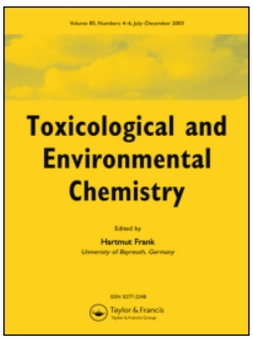

Toxicological & Environmental Chemistry

ISSN: 0277-2248 (Print) 1029-0486 (Online) Journal homepage: http://www.tandfonline.com/loi/gtec20

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To cite this article: P. Ziegler, K.S. Sree & K.-J. Appenroth (2016) Duckweeds for water remediation and toxicity testing, Toxicological & Environmental Chemistry, 98:10, 1127-1154, DOI: 10.1080/02772248.2015.1094701

To link to this article: http://dx.doi.org/10.1080/02772248.2015.1094701

Toxicological & Environmental Chemistry, 2016
Vol. 98, No. 10, 1127–1154, http://dx.doi.org/10.1080/02772248.2015.1094701

Duckweeds for water remediation and toxicity testing

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(*Received 15 April 2015; accepted 27 August 2015*)

The presence of toxic substances in wastewaters and outdoor bodies of water is an important ecotoxicological issue. The aim of this review is to illustrate how duckweeds, which are small, simply constructed, floating aquatic plants, are well suited to addressing this concern. The ability of duckweeds to grow rapidly on nutrient-rich water and to facilitate the removal of many substances from aqueous solution comprises the potential of these macrophytes for the remediation of wastewater and polluted aqueous reservoirs, while producing usable biomass containing the unwanted substances having been taken up. Their ease of cultivation under controlled and even sterile conditions makes duckweeds excellent test organisms for determining the toxicity of water contaminants, and duckweeds are important as model aquatic plants in the assessment of ecotoxicity. Duckweeds are also valuable for establishing biomarkers for the toxic effects of water contaminants on aquatic higher plants, but the current usefulness of duckweed biomarkers for identifying toxicants is limited. The recent sequencing of a duckweed genome holds the promise of combining the determination of water contaminant toxicity with toxicant diagnostics by means of gene expression profiling via DNA microarrays.

Keywords: duckweeds; water remediation; toxicity determination; biomarkers; toxicity diagnostics

1. Introduction

Duckweeds are small, simply constructed aquatic plants or macrophytes that float on the surface of quiet bodies of water. The duckweed vegetative body, or frond, is a thallus-like structure of only a few cells in thickness that represents a fusion of leaves and stems and thus the extreme reduction of an entire vascular plant. The fronds consist largely of spongy mesophyll with large air spaces that make them buoyant, and they are either rootless or bear one to several simple hairless roots on the underside. The duckweeds constitute the family Lemnaceae that consists of 37 species distributed among 5 genera (Appenroth, Borisjuk, and Lam 2013). The genera differ in the size and complexity of the fronds and in the number of roots they bear (Figure 1). The fronds reproduce predominantly in the vegetative mode, whereby daughter fronds bud off from one or two pouches in the mother fronds, while remaining attached for a time to form colonies (e.g., Figure 1(A)). Duckweed morphology and growth have been described in detail by Jacobs (1947), Landolt (1986), Lemon and Posluszny (2000), and Sree, Maheshwari, et al. (2015).

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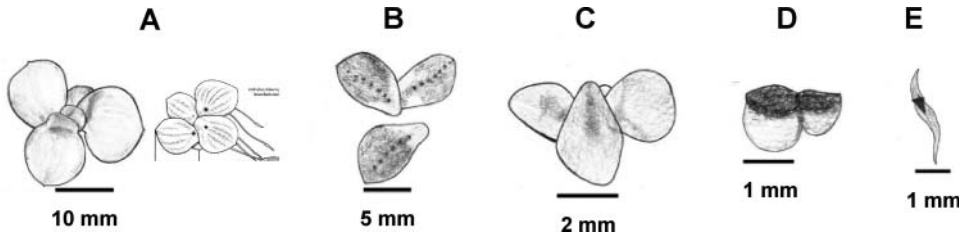

Figure 1. Five genera of Lemnaceae: (A) *Spirodela* (five-frond colony of *Spirodela polyrhiza*, with four-frond colony showing roots); (B) *Landoltia* (three fronds of *Landoltia punctata*, two attached); (C) *Lemna* (three-frond colony of *Lemna minor*); (D) *Wolffia* (mother and daughter fronds of *Wolffia arrhiza*); (E) *Wolffiella* (single frond of *Wolffiella gladiata*). Fronds of *Spirodela* bear 7–11 roots, those of *Landoltia* up to 7, and those of *Lemna* 1, while *Wolffia* and *Wolffiella* are rootless. Modified after Appenroth, Borisjuk, and Lam (2013).

Duckweeds can be of use in redressing a major environmental concern of the present day – the pollution of the hydrosphere with toxic substances. This stems on the one hand from municipal, agricultural, and industrial wastewaters. In spite of treatment facilities ranging from simple septic tanks for isolated homesteads to large, complex installations for dealing with the voluminous wastes of residential and industrial complexes, wastewaters are often discharged untreated or processed to effluents not cleared to the extent that they will have no adverse effect on the surroundings into which they are released. Leachates from bunkered solid wastes, fertilizer spread on fields, and pesticides sprayed on crops can also contaminate ground water and water reservoirs through the action of rain and runoff from heavy rainfalls. Duckweeds can help to remediate wastewater itself and contaminated water reservoirs by taking up and facilitating the removal of excess macronutrients and a large variety of xenobiotic substances from aqueous solution. The biomass produced by the remediative duckweed growth contains the unwanted substances having been taken up, and can be used for fodder or fuel. On the other hand, toxic substances taken up by duckweeds have deleterious effects on the duckweeds themselves, and these effects can be used to indicate the presence of toxic substances in any waters of interest. In the following, the removal of contaminants from water mediated by duckweeds is examined first. The inhibition of duckweed growth observed upon exposure to toxic substances is then presented as the basis of widespread toxicity testing procedures using duckweed as a test organism, and some ecotoxicological insights obtained with such procedures are discussed. Morphological, anatomical, physiological, and molecular responses of duckweeds to toxic water contaminants are then examined in terms of biomarkers for toxicity, and the usefulness of these biomarkers for identifying the agents of toxicity is evaluated. To conclude, the feasibility of establishing comprehensive diagnostic toxicity testing with duckweeds on the basis of gene expression profiling is discussed. The aim is not to provide an exhaustive compilation of findings relevant to these topics, but rather to point to the potential and limitations of using duckweeds for water remediation and toxicity testing. Recent references pertinent to the issues of discussion will serve as sources of background knowledge respective of the topics at hand.

While the present review focuses on duckweeds, these organisms are not the only macrophytes that can remove unwanted substances from water or be used for toxicity testing. And duckweeds live in nature in association with many other aquatic life forms, including fish, crustaceans, insects, algae, and bacteria. The relation of duckweeds to these other plants and life forms in terms of water remediation and toxicity will be evident in some of the highlighted studies.

2. Remediation of contaminated waters

Wastewaters and man-made or naturally occurring surface waters can be unsuitable for consumption and irrigation and/or the health and proliferation of naturally occurring freshwater organisms due to the presence of excessive macronutrients and toxic heavy metals and organic xenobiotic compounds. Duckweeds can improve water quality by removing or facilitating the removal of these deleterious substances from the water.

2.1. Removal of macronutrients

Wastewater from domestic, municipal, and agricultural sources often contains high concentrations of ammonium (NH_4^+), nitrates (NO_3^-), and phosphates (PO_4^-) even after the anaerobic breakdown of complex organic material in treatment facilities. These macronutrients may lead to eutrophication of surface waters when present in large amounts in the aquatic environment, but they are readily removed by duckweeds growing on wastewaters and polluted natural waters (Landesman, Fedler, and Duan 2011).

Duckweeds take up NH_4^+ and NO_3^- through both their roots and the lower surface of their fronds (*Lemna minor*: Cedergreen and Madsen 2002), and may prefer NH_4^+ to NO_3 (*Landoltia punctata*: Fang et al. 2007). High NH_4^+ concentrations in the environment are toxic to plants, animals, and even humans (Britto and Kronzucker 2002). However, *L. minor* has been reported to take up NH_4^+ readily and grow well at concentrations of the ion of up to 84 mg/L (Zhang et al. 2014; Huang et al. 2013; Wang, Yang, et al. 2014), although still higher NH_4^+ concentrations lead to growth rate reduction and photosynthetic pigment loss. Their ability to take up and tolerate relatively high levels of NH_4^+ makes duckweeds particularly suited to the remediation of wastewater from domestic and agricultural sources that often contain considerable amounts of this ion. Over 90% of the NH_4^+, 70% of the NO_3^-, and from 33% to 85% of the PO_4^- present in diluted university wastewater in Ghana (Awuah et al. 2004), anaerobically treated domestic wastewater in Egypt by (El-Shafei et al. 2007), and settled domestic wastewater in Israel (Ben-shalom et al. 2014) were removed by *Spirodela polyrhiza*, *Lemna gibba*/*L. minor*, and *L. gibba*, respectively. These studies showed that the treatments of the wastewaters with duckweeds also maintained a neutral pH, reduced chemical and biological oxygen demand, and removed suspended solids, mosquito larvae, and coliform bacteria.

