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Effect of ultrasonication on algal and bacterial growth in fresh water

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TABLE OF CONTENTS

1 LIST OF ABBREVIATIONS

app. – approximately

bw – body weight

CA - Cetrimide agar

CCA - Chromogenic coliform agar

CFU – Colony forming unit

Chl a – Chlorophyll a

CV – Chlorella vulgaris

DO – Dissolved oxygen

FTU - Formazin turbidity unit

GDWQ - Guidelines for drinking water quality

HPC – Heterotrophic plate count

HWT - Household Water Treatment

LD_{50} – median lethal dose

LPS - lipopolysaccharides

MSA - Mannitol salt agar

OT – Only transparent colonies

SPC – Specific conductance

TC – Total coliform

USD – Ultrasonic device

USEPA – United States Environmental Protection Agency

vs. - versus

WHO – World Health Organization

WSP - Water Safety Plans

2 INTRODUCTION

Radiation is a common phenomenon in nature: cosmic radiation, background radiation, numerous animals using echolocation for communication and orientation. However due to the development in anthropogenic activity in the last decades, a rapid increase in the level of abiotic noise and radiation occurred. Accordingly, the effect of these factors on organisms expanded which is most likely to facilitate biological changes in them, even mutations. That is why in modern microbiology and environmental sciences the study of such effects on microorganisms is of current interest. Knowledge obtained from such research can be used in implementation of new technologies in microbiology as well as other fields of studies. One of the types of radiation that can influence microorganisms is ultrasound. Ultrasonic waves possess high mechanical energy and therefore are able to cause a number of physical, chemical and biological phenomena (Antusheva, 2013).

During the propagation of an ultrasonic wave in a liquid, an alternating sound pressure occurs with an amplitude reaching several atmospheres. It happens even if the wave's intensity is relatively low. Under the action of this pressure fluid alternately undergoes compression and rarefaction (Hielscher, 2012). Low pressure during rarefaction allows ultrasonic waves to create "breaks" in the fluid: tiny bubbles filled with gas and steam. These bubbles are called cavitation, and the phenomenon became known as ultrasonic cavitation (Hielscher Ultrasonics GmbH, 2015). A shock wave that develops enormous pressure follows the collapse of a cavitation. If the shock wave encounters an obstacle, it can cause damage to its surface. Because there are a lot of cavitation bubbles formed during ultrasonication, and they are collapsing thousands of times per second, it can produce considerable damage (LLC "Matrix-production Plus" (a), 2014). Evidently, cavitation in a medium is the main cause of the destructive action of ultrasound on microorganisms. That is why ultrasonication in microbiology is mainly associated with cell disruption (LLC "Matrix-production Plus" (b), 2014).

The ability of ultrasound to break cell membranes found application in biological research, for example, for the extraction of lipids, proteins or enzymes from cells. Ultrasound is also used for the destruction of intracellular structures such as mitochondria and chloroplasts in order to study the relationship between their structure and functions (Pernet & Tremblay, 2003). Also nowadays ultrasound is widely used as a chemical-free way of water treatment. Main advantage of ultrasound usage in this field is that it is relatively cheap, extremely easy to install and operate (SonicSolutions LLC, 2009).

World Health Organization (WHO) is an international authority for drinking water quality. Among its many activities, it provides information about water safety, treatment and purification techniques and water related health risks to water suppliers, communities and individual households. Probably the most important document on the matter issued by WHO is "Guidelines for drinking water quality" (GDWQ). It includes water quality standards and assessment from microbial, chemical and radiological points of view as well as Water Safety Plans (WSP).

There are many ways through which water intended for drinking can be contaminated. The contamination can occur at any stage of drinking water processing: at the source, during transportation, treatment or storage. Waters can be polluted with chemicals or bacteria, both naturally and due to human activity. WSP is a strategy using risk assessment and management approach to avoid such

occurrences. Its aim is to ensure that water intended for drinking is safe for human consumption and meets all the regulatory requirements. GDWQ also considers the implementation of WSP and the standards in various supply types and systems, in particular community and household water systems, that are of interest in this research (World Health Organization (b), 2008).

"Evaluating household water treatment options" is a document issued by WHO that complies with GDWQ and is a part of WSP. It gives the reader an insight on microbiological performance of various Household Water Treatment (HWT) technologies. The document contains microbiological performance targets for each of WHO approved HWT methods. It also includes treatment technologies' performance evaluation guidelines. Selected indicators and pathogens selected for the evaluation of water quality are: Fecal coliforms *(Escherichia coli (E. coli)* and *Klebsiella), Campylobacter, Vibrio cholera, Enteroviruses, Rotaviruses, Cryptosporidium* and *Giardia intestinalis. Campylobacter, Rotaviruses* and *Cryptosporidium* are used as main indicators in this research, however *E. coli* is mentioned as a recommended alternative (World Health Organization (c), 2011).

WHO approved methods for HWT include: thermal technologies, filtering, settling, ultraviolet radiation, coagulation–flocculation and precipitation, ion exchange, chemical disinfection, adsorption and combinations of some of these methods. Ultrasonication is not included in the list; however, the performance of any treatment method can be evaluated.

WSPs implementation prevents waterborne diseases, and failure to ensure drinking water safety might result in outbreaks of infectious diseases (World Health Organization (a), 2008). Potential health problems that can result from microbial contamination can be so severe that its control must never be compromised. Waterborne diseases situation is especially problematic in areas with warm climate.

In spring 1994 over two million Rwandans fled to neighboring countries of the Great Lakes region of Africa from a genocidal mass slaughter (United Nations High Commissioner for Refugees, 2000). Refugees organized many camps in the area, some of them containing several hundred thousand people. One of the camps was located near Lake Kivu. At the time of their arrival there was no infrastructure to transport and purify water. Even though some agencies took action and organized water chlorination, most of the refugees consumed untreated water. July 1994 marked the outbreak of cholera and other diseases among the inhabitants of the camps. The death rate of 25-30 per 10000 per day in July - August 1994 was caused by the epidemics of diarrheal disease provoked by *Vibrio cholerae* O1 and *Shigella dysenteriae* type 1. It was concluded that the infection had happened through the consumption of bacteria contaminated lake water. The absence of designated defecation areas as well as disorganized water transportation and storage speeded up the disease propagation (Goma Epidemiology Group, 1995). If WSP was implemented on time, the tragedy of losing more than 40000 people could be avoided.

However bacterial contamination is not the only concern: algal blooms and cyanobacteria presence in both saline and fresh waters might pose a risk as well. Some of the algae residing in lakes were proved to cause skin irritation in swimmers. Most of the complaints stated that skin under swimsuit was more affected due to increased algae accumulation there and cell disruption through friction between skin and the garment. Species causing the irritation were discovered to be *Uroglena* and *Gonyostomum semen*. However, fresh water algae's toxicity is considerably lower than that of cyanobacteria due to the absence of effective accumulation mechanisms. Therefore the main concerns linked to the algae presence in drinking water are taste and odor (World Health Organization (e), 2003, pp. 146-147).

In contrast, cyanobacterial blooms had been widely recognized as a health risk for humans as well as wildlife. Cyanobacteria are photosynthetic bacteria inhabiting surface waters. They can be unicellular, colonial or filamentous and are represented by a great variety of species. Some of the most common species include *Anabaena*, *Microcystis* and *Oscillatoria* (World Health organization (f), 2015, pp. 25-31). Cyanobacteria produce a number of unusual metabolites, natural function of which is not yet discovered. Although blue-green algae are aquatic organisms, these metabolites oftentimes appear more harmful for terrestrial rather than aquatic biota. Most of cyanobacteria produce one or more of a number of substances that are potentially toxic; those are called cyanotoxins. For example, *Anabaena* produce microcystins, anatoxin-a, anatoxin-a(S) and saxitoxins; *Microcystis* produce microcystins; and *Oscillatoria* produce microcystins, anatoxin-a and aplysiatoxins. All known cyanobacteria contain lipopolysaccharides (LPS) in the cell wall; LPS are potentially irritant and affect any exposed tissue (World Health Organization (d), 1999, p. 57). Cyanotoxins are produced within the cell. Even though they can be both intracellular and extracellular, studies have shown that under natural conditions toxins produced by a healthy population are more likely to stay inside cyanobacterial cell. Thus, the release of harmful substances follows the disruption of cyanobacterial cells often occurring after the end of an algal bloom (World Health Organization (d), 1999, p. 95).

There are several ways in which cyanotoxins can affect mammals. For example, microcystins are hepatotoxic (liver-damaging); anatoxin-a, anatoxin-a(S) and saxitoxins are neurotoxic (damage nerve tissue); aplysiatoxins and LPS are irritant for skin (World Health Organization (d), 1999, p. 57).

The cyanotoxin group most frequently found within the environment are microcystins. In mammals they primarily affect the liver after toxin polluted liquid had been ingested or introduced intravenously. Exposure to high amounts of these toxins causes disruption of liver cell structure, a loss of sinusoidal structure, increase in liver weight, hemodynamic shock, heart failure and death. Other organs affected are the kidneys, lungs and intestines. The intraperitoneal (body cavity) injection LD_{50} is 50 to 60 $\mu g/kg_{bw}$ in mice, while the oral LD_{50} is 5000 to 10900 $\mu g/kg_{bw}$ in various mice strains (World Health Organization (d), 1999, pp. 136-137).

Neurotoxins, found in some cyanobacteria, in especially high doses can cause muscle weakness, convulsions, paralysis, respiratory distress, asphyxiation and death in mammals. Intraperitoneal LD_{50} in mice of anatoxin-a is 375 $\mu g/kg_{bw}$; the oral LD_{50} in mice for anatoxin-a is greater than 5,000 $\mu g/kg_{bw}$. Anatoxin-a(S) is more toxic, having intraperitoneal injection LD_{50} equal to 20 $\mu g/kg_{bw}$; no oral toxicity data is available. Saxitoxins have been the cause of paralytic shellfish poisoning in humans. The symptoms of this condition can vary from tingling about the lips to paralysis and death from respiratory failure. Intraperitoneal LD_{50} of saxitoxins is 8 to 10 $\mu g/kg_{bw}$; oral LD_{50} is 263 $\mu g/kg_{bw}$ (World Health Organization (d), 1999, pp. 143-145). Obviously, cyanotoxins are capable of causing a vast diversity of mild to severe health conditions, and their control in drinking water as well as water for dialysis is crucial.

In 1994 one of the Brazilian hospitals used for dialysis water that had 19 µg/l microcystin. 131 patients were exposed to the treatment with polluted water; 89% experienced visual disturbances, nausea, and vomiting; 100 people acute liver failure, 76 of them died. It was discovered that the water treatment method used by the hospital was incomplete and didn't include chlorine residues removal which led to the improper quality of the dialysis water (Carmichael et al., 2001).

In 2015 half a million inhabitants of the town of Toledo were without bathing and drinking water for 10 days in August due to the detection of microcystin in Lake Erie, USA. Drinking water had to be provided via water tanks as these toxins can be quite tricky to detect and remove; some treatment methods (carbon filter, heating, UV) work for one group pf cyanotoxins, but not the other (Plumer, 2015).

In order to avoid cyanotoxins intake, a proper water treatment has to be performed for all the water intended for human consumption. Cyanotoxin concentration is reduced in water in several steps. First, the intact cyanobacteria are removed primarily through coagulation/sedimentation, filtration and/or dissolved air floatation. Slow sand filtration and riverbank filtration are also effective for cyanotoxin elimination. However, the other listed methods cannot remove toxin molecules that are much smaller in size than cyanobacterial cell. Thus, the next step is to rid the water of the extracellular cyanotoxins. Every cyanotoxin responds differently to various methods of treatment. However, ozonation, chlorination or treatment with activated carbon allow to remove 80% or more of the present cyanotoxins. One of the most effective, yet expensive, methods of extracellular cyanotoxin removal is treatment with activated carbon. As long as the guideline value of 1µg microcystin-LR (the most toxic compound in the group) per liter is not exceeded, the water does not pose a risk of cyanotoxin poisoning (World Health organization (f), 2015).

Now, ultrasound cannot remove toxins or any other solutes from the water, but theoretically it can suppress the growth of microorganisms through cells disruption or disintegration. For example, some of the cyanobacteria exposed to ultrasonication experience the destruction of organelles but not of the cell wall. Thus, toxins stay captured inside the dead cell. Also some research studies state that ultrasonication is not harmful for bigger organisms, including humans (SonicSolutions LLC, 2009). These properties make it perfect for usage in water purification.

The company Dr. Raubenheimer GmbH located in Hanover, Germany, is celling various ultrasonic devices for multiple purposes. Ultrasonic algae control devices are among the products. The company has numerous positive feedbacks from previous installations on ponds, fish farms, etc. The next foreseeable objective of Dr. Raubenheimer GmbH is to supply their devices to African countries for installation in water tanks for purification as a part of charity initiative. However, before doing so a research must be conducted determining whether the available devices are suitable for the purpose or not.

Three devices with different frequencies and power intake were tested within this experiment. Water of a regional Lower Saxony lake (Steinhuder Meer) was used for testing. The objective of this work is to determine: what kind of effect these devices have on the microbiological content of the water, if any; which device shows the best performance; what the water quality before and after sonication is with regard to drinking water quality standards; what the best device available for disinfection of water in water tanks is.

3 MATERIALS AND METHODS

3.1 Experimental setup

3.1.1 Experimental design and general information

The experiment took place during July-Autumn 2015 in Wolfenbuettel, Germany. Eight 45L containers were placed in an outside greenhouse along one wall, an ultrasonic device installed in each of them, from now on referred to as test containers. Eight 22L containers were placed along the opposite wall, from now on referred to as control containers. The installation can be seen in Picture 1.

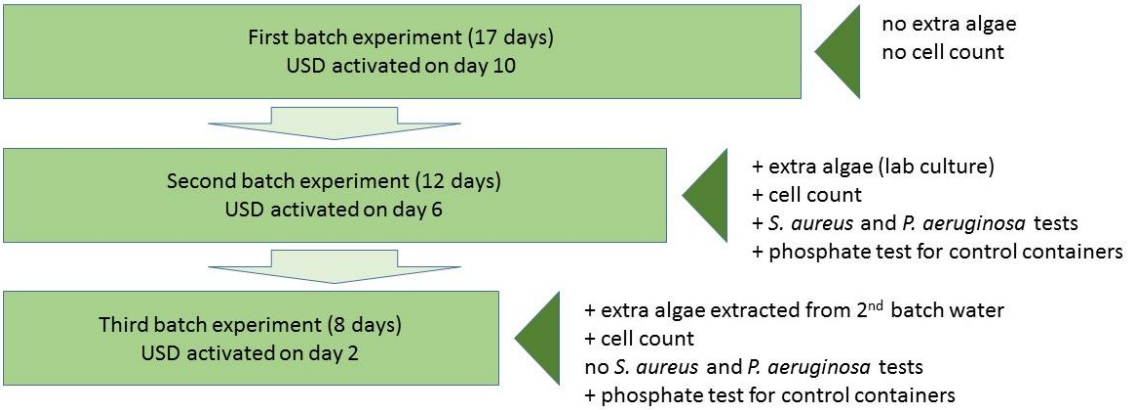


Chart 1. Experimental design.

The experimental design can be seen on Chart 1. There were three batch experiments within this research, new water delivery organized for each of them; approximately the same procedure was carried out each time. Same measurement procedures as well as testing strategies were applied every time. However, some practices implemented during the first batch experiment were found to be insufficient to provide the needed information. Therefore, cell count was introduced in second batch experiment to support algae population data provided by AlgaeTorch. Also phosphate testing in the first experiment had been done only for test containers, and later it was done for both test and control batches. Another change that followed the first experiment was introduction of selective analysis for *Staphylococcus aureus* (*S. aureus)* and *Pseudomonas aeruginosa* (*P. aeruginosa).* However, it was not performed for the last experiment due to the interruption in the supply of medium. It was decided to use extra algae culture rather than try to grow the one present in the delivered water, because the first experiment showed that the algae had been dying before the treatment was applied. These changes are explained in detail further.

Water from a shallow eutrophied lake (Steinhuder Meer in Lower Saxony, Germany) was delivered on thrice in a 1000L tank and pumped in the above mentioned containers: 40l in each test container and 20l in each control container. Devices with frequencies 40.7 kHz, 37-45 kHz and 29-37 kHz and power

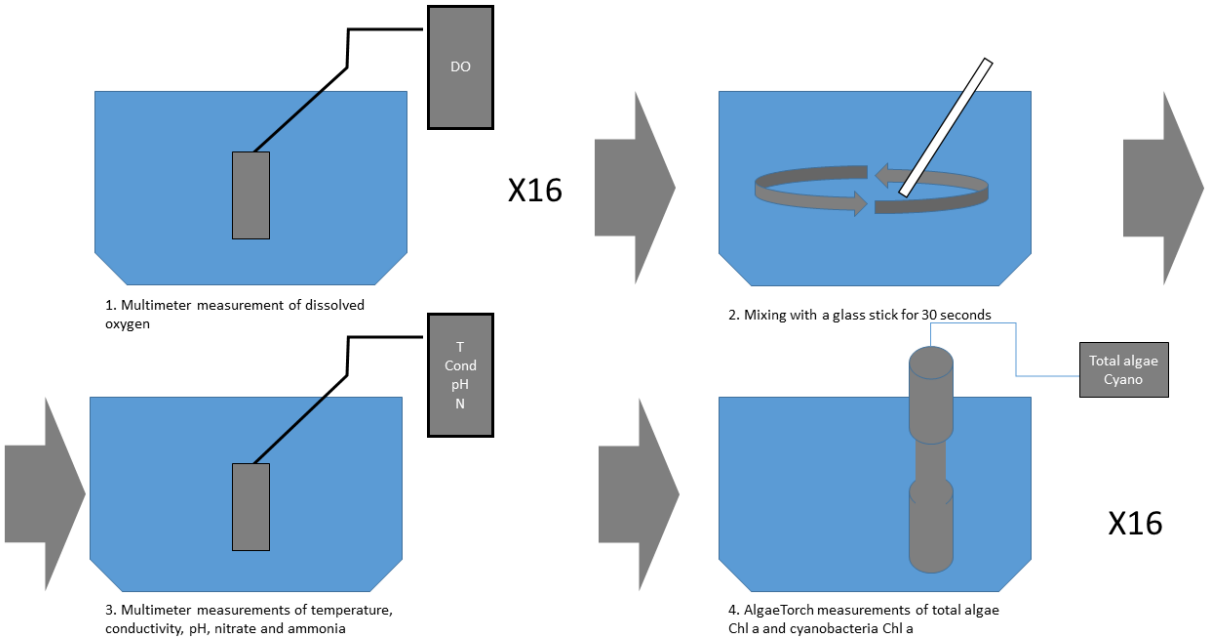
output 8W were tested respectively within 1st, 2nd and 3rd experiment. Thus, the power density was 8W/40l=0.2W/l.

Picture 1. The installation for the experiment in the greenhouse – on the left; close-up of a test container – on the right.

3.1.2 First batch experiment

The first batch experiment lasted for a total of 17 days. Every day apart from weekends at approximately the same hour the same measurement procedure was carried out (everyday measurement procedure), as can be seen in Picture 2. It involved:

- measurement of dissolved oxygen (% and mg/l) using Professional Plus Multiparameter Instrument (YSI, USA) in each of the 16 containers;
- mixing of water using a glass stick for 30 seconds;
- the same multiparameter probe measurements for temperature (°C), conductivity (SPC), pH, nitrate (mg/l) and ammonia (mg/l);
- AlgaeTorch measurements for total algae (Chl a), cyanobacteria (Chl a) and turbidity (FTU);
- repetition for each of the 16 containers.

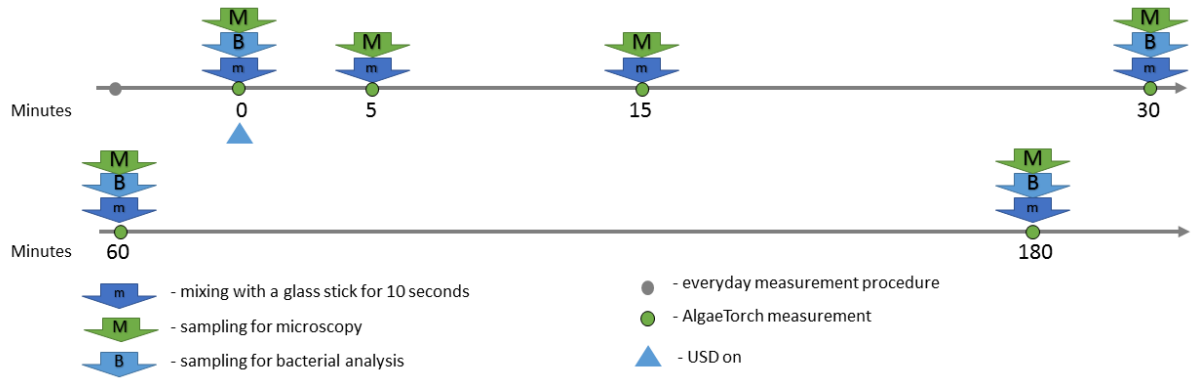


Picture 2. Everyday measurement procedure. It consists of four steps; step one was repeated for each of the 16 containers,

afterwards steps 2-4 were repeated for each of the 16 containers. Drawings for steps 1 and 3 represent multiparameter instrument measurements; step 2 drawing represents mixing; step 4 drawing represents AlgaeTorch measurement.

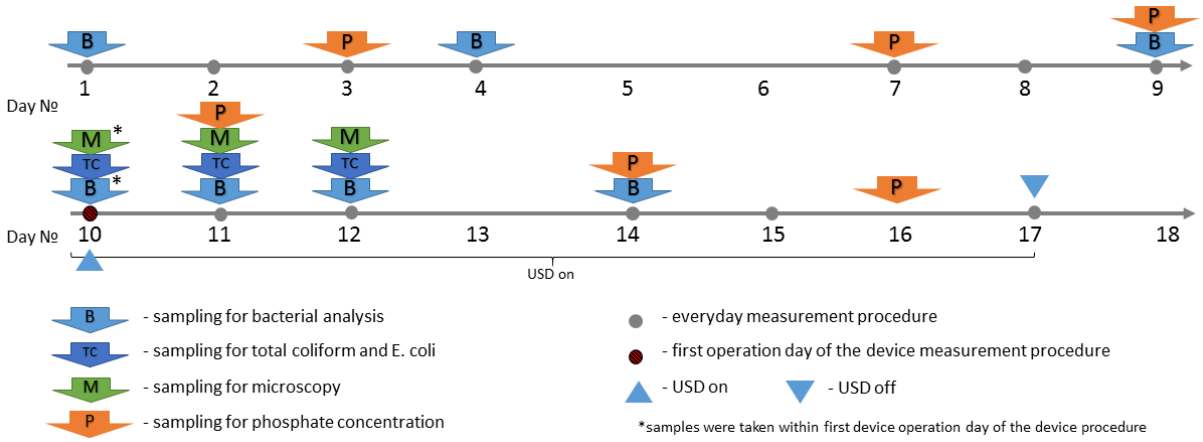
The ultrasonic device was turned on for the first batch experiment on day 10. Then the device with permanent frequency of 40.7 kHz was used. A special measurement procedure was established for the first operation day of the device that can be seen on Picture 3. It included the following:

- everyday measurement procedure was carried out;
- ultrasonic device was turned on;
- contents of the containers were mixed with glass stick for 10 seconds;
- a sample of 15ml was taken from each test container;
- AlgaeTorch measurement was carried out;
- previous 3 steps were repeated for each test container 0, 5, 15, 30, 60 and 180 minutes following the activation of device.



Picture 3. First operation day of ultrasonic device procedure. Numbers represent time after USD activation in minutes. A sample of 15ml was taken at each measurement time; the scheme depicts what the sample was used for: arrow with "M" on it means that sample was used for bacterial analysis. Arrow with small "m" shows when a 10 seconds mixing with glass stick was performed. Mixing was always carried out prior to sampling.

