DUCKWEED FORUM

Newsletter for the Community of Duckweed Research and Applications

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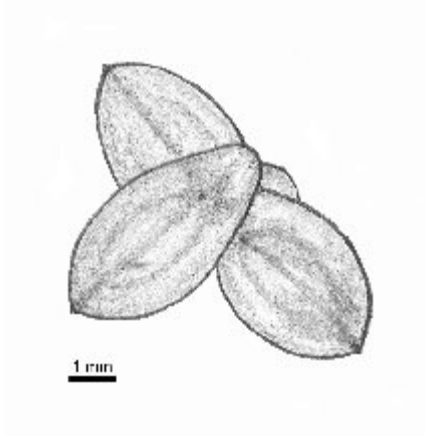
Edited by the International Steering Committee on Duckweed Research and Applications

Two duckweed species, *Spirodela polyrhiza* (Giant duckweed) and *Wolffia globosa*. The related genera occupy the lowest and the highest branches of the evolutionary tree of Lemnaceae family, respectively. Duckweeds beautifully share their waters with its relative, *Pistia stratiotes* (Water lettuce) belonging to Araceae. Credit: Dr. K. Sowjanya Sree, Delhi, India.

Information about ISCDRA and prior issues are available at
http://lemnapedia.org/wiki/ISCDRA

Science meets Art

Lemna aequinoctialis is distributed throughout the warmer zones of the world and exists also in some places of the temperate zone. In South-East Asia it is the dominant *Lemna* species. It is very often interchanged with other *Lemna* species, e.g. *Lemna minor*.



Lemna aequinoctialis Welw
Drawing by Dr. K. Sowjanya Sree

Letter from the editor

Dear Friends of Duckweed,

You are reading the first issue of the "Newsletter" produced by the "International Steering Committee on Duckweed Research and Applications" (ISCDRA) which was newly elected at the 3rd International Conference on Duckweed Research and Applications in Kyoto, July 3-6 this year. We use this opportunity to start with a new design.

"Forum" originally, in the Roman Empire, was meant to be a place where people came together to discuss important things, e.g. for judicature. Also nowadays it remains the right place for competent discussions by members of a community. With the title "**Duckweed Forum**" we want to emphasize that all those interested in duckweeds are invited to contribute and to share their experiences with rest of the duckweed community. Therefore, we introduced a new category, "Discussion Corner". In the present issue we open a discussion on the following topics: the taxonomic status of duckweeds (family versus subfamily) and meaning of the term "clone" under conditions of long-term stock cultivation.

However, there are also established categories in the present "Newsletter". Eric Lam, Rutgers University, News Brunswick and member of the ISCDRA, writes about the attractions of duckweed (especially from the genus *Wolffia*) as a model plant for basic research, and Masaaki Morikawa, Hokkaido University, Sapporo, about the interaction of duckweeds with microorganisms. We also offer again the section "Science meets Art", this time introducing the species *Lemna aequinoctialis* Welw., "Useful Methods" about nutrient media for *in vitro* cultivation of duckweeds, and "From the Database" with duckweed-related papers published in the last three months.

Some of the contributions have the clear aim to create and support scientific standards for the duckweed community which will facilitate those joining us from other fields. We have to continue with this effort.

Best wishes to all of you.

On behalf of the Steering Committee (ISCDRA),

Klaus-J. Appenroth, Head

Discussion Corner

What is a duckweed clone?

In the previous special edition that was dedicated to the 3rd International Conference on Duckweed Research and Applications, in Kyoto, July 3-6, 2015, we reported about whole genome sequencing of four more Lemnaceae clones, *Spirodela polyrhiza* 9509, *Lemna minor* 5500 and 8627, and *Lemna gibba* "7742a, better known as G3". We added the additional "a" to the clone's ID number after a discussion between the ISCDRA and RDSC. The background is that the historically often-used clone *Lemna gibba* G3 is kept at several places for a long time. As an example, in the stock collection of the Rutgers University (RDSC) there are in total 6 G3-related accessions, in Jena are also two accessions related to G3. The now sequenced *Lemna gibba* G3 was used by Dr. Janet Slovin, now USDA under the number DWC131 and we are not able to track its whole history through various laboratories over the decades. Janet only confirmed that it is *Lemna gibba* G3 and that she had procured it from Dr. Cleland. Obviously, the genotype of a strain can change during *in vitro* cultivation over time. This is trivial after generative propagation. As an example, Dr. Jitendra Khurana, Delhi University, keeps a *Lemna gibba* in his stock collection originated from G3 seed germination. However, even under exclusive vegetative propagation, spontaneous alterations in the genome over a longer period of cultivation time is possible although the extent has hardly been investigated in duckweed. With the advent of economical resequencing of well characterized genomes such as *Arabidopsis thaliana*, this type of studies are now possible at high resolution at both the genomic and epigenomic levels once a good quality reference genome is produced for *L. gibba* species.

What shall we do with DWC131? Shall we give any existing number? Shall we give a new number of the International system? The answer is not only important for that clone but of general significance. As we have to establish strict scientific standards for our duckweed community, we are asking all experts to give their opinion. Please, write a short comment (not more than 500 words) by the 15th of December for our January 2016 newsletter and send it to a member of the ISCDRA.

Eric Lam, Klaus-J. Appenroth

Duckweed: family or subfamily?

At the time when Elias Landolt published his famous book "The family of Lemnaceae – a monographic study" (1986), the taxonomic position of duckweed within the system of Angiosperm was not quite clear but it was accepted without discussion that there is a plant family, called Lemnaceae. Meanwhile it is clear, that this taxonomic group is monophyletic with Araceae. Some scientist consider by this reason duckweed as part of the Araceae family, as subfamily called *Lemnoideae*. Alternatively, it is possible to keep the taxonomic level of a plant family, called Lemnaceae.

As a scientific community we need to establish common scientific standards. We will have to face confusion and embarrassment when some of us talk about a plant family while others refer to it as a subfamily. What shall we decide? Of course, it ought to be based on sound rationale and when in doubt, agree on a set of objective criteria that can be resolved- perhaps at the genome sequence level in the near future, now that a number of duckweed genome resources are available.

The arguments for keeping the term Lemnaceae and consider duckweed as a plant family are summarized in two scientific papers:

http://onlinelibrary.wiley.com/doi/10.1111/plb.12248/pdf

http://www.cibj.com/Upload/PaperUpLoad/7e114905-8fc9-4573-9045-f2115f7a67b5.pdf

These papers may help you to put forward arguments in favour or against one of the opinions mentioned above.

We ask all experts to give their comments so that we can make a decision. Please respond by the 15th of December so that we can publish the opinions in the January, 2016 issue of this newsletter.

DUCKWEED

International Steering Committee on
Duckweed Research and Applications

Duckweed futures: duckweed's renaissance as a model system for Plant Biology?

Eric Lam, *Rutgers the State University of New Jersey, New Brunswick, NJ 08901, USA*

"*Anything found to be true of E. coli must also be true of elephants*" – Jacques L. Monod

Biology has been going through rapid advances since Darwin's publication of his famous book *Origin of the Species* more than 150 years ago. In addition to outlining in great clarity the Theory of Evolution, this remarkable work also posited the universality of mechanistic principles in Biology. This resonated at the molecular and biochemical levels more than a century later when Jacob and Monod helped set the foundations for Molecular Biology by elucidating the enzymes and pathways that dictate expression of physiological traits in the bacterium *E. coli*. The famous quote by Jacques Monod above summed up the unifying theme in Biology that basic principles of Nature, such as gene regulation mechanisms, are likely to be universal. This implies that elucidating complex biological mechanisms using "model" organisms that are more tractable can benefit our understanding of other organisms. The workhorse for Molecular Biology, the human gut bacterium *E. coli*, is the first example of a model organism in the era of modern molecular genetics. Prominent models in the animal kingdom are the fruit fly Drosophila, the nematode worm *C. elegans*, the laboratory mouse, and more recently the zebra fish. Using these models to expedite scientific investigations, many important advances in Biology and Medicine have been achieved in the past century.

The use of model plants to elucidate fundamental processes in Biology also has a rich history. To tackle the mechanism of inheritance, Gregor Mendel used the garden pea as his model organism in the 1800's to derive the law of segregation for sexual reproduction and trait inheritance that underpin modern Genetics. Using maize as her model system in the early part of the 20th Century, Barbara McClintock devised cytogenetic approaches to demonstrate recombination through crossover events in meiosis and to decipher the mobile nature of genetic elements in genomes, which earned her the Nobel Prize for Physiology or Medicine in 1983. In more recent years, *Arabidopsis thaliana* (mouse-ear cress) has taken its place as a powerful model plant system since the mid-1980s and its completed genome sequence in 2000 ushered in the genomics era for Plant Biology. Its small stature, fast generation time, and relatively small genome size are key characteristics that made Arabidopsis a superior model plant for molecular genetic and genomic approaches. The successful application of Arabidopsis to tackle complex biological pathways such as floral development and disease resistance by early pioneers Ellliot Meyerowitz and Frederick Ausubel, respectively, inspired many scientists to begin working with this model plant between 1980 and 2000, many are young scientists with training from non-plant fields. This "melting-pot" era of Arabidopsis has contributed greatly to our

understanding of plant processes and influenced generations of plant scientists. For Plant Biology, I believe duckweed's time as an important model plant could be arriving again.

What makes duckweed special?

