

# Green Liver Systems® for Water Purification: Using the Phytoremediation Potential of Aquatic Macrophytes for the Removal of Different Cyanobacterial Toxins from Water

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Received 12 April 2015; accepted 26 June 2015; published 29 June 2015

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## Abstract

The protection and reasonable use of freshwater is one of the main goals for our future, as water is most important for all organisms on earth including humans. Due to pollution, not only with xenobiotics, but also with nutrients, the status of our water bodies has changed drastically. Excess nutrient load induces eutrophication processes and, as a result, massive cyanobacterial blooms during the summer times. As cyanobacteria are known to produce several toxic secondary metabolites, the so-called cyanotoxins, exhibiting hepato-, neuro- and cell-toxicity, a potential risk is given, when using this water. There is an urgent need to have a water purification system, which is able to cope with these natural toxins. Using aquatic plants as a Green Liver, the Green Liver System®, was developed, able to remove these natural pollutants. To test the ability of the Green Liver System®, several cyanobacterial toxins including artificial and natural mixtures were tested in a small-scale laboratory system. The results showed that within 7 - 14 days a combination of different aquatic macrophytes was able to remove a given toxin amount (10 µg·L<sup>-1</sup>) by 100%. The phy-

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**How to cite this paper:** Pflugmacher, S., Kühn, S., Lee, S.-H., Choi, J.-W., Baik, S., Kwon, K.-S. and Contardo-Jara, V. (2015) Green Liver Systems® for Water Purification: Using the Phytoremediation Potential of Aquatic Macrophytes for the Removal of Different Cyanobacterial Toxins from Water. *American Journal of Plant Sciences*, 6, 1607-1618.  
<http://dx.doi.org/10.4236/ajps.2015.69161>

phytoremediation technology behind the Green Liver Systems® uses the simple ability of submerged aquatic plants to uptake, detoxify and store the toxins, without formation and release of further metabolites to the surrounding water.

## Keywords

Green Liver System®, Phytoremediation, Aquatic Macrophytes, Water Purification, Biotransformation, Metabolism, Cyanobacterial Toxins

## 1. Introduction

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 water, such as snow, ice and glaciers (1.75% - 2.0%), and therefore not immediately available, as well as 0.7% - 0.8% as groundwater and soil moisture [1].

The access to clean water is a very critical issue for the survival of all living organisms on earth, especially humans. Unfortunately there is on-going pollution of our water resources, by anthropogenic substances or an overdue of nutrient input leading to so-called eutrophication. As freshwater is a renewable but limited resource on earth, it is necessary to take special care on it. The natural way to renew freshwater is the water cycle, *via* evaporation, cloud formation and precipitation as rainfall. However, if human activities take more freshwater than that recycled within the water cycle, we sooner or later will run into a freshwater deficiency, possibly damaging not only our environment but also human health. Looking at freshwater as a limited source, tools need to be developed to purify water in a sustainable and reliable way. One of these possible ways is the use of Green Liver Systems®, using the phytoremediation potential of aquatic submerged plants.

### What is a Green Liver System® and how does it work?

In general, biotransformation occurs in three phases: phase I, the transformation, done by enzymes such as cytochrome P450 mono-oxygenases; phase II, the conjugation done by enzymes such as glutathione S-transferases taking cell internal compounds like amino acids, sugar or glutathione for conjugation reactions, and phase III, the excretion (animals) or emplace/storage (plants).

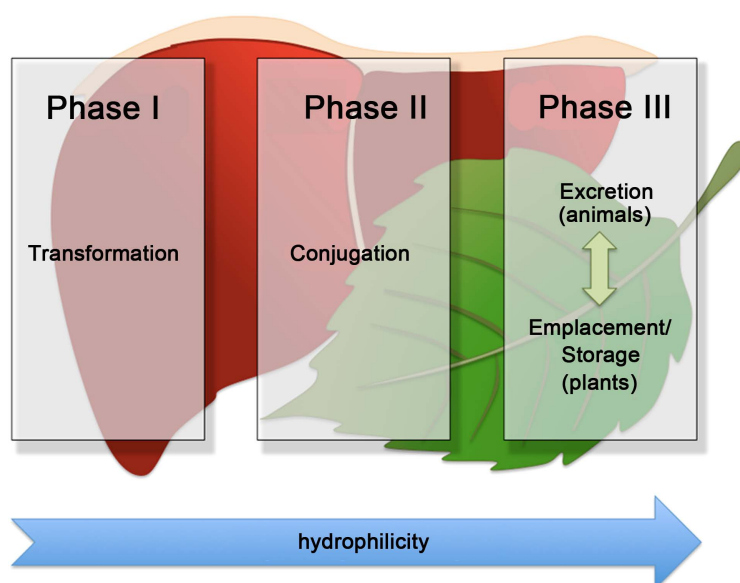
In the early 1990's researchers discovered that the metabolism of organic compounds does share many similar processes in animal liver compared to plants [2] [3]. These processes, which liver and plants share, are: 1) the capability to metabolize and detoxify xenobiotics, 2) specific metabolic pathways and biotransformation enzymes, and 3) the common aim to transform lipophilic xenobiotics to hydrophilic compounds for better transport and excretion (Figure 1).