Following the first day of ultrasonic treatment the containers were watched for another 6 days. Measurement procedure (Picture 2) was carried out every working day. In addition to that, samples taken on 1st, 4th, 9th, 10th, 11th, 12th and 14th were tested for bacterial growth; on day 10 samples were taken at 0, 30, 60 and 180 minutes of USD operation as a part of the first operation day of USD procedure (Picture 3). Mixed samples from test containers were tested for *Escherichia coli (E. coli)* and total coliform (TC) on 10th, 11th and 12th days. Each of the samples taken of the first day of the device operation as well as samples taken on 11th and 12th days underwent a microscopy analysis. Samples for phosphate measurement were taken on 3rd, 7th, 9th, 11th, 14th and 16th days from each of the test containers. The experiment setup scheme can be seen in Picture 4.



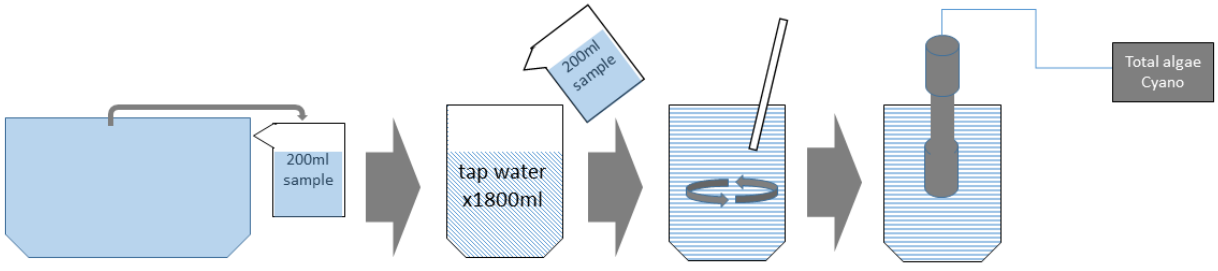
Picture 4. First batch experiment setup scheme. Numbers represent time after water delivery in days, day 1 being the day of delivery; arrows represent a variety of samplings; dots on a line represent measurement procedures. Bacterial analysis and TC and E. coli samplings were carried out only for test containers; phosphate and microscopy samples were taken from both test and control containers.

After the last set of measurements was taken on day 17, the containers were emptied and rinsed with tap water to be ready for the second experiment.

3.1.3 Second batch experiment

Second batch experiment lasted for a total of 12 days. This time containers were covered with transparent cooking film all the time except during the measurements in order to avoid excessive evaporation. Measurement procedure (Picture 2) was carried out daily on every working day in the period from day 1 until day 9. In addition, AlgaeTorch measurements were carried out on day 12.

On 2nd day *Chlorella vulgaris (CV)* culture cultivated in laboratory was added to test containers to reach the concentration of approximately 0.5 mill cells/ml. The algae concentration appeared to be too high for AlgaeTorch device to measure making dilution steps necessary. In order to measure total algae and cyanobacteria concentrations and turbidity, 200ml samples were taken from containers, mixed in the laboratory with tap water 1:10 and measured with AlgaeTorch. The procedure is depicted in Picture 5.

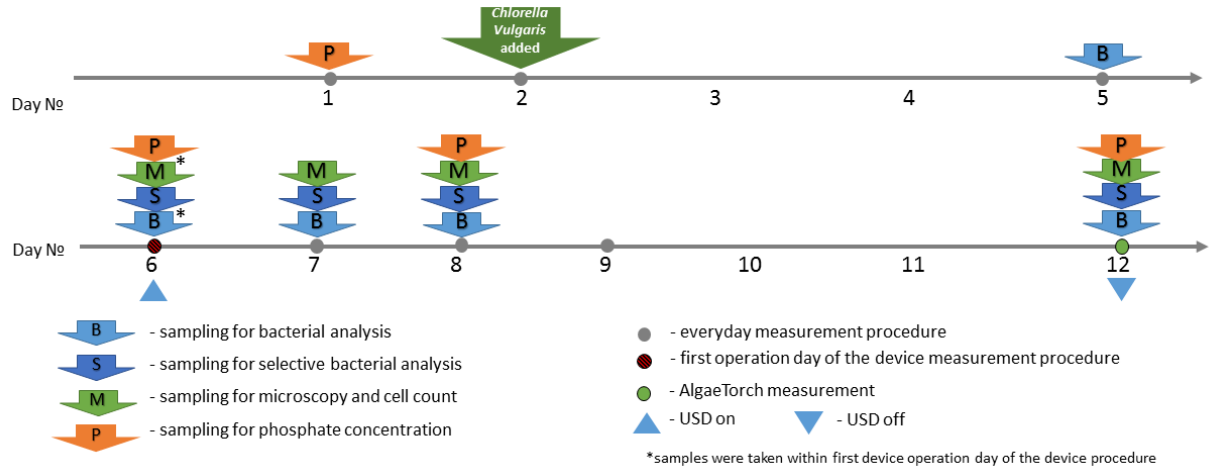


Picture 5. Diluting a sample for AlgaeTorch measurement.

Ultrasonic device with fluctuating frequency of 37-45kHz was turned on in the period of day 6 – day 12. The first operation day of the device procedure was identical to the one presented on Picture 3, however AlgaeTorch measurements were not carried out in the greenhouse. Instead samples of 200ml

were taken and procedure from Picture 5 was carried out. Also cell count was introduced at this point; it was performed for each sample taken on day 6 as well as on days 7, 8 and 12.

In this experiment samples for phosphate were taken on days 1, 6, 8 and 12 from both test and control containers. Samples for bacterial analysis were taken on days 5, 6, 7, 8 and 12; on the 6th day (USD on) the sampling was performed 0, 30, 60, 180 minutes after starting the device (Picture 3). Samples taken from test containers on days 6, 7, 8 and 12 were tested for *E. coli* and total coliform, *S. aureus* and *P. aeruginosa*. The procedure can be seen in Picture 6.



Picture 6. Second batch experiment setup scheme. Numbers represent time after water delivery in days, day 1 being the day of delivery; arrows represent a variety of samplings; dots on a line represent measurement procedures. Arrow with "S" on it represents selective bacterial analysis; it includes tests for TC and E. coli, P. aeruginosa and S. aureus. Microscopy, bacterial analysis and selective bacterial analysis samplings were carried out only for test containers; phosphate samples were taken from both test and control containers.

On the last day of the experiment containers were emptied and rinsed with tap water before new water arrival.

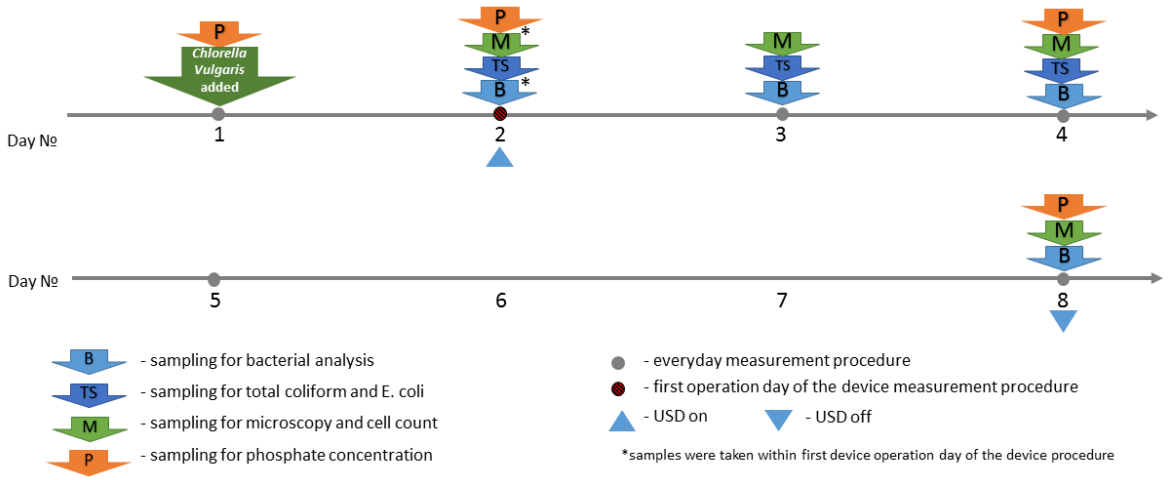
3.1.4 Third batch experiment

The third batch experiment lasted 8 days. Some *CV* culture separated from second batch water through centrifugation and sedimentation was added to the containers on day 1 giving the algae concentration of about 250000cells/ml. Ultrasonic device with fluctuating frequency of 29-37kHz had been turned on in the period of day 2 – day 8.

The procedure was similar to the one applied during the second batch experiment with minor changes: tests for *E. coli* and total coliform were run for both test and control containers, while tests for *S. aureus* and *P. aeruginosa* were eliminated. As the algae concentration was within the values that can be measured by AlgaeTorch, the measurements were taken in the greenhouse directly in the containers.

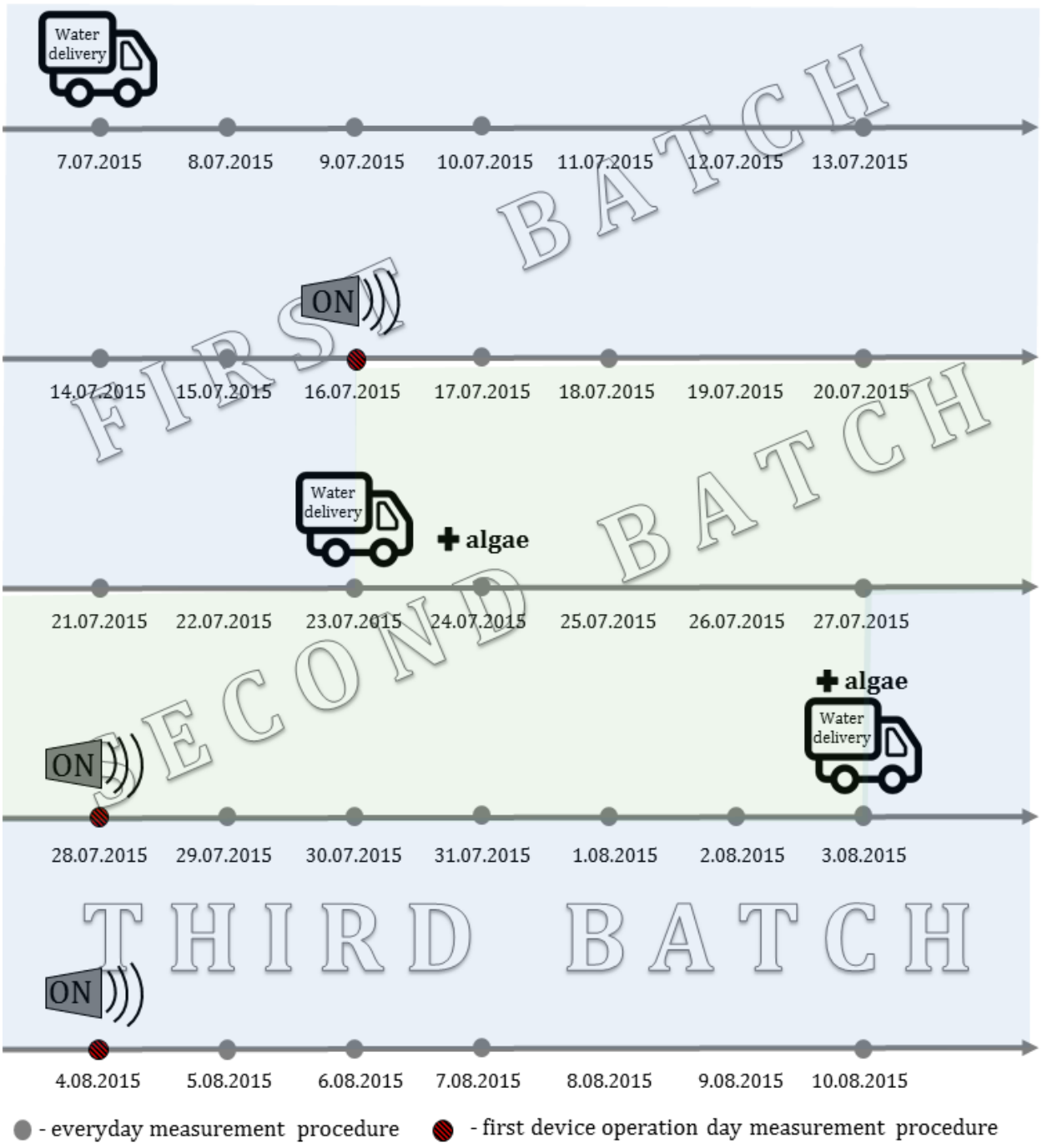
In this experiment samples for phosphate were taken on days 1, 2, 4 and 8 from both test and control containers. Samples for bacterial analysis were taken on days 2, 3, 4 and 8; on day 2 sampling was performed 0, 30, 60, 180 minutes after starting the device (Picture 3). Samples taken from test containers on days 2, 3 and 4 were tested for *E. coli* and total coliform. Cell count was carried out on all

samples taken on day 2 as well as on days 3, 4 and 8. A scheme for the procedure can be seen on Picture 7.



Picture 7. Third batch experiment setup scheme. Numbers represent time after water delivery in days, day 1 being the day of delivery; arrows represent a variety of samplings; dots on a line represent measurement procedures. Microscopy, bacterial analysis and TC and E. coli samplings were carried out only for test containers; phosphate samples were taken from both test and control containers.

3.2 Experimental timeline



Picture 8. Experiment timeline. Water delivery, addition of algae and activation of USD are marked on the scheme with icons. Dots on the line represent measurement procedures (procedures description can be found on Picture 2 and 3). Areas with different background colors represent 1st, 2nd and 3rd batch experiments.

3.3 List of materials

- 100 ml, 200ml and 1L graduated cylinders produced by VIT LAB, Fortuna W.-Germany, Hirschmann EM Technicolor W.-Germany;
- 22L and 45L rectangular plastic containers;
- 250 ml Erlenmeyer flasks produced by Blau Brand, Germany, with cotton stoppers;
- 50 ml Florence flasks produced by Blau Brand, Germany, with plastic stoppers;
- Air pump, model ACO-9630, HAILEA Ltd., China;
- AlgaeTorch, bbe Moldaenke GmbH, Schwentinental, Germany – for total chlorophyll a concentration, cyanobacteria chlorophyll a concentration and turbidity;
- Autoclave, Systec DE-65, Linden, Germany; program 10, effective time 20 minutes at 121°C;
- Bunsen burner;
- Centrifuge, Sorvall RC BIOS, Thermo scientific, USA;
- Deionizer, Seradest SD2000, Ransbach-Baumbach, Germany;
- Glass bottles with plastic caps (1l x 8; 5l x 2);
- Glass mixing stick;
- Glass test tubes with metal caps;
- Hot water bath, Koettermann, Uetze, Germany;
- Incubators, WTC binder at 28°C, USA; Memmert at 36°C, Schwabach, Germany;
- Light incubator, 28°C, 5500LUX, Binder, USA;
- Magnetic stirrer;
- Mechanical pipettes 0.5ml and 1ml, Eppendorf, USA;
- Mechanical pipette 9ml, Thermo Labsystems, USA;
- Metal spreader;
- Microscope, Carl Zeiss Microscopy GmbH, Germany,
- Mixing spoon;
- Non-sterile pipette tips;
- Spectrometer UNICAM UV/Vis UV2, USA;
- Plastic non-reusable petri dishes, VWR, Leuven, Belgium;
- Professional Plus Multiparameter Instrument, YSI, USA;
- Scales, Sartorius, Braunschweig, Germany;
- Sterile pipette tips;
- Ultrasonic devices (40.7kHz, 37-45kHz, 29-37kHz).

3.4 Testing strategies

3.4.1 General information

All chemicals were purchased from VWR Chemicals (Leuven, Belgium) unless otherwise noted. *Chlorella vulgaris* culture were obtained from Ostfalia University laboratory. All laboratory tests were carried out at room temperature and in semi-sterile conditions, while the experiment itself took place in an outside

greenhouse during early summer season in Wolfenbuettel, Germany. The ultrasonic devices used were produced and delivered by Dr. Raubenheimer GmbH, Hanover, Germany.

3.4.2 Field measurements

Temperature (˚C), pH, dissolved oxygen (mg/l and %), conductivity (SPC), nitrate and ammonia (mg/l) concentrations in the containers were measured with Professional Plus Multiparameter Instrument produced by YSI, USA, according to the instructions provided with the instrument.

Monitoring of chlorophyll a (Chl a) was employed in order to estimate the total algae and cyanobacteria concentrations. AlgaeTorch produced by bbe Moldaenke GmbH in Schwentinental, Germany, was used for this purpose.

3.4.3 Medium preparation

3.4.3.1 Double ES medium for Chlorella vulgaris

16 liters of double ES (2ES) medium were needed for the experiment in order to obtain stem cultures for inoculation. Eight one liter glass bottles and two five liter glass bottles were used for medium preparation. The recipe of Basal medium was obtained from Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG), however nutrients were doubled. First, soil extract was cooked: two 1l bottles were filled with 1/3 soil dug up right outside the laboratory and 2/3 deionized water, and then autoclaved at 121°C for 20 minutes. Second, the amount of ingredients needed for preparation of the solution were calculated as can be seen in Table 1. Third, the right amounts of ingredients were measured with graduated cylinders and mixed in six 1l bottles and two 5l bottles according to the obtained recipes.

	1l	5l	Total, 16l
KNO_3	40ml	200ml	640ml
K_2HPO_4	40ml	200ml	640ml
$MgSO_4$	40ml	200ml	640ml
Soil extract	60ml	300ml	960ml
Micronutrient solution	10ml	50ml	160ml
Deionized water	810ml	4050ml	12960ml

Table 1. Recipes for preparation of double ES medium in 1 liter vessel, 5 liter vessel and 16 liter vessel.

The contents of the bottles were mixed; medium was autoclaved at 121°C for 20 minutes.

3.4.3.2 Double ES agar

1000ml of 2ES agar were needed for the experiment. One liter of earlier prepared 2ES medium was spread in five 250ml Erlenmeyer flasks, 200ml in each flask measured with graduated cylinders. Using scales 3g of agar was weighted and added to each flask, creating the agar solution of 15g/l. The flasks were closed with cotton stoppers and autoclaved at 121°C for 20 minutes. Later, the solution was spread into plastic petri dishes, approximately 10ml per dish. Ready agar plates were stored in a fridge for no longer than 1 month.

3.4.3.3 Sodium chloride *solution and nutrient agar DEV*

Bacterial count was performed many times during this experiment; as a very big amount of medium was needed, it was prepared in parts. In this section a preparation of 1l NaCl solution and 1l nutrient agar DEV (from now on referred to as NA) will be described.

A solution of 9g/l sodium chloride in deionized water was used for preparing sample dilutions. Using scales 9 grams of NaCl were weighted and mixed with 1l of deionized water in 1l glass bottle. Then, using a 10ml mechanical pipette with reusable plastic tip the solution was spread in 110 glass test tubes, 9 ml in each tube. Those tubes were closed with metal caps and autoclaved at 121°C for 20 minutes. Tubes were stored at room temperature for no longer than 3 days.

Nutrient agar (NA) DEV (VWR Chemicals, Belgium) was cooked using the concentration suggested on the box (43g/l), however a different preparation procedure was applied. As the ingredients of NA can vary, here is the composition of the used powder: peptone from meat, 10g/l; meat extract, 10g/l; sodium chloride, 5g/l; agar, 18g/l. 8.6 grams of dry mix were weighted and put in each of five 250ml Erlenmeyer flasks used; using graduated cylinders 200ml of deionized water were added to each flask. Then, flasks were closed with cotton or metal stoppers and autoclaved at 121°C for 20 minutes, no mixing applied. After the autoclaving, the medium was spread in plastic petri dishes. If the preparation of agar plates was not performed immediately after autoclaving, hot water bath at 100°C was used for melting the agar. Ready agar plates were stored at room temperature for 24 hours. After that they were used or placed in a fridge. Plates were stored in the fridge for no longer than 7 days.

3.4.3.4 *Readymade medium*

Agar plates for enumeration of *E. coli* and total coliform, *P. aeruginosa* and *S. aureus*, Chromogenic Coliform Agar (CCA), Cetrimide Agar (CA) and Mannitol Salt Agar (MSA) respectively, were purchased from Oxoid, Wesel, Germany.

3.4.4 Algae cultivation

3.4.4.2 *Chlorella vulgaris cultivation*

Chlorella vulgaris (CV) culture available in the laboratory was mixed with freshly made 2ES medium in proportion 1:10 in six 1l glass bottles and one 10l bottle. The 10l bottle was equipped with aeration system – plastic tube with dispenser on the end connected to air pump – and placed on a windowsill. Six 1l bottles were put in an incubator working at 23°C and equipped with illumination system; aeration system consisting of air pump with 6 outlets, plastic tubes and dispensers was installed. One of the air pump outlets was out of work, so one of the bottles was not aerated. Mixing was provided by a magnetic stirrer. Installations can be seen on Picture 1.

Picture 9. Six 1l bottles containing CV in 2ES medium cultivated in an illuminated incubator at 23°C and equipped with aeration and mixing systems – on the left; 10l bottle containing CV in 2ES medium cultivated on a windowsill and equipped with aeration system – on the right.

After 18 days of harvesting cultures from all the vessels were mixed in one 20l vessel with a tap near the bottom and equally distributed to the containers.

3.4.4.3 Algal growth test

For this experiment it was essential to determine whether microalgae contained in the sample were still capable of growth even after being subjected to US treatment procedure. Self-made 2ES agar plates were used for this purpose. Sample of 1ml volume was spread on the agar, all the plates were placed in the illuminated incubator for 7 days. The result of the test was marked positive (growth of green algal colonies present) or negative (growth of green algal colonies absent).

3.4.5 Detection and enumeration of bacteria

3.4.5.2 Total microbial detection and enumeration

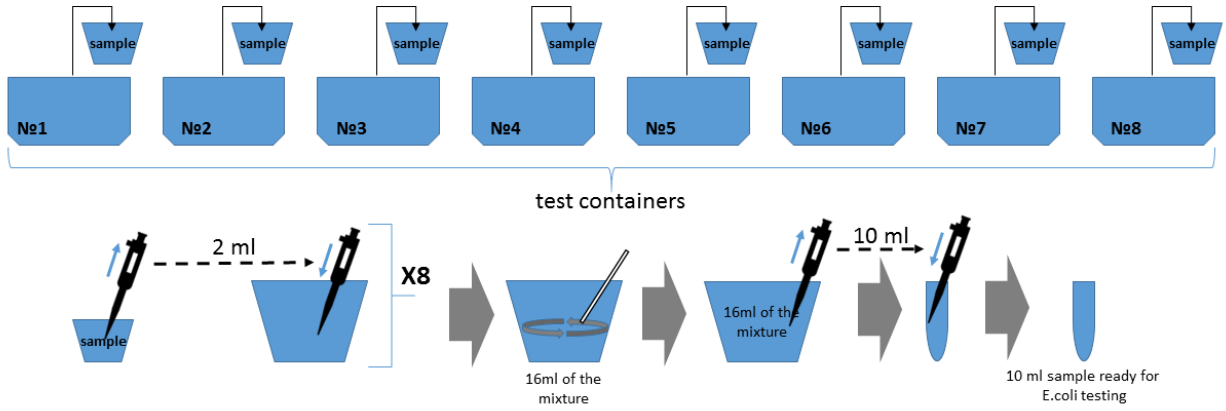
First, dilutions of a sample were prepared. At first eight dilutions (d_1 – d_8) were prepared for each sample; later it was decided to use d_0, d_3 and d_5 for testing.

Next, plates were labeled and inoculated in semi-sterile conditions. 0.5ml of a sample were placed on agar and spread with metal spreader; the spreader was flamed before and after each use on Bunsen burner. For each dilution of a sample two plates were prepared; one of them would later go to a 28°C incubator, the other to 36°C one. Samples were incubated for 48h, then colony count was performed.

