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# DUCKWEED FORUM

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International Steering Committee on Duckweed Research and Applications

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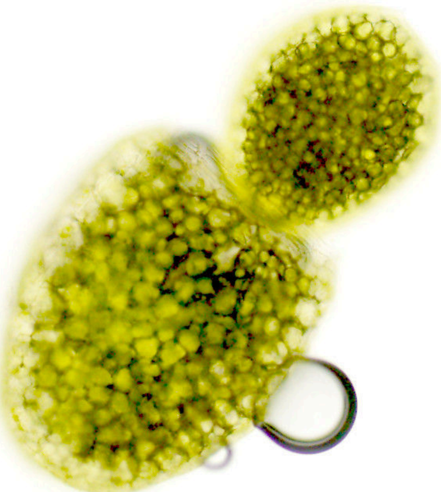
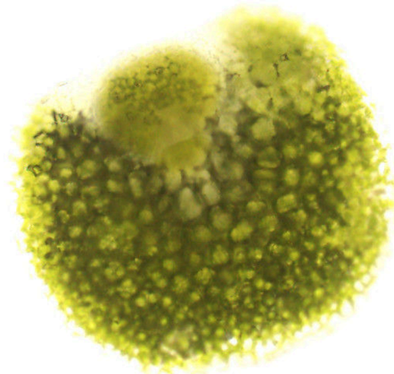
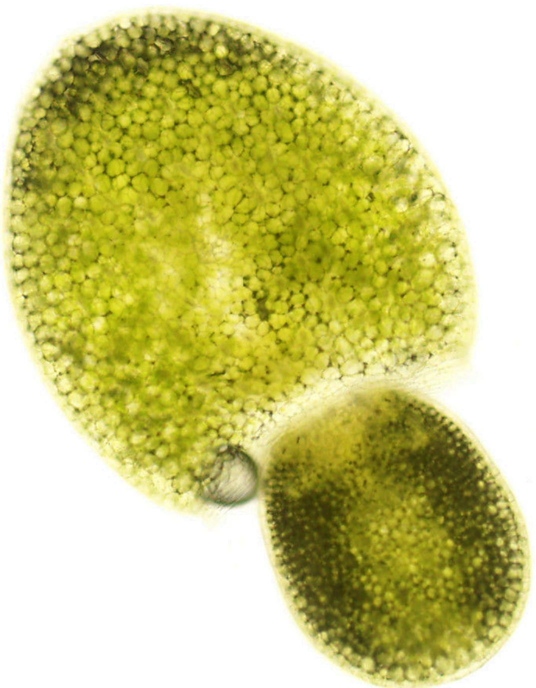


*Wolffia globosa* 9297



*Wolffia microscopica* 2005



*Wolffia australiana* 8730

*Wolffia brasiliensis* 8116

1 mm

These four duckweed species belong to the genus *Wolffia*, with a high reduction in their morphology. *Wolffia globosa* is a common and sometimes even a dominant species in South and Southeast Asia and is also found in South Africa. Whereas the re-discovered species *W. microscopica* is endemic to India, Bangladesh and Pakistan. *Wolffia australiana*, having the smallest genome in this genus, could be found in Australia and New Zealand, while *W. brasiliensis* appears to be a species dispersed throughout the warmer regions of the American continents.
Photographs taken by Dr. Eric Lam at the Rutgers Duckweed Stock Cooperative (Rutgers University).

# Useful methods 6: LemnaTec Scanalyzer

a Versatile Tool for Duckweed Research and Testing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## Duckweed assessments

Duckweed growth inhibition tests are widely used for risk assessments in many areas. For instance, testing plant toxicity of chemicals, including plant protecting agents and other substances used in agriculture and gardening. Moreover such tests deliver ecotoxicological data on water and soil contamination.

The tests largely rely on repeated counting of frond numbers throughout the test period, in particular focusing on the change in frond development after application of the testing substance. Effects of testing substances on growth and development of fronds are evaluated in comparison to the development of untreated fronds growing in parallel.

## How could LemnaTec help my routine work?

LemnaTec was founded in 1998, as a spin-off from RWTH Aachen, to develop a digital imaging system to automate frond counting in duckweed tests. Fronds, displayed on vessels with growth media typically used in duckweed assays, are imaged by the LemnaTec Scanalyzer platform. The resulting RGB images are automatically stored and analyzed by the LemnaTec's software that generates output tables containing all of the measured parameters. By repeating this procedure with multiple samples on subsequent days throughout the screening period, it is possible to monitor and compare temporal development of various treatments or sample-types. In particular, dynamic changes after applying testing materials to the duckweed can be monitored in a multi-factorial manner.

