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Green Liver Concept and Green Liver Systems –A Sustainable Way for Future Water Purification

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Abbreviations: cDNA: c Deoxyribonucleic Acid; US-EPA: United States Environmental Protection Agency; PAH: Polyaromatic Hydrocarbons; PCB: Polychlorinated Biphenyls; MC-LR: Microcystin-LR

Keywords: Biotransformation; Green Liver Concept; Green Liver Systems®; Metabolites, Toxicity, Wetlands

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The Need for Sustainable Water Purification

Water covers our planet by roughly 70%, but most of it is saline. The amount of freshwater on our planet is only 2.5 – 2.75 % including frozen, thus not immediately available water, as snow, ice and glaciers (1.75 – 2.0%), as well as 0.7 – 0.8 % as groundwater and soil moisture. Essentially, less than 0.01% available water as surface water is located in lakes swamps and rivers [1]. The protection and reasonable use of freshwater is one of the main goals for our future, as water is the most important resource for all organisms on earth including humans. Due to the ongoing pollution of our aquatic ecosystems, not only with xenobiotics, but also with nutrients, the status of our water bodies are changing drastically.

Where we are at the moment

Looking at xenobiotic metabolism in animals and plants, the enzymes working in phase I and phase II of the biotransformation pathway, like cytochrome P450 monooxy - genases, glutathione S-transferase, glucuronosyltransferases are found in animal liver, as the main organ for biotransformation, are working in a very similar way [2]. Phase I is called the transformation phase and phase II the conjugation phase [2, 3]. The main difference between plant and animal xenobiotic metabolism is seen in phase III, where animals can excrete/eliminate, the formed metabolites via urine and faeces, whereas plants will emplace/store the formed metabolites (Figure.1). This phase, also called sequestration phase, can lead to several terminal fates of xenobiotics in plants such as storage in cell vacuole, storage in the apoplast or covalent binding to cell wall fractions such as celluloses and hemicelluloses. Therefore, an active transport for the formed metabolites to reach the vacuole or the apoplast is necessary, to move them through plasma membranes or the tonoplast [4]. For glutathione conjugates this will be done and controlled e.g. by glutathione pumps. Also in animals theseglutathione pumps are active to help in eliminating conjugated xenobiotics.

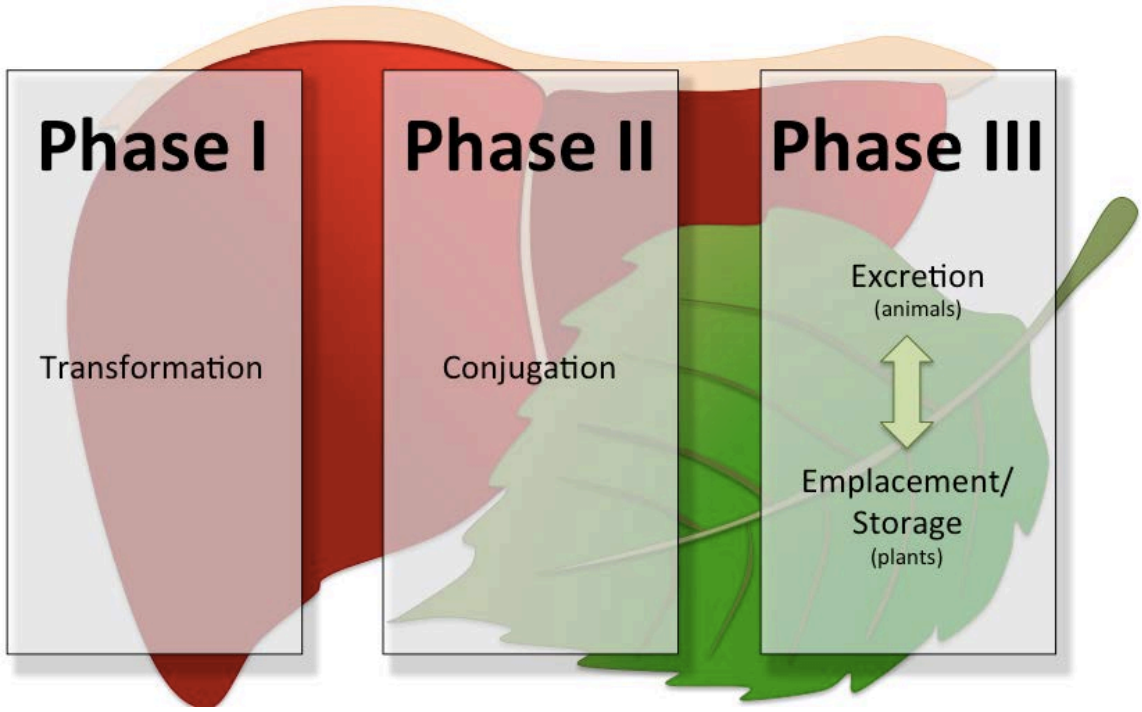

Figure 1: Biotransformation consisting of three phases: phase I (transformation) mainly catalysed by Cytochrome P-450 monooxygenases, phase II (conjugation) catalysed by glutathione S-transferase, glucuronosyl- and glucosyltransferases and phase III excretion (animals) or emplacement/storage (plants).