The biomass produced by the growth of duckweed on nutrient-rich wastewaters can be used for fodder, biofuel production, and fertilizer (e.g., Cheng and Stomp 2009; Cui and Cheng 2015), and many of the studies of nutrient uptake from agricultural wastewater have focused on biomass production. Pilot-scale studies have recorded efficient removal of total nitrogen, NH_4^+, and PO_4^- from nutrient-rich pig farming and urban wastewaters resulting in the accumulation of high amounts of biomass (see Table 1). These studies also illustrate that duckweeds can often be profitably grown directly on raw or anaerobically treated wastewater, which must, however, in some cases be diluted for efficient nutrient uptake and growth. Many of the projected yearly yields reported in such studies exceed the best national averages reported for land-based crops (see Ziegler et al. 2015). Duckweeds can thus outperform conventional land crop plants in biomass production while remediating wastewater without appropriating productive land for terrestrial crops.

Duckweeds growing rapidly on nutrient-rich waters are obviously taking up and assimilating the nutrients. However, discrepancies between the rates of removal from the water and those of actual incorporation into plant tissues have long suggested that denitrifying bacteria associated with the plant rhizosphere may actually be the main agents

Table 1. Yields of duckweeds growing on wastewaters. They are calculated on a ton dry weight per hectare and year basis from the best growth rates and yield data reported on in the cited references. The yields serve as indicators of the extent to which macronutrients can be removed from wastewater by duckweeds.

Duckweed		Biomass yield	
Species	Growing on	*t* (DW/ha/yr)	Reference
Spirodela polyrhiza	Swine wastewater	36.9	Xu, Cheng, and Stomp (2012)
	Swine wastewater	45.2	Xu et al. (2011)
Landoltia punctata	Swine wastewater	68.0	Mohedano et al. (2012)
	Swine wastewater	117	Cheng, Bergmann, et al. (2002) *
Lemna minor	Manured ponds	12.8	Ge et al. (2012)
	Swine wastewater	104	Cheng, Landesman, et al. (2002)
Lemna gibba	University sewage	33.9	Mohapatra et al. (2012)
	University wastewater	131	Verma and Suthar (2014)
Lemna japonica	Farmland runoff	32.9	Zhao, Fang, et al. (2015)
Wolffia arrhiza	Model wastewater	23.3	Soda et al. (2013)

*The authors refer to *Spirodela punctata*, which is now termed *Landoltia punctata* (see Les and Crawford 1999).

effecting nitrate removal from soils and aquatic environments (see Reddy and DeBusk 1985). Lu et al. (2014) isolated root exudates from *S. polyrhiza* and *L. minor* that stimulated bacterial denitrification in the growth medium, and identified fatty acid methyl esters and fatty acid amides as the active components. The above-mentioned reports of El-Shafei et al. (2007) and Ben-shalom et al. (2014) also discussed volatilization, adsorption, and sedimentation as additional nitrogen removal mechanisms.

Duckweed cultivation on nutrient-rich wastewaters has illustrated the diversity of the potential of these organisms for water remediation and for utilization of the remediative growth. Bergmann et al. (2000a) screened the growth and protein production of 41 geographical duckweed isolates on synthetic swine lagoon wastewater. This led to the selection of genotypes of each of *L. minor*, *L. gibba*, and *L. punctata* that were particularly suited to removing NH_4^+ and PO_4^- from swine lagoon effluent and promising for growth and biomass production (Bergmann et al. 2000b; Cheng, Bergmann, et al. 2002; Cheng, Landesman, et al. 2002). More recently, Zhao et al. (2015) showed that a *Lemna japonica* strain grown on a mixture of domestic sewage and agricultural runoff removed more total nitrogen and phosphorus from the wastewater and produced more protein-rich and P-rich biomass than did *L. punctata*, *S. polyrhiza*, and *Wolffia globosa* clones. These studies illustrate the importance of investing in the selection of a duckweed ecotype best suited for the particular wastewater remediation project that is of interest.

2.2. Removal of heavy metals, arsenic, and selenium

Heavy metals are released into the environment from both natural and anthropogenic sources, predominantly from mining and industrial activities. They constitute serious health risks to humans, animals, plants, and microbes (Duruibe, Ogwuegbu, and Egwurugwu 2007). Heavy metals disturb the metal homeostasis of the organisms they invade, bind inappropriately to proteins, and displace other metal ions from their natural binding sites. They disrupt signal transduction pathways important for growth and development

and elicit destructive oxidative action on proteins, DNA, and lipids (Jomova and Valko 2011; Hossain et al. 2012). Duckweeds are one of the numerous macrophytes that can take up heavy metals from aqueous solution and are being used for the heavy metal phytoremediation of aquatic ecosystems (Rai 2009).

Few studies of heavy metal removal from contaminated waters by duckweeds have been carried out using wastewater itself. Teixira et al. (2014) showed the accumulation of up to 19 mg iron (Fe)/tissue dry weight (DW) in *L. minor* from an Fe-rich discharge from an abandoned coal mine in Portugal, and Iram et al. (2012) determined bioconcentration factors (BFCs) ranging from 1760 to over 18,000 for the uptake of zinc (Zn), manganese, and Fe, respectively, by the same duckweed from bio-treatment ponds of sewage water from offices and hotels in Pakistan. However, most investigations have studied the take-up of metals from culture medium or metal-free water samples spiked with the metals at concentrations deemed to be representative of particular contaminated waters. This facilitates the application of known metal concentrations and the quantification of uptake rates with a view to understanding metal take-up in order to make practical use of this knowledge in the future.

Shi et al. (2011) showed that copper (Cu) in the form of both soluble Cu^{2+} ions and copper oxide (CuO) nanoparticles (NPs) was taken up by *L. punctata* from culture medium and incorporated into the frond tissue, whereby much more of the CuO-NPs were accumulated (up to 800 μg/g DW) than was the soluble metal ion. Chaudhuri et al. (2014) determined that *L. minor* and *S. polyrhiza* accumulated up to 4.8 and 5.8 mg cadmium (Cd)/g DW, respectively, from pond water spiked with 2 mg/L of the heavy metal, and Uysal (2013) showed *L. minor* to take up chromium ions (Cr^{6+}) to 4.4 mg/g DW from water in a continuous flow system considered to be indicative of large-scale wastewater treatment ponds and natural wetland water remediation systems. Megateli, Semsari, and Couderchet (2009) found that *L. gibba* took up all of the Zn, 90% of the Cu, and 85% of the Cd from a nutrient solution spiked with the 10 μg/L of each of the heavy metals.

Wastewater and environmental water often contain multiple metals. Sekomo et al. (2012) found that *L. minor* took up over 50% of the Cr and about 40% of the Zn from a nutrient solution spiked with multiple metals at concentrations approximating those found in aerobically pre-treated textile wastewater, but lead (Pb), Cd, and Cu were taken up to a much lesser extent. Ücüncü et al. (2013) determined that over 90% of each of the Cr and Pb, but less than 50% of the Cu, were removed by *L. minor* from greenhouse cultivation pool water spiked with mixtures of the metals at concentrations exceeding those considered acceptable for Turkish inland waters.

Although duckweeds can take up and remove heavy metals from solution, they only do this effectively when the metal concentrations present in the waters are not seriously toxic to the macrophytes. The studies presented above showed that heavy metal uptake rates decreased when the metal concentrations exceed certain values. Appenroth et al. (2010) determined that while both *S. polyrhiza* and *L. minor* accumulated considerable nickel (Ni) at high Ni concentrations, the duckweeds only grew well at much lower concentrations of the metal at which Ni take-up was insignificant. This shows that the physiological potential of duckweeds to take up heavy metals may not always translate into effective water remediation in practice.

Other substances related to metals can also be taken up by duckweeds. Arsenic (As) is a toxic metalloid found in natural waters upon release into the environment through the agricultural use of pesticides and wood preservatives and industrial processes such as mining and alloying. Goswami et al. (2014) determined that *L. minor* removed 70% of the As^{3+} from a 0.5 mg/L solution of As_2O_3 and accumulated 0.65 mg of the metalloid/g

fresh weight (FW). The uptake of As compounds by duckweeds may be influenced by bacterial communities associated with the fronds. Xie, Su, and Zhu (2014) determined that As^{3+} in the medium of *Wolffia australiana* was rapidly oxidized to As^{5+} in the presence of such microorganisms. This reduces the amount of As^{3+} available for uptake by the duckweeds (via aquaporins), while the uptake of the resultant As^{5+} via cell membrane phosphate transporters is inhibited by high ambient PO_4^- concentrations. The remediative value of duckweeds in removing As from contaminated waters must thus be assessed in terms of associated bacterial flora and the PO_4^- content of the water. Even non-living duckweed can remove As and possibly also metals from aqueous solution: dried and shredded fronds of *L. minor* gathered from natural watersheds adsorbed up to 20 μg As^{5+}/g from a 0.4 mg/L solution (Romero-Guzman et al. 2013). While a chalcogen and not a metal, selenium (Se) is a toxin that can accumulate in surface waters via industrial discharge and agricultural runoff. Mechora, Stibilj, and Germ (2015) determined that *L. minor* is particularly suited to selenite (Se^{4+}) uptake, as it accumulated up to 19.5 mg/g DW of this ion from a 10 mg/L solution, more than other macrophytes involved in wastewater remediation.

