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History and sensitivity comparison of the *Spirodela polyrhiza* microbiotest and *Lemna* toxicity tests

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ABSTRACT

Key-words: *duckweed toxicity tests, sensitivity comparison, Lemna minor, Spirodela polyrhiza microbiotest*

The history of toxicity tests with duckweeds shows that these assays with free-floating aquatic angiosperms are gaining increasing attention in ecotoxicological research and applications. Standard tests have been published by national and international organizations, mainly with the test species *Lemna minor* and *Lemna gibba*. Besides the former two test species the great duckweed *Spirodela polyrhiza* is to date also regularly used in duckweed testing. Under unfavorable environmental conditions, the latter species produces dormant stages (turions) and this has triggered the attention of two research groups from Belgium and Greece to jointly develop a "stock culture independent" microbiotest with *S. polyrhiza*. A 72 h new test has been worked out which besides its independence of stock culturing and maintenance of live stocks is very simple and practical to perform, and much less demanding in space and time than the conventional duckweed tests. Extensive International Interlaboratory Comparisons on the *S. polyrhiza* microbiotest showed its robustness and reliability and triggered the decision to propose this new assay to the ISO for endorsement and publication as a standard toxicity test for duckweeds. Sensitivity comparison of the 72 h *S. polyrhiza* microbiotest with the 7d *L. minor* assay for 22 compounds belonging to different groups of chemicals revealed that based on growth as the effect criterion both duckweed assays have a similar sensitivity. Taking into account its multiple advantages and assets, the *S. polyrhiza* microbiotest is a reliable and attractive alternative to the conventional duckweed tests.

RÉSUMÉ

Histoire et comparaison de sensibilité du microbiotest *Spirodela polyrhiza* et du test de toxicité *Lemna*

Mots-clés : *tests de toxicité, lentille d'eau,*

L'historique des tests de toxicité avec des lentilles d'eau démontre que ces essais réalisés avec des angiospermes aquatiques « flottants » reçoivent de plus en plus d'attention en recherche et en applications écotoxicologiques. Des tests standardisés ont d'ailleurs fait l'objet de publications par des organisations nationales et internationales, notamment avec les espèces test *Lemna minor*

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comparaison de sensibilité, Lemna minor, Spirodela polyrhiza microbiotest

et *Lemna gibba*. Outre celles-ci, la lentille d'eau *Spirodela polyrhiza* est aussi utilisée couramment pour l'entreprise de tests sur les lentilles d'eau. Lors de conditions environnementales défavorables, *S. polyrhiza* produit des stades de dormance appelés « turions ». Cette particularité a suscité la collaboration de deux groupes de recherche en Belgique et en Grèce dans le but de développer un microbiotest avec cette espèce et qui soit entièrement affranchi d'avoir à cultiver des organismes test. Un essai d'une durée de 72 h, libre de toute servitude de culture et qui s'avère simple et pratique, et beaucoup moins contraignant en termes d'espace et de temps que les tests conventionnels effectués avec des lentilles d'eau a donc été mis au point. Des comparaisons entre laboratoires résultant d'exercices d'intercalibration conduits à l'échelle internationale avec le microbiotest *S. polyrhiza* en ont montré la robustesse et la fiabilité. Ces résultats ont alors conforté la décision de proposer ce microbiotest à l'ISO pour approbation et publication comme test normalisé sur les lentilles d'eau. Une comparaison de la sensibilité du test *S. polyrhiza* d'une durée de 72 h, avec l'essai de 7 jours sur *L. minor*, a été faite sur 22 composés appartenant à différents groupes de produits chimiques. Celle-ci a révélé, sur la base du critère de croissance, que les deux tests de toxicité avaient une sensibilité similaire. Au vu de ses multiples avantages et attributs, le microbiotest utilisant *S. polyrhiza* se présente ainsi comme une alternative fiable et attrayante aux tests conventionnels entrepris avec des lentilles d'eau.

HISTORY OF PHYTOTOXICITY TESTS WITH DUCKWEEDS

The potential or the actual toxicity of chemicals on plants – the "primary producers" of all ecosystems – is, in the aquatic environment, assessed with either algae or with vascular plants.

The principles of ecotoxicology clearly state that no unique test species of either algae or macrophytes is best suited for determining the toxicity of all potential toxicants in all possible conditions of exposure, so in scientific literature there are plenty of examples of studies showing that some biological models have to be preferred over others for a number of reasons.

Wang (1989) compared the two most common phytotoxicity tests, that are the duckweed growth/mortality assay and the seed germination/root elongation test, and concluded that the duckweed test appeared to be the most sensitive in detecting phytotoxicity.

Lewis (1995) published a review on the use of freshwater plants for phytotoxicity testing which reports that aquatic vascular plants have been used less frequently than algae as test species. This is actually confirmed by a search of the EPA ECOTOX database by Dobbins *et al*. (2010) which showed that by far the majority of the aquatic phytotoxicity studies have been made on microalgae (18 700 data), *versus* 2120 on duckweeds and 1670 on other vascular plants.

Despite the fact that historically more toxicity tests have been performed with microalgae than with duckweeds, the former report nevertheless clearly shows that the latter aquatic higher plants – many of which have a cosmopolitan distribution – have already been used extensively to determine the impact of chemicals, and especially herbicides, on the aquatic primary producers.

Duckweeds are small plants with leaf-like structures (called fronds) of a size of a few mm, and with one or several roots. They are free floating on the water surface and they reproduce vegetatively.

Toxicity tests with duckweeds are mainly performed with species belonging to the family *Lemnaceae*, *i.e.* the genera *Landoltia, Lemna, Spirodela, Wolffia and Wolfiella*. Scientific literature, shows that 95% of all the tests are performed on species from the genus *Lemna*, and more in particular mainly *Lemna minor* and *Lemna gibba*.

The USEPA study of Dobbins *et al*. (2010) deals with the exploration of methods for characterizing effects of chemical stressors to aquatic plants and also includes a comparison on species sensitivity of rooted aquatic vascular plants with *Lemna'*s. The findings revealed that some of these vascular plants were more sensitive than the duckweed, whereas others were less sensitive, but virtually all the sensitivity ratios are within a factor of 10.

A detailed comparative study of duckweed and standard algal phytotoxicity tests for consumer product chemicals such as on surfactants, and on pesticides, has been made by Gausman (2006). This study confirmed the findings of Dobbins *et al*. (2010) that "toxicity is species specific and chemical specific" and that the sensitivity of algae to the investigated toxicants is either similar, lower or larger than that of duckweeds.