The idea of the Green Liver Concept was developed by Sandermann [5], pointing out the similarities of the biotransformation pathways in animals and plants. This concept was supported by the detection of similar enzyme systems, metabolite pattern and also on cDNA level, first in plant cell cultures [6, 7] and further on in several higher plants species and marine macroalgae [8].

In the early 90s, indoor air pollution was a major concern for human health perspectives [9, 10]. So the question was: how can we make use of uptake and biotransformation of potential toxic substances in a technical way? The first ideas were developed again by Sandermann et al. 1997 [11] testing the effectiveness of spider plants (*Chlorophytum comosum*) to remove formaldehyde from indoor air [11, 12]. Using plants for the removal of potentially toxic substances was already known and called phytoremediation [13]. For indoor air, several studies have been done to show the different potentials of terrestrial plant species in the removal e.g. of formaldehyde [14].

The pollution of aquatic ecosystems also occurs worldwide as a consequence of growing industry and agricultural practices. Contaminants include Polyaromatic Hydrocarbons (PAH), Polychlorinated Biphenyls (PCB), heavy metals, various pesticides and human as well as veterinary pharmaceuticals. Furthermore, due to excessive nutrient input into water bodies by wastewater and agricultural run-off, eutrophication plays a major role, leading more and more to the formation of potentially toxic cyanobacterial blooms. All these xenobiotics as well as the natural toxins from cyanobacteria, if taken up by organisms, must undergo biotransformation in order to keep the organisms healthy.

In the area of aquatic ecosystems, phytoremediation is done using natural or constructed wetland systems. Wetland systems in general are site-specific combinations trying to use physical, biological and chemical processes to remove contaminants from water. The US-EPA described natural wetland as the “earth´s kidney”, because they filter contaminants out of the water [15]. The individual components

of a wetland typically include a sediment basin, a level lip spreader, a primary grass filter, a vegetated wetland, a deep pond and finally a polishing filter. The latter e.g. can be a riparian forest buffer. The most common feature of wetlands is the fact, that generally the groundwater level is very close to the soil surface, or even shallow water covers the surface of the wetland most times of the year [16]. In a constructed wetland, the water moves slowly through the wetland giving extensive contact time with aerobic and anaerobic microorganisms present in wetland systems. In most of the transformation processes, the contaminants will undergo a biochemical conversion/transformation, with microorganisms being the driving force [17]. The higher plants in wetlands provide "only" a surface area for the growing microbial biofilm [18].

Natural and constructed wetlands have some advantages but also some disadvantages (Table. 1). One of the main advantages seems to be, that wetlands are providing a possible ecologically way of wastewater treatment. On the other hand it has been shown, that wetlands over time lose their ability to remove contaminants [19, 20].

Table 1: Advantages and disadvantages of constructed wetlands

Advantages of wetland	**Disadvantages of wetlands**
Construction relatively inexpensive	Large land area required
Ecologically way of wastewater treatment	Sometimes bad odour due to the wastewater
Tolerate big and small water volumes	Biological processes in the system not well understood
Can deal with various levels of contaminants	Die back in winter time and no purification effect
Habitat for wildlife	Restoration of wetlands difficult mainly due to water level problems
Reuse of water possible	Sometimes home of invasive species
Aesthetically pleasing for humans	Dominated by generalistic plants
	Takes 2-3 years growing season for full working action
	Management of an ecosystem is not easy
	Breeding place for mosquitos (depends on the climate zone)
	Sometimes preliminary treatment of the wastewater necessary
	Due to the high microbial activity an high amount of known and unknown metabolites are formed with unknown toxicity
	Plant harvesting and a final disposal method necessary

In short, these systems are designed to work as a specific ecosystem type, which makes the management of natural or constructed wetland difficult [21].

From a chemical and analytical point of view, what happens in a constructed wetland system? The contaminants are flushed into the system and the microbes in the biofilm are starting to work on them. In most cases, there will be a breakdown of the parent compound to different metabolites, in general this will occur extracellularly. So from the point, the "end product", the water released, will be more or less clean of the parent compound, but an unpredictable amount of known and unknown metabolites will be possibly released. Knowing the toxicity of the parent compound, the toxicity of the metabolites might not be so clear or even not known.