2.3. Removal of organic xenobiotics

A great variety of toxic organic xenobiotic compounds can be released into the environment via wastewater effluents and agricultural spraying. Some of these may actually be taken up by duckweeds. Brain et al. (2008) found that *L. gibba* accumulated up to 1.2 μg of the sulfonamide (SN) sulfamethoxazole (SMX) per gram of tissue weight from a 100 μg/L solution, and Dosnon-Olette et al. (2010) showed that both *S. polyrhiza* and *L. minor* accumulated the fungicide dimethomorph up to 41 and 26 μg/g FW from a 0.6 mg/L solution. It is not clear to what extent such compounds are metabolized to harmless products when they are taken up. Böttcher and Schroll (2007) showed that most of the phenyl urea herbicide isoproturon taken up by *L. minor* from a 58 μg/L solution (13.5 μg/g DW, corresponding to a BCF of 243) accumulated unchanged in the fronds, whereas Toyama et al. (2006) observed the crop protection agent 2,4-dichlorophenol to be both taken up and degraded by *S. polyrhiza*.

Extracellular or extraorganismic processes may also be important in the duckweed-mediated removal of organic xenobiotics from solution. Reis, Tabei, and Sakakibara (2014) found that several macrophytes, including *S. polyrhiza* and *Lemna aoukikusa* (identical with *Lemna aequinoctialis*: Borisjuk et al. 2015), completely removed the phenolic endocrine-disrupting chemicals (EDCs) bisphenol-A, 4-*tert*-octylphenol, and 2,4-dichlorophenol, that were present at concentrations found in the environment, from solution within 6 d. The fronds contained very little of the EDCs, which were considered to have largely undergone oxidative degradation by cell wall-bound peroxidases. Jansen, Hill, and Thorneley (2004) described an extracellular peroxidase activity released by *Spirodela punctata* (*L. punctata*: Les and Crawford 1999) into its growth medium in response to exposure to phytotoxic halogenated phenols that catalyzed the oxidative dechlorination of 2,4,6-trichlorophenol.

Rhizosphere-associated bacteria can be responsible for the removal of xenobiotics from contaminated waters observed in the presence of duckweeds. Toyama et al. (2006, 2009) found that both aniline and phenol removed from solution in the presence of *S. polyrhiza* were degraded by bacteria metabolically stimulated by the presence of the duckweed rhizosphere. Ogata et al. (2013) determined that the uptake of 4-*tert*-butylphenol from environmental water samples in the presence of *S. polyrhiza* did not derive from the

duckweed itself, but rather from biodegradation by the bacterium *Sphingobium fuliginis* that was stimulated by root exudates from the macrophyte. Yamaga, Washio, and Morikawa (2010) isolated phenol-degrading bacteria from the rhizosphere of *L. aoukikusa* (*L. aequinoctialis*), one of which readily colonized the surface of sterilized roots of the duckweed, formed biofilms there, and resulted in long-term removal of phenol. And Kristanti et al. (2014) demonstrated that associations of each of four nitrophenol (NP)-degrading bacterial species with the roots of *S. polyrhiza* resulted in rapid complete or near-complete removal of NPs from both synthetic nutrient medium and sewage wastewater.

2.4. The problem of water contaminant disposal

The biomass produced by duckweeds growing on wastewater or contaminated surface waters contains the unwanted water solutes that the duckweeds have taken up. When macronutrients alone represent the water contaminants, they are assimilated into nontoxic and utilizable biomass. However, heavy metals taken up by duckweeds are at best temporarily neutralized by complexation with phytochelatins (Pal and Rai 2010), and they retain their toxic character within the biomass. As indicted in Section 2.3., some toxic organic xenobiotics may also remain unchanged after being taken up, and it is a significant challenge for future research to definitively ascertain the metabolic fate of the numerous xenobiotic compounds that can be ingested by duckweeds. Duckweed biomass resulting from the phytoremediation of heavy metals and some organic xenobiotics is thus not suitable for animal fodder or fertilizer. It can be processed for biofuel production, but the heavy metal and possibly also the xenobiotic content of the residue must then still be dealt with. Combustion can effectively destroy organic xenobiotics in residual biomass, but other measures such as metal reclamation techniques will be required to guarantee the final release of heavy metal-free duckweed biomass residue.

3. Growth impairment due to the uptake of or exposure to water contaminants

As illustrated in many of the remediation studies discussed above, duckweeds cultured on wastewater, surface waters, or culture medium containing macronutrients, heavy metals or organic xenobiotics exhibit strongly retarded growth when the concentrations of the contaminating substances are sufficiently high. An example is provided in Figure 2. Toxic substances which a duckweed takes up or comes into contact with can thus have deleterious effects on the macrophytes themselves, which manifest themselves ultimately as reduced growth. Parameters of growth measured to document this reduction include frond number, area, FW, and DW. For example, Gubbins, Batty, and Lead (2011) showed the toxicity of silver (Ag) oxide NPs to *L. minor* in terms of frond number and DW, and Bian et al. (2013) observed Ag^{2+} ions to result in decreases in frond number and FW in *L. gibba*. However, Babu et al. (2003) measured only frond number and Goswami et al. (2014) only biomass in determining Cu and As toxicity to *L. gibba* and *L. minor*, respectively. Of course, the impaired growth is caused by physiological and biochemical perturbations effected by the contaminants, as will be discussed in Section 5 on biomarkers. But the mere observation of reduced growth of a duckweed in the presence of a particular water constituent is sufficient to indicate toxicity of that constituent, irrespective of the action of the constituent or the metabolic disturbance leading to the growth inhibition. Indeed, Brain and Cedergreen (2009) have cited many studies in which growth inhibition was used as the sole biomarker of toxic effect to duckweeds. The reduction of growth is

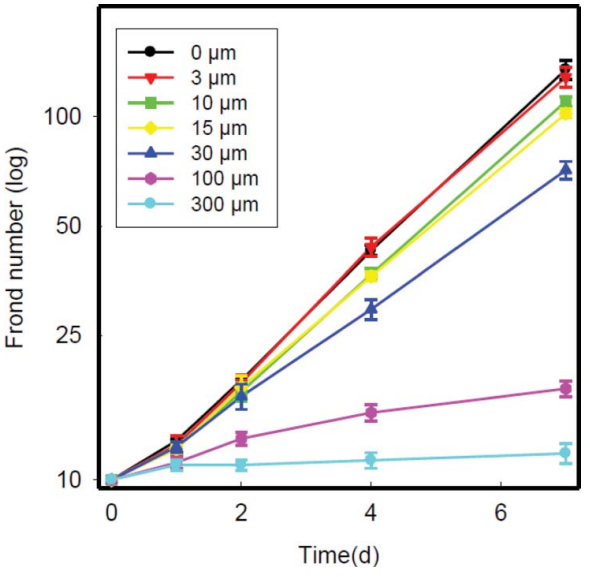

Figure 2. The influence of selenate (SeO_4^{2-}) on the growth of *Lemna minor*. Ten fronds of *L. minor* (3-frond and 4-frond colonies) were inoculated onto 300 mL portions of culture medium in beakers (surface area 50 cm^2) containing the indicated concentrations of Na_2SeO_4 and cultivated for one week as specified in the ISO (2005) toxicity testing protocol (see Section 4.1). The number of fronds observed after 1, 2, 4, and 7 d of culture was recorded. Selenate concentrations upwards of 30 µM seriously inhibited growth.

easily determined and forms the basis of standardized toxicity testing using duckweed as a test organism.