Size and its aquatic habitat are key attributes that have made duckweed, plants in the Lemnaceae family, an attractive plant model for physiological and biochemical studies between the1950s to mid-1990s. As the world's smallest angiosperms that prefer to propagate asexually, duckweed can be studied under well-defined conditions with modest demand for space, time or cost. Duckweed also can readily take up isotopically labeled compounds from its growth medium to facilitate studies of metabolic pathways. Taking advantage of these special characteristics, past studies with duckweed had made fundamental contributions to a number of important fields in Plant Biology. For example, Hillman and co-workers used duckweed as the model plant to study the basic principles underlying photoperiodic time measurement and circadian rhythms in the 1950s (1). Using radioisotope labeled precursors and mass spectrometry, duckweed also enabled the first systematic biochemical characterization of auxin biosynthetic pathways in a higher plant (2,3). This series of studies directly demonstrated that auxins could be simultaneously produced via two distinct pathways involving either tryptophan or anthranilate, a conclusion later supported by genetic mutants in maize and Arabidopsis. Finally, taking advantage of the natural aquatic habitat of duckweed, a "phytostat" was constructed and used to characterize the metabolic pathways for sulfur metabolism in a higher plant model for the first time (4). This remains a case study in Plant Biology that deployed a chemically defined system for investigating metabolism with whole plants.

So, if duckweed was so useful, why has its level of use for basic Plant Biology research been relatively low for the past 2 decades? In contrast to the surge of duckweed studies on applied research areas such as Biotechnology, Environmental Remediation, and Exotoxicology, the number of reported duckweed-enabled studies in basic Plant Biology has actually declined during this period (5). Two main factors are likely important for this decline in interest of working with duckweed: 1) Due to the small size of its flowers and its aquatic habitat, duckweed are not easily amenable to classical genetic studies. This is in clear contrast to the rising model plant Arabidopsis during the '80s and '90s where relative ease of forward genetics with this species allowed many important plant biology questions to be tackled quickly. 2) Unlike important crop plants such as maize and soybean, there was little commercial driver to encourage support of research in duckweed from either government or industrial sources.

As Marvin Edelman pointed out in his summary of duckweed state-of-affairs in the first issue of the ISCDRA Newsletter this year (5), the recent advances in duckweed genomics should mitigate these barriers and attract renewed interest in duckweed research globally. In addition, the rising need for environmentally sustainable crops that can complement traditional agriculture to meet the anticipated demand of a growing world population should provide an economic driver for renewed interest to support duckweed research as well. For the rise of commercial interest in developing a

duckweed-based economy, I would refer interested readers to the last 3 Newsletter issues on Argentina's MamaGrande Co., the Spa-Agriquatic Group's work in India and the focus on creating a source of protein-on-demand by GreenOnyx in Israel as examples (Issues 7 to 9 of the ISCDRA Newsletter). In this Perspective article, I would like to elaborate my thoughts on the rise of duckweed again as a powerful scientific tool for basic Plant Biology research.

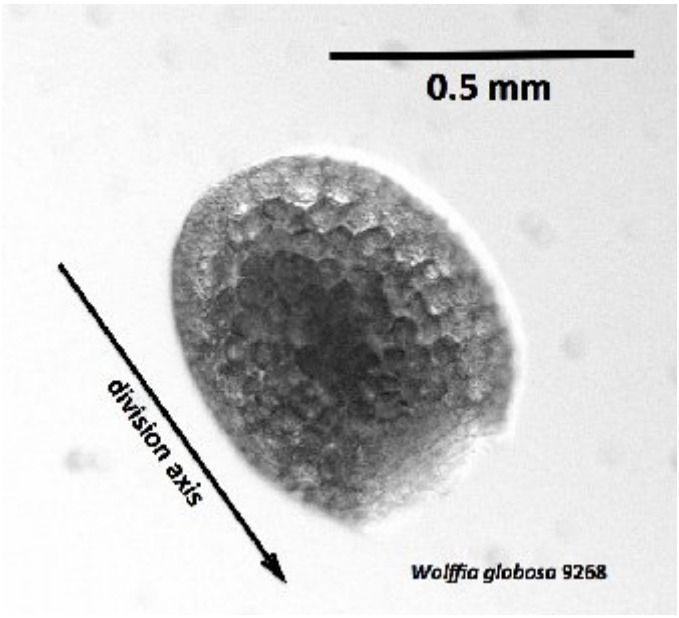
What important questions can be more easily studied by using duckweed as a model?

With today's advanced analytical technologies and rapidly increasing capacity in data acquisition and processing, what are some of the exciting research areas that duckweed may be more suitable as an experimental system than any other plant model? Here, I would like to elaborate on one "Grand Challenge" type of research topic that duckweed should be very well suited for and deserve our community's consideration.

Organismal Systems Biology: production of comprehensive maps of components and interactions at a whole plant level.

Duckweed's aquatic life style, fast growth rate, small size and simple architecture should greatly facilitate organismal level studies to produce deep "systems" datasets. While "omics" type of data collection has become common-place in recent years for many plant species, due in part to the rapid improvement in DNA sequencing and spectroscopic methods, their full integration for pathway modeling or network analysis remains a huge challenge for Biology. This difficulty lies in a number of parameters that are difficult to ascertain and quantify for most plant models: 1) The inherent complexities for multicellular organisms where many distinct cell types at various stages of development could be coexisting at any given time point of sample collection. Thus, the more tissue/cell types and developmental stages, the more complex the system that one has to consider in terms of mapping the quantitative changes observed to their respective locations in the plant. 2) Differential access of distinct organs of land plants to either air (e.g. leaves) or soil (e.g. roots) creates inhomogeneity in the system in terms of exposure to nutrients and environmental effectors. 3) Large genome size and extensive gene families for many key regulators further confound the effort to functionally define the roles of specific genes and their associated products. These issues could be minimized by using the simple aquatic monocot duckweed as a model plant, especially with species in the *Wolffia* genus.

A common Wolffia species, *W. globosa*, is shown in the figure below to illustrate several points. Wolffia species are rootless and they are the smallest amongst duckweeds with diameters of the single frond at less than 1 mm. There are 11 distinct species that have been identified from various locales in the globe and their genome sizes have been estimated by flow cytometry to vary from about 400 Mb (*W. australiana*) to 2 Gb (*W. arrhiza*), comparing to the size of the *S. polyrhiza* genome at 158 Mb (6). Based on the extreme abbreviation of the plant's architecture in Wolffia species, it is likely that this genus represents the most derived of the duckweeds from the ancestral *Spirodela* genus. In addition



to the lack of roots and multiple attached fronds as in the case of Spirodela, there is a simple axis of budding that can be readily discerned. While there is an epidermal layer and internal mesophyll cells, there are no vascular tissues. Although the number of distinct cell types in the meristematic region of Wolffia would need more characterization in the future, it is clear that the total number of cell types in Wolffia is much smaller than in most angiosperms. The preferred life style of asexual propagation and neoteny (persisting in the juvenile phase of development) further simplify the study of the life cycle of duckweeds such as Wolffia since they normally do not undergo transition to the adult reproductive phase.

As an aquatic plant, Wolffia can be grown in defined aqueous media with mixing to minimize heterogeneity in exposure of different regions of the plant to diverse environments such as air and soil. This simplicity and convenience of culture conditions further allow the "system" of assay to be quantitatively defined. As has been demonstrated in previous work with duckweed several decades ago (3), metabolites can be readily taken up from the media and thus isotopically labeled precursors can be used to help define pathways of metabolism and signalling intermediates. In combination, all these advantages make duckweeds an excellent model system for plant metabolomics that could be used to address fundamental questions in Plant Biology.

Lastly, the simplified architecture and streamlined life-style would indicate that there are likely fewer genes required to maintain the duckweed genomes. Indeed, the first draft of the *S. polyrhiza* 7498 genome revealed a set of 19,623 annotated genes (7). Our recent work with a higher resolution chromosome map of *S. polyrhiza* 9509 confirmed this low level (<20,000) of gene count (unpublished). This is significantly smaller in number compared to the model dicot *Arabidopsis thaliana* (27,416 genes), which has a similar genome size as compared to *S. polyrhiza*. As a reference monocot, the fast growing model grass *Brachypodium distachyon* Bd21 has over 31,000 annotated genes. The significantly lower gene count (~30% less than Arabidopsis) means that there should be fewer genes and proteins in the duckweed model plant than other plants. This in turn should make the work of tracking and quantifying changes in the transcriptome and proteome of duckweed an easier task. In addition, the subsequent challenge to integrating these large datasets for modeling interactions and signalling pathways should be a simpler problem due to a significantly smaller number of potential variables. A nice discussion for the approaches and rationale in Plant Systems Biology can be found in a recent review on network analysis (8).

Practical timeline and possible bottlenecks for Systems Biology studies with duckweed

Recently, my collaborators and I have successfully shown that a high fidelity reference genome for *S. polyrhiza* 9509 can be obtained *de novo* by using a combination of NGS (next generation sequencing) and single molecular physical mapping techniques (reported in the recent Kyoto duckweed conference). Using this refined chromosome sequence map, general Systems Biology-related questions in Plant Biology can now be readily addressed with this species. In addition to gaining confidence in the annotated and mapped genes for the Spirodela genome, our study also complete the description of the genome-wide cytosine methylation pattern and the transcript-based mapping of small RNAs (microRNAs and siRNAs) that are likely important for gene regulation. The annotated gene set can be used to guide proteomic studies while metabolomics can also be applied to study this plant model. If sufficient funding in basic research with Duckweed can be forthcoming from government or NGO sources, such as the recent duckweed protein project in the Netherlands that was funded by the Wellcome Trust in the UK (9), I believe exciting Systems Biology studies involving various "omics" strategies produced with duckweed can be completed in 2 to 3 years' time.