As an example, the cyanobacterial toxin and heptapeptide microcystin-LR (MC-LR) can be used. In a

traditional wetland system, using mostly aquatic bacteria, this heptapeptide would be broken down to a whole bunch of metabolites (Figure. 2). Because of the extracellular metabolisms of the toxin by the aquatic bacteria, these metabolites are still in the water body [22-25].

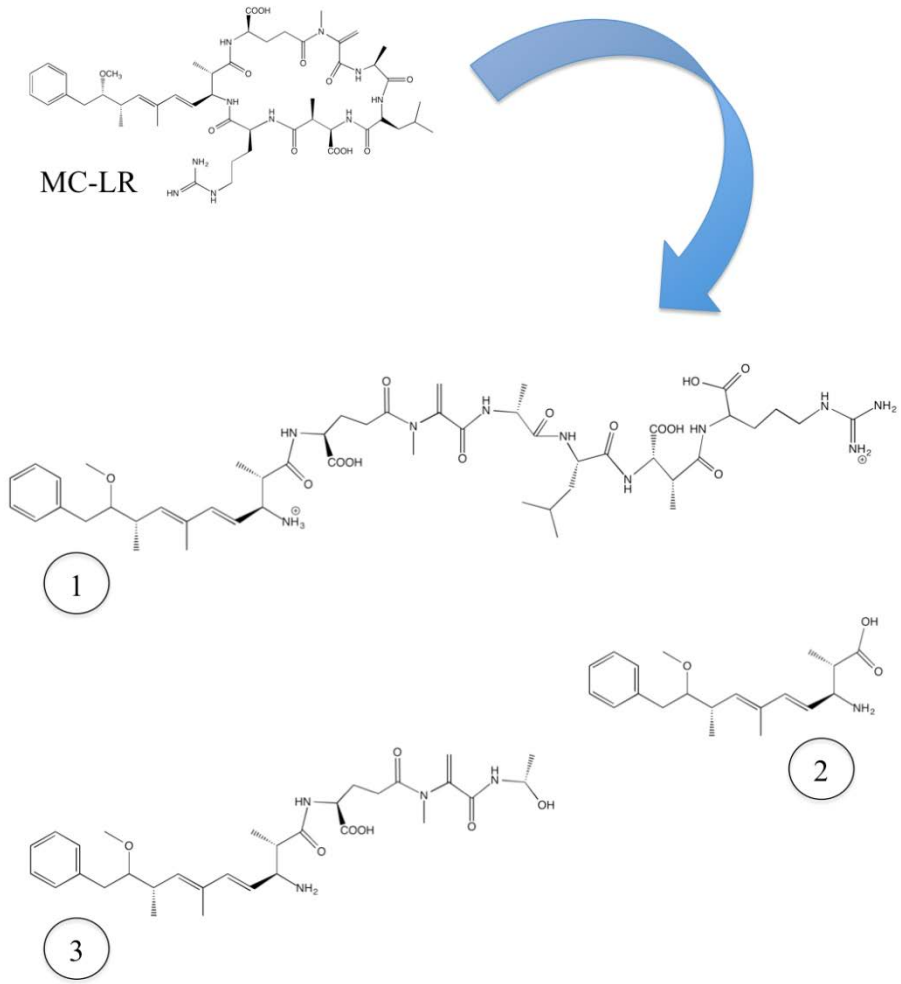

Figure 2: Potential extracellular formation of metabolites from the cyanobacterial toxin MC-LR *via* aquatic bacteria in traditional constructed wetlands. 1) ring-opening at the ADDA-arginine bound, 2) formation of a tetrapeptide, 3) ADDA moiety according to [25].

Taking the pros and the cons of constructed wetlands into account, a new development was necessary trying to overcome the negative points of constructed wetlands. Therefore, a complete artificial system was designed exhibiting the following main characteristics:

- Using aquatic plants for phytoremediation
- Minimise the microbial input
- The system should be effective but not producing metabolites in the water phase
- Easy to handle
- Customisable to the contaminants and needs
- Inexpensive

Green Liver Systems®

Based on the results from [5, 8] on the biotransformation capacity of plants, an artificial system, called the Green Liver System® was developed. As a basis, submerged aquatic

macrophytes were used, preferably those not rooting or having only small roots, to minimise bacterial influence. To cut off the microbial part in a Green Liver System, it should have no sediment therefore plants not rooting are preferred.

Furthermore, the ability of aquatic macrophytes to take up contaminants from the water is used as an ecosystem services, as well as their ability to bio transform and metabolise these contaminants within the plant cells. Again here the MC-LR can be used as an example (Figure. 3). The MC-LR is metabolised within plant cell *via* the glutathione S-transferase pathway [26]. The first metabolite formed is a glutathione conjugate, which is further degraded in the plant cells to a γ-glutamyl cysteine conjugate and a cysteinyl conjugate (Figure. 3) [27]. All these conjugates are not further released from the plant cells as long as the plants are not decaying. This internal metabolism is the big advantage of this Green Liver System®, because no metabolites were formed externally and distributed in the water phase.