Another general indicator of toxicity to duckweeds related to growth impairment is reduction in the content of photosynthetic pigments. This reduction – often readily apparent to the naked eye – reflects a decrease in the light-gathering potential underlying the photosynthetic capacity of duckweeds as photoautotrophic organisms. Most of the phytoremediation studies described above took changes in photosynthetic pigment content into account in determining toxicity, and Brain and Cedergreen (2009) have compiled a number of further examples to this effect. Naumann, Eberius, and Appenroth (2007) ranked the toxicity of 10 heavy metal ions to *L. minor* based on reductions in chlorophyll and carotenoid contents as well as on decreases in growth parameters (see Table 2). Demim et al. (2013) showed that certain combinations of heavy metal ions were particularly effective in inhibiting both growth and chlorophyll and carotenoid contents of *L. gibba*, and Shi et al. (2011) found that, while comparable doses of Cu in soluble form and as CuO-NPs similarly inhibited the growth of *L. punctata*, the NPs were more effective in decreasing the chlorophyll content of the fronds. Lahive, O'Halloran, and Jansen (2011) used chlorophyll absorbance in addition to biomass and frond number growth rates to show that different duckweed species exhibit different sensitivities to $ZnSO_4$, which generally affected chlorophyll absorbance more than the growth parameters. Megateli, Semsari, and Couderchet (2009) observed that low concentrations of each of Cu and Cd resulted in strong decreases in the chlorophyll/pheophytin ratio D665/D665a, an indicator of physiological stress (Lopez, Retuerto, and Carballeira 1997), as well as in the growth rate of *L. gibba*. On account of its close relationship with growth, chlorophyll content is sometimes included as a measurement parameter in standardized duckweed toxicity testing.

Table 2. Toxicity of heavy metals to *Lemna minor*. Ten fronds of *Lemna minor* L., clone St, were cultivated in the presence of various concentrations of heavy metals according to the ISO 20079 test protocol as described by Naumann, Eberius, and Appenroth (2007). The EC_{50} values (i.e., the metal concentrations effective in inhibiting the increment of the investigated duckweed growth parameter by 50% in comparison to the metal-free control) respective of dry weight and chlorophyll content (mean concentrations and concentration ranges observed; $n = 6$) are shown for each metal.

Metal		EC50 (mg/L)	
Name	Ion	Dry weight	Chlorophyll content
Arsenic	AsO_4^{3-}	73.7 (53.2−101)	8.18 (6.37−12.3)
Arsenic	AsO_2^{-}	2.18 (2.16−2.20)	2.04 (1.99−2.17)
Cadmium	Cd^{2+}	0.241 (0.151−0.384)	0.102 (0.086−0.125)
Chromium	CrO_4^{2-}	2.30 (0.87−8.50)	0.155 (0.028−2.77)
Cobalt	Co^{2+}	0.542 (0.429−1.201)	0.163 (0.152−0.175)
Copper	Cu^{2+}	0.157 (0.120−0.181)	0.136 (0.121−0.153)
Mercury	Hg^{2+}	0.221 (0.162−0.301)	0.135 (0.092−0.205)
Nickel	Ni^{2+}	0.655 (0.374−1.147)	0.191 (0.172−0.210)
Silver	Ag^{2+}	0.031 (0.026−0.037)	0.037 (0.17−0.065)
Thalium	Tl^{+}	0.338 (0.241−0.433)	0.277 (0.263−298)
Zinc	Zn^{2+}	1.05 (0.51−2.31)	0.601 (0.316−1.08)

Note: Data taken from Naumann, Eberius, and Appenroth (2007).

4. Toxicity testing

The toxicity of chemicals found in wastewaters and surface waters is widely tested on duckweeds as model aquatic higher plants. Standardized test procedures reveal such toxicity in terms of the growth inhibition and photosynthetic pigment depletion of the duckweed test organisms discussed in Section 3**.** If a particular substance proves to be toxic to duckweed in such a test, it may be considered to be a potential toxin for all aquatic higher plants. The potential toxicity of the substance to aquatic habitats in a more comprehensive context can be assessed when duckweed testing is complemented by toxicity tests on other aquatic life forms.

4.1. Standardized tests of toxicity to duckweeds

L. minor was proposed in 1979 to be a "representative" aquatic macrophyte for assessing the environmental safety of chemicals (see EC 2007). On the basis of experience in duckweed toxicity testing up till the end of the 1980s (see Wang 1990), duckweed test methods have been recommended by a number of national and international organizations (see EC 2007), including the Organization of Economic Cooperation and Development (OECD: OECD 2006) and the International Organization for Standardization (ISO: ISO 2005). These methods are equally useful for identifying toxicity present in wastewaters and surface waters and for determining the toxicity of substances that may be found in such waters. The choice of duckweeds as model organisms for higher aquatic plants is based on characteristic attributes of these macrophytes that make them particularly suited to toxicity testing.

In addition to their small size and rapid growth, duckweeds are particularly easy to cultivate in simple flasks or dishes on liquid medium. Duckweeds are grown under

controlled laboratory conditions for standardized toxicity testing, and this avoids the impact of unfavorable environmental conditions that might adversely affect the duckweed, thus confining the source of any adverse effects to contaminants added to the culture medium. Their predominantly vegetative reproduction enables them to be grown indefinitely as genetically homogeneous clonal colonies, and their high surface-to-volume ratio and lack of cuticle on their surface in contact with water ensure ready contact with substances dissolved in the culture medium (see EC 2007). Their amenability to sterilization is important for differentiating between toxic effects specific for the higher plant and those affecting associated microorganisms.

Only 2 of the 37 known duckweed species are specified for use in the toxicity tests, the *Lemna* species *L. minor* (in Canada and Europe) and *L. gibba* (in the USA). This is in accordance with the original proposal from 1979 and recommendations based on the historical data as to duckweed toxicity testing obtained with these species (Wang 1990). Although other species of *Lemna* and other genera also have been used for toxicity testing, it is convenient to have defined standard species for ensuring comparative test results between scientific and governmental organizations. In some cases, particular clones are recommended for testing (e.g., *L. minor* Landolt clones 8434 and 7730 by Environment Canada (EC) (EC 2007)), in other test specifications they are not (e.g., ISO 2005).

The inhibition of growth as the overall indicator of toxicity to duckweeds due to noxious substances as discussed in Section 3 finds application in the use of the growth parameters frond number, frond area, FW, and DW as end points of effect in all standardized duckweed toxicity tests. The evaluation of two end points is usually required for the test result, and in some cases, these are both growth parameters, such as the measurement of frond number and DW required by the EC protocol (EC 2007). In other protocols, the chlorophyll content that reflects the total photosynthetic capacity of the plant (see Section 3) can also be used. For example, the ISO specifies the assessment of either frond area, DW, or chlorophyll content in addition to frond number (ISO 2005). The inhibition of growth or pigment accumulation (see, e.g., Figure 3) is expressed in standardized value form, such as the IC_{50} or EC_{50}, the inhibitory or effective concentration required to inhibit the development of the end-point parameter by 50% (examples illustrating heavy metal toxicity are shown in Table 2, and herbicide toxicities in Table 3). Cedergreen, Abbaspoor, et al. (2007) have pointed out that non-growth parameters such as chlorophyll content may behave differently to purely growth parameters, especially in the presence of mixtures of toxic substances. Chlorophyll content can thus complement, but not replace, growth parameters such as frond number or weight as toxicity biomarkers.

The various toxicity testing guidelines most basically provide for the duckweeds to be cultivated for one week in a single batch of defined medium containing the test substances (static test). A good example is the ranking of the toxicity of 10 heavy metal ions to *L. minor* using the ISO 20079 testing methodology described by Naumann, Eberius, and Appenroth (2007). However, provisions are also made for replenishing the medium at intervals (semi-static test) or continually (flow-through test) to ensure more consistent exposure to toxicants, especially when these are short-lived in aqueous solution. Brain and Solomon (2007) published a protocol for conducting simple daily nutrient replenishment tests with *L. gibba* to optimize toxicant exposure in semi-static procedures. An example of advantages of a flow-through assay in comparison with a batch assay was illustrated by Clement, Delhaye, et al. (2014) with a microcosm of microalgae, *L. minor*, daphnids, amphipods, and chironomids, using Cd as a model toxicant, over a time span of 21 d. The growth and fitness of all the tested organisms were improved in the flow-through setup in comparison to the batch procedure, and the physicochemical and

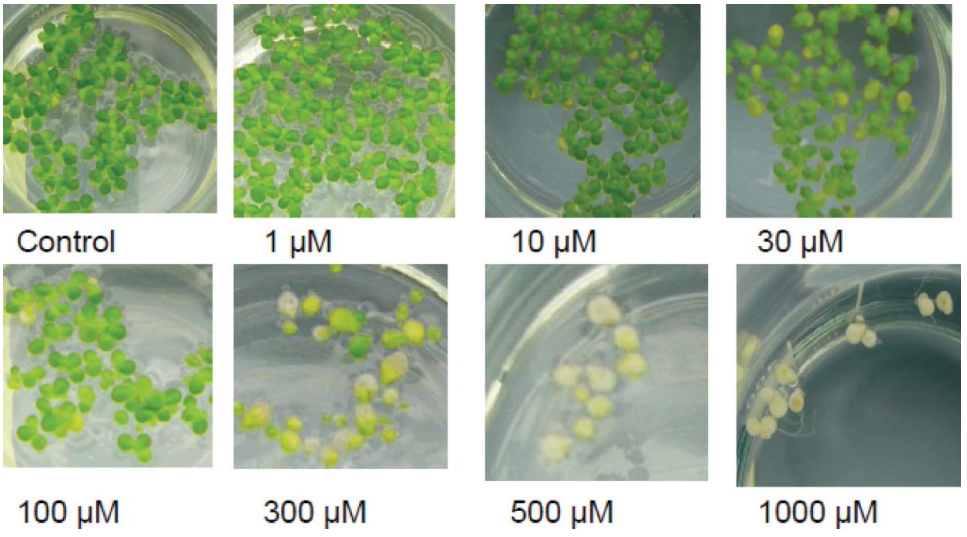

Figure 3. The results of a test of the toxicity of selenite (SeO_3^{2-}) on *Lemna minor* carried out according to the ISO (2005) protocol. Ten fronds of *L. minor* (3-frond and 4-frond colonies) were inoculated onto 300 mL portions of culture medium in beakers (surface area 50 cm^2) containing the indicated concentrations of Na_2SeO_3 and cultivated for one week as specified in the ISO (2005) toxicity testing protocol. The frond number present after this time was markedly reduced at selenite concentrations upwards of 30 µM (compare Figure 2), and loss of chlorophyll in the fronds still present was particularly noticeable at >100 μM selenite.