Additional resources that I believe would be critically important to further push the limits of the duckweed model system are: 1) Production of a reference genome for species from the *Wolffia* genus as a comparative model to the Greater Duckweed *S. polyrhiza* and for its simplistic structure; 2) Optimization of a rapid and efficient transformation method for *Spirodela polyrhiza* so that powerful genome editing strategies such as that of CRISPR/Cas9 can be brought to bear on the duckweed system; and 3) Creating a reliable and facile crossing method using selectable marker lines of duckweed to enable trait analysis and phenotype stacking strategies. Obviously, there is a lot of work to be done and my hope is that with more outreach/education to raise awareness and publically accessible resources, many new duckweed researchers will be attracted to join our community in the near future. A vibrant, open, and supportive community will definitely be an important factor to facilitate this renaissance of the duckweed model system for Plant Biology.

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Mutualisms between Duckweed and its associated bacteria

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The number of prokaryotes is estimated approximately 10^{30} cells on the earth and total weight of them becomes 10^{17} g, which is 1,000 times more than total human weight. These numerical values may allow us to say, "The earth is a planet of prokaryotes." When we look into our body, the cell numbers of normal micro-flora on skin, oral, and gut are 100 trillion and are more than the number of body human cells ~60 trillion. It is no doubt that community structure of the micro-flora affects in part on our health and even behaviors. Similar to a human society, there are certainly good and bad players in the micro-flora.

One of the most well-known beneficial gut bacteria is *Bifidobacterium* sp. that can eliminate pro-carcinogens and suppresses tumors. *Lactobacillus acidophilus* hydrolyzes the plant poly-↑-glycosides and results in the generation of potential anti-carcinogenic and anti-mutagenic substances in the form of flavonoids such as quercetin. We should also appreciate human gut bacteria for producing bacteriocins and epidermal bacteria for producing organic acids that prevent harmful pathogen infection. These bacteria that live in or on the human body often forms biofilms in order to stay and survive under stressful conditions. The biofilm is defined as microbial community structure formed on either biotic or abiotic solid surfaces.

There are also mutualistic biofilms that can benefit plant growth. *Acinetobacter calcoaceticus* P23 is one of the first reported plant growth-promoting (PGP) bacteria from duckweed. P23 is capable of adhering to and forming the biofilm on the surface of *Lemna minor,* a small duckweed, and enhance the plant growth by two-fold. Indole acetic acid (a plant growth hormone "auxin"), ACC deaminase (a inhibitor of ripening hormone "ethylene" production), siderophore (an iron solubilizer), organic acids (phosphorous solubilizer), and anti-fungal agents are general PGP compounds produced by plant-associated bacteria. However, P23 produces unique exopolysaccharide (PGP-AC23) as PGP compound. Bacterial exopolysaccharide often functions as a matrix glue of the biofilm. There is no report so far that a soil plant grows faster upon receiving microbial exopolysaccharides.

During purification of PGP-AC23, yeast mannan, →-polymannose, has been found by chance to increase the growth of duckweed. On the other hand, glucomannan derived from a plant Konjac does not harbor such activity. It may be worth to note that glucomannan is a copolymer of glucose, acetylglucose, and ↑-mannose. Based on the observation that the amount in the culture medium is not apparently decreased during the growth of duckweed, yeast mannan is supposed to function as a

surface stimulator compound that triggers signal transduction resulting to the growth promotion. Moreover, P23 significantly increases chlorophyll content of *L. minor*. This activity enables *L. minor* to grow normally under half light intensity and poor nutrient conditions. I hope that PGP bacteria will contribute to effective production of duckweed and concomitant low cost water purification.

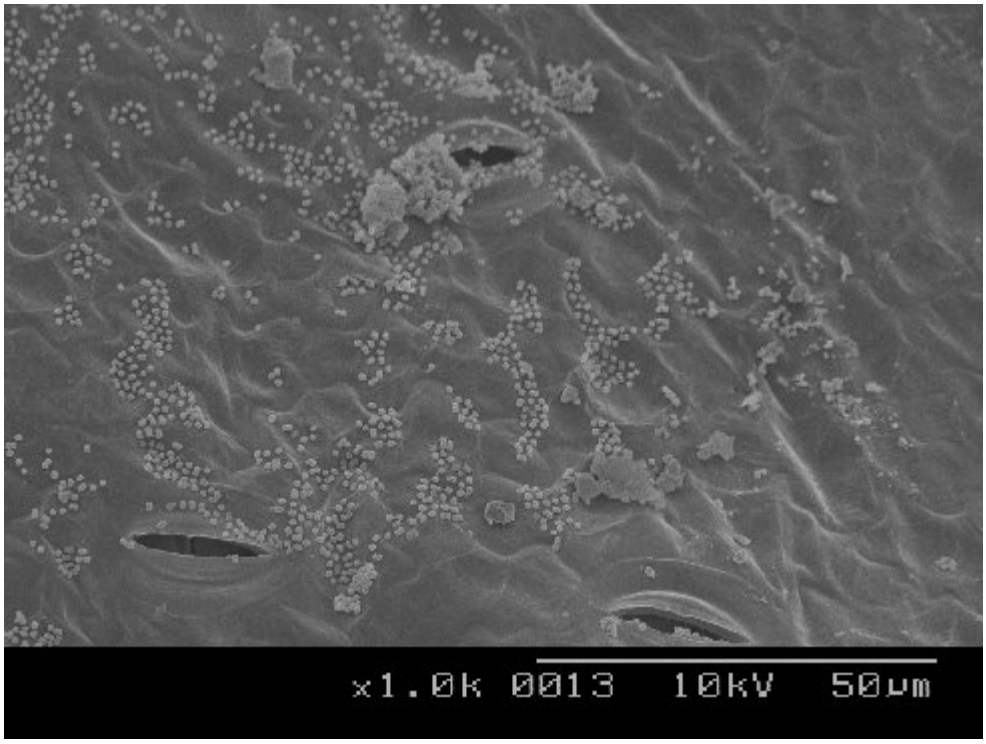

Fig. *Acinetobacter calcoaceticus* P23, a growth-promoting bacterium, cells which colonizes the upper surface of a *Lemna minor* frond.

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Useful methods 3: Media for in vitro-cultivation of duckweed

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There is a large number of cultivation media in use for duckweed. This simply shows that most duckweed can adapt to a broad range of conditions. This does not mean that physiological responses are always the same. In order to support scientific standards for the duckweed community, we compiled the most useful nutrient media here in form of lab protocols, including the stock solutions used. All protocols are for autotrophic cultivation. If mixotrophic cultivations are required, either glucose (50 mM) or sucrose (25 mM) should be added. We have never added vitamins or other organics as green plants produce it themselves.

Final concentrations are given in molar concentrations – and should be given in scientific publications. Only then different nutrient media can be compared and the data do not depend e.g. on the amount of crystal water of the substances.

Most of the original protocols were published before FeEDTA or FeNaEDTA were available on a commercial basis. In most cases, these substance are much easier to handle than other sources of iron. Only when possible chelating effects may have an influence on the physiological effects under investigation (e.g. flowering) one should take care. All nutrient media should be autoclaved before use.

The numbers in brackets are the molecular weight/ mass in g mol^{-1}, which might help in calculations.

Modified Steinberg-Medium

Ref.: R. A. Steinberg (1946) Mineral requirements of *Lemna minor*. Plant Physiology **21**, 42-48.

Modified 1998 by R. Altenburger, Dept. of Chemical Ecotoxicology, UFZ Centre for Environmental Research, Leipzig. Germany. Described in ISO standard (ISO 20079).

This nutrient medium is recommended for investigating the effects of substances that might be chelated, e. g. heavy metals. Therefore, the concentration of chelators is so low that the optimal growth of duckweed is not possible. If you want to use this medium but want to have higher growth rates, the FeEDTA and $EDTANa_2$ concentrations should be increased fivefold. $EDTANa_2$ was added to keep the same ration of chelated and free EDTA as in the original protocol.

From stock solution 1 to 3, 20 ml per litre ready-to-use media were used, from stock solution 4 and 5

only 5 ml per litre.

Stock	Compound	stock concentration		final concentration
1	KNO_3 (101.1)	173mM	17.50 g/L	3.46 mM
	KH_2PO_4 (136.1)	33mM	4.50 g/L	0.66 mM
	$K_2HPO_4x3H_2O$ (228.2)	0.63 g/L	3.6mg/L	72 µM
2	$MgSO_4x7H_2O$ (246.5)	20.5mM	5.00 g/L	0.41 mM
3	$Ca(NO_3)_2x4H_2O$ (236.1)	62.5mg/L	14.75 g/L	1.25 mM
4	H_3BO_3 (61.8)	0.388mM	120 mg/L	1.94 µM
	$ZnSO_4x7H_2O$ (287.5)	0.126mM	180 mg/L	0.63 µM
	$Na_2MoO_4x2H_2O$ (241.9)	36 µM	44 mg/L	0.18 µM
	$MnCl_2$ x $4H_2O$ (197.9)	0.182mM	180 mg/L	0.91 µM
5	FeNaEDTA (367.0)	0.562mM	1.031 g/L	2.81 µM
	EDTA-Na_2 (372.24)	0.244mM	0.454 g/L	1.22 µM

The pH was adjusted to 5.5, e.g. by using 1 % (v/v) HCl.

Murashige-Skoog-Medium (1/10 MS-medium)

Ref.: Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bio assay with tobacco tissue cultures. Physiol. Plant. **15**, 473-497.

This medium is especially popular for stock cultivations. Several of the components in stock solution 6 might not be required at all, e.g. KI. Landolt and Kandeler (1987) cited this version of the MS-medium according to Jacobs (1947) and mentioned that it is very rich of nitrate and potassium. They also criticized the complete lack of sodium and the low concentrations of trace elements.