After the counted plates were discarded into utilization bags and autoclaved at 121°C for 20 minutes for sterilization.

3.4.5.3 Total Coliform and Escherichia coli detection and enumeration

Enumeration of *Escherichia coli* (E. coli) and coliform bacteria were mainly analyzed according to ISO 9308-1, 2014, however 10ml of the sample underwent membrane filtration instead of 100ml. Mixed samples were prepared for the testing: scheme for mixed sample preparation for E. coli testing can be seen on Picture 10.



Picture 10. Mixed sample preparation for E. coli test. Eppendorf 1ml mechanical pipette was used for this test.

3.4.5.4 Pseudomonas aeruginosa and Staphylococcus aureus detection and enumeration

First, some colonies from NA were placed on CA and MSA in order to determine if those might be *Pseudomonas aeruginosa (P. aeruginosa)* or *Staphylococcus aureus (S. aureus)*. When it was proven that samples do contain those bacteria, CA and MSA plates were inoculated with 0.5ml samples using 1ml mechanical pipette and metal spreader and incubated at 36°C for 24h. Mixed samples were prepared for testing: equal in volume samples were taken from each test container and mixed in one vessel. Later the needed amounts were pipetted onto the plates.

3.4.5.5 Phosphate test

Phosphate was analyzed according to DIN Methods, DIN ISO 15923-1, 2012.

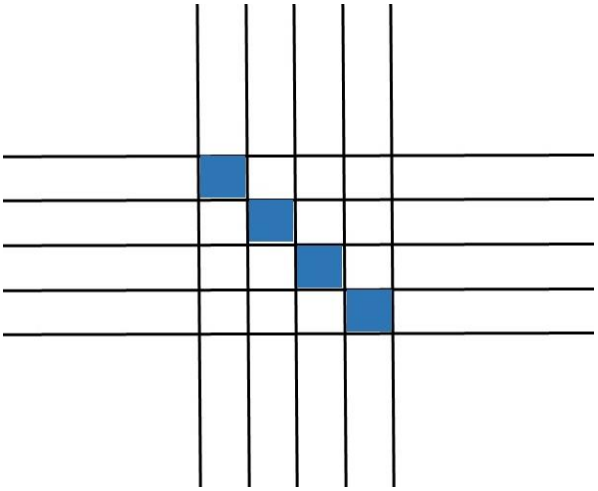
3.4.6 Microscopy and cell count

Microscopy samples were prepared. First, a plastic tube with a sample was shaken in order to evenly distribute particles. Second, a metal loop was used to place a drop of the sample on a glass slide; then the drop was covered with coverslip. Third, a drop of oil was applied on top of the cover glass. The samples underwent microscopy analysis; Zeiss microscope was used equipped with 100x oil objective lens and 10x oculars. Pictures were taken with a camera through an ocular.

Thoma cell counting chamber (depth 0.100mm, size 0.0025mm^2) was used for enumeration of microalgae. Undiluted samples were used each time, placed under the coverslip with metal loop. Only visibly healthy algae cells were counted in groups of 4 medium squares in a diagonal in each of two big squares (see Picture 11); then the following formula was applied for calculating the number of cells per milliliter:

$$\frac{Total\ cells\ counted}{Number\ of\ squares} \times 250000 = \frac{TCC}{4 \times 2} \times 250000 = TCC \times 31250$$

$$Cells\ per\ ml = TCC \times 31250$$



Picture 11. Areas of Thoma chamber in which cells were counted highlighted with blue color.

3.5 Meeting WHO standards

"Evaluating household water treatment options" (World Health Organization (c), 2011) document contains microbiological performance targets for each of WHO approved HWT methods expressed in terms of $\log_{10}$ reductions; it is computed as

$$log_{10}(\frac{C_{\ untreated\ water}}{C_{\ treated\ water}}),$$

where C is microbe concentration in water. In this research the performance of ultrasonication is calculated using the formula above based on *E. coli* concentrations and HPC results. The highest of all obtained concentrations on first and last days of ultrasonication will be used when calculating the microbial performance. The obtained results are as well compared to WHO and USEPA drinking water standards that can be seen in the table below.

	C: Standard value
Cyanobacteria	Not present
E. coli	< 1CFU/100ml
TC	< 1CFU/100ml
HPC	< 500CFU/ml
Nitrate	< 10mg/l
pH	6.5-9.5
Turbidity	< 1FTU

Table 2. WHO and USEPA drinking water standards (World Health Organization, 2008; USEPA, 2009).

3.6 Error

Graphs were created in order to present the results of this experiment using Microsoft Office Excel 2013. Standard error of the mean had been chosen to depict the variation of values; chart tool "Error bars → Standard error" was used to do so. The error amounts shown on charts are calculated using the following equation:

$$S.E. = \sqrt{\frac{\sum_{s=1}^{m}\sum_{i=1}^{n} y_{is}^2}{(n_y - 1)(n_y)}}$$

where s - series number, i - point number in series s, m - number of series for point y in chart, n = number of points in each series, y_{is} - data value of series s and the i^{th} point, n_y = total number of data values in all series (Microsoft, 2015).

4 RESULTS

4.1 First batch

4.1.1 Physical parameters development

The device with constant frequency of 40kHz was used in this experiment. During 17 days of the experiment water temperature stayed within the range of 17-25°C. Temperatures in test and control containers were similar during first batch experiment. However, after 16 July, 2015, when temperature started rising, water in test containers had higher temperatures (1-2°C difference) every day until 23 July, 2015 (for example, 17 July, 2015: test 23°C, control 21°C). The reason for this temperature difference might be the position of test and control containers on opposite sides of a greenhouse they were kept in. Another possibility is the warming up of test waters by USD produced energy. The average water temperature was 21.14°C.

Dissolved oxygen (DO) value before turning on USD in both test and control containers was fluctuating around 7mg/l. On 16 July, 2015 DO in control containers peaked at 9.4mg/l, however returned to 7mg/l on the next day. After USD was on, DO in test containers dropped to 5.5-6mg/l and stayed at that value until the end of the experiment. At the same time DO values in control containers stayed around 6.5-7mg/l. Difference in DO was probably due to the fact that ultrasonication makes small oxygen bubbles coalesce and form bigger bubbles and therefore leave liquid faster.

On graphs for temperature and DO on Figure 1 the inverse proportion of temperature and DO can be noticed: on 18 July, 2015 and 21 July, 2015 there are two peaks of high water temperatures (24-25°C), and on the same dates the DO graph shows lower values (app. 5.5mg/l). However, these changes in DO were not very notable.

Specific conductivity graphs for test and control containers presented on Figure 2 showed similar development: it was rising from 8 July, 2015 to 23 July, 2015. Same as the temperature, throughout the experiment conductivity was slightly higher in test containers before and after USD activation (15 July, 2015: test 356µS/cm, control 350µS/cm; 21 July, 2015: test 364µS/cm, control 359µS/cm). Conductivity in test containers variation from the average was much higher than that in control containers.

Turbidity values in test containers were slightly lower than in control containers for the whole first batch experiment. It might be due to the difference in container sizes (22l control containers and 44l test containers): the same mixing procedure lasting 30 seconds was applied to both types of containers. After turning on USD turbidity in test containers have slightly been going down for 2 days while turbidity in control containers have been going up for the same two days. It might be due to the increased sedimentation caused by ultrasonication.

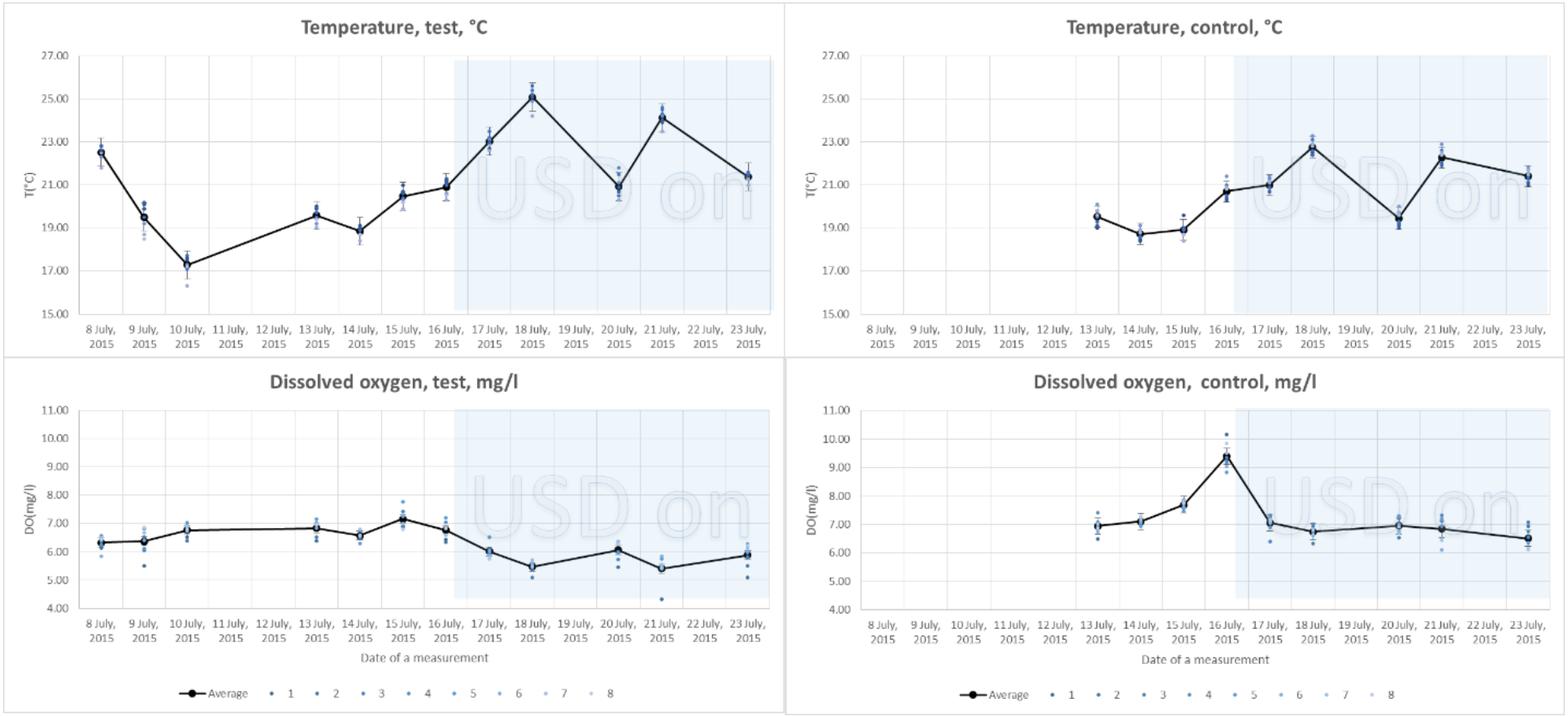


Figure 1. Temperature changes (°C) and Dissolved Oxygen (mg/l) development over time (twelve measurements within sixteen days for test containers, nine measurements within eleven days for control) in the containers (test containers on the left, control containers on the right) during the experiment. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 8 July, 2015 until 23 July, 2015 within the first batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

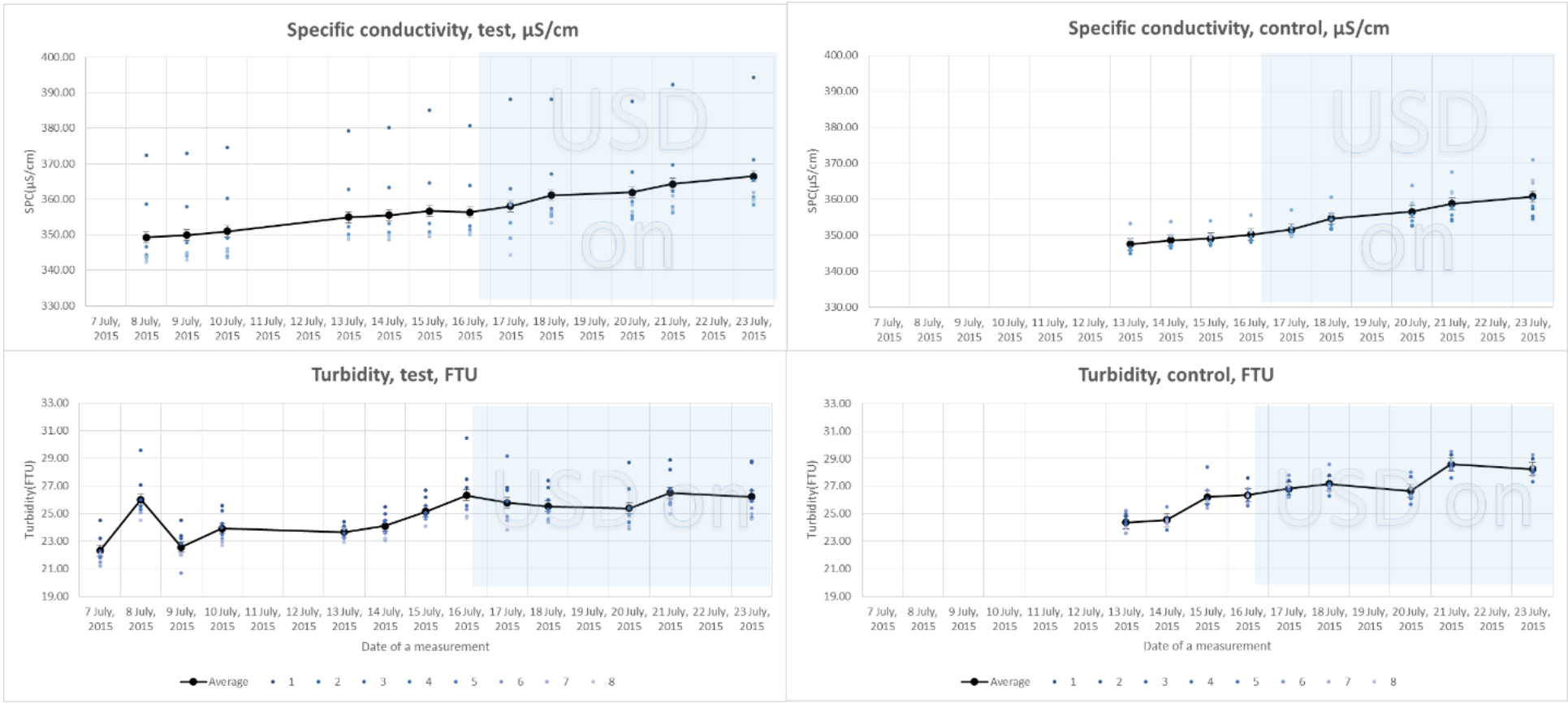


Figure 2. Specific conductivity (µS/cm) and Turbidity (FTU) development over time (twelve measurements within sixteen days for test containers, nine measurements within eleven days for control) in the containers (test containers on the left, control containers on the right) during the experiment. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 8 July, 2015 until 23 July, 2015 within the first batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

Average measured pH values are alkaline and fall between 7.7 and 8.5 for test containers and 8.3 and 8.8 for control containers. As the measured pH is higher than neutral pH level and the experiment took place in July, it can be concluded that water for the experiment had been pumped from the upper layer of a stratified lake (Fondriest Environmental Inc., 2015). Water in test containers had generally higher pH than water in control containers both before and after USD activation. The pattern of pH development in test containers repeats that of control containers (Figure 3).

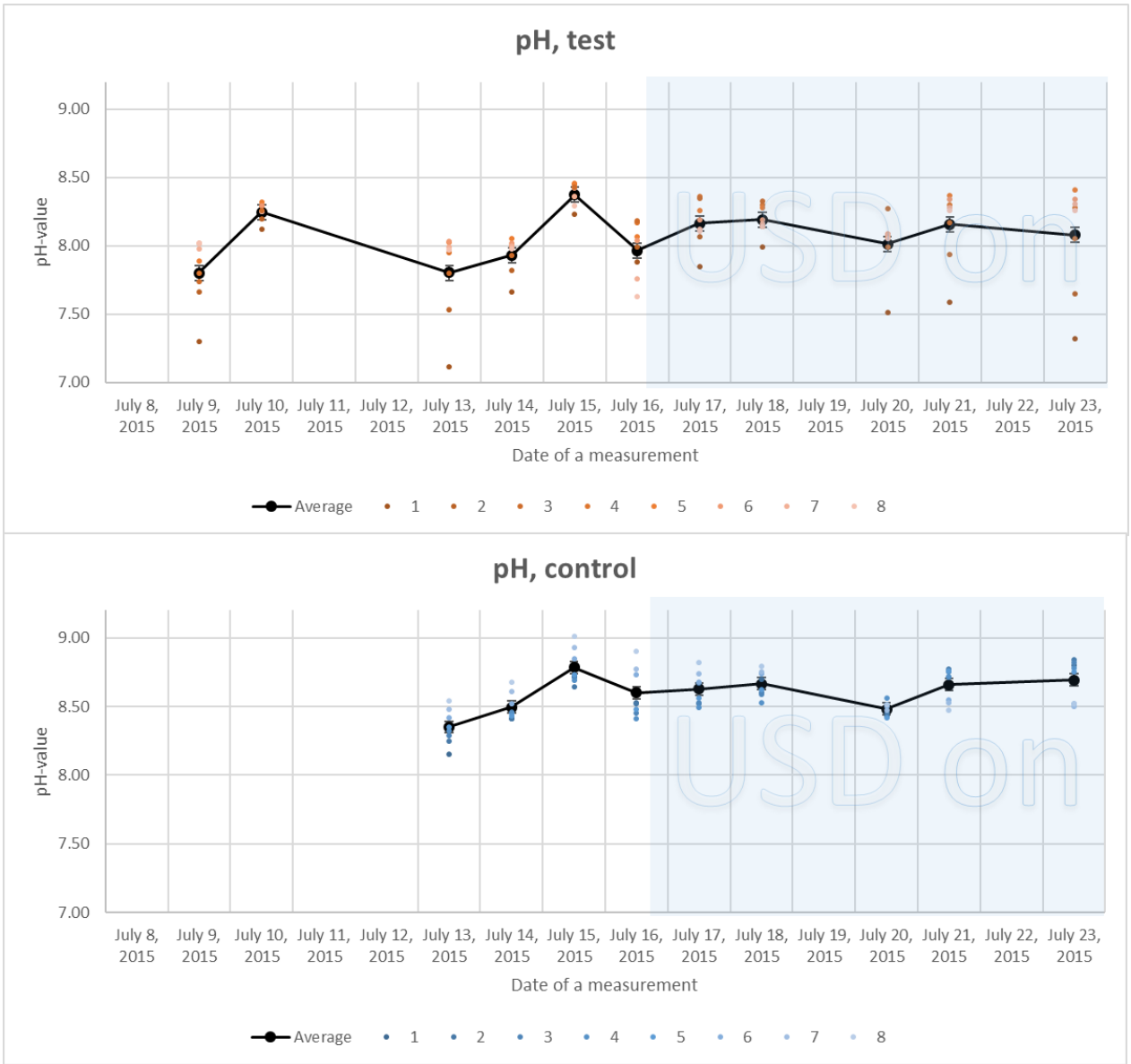


Figure 3. pH development over time (eleven measurements within sixteen days for test containers, nine measurements within eleven days) in the containers (test containers on the top, control containers on the bottom) during the experiment. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the top) and eight control containers (bottom) in the period from 9 July, 2015 until 23 July, 2015 within the first batch experiment. Error values are calculated using formula for standard error of the mean (See part ***Error! Reference source not found.****, page* ***Error! Bookmark not defined.****). Blue field represents the time when USD was operating.*

4.1.2 Nutrient development

Phosphate concentration was low in the delivered water. It dropped from 0.014mg/l on 9 July, 2015 to 0.003mg/l six days later. After 15 July, 2015 the phosphate concentration in test containers was fluctuating around the value of 0.005mg/l during the time of device operation, in some containers the concentration appeared to be 0mg/l on 20 July, 2015.

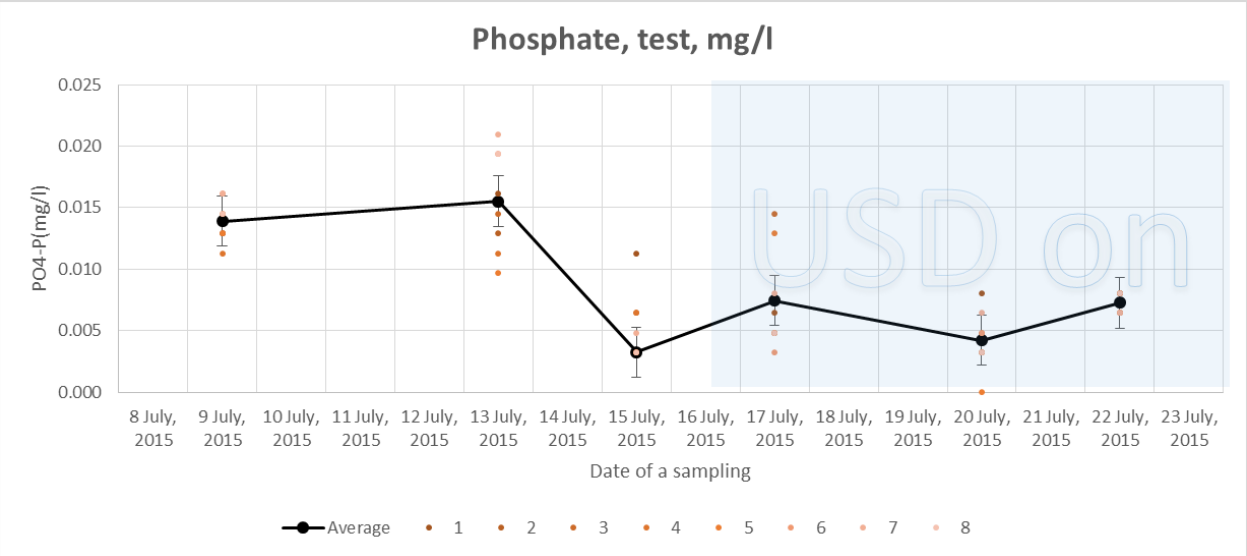


Figure 4. Phosphate (mg/l) development over time (six measurements within seventeen days) in the test containers during the experiment. Dots represent every single value obtained through measurements. The line represents mean values of eight test containers every day. Phosphate concentration was measured in the period from 9 July, 2015 until 22 July, 2015 every 2-4 days within the first batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

Nitrate concentration in test container was rapidly declining for the first three days of the experiment (from 0.67mg/l on 8 July, 2015 to 0.4mg/l on 10 July, 2015). However, in the period of 13 July, 2015 – 23 July, 2015 values for both test and control containers were fluctuating around 0.45mg/l. USD didn't seem to have any effect on nitrate concentration in the water.

Similarly, ammonia graphs for test and control containers follow the same trend; however, test containers graph looks more disturbed: The variation of values from the mean is much higher in test containers (Figure 5). Ammonia concentration is generally higher in test containers throughout the whole experiment.

All in all, the nutrient levels in the tested water were normal for lake water collected in mid-summer and sufficient to ensure algal growth.