Table 3. Toxicity of herbicides to *Lemna aequinoctialis* (*L. paucicostata*). Three fronds of the duckweed were grown on Hoaglands medium on a miniaturized scale under standardized conditions in an incubator, and growth was determined over 7 d as described by Michel et al. (2004). The EC_{50} values were calculated as the median effective concentrations of the herbicides found to reduce the control growth of the duckweed (as increase of total frond area) by 50%.

Herbicide		
Name	Affects	**EC_{50} (μM)**
Chlorsulfuron	Acetolactate synthase	0.005 ± 0.0002
Alachlor	Long chain fatty acid elongases	0.052 ± 0.004
Isoproturon	Photosystem II D1 protein	0.349 ± 0.027
Paraquat	Photosystem I	0.617 ± 0.060
4,6-Dintro-o-cresol	Transmembrane proton gradient	3.022 ± 0.201
Chlorpropham	Microtubule organization	4.928 ± 0.325
Glufosinate	Glutamine synthase	22.53 ± 0.669
S-ethyl dipropyl carbamothiolate	Long chain fatty acid elongases	62.34 ± 9.574
Naphtalam	Auxin effluent receptor	127.6 ± 5.308
Glyphosate	Enopyruvylshikimate synthase	387.5 ± 27.73

Note: Data taken from Michel et al. (2004); the data shown in the table were selected to illustrate the range of the EC_{50} values found for the 26 herbicides investigated.

biological system parameters showed less variation. However, the technical requirements of the flow-through technique will restrict its practical application. Many of the investigations having been carried out to assess the uptake of heavy metals and organic xenobiotics by duckweeds (see Section 2) and to elucidate the toxic action of these substances on the duckweeds (see Section 5) have also made use of the methodologies developed for toxicity testing, or procedures similar to them, as a cultivation framework for the uptake or mechanistic studies.

It is of interest that testing with duckweeds can serve not only to establish the presence of toxicity, but also to document its removal. A good example of this is the finding of Cayuela et al. (2007) that extracts of olive mill waste, a major pollutant in many Mediterranean regions with high concentrations of toxic phenols, strongly inhibited the growth of *L. gibba*. Composting of the olive mill wastes with other agricultural material progressively and dramatically reduced the toxicity of the waste extracts according to the same test protocol. The duckweed toxicity test thus served to assess both the toxicity of olive mill wastes and the degree of maturity of the waste composts. Testing with duckweeds can also help to assess the actual threat posed by hazardous chemicals. Although the statin pharmaceuticals, atorvastatin and lovastatin, were found to elicit toxic effects on *L. gibba* in standardized tests, they were considered to pose little risk to the duckweed at environmentally relevant concentrations (Brain et al. 2006).

4.2. Limitations of the standardized toxicity tests

While the standardized toxicity tests all operate on similar principles, they still make use of two different test species and varied cultivation and measurement parameters. The goal of standardized testing must be the establishment of a uniform test procedure that employs a single duckweed species - and clone of this species - and is carried out according to a generally accepted protocol to ensure universally comparative and reproducible test results. On the other hand, the observation of considerable differences in the growth of 41 different duckweed clones growing on toxic swine wastewater (Bergmann et al. 2000b; see Section 2.1) raises the question of whether toxicity testing with only one (or two) duckweed species is realistic for assessing toxicity. There is no practical alternative to testing with a single, "standardized" duckweed species or clone for initially identifying the presence of toxicity. However, it must be realized that other duckweeds might be better suited to quantifying this toxicity, and that these test organisms would have to be screened for.

The standard experimental conditions employed in any standardized duckweed toxicity test will not accurately reflect the actual environmental conditions under which the test organisms live in nature. The toxicity of a given compound in the field will always be influenced by the environmental conditions actually effective there. Rosenkrantz et al. (2013) have drawn attention to this in showing that the pH of the culture medium significantly influenced the toxicity of four sulfonylurea herbicides to *L. gibba*. Dosnon-Olette et al. (2010) showed that plant density had a significant influence on the toxicity of the pesticide dimethomorph to *L. minor* and *S. polyrhiza* and on its uptake by the duckweeds. Although standardized toxicity tests must adhere to their protocols, ambient conditions should always be taken into account when environmental quality standards are to be derived from the test results, particularly when they deviate significantly from the growth conditions specified for the standard tests. The implementation of this consideration in ecotoxicity assessment is a challenge for the future.

Even though duckweeds have proved to be a convenient and versatile organism for toxicity testing, as free-floating monocots they are not necessarily sufficient for risk assessment for macrophytes in general. Dicotyledonous aquatic plants may differ considerably from monocotyledonous ones in morphology and physiology, and a duckweed can also not properly represent a rooting aquatic monocot. Mohr et al. (2013) pointed to this in investigating the suitability of the dicotyledonous water milfoil *Myriophyllum spicatum* for toxicity testing in test systems in which the plant floated freely, was rooted in the aquatic sediment, and was a component of a species microcosm. In addition, the action of toxic xenobiotics on aquatic plants may differ from that on terrestrial plants. Cedergreen, Kudsk, et al. (2007) showed that two terrestrial plant species proved more sensitive to numerous herbicides than did *L. minor* and an alga, emphasizing that terrestrial non-target plants should generally be included in herbicide risk assessment.

While testing with duckweed alone to assess the toxicity of waters or substances present in them is valuable from the viewpoint of higher aquatic plants, it assumes greater ecological significance when it is combined with testing of other life forms also inhabiting natural aquatic habitats. An example of an investigation of heavy metal toxicity in this regard is the study of Levard et al. (2013), who showed that the environmentally relevant sulfidation of AgO-NPs, which are produced industrially to exploit their conductive, optical, and antimicrobial properties, considerably reduced the toxicity of the pristine nanoparticles to fish embryos and nematode worms in addition to *Lemna minuta*. In contrast to such investigations, in which the individual species were separately tested, microcosms of multiple species can be subjected to water contaminants in the same reaction volume. For example, Clément, Delhaye, et al. (2014) examined the effects of Cd on a community of algae, daphnids, amphipods, and chironomids in addition to *L. minor* in demonstrating the advantages of flow-through over batch culture. In the following, some further insights into environmentally relevant toxicity gleaned from tests with duckweeds together with other aquatic organisms are presented.

4.3. Tests of toxicity to duckweeds along with other aquatic life forms

Toxicity testing with duckweeds together with other aquatic organisms has been useful in assessing the ecotoxicological potential of complex wastewaters. For example, coking wastewater derived from processes such as coal carbonization, coal gas purification, and chemical product refining involved in producing coke contains numerous toxic and hazardous substances such as metals, phenols, cyanides, polycyclic aromatic compounds, and heterocyclic substances. Zhao et al. (2014) showed that raw coking wastewater was highly toxic to *L. minor* according to the OECD (2006) protocol, as well as to bacteria, green alga, a crustacean, and zebrafish embryos. Sequential primary, biological, and clarifier treatment of the wastewater reduced the toxicity to all organisms to essentially nil.

Disposal of solid municipal and industrial waste in landfills seeks to isolate this material from the environment at large, but toxic aqueous material can emanate from the landfill to contaminate surrounding waters. Clement, Guillen, et al. (2014) used *L. minor*, both alone and in a microcosm of other pelagic and benthic freshwater species, to assess the toxicity of complex leachates from seaport sediments that were highly contaminated with metals, polycylic aromatic hydrocarbons, tributyltin, and chlorides. The duckweed, together with an amphipod, was more sensitive to high leachate concentrations than were algae, daphnids, and chironomids, and its growth was inhibited more strongly as a single-species test organism than as a microcosm component. This shows both the comparative sensitivity of the duckweed as a test organism, and that toxic effects on isolated test

organisms may not be representative of the organism in its natural association with freshwater organisms.