In his extensive review on "The *Lemnaceae*, or duckweeds: a review of the descriptive and experimental literature", Hillman (1961) indicates that duckweeds have already been used since the 1930s to assess the effects not only of herbicides but of a variety of pesticides and inorganic and organic compounds. During the 1940s and 1950s the early applications of duckweeds in the field of toxicology were centered on herbicide and fertilizer development. A criticism of Hillman (1961) on this early toxicology work is that the outcome of these investigations are highly dependent on control conditions and that little attention is paid to what optimal growth conditions for the controls should be.

Wang (1986) tested several aquatic pollutants on duckweeds, including heavy metals, and in 1990 he published a literature review on the subject, with more than 100 references.

Chaudhary and Sharma (2014) consider duckweeds as an ideal test system for screening and biomonitoring of the complex effluents of wastewaters.

The sensitivity of duckweeds to toxicants has in several studies been compared with that of other model organisms such as macrophytes, algae fish and crustaceans. Hoffman *et al*. (2002) collected the available information and concluded that the reported studies presented somewhat contradictory results, as had actually also already been underlined by Wang (1990).

Since the turn of the century, duckweeds, which are known to be the "fastest growing angiosperms", are gaining more and more attention in ecotoxicological research. In the Journal of the "International Steering Committee on Duckweed research and Applications (ISCDRA)", Edelman (2015) reports that over the last 20 years, more than 300 studies have been published on toxicity tests with duckweeds on either chemicals or waste waters. It is therefore no surprise that toxicity tests with duckweeds have over the years been included in environmental legislation and guidelines.

In the USA, duckweed test methods are published by the American Public Health Association (APHA *et al*., 1995), the American Society for Testing and Materials (ASTM, 1991) and the United States Environmental Protection Agency (USEPA, 1996). In Canada, Environment Canada issued a biological test method for measuring the inhibition of growth using the freshwater macrophyte *Lemna minor* (Environment Canada, 2007). In Europe duckweed test standards are published by the Association Française de Normalisation (AFNOR, 1996) and the Swedish Standards Institute (SIS, 1995).

At the international level, the International Organization for Standardization (ISO) published a standard on a growth inhibition test on the duckweed *Lemna minor* (ISO, 2005), and the Organization for Economic Cooperation and Development (OECD) issued a revised guideline for a *Lemna* sp. growth inhibition test (OECD, 2006).

Although duckweed tests are used for a variety of applications – often as part of a battery of toxicity tests – they are also mandatory for risk assessment of herbicides and plant regulators with regard to the placing of plant protection products on the market (EC Reg 1107/2009).

In their detailed brochure on the *Lemna minor* test procedure, Environment Canada (2007) describes the numerous reasons why duckweed species are "uniquely useful for toxicity tests":

- they have a small size;
- they have a relative structural simplicity and a rapid growth;
- they reproduce vegetatively with genetically homogenous populations, which eliminate effects due to genetic variability;
- they can be disinfected and grown in a liquid medium as well as on agar;
- they can be cultured in controlled conditions more easily than most other angiosperms;
- they have a high surface area to volume ratio;
- they are excellent accumulators of a number of metallic elements;
- they are especially susceptible to surface-active substances, hydrophobic compounds and similar substances that concentrate at the air-water interface;

– they can be used in tests with solutions renewal and with colored or turbid samples.

Until the time that toxicity tests with duckweeds were standardized, the experimental conditions used by the scientists for their assays covered, besides the choice of different test species, a wide range of procedures with regard to the culture and the test medium, the test containers, the temperature and illumination, the exposure time, and the effect parameters used to evaluate the impact of the analyzed toxicant or sample on the duckweed.

Presently most of the standard methods referred to above make use of *Lemna minor* or *Lemna gibba* as the test species, and the test procedures measure growth inhibition as the effect parameter. Growth is determined by counting after 7 days exposure the number of fronds which develop from the colonies inoculated at the start of the test. The assays are performed in test vessels containing a specific nutrient medium, which are incubated at 25 °C and with continuous illumination (usually 85–125 $\mu E \cdot m^{-2} \cdot s^{-1}$). Additional effect parameters which are measured (or have to be measured) are frond area, dry weight, fresh weight and chlorophyll.

TOXICITY TESTS WITH *SPIRODELA POLYRHIZA*

Although as mentioned above the majority of toxicity tests with duckweeds have been performed with either *Lemna minor* or *Lemna gibba*, other duckweed species, and in particular *Spirodela polyrhiza*, have over the last decades also already been used extensively for toxicity testing of chemicals as well as of natural samples.

The "giant duckweed" *S. polyrhiza* is, as indicated by its common name, substantially larger than other duckweeds, with fronds of up to 1 cm in comparison to only a few mm for most other duckweed species. Morphologically *S. polyrhiza* has several roots, whereas *L. minor* and *L. gibba* have one root only. Duckweeds reproduce by asexual budding but in unfavorable conditions for growth, such as drought or winter, "dormant stages" (called "turions") are produced. Turions are specialized fronds which are smaller and thicker than the fronds, and which sink to the bottom and stay dormant until favorable conditions for growth return.

Taking into account their importance as pollutants of the aquatic environment, heavy metals in particular have been the subject of specific research with *S. polyrhiza* on both their toxic effects and their bioaccumulation potential. Studies have been performed on individual metals such as cadmium (Charpentier *et al*., 1987; Sinha *et al*., 1995) or on cadmium and copper (Saadi *et al*., 2002). The effects of nickel were investigated by Xylander *et al*. (1993) and of arsenic by Zhang *et al*. (2011). Extensive fundamental investigations on the toxicity and the accumulation of chromium by *S. polyrhiza* have been performed by Appenroth *et al*. (2003, 2008) and Kaszycki *et al*. (2005).

Quite a number of comparative studies on the toxicity of metals and of various organic compounds have also been performed with *S. polyrhiza* in combination with one or several other duckweed species, such as *Lemna minor* and *Lemna gibba*, and with other plant and animal test species.

Table I gives an overview of these studies and of the involved test species and chemicals.

Other studies have addressed the toxicity of natural samples to the giant duckweed. Lakachauskiene *et al*. (1997) *e.g.* report that *S. polyrhiza* has been used since 1989 as an ecotoxicological biotest in the Institute of Botany in Vilnius, Lithuania, to estimate the "precipitation toxicity" in two regions, the toxicity of waste waters of industrial galvanic departments and the state of pollution of water reservoirs. In Germany, the suitability of *S. polyrhiza* was investigated for assessing the toxicity of a municipal landfill leachate and of two leachates of copper mining residue (Sallenave and Fomin, 1997). Phytotoxicological assessments have been made with giant duckweeds in India, on wetlands (Lovesan and Sivalingham, 2012, 2013) and on chrome liquor from the tanning industry (Singh and Malaviya, 2013).

With regard to the effect levels reported in these publications, a comparison of EC50 values for *e.g.* the same metal shows that the data can differ substantially; this was actually also the case for the toxicity studies on *Lemna* species before they were standardized. For toxicity

Table I
Comparison toxicity studies of Spirodela polyrhiza with other biological models for various inorganic and organic chemicals.