Beside all similarities, animal and plant biotransformation differs mainly in phase III. In animals, as well as in the human liver, the final step is excretion *via* urine and faeces, whereas in plants, this ultimate step comprises of compartmentalisation in cell wall fractions or in the vacuoles.

For a long time, different plant species have been used for phytoremediation purposes of air, soil and water pollution (Table 1).

### What is the difference between constructed wetlands and the Green Liver System®?

A constructed wetland system is a site-specific combination using physical, biological and chemical processes to remove nutrients, bacteria, pesticides, and organic matter from runoff. The individual components of the system typically include: sediment basin, a level lip spreader, a primary grass filter, a vegetated wetland, a deep pond and a polishing filter; the last can be a riparian forest buffer. Wetlands can have different characteristics such as the salinity of the water, soil types, and the types of plants and animals living in such a wetland. The most common feature of all wetlands is that the groundwater level is very near to the soil surface or shallow water covers the surface at least for parts of the year. This makes constructed wetland very variable and so far no single wetland classification system would count for the manifold aspects of this man-made specific ecosystem type. Wetlands are transitional areas between terrestrial and aquatic systems. They contain a certain characteristic fauna and flora specifically adapted to the area conditions. For many years, wetland-use was limited to activities that did not adversely affect the ecosystem and its productivity. However, pressure on wetlands has in-



**Figure 1.** Three phases of biotransformation in animals and plants leading to an increase in hydrophilicity of the substance taken up. This will enable the organisms to excrete the metabolites in animals and the emplacement/storage in plants.

**Table 1.** Examples of aquatic (free-floating and submerged) macrophytes, tested for the removal of different environmental contaminants.

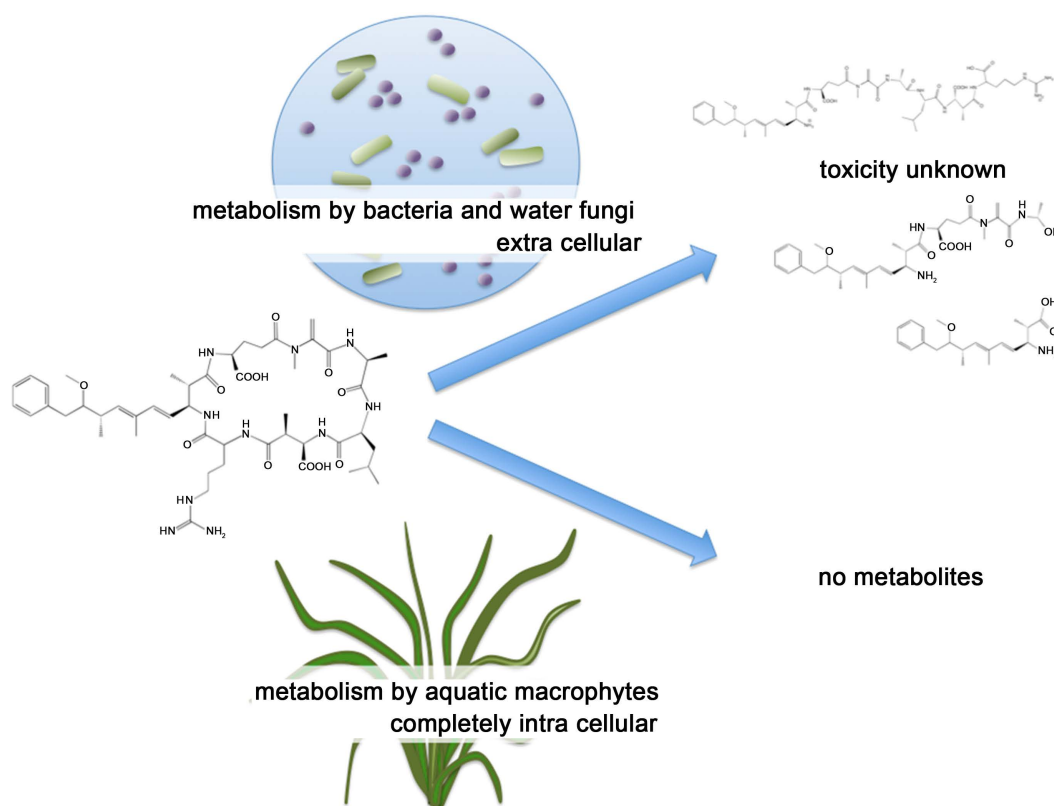
Plant species (common name)	Plant species (scientific name)	Contaminant	References
Duckweed	<i>Lemna gibba</i> <i>Lemna minor</i> <i>Lemna trisulca</i>	heavy metals nutrients phenol, 2,4,5-trichlorophenol	[18]-[20]
Duckweed	<i>Spirodela polyrrhiza</i>	Nutrients Heavy metals DDT, chlorobenzenes, organochlorine compounds	[21]-[23]
Water velvet	<i>Azolla pinnata</i> <i>Azolla caroliniana</i> <i>Azolla filiculoides</i>	heavy metals nutrients	[24] [25]
Water hyacinth	<i>Eichhornia crassipes</i>	Nutrients Ethion, dicofol, pentachlorophenol	[26] [27]
Coontail	<i>Ceratophyllum demersum</i>	Trinitrotoluene, hexahydro-1,3,5,7,-tetranitro-1,3,5,7-tetrazocine, chlorobenzenes Nitrogen and phosphorus	[21] [22] [28]
Canadian waterweed	<i>Elodea canadensis</i> <i>Elodea nutallii</i> <i>Elodea densa</i>	$^{137}\text{CS}$ , $^{60}\text{Co}$ , $^{54}\text{Mn}$ Nitrogen and phosphorus, phenanthracene, DDT, hexachloroethane	[21] [22]
Fennel pondweed	<i>Potamogeton perfoliatus</i> <i>Potamogeton pectinatus</i> <i>Potamogeton nodosus</i> <i>Potamogeton crispus</i>	Cd, Pb, Cu, Zn, explosives, phenol	[29]-[31]
Water milfoil	<i>Myriophyllum aquaticum</i> <i>Myriophyllum exalbescent</i> <i>Myriophyllum verticillatum</i> <i>Myriophyllum propinquum</i>	$^{137}\text{CS}$ , $^{60}\text{Co}$ , $^{54}\text{Mn}$ , Simazine, DDT, perchlorate, TNT	[28] [32] [33]