Non-target aquatic organisms are often exposed to herbicides and fungicides when these chemicals gain access to the hydrosphere via rain runoff after spraying and spray drift. Gatidou, Stasinakis, and Iatrou (2015) investigated the toxicity of the substituted urea herbicides, diuron, linuron, and monolinuron on *L. minor* and the luminescent bacterium *Vibrio fischeri*. The growth of the duckweed was much more sensitive to the test compounds than was the light output of the bacterium, and combinations of the test compounds resulted in additive effects, due to the common phenylurea mode of action the herbicides. Chambers et al. (2014) reviewed the human and ecological risks associated with the environmental presence of epoxiconazole, an azole fungicide used as a crop protection agent. *L. gibba* proved to be clearly more sensitive to the fungicide than humans and terrestrial vertebrates, as well as aquatic vertebrates and invertebrates and algae. Since the response of *L. gibba* to triazole pesticides was deemed to be fairly representative of higher plants in general, the toxicity test data for the duckweed are valuable for setting limits for the tolerable presence of epoxiconazole and related compounds in the environment.

While standardized ecotoxicological test procedures aim at providing constant test substance concentrations over the whole test duration, the exposure of aquatic plants to pesticides often takes the form of a pulse occasioned by discrete rain and spraying events. Rosenkrantz, Baun, and Kusk (2013) examined the effect of short-term, high-exposure pulses of four sulfonylurea herbicides on *L. gibba* in comparison to that of a week of continual exposure as specified by the OECD guidelines. The growth rate of *L. gibba* exposed to relatively high concentrations of the herbicides for 24 h initially decreased more strongly than it did upon exposure to lower concentrations under the standardized 7-d test conditions. However, it subsequently recovered to the levels of the non-treated controls during the 6 d period following the removal of the herbicide. On account of this, the authors concluded that although the pulse-exposure test may more accurately represent naturally occurring herbicide concentration fluctuations, it need not supplant the existing standardized testing procedures.

Antibiotics are of concern because of their copious consumption and release into the environment, and duckweeds can be better indicators of their toxicity than other aquatic organisms. Wagil et al. (2014) determined the presence of the fluoroquinolones enrofloxacin (ENR), norfloxacin (NOR), and ciprofloxacin (CIP) in water samples, and tested their toxicity on marine bacteria, green algae, and crustaceans in addition to *L. minor*. The duckweed proved to be the organism most sensitive to the antibiotics, and contributed to the assessment of ENC and NOR as being of moderate, and CIP of high, environmental risk. SNs are antibiotics that are used widely in veterinary medicine and escape into the environment; a modern strategy to prevent them from doing so is electrochemical oxidation. Fabianska et al. (2014) tested the toxicity of five SNs and their oxidation products on *L. minor* and *Scenedesmus vacuolatus*. Whereas the algae test revealed no signs of toxicity, the duckweed growth was inhibited by both the SN substrates and their oxidation effluents. This shows that the electrochemical process must be carried out to complete mineralization to avoid SN derivatives from posing an ecotoxicity threat.

Duckweeds are not always the most sensitive ecotoxicity marker, as in the case of beta-blockers (BB), a group of extensively used pharmaceuticals that accumulate in and are of potential harm for the environment. Maszkowska et al. (2014) examined the effect of propranolol, metoprolol, and nadolol on an ecotoxicological test battery including marine and soil/sediment bacteria, green algae, and *L. minor*. The growth of the duckweed and the bacteria was not affected by the BB at concentrations of up to 100 mg/L,

whereas the reproduction of the green algae *S. vacuolatus* was inhibited. Nevertheless, the risks posed by these compounds to aquatic life forms were regarded to be of minor importance, since sorption restricts the action of the BB. In a similar vein, some toxicity tests with duckweeds and other organisms have indicated that particular pollutants do not pose a significant risk to the aquatic environment. Stolte et al. (2013) determined high No Observed Effect Concentrations for each of the artificial sweeteners, acesulfame, cyclamate, saccharin, and sucralose, on the growth of *L. minor*, the reproduction of the green alga *S. vacuolatus*, and the mobility of the water flea *Daphnia magna*.

5. Biomarkers of toxic effect

Although growth and photosynthetic pigment content reduction indicate overall toxicity to duckweeds, they in themselves do not point to the nature of the toxicity or the identity of the toxic agent. Observations and investigations on the morphological, histological, physiological, and biochemical levels can illustrate the structural and metabolic changes elicited in duckweeds by particular water contaminants. Structures or processes on these levels that have been correlated or causally linked to biological effects measured upon exposure of plants to xenobiotic stresses constitute biomarkers (Brain and Cedergreen 2009). Morphological biomarkers include the parameters of growth and developmental features that can be readily observed. They can be complemented by ultrastructural features revealed by microscopy. Photosynthesis pigments and processes, reactive oxygen species (ROS) and ROS-scavenging enzymes, stress proteins and phytochelatins, and pathway-specific enzymes and metabolites are physiological/biochemical markers that respond in various ways to the presence of toxic substances and stressors, whereas gene expression can also respond to any stress situation. Brain and Cedergreen (2009) have compiled extensive stress-related biomarker data for a number of aquatic plants, including duckweeds, in addition to evaluating the advantages and disadvantages of individual biomarkers for toxicological testing. Growth parameters and photosynthetic pigment contents as global biomarkers of toxicity to duckweeds have been discussed in Section 4.1. In the following, a selection of further biomarkers observed with duckweeds in response to water contaminants is described, based on recent studies where possible. These biomarkers are pollutant-specific, responding to water contaminants investigated within the context of the controlled cultivation conditions typical of standardized toxicity testing, and do not relate to non-pollutant environmental stressors. The usefulness of these biomarkers for identifying substances actually responsible for toxicity is also discussed.

5.1. Morphological and ultrastructural biomarkers

A growth-related morphological phenomenon in duckweeds related to xenobiotic toxicity has been described by Topp et al. (2011) and Henke, Eberius, and Appenroth (2011) for *L. minor*. Treatment with each of 10 heavy metals and Se resulted in the release of daughter fronds from the mother frond before they had attained maturity, resulting in colony disintegration. This abscissive phenomenon, which was often less sensitive to the metals than overall growth, may be a biomarker for heavy metals. It has, however, also been observed with *L. minor* in complex surface waters not containing high concentrations of these metals (e.g., Brkanac et al. 2014).

Exposure to heavy metals affects the chloroplast ultrastructure of duckweeds as revealed by transmission electron microscopy. Appenroth et al. (2010) found that chloroplasts of both *S. polyrhiza* and *L. minor* developed massive starch inclusions

characteristic of amyloplasts in response to nickel (Ni^{2+}), presumably due to the reduced export of photosynthate from the plastids due to effects of the metal. Similar effects were noted by Sree, Keresztes, et al. (2015) with *L. minor* exposed to cobalt ions (Co^{2+}). Although they did not detect this starch accumulation, Basile et al. (2015) noted structural and organizational aberrations in *L. minor* chloroplasts due to exposure to Cd, Cu, Cr, Pb, and Zn in both actual contaminated wastewater and spiked *in vitro* culture medium. Lalau et al. (2015) reported similar findings with *L. punctata* in response to exposure to CuO-NPs. While chloroplast aberrations may result from the effects of many stressors, starch accumulation may be specific for exposure to at least some heavy metals.

5.2. Physiological biomarkers

Impairments of photosynthetic activity or respiration are physiological indicators of toxic effect that are more process-specific than mere overall decreases in photosynthetic pigment contents or ratios. However, few studies have been carried out in this regard. Drinovec et al. (2004) showed that the presence of each of Cu, Cd, and Zn resulted in a decrease in delayed chlorophyll fluorescence intensity (DFI) measured upon illumination of *L. minor*. DFI was considerably more affected than the rapid fluorescence Fv/Fm ratio widely used to evaluate photosynthetic performance, and its decreases were much more rapidly evident than was the accompanying inhibition of growth. Mitrovic et al. (2004) measured a decrease in photosynthetic O_2-evolution (POE) in *L. minor* in response to exposure to anatoxin-a, a phytotoxic neurotoxin released into lakes and rivers by cyanobacteria blooms. Mechora, Stibilj, and Germ (2015) showed that both photochemical efficiency (as the Fv/Fm ratio) and respiratory potential (as terminal electron transport system activity: ETS) were reduced in *L. minor* exposed to high concentrations of selenite. Decreased DFI, Fv/Fm, POE, and ETS thus point to serious impairments of fundamental plant physiology processes in duckweeds.

5.3. Molecular biomarkers

All chemical stressors have their initial toxic effect at the molecular level, and biomarkers at this level should thus be the earliest and most sensitive indicators of toxic effect (Hightower 1998). Responses of enzymes, proteins, and metabolites in duckweeds to chemical stressors are examined in the following sections.