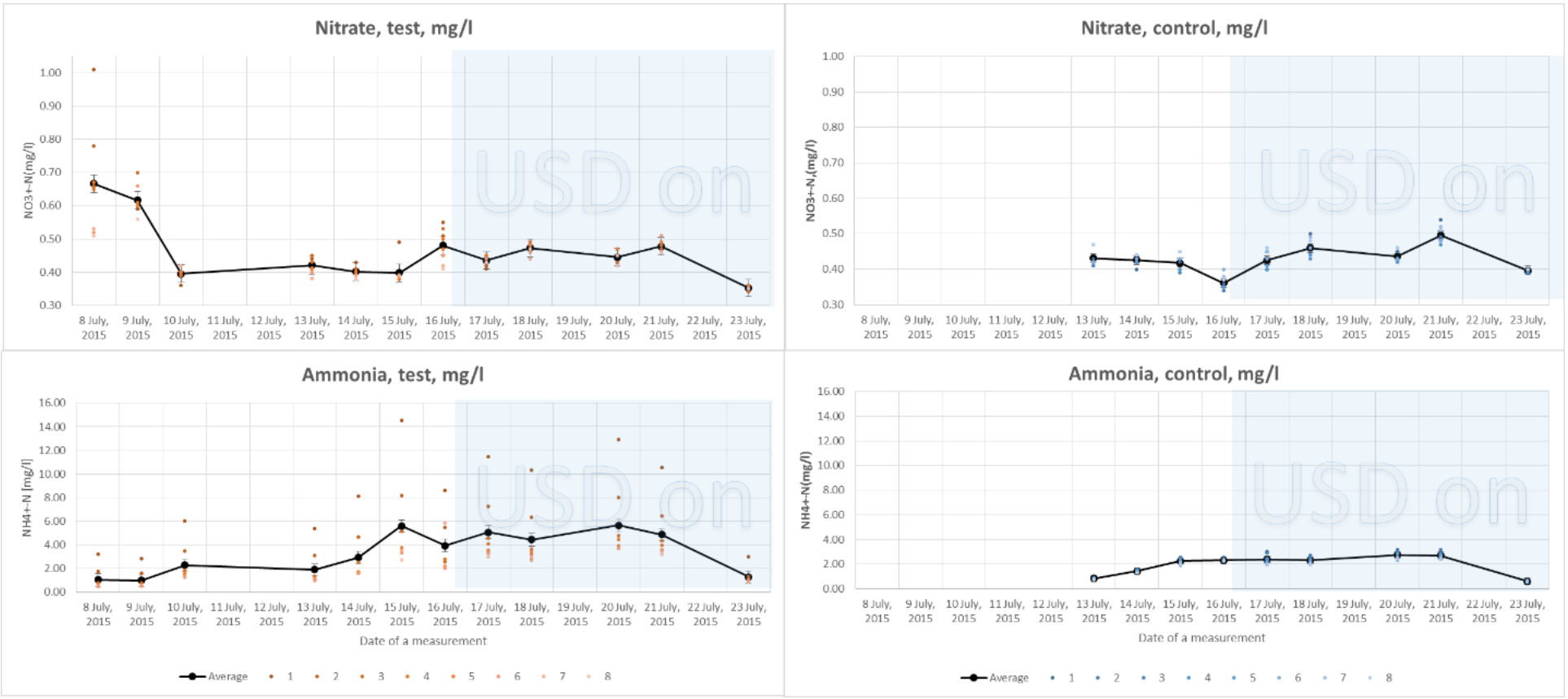


Figure 5. Nitrate (mg/l) and Ammonia (mg/l) development over time (twelve measurements within sixteen days for test containers, nine measurements within eleven days for control) in the containers (test containers on the left, control containers on the right) during the experiment. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 8 July, 2015 until 23 July, 2015 within the first batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

4.1.3 Population parameters

4.1.3.1 Algae and cyanobacteria growth throughout the experiment

It can be seen from Figure 6 that total algae concentration was growing in both test and control containers before USD activation while cyanobacteria concentration was more or less static throughout the experiment in both types of containers. After the activation of USD total algae growth in test containers slowed down, however total algae Chl a in control containers went down after 18 July, 2015 as well. Also as can be seen on Figure 5, phosphate levels decreased significantly by 15 July, 2015; taking into account this fact as well as the slowing growth in control containers, it can be concluded that PO_4^- level is responsible for the growth deceleration. The variation of measured values in test containers was higher than in control containers.

A slight drop in total algae Chl a can be seen in control containers on 20 July, 2015. That day of the experiment was marked by a temperature drop by 4˚C compared to the previous day. It might have been the reason for such a rapid downturn, however the water temperature of 21˚C stands within the margins of optimum temperature for algal growth (Lavens & Sorgeloos, 1996).

As shown in Figure 7, both total algae and cyanobacteria Chl a was higher in test containers in the period of 13 July, 2015 – 23 July, 2015. The development of total algae in test and control containers is similar except for low value of 65 µg/l Chl a in control containers on 20 July, 2015 that might be linked to temperature drop on that day. Total algae concentration in both types of containers started reducing on 21 July, 2015. At the same time, cyanobacteria showed slight growth in test containers and reduction in control containers in the period from 13 July, 2015 till 23 July, 2015.

On 23 July, 2015 both total algae and cyanobacteria Chl a were higher in test containers; growth patterns for test and control groups looked similar and didn't change after USD activation.

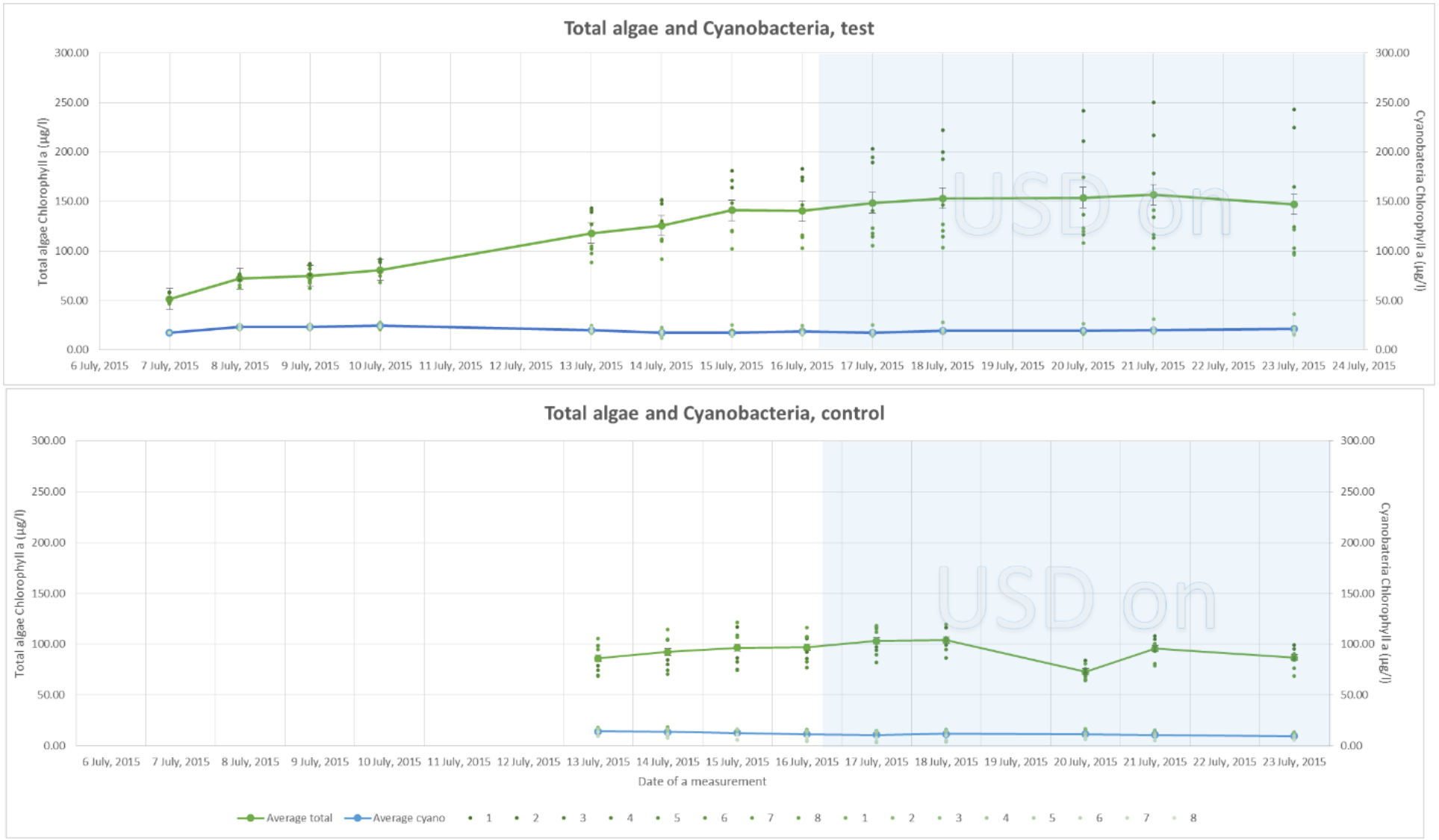


Figure 6. Total algae Chl a (µg/l) and Cyanobacteria Chl a (µg/l) development over time (thirteen measurements within seventeen days for test containers, nine measurements within eleven days for control containers) in the containers (test containers on the top, control containers on the bottom) during the experiment. Dots represent every single value obtained through measurements. Green lines represent mean values of total algae chlorophyll a taken in eight test containers (top graph) and eight control containers (bottom graph) in the period from 7 July, 2015 until 23 July, 2015 within the first batch experiment. Blue lines represent mean values of cyanobacteria chlorophyll a taken in eight test containers (top graph) and eight control containers (bottom graph). Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

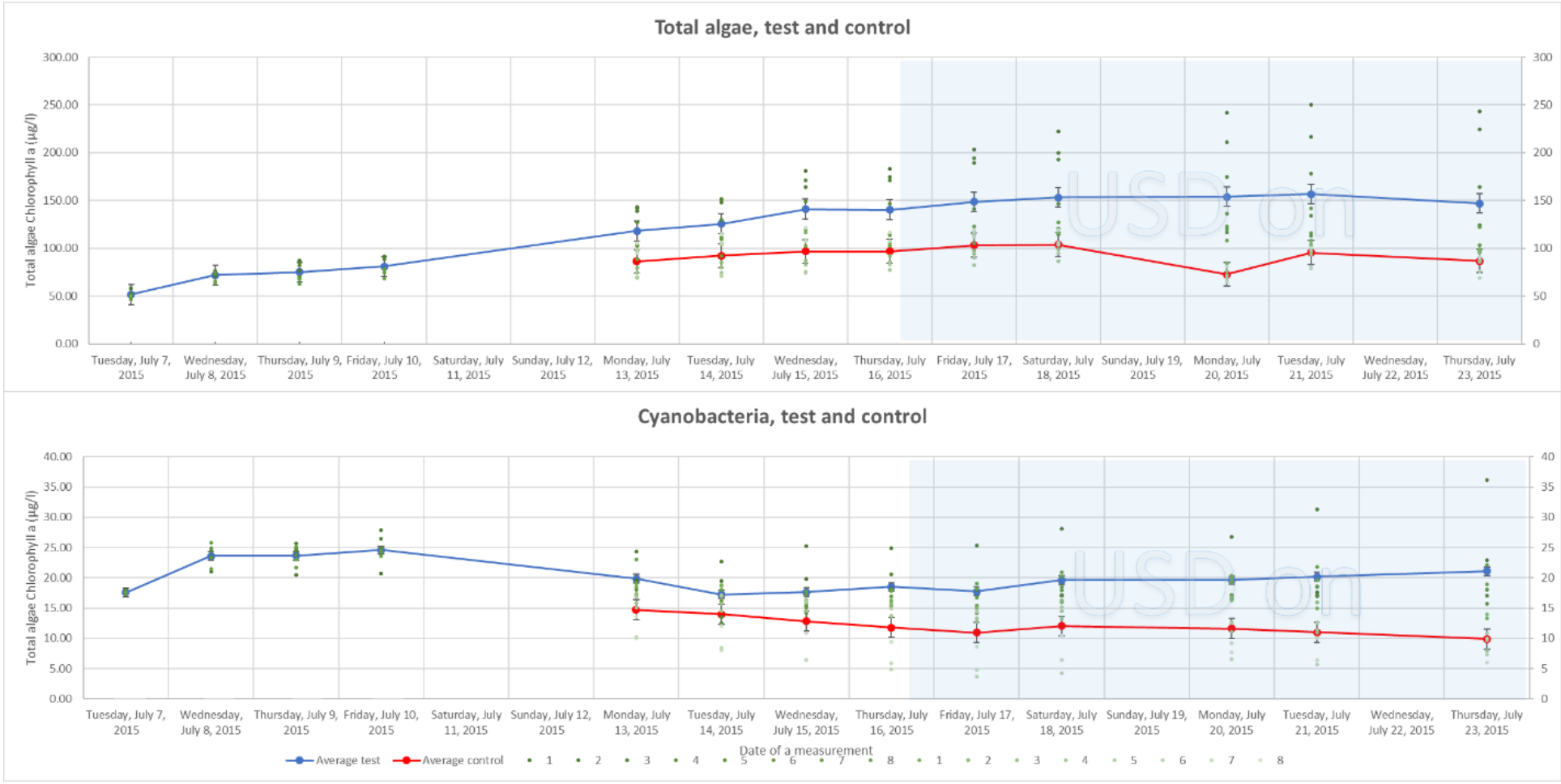


Figure 7. Total algae Chl a (µg/l) and Cyanobacteria Chl a (µg/l) development over time (thirteen measurements within seventeen days for test containers, nine measurements within eleven days for control containers) during the experiment. Dots represent every single value obtained through measurements. Blue lines represent mean values of total algae (top graph) and cyanobacteria (bottom graph) chlorophyll a taken in eight test containers in the period from 7 July, 2015 until 23 July, 2015 within the first batch experiment. Red lines represent mean values of total algae (top graph) and cyanobacteria (bottom graph) chlorophyll a in eight control containers. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

4.1.3.2 *Algae and cyanobacterial growth and turbidity development on the first three days of the device operation*

Results of the measurements performed within first operation day of ultrasonic device procedure can be seen on Figures 8, 9 and 10, as well as the measurements done 24 and 48 hours following the USD activation. Total algae Chl a, cyanobacteria Chl a and turbidity did not show drastic changes within first day of the device operation. However, some slight changes in cyanobacteria Chl a can be seen 48h after USD activation: it is higher comparing to all the previous measurements.

Another noticeable change is in a slight total algae Chl a and turbidity values' increase between 1h and 3h measurements.

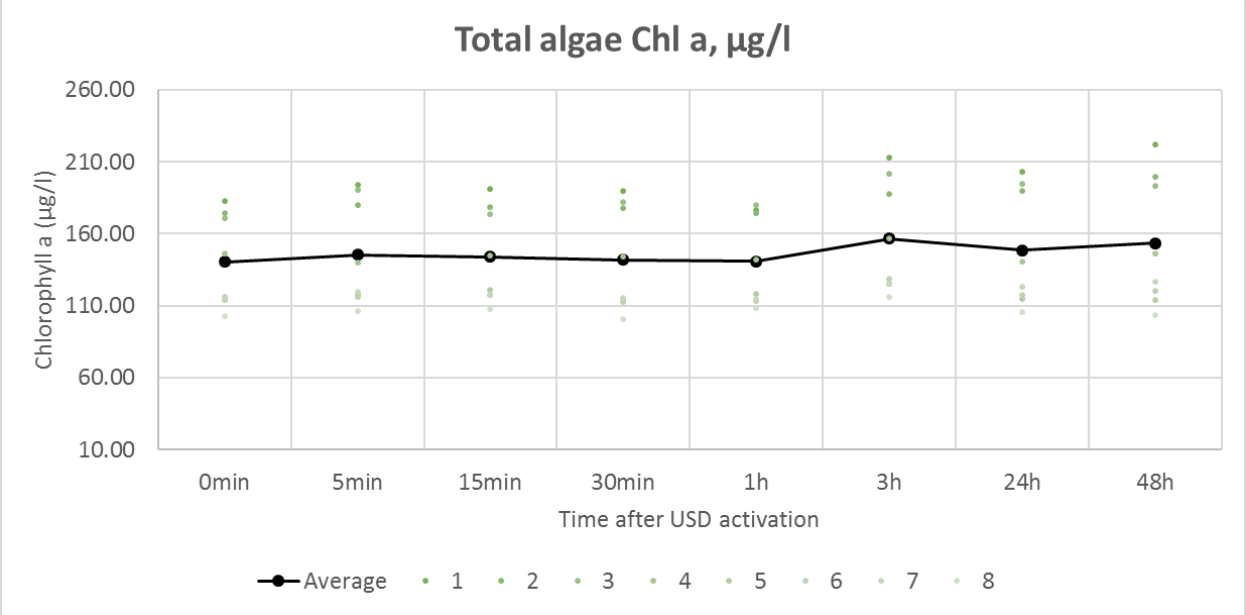


Figure 8. Total algae Chl a (µg/l) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of total algae chlorophyll a taken in eight test containers on 16 July, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 17 July, 2015 and 18 July, 2015 within the first batch experiment.

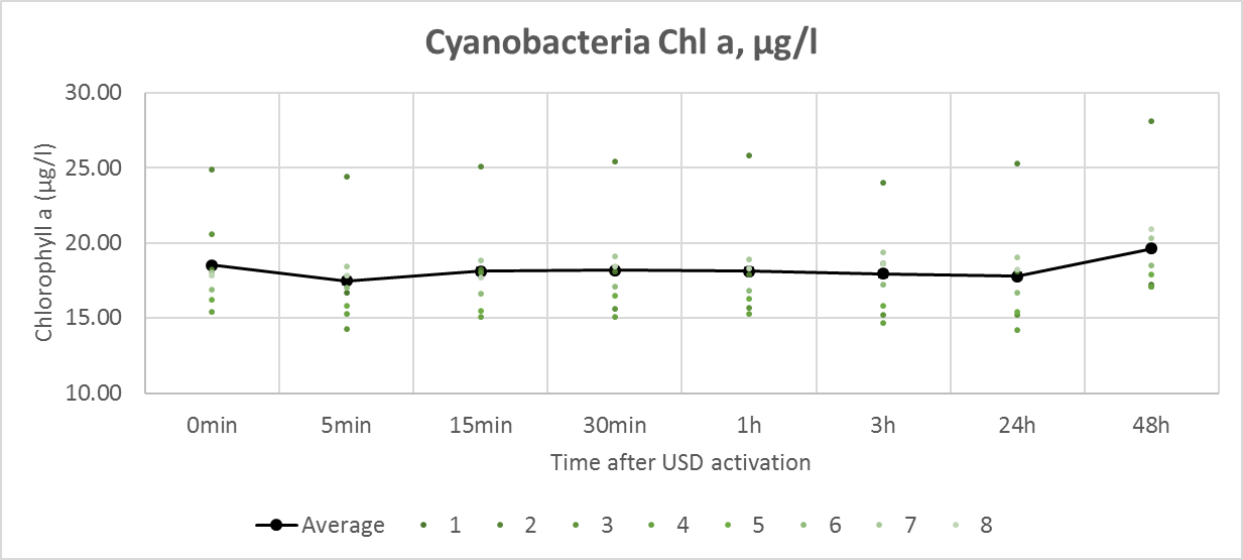


Figure 9. Cyanobacteria Chl a (µg/l) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of cyanobacteria chlorophyll a taken in eight test containers on 16 July, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 17 July, 2015 and 18 July, 2015 within the first batch experiment.

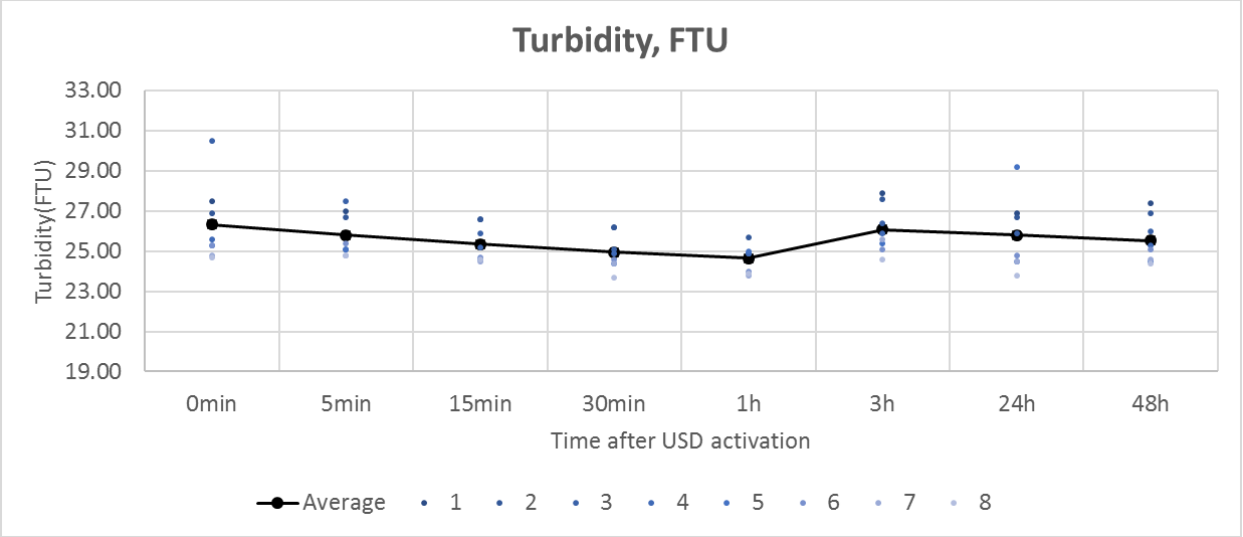


Figure 10. Turbidity (FTU) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of cyanobacteria chlorophyll a taken in eight test containers on 16 July, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 17 July, 2015 and 18 July, 2015 within the first batch experiment.

4.1.3.3 Microscopy results

Among all the microalgae and cyanobacteria cells detected were members of the following species: *Asterionella, Anabaena* (potentially toxic)*, Coelastrum, Desmodesmus, Fragilaria crotonesis, Microcystis* (potentially toxic)*, Nitzschia, Oscillatoria* (potentially toxic)*, Pediastrum simplex, Pseudanabaena, Scenedesmus and Spirogyra* (Van et al., 2006).

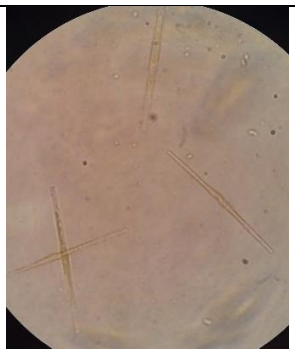
16 July, 2015 USD off	 *Pseudanabaena*, damaged	 *Nitzschia*, damaged	 *Nitzschia*, damaged	 *Desmodesmus*, healthy
17 July, 2015 USD on	 *Pseudanabaena*, damaged	 *Nitzschia*, damaged	 *Spirogyra*, damaged	 *Desmodesmus*, damaged

Table 3. The table presents pictures taken during microscopy analysis before and after USD activation within first batch experiment. Each picture is provided along with the name of the specie, health status of the microalgae (Van Vuuren et al., 2006).

All the algae species except for *Desmodesmus* found in the samples were damaged before USD activation. However most of the *Desmodesmus* cells found during microscopy analysis 24h after USD activation were damaged. There were no changes in algae cells' state noticed during the first day of USD operation.

4.1.3.4 Bacterial growth

Heterotrophic plate counts (HPC) results for the same samples at different temperatures (28 and 36˚C) were similar. HPC in the period from 7 July, 2015 till 16 July, 2015 performed before USD activation showed bacterial concentration of more than $300x10^5$ CFU/ml. However already after 30 minutes of USD operation the reduction in bacterial concentration can be seen: some of the d_5 dilutions had only transparent colonies after 48h incubation. With the increase in time of ultrasonication, HPC visibly decreases (from more than $300x10^5$ CFU/ml on 7 July, 2015 to less than 10^3 CFU/ml on 20 July, 2015). The development of HPC for the first batch experiment with photo examples is shown in Table 4.