	Other species	Toxicant(s)	References
Spirodela	*Lemna*	several herbicides	Hudson and Bee Hudson, 1957
Spirodela polyrhiza	*Lemna minor*, *Lemna gibba*	Cu, Zn, organic biocides	Lakatos *et al*., 1993
Spirodela polyrhiza	*Azolla pinnata*	Cd, Cr, Co, Cu, Ni, Pb and Zn	Gaur *et al*., 1994
Spirodela polyrhiza	*Azolla pinnata*	Cd	Gaur and Noraho, 1995
Spirodela polyrhiza	*Eichhornia crassipes*, *Salvinia molesta*, *Pistia stratiotes*, *Azolla nilotica* *Lemna paucicostata,* *Hydrilla verticillata*, *Ceratophyllum demersum* *Najas graminea*	p-Hydroxybenzoic acid, anisic acid, salicylic acid, coumaric acid, fumaric acid, tannic acid, gallic acid, chlorogenic acid, vanillic acid, caffeic acid and ferulic acid	Pandey, 1996
Spirodela polyrhiza	*Tradescantia*	Cu, Cr (VI), Fe, Ni, Zn	Montvydienė *et al*., 1999
Spirodela polyrhiza	*Lepidium sativum*, *Tradescantia*	heavy metals	Montvydienė *et al*., 2000
Spirodela polyrhiza	*Lepidium sativum*	Cu, Cr, Cd, Ni, Mn, Zn and Pb	Montvydienė and Marčiulionienė, 2004
Spirodela polyrhiza	*Lepidium sativum*	Cu, Cr, Zn, Ni	Montvydienė and Marčiulionienė, 2007
Spirodela polyrhiza	*Lemna minor*	Chromate, sulphate	Appenroth *et al*., 2008
Spirodela polyrhiza	*Lemma aequinoctialis*	Pb	Xia *et al*., 2009
Spirodela polyrhiza	*Lemna minor*	Ni	Appenroth *et al*., 2010
Spirodela polyrhiza	*Lemna minor*	metazachlor	Müller *et al*., 2010
Spirodela polyrhiza	*Lemna minor*	Pb	Leblebici and Aksoy, 2011
Spirodela polyrhiza	*Lemna gibba*, *Lemna minor*, *Daphnia magna*	Cr, Zn	Dvořák *et al*., 2012.
Spirodela polyrhiza,	*Landoltia punctata*, *Lemna minor*	paraquat	Wang *et al*., 2013
Spirodela polyrhiza	*Lemna minor*, *Lemna gibba*	Cu, Cd	Doganlar, 2013

tests with *S. polyrhiza* the differences are due to the different test conditions and the different nutrient media, as underlined by Appenroth *et al*. (2010) in their study on the toxicity of nickel to *S. polyrhiza*.

A variety of nutrient media have indeed been employed for the culturing of the duckweeds and/or the performance of the assays with *S. polyrhiza,* such as *e.g.* Tellier's mineral nutritive solution, Hutner's nutrient medium, Algal Assay Procedure medium, Hoagland's nutrient solution, modified Homes culture medium or modified Steinberg medium. The test conditions with regard to temperature and illumination, and the duration of the assays (from 1 days up to

Table I
Continued.

	Other species	Toxicant(s)	References
Spirodela polyrhiza	*Hyalella azteca*, *Daphnia magna*, *Lemna minor*, *Ceriodaphnia dubia*, *Lymnea stagnalis*, *Pseudokirchneriella subcapitata*, *Chironomus tentans*, *Aeolosoma*, *Brachydanio rerio*, *Pimephales promelas*, *Chlamydomonas reinhardtii*, *Oncorhynchus mykiss*, *Chlamydomonas acidophila*, *Philodina acuticornis*	Co	Canadian Environmental Protection Act 1999. 2013.

14 days) were also different from one study to the other, as well as the criteria used to evaluate the toxic impact. Effect parameters ranged from calculation of growth rates by counting of the colonies or the frond numbers, determination of wet weight or dry weight biomass and chlorophyll content.

It is worth mentioning here that besides its use as an interesting test species for toxicity testing, the attention for *S. polyrhiza* has over the last decades also turned to other domains of interest. The major reason for this is their very rapid growth with a doubling time of less than 30 hours in optimal circumstances. This is nearly twice as fast as other "fast" growing flowering plants, and more than double that of conventional agricultural crops. A substantial number of publications address and report the usefulness of duckweeds for bioremediation of waste waters and in particular the treatment of sewage from agricultural operations with harvesting of biomass for biofuel feedstocks.

Over the last few years, attention has furthermore focused on *S. polyrhiza* for fundamental research, more specifically the unraveling of its genome sequence (Wang *et al*., 2014). The giant duckweed was selected for genome sequencing because it has the smallest genome in the family of *Lemnaceae.* The findings on the *S. polyrhiza* genome will in the near future be very useful for fundamental research on genomics, systematics, genetics and biochemistry, as well as for applied industrial research.

DEVELOPMENT OF A "STOCK CULTURE FREE" MICROBIOTEST WITH *SPIRODELA POLYRHIZA*

Whereas toxicity tests with "terrestrial" plants can be started from the "dormant" life stages (seeds), toxicity test with duckweeds require continuous culturing and maintenance of live stocks, with the inherent biological, technical and financial costs. However, as mentioned above, when environmental conditions are no longer favorable for growth, some duckweed species can produce "turions"(see photo in Figure 1).

One of the first scientists who studied turion formation in duckweeds is Jacobs (1947) who published an "Ecological life history of *Spirodela polyrhiza* with emphasis on the turion phase". A substantial number of fundamental studies have been made as of the 1980s on the induction of turions and on their dormancy and germination in duckweeds and in particular on *S. polyrhiza.* The choice of *S. polyrhiza* for these studies is due to the easy induction of turions which makes them an ideal model system for dormancy in plants. One of the most active

Figure 1
Spirodela polyrhiza colonies with (smaller and darker) turions.