creased as people turn them into agricultural and industrial land, domestic and industrial water or ground for dumping waste [4]. Therefore some wetland resources were slowly being depleted or degraded, thus not contributing to the overall purification success. The management of wetlands, which started roughly over 10 years ago, seems to be difficult from a human need, cultural value, conservation need and also from the pure technical side to run such systems in a sustainable way.

In most cases the phytoremediation in constructed wetlands is in fact done by bacteria living in biofilms at the root part of wetland plants, producing metabolites of the parent compound (Figure 2). Sometimes these metabolites may be more toxic than the parent compound, or in many cases, the toxicity of the metabolites formed is not sufficiently studied and understood. An example of this is the bacterial breakdown of the cyanobacterial toxin microcystin-LR [5].

In the Green Liver System<sup>®</sup>, in most cases submerged aquatic plants having no or only small root systems were used to minimize the formation of metabolites outside of the plant. The main principle of the Green Liver System<sup>®</sup> is that xenobiotics/toxins are completely taken up by the plant and subsequently cell-internally metabolized within the plant. The metabolites are then, according to phase III of the biotransformation process, stored away in cell wall fractions or the plant vacuoles and not released again into the surrounding water phase. The positive effect is that the metabolites are properly packed away within the plant as long as the plant is not degrading. To achieve a sustainable removal of contaminants and according metabolites, macrophytes have to be harvested from time to time to avoid a possible re-contamination.

**The aim of this study** was to test several aquatic plants within a small laboratory Green Liver System<sup>®</sup> for their ability to remove different cyanobacterial toxins, as pure substances, as well as in artificial mixtures and a cyanobacterial crude extract from Lake Amatitlán, derived from a natural bloom. To ascertain a more complete picture, a balance of the fate of the toxins within the system was done.



**Figure 2.** Difference between conventional wetland systems and the newly developed Green Liver System<sup>®</sup> using the cyanobacterial toxin microcystin-LR as an example. The production of metabolites [5] from MC-LR with unknown toxicity in conventional wetlands by bacterial biofilms is the main difference, as in the Green Liver System the metabolisation is performed within the plant cells, with no release of metabolites into the surrounding water body.

## 2. Material and Methods

### 2.1. Plant Material

Aquatic plants, *Ceratophyllum demersum*, *Elodea canadensis*, *Myriophyllum spicatum* were purchased from Extraplant (Extragroup GmbH, Münster, Germany). All species were identified according to Casper and Krausch [6] and Orchard [7] and verified through ITIS classification standards (<http://www.itis.usda.gov>) in order to reach current taxonomic status of species. Plants were cultivated prior to exposure in 100 L tanks, over two weeks for acclimatization, under a photoperiod of 14:10 (dark/light), a temperature of  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and an irradiance of  $100 \text{ mE}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The culture medium was made according to Nimptsch and Pflugmacher [8] with a pH of 8.

### 2.2. Exposure Experiments

A model Green Liver System<sup>®</sup> was built using 4 mm glass with a total volume of 60 L. The system was split in six compartments each having a size of  $13 \times 30 \text{ cm}$ , connected to each other (Figure 3). To circulate the water, an Eheim pump Typ 2048 with a flow of  $10 \text{ L}\cdot\text{min}^{-1}$  was used connected by Teflon tubes without a filter unit. The bottom of the system was filled with 1.5 cm quartz sand, washed and sterilized before use. Aquatic submerged macrophytes were planted into the sterilized sand. Compartment 1 and 2 was planted with *E. canadensis*, compartment 3 and 4 with *C. demersum* and compartment 5 and 6 with *M. spicatum*. Each plant species had a total biomass of 150 g in the respective compartment of the system. Exposure experiments took two weeks in total for each treatment and two weeks without plants and sand as a control for natural and/or bacterial degradation. After 1 day, 3 days, 7 days and 14 days, samples of water 200 mL were taken. On day 14 in addition to the water samples all plants (150 g for each plant species) and the sand (1 kg) were sampled.