Stock	Compound	stock concentration	final concentration
1	KNO_3 (101.11)	752mM	76.0g/L	2.06 mM
	NH_4NO_3 (80.04)	824mM	65.95g/L	1.88 mM
2	$CaCl_2x2H_2O$ (147.02)	120mM	17,6g/L	0.3 mM
3	$MgSO_4x7H_2O$ (246.48)	60mM	14.79g/L	0.15 mM
4	KH_2PO_4 (136.09)	50mM	6.80g/L	125 µM
5	Fe(III)EDTA (367.0)	4mM	1.468g/L	10 µM
6	H_3BO_3 (61.83)	4mM	247mg/L	10 µM
	$MnSO_4xH_2O$ (169.02)	4mM	676mg/L	10 µM
	$ZnSO_4x7H_2O$ (287.5)	1.2mM	345mg/L	3 µM
	KI (166.0)	0.2mM	33.2mg/L	0.5 µM
	$Na_2MoO_4x2H_2O$ (241.95)	0.04mM	9.7mg/L	0.1 µM
	$CuSO_4x5H_2O$ (249.68)	0.004mM	1mg/L	0.01 µM

From each stock solution 2.5 ml per litre final medium.

N-Medium

Ref: K.-J. Appenroth, S. Teller, M. Horn "Photophysiology of turion formation and germination in *Spirodela polyrhiza*" - Biologia Plantarum **38**, 95 - 106 (1996).

This medium was developed in our Institute and used for several decades for all duckweed species. The original low concentration of phosphate (60 µM) was used to induce turions formation. As this is rather a special case, we increased the phosphate concentration to 150 µM as given in the table. We never found nutrient media that supported a faster growth of duckweed than this one. When this nutrient medium should be used for stock cultivation, we increase the KH_2PO_4 concentration to even

1 mM, add 50 mM glucose and solidify by 0.9 % agar. Even after several decades of cultivation, the plants do not miss the trace elements that are not supplied. Most probably, even in chemicals of p.A.-quality, these elements are present as contaminations.

Stock	Compound	stock concentration		final concentration
1	KH_2PO_4 (136.1)	30 mM	4.083 g/L	0.15 mM
2	$Ca(NO_3)_2 \cdot 4H_2O$ (236.2)	0.2 M	47.23 g/L	1 mM
3	KNO_3 (101.1)	1.6 M	161.8 g/L	8 mM
	H_3BO_3 (61.83)	1 mM	61.8 mg/L	5 µM
	$MnCl_2 \cdot 4H_2O$ (197.9)	2.6 mM	514.5 mg/L	13 µM
	$Na_2MoO_4 \cdot 2H_2O$ (241.95)	80 µM	9.4 mg/L	0.4 µM
	$MgSO_4 \cdot 7H_2O$ (246.48)	0.2 M	49.30 g/L	1 mM
4	Fe(III)EDTA (345.07)	5 mM	1.725 g/L	25 µM
	or			
	FeNaEDTA (MW 367.1)	5 mM	1.835 g/L	25 µM

From each stock solution 5 ml is used for 1 L nutrient medium. The nutrient medium might be adjusted to pH 5.5 but this is normally omitted.

Schenk-Hildebrand medium (SH-medium)

Ref.: Schenk, R. U. and A. C. Hildebrandt, 1972, *Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures*.

Can. J. Bot. 50:199-204.

SH-Medium is not even described in Landolt and Kandeler (1987). However, following recommendation by Dr. Ann Stomp, USA Elias Landolt introduced this medium in his lab and was very satisfied with it for stock cultivation. This medium is also commercially available. As in many other cases, several of the trace elements (stock solution 3) might have no function for duckweed

cultivation.

Stock	Compound	stock concentration	final concentration
1	$CaCl_2 \cdot 2H_2O$ (147.02)	136mM	20 g/L	0.68 mM
2	KNO_3 (101.1)	2.48M	250 g/L	12.4mM
	$MgSO_4 \cdot 7H_2O$ (246.48)	162mM	40 g/L	0.8mM
	$(NH_4)H_2PO_4$ (115.03)	260mM	30 g/L	1.3mM
3	$MnSO_4 \cdot H_2O$ (169.02)	5.92mM	1 g/L	30µM
	H_3BO_3 (61.83)	8.1 mM	0.5 g/L	40µM
	$ZnSO_4 \cdot 7H_2O$ (287.6)	0.35mM	0.1g/L	1.74µM
	KI (166)	0.6mM	0.1g/L	3µM
	$CuSO_4 \cdot 5H_2O$ (249.68)	80.1 µM	0.02 g	0.4µM
	$Na_2MoO_4 \cdot 2H_2O$ (241.95)	41µM	0.01 g	0.2µM
	$CoCl_2 \cdot 6H_2O$ (237.96)	42µM	0.01 g	0.21µM
4	FeNaEDTA (367.1)	5.39mM	1.98g/L	0.26mM
	Na_2EDTA (372.24)	0.55µM	0.204g/L	2.75µM

Five ml from each stock solution has to be used for one litre ready-to-use nutrient medium.

Hoagland medium

Ref.: We describe the nutrient medium as given by Landolt and Kandeler (1987), p. 60-62 as the

original paper was published already 1920. This might be the reason why so many modifications of this medium exists, e.g. Venkataraman et al. (1970).

Stock	Compound	stock concentration		final concentration
1	KNO_3 (101.1)	2M	202g/L	10mM
2	$Ca(NO_3)_2x4H_2O$ (236.15)	0.8M	189g/L	4mM
3	KH_2PO_4 (136.1)	0.2M	27.2g/L	1mM
4	$MgSO_4x7H_2O$ (246.5)	0.4M	98.6g/L	2mM
	ZnSO4x7H2O (287.5)	0.3mM	86.2mg/L	1.5µM
	H_3BO_3 (61.8)	10mM	618mg/L	50µM
	$MnCl_2x4H_2O$ (197.91)	2mM	396mg/L	10µM
	$CuSO_4x5H_2O$ (249.68)	80µM	20mg/L	0.4µM
	$Na_2MoO_4x2H_2O$* (241.9)	1mM	242mg/L	5µM
5	Iron(III)tartrate** (555.9)	4mM	2.2g/L	20µM

* Used instead of MoO_3

** This suabtance is difficult to solve. Heat shortly and stir over night. Venkataraman et al. (1970) replaced this substance by Iron(III)citrate and often added Na_2EDTA, 10 µM to the final medium.

Five ml from each stock solution has to be used for each litre of ready-to-use nutrient medium.

Bonner-Devirian medium

Ref.: Bonner, J., and P. S. Devirian: Growth factor requirements of four species of isolated roots. Amer. J. Bot. 26: 661-665 (1939).

This nutrient medium has rather low concentrations. We have only good experiences with this nutrient medium cultivating *Lemna trisul*ca.

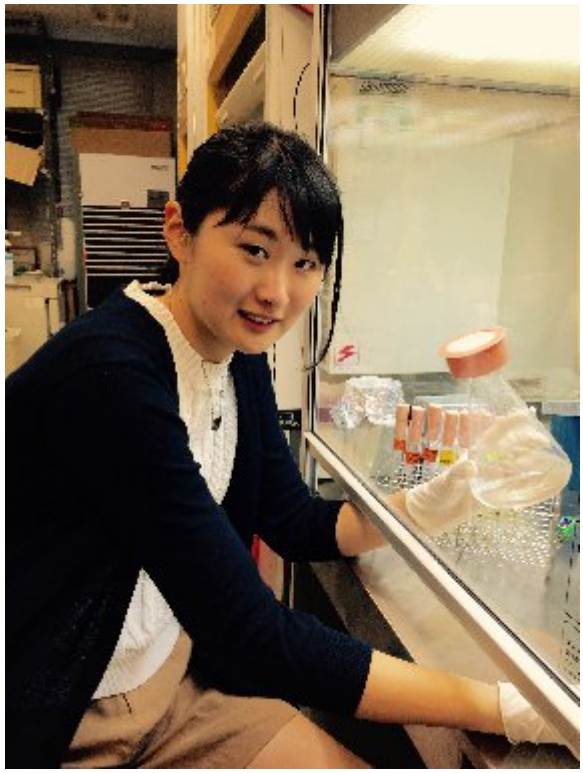
Stock	Compound	stock concentration		final concentration
1	KNO_3 (101.1)	0.168M	17g/L	168mM
2	$Ca(NO_3)_2x4H_2O$ (236.15)	0.2M	47.2g/L	1mM
3	KH_2PO_4 (136.1)	29.4mM	4g/L	0.147mM
	KCl (74.55)	161mM	12g/L	0.8mM
4	$MgSO_4x7H_2O$ (246.5)	0.4M	98.6g/L	2mM
	ZnSO4x7H2O (287.5)	0.3mM	86.2mg/L	1.5µM
	H_3BO_3 (61.8)	10mM	618mg/L	50µM
	$MnCl_2x4H_2O$ (197.91)	2mM	396mg/L	10µM
	$CuSO_4x5H_2O$ (249.68)	80µM	20mg/L	0.4µM
	$Na_2MoO_4x2H_2O$ (241.9)	1mM	242mg/L	5µM
5	Iron(III)tartrate* (555.9)	4mM	2.2g/L	20µM

* This substance is difficult to solve. Heat shortly and stir over night. Venkataraman et al. (1970) replaced this substance by Iron (III) citrate and often added Na_2EDTA, 100 µM to the final medium to study flower induction of *Wolffia microscopica*.

Five ml from each stock solution has to be used for each litre of ready-to-use nutrient medium.

Student Spotlight- Ami Kawahata

Undergraduate at Hokkaido University, Japan



I am doing research on rhizoremediation by employing team power of *Lemna minor* and associated microbes. I have been interested in microbiology since my high school days, and that motivated me to enter Department of Biology, Hokkaido University (Japan). I think the attractiveness of microbiology is that we cannot see microbes by eyes, but certainly they exist as the essential basis of our nature and life. Moreover, there are still many undiscovered species, whose functions are unidentified. Research of such unknown bacteria and their application is my biggest interest.