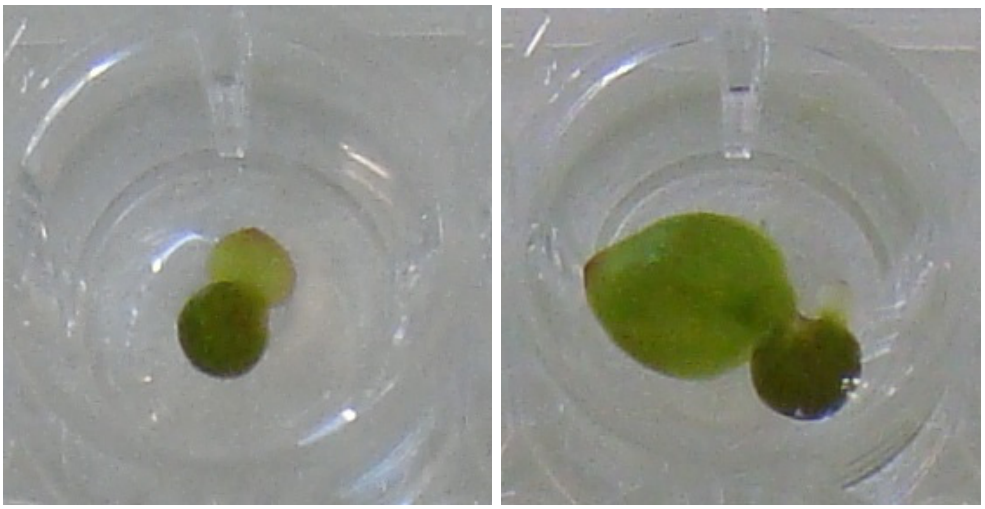
scientists in duckweed research who has performed extensive investigations on *S. polyrhiza* turions is Prof. Appenroth from the University of Jena in Germany. As of 1988. Appenroth has published with his research team and with scientists from various laboratories in different countries more than 30 papers on turion research.

Taking into account the importance of duckweeds in freshwater environments and the possibility of using turions as the starting material for a toxicity test, research has been performed by MicroBioTests Inc. – a spin-off company of the Laboratory for Environmental Toxicology and Aquatic Ecology at the Ghent University in Belgium – on the development of a "stock culture independent" microbiotest with *S. polyrhiza.* As of the 1980s, the latter laboratory has performed research on simple and practical microbiotests which are all based on "dormant" or "immobilized" stages of the test organisms, and which are hence independent of culturing and maintenance of the live stocks of the test species. A whole battery of microbiotests with test species belonging to several phylogenetic groups has gradually been worked out and subsequently commercialized by the spin-off company MicroBioTests under the generic name "Toxkit microbiotests".

The interest for an additional microbiotest with duckweeds was also triggered by the discovery that in the studies cited above on toxicity tests with *S. polyrhiza*, one study (Montvydienė and Marčiulionienė, 2004) indicated that the assays had been started "with turions in the initial stage of first leafy stem development".

Research on a *S. polyrhiza* microbiotest has been performed for several years jointly by the research team of MicroBioTests with the research group of the Laboratory of Ecology and Environmental Sciences at the Agricultural University of Athens in Greece, which has extensive know how on duckweed culturing and toxicity testing with duckweeds. The investigations first focused on the biological and technical aspects of production of *S. polyrhiza* turions and on the storage medium and the storage conditions for the turions for their successful germination after increasing periods of storage.

Once appropriate conditions for the former aspects had been found, the research was focused on the development of a methodology for the *Spirodel*a microbiotest. The first procedure consisted of a 3 days germination of the turions in a Petri dish, in Steinberg medium, at 25 °C and with 6000 lux continuous illumination. Two germinated turions were transferred into each cup of a 6×4 multiwell containing Steinberg medium and the respective toxicant concentrations. The multiwell was incubated for 3 days at 25 °C and 6000 lux continuous illumination. A photo of the multiwell taken with a digital camera was transferred to a computer, for measurement of the areas of the first fronds of the germinated turions in each cup, with an Image Analysis programme. Data treatment and calculation of the 72 h EC50 was performed



(a) (b)

Figure 2
(a) Control cup with germinated turion at the start of the toxicity test. (b) First frond after 72 h incubation.

on specific Excel sheets. Figure 2a shows a photo of a germinated turion at the start of the toxicity test, and Figure 2b a photo of the first frond in a control cup after 3 days incubation.

The 72 h EC50 of the *S. polyrhiza* microbiotest performed according to the procedure described above was compared with the 7d EC50 range of the *Lemna* minor assay for the 2 reference chemicals (3,5 dichlorophenol and KCl) prescribed by ISO standard 20079. This comparison revealed that the EC50's of the new microbiotest were within the acceptability range for both reference chemicals indicated in this ISO standard.

In order to evaluate the ease of performance and the practicality of the *S. polyrhiza* microbiotest and its interlaboratory precision, a preliminary ringtest was then organized on the reference chemical KCl. The outcome of this exercise with 6 laboratories was very successful (an interlaboratory CV of 6%) and the comments and suggestions of the participants allowed to refine and improve the test methodology.

An adapted procedure was worked out which makes use of multiwells with 6×8 cups with only one germinated turion per cup, which allows an easier area measurement of the first frond of each turion. Growth of the first fronds was selected as the best effect criterion and is determined by taking a digital photo of the multiwell at the start and at the end of the test, on which measurement of the area of the first frond in each cup can be made by Image analysis. Calculation of the growth of the first fronds (*i.e.* the *t*72 h–the t_0 h area) was found to substantially increase the sensitivity of the test in comparison to the original test procedure.

A second International Interlaboratory Comparison with the adapted test procedure was subsequently organized with the same reference chemical KCl. Fifty six laboratories, institutes, organizations and companies from 22 countries participated in this second ringtest which was again very successful. Based on the results and the comments of this exercise one single validity criterion for the *Spirodela* microbiotest was eventually selected, *i.e.* that the growth of the first fronds in the controls at the end of the 3 days exposure should be at least 10 mm^2. A detailed report on this International Interlaboratory Comparison of the *Spirodela* duckweed microbiotest can be found on the website www.microbiotests.be.

According to the participants in the first and the second International Interlaboratory Comparison exercises, the test procedure of the "stock culture free" *Spirodela polyrhiza* assay is very simple and practical. Since the results of these ringtests furthermore showed that this toxicity test has a high degree of reliability and robustness, it was decided to work out a proposal for submission to the ISO for publishing this new test as an International Standard.

The requirement of ISO 5725-1 (1994) which addresses "accuracy (trueness and precision) of measurement methods and results", however, stipulates that "supporting data" for the accuracy of any new method "must originate from laboratories which have a previous experience with the application of the proposed test procedure". Yet, this was not the case with the 2 ringtests which had already been organized since all the participants had in fact been "first time users" of the *Spirodela* microbiotest.

So in order to abide by the conditions for an ISO standard, an additional ringtest was needed and a call was issued to 10 laboratories from different countries, which had participated in the second ringtest. Consistent results were again obtained in this 3rd ringtest and a detailed report on this exercise can be found on the website www.microbiotests.be.

A proposal for "Determination of the growth inhibition effects of waste waters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent microbiotest" has then therefore been submitted to ISO in 2014. Since the majority of the votes on the proposed new test method from the National Standardization Organizations members of ISO were positive, the proposal has been approved by ISO for further evaluation as a "new project" (ISO/NWIP).

