises on the Toxkits have shown that the sensitivity of the bioassays performed with the microbiotests (Daphtoxkits, Algaltoxkit, Rotoxkit, Thamnotoxkit, Ostracodtoxkit and Spirodela duckweed toxkit) is similar to that of the conventional toxicity tests. Using neonates hatched from cysts for toxicity tests reduces variability in test sensitivity and reduces failure due to excessive control of mortality (Snell *et al.*, 1991). Table 5 gives a brief description of Toxkit microbiotests for freshwater and freshwater sediments with regard to the incubation and testing conditions. Except for

Table 5. Toxkit microbiotests for freshwater and freshwater sediments.

Test	Test species	Hatching/Incubation conditions	Testing conditions	Culturing and dilution medium
Toxi-Screening kit	Freeze-dried luminescent bacteria *Aliivibrio fischeri*	–	At ambient temperature within the range 15 °C–25 °C	Control water
Chronic protoxkit F	Ciliate *Tetrahymena thermophila*	–	In darkness at 30 °C	Protoxit medium
Acute rotoxkit F	Rotifer *Brachionus calyciflorus*	At 25 °C, under continuous illumination (light source of min. 3000–4000 lux), 16–18 h	At 25 °C in darkness, for 24 h	Moderately hard synthetic water, US EPA formula
Short-chronic rotoxkit F	Rotifer *Brachionus calyciflorus*	At 25 °C, under continuous illumination (light source of min. 3000–4000 lux), 16–18 h	At 25 °C in darkness, for 24 h	Moderately hard synthetic water, US EPA formula
Daph toxkit F magna	Cladoceran crustacean *Daphnia magna*	At 20–22 °C under continuous illumination of min. 6000 lux, 72 h	At 20°C, in darkness	ISO medium, formula according to ISO 6341
Thamno toxkit F	Anostracan crustacean *Thamnocephalus platyurus*	At 25 °C, under continuous illumination (light source of min. 3000–4000 lux), 20–22 h	At 25 °C in darkness, for 24 h	Moderately hard synthetic water, US EPA formula
Ostracod toxkit F	Benthic ostracod crustacean *Heterocypris incongruens*	At 25 °C, under continuous illumination (light source of min. 3000–4000 lux), 52 h	At 25 °C, in darkness, for 6 days	Moderately hard synthetic water, US EPA formula
Ceriodaphtoxkit	Cladoceran crustacean *Ceriodaphnia dubia*	At 25 °C, under continuous illumination of min. 6000 lux, about 80 h	At 25 °C, in darkness, for 24 h	Moderately hard synthetic water, US EPA formula
Rapid toxkit	Anostracan crustacean *Thamnocephalus platyurus*	At 25 °C, under continuous illumination (light source of min. 3000–4000 lux), for 30 h (minimum) to 45 h (maximum)	For 15 to 30 min at 25 °C in darkness	Moderately hard synthetic water, US EPA formula
Algal toxkit F	Green micro-algae *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*)	–	23 °C + 2 °C with constant uniform illumination from cool white fluorescent lamps: 10 000 lux for sideway illumination of the holding tray or 3000–4000 lux for bottom illumination	Algal culturing medium with deionized water, according to the formula of the OECD guideline 201 and the ISO/DIS guideline 8692
Spirodela duckweed toxkit	Macrophyte *Spirodela polyrhiza*	At 25 °C with continuous "top" illumination (at least 6000 lux at the surface of the petri dish)	At 25 °C with continuous illumination of 6000 lux (at the top of the multiwell), 3 days (72 h + 1 h)	The growth medium is the "Steinberg medium" prescribed by ISO for Lemna toxicity tests (ISO 20079)

algal and duckweed tests which require illumination, the tests are for the most part performed in darkness, which creates stable conditions for difficult substances and mixtures. Synthetic media are used for culturing and dilution thereby facilitating water quality analysis. Standard operational procedures prescribe in detail and step by step the implementation of the microbiotests.

4 Conclusion

The review of the literature sources and regulatory documents on the standardized biological test species and their cultures presently applied for establishing the extent of environment toxicity and safety testifies to a great diversity of approaches and living organisms being in practical use. Nevertheless, we have to acknowledge that their use and application globally and in Russia lacks uniformity or co-ordinated monitoring.

Test organism species and strains as well as the quality of collections vary across organizations. The organisms used for biotesting are most comprehensively represented in universities and research centers but they reflect significant variability aligned to individual interests and research focus of the investigators. It is regretful that, once laboratories are restructured or shutdown, the collections are often not maintained and no longer exist.

The insufficient number of ecoanalytical laboratories with standardized cultures of test organisms impedes toxicological research progress and the generation of pertinent information on environmental risk assessment which is essential for the ecosystem health. The use of biological tools from odd sources, without the guaranteed species identification and standard-compliant sensitivity to reference substances does not further the unification of the approaches to environmental assessment of natural media, wastes and other materials.

The relevance and need of creating coordination center(s) for the dissemination of test cultures is obvious. Research infrastructure in the form of a centralized network of users would provide users verified test cultures supported with reference data.

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