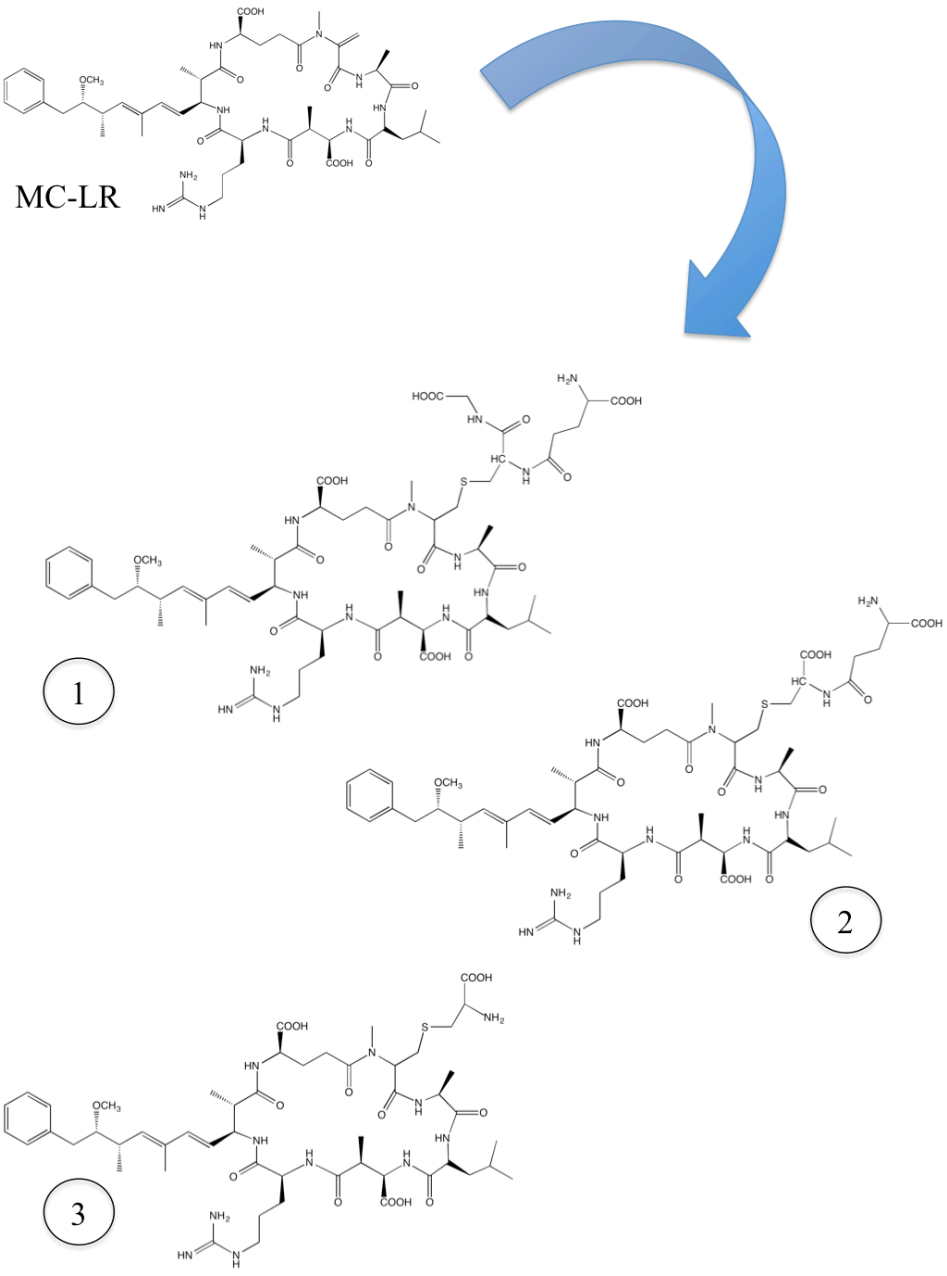

Figure 3: Plant cell internally formed metabolites of MC-LR starting with 1) glutathione conjugate, 2) γ-glutamylcysteine conjugate and 3) cysteinylconjugate [27]

The first Green Liver System® was designed in a laboratory style manner and a size of 60 x 20 x 20 cm made of acrylic glass [28]. The space in the system was divided with barriers into six compartments allowing water to flow from one compartment into the next, but giving a separation to the plants. The follow up system was made of glass having a size of 220 x 80 x 60 cm (Figure. 4). The design of this bigger system followed the original ones, only the compartment barriers were re-designed having round edges to minimise water velocity or turbulences in the system and giving more space for the water to flow between the compartments.