Three exposure scenarios were conducted. The Green Liver System was first exposed to pure toxins MC (-LR, YR and -RR, each at a concentration of  $10 \mu\text{g}\cdot\text{L}^{-1}$ ) and anatoxin-a ( $5 \mu\text{g}\cdot\text{L}^{-1}$ ), respectively for 14 days. Then an artificial mix of the pure MC congeners at a total concentration of  $30 \mu\text{g}\cdot\text{L}^{-1}$  was applied to the system for 14 days. Lastly, the system was tested with a crude bloom extract with an initial MC exposure concentration of  $16 \mu\text{g}\cdot\text{L}^{-1}$ . In all exposure scenarios, the toxin concentration in media was analysed over 14 days and the toxin content inside the plants after the 14<sup>th</sup> day.



Figure 3. Laboratory Green Liver System in working progress with cyanobacterial crude extract (right Erlenmeyer).



## 2.3. Cyanobacterial Toxins and Cyanobacterial Crude Extract

The used cyanobacterial toxins (microcystins (MC): MC-LR, -RR and -YR) were purchased from Alexxis GmbH (Grünberg, Germany) having a purity of 97% - 98%. Anatoxin-a was used as a purified substance provided by Tocris Bioscience (Strathmore Road Natick, MA, USA). The purchased toxins, in solid form were dissolved in 70% methanol. These toxins were also used as reference material on the LC-MSMS system. Cyanobacterial bloom, material mainly consisting of *M. aeruginosa*, was collected in Lake Amatitlán, Guatemala [9]. From the re-concentrated bloom material obtained in field, cell-free crude extract was made. Extraction was conducted with deionized water and to provoke cell lysis, bloom material followed a freeze (−40°C) thaw (22°C) cycle. This procedure was repeated three times. On every occasion, material was centrifuged at 2880 ×g for 1 h at 4°C. The solution was then mixed for 4 h and again centrifuged for 1 h at 2880 ×g. All resulting supernatants were combined to obtain a homogeneous cell-free crude extract and stored at −20°C until MC quantification with LC-MS/MS. The resulting crude extract contained a total MC concentration of  $96.5 \pm 5 \mu\text{g}\cdot\text{L}^{-1}$ .

## 2.4. Toxins Extraction from Exposure Media

### 2.4.1. Microcystins

To reach detectable MC concentrations for toxin analysis *via* LC-MSMS a pre-concentration of the MC congeners *via* solid-phase-extraction (SPE) was done beforehand using reversed-phase cartridges (Sep-Pak® tC18, 400 mg sorbent, Waters, Ireland). Samples were passed through the SPE tubes for toxin enrichment followed by eluting with 5 mL of 99% methanol (MeOH). Subsequently, all methanol was removed in a vacuum centrifuge (Concentrator plus/Vacufuge® plus, Eppendorf AG, Germany) at a temperature of 30°C and finally re-dissolved with 500 µL HPLC-grade MeOH.

### 2.4.2. Anatoxin-a

Water samples were centrifuged at 5000 ×g for 15 min at 4°C and water was settled at pH  $7.0 \pm 0.2$  with 1 M NaOH. The remaining anatoxin-a in the exposure media was extracted and concentrated with Strata WCX SPE columns using the same procedure as described above. Solid phase-extracted water samples were immediately processed for the toxin analysis *via* LC-MS/MS.

## 2.5. Toxin Extraction from Plant Material

### 2.5.1. Microcystins

Plant material was ground in liquid nitrogen to a fine powder. This powder was transferred into 50 mL falcon tubes and 70% MeOH was added to start the extraction. The slurry was shaken for 1 h at room temperature, then ultrasonic treatment for 1 min followed by turnover shaking for 24 h. After centrifugation at 12,500 ×g for 10 min at 4°C, the supernatant was collected and the pellet subjected to a second MeOH extraction step equal to the first extraction procedure. The resulting supernatant was pooled with the first and evaporated to dryness at 35°C under a constant nitrogen flow. Samples were re-dissolved in 1 mL HPLC-grade MeOH for quantification by LC-MS/MS.

### 2.5.2. Anatoxin-a

Anatoxin-a was extracted from plant tissue according to Rellan *et al.* [10] with slight modifications. The frozen plant material was ground in liquid nitrogen to a fine powder. Anatoxin-a was extracted by mixing 0.2 g of pulverized plant tissue in 1 mL of acidified 70% MeOH containing 0.1% trifluoroacetic acid (TFA). Tissue samples were continuously shaken in the dark for 3 h at room temperature using overhead shaker, Intelli-Mixer RM- 2 (Neolab, Heidelberg, Germany). Extracts were then centrifuged at 10,600 ×g for 10 min at 4°C and the supernatant was adjusted to pH  $7.0 \pm 0.2$  with 1 M sodium hydroxide (NaOH) before SPE using a weak cation exchanger. SPE columns, Strata WCX, 3 mL with 200 mg packing (Phenomenex, CA, USA), were conditioned with equal volumes of MeOH and water. Sample loading and elution with 100% MeOH containing 0.2% TFA were carried out under gravity. Afterwards, the eluates were completely dried under constant nitrogen flow. The final residues were reconstituted in 200 µL of 70% MeOH and the anatoxin-a content was immediately determined by LC-MS/MS.