During my undergraduate, I met Prof. Masaaki Morikawa, who works widely on functional analysis and application of environmental microbes, and I decided to begin my research in his laboratory.

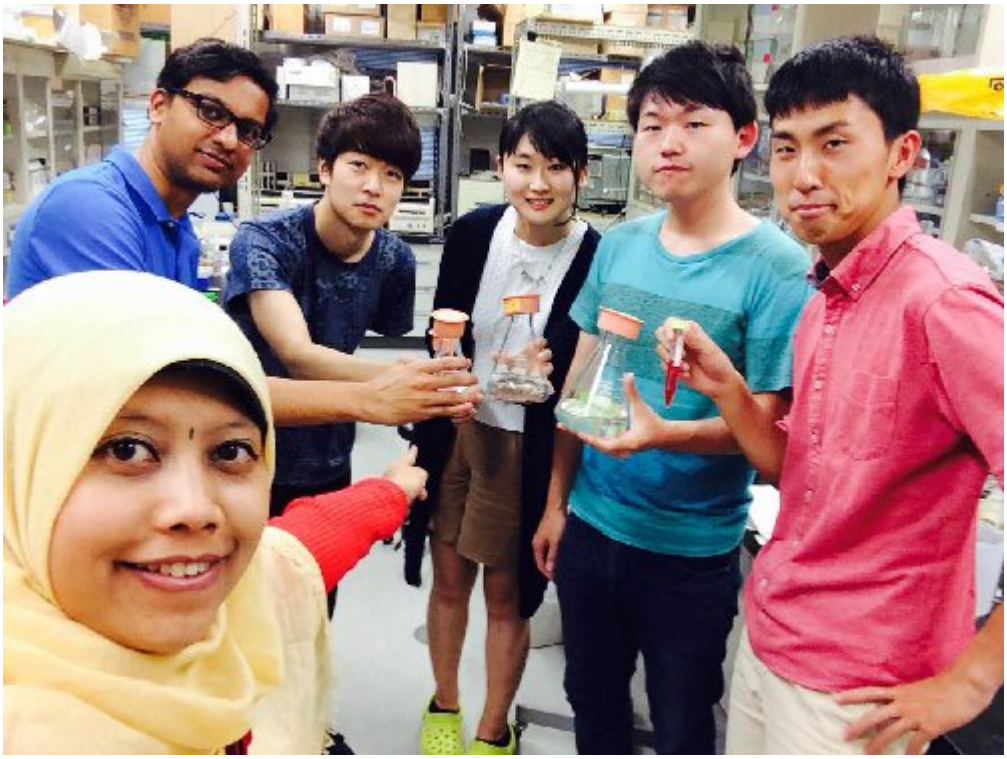
In the Morikawa lab, we are working on the isolation of plant growth-promoting bacteria (PGPB) and analysis of their functional mechanisms. One of the isolated bacteria, *Acinetobacter calcoaceticus* P23, had high ability to attach to the surface of plants and promoted the growth of *Lemna minor* by two folds. What excited me was that the plant growth-promoting factor of P23 was a novel compound and its functional mechanism was also new. *A. calcoaceticus* is a popular soil bacterium and is also reported for plant growth-promoting activity. However, aquatic bacterium P23 has very unique features. The interaction between duckweed and its associated bacteria looked so attractive to me.

The Morikawa laboratory is not only working of PGPB, but also on rhizoremediation technology by *Lemna* and useful bacteria. Rhizoremediation is a type of environmental remediation technology which utilizes symbiosis of pollutant degrading-rhizobacteria and their host plants. (In the case of duckweed, fronds and roots are similarly submerged to the water. It is difficult to define the "rhizosphere" of the duckweed but for now I would like to use the term "rhizoremediation".) The mutual benefits between plants and bacteria make them easier to live in poor nutrient and polluted environment that results in more effective and sustainable degradation of pollutants.It was also very interesting for me to know that there is a low cost, ecofriendly way of environment remediation using duckweed.

Now, I am developing the rhizoremediation technology of estrogen, one of the endocrine disrupting hormones (EDCs) for my master course thesis. The pollution by EDCs is still a serious problem worldwide. Some reports show that conventional activated sludge system cannot completely eliminate them. My goal is to establish a new rhizoremediation technology which applies the rationally designed duckweed and useful rhizobateria symbiotic system to complement the activated sludge system in wastewater treatment.

Attending the 3rd ICDRA in Kyoto was a fantastic experience for me. Giving a presentation in front of experts who have been leading the duckweed research and application made me very nervous, but finally I could get many useful discussions and comments for my future work. It was also very stimulating to hear presentations from different research fields of duckweed. Once again I was very much attracted and addicted to the world of duckweed.

I am so excited that I could join the researchers network of duckweed, and now I am trying my best to follow them. Furthermore, my dream is to spread the attractiveness of duckweed-microbe association to the world!



Lab team from left: Desi Utami, Rahul Jog,Keita Kagemoto, Ami Kawahata, Ayumu Kuramoto,Takahiro Kato.

From the Database

Highlights

Expression of H5 hemagglutinin vaccine antigen in common duckweed (*Lemna minor*) protects against H5N1 high pathogenicity avian influenza virus challenge in immunized chickens. Bertran, K., Thomas, C., Guo, X., Bublot, M., Pritchard, N., Regan, J.T., Cox, K.M., Gasdaska, J.R., Dickey, L.F., Kapczynski, D.R. et al.

VACCINE 33: 3456-3462 (2015)

High-Yield Expression of M2e Peptide of Avian Influenza Virus H5N1 in Transgenic Duckweed Plants. Firsov, A., Tarasenko, I., Mitiouchkina, T., Ismailova, N., Shaloiko, L., Vainstein, A., Dolgov, S.

MOLECULAR BIOTECHNOLOGY 57: 653-661 (2015)

(Both abstracts see under Molecular Biology)

Biotechnology

Engineering *Corynebacterium crenatum* to produce higher alcohols for biofuel using hydrolysates of duckweed (*Landoltia punctata*) as feedstock

Su, H., Jiang, J., Lu, Q., Zhao, Z., Xie, T., Zhao, H., Wang, M.

Microbial Cell Factories 14: 199 (2015)

Early trials have demonstrated great potential for the use of duckweed (family Lemnaceae) as the next generation of energy plants for the production of biofuels. Achieving this technological advance demands research to develop novel bioengineering microorganisms that can ferment duckweed feedstock to produce higher alcohols. In this study, we used relevant genes to transfer five metabolic pathways of isoleucine, leucine and valine from the yeast Saccharomyces cerevisiae into the bioengineered microorganism Corynebacterium crenatum. Experimental results showed that the bioengineered strain was able to produce 1026.61mg/L of 2-methyl-1-butanol by fermenting glucose, compared to 981.79mg/L from the acid hydrolysates of duckweed. The highest isobutanol

yields achieved were 1264.63mg/L from glucose and 1154.83mg/L from duckweed, and the corresponding highest yields of 3-methyl-1-butanol were 748.35 and 684.79mg/L. Our findings demonstrate the feasibility of using bioengineered C. crenatum as a platform to construct a bacterial strain that is capable of producing higher alcohols. We have also shown the promise of using duckweed as the basis for developing higher alcohols, illustrating that this group of plants represents an ideal fermentation substrate that can be considered the next generation of alternative energy feedstocks.

Simultaneous saccharification and fermentation of steam exploded duckweed: Improvement of the ethanol yield by increasing yeast titre

Zhao, X., Moates, G.K., Elliston, A., Wilson, D.R., Coleman, M.J., Waldron, K.W.

BIORESOURCE TECHNOLOGY 194: 263-269 (2015)

This study investigated the conversion of *Lemna minor* biomass to bioethanol. The biomass was pre-treated by steam explosion (SE, 210 degrees C, 10 min) and then subjected to simultaneous saccharification and fermentation (SSF) using Cellic (R) CTec 2 (20 U or 0.87 FPU g (1) substrate) cellulase plus beta-glucosidase (2 U g (1) substrate) and a yeast inoculum of 10% (v/v or 8.0 x 10(7) cells mL (1)). At a substrate concentration of 1% (w/v) an ethanol yield of 80% (w/w, theoretical) was achieved. However at a substrate concentration of 20% (w/v), the ethanol yield was lowered to 18.8% (w/w, theoretical). Yields were considerably improved by increasing the yeast titre in the inoculum or preconditioning the yeast on steam exploded liquor. These approaches enhanced the ethanol yield up to 70% (w/w, theoretical) at a substrate concentration of 20% (w/v) by metabolising fermentation inhibitors.

The influence of light intensity and photoperiod on duckweed biomass and starch accumulation for bioethanol production

Yin, Y., Yu, C., Yu, L., Zhao, J., Sun, C., Ma, Y., Zhou, G.

BIORESOURCE TECHNOLOGY 187: 84-90 (2015)

Duckweed has been considered as a valuable feedstock for bioethanol production due to its high biomass and starch production. To investigate the effects of light conditions on duckweed biomass and starch production, *Lemna aequinoctialis* 6000 was cultivated at different photoperiods (12:12, 16:8 and 24:0 h) and light intensities (20, 50, 80, 110, 200 and 400 mu mol m(-2) s(-1)). The results showed that the duckweed biomass and starch production was increased with increasing light intensity and photoperiod except at 200 and 400 mu mol m(-2) s(-1). Considering the light cost, 110

mu mol m(-2) s(-1) was optimum light condition for starch accumulation with the highest maximum growth rate, biomass and starch production of 8.90 g m(-2) day(-1), 233.25 g m(-2) and 98.70 g m(-2), respectively. Moreover, the results suggested that high light induction was a promising method for duckweed starch accumulation. This study provides optimized light conditions for future industrial large-scale duckweed cultivation.

Fermentation of swine wastewater-derived duckweed for biohydrogen production

Xu, J., Deshusses, M.A.