5.3.1. Detoxification enzymes

As described by Brain and Cedergreen (2009), plant enzymes metabolize drugs, herbicides, and other organic xenobiotics to non-phytotoxic products. The extracellular peroxidase (POD) reported by Jansen, Hill, and Thorneley (2004: see Section 2.3) to be released by *S. punctata* in response to exposure to phytotoxic halogenated phenols was able to oxidatively dechlorinate 2,4,6-trichlorophenol and thus detoxify the phenol. The decrease in POE observed by Mitrovic et al. (2004) in *L. minor* upon exposure to anatoxin-a was accompanied by a significant increase in the activity of both POD and glutathione-S-transferase, which conjugates the toxin to glutathione. Although many environmental contaminants can act as substrates for the detoxifying enzymes, the release of extracellular *S. punctata* POD was specific for the halogenated phenols and not for other stress-inducing xenobiotics including heavy metals, elicitors, herbicidal auxin analogs, and bioactive phenols.

5.3.2. ROS-scavenging enzymes

Reactive oxygen species (ROS) are generated in plants in response to many stressors, including unfavorable environmental conditions, pathogenic organisms, and UV radiation, and their formation induces enzyme activities to combat their oxidative effects (Brain and Cedergreen 2009). Exposure of *L. minor* to each of $CuSO_4$ and folpet, which are used to control mildew and other fungal diseases in grapes, led to an increase in the activities of catalase (CAT), ascorbate (AP), guaiacol (GP), and pyrogallol peroxidase (PP) (Teissere and Guy 2000; Teisseire and Vernet 2001). The coordinated stimulation of enzyme activities known to be involved in ROS scavenging suggested that the toxic effects of both Cu^{2+} ions and folpet derive from ROS formation. Babu et al. (2003) then showed that exposure of *L. gibba* to Cu indeed led to enhanced ROS production, which was accompanied by enhanced superoxide dismutase (SOD) and glutathione reductase (GR) enzyme activity levels. The polycyclic aromatic hydrocarbon 1,2-dihydrooxyanthraquinone, which often occurs together in the aqueous environment with Cu, led to a synergistic up-regulation of SOD and GR activities in *L. gibba* in the presence of Cu on account of enhanced ROS production (Babu, Tripuranthakam, and Greenberg 2005). Razinger et al. (2008) showed that the total antioxidative potential of *L. minor* was enhanced upon exposure to low concentrations of Cd, but was weakened at higher concentrations resulting in growth inhibition, at which the activities of CAT, AP, GP, and GR all decreased. On the basis of increased SOD, CAT, and POD activities, Hu et al. (2013) determined that ZnO-NPs were harmful to *S. polyrhiza* at relatively high concentrations. Since much lower concentrations of $ZnSO_4$ gave the same results, Zn^{2+} released from the NPs may be responsible for the toxic effects of these particles.

Even though numerous environmental and biotic stressors elicit ROS production and the induction of antioxidant defenses in addition to water contaminants, most of them can be excluded from duckweeds grown under controlled laboratory conditions. The induction of ROS-scavenging enzymes can thus point to heavy metals as possible toxicants within the confines of standardized duckweed toxicity testing.

5.3.3. Stress-induced proteins

Environmental stressors often lead to the upregulation of "heat-shock" proteins in plants which are often constitutively expressed in small amounts and function in molecular chaperoning. The increased levels of these proteins then play an important role in protein repair and the prevention of protein damage (Hightower 1998; Brain and Cedergreen 2009). Treatment of *L. minor* with Cd resulted in the accumulation of the heat-shock protein Hsp70 (Ireland et al. 2004), and Santos et al. (2006) observed the accumulation of multiple heat-shock proteins upon exposure of the same duckweed to sodium arsenite ($NaAsO_2$). Basile et al. (2015) described Hsp70 induction in *L. minor* exposed to river water heavy metals, and Tukai et al. (2011) found that the polycyclic aromatic hydrocarbon anthracene and the herbicide chloridazon also stimulated the biosynthesis of Hsp70 in this duckweed.

As with ROS production and the induction of ROS-scavenging enzymes, the accumulation of heat-shock proteins can be elicited by many non-chemical stressors, such as UV radiation, high temperatures and osmotic values, hypoxia, and anoxia. Since these are also eliminated under the standardized growth conditions, duckweed Hsp70s can be toxicity biomarkers for heavy metals or certain organic xenobiotics in the context of duckweed toxicity testing.

5.3.4. Phytochelatins

Phytochelatins are enzymatically synthesized heavy metal-binding plant peptides involved in heavy metal take-up and detoxification via complexing and storage in vacuoles (Pal and Rai 2010). Yin, Zhou, and Lu (2002) showed that the small phytochelatins P2 and P3 accumulated strongly upon exposure of *L. aequinoctialis* to Cd, and Zhang et al. (2012) found that As taken up by *W. globosa* was complexed with mainly P3 and P4 phytochelatins. In both these studies, the accumulation of and tolerance to As by the duckweed were strongly inhibited in the presence of 1-buthionine sulfoximine, a potent inhibitor of phytochelatin synthase. Since phytochelatins are highly specific for heavy metals, they are good biomarkers for indicating exposure to heavy metals.

5.3.5. Pathway-specific metabolites

Both Babu et al. (2003) and Akhtar et al. (2010) found that exposure of *L. gibba* to Cu^{2+} ions induced the synthesis of UV-absorbing flavonoid compounds, mainly flavones, and a corresponding increase in chalcone synthase (CHS), the gateway enzyme of the flavonoid-synthesizing phenylpropanoid pathway. The Cu^{2+} ions were as effective in these regards as UV radiation, a well-known elicitor of CHS and flavonoid synthesis. Megateli, Semsari, and Couderchet (2009) determined that proline, an organic osmolyte that accumulates in plants in response to environmental stress (Kishor et al. 2005), showed a transient accumulation in *L. gibba* exposed to Cd, Cu, and Zn. Although both flavonoid production and proline accumulation can be stimulated by high light intensities, photosynthetic electron transport (PET) inhibition, pathogen attack, wounding, low temperatures, and nutrient deficiencies in addition to UV radiation (Kishor et al. 2005; Brain and Cedergreen 2009; Akhtar et al. 2010), heavy metals are the only environmental contaminants known to elicit these effects. Since non-contaminant stressors are eliminated under controlled cultivation conditions, the induction of CHS and the accumulation of flavonoids and proline can point specifically to the presence of heavy metal toxicity in standardized duckweed toxicity tests.

Statin pharmaceuticals for the treatment of hypercholesterolemia inhibit 3-hydroxy-3-methylglutaryl coenzyme A-reductase in plants, which regulates cytosolic isoprenoid biosynthesis in the mevalonic acid (MVA) pathway. Brain et al. (2006) found that exposure of *L. gibba* to atorvastatin and lovastatin elicited a decrease in the concentrations of stigmasterol and β-sitosterol, critical plant membrane components downstream in the MVA pathway that regulate morphogenesis and development and could function as biomarkers for exposure to the statins.

SNs used to treat human and animal diseases and infections act as structural analogs of *p*-aminobenzoic acid (*p*ABA) to inhibit the enzyme dihydropteroate synthase (DHPS) in the folate biosynthetic pathway. Brain et al. (2008) showed that exposure of *L. gibba* to the sulfonamide SMX resulted in an increase in the content of *p*ABA in a concentration-dependent manner that was 20-fold more sensitive in indicating toxicity than growth inhibition. The accumulation of *p*ABA as the substrate of blocked DHPS activity showed this metabolite to be a highly SN-specific biomarker.

5.4. Gene expression and DNA damage

Although changes in biomarkers associated with toxicity to duckweeds will ultimately derive from changes in gene expression, only few studies have actually illustrated this. Akhtar, Lampi, and Greenberg (2005) identified six genes that showed altered expression

in response to Cu in *L. gibba*. The transcript levels of the genes encoding callose synthase, heat-shock protein 90, serine decarboxylase, and the biotin carboxylase subunit of acetyl-coenzyme A carboxylase were enhanced, whereas those of the genes encoding the HAP5 subunit of a heme-activated protein transcription factor and the chloroplast nucleoid DNA-binding protein CND41 were decreased. Akhtar et al. (2010) also showed that Cu, as well as the PET inhibitors 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone and 1,2-dihydroanthraquinone, resulted in increased transcription of the genes encoding the flavonoid biosynthetic enzymes chalcone synthase and chalcone isomerase in the context of PET chain reduction leading to flavonoid accumulation. Santos et al. (2006) observed that the exposure of *L. minor* to 50 μM sodium arsenite resulted in a strong increase in the formation of large ubiquitin−protein conjugates paralleled by increases in the levels of transcripts coding for ubiquitin pathway components (polyubiquitin, E1 and E2, and the β-subunit and ATPase subunits of the 26S proteasome). Arsenite damage to proteins by reaction with sulfhydryl groups thus induced a fortification of the ubiquitin/proteasome pathway to remove the damaged proteins.

Water contaminants have been shown to result in damage to duckweed DNA, as revealed by strand breaks in the Single-Cell Gel Electrophoresis (or Comet) assay. Radic et al. (2010) proposed this assay with *L. minor* to be a sensitive indicator of genotoxic effects in surface waters and wastewaters on the basis of investigations with stream water near a pharmaceutical and food industry complex in Croatia. The Comet assay with the same duckweed was also used to monitor the genotoxicity of leachate from landfill from municipal and industrial wastes in Croatia (Brkanac et al. 2014) and from a copper mining and smelting complex in Serbia (Radic et al. 2014). Although oxidative stress was suggested to account for some of the observed DNA damage, the relatively low heavy metal concentrations present in the water samples investigated indicated that other, unidentified components of the samples may have been more important for the genotoxicity.