## 2.6. Toxin Extraction from Sand

The wet sand was centrifuged at 20,000  $\times g$  on a Sorval centrifuge to remove interstitial water as best as possible. The supernatant was collected. The sand was then washed with 1.5 L of 70% MeOH, the volume was reduced to 100 mL using a rotary evaporator. The 100 mL samples were applied to SPE Oasis MCX columns for pre-cleaning and concentration.

## 2.7. Analytics of Cyanobacterial Toxins

Determination and quantification of the MCs and congeners MC-LR, -RR, and -YR were performed by LC-MS/MS (Alliance 2695 UHPLC combined with a Micromass Quattro micro<sup>TM</sup>, Waters). The chromatographic separation was carried out on a reverse phase column using a Kinetex<sup>TM</sup> C18 column (2.1  $\times$  50 mm, 2.6  $\mu m$  pore size, Phenomenex). The mobile phase consisted of solution A, Milli-Q water containing 0.1% TFA and 5% acetonitrile (ACN) and solution B (ACN containing 0.1% TFA) at a flow rate of 0.2 mL $\cdot$ min<sup>-1</sup>. The linear gradient conditions were as follows: 0 min 65 % A; 3.75 - 7 min 35% A and 7.8 - 12 min 100% A. The column oven temperature was set at 40°C with an injection volume of 20  $\mu$ L. Elution peaks for each of the MCs congeners were observed at 7.1 min for MC-RR, 7.34 min for MC-YR and 7.44 min for MC-LR. Desolvation gas N<sub>2</sub> was set as trigger gas and Argon (Ar) as collision gas. Parent compound and its fragment ions, respectively, were scanned at the following mass-to-charge ratio ( $m/z$ ): MC-LR 995.5  $\rightarrow$  135.1; MC-RR 519.9  $\rightarrow$  135.3 and MC-YR 1045  $\rightarrow$  135. ESI+ conditions for all MCs were set as follows: capillary voltage of 3 kV, source temperature of 120°C, desolvation temperature of 500°C and cone gas flow-rate of 100 L $\cdot$ h<sup>-1</sup>. For MCs congeners MC-LR and MC-YR collision energy was 65, cone voltage was 60 V and for MC-RR collision energy was 35, cone voltage was 20 V. Desolvation gas flow-rate was 1000 L $\cdot$ h<sup>-1</sup>. Calibrations were linear ( $R^2 = 0.999$ ) between 5 and 500  $\mu g \cdot L^{-1}$ . Limit of detection (LOD) was set at 1 ng $\cdot$ mL<sup>-1</sup> (signal to noise S/N > 3) and limit of quantification at 5 ng $\cdot$ mL<sup>-1</sup> (S/N > 5) for all MCs congeners.

Chromatographic separation of anatoxin-a was realized with the Kinetex<sup>TM</sup> HILIC column (2.6  $\mu m$ , 2.1  $\times$  100 mm) on the same equipment as for the MCs. Column oven temperature was set to 30°C and the injection volume was 2  $\mu$ L at a flow rate of 0.2 mL $\cdot$ min<sup>-1</sup> using an isocratic run with 75% ACN, 22.5% H<sub>2</sub>O and 2.5% 50 mM ammonium formate with 0.32% formic acid over 8 min.

For the subsequent MSMS detection the MRM mode (positive mode) was used with an  $m/z$  of 166.2 91.1, 131.2 and 149.2 for anatoxin-a. Calibrations were linear ( $R^2 = 0.999$ ) between 5 and 250  $\mu g \cdot L^{-1}$ . LOD and LOQ were 1 and 5  $\mu g \cdot L^{-1}$  respectively.