INTERNATIONAL JOURNAL OF HYDROGEN ENERGY 40: 7028-7036 (2015)

Duckweed harvested from a swine wastewater treatment system was evaluated for its potential as a fermentation feedstock for the production of biohydrogen. The effects of pretreatment and fermentation conditions on biohydrogen production were investigated in laboratory-scale batch experiments. The results showed that mild acidic thermal pretreatment (1% H2SO4 and 85 degrees C for 1 h) was more effective in improving the fermentability of duckweed than either thermal or mild alkaline thermal pretreatments. Fermentation of acid-pretreated duckweed resulted in a biohydrogen production of up to 75 mL H-2 per g dry duckweed in 7 days (at a H2 concentration of 42%), which is comparable with other plant biomass. Overall, the results show that small aquatic plants such as duckweed can be promising substrate for biohydrogen production

Ecology

Aquatic macrophyte *Lemna trisulca* (L.) as a natural factor for reducing anatoxin-a concentration in the aquatic environment and biomass of cyanobacterium *Anabaena flos-aquae* (Lyngb.) de Breb

Kaminski, A., Chrapusta, E., Bober, B., Adamski, M., Latkowska, E., Bialczyk, J.

ALGAL RESEARCH-BIOMASS BIOFUELS AND BIOPRODUCTS 9: 212-217 (2015)

This study evaluated the interaction between the aquatic macrophyte Lemna trisulca and the cyanobacterium Anabaena flos-aquae during co-cultivation. The macrophyte drastically inhibited the biomass accumulation of the cyanobacterium. After 32 days of co-cultivation of both organisms, the cyanobacterial biomass accumulation decreased by 86.9% compared with the control. By contrast, the biomass of the macrophyte increased by 13.1% compared with the control. L. trisulca had a stabilizing effect on the media pH. The cultivation of the macrophyte, cyanobacterium or both

organisms simultaneously increased the pH of the media from the initial value of 7.0 to 7.6, 8.9 and 7.9 units, respectively. Both organisms demonstrated differences in their amounts and rates of nitrate uptake. Over 32 days of cultivation, the macrophyte readily adsorbed phosphate ions from the media, resulting in a systematic increase in the N/P ratio, whereas the cultivation of the cyanobacterium alone resulted in a decrease in the N/P ratio to 14.7 (from the initial N/P= 21.2). The co-cultivation of both organisms led to an increase in the N/P ratio. Furthermore, under this condition, reductions in anatoxin-a (ANTX-a) concentrations by 78.6% in the media and by 99.9% in the cyanobacterial cells were detected. Presumably, L. trisulca simultaneously accumulated and degraded the toxin inside of its tissues. On day 4 of co-cultivation, the macrophyte contained 150.7 mu g of ANTX-a . g(-1) DW, and after 32 days, it was not detected. During 32 days of A. flos-aquae and L. trisulca cocultivation, no increases were observed in the total phenolic concentrations in the media or in the extractable fractions for both organisms. Our results suggest that L. trisulca is a natural factor that may be used for the biological inactivation of this cyanobacterium and its toxin. These results have an ecological significance for the prevention of cyanobacterial blooms in water ponds.

Humid microclimates within the plumage of mallard ducks (*Anas platyrhynchos*) can potentially facilitate long distance dispersal of propagules

Coughlan, N.E., Kelly, T.C., Davenport, J., Jansen, M.A.K.

ACTA OECOLOGICA-INTERNATIONAL JOURNAL OF ECOLOGY 65-66: 17-23 (2015)

Birds as carriers of propagules are major agents in the dispersal of plants, animals, fungi and microbes. However, there is a lack of empirical data in relation to bird-mediated, epizoochorous dispersal. The microclimate found within the plumage likely plays a pivotal role in survival during flight conditions. To investigate the potential of epizoochory, we have analysed the microclimatic conditions within the plumage of mallard ducks (Anas platyrhynchos). Under similar ambient conditions of humidity and temperature, a sample of mallards showed a consistent microclimatic regime with variation across the body surface. The highest (mean) temperature and specific humidity occurred between feathers of the postpatagium. The lowest humidity was found between feathers of the centre back and the lowest temperature in the crissum. Observed differences in plumage depth and density, and distance from the skin, are all likely to be determining factors of microclimate condition. Specific humidity found within the plumage was on average 1.8-3.5 times greater than ambient specific humidity. Thus, the plumage can supply a microclimate buffered from that of the exterior environment. Extrapolating survival data for Lemna minor desiccation at various temperature and humidity levels to the measured plumage microclimatic conditions of living birds, survival for up to 6 h can be anticipated, especially in crissum, crural and breast plumage. The results

are discussed in the context of potential long distance epizoochorous dispersal by A. platyrhynchos and similar species

Feed and Food

Survey of duckweed diversity in Lake Chao and total fatty acid, triacylglycerol, profiles of representative strains

Tang, J., Li, Y., Ma, J., Cheng, J.J.

PLANT BIOLOGY 17: 1066-1072 (2015)

Lemnaceae (duckweeds) are widely distributed aquatic flowering plants. Their high growth rate, starch content and suitability for bioremediation make them potential feedstock for biofuels. However, few natural duckweed resources have been investigated in China, and there is no information about total fatty acid (TFA) and triacylglycerol (TAG) composition of duckweeds from China. Here, the genetic diversity of a natural duckweed population collected from Lake Chao, China, was investigated using multilocus sequence typing (MLST). The 54 strains were categorised into four species in four genera, representing 12 distinct sequence types. Strains representing Lemna aequinoctialis and Spirodela polyrhiza were predominant. Interestingly, a surprisingly high degree of genetic diversification within L.aequinoctialis was observed. The four duckweed species revealed a uniform fatty acid composition, with three fatty acids, palmitic acid, linoleic acid and linolenic acid, accounting for more than 80% of the TFA. The TFA in biomass varied among species, ranging from 1.05% (of dry weight, DW) for L.punctata and S.polyrhiza to 1.62% for Wolffia globosa. The four duckweed species contained similar TAG contents, 0.02% mgDW-1. The fatty acid profiles of TAG were different from those of TFA, and also varied among the four species. The survey investigated the genetic diversity of duckweeds from Lake Chao, and provides an initial insight into TFA and TAG of four duckweed species, indicating that intraspecific and interspecific variations exist in the content and composition of both TFA and TAG in comparison with other studies.

Molecular Biology

Expression of H5 hemagglutinin vaccine antigen in common duckweed (*Lemna minor*) protects against H5N1 high pathogenicity avian influenza virus challenge in immunized chickens

Bertran, K., Thomas, C., Guo, X., Bublot, M., Pritchard, N., Regan, J.T., Cox, K.M., Gasdaska, J.R., Dickey, L.F., Kapczynski, D.R. et al.

VACCINE 33: 3456-3462 (2015)

A synthetic hemagglutinin (HA) gene from the highly pathogenic avian influenza (HPAI) virus A/chicken/Indonesia/7/2003 (H5N1) (Indo/03) was expressed in aquatic plant Lemna minor (rLemna-HA). In Experiment 1, efficacy of rlemna-HA was tested on birds immunized with 0.2 mu g or 23 mu g HA and challenged with 10(6) mean chicken embryo infectious doses (EID50) of homologous virus strain. Both dosages of rLemna-HA conferred clinical protection and dramatically reduced viral shedding. Almost all the birds immunized with either dosage of rLemna-HA elicited HA antibody titers against Indo/03 antigen, suggesting an association between levels of anti-Indo/03 antibodies and protection. In Experiment 2, efficacy of rlemna-HA was tested on birds immunized with 0.9 mu g or 2.2 mu g HA and challenged with 10(6) EID50 of heterologous H5N1 virus strains A/chicken/Vietnam/NCVD-421/2010 (VN/10) or A/chicken/West Java/PM-WU/2006 (PWT/06). Birds challenged with VN/10 exhibited 100% survival regardless of immunization dosage, while birds challenged with PWT/06 had 50% and 30% mortality at 0.9 mu g HA and 2.2 mu g HA, respectively. For each challenge virus, viral shedding titers from 2.2 mu g HA vaccinated birds were significantly lower than those from 0.9 mu g HA vaccinated birds, and titers from both immunized groups were in turn significantly lower than those from sham vaccinated birds. Even if immunized birds elicited HA titers against the vaccine antigen Indo/03, only the groups challenged with VN/10 developed humoral immunity against the challenge antigen. None (rLemna-HA 0.9 mu g HA) and 40% (rLemna-HA 2.2 mu g HA) of the immunized birds challenged with PWT/06 elicited pre-challenge antibody titers, respectively. In conclusion, Lemna-expressed HA demonstrated complete protective immunity against homologous challenge and suboptimal protection against heterologous challenge, the latter being similar to results from inactivated whole virus vaccines. Transgenic duckweed-derived HA could be a good alternative for producing high quality antigen for an injectable vaccine against H5N1 HPAI viruses.

High-Yield Expression of M2e Peptide of Avian Influenza Virus H5N1 in Transgenic Duckweed Plants

Firsov, A., Tarasenko, I., Mitiouchkina, T., Ismailova, N., Shaloiko, L., Vainstein, A., Dolgov, S.

MOLECULAR BIOTECHNOLOGY 57: 653-661 (2015)

Avian influenza is a major viral disease in poultry. Antigenic variation of this virus hinders vaccine development. However, the extracellular domain of the virus-encoded M2 protein (peptide M2e) is nearly invariant in all influenza A strains, enabling the development of a broad-range vaccine against them. Antigen expression in transgenic plants is becoming a popular alternative to classical expression methods. Here we expressed M2e from avian influenza virus A/chicken/Kurgan/5/2005(H5N1) in nuclear-transformed duckweed plants for further development of avian influenza vaccine. The N-terminal fragment of M2, including M2e, was selected for expression.