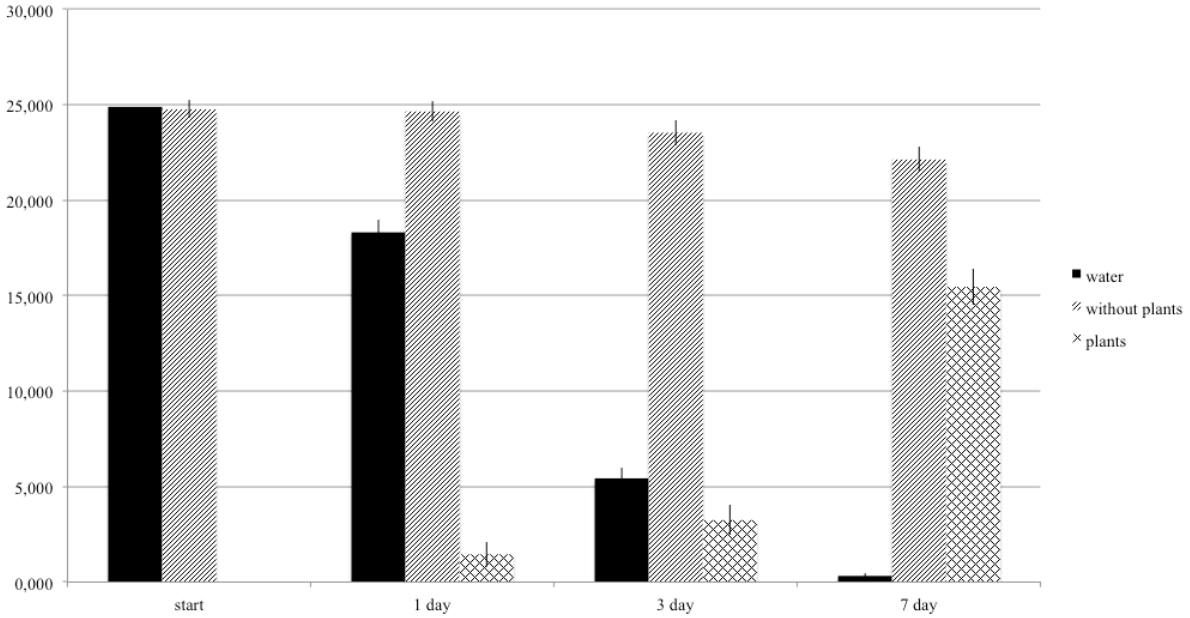

Figure 4: Example of the removal of benzo(a)pyrene in the laboratory system using the macrophytes *C. demersum*, *E. densa* and *M. aquaticum*. The black bars resemble the decline of benzo(a)pyren from the water phase during time, the dashed bars resemble the bacterial degradation in the laboratory system without plants and the crossed bars resemble the concentration of benzo(a)pyren detected in the plants itself (sum of all three plant species).

Within these laboratory systems a set of aquatic macrophytes were tested in their ability and effectiveness to reduce different contaminants from water. Concerning the aquatic macrophytes, plants from different taxonomic classes were tested: macroalgae, bryophytes, aquatic ferns and higher vascular macrophytes (Table. 2). The exposure time of these plants in different laboratory systems differ between 24h and 7 d.

Table 2: Aquatic plants from different taxonomic groups tested so far, for the possible use in a Green Liver System®

Scientific name	Common name	Reference
Higher vascular plants		
Ceratophyllum demersum	Coontail, Hornworth	[26] [29] [30] [31] [32]

Myriophyllum aquaticum *Myriophyllum spicatum* *Myriophyllum elatinoides* *Myriophyllum hippuroides* *Myriophyllum mattogrossense* *Myriophyllum quitense* *Myriophyllum verticillatum*	Parroth´s Feather	 [28] [33] [34] [35]
Elodea canadensis	Waterweed	[36]
Egeria densa	Large Flowered Waterweed	[37]
Eichhornia crassipes	Common Water Hyacinth	[38]
Limnoohila sessiliflora	Ambulia	[39]
Potamogeton perfoliatus *Potamogeton gayi*	Clasping-leaf Pondweed	[39]
Hydrilla verticillata	Esthwaite Waterweed	[28] [37]
Lemna gibba	Gibbous Duckweed	[40]
Lemna minor	Common Duckweed	[41]
Spirodela intermedia *Spirodela oligorhiza*	Greater Duckweed	[42] [43]
Wolffia arrhiza	Spotless Watermeal	[44]
Aquatic ferns		
Azolla filiculoides *Azolla caroliniana*	Mosquito Fern	[38] [45]
Salvinia auriculata	African payal	[46]
Lomariopsis lineata		[47]
Ceratopteris thalictroides	Indian fern	[28]
Aquatic bryophytes		
Fontinalis antipyretica	Common Water Moss	[47]
Riccia fluitans	Crystalwort	[47]
Taxiphyllum barbieri	Java Moss	[47]
Vesicularia dubyana	Christmass moss	[48]
Macroalgae		
Cladophora aegagrophila (*Aegagropila linnaei)*	Marimo	[49]
Cladophora glomerata		[50]
Chladophora fracta		[44]
Chara intermedia	Stonewort	[51]
Nitellopsis obtusa	Starry Stonewort	[52]