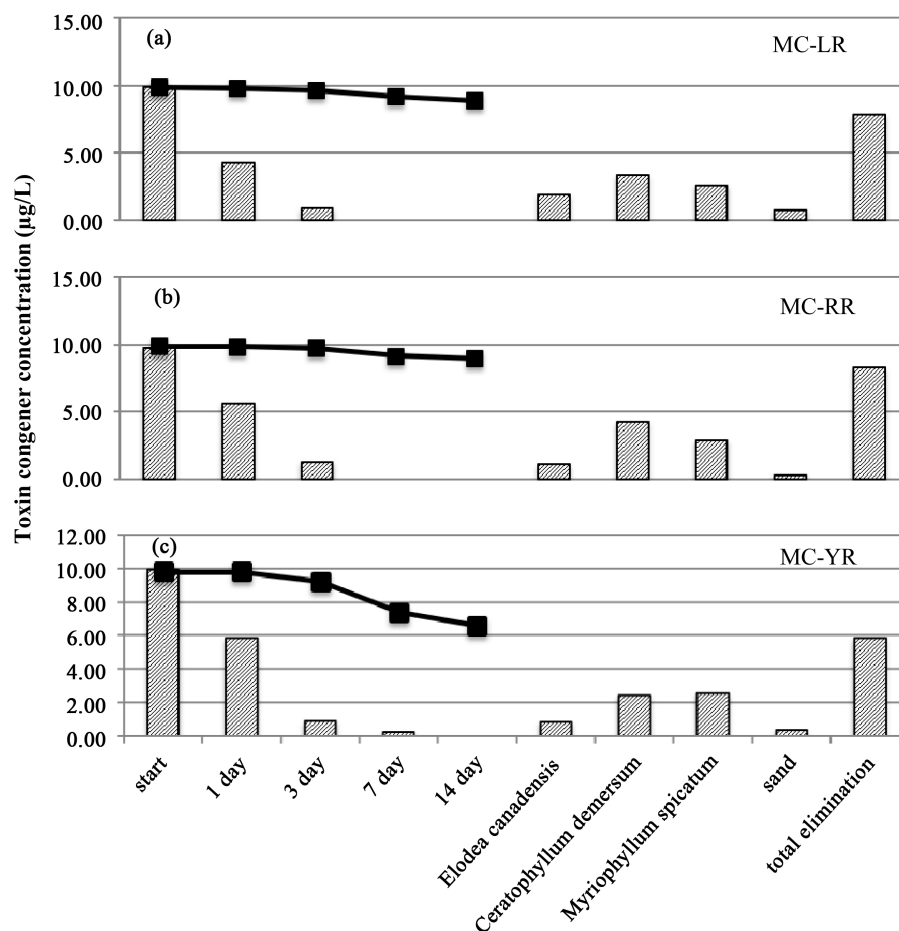
## 3. Results

During each of the two weeks experiments, none of the used aquatic macrophytes showed any visible morphological damage like leaf loss or colour change.

### 3.1. Microcystins (Hepatotoxins)

Monitoring the distribution of MC-LR with the Green Liver System<sup>®</sup> showed a decrease of this toxin congener during the first day by more than 50% in the water phase with a continued decrease on day 3 (**Figure 4(a)**). On day 7 and day 14 no MC-LR was detectable in the water phase. The toxin content in the plants was well measurable after the 14 days, showing the highest concentration in *C. demersum*, followed by *M. spicatum* and *E. canadensis*. The total amount of toxin taken up by all plant species together was 79% of the originally applied toxin concentration of 10  $\mu g \cdot L^{-1}$ . The other tested microcystin congeners (MC-RR and MC-YR) showed a similar behaviour in the laboratory scale Green Liver System<sup>®</sup> (**Figure 4(b)** and **Figure 4(c)**). The total MC-RR uptake was 85% and 59% for MC-YR of the initially applied toxin concentration after 14 days.

In the sand fraction, a binding of the toxin was detected between 7.9% (MC-LR), 3.3% (MC-RR), and 3.2% (MC-YR). In the parallel experiment, without plant material and sand, the natural/bacterial degradation for the different toxin congeners ranged from 32.9% (MC-YR) to 9.3% (MC-RR) during the 14 day experimental period. UV degradation and massive changes in pH as possible causes could be rejected as possible causes for degradation as this was strictly controlled. The respective toxin congeners were quickly reduced during the first day and then successively further reduced until the end of the experimental period (**Figures 4(a)-(c)**).



**Figure 4.** Distribution of three common microcystins (pure toxin mixture) in the laboratory Green Liver System® over a time period of 14 days in exposure medium and after 14 days in tissue of exposed plants and attached sand. Solid line resembles the system without plants and sediment, therefore only the natural/bacterial toxin degradation. The total MC-LR taken up is 79% (a), 85% for MC-RR (b) and 59% for MC-YR (c).

### 3.2. Anatoxin-a (Neurotoxin)

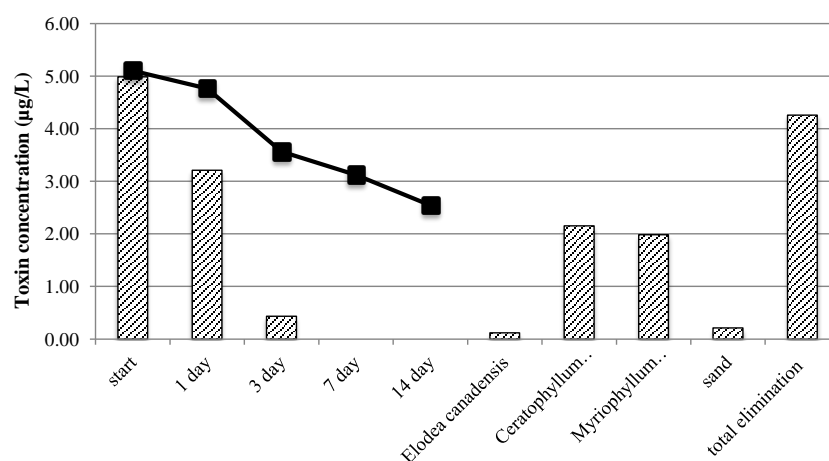
Using anatoxin-a, a small molecule compared to microcystins, in the lab scale Green Liver System®, the total anatoxin-a uptake was 86% of the initially applied toxin concentration after 14 days (**Figure 5**). The highest reduction step was observed between day 3 and day 7. Anatoxin-a was mainly taken up by *C. demersum* and *M. spicatum* and to a much lower extent by *E. canadensis*. The sand fraction resembling the sediment part showed a binding of 4.3% of the total anatoxin-a supplied. Natural and/or bacterial degradation in the laboratory showed a decrease of anatoxin-a by 50.2% during 14 days.