	D_0, CFU/ml		D_3, CFU/ml		D_5, CFU/ml	
	28°C	36°C	28°C	36°C	28°C	36°C
7 July, 2015	>300, YC	>300, YC	>300	>300	>300	>300
10 July, 2015	>300, YC	>300, YC	>300	>300	>300	>300
15 July, 2015	>300, YC	>300, YC	>300	>300	>300	>300
16July, 2015 0m	>300, YC	>300, YC	>300	>300	>300	>300

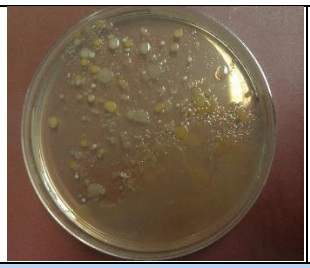
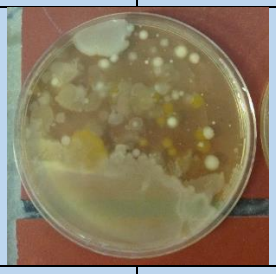
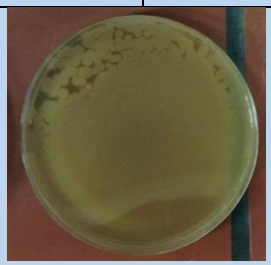
Photo example						
Ultrasonic devices turned on						
16 July, 2015 30m	>300, YC	>300, YC	>300	OT to >300	OT to >300	OT to >300
16 July, 2015 1h	>300, YC	>300, YC	>300	>300	OT to 2	0 to 22
16 July, 2015 3h	>300	>300, YC	0 to >300	0 to >300	0 to OT	0 to 2
Photo example						
17 July, 2015 24h	>300	>300	0 to >300	OT to >300	0 to 22	0 to 2
18 July, 2015 48h	>300, YC	>300, YC	>300	36 to >300	0 to >300	0 to >300
20 July, 2015	>300	>300	OT	OT	0 to OT	0 to OT
Photo example						

Table 4. Bacterial analysis during the first batch experiment (7 July, 2015 – 20 July, 2015): rows with white background represent samples collected before turning on USD while rows with blue background represent samples collected during USD operation. On each date three dilutions (d_0, d_3 and d_5) of each of the 8 samples were incubated at two temperatures (28°C and 36°C) for 48 hours. The presented results are the ranges in which values varied for each sampling date; results are presented in CFU/ml. OT stands for Only Transparent colonies.

In addition to HPC, selective test for TC and *E. coli* was performed. The results of the test performed right before USD activation, 24h and 48h later can be seen in Table 5. While TC is more than 300 CFU/100ml in the period of 16 July, 2015 – 18 July, 2015, *E. coli* concentration was highest 24h hours after USD activation (40CFU/100ml) and equal on day 1 and day 3 of measurements (10CFU/100ml).

	16 July, 2015	17 July, 2015	18 July, 2015
E. coli, CFU/100ml	10	40	10
TC, CFU/100ml	>300	>300	>300

Table 5. Total Coliform and Escherichia coli detection and enumeration test results for test containers during the first batch experiment (16 July, 2015 – 18 July, 2015).

4.1.4 Meeting drinking water standards

Log_{10} reduction was lower than required when calculated both using *E. coli* and HPC as a reference measurement. Out of all the values measured, only nitrate concentration and pH met WHO drinking water standards.

	$C_{untreated\ water}$, CFU/l	$C_{treated\ water}$, CFU/l	Log_{10} reduction	Log_{10} reduction required	Standard
E. coli	10^2	10^2	0	1	< 1CFU/100ml
HPC	$3x10^{10}$	10^6	4.48	4.78	< 500CFU/ml

Table 6. Evaluation of microbial performance of ultrasonication according to WHO (World Health Organization (c), 2011).

	Before USD on	**24h of USD**	**48h of USD**	**1 week of USD**	**Standard**
Cyano Chl a	17.8µg/l	19.2µg/l	20.8µg/l	22.1µg/l	Not present
E. coli	10CFU/100ml	40CFU/100ml	10CFU/100ml	-	< 1CFU/100ml
TC	>300 CFU/100ml	>300 CFU/100ml	>300 CFU/100ml	-	< 1CFU/100ml
HPC	$>300x10^5$CFU/ml	$22x10^5$CFU/ml	$>300x10^5$CFU/ml	10^3CFU/ml	< 500CFU/ml
Nitrate	0.47mg/l	0.45mg/l	0.48mg/l	0.36mg/l	< 10mg/l
pH	8.0	8.1	8.1	8.1	6.5-9.5
Turbidity	25.6FTU	25.9FTU	25.1FTU	25.4FTU	< 1FTU

Table 7. First batch experiment values compliance with WHO drinking water standards. The table contains average values for cyanobacteria Chl a(µg/l), E. coli(CFU/100ml), TC(CFU/100ml), HPC(CFU/ml), nitrate(mg/l), pH and turbidity(FTU) of eight test containers. Values that meet the standard are highlighted with green; values that exceed the standard are highlighted with red.

4.2 Second batch

4.2.1 Physical parameters development

USD with fluctuating frequency of 37-45kHz was used this time. The second batch experiment lasted 9 days; during this time the temperature of water in all the containers was changing in the range of 17-24˚C, average temperature being 19.86°C. Test and control temperature graphs on Figure 11 show similar pattern, however after the activation of USD temperature in test containers is approximately 1˚C higher. The same phenomenon was observed during the first batch experiment which proves that the energy passed on to water through USD and not the position of containers in the greenhouse is the reason for water warm up.

Throughout the second batch experiment dissolved oxygen concentration in control containers almost always had been notably higher than in test containers showing 1-3mg/l differences. A similar scene can be seen on first batch DO graph, however with lower variation between test and control values. The

difference in DO during second batch experiment was the highest after USD activation (for example, 29 July, 2015: test 4.7mg/l, control 8.6mg/l); same as in first batch case, most probable reason is the faster oxygen bubbles removal in the presence of ultrasound waves.

The rapid increase in conductivity and turbidity from 23 July, 2015 to 28 July, 2015 is due to the addition of *CV* culture to the containers on 24 July, 2015. After the adjustment of the culture both values stayed almost constant starting 27 July, 2015 and until the end of the experiment.

Specific conductivity was higher in test containers after the adjustment of culture: approximately 380µS/cm in test containers and 360µS/cm in control containers in the period from 27 July, 2015 until 31 July, 2015.

On the opposite, turbidity values on 28 July, 2015 – 3 August, 2015 were higher in control containers (app. 100FTU test vs. app. 120FTU control). Similar to the first batch case, higher turbidity of control samples might be due to the different containers' sizes given same mixing procedure (test 40l, control 20l).

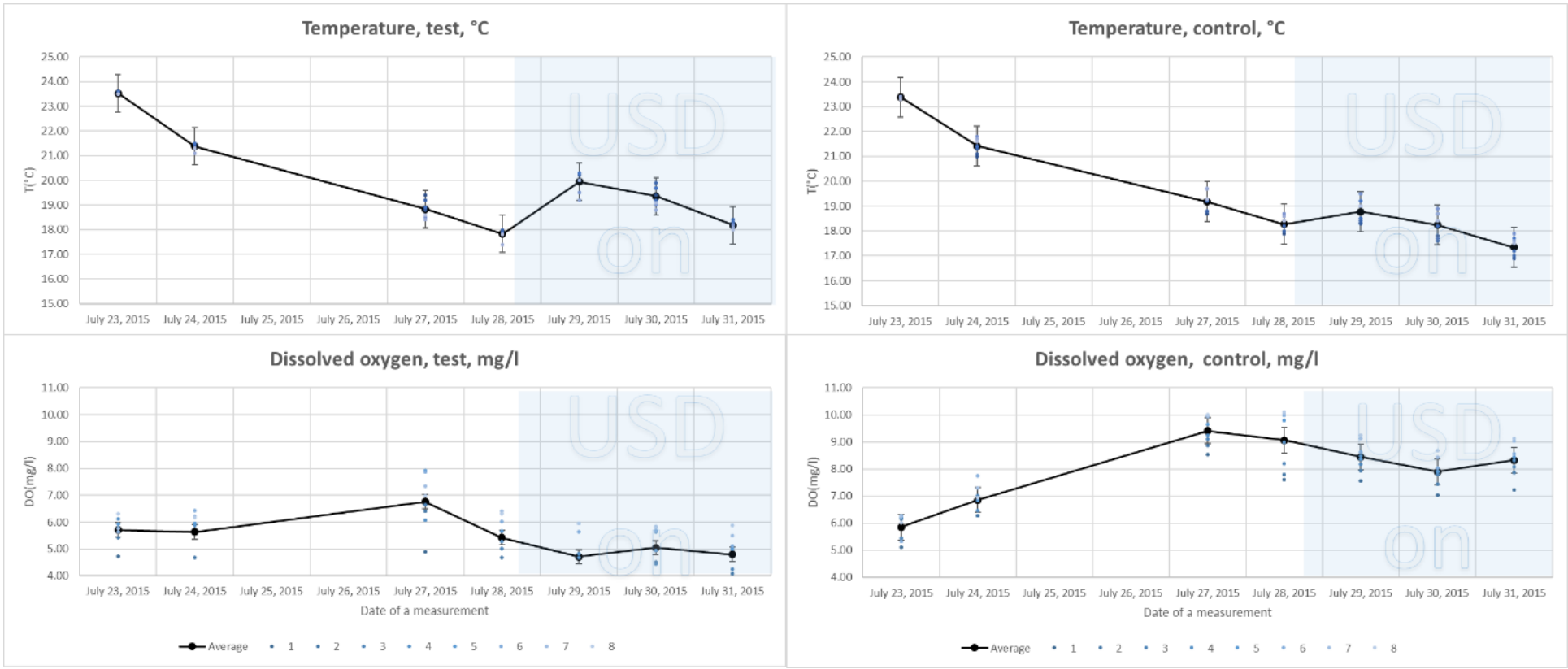


Figure 11. Temperature changes (°C) and Dissolved Oxygen (mg/l) development over time (seven measurements within nine days) in the containers (test containers on the left, control containers on the right) during the second batch experiment. Extra CV culture had been added on 24 July, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 23 July, 2015 until 31 July, 2015 within the second batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

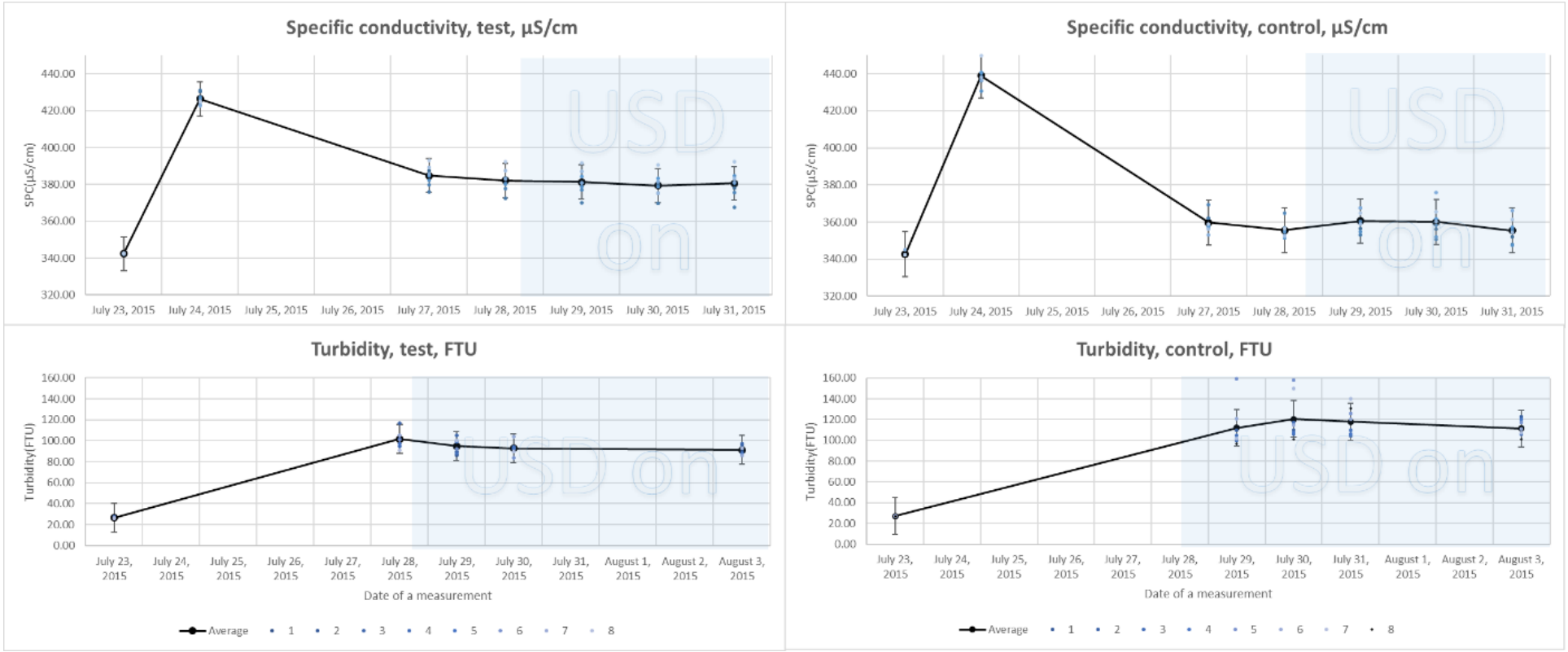


Figure 12. . Specific conductivity (µS/cm) and Turbidity (FTU) development over time (seven measurements within nine days for conductivity, five measurements within twelve days for turbidity) in the containers (test containers on the left, control containers on the right) during the experiment. Extra CV culture had been added on 24 July, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 23 July, 2015 until 31 July, 2015 for SPC and until 3 August, 2015 for turbidity within the second batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

During the first two days of the experiment pH in both groups of containers is approximately the same and is between 9-9.5. However, by 27 July, 2015 the test value drops 0.6 points and control value grows by 0.2 points. pH development trend is the same in the period from 27 July, 2015 until 31 July, 2015, however the value is notably higher in control containers: 8.9 test vs. 9.8 control on 28 July, 2015.

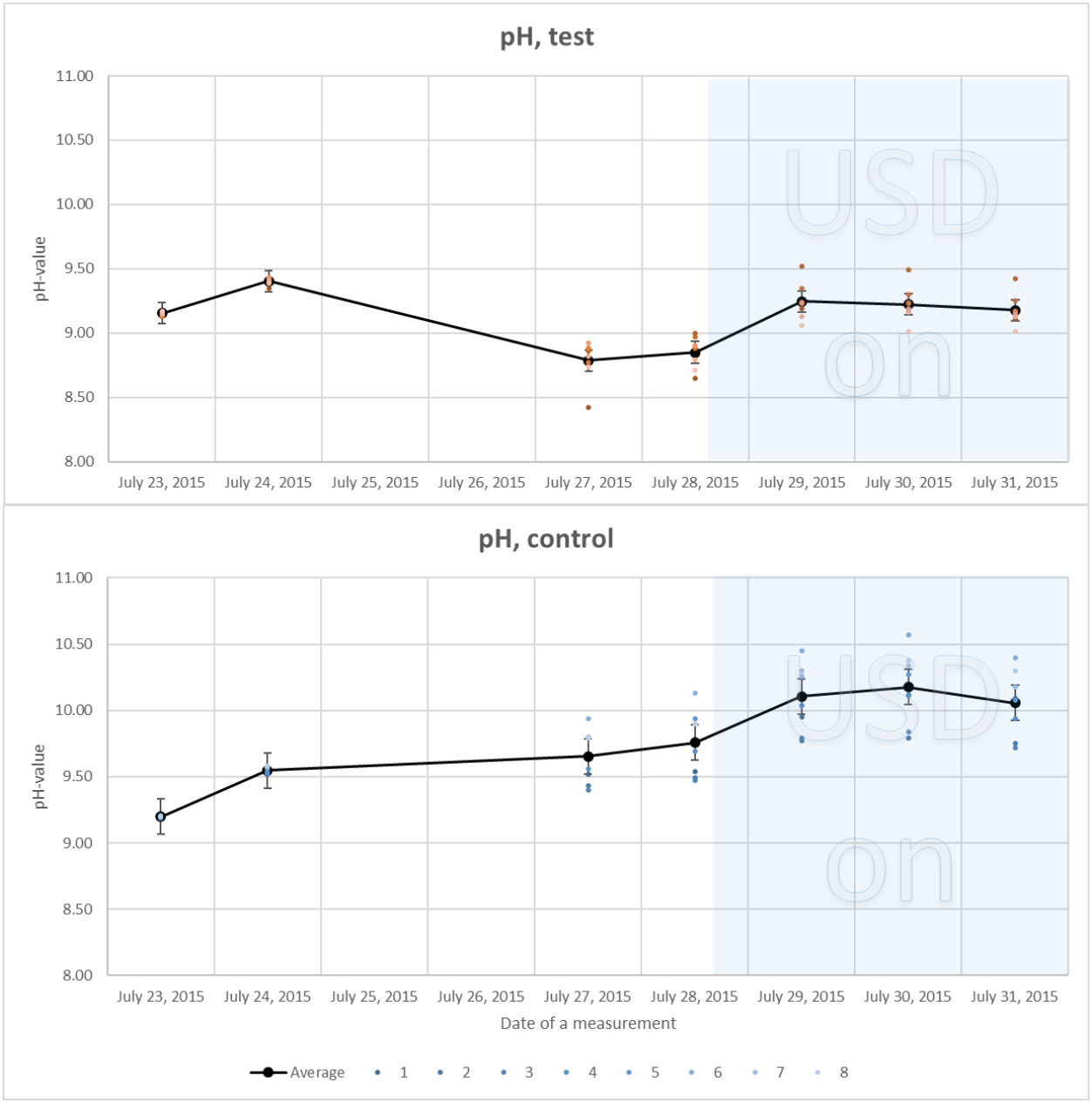


Figure 13. pH development over time (seven measurements within nine days) in the containers (test containers on the top, control containers on the bottom) during the experiment. Extra CV culture had been added on 24 July, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left top) and eight control containers (bottom) in the period from 23 July, 2015 until 31 July, 2015 within the second batch experiment. Error values are calculated using formula for standard error of the mean. Blue field represents the time when USD was operating.

4.2.2 Nutrients development

The rise in nitrate concentration from 0.4mg/l on 23 July, 2015 to 0.8mg/l on 24 July, 2015 and in ammonia from 0.5g/l on 23 July, 2015 to 1.4mg/l on 24 July, 2015 is caused by the addition of *CV* culture on the second day of the experiment. Figure 14 shows that after the weekend, nitrate concentration stays in the range of 0.4-0.5mg/l in test containers and around 0.6mg/l in control containers from 27 July, 2015 until 30 July, 2015. On the last day of the experiment the value drops to 0.4mg/l in control group.

On the other hand, ammonia concentration is slightly higher in test containers. After the adjustment of the culture the value stabilizes and appears to be approximately 1.5mg/l in test containers and 1.25mg/l in control containers in the period of 27 July, 2015 – 31 July, 2015. These numbers are about 3 times higher than in the first batch experiment. The most probable reason for it is that the *CV* culture added on day 2 had high ammonia and nitrate concentrations.

Phosphate value slowly decreases from day 1 to day 6 of the experiment, however rises again rapidly within the first two days of USD activation: from 0.005mg/l on 28 July, 2015 to 0.015mg/l on 30 July, 2015 in test containers and from 0.010mg/l on 28 July, 2015 to 0.020mg/l on 30 July, 2015 in control containers. As the increase in PO_4^- level happened simultaneously in test and control group, it cannot be due to the USD activation. Later phosphate concentration had been decreasing in both test and control containers until it reached 0.005mg/l on 3 August, 2015 in both groups.

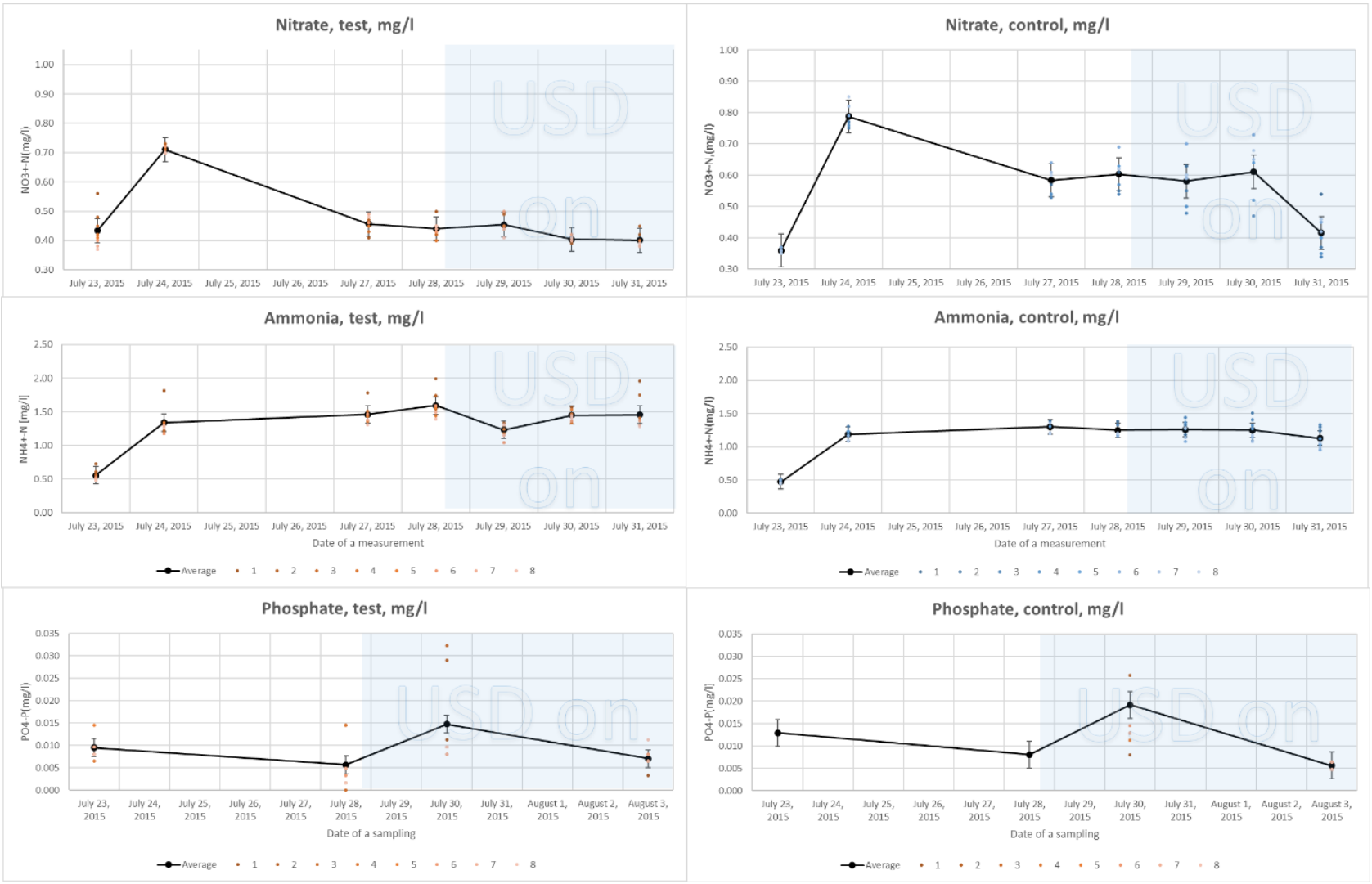


Figure 14. Nitrate (mg/l), Ammonia (mg/l) and Phosphate (mg/l) development over time (seven measurements within nine days for nitrate and ammonia, four measurements within twelve days for phosphate) in the containers (test containers on the left, control containers on the right) during the experiment. Extra CV culture had been added on 24 July, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 23 July, 2015 until 31 July, 2015 for nitrate and ammonia and until 3 August, 2015 for phosphate within the second batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

4.2.3 Population parameters

4.2.3.1 Algae and cyanobacteria growth throughout the experiment

The rapid growth of total algae that can be observed on Figure 15 is caused by the addition of *CV* culture on 24 July, 2015. In this experiment the concentration of algae on the day of USD activation is approximately 3 times higher than during the first batch experiment; also the difference between total algae and cyanobacteria Chl a is much higher this time.

The top graph presented on Figure 16 shows the difference between test and control total algae development. It is clear that test and control groups develop almost identically until 28 July, 2015. However, after the USD activation patterns change drastically: from 28 July, 2015 until 3 August, 2015 total algae Chl a concentration in control containers grows from 470µg/l to 670µg/l while the same value in test containers decreases from 430µg/l to 180 µg/l. Unlike in the first experiment, the difference between test and control total algae Chl a concentrations on the last day of the experiment is extremely prominent.