The removal of contaminants from the water was tested using benzo (a) pyrene (Figure. 4) and fluoranthrene (PAH), 3-chlorobiphenyl and Arachlor 1224 (PCB), paracetamol (acetaminophen), diclofenac, ibuprofen (human pharmaceuticals), oxytetracycline and methyltestosterone (veterinary pharmaceuticals), different microcystins (MC-LR (Figure. 5), -RR, -YR, -LF), anatoxin-a, cylindrospermopsin, BMAA (cyanobacterial toxins) and isoproturon, atrazine and cypermethrin (Figure. 6).

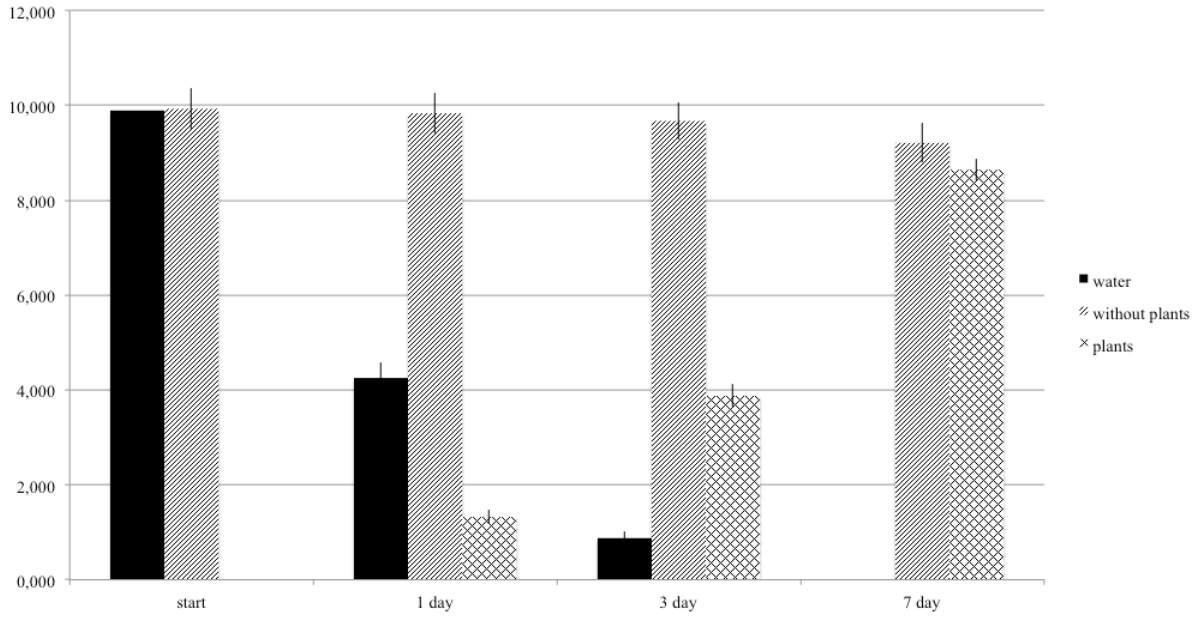

Figure 5: Example of the removal of MC-LR in the laboratory system using the macrophytes *C. demersum*, *E. densa* and *M. aquaticum*. The black bars resemble the decline of MC-LR from the water phase during time, the dashed bars resemble the bacterial degradation in the laboratory system without plants and the crossed bars resemble the concentration of MC-LR detected in the plants itself (sum of all three plant species).