### 3.3. Artificial toxin Mixture and Cyanobacterial Crude Extract

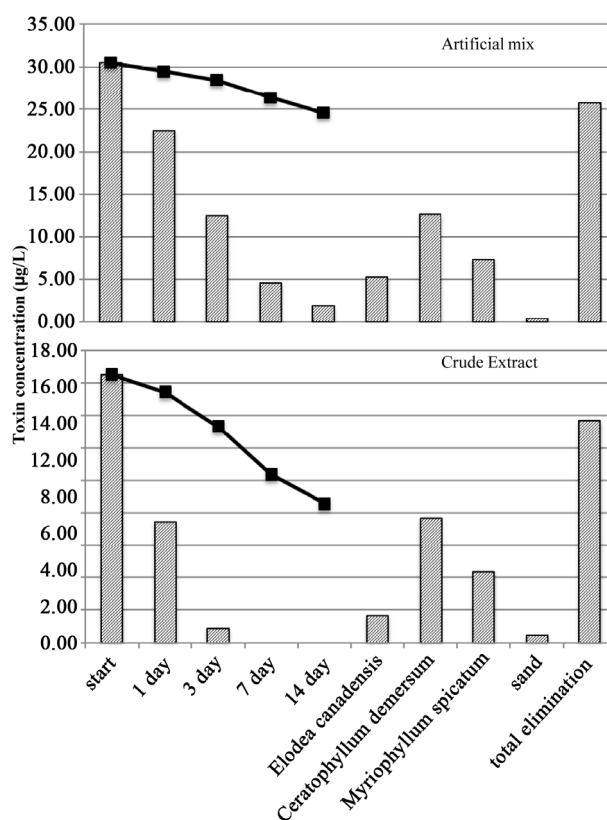
The prepared artificial mixture of cyanobacterial MCs (**Figure 6(a)**) applied to the laboratory-scale Green Liver System® showed similar behaviour to the microcystins as single congeners. The total microcystin uptake of 84.4% of the initially applied mixed toxin concentration of  $30.48 \mu\text{g}\cdot\text{L}^{-1}$  after 14 days was seen. Principal uptake in plants was detected in *C. demersum*, followed by *M. aquaticum* and *E. canadensis*. In the sand fraction 1.40% of the toxin mixture amount was found. Bacterial/natural toxin degradation was slow as seen in the single congener exposures with a total of 28.11% toxin degradation after 14 days.

In the experiment using cyanobacterial crude extract prepared from a bloom in Lake Amatitlán (Guatemala) (**Figure 6(b)**), the total uptake of the microcystin-congeners present in this extract was 82.7%. The main uptake of the two congeners present in this extract (MC-LR and MC-RR), were observed in *C. demersum* followed by





**Figure 5.** Distribution of anatoxin-a (pure toxin) in the Green Liver System over a time period of 14 days in exposure medium and after 14 days in tissue of exposed plants and attached sand. Solid line resembles the system without plants and sediment, therefore only the natural/bacterial degradation. The total anatoxin-a uptake is 86% of the initially applied toxin concentration after 14 days.



**Figure 6.** Distribution of microcystins from an artificial mixture and a cyanobacterial crude extract in the Green Liver System<sup>®</sup> over a time period of 14 days in exposure medium and after 14 days in tissue of exposed plants and attached sand. Solid line resembles the system without plants and sediment, therefore only natural decomposition and possibly bacterial degradation. The total microcystin uptake is 75% of the initially applied toxin concentration after 14 days in exposure medium and after 14 days in tissue of exposed plants and attached sand. Distribution of a cyanobacterial crude extract containing MC-LR, MC-RR and traces of MC-YR an artificial mixture in the Green Liver System<sup>®</sup> over a time period of 14 days in exposure medium and after 14 days in tissue of exposed plants and attached sand. Solid line resembles the system without plants and sediment, therefore only the bacterial degradation. The total microcystin elimination is 83% of the initially applied toxin concentration after 14 days.

*M. aquaticum* and *E. canadensis*. Also, here a clear uptake ranking was observed: MC-RR (76.2% of the applied  $5.21 \mu\text{g}\cdot\text{L}^{-1}$ ) and MC-LR (68.6% of the applied  $11.32 \mu\text{g}\cdot\text{L}^{-1}$ ) taking all three plants into account. In the sand fraction 2.6% of the total applied toxin concentration was detected. Natural/bacterial toxin degradation under laboratory conditions was 51.8% after 14 days.