SENSITIVITY COMPARISON OF THE *SPIRODELA POLYRHIZA* MICROBIOTEST WITH THE *LEMNA* TOXICITY TESTS

The *Spirodela polyrhiza* microbiotest besides being very simple and easy to perform, has several advantages over the conventional duckweed tests:

(1) the assay does not require culturing or maintenance of live stocks of the test species, and can be performed "anytime, anywhere" by use of stored turions;
(2) stored turions have a shelf life of several months with a high germination success;
(3) the microbiotest requires minimal bench and incubation space, and minimal equipment;
(4) the test does not require manipulation of the organisms during or at the end of the test;
(5) the area measurements of the first fronds do not need to be made immediately and can be postponed to an appropriate timing;
(6) the area measurements by image analysis are very rapid and precise, and take less than one hour for a complete test.

Table II gives an overview of the characteristics of the test protocols of the "conventional" *Lemna* toxicity tests, as described in ISO 20079 (2005) and in OECD Guideline 221 (2006), and of the *Spirodela polyrhiza* microbiotest (ISO/NP 20227 (2015).

This Table shows that the *S. polyrhiza* microbiotest test has a number of advantages over the "conventional" *Lemna* tests :

- the duration of the microbiotest is shorter, the more so if considering the pre-culturing requirements;
- it is based on more replicates (8 instead of 3 or 6);
- it requires a much lower volume of test sample;
- with regard to precision, the ISO protocol does not report any indications, while the OECD method refers to a publication which is not easily available; in turn the intra- and interlaboratory variabilities of the *Spirodela* ringtest(s) are treated in detail in the reports given in www.microbiotests.be.

Irrespective of the simplicity and practicality of the "stock culture independent" *S. polyrhiza* microbiotest, its sensitivity to chemicals and natural samples should be at least "similar" to that of the standard duckweed tests with *Lemna* species in order to be taken into consideration as a possible (interesting) alternative duckweed assay.

At the time of development of the first test protocol described above, a first sensitivity evaluation had been made on 18 chemicals, with comparison of the EC50's with those of studies on *Lemna* tests found in scientific literature. A linear comparison of the data pairs was made and showed a very nice correlation between the respective EC50's (R^2 = 0.95).

Table II
Characteristics of the duckweed test protocols of ISO 20079, OECD Guideline 221 and the Spirodela polyrhiza microbiotest.

	ISO 20079:2005 (a)	OECD Guideline 221 (b)	ISO/NP 20227 (c)
Species	*Lemna minor*	*Lemna gibba*, *Lemna minor*	*Spirodela polyrhiza*
End point	Growth inhibition	Growth inhibition Additional response variable: yield	Growth inhibition
Measurements *	Frond number *plus* at least one among frond area, chlorophyll, dry weight	Frond number *plus* at least one among frond area, dry weight, fresh weight	First frond area
Stock culture	Yes	Yes	No
Field organisms	No	Yes	No
Pre-cultures	7–10 d prior to the test	At least 8 weeks (field collected), at least 3 weeks (culture) 7–10 days under the conditions of the test	3 d turions germination
Start of the test	10–16 fronds (2–3 fronds per colony) in 100 mL test vessels	9–12 fronds (2 . 4 fronds per colony) in 100 mL test vessels	1 turion per cup in a 6 × 8 cups multiwell
Test conditions	Static	Static, semi-static or flow-through	Static
Nutrient medium	Modified Steinberg medium	Modified Steinberg medium; also Swedish Standard growth medium for *Lemna minor* and 20X AAP medium for *L. gibba*	Modified Steinberg medium
Sample volume	100 mL	100 mL	1 mL
Replicates	3 for test sample, 6 for test control (6 for sample and control for limit test)	3	8
Test concentrations	5	5	5
Temperature	24 ± 2 °C	24 ± 2 °C	25 ± 1 °C
Light	85–125 $\mu E \cdot m^{-2} \cdot s^{-1}$	85–135 $\mu E \cdot t \cdot m^{-2} \cdot s^{-1}$	6 000 lx (corresponding approximately to 85 $\mu E \cdot m^{-2} \cdot s^{-1}$)
Duration	7 d	7 d	3 d (72 ± 1) h
Sequence of measurements	All parameters: start and end of test Frond number in controls: at least every 48 to 72 h Chlorophyll or dry weight: random control measurements	Frond number and chosen additional variable: start and end of test	First frond area at start and end of test

Table II
Continued.

	ISO 20079:2005 (a)	OECD Guideline 221 (b)	ISO/NP 20227 (c)
Validity criteria	7 fold increase in frond number in the control in 7 days	7 fold increase in frond number in the control in 7 days	Mean growth of the first fronds in the controls must be at least 10 mm^2 after 3 days
Reference toxicant	3,5-Dichlorophenol, Potassium chloride	3,5-Dichlorophenol	3,5-Dichlorophenol, Potassium chloride
Precision	Not indicated	Referred to in: Sims I., Whitehouse P. and Lacey R. (1999) The OECD Lemna Growth Inhibition Test. Development and Ring-testing of draft OECD Test Guideline. R&D Technical Report EMA 003. WRc plc - Environment Agency.	Repeatability and reproducibility of the microbiotest with the reference toxicant KCl have been determined in International Interlaboratory Comparisons (Reports in www.microbiotests.be)

(a) Water quality – Determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) – Duckweed growth inhibition test
(b) *Lemna* sp. Growth Inhibition Test. OECD Guideline 221, 2006.
(c) Water quality – Determination of the growth inhibition effects of waste waters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent microbiotest

Yet, additional sensitivity comparisons are needed in this regard because the EC50's for the *Lemna* tests used for this first comparison originate from publications with 2 different test species (*Lemna minor* or *Lemna gibba*) and with different exposure times and different effect parameters.

Research was therefore initiated to make a more strict sensitivity comparison of the *S. polyrhiza* microbiotest with 7 d *Lemna minor* assays in which "frond number"has been used as the effect parameter.

Literature data were therefore collected for *Lemna minor* 7d EC50's based on frond number, and additional *Lemna minor* tests on a number of compounds were performed in the Laboratory of Ecology and Environmental Sciences at the Agricultural University of Athens in Greece, and on *Spirodela* tests in the latter laboratory and in the company MicroBioTests Inc.

Eventually EC50 data were found or were generated for 20 inorganic and organic chemicals for both the 7d *Lemna minor* and the 72 h *Spirodela* microbiotest, both based on growth as the effect criterion.

Table III reports the EC50 data pairs for 9 herbicides, ordered in increasing value of the *Spirodela* test. The table shows that the sensitivity of both duckweed tests for all these chemicals is very similar. For several herbicides the 72 h giant duckweed microbiotest is actually comparable or more sensitive than the 7d *L. minor* assay.