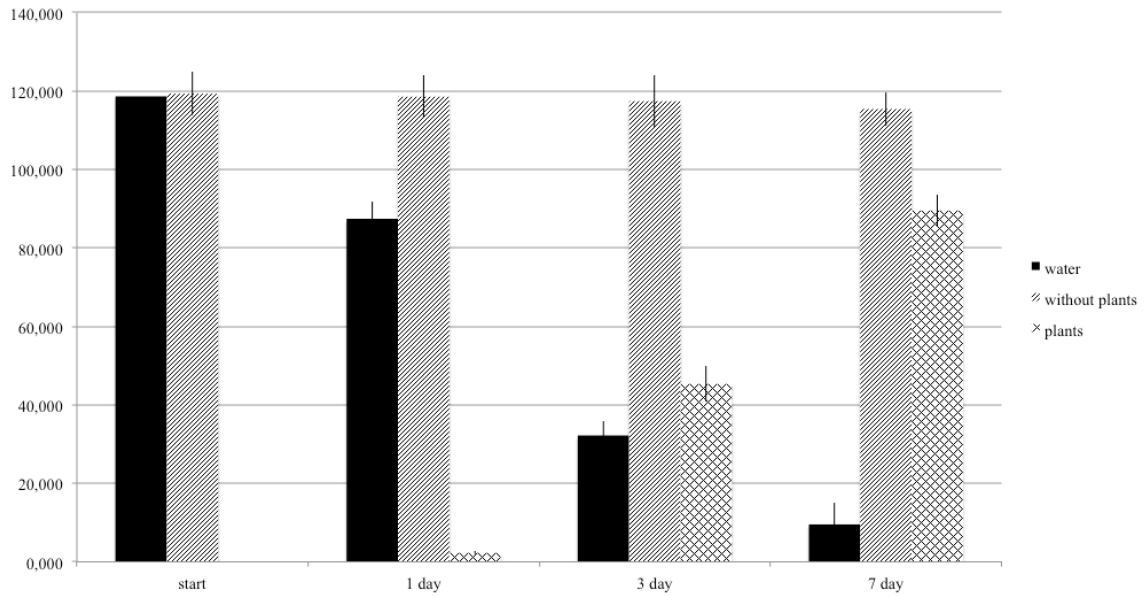

Figure 6: Example of the removal of cypermethrin in the laboratory system using the macrophytes *C. demersum*, *E. densa* and *M. aquaticum.* The black bars resemble the decline of cypermethrin from the water phase during time, the dashed bars resemble the bacterial degradation in the laboratory system without plants and the crossed bars resemble the concentration of cypermethrin detected in the plants itself (sum of all three plant species).

The removal efficiency in this laboratory system for benzo (a) pyren showed that after 7 days this PAH was removed by 98.7 % from the water phase. This still exceeds the German drinking water regulation limit of 0.01 □gbuŁ is significantly reduced. Of the total amount of benzo (a) pyren the plants were exposed to, merely 38% was detected intracellularly. Compared to a partly microbial degradation under full laboratory conditions, the removal after 7 days is only by 10.6 % (Figure. 4).

For the cyano bacterial toxin MC-LR the results showed that this cyanobacterial toxin could already be removed within 3 day to concentrations below the WHO guideline level of 1 □gAfter 7 days the toxin was completely removed. The toxin is detected in the plants itself and will be metabolised intra cellular to glutathione-conjugates and stored in the vacuole or cell wall fractions.

The insecticide cypermethrin was significantly removed by 91.9 % within 7 day from the water phase, of which 24.5 % were detected in the plant. The system was run for 7 days without plants and only 3.2 % were degraded. So the overall removal efficiency was between 91.9 to 100 % of the contaminants within 7 days of exposure.

Figure 7: Green Liver System® constructed at the water work facilities of Hefei City at Lake ChaoHu (PR China) consisting of six compartments: 1+2 covered with *Lema minor*; 3+4 planted with *Ceratophyllum demersum*; 5+6 planted with *Phragmites australis*. (photo: S. Pflugmacher)

From laboratory to real live situation

The first pilot plant of a Green Liver System® was built in Hefei at the local water treatment plant located at Lake Chao Hu (Anhui province, PR China). The system had a size of 25 m x 10 x 1,5 m giving a final volume of 375 m^3 water (Fig. 7). It was divided into six compartments by wooden barriers. Water flow was achieved by two external pumps operating with solar panels. The problem was the massive eutrophicated Lake Chao, exhibiting a nearly year round massive cyanobacterial bloom. This water was used for drinking water production. Toxicity measurements of bloom samples from Lake ChaoHu showed the presence of different microcystin congeners such as MC-LR (58.99 µg l^{-1}), MC-YR (1.72 µg l^{-1}) and MC-RR (42.64 µg l^{-1}). Using the Green Liver System® pilot plant, between 75-85 % of the toxins could be removed, to contribute successfully to a more safe the drinking water production of the City of Hefei [28].

The second pilot plant of a Green Liver System® was built in Itacuruba (Brasil) at a local Tilapia farm within the INNOVATE project (Figure. 8). The system has a size of 100 m x 25 m x 2 m giving a final volume of 5000 m^3 water. It is divided into six compartments by curved brick stone barriers to control the water flow. The water flow in the system was realized by a natural slope. The wastewater from aquacultural ponds should be cleaned before the water is used for agricultural field irrigation or released in the nearby Itaparica reservoir. The main contaminants were oxytetracycline (a common fish antibiotic) methyl-testosterone (synchronising the juvenile fish to become male) and cyanobacterial toxins (because of the high amount of fish food and nutrients, cyanobacteria develop in the hatching ponds). Toxicity measurements form the pond water showed the presence of two cyanobacterial micocystin congeners in a concentration of 22.4 □g L^{-1} (MC-LR) and 31.2 □g L^{-1} (MC-RR). Using the Green Liver System® 100% of these cyanotoxins could be removed using three different macrophytes: *Eichhornia crassipes* (two compartments), *Egeria densa* (three compartments) and *Myriophyllum aquaticum* (one compartment).