The M2e DNA sequence fused in-frame to the 5' end of beta-glucuronidase was cloned into pBI121 under the control of CaMV 35S promoter. The resulting plasmid was successfully used for duckweed transformation, and western analysis with anti-beta-glucuronidase and anti-M2e antibodies confirmed accumulation of the target protein (M130) in 17 independent transgenic lines. Quantitative ELISA of crude protein extracts from these lines showed M130-beta-glucuronidase accumulation ranging from 0.09-0.97 mg/g FW (0.12-1.96 % of total soluble protein), equivalent to yields of up to 40 mu g M2e/g plant FW. This relatively high yield holds promise for the development of a duckweed-based expression system to produce an edible vaccine against avian influenza.

Phytoremediation

Post-treatment and reuse of secondary effluents using natural treatment systems: the Indian practices

Kumar, D., Asolekar, S.R., Sharma, S.K.

Environmental Monitoring and Assessment 187: 4792 (2015)

Paper summarizes the results of India-wide survey of natural treatment systems (NTSs) for wastewater treatment and reuse. The quality of treated wastewater from different types of NTSs was analyzed for various physico-chemical and bacteriological parameters, and needs for post-treatment were identified. Currently, about 1838 million liters per day (MLD) of wastewater is being treated using NTSs, of which the contributions of polishing ponds, waste stabilization ponds, duckweed ponds, constructed wetlands, and Karnal technology were found to be 53.39, 45.15, 0.13, 0.55, and 0.78%, respectively. Among the NTSs studied, constructed wetland was found most efficient in removal of pollutants including nitrogen, phosphorus, total coliform, and fecal coliform in the range of 76, 61, 99.956, and 99.923%, respectively. Of all types of NTSs, only constructed wetland was found to meet the total coliform count requirements (<1000 per 100ml). Of all the 108 NTSs in operation, 23 systems are producing treated effluents for irrigation; effluents from 48 systems are being discharged into river or lake, and remaining 38 systems have not found any designated use of treated effluent. The chlorination was the only post-treatment, which is being practiced at only three wastewater treatment facilities. During post-treatment, 1-2ppm of chlorine is applied to the secondary effluent irrespective of its quality. The treated effluents from different NTSs contain fecal bacteria in the magnitude of 10(3) to 10(5), which may cause the severe health impacts through contamination of groundwater as well as surface water resources.

Duckweed does not improve the efficiency of municipal wastewater treatment in *Lemna* system plants

Ozimek, T., Dabrowski, W., Florkiewicz, M.

ARCHIVES OF ENVIRONMENTAL PROTECTION 41: 47-52 (2015)

This study investigated the operation of three full-scale Lemna System surface flow municipal wastewater treatment plants, built according to the Lemna Corporation design. These plants consist of two ponds, the first aerated and the second for duckweed, with a barrier grid in the latter to ensure uniform plant distribution across its area. According to designers duckweed improves the efficiency of wastewater treatment. The three treatment plants are situated in central Poland and they differ in the occurrence of duckweed, two of them, located in Rakow and Bakowiec, operate without duckweed. and the third in Falecin Stary, Lemna minor covers ca. 90% of second pond surface. The efficiency of Lemna System wastewater treatment was found not to differ between the plants with and without duckweed. The aerated pond played the main role in reduction of pollutants in the investigated Lemna Systems.

Effects of a rhizobacterium on the growth of and chromium remediation by *Lemna minor*

Tang, J., Zhang, Y., Cui, Y., Ma, J.

ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH 22: 9686-9693 (2015)

Duckweed has shown great potential for both energy and environmental applications, particularly in wastewater treatment and fuel ethanol production. A rhizobacterium, Exiguobacterium sp. MH3, has been reported to associate with the duckweed Lemna minor for symbiotic growth. The aim of this work is to study the effects of rhizobacterium MH3 on L. minor growth and chromium (Cr) remediation. It appeared to have a synergism between the rhizobacterium MH3 and duckweed; the presence of strain MH3 promoted the growth of duckweeds by increasing both the frond number and dry weight of duckweed by more than 30 %, while duckweed in turn provided essential carbon source and energy for the growth of rhizobacterium MH3. Under Cr(VI) exposure, particularly at higher Cr(VI) concentrations, Exiguobacterium sp. MH3 significantly alleviated the harmful effects of the stress on the duckweed by promoting duckweed growth and preventing duckweed from excessive uptake of Cr. Potential mechanisms were also discussed in light of the genome sequence of strain MH3, and it was speculated that siderophores and indole-3-acetic acid (IAA) secreted by strain MH3 might contribute to promoting duckweed growth.

The influence of *Lemna* sp and *Spirogyra* sp on the removal of pharmaceuticals and endocrine disruptors in treated wastewaters

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INTERNATIONAL JOURNAL OF ENVIRONMENTAL SCIENCE AND TECHNOLOGY 12: 2327-2338 (2015)

The presence of pharmaceuticals and endocrine-disrupting chemicals (EDCs) in wastewater treatment plant effluents is an issue of great concern due to the negative effects that these compounds may have on human health and ecosystems. The present study aims to assess the capacity of two aquatic plants (Lemna sp. and Spirogyra sp.), commonly found in polishing ponds, for removing six pharmaceutical compounds (diclofenac, acetaminophen, ibuprofen, carbamazepine, clofibric acid, and propranolol), two EDCs (17 alpha-ethinylestradiol and bisphenol A), and one stimulant (caffeine) under laboratory-scale conditions. Planted and unplanted reactors fed with secondary-treated wastewater or ultrapure water in both covered and uncovered conditions were studied. The highest removal efficiencies, which ranged from 31 to 100 %, were achieved in uncovered planted systems containing secondary-treated wastewater after 20 days of incubation. The results demonstrated that non-charged compounds with a log Kow between 2 and 4 were affected by the presence of vegetation, probably due to their plant uptake, whereas negatively charged compounds were not. This highlights that the presence of plants in polishing ponds plays an important role in the removal of pharmaceuticals and EDCs.

Phytotoxicity

Zinc conferred cadmium tolerance in *Lemna minor* L. via modulating polyamines and proline metabolism

Qiao, X., Wang, P., Shi, G., Yang, H.

PLANT GROWTH REGULATION 77: 1-9 (2015)

To investigate the alleviating effects of zinc (Zn) against gradually increasing cadmium (Cd) stress in aquatic environment, dry weight, polyamines and proline contents as well as metabolic enzymes were studied in Lemna minor L. after 4 days exposure. Dry weight was significantly decreased as the concentration of Cd increased. Cd stress also increased the putrescine (Put) content, while decreasing spermidine (Spd) content, whereas no significant change was observed in spermine (Spm) content. Hence, the ratio of (Spd + Spm)/Put rapidly reduced. In addition, the activities of arginine decarboxylase (ADC), ornithine decarboxylase and polyamine oxidase (PAO) enhanced accordingly. Cd treatment also induced a continuous accumulation of proline. Meanwhile, pyroline-5-carboxylate synthetase (P5CS) activity increased initially only to decline later and ornithine delta-

aminotransferase (OAT) activity was only significantly stimulated at 4 mu M Cd, while the proline dehydrogenase (PDH) activity declined. However, Zn supplementation lowered accumulation of Put and proline contents and raised the Spd content, via decreasing the activities the ADC and PAO and keeping the activities of P5CS, OAT and PDH at the control levels, but failed to generate a statistically significant difference in content of dry weight. These results suggested that Zn application can maintain polyamines and proline homeostasis, thus conferring the tolerance of L. minor to Cd.

Subcellular distribution of uranium in the roots of *Spirodela punctata* and surface interactions

Nie, X., Dong, F., Liu, N., Liu, M., Zhang, D., Kang, W., Sun, S., Zhang, W., Yang, J.

APPLIED SURFACE SCIENCE 347: 122-130 (2015)

The subcellular distribution of uranium in roots of Spirodela punctata (duckweed) and the process of surface interaction were studied upon exposure to U (0, 5-200 mg/L) at pH 5. The concentration of uranium in each subcelluar fraction increased significantly with increasing solution U level, after 200 mg/L uranium solution treatment 120 h, the proportion of uranium concentration approximate as 8:2:1 in the cell wall organelle and cytosol fractions of roots of S. punctata. OM SEM and EDS showed after 5-200 mg/L U treatment 4-24 h, some intracellular fluid released from the root cells, after 100 mg/L U treatment 48h, the particles including 35% Fe (wt%) and other organic matters such as EPS released from the cells, most of the uranium bound onto the root surface and contacted with phosphorus ligands and formed as nano-scales U-P lamellar crystal, similar crystal has been found in the cell wall and organelle fractions after 50 mg/L U treatment 120 h. FTIR and XPS analyses result indicates the uranium changed the band position and shapes of phosphate group, and the region of characteristic peak belongs to U(VI) and U(IV) were also observed. (C) 2015 Published by Elsevier B.V.

Growth recovery of *Lemna gibba* and *Lemna minor* following a 7-day exposure to the herbicide diuron

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BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY 95: 150-156 (2015)

In agricultural catchments, aquatic ecosystems can experience a pulse exposure to pesticides. Following such exposure, non-target organisms that are not extirpated may recover. This paper investigates the potential of two duckweed species (Lemna minor and Lemna gibba) to recover from a

7-day exposure to different concentrations (0.4-208 A mu g L-1) of the herbicide diuron. There was significant inhibition in the growth and biomass after the initial 7-day exposure (e.g. frond number EC50 = 59.2 and 52.2 A mu g L-1 for L. minor and L. gibba, respectively). Following transfer to clean media, recovery (the highest concentration yielding no significant difference in the effect endpoint from the control) was observed for all effects endpoints at concentrations ranging 60-111 A mu g L-1 for L. minor and 60-208 A mu g L-1 for L. gibba. These results suggest that recovery is possible for primary producers at environmentally relevant concentrations considered significant in ecological risk assessment.