The EC50 data for 4 inorganic and organic compounds are given in Table IV, in increasing order for the *Spirodela* microbiotest. The sensitivity of both duckweed assays is similar for 3,5 dichlorophenol, and *S. polyrhiza* is slightly more sensitive than *L. minor* for aceton and KCl, but slightly less sensitive for ethanol.

Table III

Tests on herbicides EC50 values (in mg·L^{-1}) for the 3 days Spirodela polyrhiza microbiotest (growth based on area of the first frond) and the 7 days Lemna minor test (growth based on frond number).

Chemical compound	*Spirodela polyrhiza*	Origin of data	*Lemna minor*	Origin of data
Thifenylsulfuron-methyl	0.0026	OR-1	0.002	Gatidou *et al*. (2015)
Tribenuron-methyl	0.0057	OR-1	0.008	OR-2
Metribuzin	0.019	OR-1	0.070	OR-2
Lenacil	0.023	OR-1	0.035	OR-2
Tritosulfuron	0.033	OR-1	0.157	OR-2
Linuron	0.039	OR-1	0.030	OR-2
Terbutylazine	0.053	OR-1	0.132	OR-2
Imazamox	0.056	OR-1	0.036	OR-2
Metamitron	0.58	OR-1	1.182	OR-2

OR-1 = Own Research data from the Laboratory of Ecology and Environmental Sciences at the Agricultural University of Athens and from the company MicroBioTests. OR-2 = Own Research data from the Laboratory of Ecology and Environmental Sciences at the Agricultural University of Athens

Table IV

Tests on inorganic and organic compounds EC50 values (in mg·L^{-1}) for the 3 days Spirodela polyrhiza microbiotest (growth based on area of the first frond) and the 7 days Lemna minor test (growth based on frond number).

Chemical compound	*Spirodela polyrhiza*	Origin of data	*Lemna minor*	Origin of data
3,5 Dichlorophenol	3.77	OR-3	2.98	ISO20079
Acetone	5048	OR-3	9783	Cowgill *et al*. 1991
KCl	7078	Mean value of 3rd ringtest	9780	Cowgill et a. 1991
Ethanol	17760	OR-3	14950	Cowgill *et al*. 1991

OR-3 = Own Research data from the company MicroBioTests.

Table V

Tests on metals EC50 values (in mg·L^{-1}) for the 3 days Spirodela polyrhiza microbiotest (growth based on area of the first frond) and the 7 days Lemna minor test (growth based on frond number).

Chemical compound	*Spirodela polyrhiza*	Origin of data	*Lemna minor*	Origin of data
Silver (Ag^{2+})	0.083	OR-3	0.081	Naumann *et al*. 2007
Copper (Cu^{2+})	0.21	OR-3	0.330	Naumann *et al*.2007
Cadmium (Cd^{2+})	0.31	OR-3	0.323	Naumann *et al*.2007
Nickel (Ni^{2+})	0.98	OR-3	0.370	Naumann *et al*. 2007
Mercury chloride ($HgCl^{2}$)	1.52	OR-3	0.683	Naumann *et al*. 2007
Cobalt (Co^{2+})	2.03	OR-3	0.557	Naumann *et al*. 2007
Chromium (Cr^{6+})	2.13	OR-3	11.1	Cowgill *et al*. 1991
Zinc (Zn^{2+})	2.29	OR-3	0.909	Naumann *et al*. 2007
Boron (B)	6.92	OR-3	53.48	OR-2

OR-2 = Own Research data from the Laboratory of Ecology and Environmental Sciences at the Agricultural University of Athens. OR-3 = Own Research data from the company MicroBioTests.

The EC50 results for 9 metals are shown in Table V, in increasing order for the *Spirodela* microbiotest. As was the case for the analyzed herbicides and the inorganic and organic compounds, the sensitivity of the 2 assays is similar for some metals, but somewhat different for other metals, with either the giant duckweed or the *Lemna* test being more or less sensitive than the other test species. The differences are, however, quite small, except for chromium and boron, for which *Spirodela* appears to be substantially more sensitive than *L. minor*.

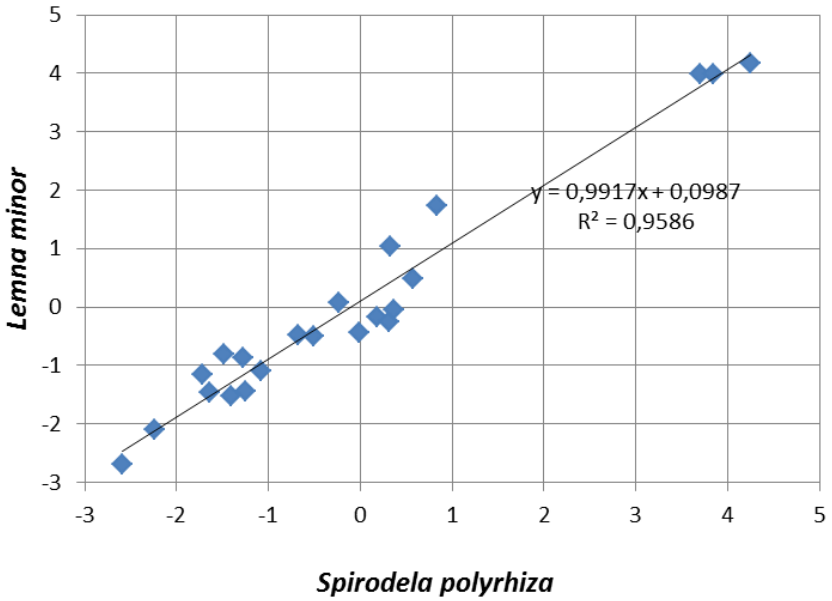


Figure 3
Data pair correlation of the Spirodela polyrhiza and the Lemna minor EC50's for the 22 chemicals listed in Tables 3–5, after transformation from decimal values to log values.

A correlation analysis of the EC50 data pairs of the 22 chemicals listed in Tables III–V has been made and is expressed graphically in Figure 3. Since the EC50's range from $\mu g \cdot L^{-1}$ to $g \cdot L^{-1}$, the data have been transformed in log values for visualization in the graph.
The regression line and the R^2 of 0.95 for chemicals belonging to different groups with different modes of action for the two concerned aquatic macrophytes confirms the "overall" good agreement of the sensitivity of the *S. polyrhiza* microbiotest and the *L. minor* assay.