5.5. How useful are biomarkers in identifying toxicants?

Biomarkers of toxicity should point to the chemical agents responsible for the toxicity, and the more specifically and exclusively they are correlated with the action of a particular toxin or related group of toxins, the better is their diagnostic value in identifying the toxic agent(s). Unfortunately, many of the duckweed biomarkers discussed above are of limited use in identifying specific water contaminants because they apply to diverse toxicant species or they have not been shown to be specific for particular toxicants. As discussed in Section 2.4, the plant growth parameters and photosynthetic pigments routinely determined in assessing toxicity in duckweed tests have no diagnostic value in water contaminant identification, as they are affected by all substances that have significant deleterious effects on the macrophytes. The morphological/ultrastructural biomarkers premature frond colony disintegration and massive starch granule accumulation (Section 5.1) observed upon heavy metal treatment have not been looked for respective of other water contaminants. Similarly, the correlation of the biomarkers DFI, POE, Fv/Fm, and ETS with heavy metals, a phytotoxic neurotoxin and selenite, respectively (Section 5.2), was only observed in studies analyzing these physiological parameters with the particular toxicants applied, and it is to be expected that such basic physiological processes will be susceptible to many more substances exerting a toxic effect on duckweeds.

However, the identification of heavy metals as toxic agents can be carried out with some degree of confidence in terms of the numerous observations of ROS production and

the induction of ROS-scavenging enzymes in response to the presence of the metals (see Section 5.3.2), although it remains to be seen whether non-metal water contaminants also lead to such responses. In this regard, the induction of heat shock proteins in duckweeds by heavy metals is not a metal-specific response, as it is also elicited by at least two organic xenobiotics (Section 5.3.3). Although phytochelatins may be on the whole a reliable biomarker for heavy metals, as the function of these compounds is highly specific for the these metals, their synthesis can also be elicited by a the metalloid As (Section 5.3.4).

Pathway-specific metabolites may constitute the best diagnostic biomarkers for water contaminants in duckweed. This is especially evident respective of the intracellular concentrations of stigmasterol/β-sitosterol and *p*ABA as they respond to statin pharmaceuticals and SNs (Section 5.3.5), due to the specific metabolic pathways that these organic xenobiotics interfere with. It may also apply to flavonoid and CHS synthesis as markers of heavy metal toxicity (see Section 5.3.5), as long as no further toxicants are identified that elicit the same responses. Extracellular POD is also a highly reliable biomarker for halogenated phenols, on account of its observed specificity for this group of phytotoxic water contaminants (Section 5.3.1).

The diagnostic usefulness of duckweed biomarkers is also determined by the particular toxicity assessment context. In some instances, the nature of the toxicant being tested for is known in advance, e.g., the presence of heavy metals in effluent from mining wastewater or leachate from industrial landfill waste. In this case, an assay for ROS-scavenging enzymes or phytochelatins can complement a standard toxicity test to provide evidence for the presence of the metals. Similarly, if particular pesticides or pharmaceuticals are at issue in wastewaters or surface waters, tests for known physiological or metabolic biomarkers for these substances can be carried out. However, in many cases, the chemical substances responsible for the toxicity established for a particular water sample will not be known (even if some of them may be suspected), and no biomarkers are known for many of the numerous toxic organic xenobiotics that can infest wastewaters and surface waters. The establishment of specific biomarkers for these substances is an important research goal in improving duckweed toxicity diagnostics. But even given this, it is not practical to set up a battery of multiple biomarker tests to encompass unknown toxic water contaminants. A new approach is required to implement diagnostic toxicity testing with duckweeds on a comprehensive scale: as will be discussed in the following section, this can be realized on the basis of gene expression biomarkers, the development of which has been unduly neglected to date (see Section 5.5).

While some biomarkers can point to a group of toxic substances, e.g., heavy metals or certain classes of pesticides or pharmaceuticals, they cannot identify the specific chemical species responsible for the toxicity. Analytical techniques such as inductively coupled plasma−optical emission spectroscopy (ICP-OES), inductively coupled plasma−mass spectrometry (ICP-MS), or gas chromatography−mass spectrometry (GS-MS) can be used to show which species of toxic water contaminant classes are actually present in the water sample being tested. However, even this does not remove the necessity to check whether the identified water constituents indeed exert toxic action in standard toxicity tests. It should also not be forgotten that toxicity biomarker data have been obtained in experiments with several different duckweed species, and that a comprehensive catalog of biomarkers would best be established first with a single species and clone, preferably that being used as the test object of a uniform toxicity test setup.

6. Outlook

Each environmental toxicant has its mode of action, and may well elicit a particular signature of altered gene expression depending on this mode. The elucidation of such signatures can now be facilitated with the knowledge of the genomic sequence for *S. polyrhiza* that has recently become available (Wang, Haberer, et al. 2014). This sequence now opens the way for developing gene expression biotoxicity markers that may be of great diagnostic value for identifying toxic agents. The possibility of isolating each of the genes encoding specific protein sequences, or expressed sequence tags corresponding to them, makes gene expression profiling using microarrays on DNA chips feasible. In this sense, duckweeds would be cultivated under both controlled conditions and upon exposure to a particular water contaminant, most appropriately according to the protocol of a standardized duckweed toxicity test. At the end of the test period, the toxicity of the solute would be assessed, and mRNA from each of the "control" and the "stressed" duckweed tissues would be transcribed into cDNA incorporating treatment-specific fluorescence markers. The two specifically labeled cDNA preparations would be hybridized with a microarray of protein-specific genomic DNA spots, and the hybridization patterns revealed upon fluorometry would yield a profile of gene expression corresponding to the action of the investigated water contaminant on the duckweed. This has been practiced for many years with other sequenced genomes, including that of the model molecular biology plant *Arabidopsis thaliana* (see, e.g., Van Zhong and Burns 2003). That it is quite feasible with *S. polyrhiza* is shown by the fact that the duckweed contains even fewer protein-encoding genes (19,623) than does *A. thaliana* (27,416: Wang, Haberer, et al. 2014).

With this methodology, gene expression profiles could be determined for every water contaminant shown to be toxic to the duckweed, and computational analysis could determine how specifically particular profiles correspond to particular toxicants. Tests of exposure to combinations of multiple contaminants in both standard nutrient medium and natural waters could reveal how valid toxicant-specific expression profiles are in tests of complex water samples. When the genomic sequences of *L. minor* and *L. gibba* are available, as they soon will be, this gene expression profiling can be carried out with the duckweed species now universally used as test organisms in toxicity testing. In the most optimistic scenario, gene expression profiles could point to the particular toxicant(s) (or, more realistically, to particular groups of mechanistically related toxicants) present in any toxic water sample in a single experiment. Even if the identification of specific toxicants turns out to be more complicated than might be hoped, this identification technology should be explored in the near future. Although the widespread use of DNA microarrays will make this expensive analytical procedure ever more affordable, it is more realistic to envisage over-regional, centralized environmental laboratories as venues for routine gene expression profile toxicity testing, rather than smaller, local institutions.

Gene expression can also be used on a much more modest scale for identifying toxic effects and agents when the expression of a limited number of genes is known to be sufficient for identifying the action of one to several particular toxicants on duckweeds. The expression of up to 30 such marker genes can be examined in each of control and contaminated water-treated duckweeds by multiplex quantitative PCR, using, for example, the GenomeLab GeXP Genetic Analysis System marketed by Beckman Coulter (Fullerton, CA, USA). An example of the use of this system in plant gene expression studies is provided by Wang et al. (2011). New approaches can also be developed for studying the expression of single genes when these are sufficient to serve as biomarkers for particular contaminants. Duckweeds can now be genetically transformed efficiently

(Canto-Pastor et al. 2015). This allows the integration of reporter genes into a duckweed genome that would react to the presence of stress-induced metabolites (e.g., ROS) and thus serve to indicate the toxic effect of the corresponding toxic water contaminant (e.g., heavy metals).

7. Conclusions

Duckweeds have proved to be useful in environmental clean-up by taking up unwanted water contaminants and facilitating their removal by associated rhizosphere microorganisms, and their concomitant susceptibility to the toxic action of these contaminants has promoted their use as model aquatic plants for toxicity testing. Although standardized toxicity testing with duckweeds as test organisms is well established and effective in indicating toxicity, the elucidation of the mechanisms leading to the toxicity of the various water components is far from complete. This is especially true with regard to the still very limited ability to use the known toxic mechanisms to accurately identify the chemical sources of the toxicity. Gene expression profiling on the basis of genomic duckweed sequence information is envisaged to be of great potential for combining toxicity determination with toxicant identification.

Disclosure statement

No potential conflict of interest was reported by the authors.

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