Comparative study on the sensitivity of turions and active fronds of giant duckweed (*Spirodela polyrhiza* (L.) Schleiden) to heavy metal treatments

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CHEMOSPHERE 132: 40-46 (2015)

Standard ecotoxicological test procedures use only active forms of aquatic plants. The potential effects of toxicants on vegetative propagules, which play an important role in the survival of several aquatic plant species, is not well understood. Because turion-like resting propagules overwinter on the water bottom in temperate regions, they could be exposed to contaminants for longer periods than active plants. Due to its turion producing capability, giant duckweed (Spirodela polyrhiza) is widely used in studying morphogenesis, dormancy, and activation mechanisms in plants. It is also suitable for ecotoxicological purposes. The present work aims to compare the growth inhibition sensitivity of active (normal frond) and overwintering (turion) forms of S. polyrhiza to concentrations of nickel (Ni), cadmium (Cd) and hexavalent chromium (Cr) ranging from 0 to 100 mg L-1. The results indicated that in general, resting turions have higher heavy metal tolerance than active fronds. Cd proved to be the most toxic heavy metal to S. polyrhiza active frond cultures because it induced rapid turion formation. In contrast, the toxicity of Ni and Cr were found to be similar but lower than the effects of Cd. Cr treatments up to 10 mg L-1 did not result in any future negative effects on turion activation. Turions did not survive heavy metal treatments at higher concentrations of Cr. Cd and Ni treatments affected both the floating-up and germination of turions but did not significantly affect the vigor of sprouts. Higher concentrations (of 100 mg L-1) Cd completely inhibited germination.

Beta-Radiation Stress Responses on Growth and Antioxidative Defense System in Plants: A Study with Strontium-90 in *Lemna minor*

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INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 16: 15309-15327 (2015)

In the following study, dose dependent effects on growth and oxidative stress induced by -radiation were examined to gain better insights in the mode of action of -radiation induced stress in plant species. Radiostrontium (Sr-90) was used to test for -radiation induced responses in the freshwater macrophyte Lemna minor. The accumulation pattern of Sr-90 was examined for L. minor root and fronds separately over a seven-day time period and was subsequently used in a dynamic dosimetric model to calculate -radiation dose rates. Exposing L. minor plants for seven days to a Sr-90 activity concentration of 25 up to 25,000 kBqL(-1) resulted in a dose rate between 0.084 +/- 0.004 and 97 +/- 8 mGyh(-1). After seven days of exposure, root fresh weight showed a dose dependent decrease starting from a dose rate of 9.4 +/- 0.5 mGyh(-1). Based on these data, an EDR10 value of 1.5 +/- 0.4 mGyh(-1) was estimated for root fresh weight and 52 +/- 17 mGyh(-1) for frond fresh weight. Different antioxidative enzymes and metabolites were further examined to analyze if -radiation induces oxidative stress in L. minor.

The influence of duckweed species diversity on ecophysiological tolerance to copper exposure

Zhao, Z., Shi, H., Duan, D., Li, H., Lei, T., Wang, M., Zhao, H., Zhao, Y.

AQUATIC TOXICOLOGY 164: 92-98 (2015)

In excess, copper is toxic to plants. In the plants, *Landoltia punctata* and *Lemna minor* grown in mixed and monoculture, the effects of exposure to varying concentrations of copper (0.01, 0.1, 0.5 and 1 mg L-1 Cu) for seven days were assessed by measuring changes in the chlorophyll, protein and malondialdehyde (MDA) content, catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity. According to results, Cu levels in plants increased with increasing Cu concentration. The level of photosynthetic pigments and crude proteins decreased only upon exposure to high Cu concentrations. However, the starch and malondialdehyde (MDA) content increased. These results suggested a stress alleviation that was possibly the result of antioxidants such as CAT and SOD, the activities of which increased with increasing Cu levels. APX activity increased in L. punctata, but decreased in L. minor, under monoculture or mixed culture conditions. In addition, the duckweed in mixed culture exhibited increased antioxidant enzyme activities which provide increased resistance to copper in moderate copper concentrations. As the copper concentration increased, the duckweed in the mixed culture limited the uptake of copper to avoid toxicity

Ecotoxicological evaluation of propranolol hydrochloride and losartan potassium to *Lemna minor* L. (1753) individually and in binary mixtures

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ECOTOXICOLOGY 24: 1112-1123 (2015)

Antihypertensive pharmaceuticals, including the beta-blockers, are one of the most detected therapeutic classes in the environment. The ecotoxicity of propranolol hydrochloride and losartan potassium was evaluated, both individually and combined in a binary mixture, by using the Lemna minor growth inhibition test. The endpoints evaluated in the single-pharmaceutical tests were frond number, total frond area and fresh weight. For the evaluation of the mixture toxicity, the selected endpoint was frond number. Water quality criteria values (WQC) were derived for the protection of freshwater and saltwater pelagic communities regarding the effects induced by propranolol and losartan using ecotoxicological data from the literature, including our data. The risks associated with both pharmaceutical effects on non-target organisms were quantified through the measured environmental concentration (MEC)/predicted-no-effect concentration (PNEC) ratios. For propranolol, the total frond area was the most sensitive endpoint (EC50 = 77.3 mg L-1), while for losartan there was no statistically significant difference between the endpoints. Losartan is only slightly more toxic than propranolol. Both concentration addition and independent action models overestimated the mixture toxicity of the pharmaceuticals at all the effect concentration levels evaluated. The joint action of both pharmaceuticals showed an antagonistic interaction to L. minor. Derived WQC assumed lower values for propranolol than for losartan. The MEC/PNEC ratios showed that propranolol may pose a risk for the most sensitive aquatic species, while acceptable risks posed by losartan were estimated for most of aquatic matrices. To the authors knowledge these are the first data about losartan toxicity for L. minor.

Growth and photosynthetic responses of *Lemna minor* L. exposed to cadmium in combination with zinc or copper

Vidakovic-Cifrek, Z., Tkalec, M., Sikic, S., Tolic, S., Lepedus, H., Pevalek-Kozlina, B.

ARHIV ZA HIGIJENU RADA I TOKSIKOLOGIJU-ARCHIVES OF INDUSTRIAL HYGIENE AND TOXICOLOGY 66: 141-152 (2015)

Metals have a variety of negative outcomes on plants, essential components of any ecosystem. The effects of CdCl2 (5 mu mol L-1), ZnCl2 (25 or 50 mu mol L-1), and CuCl2 (2.5 or 5 mu mol L-1) and combinations of CdCl2 with either ZnCl2 or CuCl2 on the growth, photosynthetic pigments, and photosystem II (PSII) efficiency of duckweed (Lemna minor L.) were investigated. All of the treatments

caused growth inhibition and remarkable metal accumulation in plant tissue after 4 and 7 days. In the combined treatments, the accumulation of each metal applied was lesser in comparison to treatments with single metals. After 4 days, all of the treatments generally diminished chlorophyll a content and decreased the maximum quantum yield (F-v/F-m) and effective quantum yield (Delta F/F'(m)) of PSII. However, after 7 days of exposure to a combination of Cd and Zn, pigment content and PSII activity recovered to control levels. A higher concentration of Cu (5 mu mol L-1) as well as Cd in combination with Cu had a prolonged inhibitory effect on photosynthetic features. Our results suggest that growth inhibition was due to the toxic effect of absolute metal quantity in plant tissue. Zn counteracted Cd uptake, as seen from the recovery of pigment content and PSII efficiency in plants exposed for 7 days to the Cd and Zn combination. Cu-induced oxidative stress led to a prolonged inhibitory effect in plants treated both with a higher concentration of Cu (5 mu mol L-1) and simultaneously with Cd and Cu. Our findings could contribute to general knowledge on anthropogenic and environmental contaminants that endanger plant communities and significantly disrupt the sensitive balance of an ecosystem by influencing photosynthetic mechanisms.

Others

Enhanced metabolic and redox activity of vascular aquatic plant *Lemna valdiviana* under polarization in Direct Photosynthetic Plant Fuel Cell

Hubenova, Y., Mitov, M.

Bioelectrochemistry 106 (Pt A): 226-231 (2015)

In this study, duckweed species *Lemna valdiviana* was investigated as a photoautotrophycally grown biocatalyst in recently developed Direct Photosynthetic Plant Fuel Cell. Stable current outputs, reaching maximum of 226±11mA/m(2), were achieved during the operating period. The electricity production is associated with electrons generated through the light-dependent reactions in the chloroplasts as well as the respiratory processes in the mitochondria and transferred to the anode via endogenous electron shuttle, synthesized by the plants as a specific response to the polarization. In parallel, a considerable increase in the content of proteins (47%) and reserve carbohydrates (44%) of duckweeds grown under polarization conditions was established by means of biochemical analyses. This, combined with the electricity generation, makes the technology a feasible approach for the duckweed farming.

Links for Further Reading

http://www.duckweed2015.cosmos.bot.kyoto-u.ac.jp Duckweed 2015 Conference site- watch for details as Kyoto, Japan conference preparations develop.
http://duckweed2013.rutgers.edu/ Past duckweed conference papers and proceedings held at Rutgers University, New Brunswick, NJ in Aug, 2013
http://Lemnapedia.org Online developing compendium of duckweed research & applications, founded by the ISCDRA.
http://InternationalLemnaAssociation.org Working to develop commercial applications for duckweed globally, Exec. Director, Tamra Fakhoorian
http://www.mobot.org/jwcross/duckweed/duckweed.htm Comprehensive site on all things duckweed-related, By Dr. John Cross.
http://plants.ifas.ufl.edu/ University of Florida's Center for Aquatic & Invasive Plants

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