#### 4. Discussion

The massive amount of cyanobacterial bloom events all over the world, also in water bodies used for drinking water purposes and spray irrigation of agricultural crops, makes it necessary to develop methods for the removal of cyanobacterial toxin burdens from these kinds of water. The World Health Organization (WHO) recommends a guideline level of  $1.0 \mu\text{g}\cdot\text{L}^{-1}$  microcystin in drinking water, however, there is no guideline value for water used for spray irrigation or recreation. Many promising possibilities, such as activated carbon [11] [12], chlorine and chloramine treatment [13], micro- and ultrafiltration [14] and ozonation [15], exist to remove cyanobacterial toxins from the water phase. However, these treatments may all have several imponderabilities such as price and practicability and also the issue of possible, and even more toxic, by-products (metabolites) has to be addressed.

The biotransformation process compromised by three phases in animals and plants are very similar and often even based on the same enzyme systems [16]. Only phase III is different in plants from animals. As animals have the possibility to excrete *via* urine and faeces, plants normally store the metabolites in vacuole or cell wall fractions. Within this storage, these compounds are no longer circulating and available for the plant metabolism and also not anymore able to provoke harmful effects to the plants.

Within the aquatic plants used in a Green Liver System<sup>®</sup>, the cyanobacterial toxins are completely taken up by the plants and no detectable metabolites, such as the microcystin-LR-mercapturic acid conjugate, as described by Schmidt *et al.* [5], were detected. Hence the system used in the present study showed a great potential for sustainable purification of cyanobacterial toxins from water bodies. Optimization might be done by using different plant species and different combinations of aquatic plants, to enhance the overall performance of the Green Liver System<sup>®</sup>.

To date two experimental large-scale systems have been built in: a) Hefei (PR China) for purification of cyanobacterial contaminated water from lake ChaoHu as raw water for drinking water production [17] and b) Itacuruba (Brazil) for purification of wastewater from an aquacultural company to remove cyanobacterial toxins, fish antibiotics (oxytetracycline) and hormones (methyltestosterone). In the Hefei Green Liver System<sup>®</sup>, *Lemna minor*, *M. elatinoides*, *Hydrilla verticillata*, and *Ceratopteris thalictroides* was used for phytoremediation and in Itacuruba, *Azolla caroliniana*, *Myriophyllum aquaticum* and *Eichhornia crassipes*. Plant selection for the Green Liver System<sup>®</sup> relies profoundly on the xenobiotics present in the water, which plants are endemic to the area and the remediation abilities of these endemic plants, therefore making each plant combination unique to where the system is built. Both the Hefei and the Itacuruba systems showed a very good performance in short terms (Hefei up to 85% cyanobacterial toxin removal; Itacuruba up to 100% of cyanotoxin and 75% of oxytetracycline removal) and now long-term measurements hopefully will show the same or even better performance.

Green Liver Systems<sup>®</sup>, are totally artificial systems, and not comparable to traditional wetlands, as the Green Liver System does not attempt to mimic aquatic ecosystems. The ecological services provided by macrophytes are uptake, biotransformation and metabolism 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<sup>®</sup> 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 in some countries (winter time) plants will decay. In that case, the contaminants probably will be released into the water body again. Therefore, techniques have to be developed to make use of the contaminated plants e.g. by micro-mining to regain the heavy metal. For nutrients such as phosphorus and nitrogen the solution for sure will be to use the plant material as fertilizers if not contaminated by other toxic compounds. 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. So closing cycles will be a future goal.

#### 5. Conclusion

In this study we find that the aquatic macrophytes, *C. demersum*, *E. canadensis*, and *M. spicatum* can adequately take up MC congeners (-LR, -RR and YR), as single purified toxin, as an artificial mix and in a natural bloom

extract. 67% - 85% of the MCs was taken up, and in combination with the natural degradation, 100% of the toxins were removed from the exposure media. The same trend was seen with anatoxin-a applied as a pure toxin. We conclude that the tested aquatic plants are suitable for use in cyanotoxin treatment in the Green Liver System®.

## Acknowledgements

This research was in part supported by the National Research Foundation of Korea Grant funded by the Korean Government (MISP) (2013, University-Institute Cooperation Program) and the Korean Institute of Science and Technology (KIST) Institutional Program (2E24280). The author would like to thank the BMBF for sponsoring the steps from laboratory to real life (BMBF; ChaoHu 02WT0529 and Innovate 01LL0904A). Furthermore, my Chinese colleague Dr. Li Kun (Anhui Environmental Protection Agency, PR China), and the Brazilian owner of the Tilapia farm Mr. Romero Magalhaes Ledo (Itacuruba, Brazil), as well as Prof. Dr. Maria do Carmo Sobral, UFPE (Recife, Brazil). Thanks to Dr. Maranda Esterhuizen-Londt for helpful discussions.

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