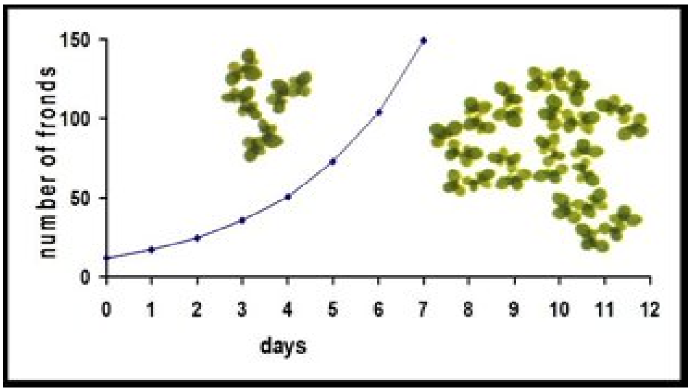


Figure 1 Example of automatically measured frond numbers over seven days.

# Image processing tools to support duckweed measurements

LemnaGrid is a computer software package with a rich library of image processing functions (grid devices). It uses an intuitive approach to create image processing and image analysis algorithms by dragging and dropping functions from a library panel into the grid designer window, and then connecting the devices in a logical manner. The user, therefore, does not have to write lines of code but simply create workflows. Thereby, users can quickly create re-usable and customizable analysis procedures without the need to learn a programming language or involve data scientists.

A basic workflow consists of four main devices: an image processing module that is a database reader (DB Reader) together with a foreground/background color picker and an image analysis module that consists of a universal converter and a database writer (DB Writer).

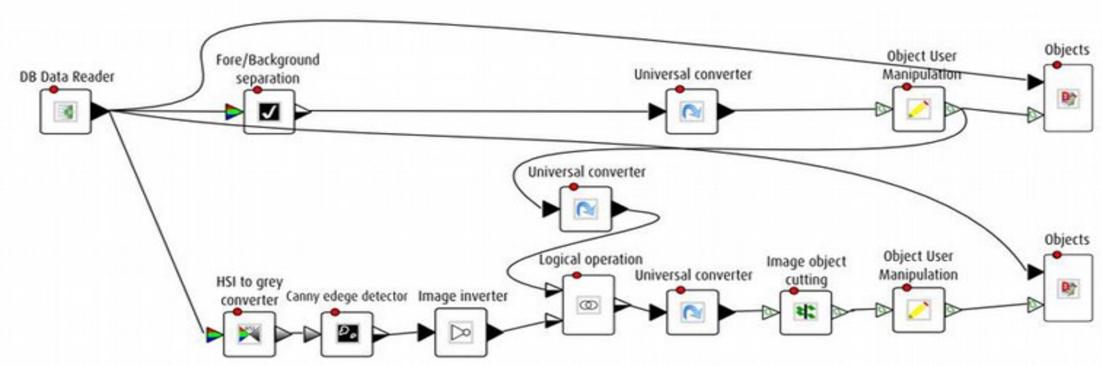


Figure 2: Example setup of a data processing pipeline for LemnaTec's image analysis built with the LemnaGrid software platform.

In this application example (Figure 2) digitized images of *Lemna* samples are loaded by the DB Reader. Due to a homogeneous background - in this case a white panel - the detection of image objects (image segmentation) can be efficiently done by defining white (R/G/B = 255/255/255 +/- 30) as the background. All other colors are taken as foreground. The result is a binary image (image mask) where white pixels represent the image object.

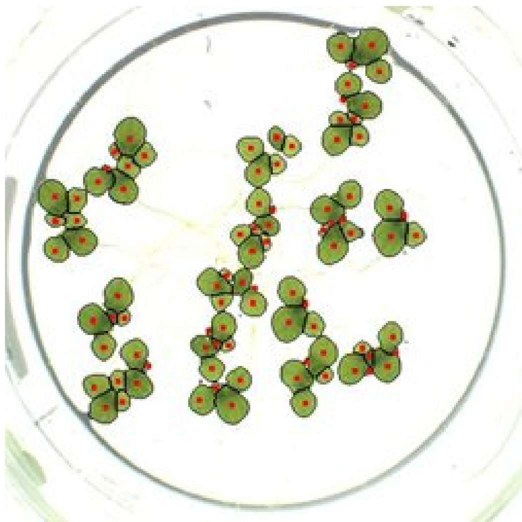

Figure 3: Duckweed fronds after analysis with LemnaGrid – red dots denote frond counts, single detected fronds are encircled by dark lines.