CONCLUSIONS

The overview of the history of toxicity tests with duckweeds confirms that these free-floating aquatic macrophytes are an important group of primary producers which deserve their place in a test battery well complementing the toxicity tests results for other members of the aquatic food chain, for an ecologically meaningful evaluation of the toxic hazard of pollutants in freshwater ecosystems.
Standard tests with *Lemna* species are prescribed and used worldwide but they are dependent on culturing/maintenance of live stocks and they require much bench space and incubation space and their performance in addition is quite time consuming.
The 72 h microbiotest with *Spirodela polyrhiza*, the development of which is treated in detail in this paper is undoubtedly for several reasons a major step forward in this regard. The new assay is not only quite simple and practical and less demanding than conventional duckweed assays, but it is also "stock culture/maintenance free" since it departs from dormant turions. Extensive International Interlaboratory Comparisons have shown the robustness and the reliability of the *S. polyrhiza* microbiotest. Last but not least, sensitivity comparisons of the 3d new microbiotest with the 7d *Lemna minor* assay on 22 compounds of different groups of chemicals have clearly indicated that based on growth as the effect criterion, both assays give a similar response for the toxic effects.
Based on all the former evidence it can thus be concluded that the 72 h *S. polyrhiza* microbiotest is an interesting alternative to the 7d conventional assay with *L. minor*, and that it was worth to propose it to the ISO for publication as a standard test for toxicity testing with duckweeds.

REFERENCES

AFNOR – Association Française de Normalisation, 1996. Détermination de l'inhibition de la croissance de *Lemna minor.* XP T:90–337.

APHA – American Public Health Association, AWWA – American Water Works Association, WEF – Water Environment Federation, 1995. Toxicity – Part 8000: 8–40 and 8–42. *Standard methods for the Examination of Water and Wastewater*, 19th edition.

Appenroth K.-J., Keresztes A., Sarvari A.E., Jaglarz S. and Fischer W., 2003. Multiple effects of chromate on *Spirodela polyrhiza* : Electron microscopy and biochemical investigations. *Plant Biology*, *5*, 315–323.

Appenroth K.-J., Luther A., Jetschke G. and Gabrys H., 2008. Modification of chromate toxicity by sulphate in duckweeds (Lemnaceae). *Aquat. Toxicol.*, *89*, 167–171.

Appenroth K-J., Krech K., Keresztes A., Fischer W. and Koloczek H., 2010. Effects of nickel on the chloroplasts of the duckweeds *Spirodela polyrhiza* and *Lemna minor* and their possible use in biomonitoring and phytoremediation. *Chemosphere*, *78*, 216–223.

ASTM - American Society for Testing and Materials, 1991. Standard guide for conducting toxicity tests with *Lemna gibba* G3. E-1415-91: 1–10.

Canadian Environmental Protection Act 1999, 2013. Federal Environmental Quality Guidelines. Cobalt. Environment Canada: 10 p.

Charpentier S., Garnier J. and Flaugnatti R., 1987. Toxicity and bioaccumulation of cadmium in experimental cultures of duckweed, *Lemna polyrhiza* L. *Bull. Environ. Contam. Toxicol*., *38*, 1055–1061.

Chaudhary E. and Sharma P, 2014. Duckweed as a Test Organism for Eco toxicological Assessment of Wastewater. *Int. J. Sci. Res.*, *3*, 2073–2075.

Cowgill U.M., Milazzo D.P. and Landenberger B.D., 1991. The sensitivity of *Lemna gibba* G-3 and four clones of *Lemna minor* to eight common chemicals using a 7-day test. *J. Water Pollut. Control Fed*. *63*, 991–998.

Dobbins L., Lewis M., Sankula S. and Thursby G., 2010. Exploration of methods for characterizing effects of chemical stressors to aquatic plants. USEPA. Office of Water, Office of Science and Technology, Washington, DC.

Doganlar Z.B., 2013. Metal accumulation and physiological responses induced by copper and cadmium in *Lemna gibba, L. minor* and *Spirodela polyrhiza*. *Chem. Speciation Bioavailability*, *25*, 79–88.

Dvořák V., Caldová J. and Trnková L., 2012. Differential Sensitivity Of the Lemnaceae Species To Chromium And Zinc. *Proceedings of ECOpole*, 2012, vol. 6, pp. 505–510.

Edelman M., 2015. The state of duckweed affairs in the literature. *J. Int. Steering Committee on Duckweed Research and Applications, 3*, 1–33.

Environment Canada. Method Development and Applications Section, Environmental Technology Centre, 2007. Biological Test Method: Test for measuring the inhibition of growth using the freshwater macrophyte, *Lemna minor*. Report EPS 1/RM/37, 2nd edition, 112 p.

Gatidou G., Stasinakis A.S. and Iatrou E.I., 2015. Assessing single and joint toxicity of three phenylurea herbicides using *Lemna minor* and *Vibrio fischeri* bioassays. *Chemosphere, 119*, 569–574.

Gaur J.P. and Noraho N., 1995. Role of certain environmental factors on cadmium uptake and toxicity in *Spirodela polyrhiza* (L.) Schleid. and *Azolla pinnata* R. Br. *Biomed. Environ. Sci*., *8*, 202–210.

Gaur J.P., Noraho N. and Chauhan Y.S., 1994. Relationship between heavy metal accumulation and toxicity in *Spirodela polyrhiza* (L.) Schleid. and *Azolla pinnata R.Br*. *Aquatic Botany*, *49*, 183–192.

Gausman M., 2006. A comparison of duckweed and standard algal phytotoxicity tests as indicators of aquatic toxicology. Institute of Environmental Sciences. A practicum submitted to the Faculty of Miami University, 39 p.

Hillman W.S., 1961. The *Lemnaceae*, or Duckweeds: A Review of the Descriptive and Experimental Literature. *Bot. Rev.*, *27*, 221–287.

Hoffman D.J., Rattner B.A., Burton G.A., Jr. and Cairns J. Jr., (Eds.). 2003. *Handbook of Ecotoxicology*. 2nd edition. CRC Press, 1290 p.

Hudson M.S. and Hudson B., 1957. A Laboratory Test for Screening Chemicals for Toxicity to Submerged Aquatic Plants. *Weeds*, *5*, 371–37.

ISO 5725-1, 1994. Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions.

ISO 20079, 2005. Water quality – Determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) – Duckweed growth inhibition test.

ISO/NP 20227, 2015 – Water quality – Determination of the growth inhibition effects of waste waters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent microbiotest.

Jacobs D.L., 1947. An ecological life history of *Spirodela polyrrhiza* (greater duckweed) with emphasis on the turion phase. *Ecol. Monogr*., *17*, 437–4467.

Jenkins J., Chow C., Choi C., Adam X.H., Cao J., Fuchs I., Schubert D., Rokhsar J., Schmutz T.P., Mayer M.K.F. and Messing J., 2014. The *Spirodela polyrhiza* genome reveals insights into its neotenous reduction fast growth and aquatic lifestyle. *Nat. Commun*., *5*, 3311.