Cyanobacteria in test and control containers behaved differently starting the first day of the experiment. The cyanobacteria Chl a in test containers increased from 19µg/l on 23 July, 2015 to 34µg/l on 28 July, 2015 while in control containers it stayed at the same level of 19µg/l in the same time slot. 24h after USD activation the concentration in test containers dropped to 24µg/l and stayed the same in control group. From 29 July, 2015 until 3 August, 2015 cyanobacteria Chl a was slowly growing until it reached the value of 35µg/l. The concentration in control group changed from 20µg/l on 29 July, 2015 to 47µg/l on 3 August, 2015.

In the end of the experiment both total algae and cyanobacteria Chl a were lower in test containers; USD activation marked the change of the total algae development pattern in the containers.

Figure 15. Total algae Chl a (µg/l) and Cyanobacteria Chl a (µg/l) development over time (six measurements within twelve days) in the containers (test containers on the top, control containers on the bottom) during the experiment. Extra CV culture had been added on 24 July, 2015 to each container. Dots represent every single value obtained through measurements. Green lines represent mean values of total algae chlorophyll a taken in eight test containers (top graph) and eight control containers (bottom graph) in the period from 23 July, 2015 until 3 August, 2015 within the second batch experiment. Blue lines represent mean values of cyanobacteria chlorophyll a taken in eight test containers (top graph) and eight control containers (bottom graph). Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

Figure 16. Total algae Chl a (µg/l) and Cyanobacteria Chl a (µg/l) development over time (six measurements within twelve days) during the experiment. Extra CV culture had been added on 24 July, 2015 to each container. Dots represent every single value obtained through measurements. Blue lines represent mean values of total algae (top graph) and cyanobacteria (bottom graph) chlorophyll a taken in eight test containers in the period from 23 July, 2015 until 3 August, 2015 within the second batch experiment. Red lines represent mean values of total algae (top graph) and cyanobacteria (bottom graph) chlorophyll a in eight control containers. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

4.2.3.2 *Algae and cyanobacterial growth and turbidity development on the first three days of the device operation*

Total algae Chl a concentration was increasing from 430µg/l at the moment of USD activation to 530µg/l 30 minutes after that as depicted on Figure 17. It was probably caused by the mixing procedure: the measurement 30mins after device activation was the 4th measurement of the day, which means the water in containers by then was mixed four times. As algae tend to get attached to the walls of containers, not all the cells are distributed in the water after one mixing. More frequent mixing could lead to a more even cell distribution.

From 4th until 6th measurement 3h after USD activation the concentration drops to 450µg/l. Hereby, according to AlgaeTorch measurements total algae concentration changed from 430µg/l to 450µg/l within the first 3 hours of the device operation on 28 July, 2015. The value significantly decreased to 360µg/l on the day following device activation, however grew up to 470µg/l on 30 July, 2015.

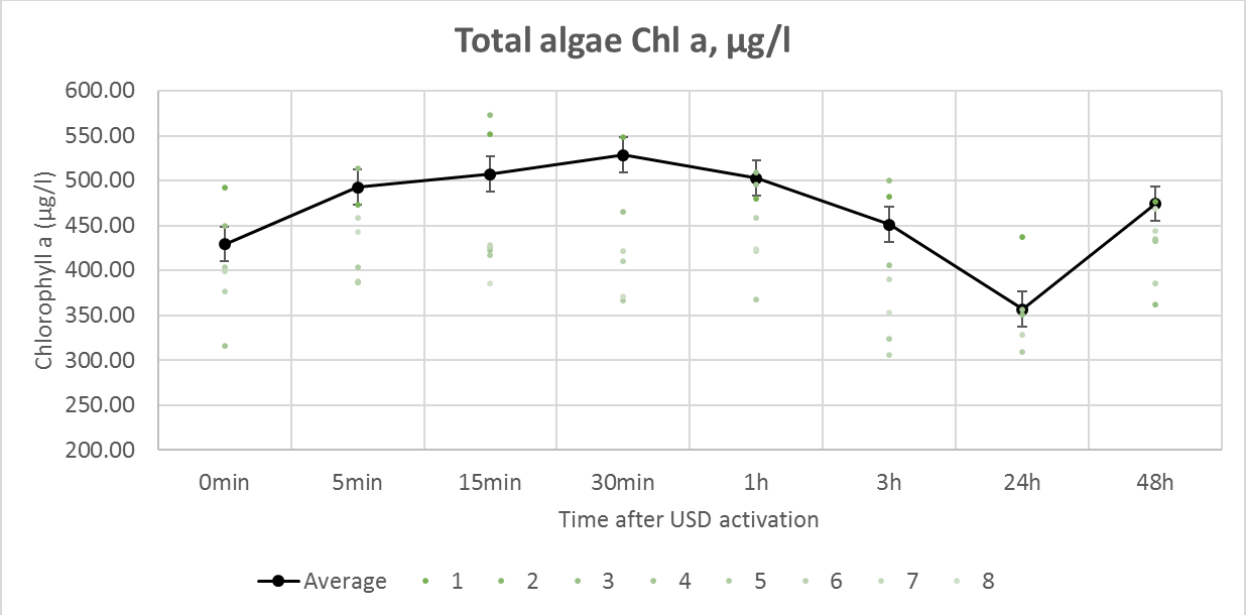


Figure 17. Total algae Chl a (µg/l) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of total algae chlorophyll a taken in eight test containers on 28 July, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 29 July, 2015 and 30 July, 2015 within the second batch experiment.

Figure 18 shows that cyanobacteria Chl a was steadily declining for the first 30 minutes of USD operation and stayed at 27µg/l until later that day. The concentration was 23µg/l and 26µg/l 24 and 48 hours after USD activation respectively.

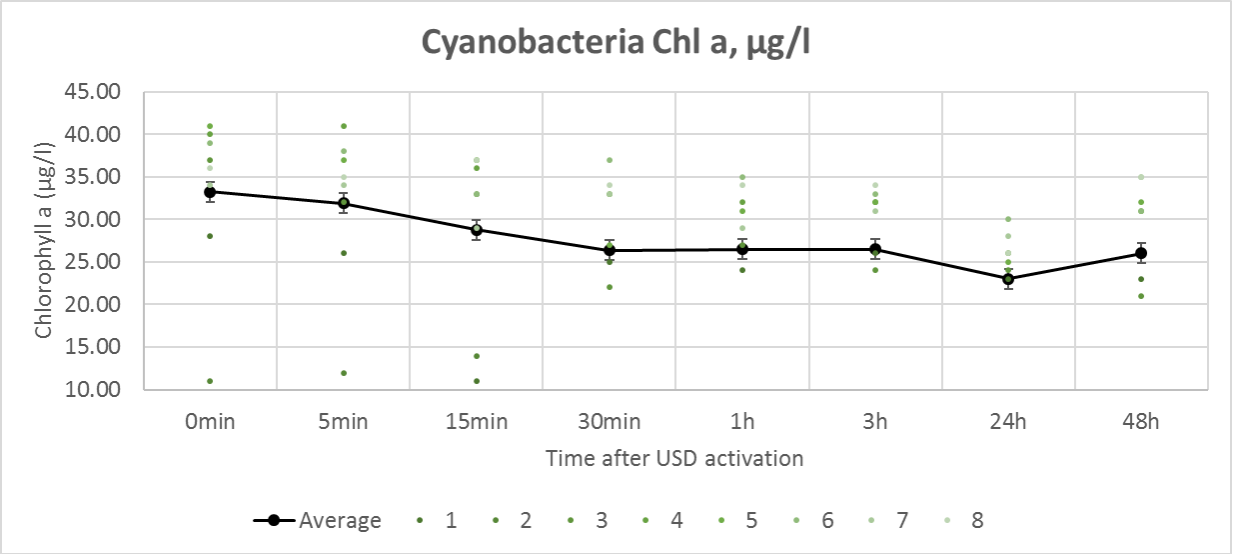


Figure 18. Cyanobacteria Chl a (µg/l) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of cyanobacteria chlorophyll a taken in eight test containers on 28 July, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 29 July, 2015 and 30 July, 2015 within the second batch experiment.

Turbidity fluctuated around 100FTU during the first 3 hours of USD operation. The value declined from 101FTU on the moment of device activation to 95FTU after 24 hours and 92FTU after 48 hours.

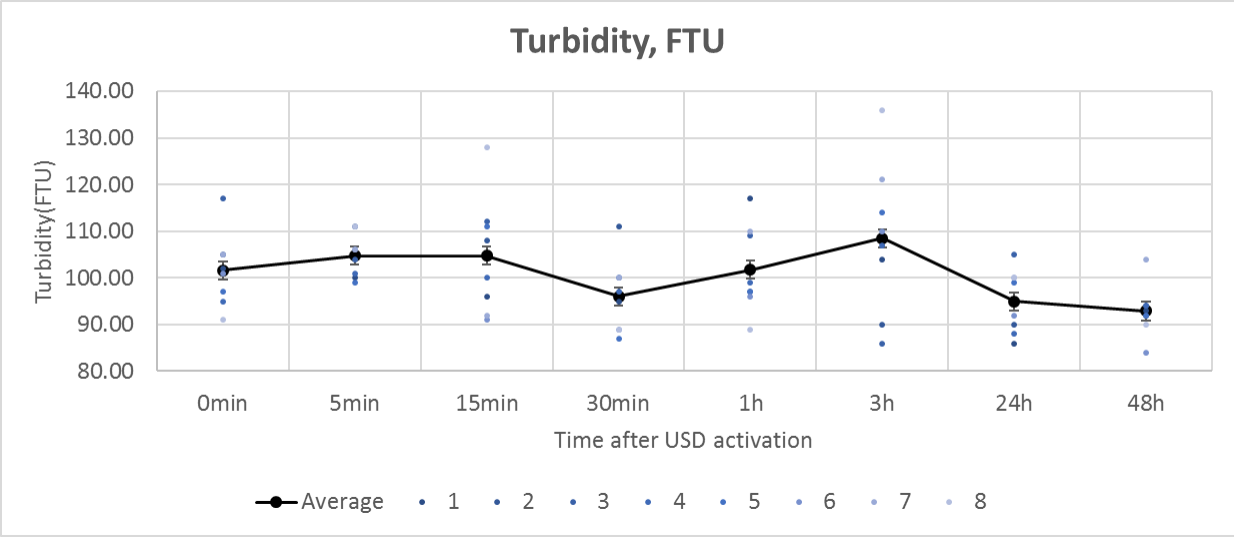
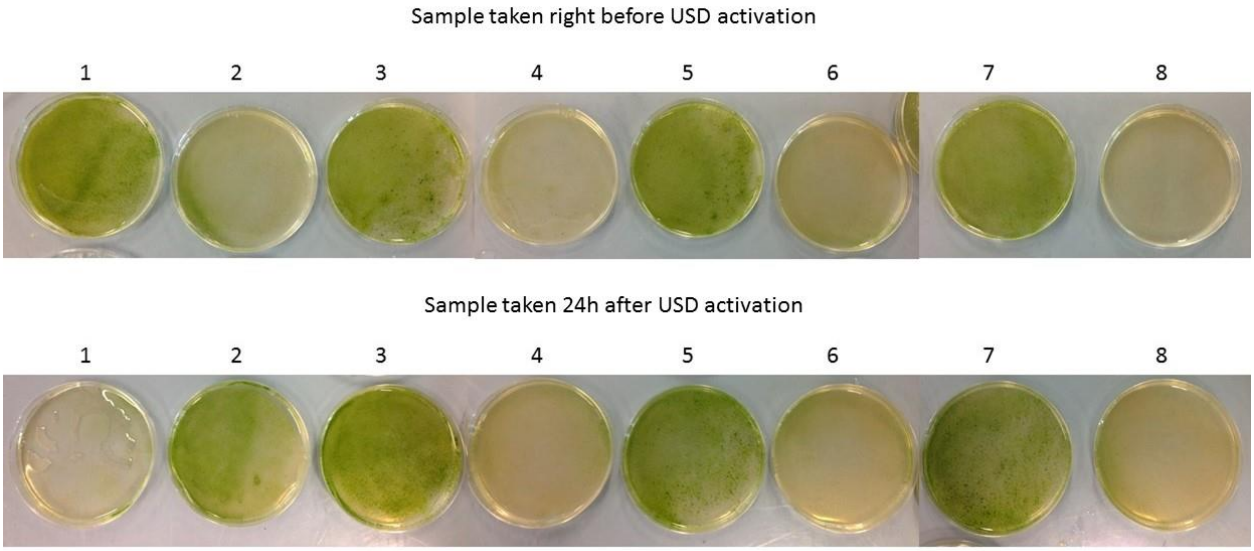


Figure 19. Turbidity (FTU) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of cyanobacteria chlorophyll a taken in eight test containers on 28 July, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 29 July, 2015 and 30 July, 2015 within the second batch experiment.



Picture 12. The results of algal growth test for eight test containers on 28 July, 2015 (top row) before USD activation and on 29 July, 2015 (bottom row) 24h after USD activation performed within the second batch experiment.

It can be seen in Picture 12 that some plates appeared greener than others, for example, plates 3, 5 and 7 for both sampling dates. It happened due to different light availability. However, green colonies were still present on plates 2, 4, 6 and 8 of 28 July, 2015 sample and plates 1, 4, 6 and 8 of 29 July, 2015 sample. Thus, algae growth test was positive for all the samples, and algae were viable both before USD activation and 24h after.

4.2.3.3 Microscopy results

Among all the microalgae and cyanobacteria cells detected were members of the following species: *Asterionella, Anabaena* (potentially toxic)*, Chlorella vulgaris, Coelastrum, Desmodesmus, Fragilaria crotonesis, Microcystis* (potentially toxic)*, Nitzschia, Oscillatoria* (potentially toxic)*, Pediastrum simplex, Pseudanabaena, Scenedesmus and Spirogyra* (Van et al., 2006). Despite the results obtained through AlgaeTorch measurements presented on Figure 17, microscopic cell count proved steady degradation of algae from the very moment of the device activation. Chl a was probably still in the water even though the algal cells were increasingly deteriorating (see Discussion). According to the microscopy analysis the population gradually declined from 457000cells/ml right before USD activation to 187000cells/ml six days later. All in all, the final population size was 59% smaller than the initial population according to the cell count.

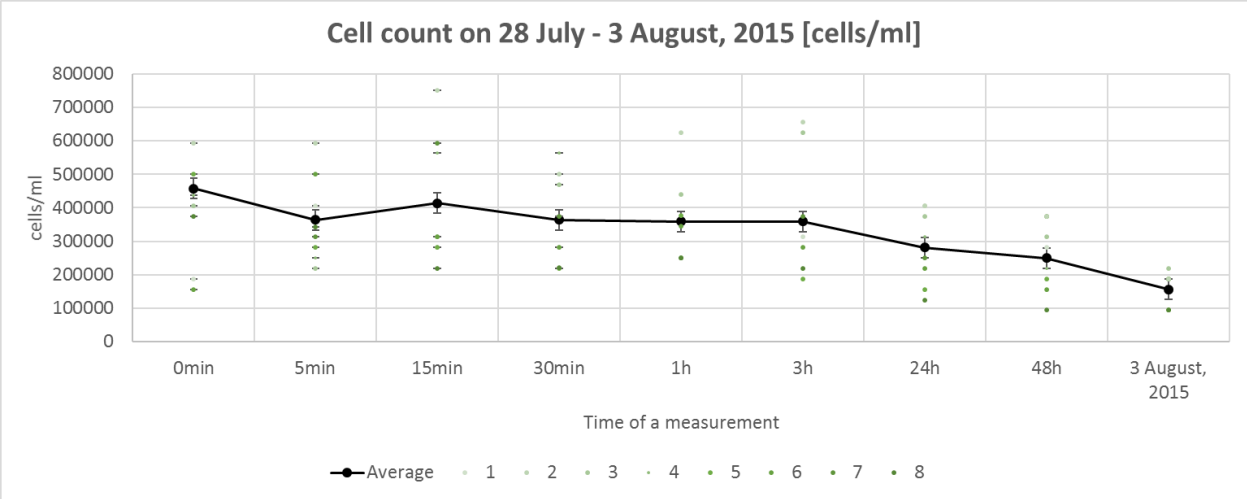


Figure 20. Colony count (cells/ml) development over time on the first three days and on day 7 of the device operation. The meanings of times on x-axe are the following: 0min – 3h are cell counts done on 28 July, 2015 on given times following the activation of USD; 24h = 29 July, 2015; 48h = 30 July, 2015. The line represents the mean values of cell count in eight test containers in the period from 28 July, 2015 until 3 August, 2015 within the second batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16).

However, microscopy analysis has not revealed changes in cells' condition until 24h after the device activation. On the photos presented in Table 8 the damage caused by USD can be seen: almost all the algae species detected underwent changes in either cell wall or organelles' condition. Only *CV* cells did not seem damaged by ultrasonication.

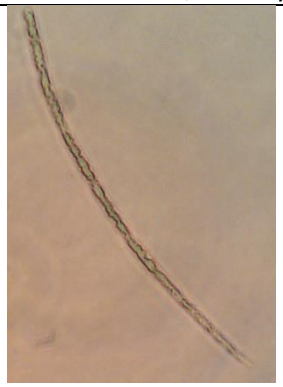
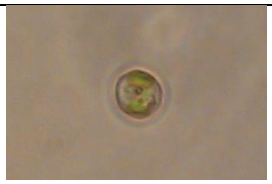
28 July, 2015 USD off	 *Pseudanabaena*, healthy	 *Desmodesmus* and *CV*, healthy	 *Nitzschia*, healthy
29 July, 2015 USD on	 *Pseudanabaena*, damaged	 CV, healthy  *Desmodesmus*, damaged	 *Nitzschia*, damaged

Table 8. The table presents pictures taken during microscopy analysis before and after USD activation within second batch experiment. Each picture is provided along with the name of the specie, health status of the microalgae (Van Vuuren et al., 2006).

4.2.3.4 Bacterial growth

HPC results for the same samples at different temperatures (28 and 36˚C) were similar. The concentration of bacteria before USD activation in this experiment was higher comparing to the first batch being 56x10^{5}CFU/ml comparing to more than 300x10^{5}CFU/ml (Table 9). Also in this batch seeding on nutrient agar of some samples contained pink colonies absent in the previous experiment. HPC showed that bacteria population was declining before USD activation from 56x10^{5}CFU/ml on 27 July, 2015 to 26x10^{5}CFU/ml on 28 July, 2015 before the device activation. Following the activation of USD, the bacterial count results were varying around the same values. Significant decrease in bacterial population could be observed one and two days following the start of ultrasonication: most of the samples showed less than 1x10^{5}CFU/ml on 29 July, 2015 and less than 1x10^{3}CFU/ml at 28˚C on 30 July, 2015. However, on the last day of the second batch experiment bacterial population grew again to more than 100x10^{5}CFU/ml.

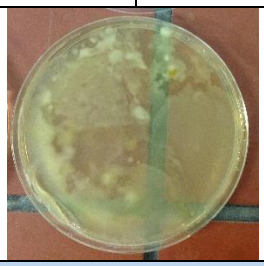
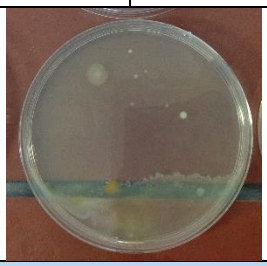
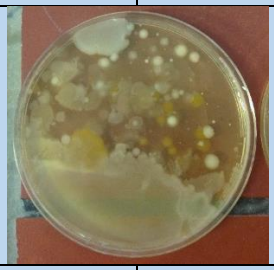
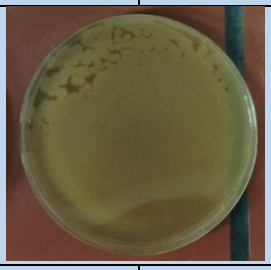
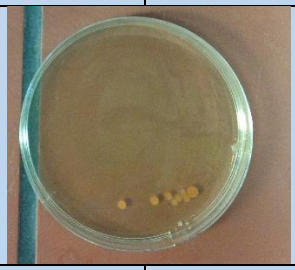
	D_0, CFU/ml		D_3, CFU/ml		D_5, CFU/ml	
	28°C	36°C	28°C	36°C	28°C	36°C
27 July, 2015	>300, YC	>300, YPC	>300, YC	>300, YC	OT	OT to 56
28 July, 2015 0m	>300, YPC	>300, YPC	OT to 38	OT to 50	0 to OT	0 to 26
Photo example						
Ultrasonic devices turned on						
28 July, 2015 30m	>300, YC	>300, YC	176 to >300	136 to >300	0 to 176	0 to 8
28 July, 2015 1h	>300, YC	>300, YC	22 to >300	10 to >300	OT to 16	0 to 22
28 July, 2015 3h	>300, YC	>300, YC	24 to 172	34 to 128	0 to 10	OT to 12
Photo example						
29 July, 2015 24h	>300, YC	>300, YC	OT to >300	OT to 84	0 to OT	0 to 10
30 July, 2015 48h	>300, YC	>300, YC	OT	OT to 34	0 to OT	0 to 4

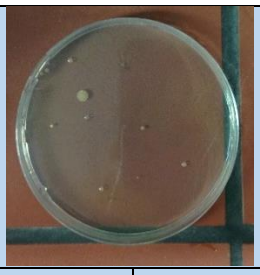
Photo example						
3 August, 2015	>300, YC	>300, YC	OT to 256	OT to 200	OT to 96	OT to 106

Table 9. Bacterial analysis during the second batch experiment (23 July, 2015 – 3 August, 2015): rows with white background represent samples collected before turning on USD while rows with blue background represent samples collected during USD operation. On each date three dilutions (d_0, d_3 and d_5) of each of the 8 samples were incubated at two temperatures (28°C and 36°C) for 48 hours. The presented results are the ranges in which values varied for each sampling date; results are presented in CFU/ml. OT stands for Only Transparent colonies.

Along with HPC, selective tests for TS, *E. coli, S. aureus* and *P. aeruginosa* were performed; their results can be seen in Table 10. *E. coli* population happened to decrease from 160CFU/100ml on 28 July, 2015 to less than 1CFU/100ml on the final day of the experiment. Total coliform however stayed at the same level of more than 300CFU/100ml in the period of 28 July, 2015 – 3 August, 2015. *S. aureus* however detected among colonies HPC showed to be less than 1CFU/ml during selective analysis. *P. aeruginosa* grew from 5 to 7CFU/ml from day 1 to day 2 of USD operation, however later died down and became undetectable in the sample on 3 August, 2015.

	28 July, 2015	29 July, 2015	30 July, 2015	3 August, 2015
E. coli CFU/100ml	160	40	10	>1
Total Coliform CFU/100ml	>300	>300	>300	>300
S. aureus CFU/ml	>1	>1	>1	>1
P. aeruginosa CFU/ml	5	7	3	>1

Table 10. Total Coliform, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa detection and enumeration test results for test containers during the second batch experiment (28 July, 2015 – 3 August, 2015).

4.2.4 Meeting drinking water standards

Log_{10} reduction was lower than required when calculated using HPC as a reference measurement. However, *E. coli* based evaluation showed this treatment to be efficient. Out of all the values measured, only nitrate concentration, pH and *E. coli* concentration met WHO drinking water standards.

	$C_{untreated\ water}$	$C_{treated\ water}$	Log_{10} reduction	Log_{10} reduction required	Standard value
E. coli, CFU/l	$16x10^2$	$1x10^{-3}$	6.2	6.2	< 1CFU/100ml
HPC, CFU/l	$56x10^8$	$106x10^8$	-2.03	4.05	< 500CFU/ml

Table 11. Evaluation of microbial performance of ultrasonication according to WHO (World Health Organization (c), 2011).