By converting all binary large objects (blobs) in the binary image into a list of objects with the Universal Converter, objects are counted and parameters for each object are determined, including its surface area (figure 3). Other morphometric parameters include center of mass, convex hull area, compactness, or bounding box dimensions such as width and height. The result of the analysis is written to the database with the DB Writer and can be retrieved from there for further analysis.

In order to determine the number of fronds, the processing pipeline converts the color image into an intensity image with the HSI to gray converter and uses the Canny Edge Detector to find edges between fronds. The result is used to modify the image mask with the Image Inverter and Logical Operator. In a next step the Image Object Cutter segments objects into smaller constituents, in this case *Lemna* fronds. Frond detection is a challenging task, because depending on the growth stage, age and physiology a clear edge between fronds may be hard to detect. Therefore the result is returned as a predicted solution to the user, and using the Object User Manipulation device, the user can refine the output.

Taken together, LemnaGrid enables an automated evaluation, but still leaves the possibility that users refine results in complex analysis cases.

## Parameters derived from image processing

Image processing tools are more accurate and reproducible than visual inspection by human operators. Different from visual scoring that frequently delivers qualitative data, image processing enables capturing more quantitative parameters beyond the frond count. The processing pipeline can deliver numbers that describe sizes, shapes, and colors of the fronds in addition to the frond counts. These parameters link to biologically and eco-toxicologically relevant data and describe how phenotypic changes – in comparison to untreated healthy fronds – occur after exposure to toxic substances (Figure 4).

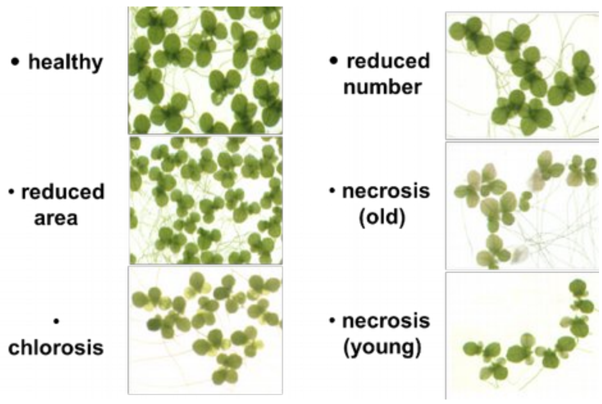


Figure 4 Typical phenotypic changes of stressed fronds in comparison to healthy fronds

Frond counts, and particularly the change of them, are indicative of growth and multiplication processes, or if death occurred when the frond number decreased (Figure 1). Using image processing, the area of the fronds can be determined in order to assess growth more precisely than it is possible by just counting. The dynamics of the overall area of the visible fronds similarly link to growing or decreasing populations. In addition, area data on single fronds, and their distribution within the population, can reveal information on individual growth processes. As large numbers of small fronds might have the same overall area as small numbers of large fronds, it is important to combine parameters in order to comprehensively assess the developmental status of the fronds and their response to testing factors and substances.

Image analysis can provide information on surface colours, too, and thereby assist the estimation of whether fronds are healthy or stressed. Chlorosis and necrosis of frond evoke characteristic colour changes that can be quantified with image analysis tools. Such quantification enables precise calculation of the degree of chlorosis or necrosis and thereby helps to assess viability. Viability assessment in turn enables one to distinguish between living and dead fronds, which is important for rating the impact of testing substances on plant survival. Finally image analysis determines shape description that helps to detect stress-related shape changes.