Jiang H.S., Li M., Chang F.Y., Li W. and Yin L.Y., 2012. Physiological analysis of silver nanoparticles and $AgNO_3$ toxicity to *Spirodela polyrhiza*. *Environ. Toxicol. Chem.*, *31*, 1880–1886.

Kaszycki P., Gabrys H., Appenroth K.J., Jaglarz A., Sedziwy S., Walczak T. and Koloczek H., 2005. Exogenous applied sulphate as a tool to investigate transport and reduction of chromate in the duckweed *Spirodela polyrhiza*. *Plant, Cell and Environment*, *28*, 260–268.

Lakatos G., Mészaros I., Bohatka S., Szabo Z., Makadi M., Csatlos M. and Langer B., 1993. Application of *Lemna* species in ecotoxicological studies of heavy metals and organic biocides. *Sci. Total Environ.,* 773–778.

Lakachauskiene R., Montvydiene D. and Marchiulioniene D., 1997. Duckweed as an ecotoxicological biotest. *Lietuvos Mokslu Akademija.Ekologija*, *1*, 54–59.

Leblebici Z. and Aksoy A., 2011. Growth and lead accumulation capacity of *Lemna minor* and *Spirodela polyrhiza (Lemnaceae):* Interactions with nutrient enrichment. *Water, Air, Soil Pollut., 214*, 175–184.

Lewis M.A., 1995. Use of freshwater plants for phytotoxicity testing: a review. *Environ. Pollut., 87*, 319−336.

Loveson A. and Sivalingam R., 2012. Phytotoxicological assessment of two wetlands in Eloor, Kochi using aquatic macrophyte *Spirodela polyrhiza*. *J. Env. Sci. Toxicol. Food Technol., 1*, 44–49.

Loveson A. and Sivalingam R., 2013. Phytotoxicological assessment of two backwater wetlands in Kannamaly, Ernakulam using aquatic macrophyte *Spirodela polyrhiza*. *J. Environ. Anal. Toxicol*., *3*, 1–5.

Montvydienė D. and Marčiulionienė D., 2004. Assessment of toxic interactions of heavy metals in a multicomponent mixture using *Lepidium sativum* and *Spirodela polyrrhiza*. *Environ. Toxicol.*, *19*, 351–358.

Montvydienė D. and Marčiulionienė D., 2007. Assessment of Toxic Interaction of Metals in Binary Mixtures Using *Lepidium sativum* and *Spirodela polyrrhiza*. *Polish J. Environ. Stud*., *16*, 777–783.

Montvydienė D., Lakačauskienė R. and Marčiulionienė D., 1999. Investigations of Toxic and Genotoxic Effects of Heavy Metals and their Model Mixture on Plants. *Acta Zoologica Lituanica*, *9*, 3–11.

Montvydienė D., Lakačauskienė R. and Marçiulionienė F., 2000. Assessment of toxicity of heavy metal model and natural mixtures for higher plants. *J. Botanica Lithuanica*, *6*, 281–297.

Müller R., Berghahn R. and Hilt S., 2010. Herbicide effects of metazachlor on duckweed (*Lemna minor* and *Spirodela polyrhiza*) in test systems with different trophic status and complexity. *J. Environ. Sci. Health, Part B*, *45*, 95–101.

Naumann B., Eberius M. and Appenroth K.-J., 2007. Growth rate based dose-response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test (ISO 20079) with *Lemna minor* L.Clone St. *J. Plant. Physiol*., *164*, 1656–1664.

OECD Guidelines for the Testing of Chemicals, 2006. Guideline 221. *Lemna* sp. Growth Inhibition Test.

Pandey D.K., 1996. Relative toxicity of allelochemicals to aquatic weeds. *Allelopathy J.*, *3*, 241−246.

Saadi A., Guerbet M. and Garnier J., 2002. Influence of diethyldithiocarbamate on cadmium and copper toxicity to freshwater microphyte *Spirodela polyrhiza*: short communication. *Water SA*, *28*, 107−110.

Sallenave R. and Fomin A., 1997. Some Advantages of the duckweed Test to Assess the toxicity of environmental samples. *Acta hydrochim. hydrobiol*., *25*, 135–140.

Singh A. and Malaviya P., 2013. Phytotoxic effect of chrome liquor on growth and chlorophyll content of *Spirodela polyrrhiza* L. Schleid. *J. Appl. Nat. Sci.*, *5*, 165–170.

Sinha S., Rai U.N. and Chandra P., 1995. Modulation of cadmium uptake and toxicity in *Spirodela polyrrhiza* (L.) Schleiden due to malathion. *Environ. Monit. Assess.*, *38*, 67–73.

SIS – Swedish Standards Institute, 1995. Water quality – Determination of growth inhibition (7d) of *Lemna minor* duckweed SS 02 82 13, 15 p.

USEPA – United States Environmental Protection Agency, 1996. Aquatic plant toxicity test using *Lemna* spp. Tiers I and II. Ecological Effects Test Guidelines OPPTS 850.4400, pp. 96–156.

Wang W., 1986. Toxicity tests of aquatic pollutants by using common duckweed. *Environ. Pollut*., *11*, 1–14.

Wang W., 1989. *Toxicity assessment of the aquatic environment using phytoassay methods*. Illinois Department of Energy and Natural Resources, SWS Contract Report 455, 76 p.

Wang W., 1990. Literature review on duckweed toxicity testing. *Environ. Res.*, *52*, 7–22.

Wang W., Haberer G., Gundlach H., Gläßer C., Nussbaumer T., Luo M.C., Lomsadze A., Borodovsky M., Kerstetter R.A., Shanklin J., Byrant D.W., Mockler J.C., Appenroth K-J., Grimwood J., Jenkins J., Chow J., Choi C., Adamp C., Cao X-H. and Fucks J., 2014. The *Spirodela polyrhiza* genome reveals insights into its neotenous reduction fast growth and aquatic lifestyle. *Nat. Commun.*, *5*, 1–21

Wang L.-L., Zhang G.-F., He X., Ge Feng F. and Zhou J-Y., 2013. Toxic effects of herbicide paraquat on different species of *Lemnaceae*. *Chin. J. Ecology*, *6*, 1551–1556.

Xia Z.-Y., Zhang M.-O. and Wang L.F., 2009. Toxicity Action of Pb(II)with different concentrations on *Lemma aequinoctialis* and *Spirodela polyrrhiza*. *Environ. Sci. Technol.*, *6*, 123–129.

Xylander M., Augsten H. and Appenroth K.-J., 1993. Influence of nickel on the life cycle of the duckweed *Spirodela polyrhiza* (L.) Schleiden. *J. Plant Physiol*., *142*, 208–213.

Zhang X., Hu Y., Liu Y. and Chen B., 2011. Arsenic uptake, accumulation and phytofiltration by duckweed (*Spirodela polyrhiza* L.). *J. Environ. Sci.*, *23*, 601–606.

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