	Before USD on	**24h of USD**	**48h of USD**	**6 days of USD**	**Standard**
Cyano Chl a	33.5µg/l	24.0µg/l	23.0µg/l	25.0µg/l	Not present

E. coli	160CFU/100ml	40CFU/100ml	10CFU/100ml	>1CFU/100ml	< 1CFU/100ml
TC	>300CFU/100ml	>300CFU/100ml	>300CFU/100ml	>300CFU/100ml	< 1CFU/100ml
HPC	$26x10^5$CFU/ml	$10x10^5$CFU/ml	$4x10^5$CFU/ml	$106x10^5$CFU/ml	< 500CFU/ml
Nitrate	0.44mg/l	0.44mg/l	0.41mg/l	-	< 10mg/l
pH	8.9	9.2	9.2	-	6.5-9.5
Turbidity	101FTU	92FTU	90FTU	93FTU	< 1FTU

Table 12. Second batch experiment values compliance with WHO drinking water standards. The table contains average values for cyanobacteria Chl a(µg/l), E. coli(CFU/100ml), TC(CFU/100ml), HPC(CFU/ml), nitrate(mg/l), pH and turbidity(FTU) of eight test containers. Values that meet the standard are highlighted with green; values that exceed the standard are highlighted with red.

4.3 Third batch

4.3.3 Physical parameters development

In the third batch experiment USD with fluctuating frequency of 29-37kHz was tested. This experiment was the shortest, lasting only 8 days. Some algal culture separated from the second batch water was added to the containers on the first day. Test and control water temperature patterns look, as expected, the same. Similar to first and second batch tests, temperature in test containers becomes app. 1˚C higher following the activation of USD. Average temperature was 23.89°C.

Dissolved oxygen in this experiment's test containers is much lower than in the previous experiments: it is varying from 2 to 3.5mg/l comparing to ranges of 5-7mg/l and 4-7mg/l during first and second batch respectively (Figures 1 and 11). Same as it happened in the first two batches, DO decreases in test group following the USD activation (1-2mg/l difference in concentration comparing to control samples). The inverse proportion of temperature and DO is prominent from 4 August, 2015 until 7 August, 2015 on bottom graphs presented on Figure 21. The same phenomenon was observed during the first batch experiment. As it was in all the experiments, DO is generally higher in control containers.

Specific conductivity was generally higher in test containers. It was steadily growing throughout the experiment: from 350µS/cm on 3 August, 2015 to 380µS/cm on 10 August, 2015 in test containers, from 350µS/cm on 3 August, 2015 to 355µS/cm on 10 August, 2015 in control containers. Thus, the change of conductivity in the test group is more prominent.

Turbidity in test containers was declining from 5 August, 2015 until 10 August, 2015 (20FTU to 14FTU). During the same period of time turbidity in control containers stayed about the same: app. 19FTU.

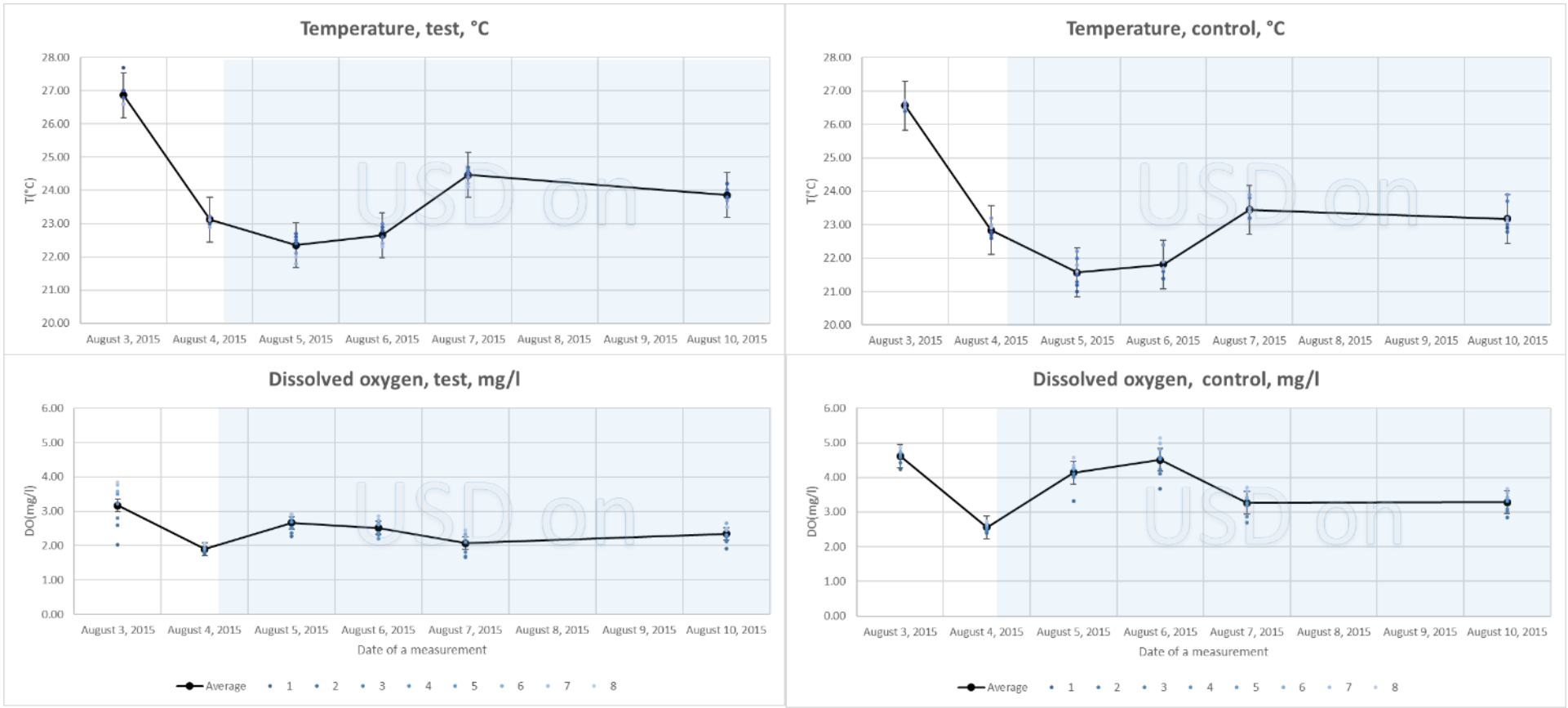


Figure 21. Temperature changes (°C) and Dissolved Oxygen (mg/l) development over time (six measurements within eight days) in the containers (test containers on the left, control containers on the right) during the third batch experiment. Extra CV culture had been added on 3 August, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 3 August, 2015 until 10 August, 2015 within the third batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

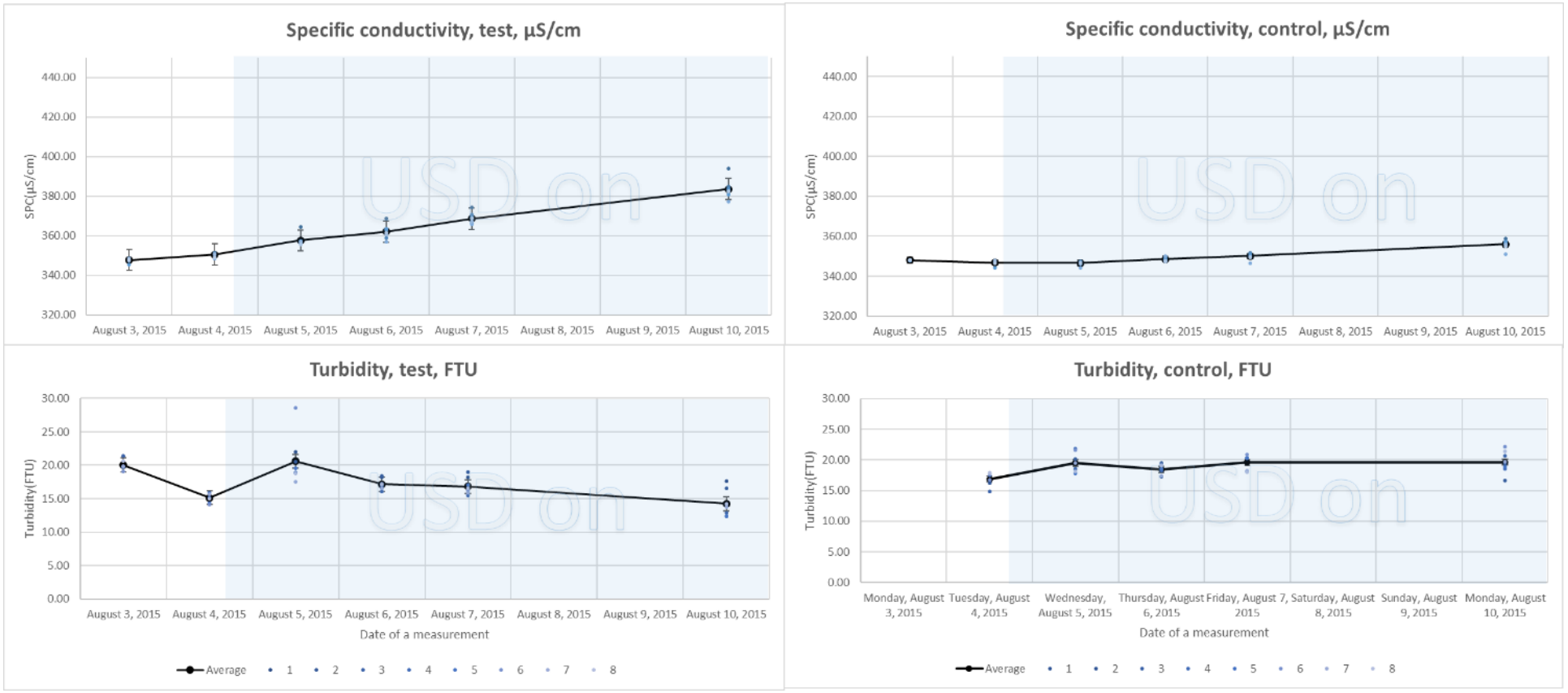


Figure 22. Specific conductivity (µS/cm) and Turbidity (FTU) development over time (six measurements within eight days) in the containers (test containers on the left, control containers on the right) during the experiment. Extra CV culture had been added on 3 August, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 3 August, 2015 until 10 August, 2015 within the third batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

pH level was in the same range as in the previous experiments: alkaline. The graphs on Figure 23 are showing that test and control groups had similar development of pH. However, after the activation of USD pH in test containers was decreasing faster: from 8.8 on 4 August, 2015 to 8.4 on 5 August, 2015 to 8.2 on 10 August, 2015. In the control group the value changed from 9.1 on 4 August, 2015 to 8.8 on 10 August, 2015.

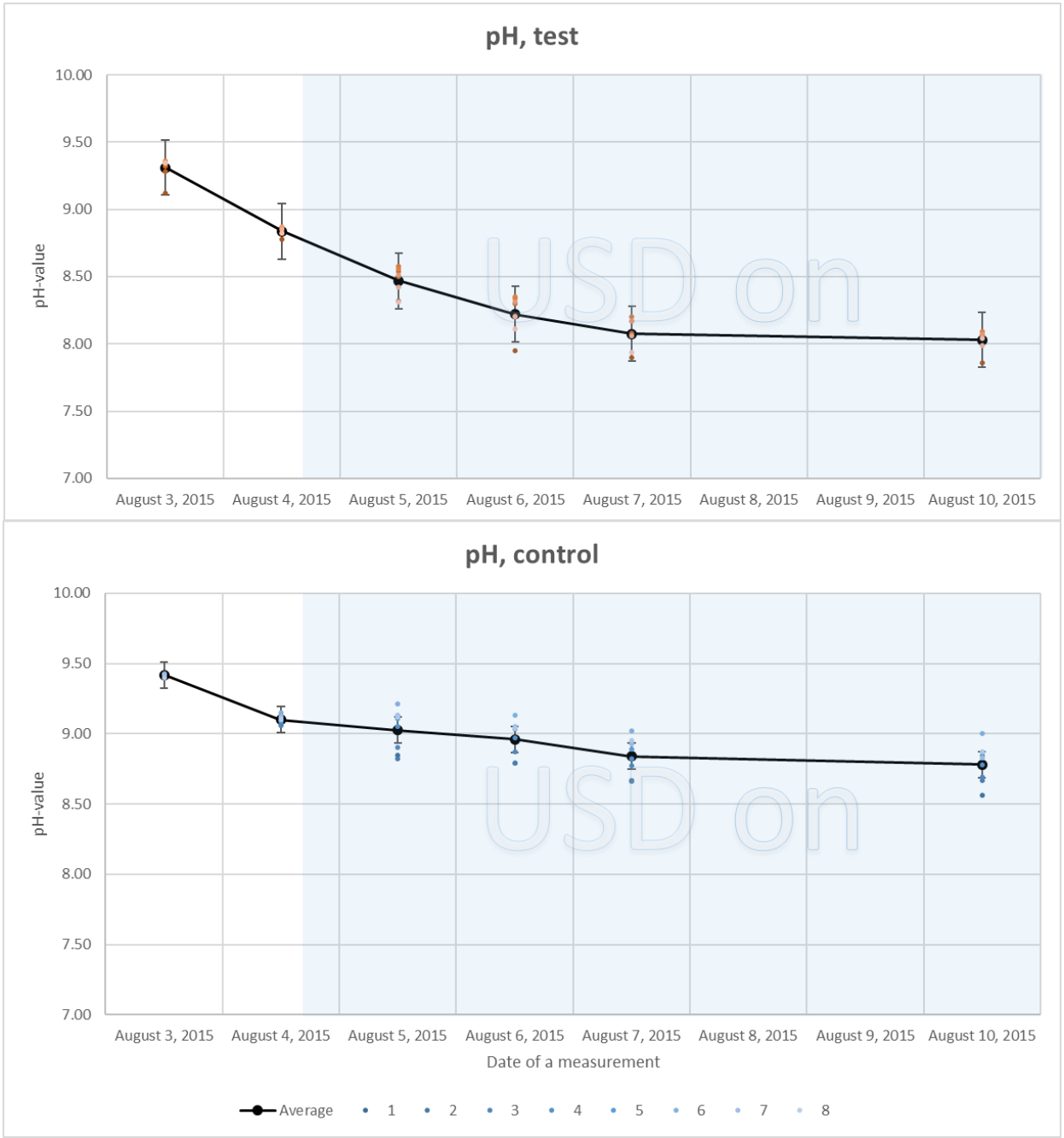


Figure 23. pH development over time (six measurements within eight days) in the containers (test containers on the top, control containers on the bottom) during the experiment. Extra CV culture had been added on 3 August, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left top) and eight control containers (bottom) in the period from 3 August, 2015 until 10 August, 2015 within the third batch experiment. Error values are calculated using formula for standard error of the mean. Blue field represents the time when USD was operating.

4.3.4 Nutrients development

Nitrate concentrations in test and control containers developed in a similar way throughout the experiment. They started at the same values during the first two days (0.44mg/l and 0.36mg/l respectively), however after the USD activation nitrate values in control containers happened to be approximately 0.1mg/l higher: for example, on 6 August, 2015 0.56mg/l test, 0.68mg/l control.

Ammonia concentration in test containers grew from 0.46mg/l on day 1 to 1.31mg/l on 6 August, 2015, and stared declining after that going down to 0.94mg/l on the last day of the experiment. Ammonia concentration in control containers did not show same drastic changes, however grew from 0.43mg/l on day 1 to 0.73mg/l on day 3, declining to 0.59mg/l on the final day of the third batch experiment. Clearly, there was more ammonia in test containers starting 4 August, 2015.

Phosphate concentration in test and control containers was approximately the same on the first day of the experiment: 0.015mg/l and 0.021mg/l respectively. It notably grew in test group being 0.076mg/l on 6 August, 2015 and stayed unchanged in control. On 10 August, 2015 the phosphate concentration was 0.056mg/l in test group and 0.038mg/l in control group.

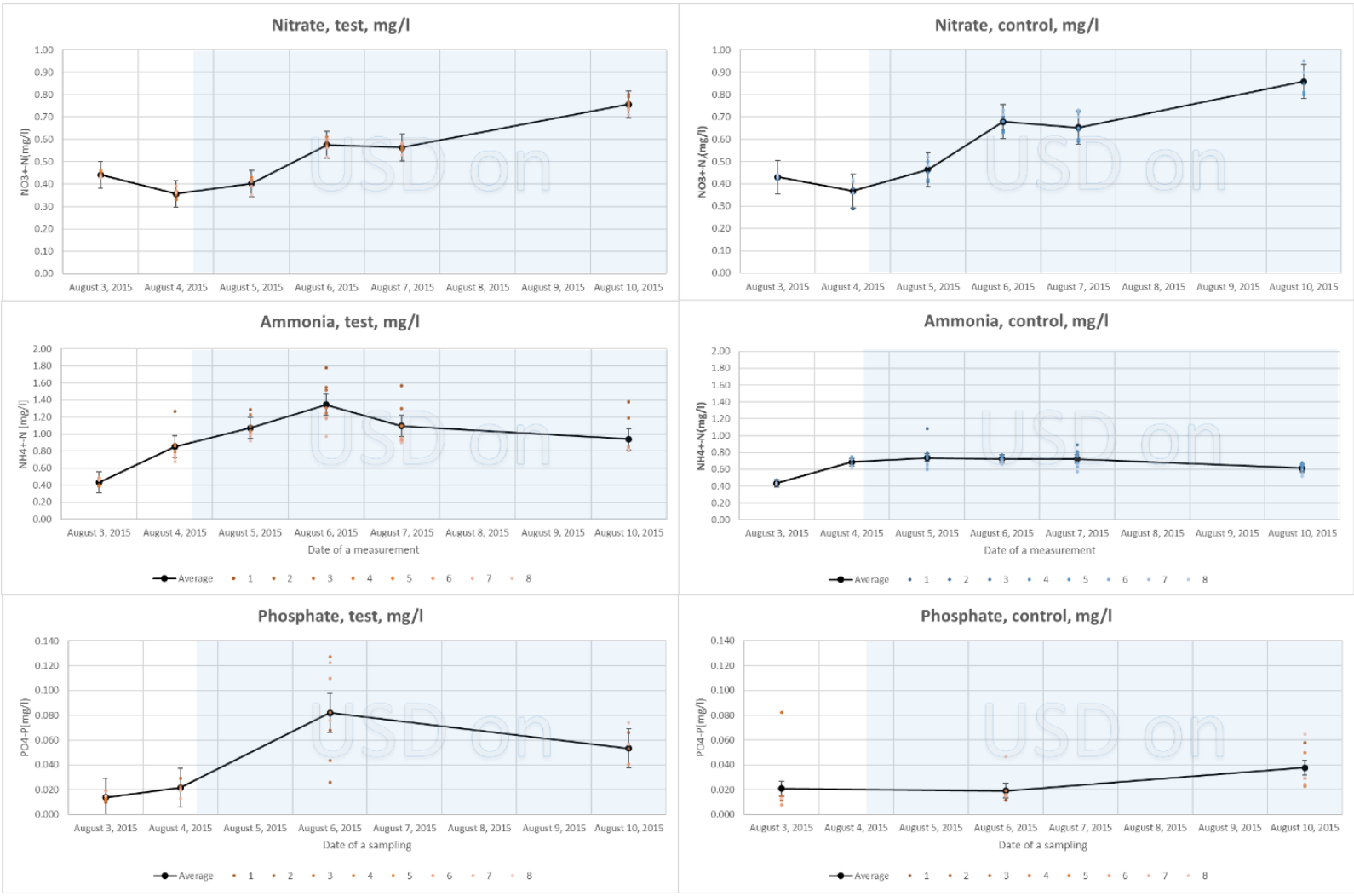


Figure 24. Nitrate (mg/l), Ammonia (mg/l) and Phosphate (mg/l) development over time (six measurements within eight days for nitrate and ammonia, four/three measurements within eight days for phosphate) in the containers (test containers on the left, control containers on the right) during the experiment. Extra CV culture had been added on 3 August, 2015 to each container. Dots represent every single value obtained through measurements. The lines represent mean values taken in eight test containers (graphs on the left side) and eight control containers (right side) in the period from 3 August, 2015 until 10 August, 2015 within the third batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

4.3.3 Population parameters

4.3.3.1 Algae and cyanobacteria growth throughout the experiment

Graphs presented on Figure 25 show almost identical development of cyanobacteria population in test and control groups; the developments of total algae are similar as well. However, Figure 26 gives a more proper comparison of the values.

On the top graph of Figure 26 the development of total algae Chl a within the third batch experiment can be seen. Starting at almost the same value of app. 110µg/l on 4 August, 2015, test and control graphics intersect twice after USD activation. The total algae Chl a concentration declines from 160µg/l on 5 August, 2015 to 80µg/l on 10 August, 2015 while change in control values on the same dates is 150µg/l to 110µg/l. However according to the standard error evaluation the variation between test and control are not significant.

On the other hand, cyanobacteria Chl a on the last day of the experiment is a lot lower in test containers with average values being 41µg/l for control and 28µg/l for test. The concentration was growing in test containers up to 7 August, 2015 and started declining after, while in control containers in never stopped growing however slowly.

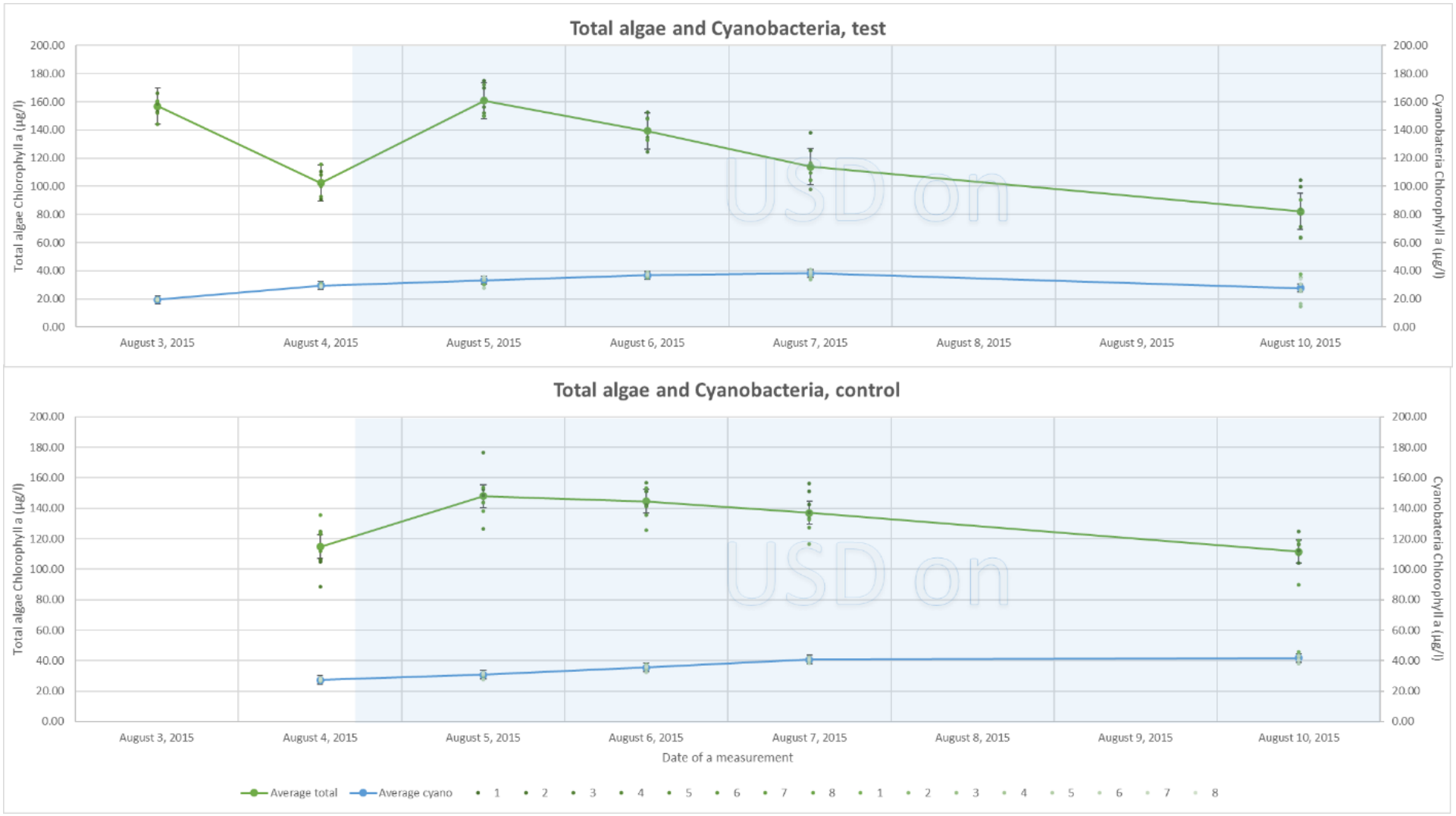


Figure 25. Total algae Chl a (µg/l) and Cyanobacteria Chl a (µg/l) development over time (six measurements within eight days) in the containers (test containers on the top, control containers on the bottom) during the experiment. Extra CV culture had been added on 3 August, 2015 to each container. Dots represent every single value obtained through measurements. Green lines represent mean values of total algae chlorophyll a taken in eight test containers (top graph) and eight control containers (bottom graph) in the period from 3 August, 2015 until 10 August, 2015 within the third batch experiment. Blue lines represent mean values of cyanobacteria chlorophyll a taken in eight test containers (top graph) and eight control containers (bottom graph). Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

Figure 26. Total algae Chl a (µg/l) and Cyanobacteria Chl a (µg/l) development over time (six measurements within eight days) during the experiment. Extra CV culture had been added on 3 August, 2015 to each container. Dots represent every single value obtained through measurements. Blue lines represent mean values of total algae (top graph) and cyanobacteria (bottom graph) chlorophyll a taken in eight test containers in the period from 3 August, 2015 until 10 August, 2015 within the third batch experiment. Red lines represent mean values of total algae (top graph) and cyanobacteria (bottom graph) chlorophyll a in eight control containers. Error values are calculated using formula for standard error of the mean (See p. 16). Blue field represents the time when USD was operating.