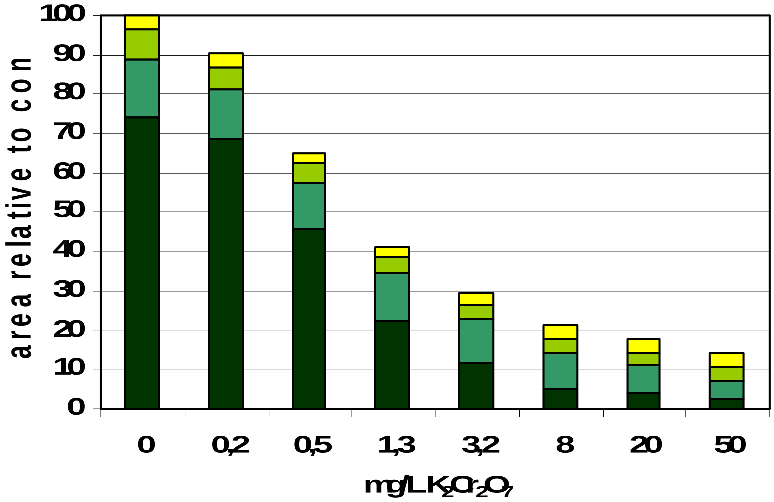


Figure 5: Frond area and color class fractions in dependency of increasing concentrations of a toxic substance (potassium dichromate).

In a pilot study, fronds were exposed to potassium dichromate in concentrations of 0.2 to 50 mg per liter of cultivation medium and compared to fronds growing without the testing substance (Figure 5). Already at the lowest concentration tested markedly diminished frond area can be observed, and the growth inhibition increased further with higher levels of potassium dichromate in the growth medium. Coincident with growth reduction, remaining frond area changed from healthy green to pale and yellow, indicating that large fractions of the remaining fronds undergone damage and were no longer viable. At control cultivation without toxins, a baseline of around 30% frond area having colour changes from green towards pale green and yellow were observed. This fraction grew to more than 75% at high concentrations of potassium dichromate. The results indicate that potassium dichromate not only inhibits the growth of new fronds, but also damages the existing fronds by reducing viable area.

Combining multiple duckweed characterizing factors derived from image processing together with biochemical data provides comprehensive sets of information in toxicology studies, for example in the assessment of phytotoxicity of cobalt ions by Sree et al. (2015).

## Applications in research and monitoring

Duckweed assays are used in wastewater management in order to monitor whether substances with toxicity to plants remain after wastewater treatment processes. A research group in Lisbon analyzed the efficiency of wastewater cleaning with ecotoxicological test with different organisms, including *L. minor* (Mendonça et al., 2013). At different days throughout the week, and also at different times throughout the days, the observed toxicity of influent water changed due to wastewater composition. Primary and particularly secondary treatment steps in the cleaning procedure turned out to efficiently clean the water and eliminating toxic compounds. The results proved that such monitoring procedures are valuable tools for environmental management.

Nanoparticles are broadly used, e.g. for surface coatings or solar cells, but they are prone to have toxic effects on organisms. A research consortium in Lisbon, partly comprising the same researchers as the group mentioned in the previous paragraph, tested phytotoxicity of such particles using a *L. minor* assay (Picado et al., 2015). Although the tested particles were non-toxic to *L. minor*, there was toxicity to other organisms such as bacteria, algae, or crustaceae.

Chemical compounds of catalysts are potential sources of environmental pollution and damage; therefore ecotoxicological tests are meaningful assays to measure hazard potentials of such substances. A consortium of German and Polish researchers measured the toxic effect of Methyltrioxorhenium and derivatives thereof, which are suitable for use as catalysts in petrol chemistry (Stolte et al., 2015). Not only toxicity towards higher plants as determined with a Scanalzyer-assisted duckweed assay, but toxicity to various organisms led to the conclusion that preventing release of and exposure to such chemicals is essential.

Similarly, German and Polish researchers assessed ecotoxic properties of sweetener substances, again including a duckweed assay that should indicate toxicity of the tested substances towards plants (Stolte et al., 2013). As artificial sweeteners became popular as replacements for sugar, they occurred as anthropogenic trace substances in aquatic environments but their toxicity data are scarce. Assessing duckweed together with algae and water fleas, the researchers reported that common artificial sweeteners tested as well as the natural sweetener stevioside do not exhibit toxicity to such organisms, although additional data may be needed for more complete assessment.

Published literature on ecotoxicology studies relying on LemnaTec duckweed assays not only comprises these examples of water management, nanoparticle studies, catalyst assessment, and food additive characterisation, but there are examples available from herbicide testing (Grossmann and Ehrhardt, 2007; Tresch et al., 2008; Grossmann et al., 2012), natural compound assays (Meepagala et al., 2005; Diers et al., 2006; Cantrell et al., 2007; Cerdeira et al., 2012), homeopathic substance studies (Scherr et al., 2009; Jäger et al., 2010; Jäger et al., 2011) and other applications.

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