Figure 8: Green Liver System® constructed at the facilities of a Tilapia farm in Itacuruba (Brasil) consisting of six compartments: 1+2 covered with *Eichhornia crassipes*; 3+5 planted with *Egeria densa* and compartment 6 planted with *Myriophyllum aquaticum*. In the far back the Itaparica reservoir can be seen. (Photo: S. Pflugmacher)

Risk assessment of Green Liver Systems®

Taking the pros and cons of constructed wetlands into account, what is the general risk of using artificial Green Liver Systems®? In this case we have to separate the risks of the system itself and the risk which the system might provoke to the surrounding environment.

a) Possible risks for the Green Liver System®

Plant fitness

First of all, the Green Liver System needs healthy and good growing macrophytes. So the fitness of the plants is correlated with the efficiency of the whole system. The risk would therefore be that the plants are not growing well.

Allelopathic effects

Plants will interact with each other and allelopathic effects might disturb the system, leading to a decline of one of the species. (To prevent this all plants sets are tested in our laboratory system.)

Flooding and drought

Within this, rapid changing water levels might flush through the system and remove the plants, as well as a strong drought might decrease the water level, leading to an increase of water temperature and the submerged macropyhtes might die.

Animals invading the system

In some regions, there is a high possibility that due to birds, the Green Liver System (R) might be invaded my fish. Some of these fish, e.g. Tilapia, are plant feeders and might significantly reduce the macrophytic biomass in the system leading to a decline in its overall efficiency. Also animals like goats or sheep can contribute to the reduction of the biomass.

Plants invading the system

Considering the same carriers (birds), plants can also be transferred into the system, such as *Lemna* or *Azolla* species. They can grow and cover the water surface, hindering the sunlight to penetrate the water column and leading to a decline of the submerged macrophytes.

Rapid change of contaminants

The removal of the contaminants is highly dependent on the abilities of the chosen macrophytes. The Green Liver Systems (R) are normally customized to the needs in the specific case. A rapid change in the contaminant composition of the water, might lead to a decline in the overall efficiency.

b) Possible risks, which might be posed by the Green Liver System® for the surrounding environment

Development of methane emission

Dying plants, due to massive contamination, anoxic situations or turbid water might lead to an increase in methane production, which might have negative effects on the surrounding environment.

Development of pests

Depending on the design, the Green Liver System® is a pond based system. The water body might be a place where e.g. pests such as mosquitoes might develop, as the system has normally no fish (predators) present feeding on the insect larvae.

Contaminated plants

Due to the fact, that the plants within a Green Liver System(R), take up the contaminants into their cells (vacuole, apoplast and cell wall fractions), the plants are with time highly contaminated. Therefore, it is not recommended that these plants are used as animal food or fertilizer on agricultural fields.

Management of Green Liver Systems®

The fact that Green Liver Systems® are totally artificial systems containing not more than three different aquatic macrophytes, the management of this system is more easy than with constructed wetland or natural wetland systems. The main task is to ensure constant water flow through the system and to prevent flooding and drought situations. In a long-term working Green Liver System®, the plants have to be replaced from time to time to keep the efficiency of the system high. This normally should be done partially to keep the system continuously working.

Constant monitoring of the contaminants would be necessary to ensure the removal efficiency of the system. Depending on the concentrations of the contaminants in the inflow of the system, weekly monitoring on a long term basis seems sufficient. In the beginning, this monitoring should be more frequently.

Conclusion

Green Liver Systems®, as totally artificial systems, are not comparable to traditional wetlands, which are using submerged aquatic macrophytes. The ecological services provided by these macrophytes are uptake, biotransformation and metabolisation as well as the main feature, the storage of the metabolites in vacuole and cell wall fractions, so no metabolites will be formed outside of the plant cells. Green Liver Systems® make use of the beneficial parts of phytoremediation as a sustainable Green Technology. Of course, the used macrophytes are not able to deal endlessly with contaminants and also due to climate factors (winter time) plants will decay. In this case, the contaminants probably will be released into the water body again. Therefore, techniques have to be developed to make use of the contaminated plant material. For special heavy metals, e.g. copper, the solution might be micro-mining to regain the metal. For nutrients such as phosphorus and nitrogen the solution for sure will be to use the plant material as fertilizers. With other contaminants such as PAH, PCB, pesticides or cyanobacterial toxins a possible solution might to use the plant material as basis for the bio-diesel production. But closing these cycles will be a future goal.

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