4.3.3.2 Algae and cyanobacterial growth and turbidity development on the first three days of the device operation

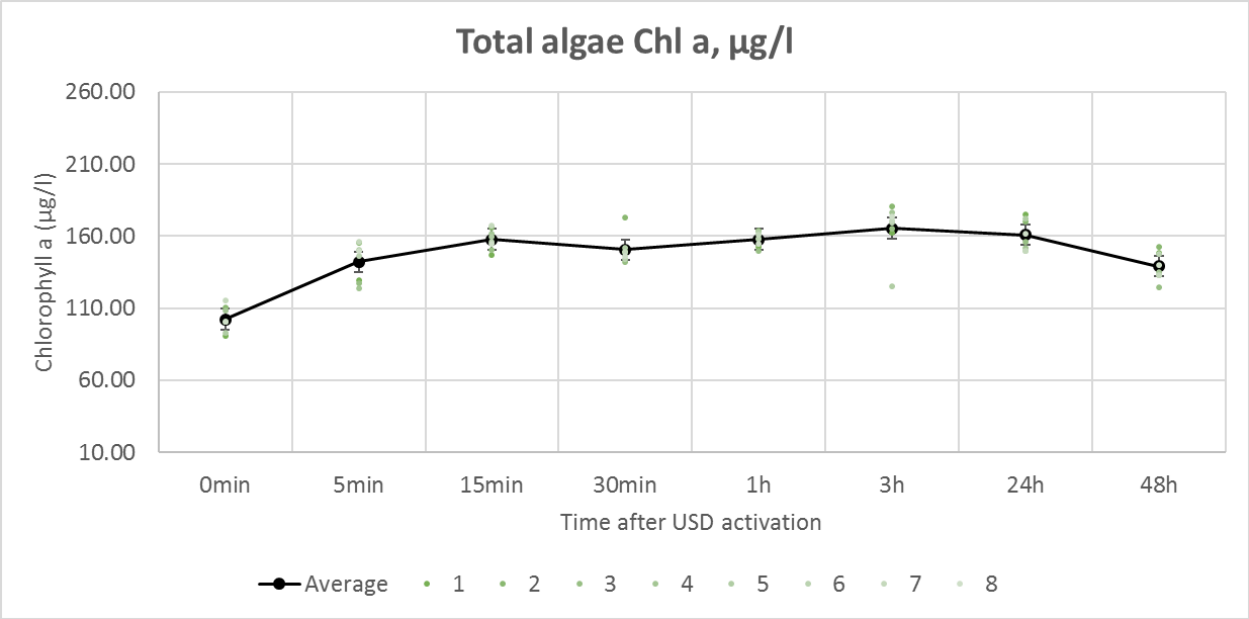


Figure 27. Total algae Chl a (µg/l) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of total algae chlorophyll a taken in eight test containers on 4 August, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 5 August, 2015 and 6 August, 2015 within the third batch experiment

Total algae concentration was growing for the first 15 minutes of the device operation and then was fluctuating around the value of 160µg/l until 5 August, 2015. The value decreased to 140µg/l by 6 August, 2015. Cyanobacteria on the other hand was slightly declining for the first 15 minutes of USD operation, however then it was growing until 6 August, 2015 (37µg/l).

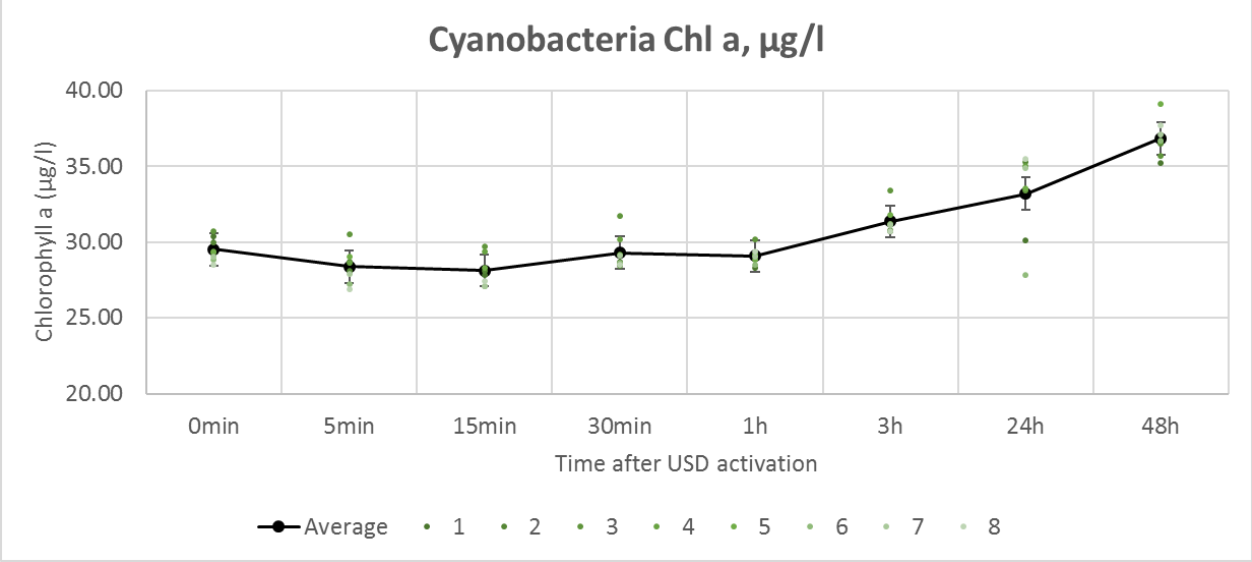


Figure 28. Cyanobacteria Chl a (µg/l) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of cyanobacteria chlorophyll a taken in eight test

containers on 4 August, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 5 August, 2015 and 6 August, 2015 within the third batch experiment.

Turbidity grew within the first 5 minutes following the device activation and later was fluctuating around the value of 20FTU until the decrease to 17FTU on 6 August, 2015. The trend of turbidity mirrors one of total algae.

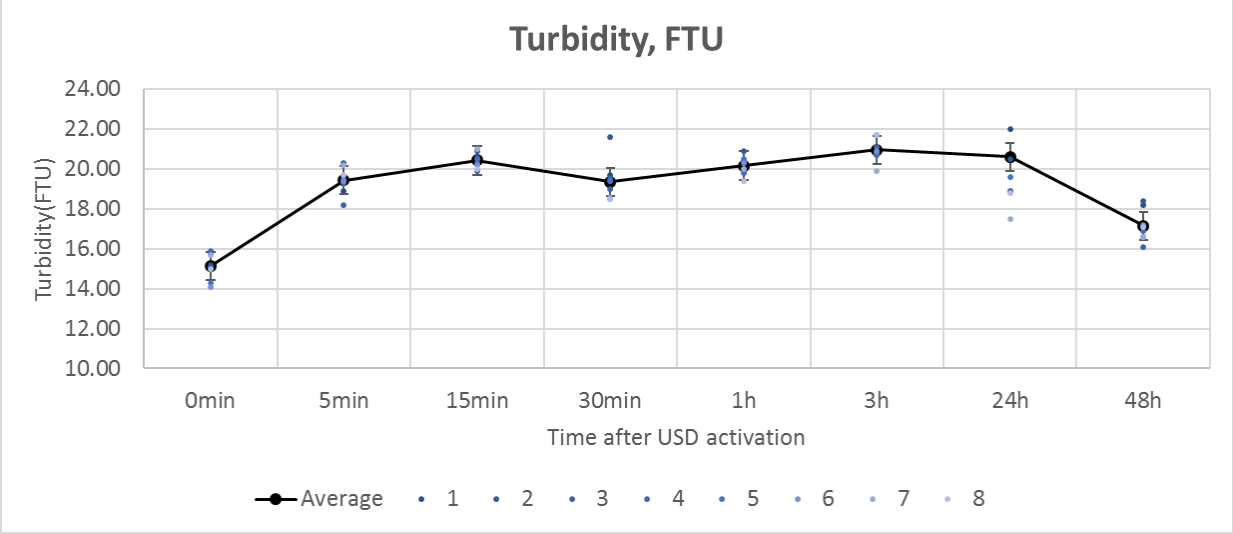
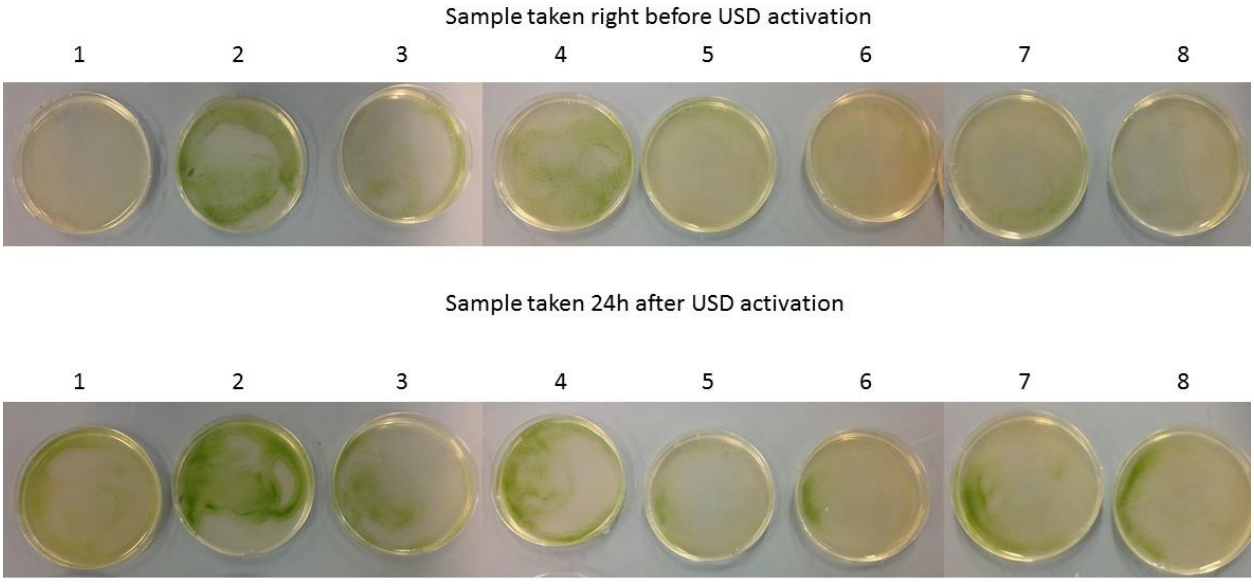


Figure 29. Turbidity (FTU) development over time during the first three days of the experiment. Dots represent every single value obtained through measurements. The line represents mean value of cyanobacteria chlorophyll a taken in eight test containers on 4 August, 2015 0, 5, 15, 30, 60 and 180 minutes after the device activation; on 5 August, 2015 and 6 August, 2015 within the second batch experiment.



Picture 13. The results of algal growth test for eight test containers on 3 August, 2015 (top row) before USD activation and on 4 August, 2015 (bottom row) 24h after USD activation performed within the third batch experiment.

Picture 13 presents the results for algae growth test performed before USD activation and 24h after USD activation during the last experiment. Some plates are greener than the others due to the difference in light availability. Same as in the second batch experiment test, all plates contained green colonies.

Therefore, algae were viable both before and 24h after the device activation. However, it can be seen that photos in Picture 12 show greener plates that photos in Picture 13. It can be explained by higher concentration of algae in the second batch experiment.

4.3.3.3 Microscopy results

Among all the microalgae and cyanobacteria cells detected were members of the following species: *Asterionella, Anabaena* (potentially toxic)*, Chlorella vulgaris, Coelastrum, Desmodesmus, Fragilaria crotonesis, Microcystis* (potentially toxic)*, Nitzschia, Oscillatoria* (potentially toxic)*, Pediastrum simplex, Pseudanabaena, Scenedesmus and Spirogyra* (Van et al., 2006). Similar to the results of AlgaeTorch measurements, cell count showed that the algae population increased from day 1 to day 2 of the device operation and was declining since. The value started at 246000cells/ml at the moment of device activation and appeared to be 180000cells/ml 24 hours after the device activation, 94000cells/ml 48 hours after USD activation and 63000 cells/ml on the last day of the third batch experiment. In general, the population size subsided by 75% according to the microscopic cell count.

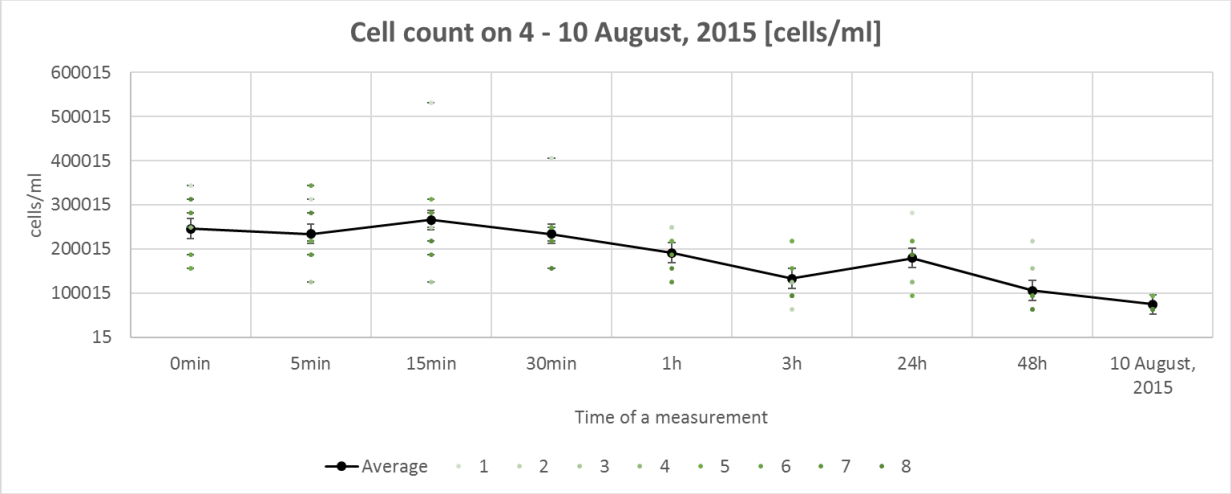


Figure 30. Colony count (cells/ml) development over time on the first three days and on day 7 of the device operation. The meanings of times on x-axe are the following: 0min – 3h are cell counts done on 4 August, 2015 on given times following the activation of USD; 24h = 5 August, 2015; 48h = 6 August, 2015. The line represents the mean values of cell count in eight test containers in the period from 4 August, 2015 until 10 August, 2015 within the third batch experiment. Error values are calculated using formula for standard error of the mean (See p. 16).

Microscopy analysis showed no change to the condition of cells during the first 24 hours of USD operation. However, on 6 August, 2015 a lot of damaged *Desmodesmus* cells were detected along with other green algae species. In contrast, cyanobacteria that were found during all the examinations after USD activation looked healthy (Table 13).

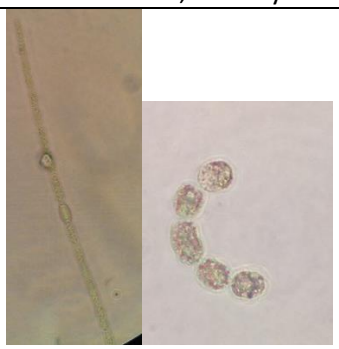
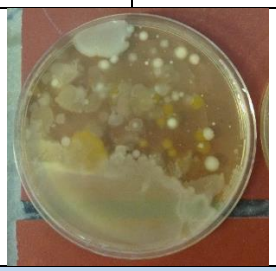
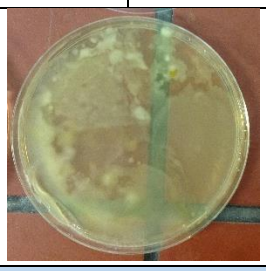
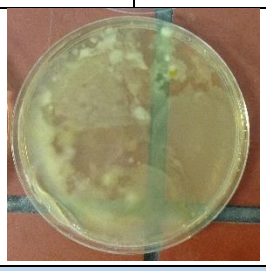
4 August, 2015 USD off	 *Anabaena*, healthy	 *Desmodesmus*, healthy	 *CV*, healthy
6 August, 2015 USD on	 *Anabaena* (left), healthy *Microcystis* (right), healthy	 *Desmodesmus*, damaged	 *CV*, healthy

Table 13. The table presents pictures taken during microscopy analysis before and after USD activation within third batch experiment. Each picture is provided along with the name of the specie, health status of the microalgae (Van Vuuren et al., 2006).

4.3.3.4 Bacterial growth

HPC results for the same samples at different temperatures (28 and 36˚C) were similar. Bacterial concentration before USD activation during the third batch experiment was in between the values of that during the first two experiments: it started with the concentration of $2x10^5$ to $300x10^5$ CFU/ml. This value declined for some samples to less than $300x10^3$CFU/ml after 30 minutes of ultrasonication and stayed around that value for 48 hours following the USD activation. Significant change can be seen on 10 August, 2015 when bacterial growth was shown to be $1x10^3$ to $60x10^3$CFU/ml in all the samples.

	D_0, CFU/ml		D_3, CFU/ml		D_5, CFU/ml	
	28°C	36°C	28°C	36°C	28°C	36°C
4 August, 2015 0m	>300	>300, YC	>300	>300	2 to >300	2 to >300
Photo example						
Ultrasonic devices turned on						
4 August, 2015 30m	>300	>300, YC	>300	OT to >300	OT to 4	0 to 14

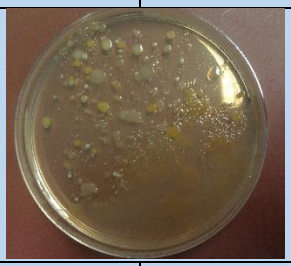
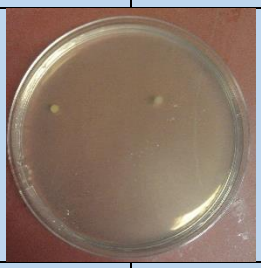
4 August, 2015 1h	>300	>300, YC	>300	OT to >300	OT to >300	0 to >300
4 August, 2015 3h	>300, YC	>300, YC	>300	OT to >300	0 to 2	OT to >300
5 August, 2015 24h	>300, YC	>300, YC	>300	OT to >300	0 to 6	0 to >300
6 August, 2015 48h	>300, YC	>300, YC	OT to >300	OT to 206	0 to 4	0 to 6
Photo example						
10 August, 2015	>300, YC	>300, YC	OT to 60	OT to 10	0	0
Photo example						

Table 14. Bacterial analysis during the third batch experiment (3 August, 2015 – 10 August, 2015): rows with white background represent samples collected before turning on USD while rows with blue background represent samples collected during USD operation. On each date three dilutions (d_0, d_3 and d_5) of each of the 8 samples were incubated at two temperatures (28°C and 36°C) for 48 hours. The presented results are the ranges in which values varied for each sampling date; results are presented in CFU/ml. OT stands for Only Transparent colonies.

Apart from HPC, TC and *E. coli* selective tests were performed on 4, 5 and 6 August for both test and control groups. As it can be seen in Table 15, *E. coli* population was declining until it became undetectable in the samples on 6 August, 2015. As this was a trend in both test and control groups, it seems that ultrasonication was not the reason for the *E. coli* dying out. As well as in the previous experiments, TC concentration did not change.

	4 August, 2015	5 August, 2015	6 August, 2015
E. coli, test, CFU/100ml	40	10	>1
E. coli, control, CFU/100ml	50	30	>1
Total Coliform, test, CFU/100ml	>300	>300	>300
Total Coliform, control, CFU/100ml	>300	>300	>300

Table 15. Total Coliform and Escherichia coli detection and enumeration test results for test (blue background) and control (white background) containers during the third batch experiment (4 August, 2015 – 6 August, 2015).

4.3.4 Meeting drinking water standards

Log_{10} reduction was lower than required when calculated using HPC as a reference measurement. However, *E. coli* based evaluation showed this treatment to be efficient. Out of all the values measured, only nitrate concentration, pH and *E. coli* concentration met WHO drinking water standards.

	$C_{untreated\ water}$	$C_{treated\ water}$	Log_{10} reduction	Log_{10} reduction required	Standard value
E. coli, CFU/l	$4x10^{2}$	$1x10^{-3}$	5.6	5.6	< 1CFU/100ml
HPC, CFU/l	$3x10^{10}$	$6x10^{7}$	2.70	4.79	< 500CFU/ml

Table 16. Evaluation of microbial performance of ultrasonication according to WHO (World Health Organization (c), 2011).

	Before USD on	**24h of USD**	**48h of USD**	**6 days of USD**	**Standard**
Cyano Chl a	28.5µg/l	32.0µg/l	36.5µg/l	26.4µg/l	Not present
E. coli	40CFU/100ml	10CFU/100ml	>1CFU/100ml	-	< 1CFU/100ml
TC	>300 CFU/100ml	>300 CFU/100ml	>300 CFU/100ml	-	< 1CFU/100ml
HPC	$>300x10^{5}$CFU/ml	$>300x10^{5}$CFU/ml	$6x10^{5}$CFU/ml	$60x10^{3}$CFU/ml	< 500CFU/ml
Nitrate	0.36mg/l	0.49mg/l	0.56mg/l	0.73mg/l	< 10mg/l
pH	8.8	8.4	8.2	8.0	6.5-9.5
Turbidity	15FTU	20.5FTU	17.1FTU	14.2FTU	< 1FTU

Table 17. Third batch experiment values compliance with WHO drinking water standards. The table contains average values for cyanobacteria Chl a(µg/l), E. coli(CFU/100ml), TC(CFU/100ml), HPC(CFU/ml), nitrate(mg/l), pH and turbidity(FTU) of eight test containers. Values that meet the standard are highlighted with green; values that exceed the standard are highlighted with red.

5 DISCUSSION

Temperature values in all three experiments were within the same range of 17-27˚C; however, average temperatures of test waters for each experiment were as follows: t_{av1}=21.14˚C, t_{av2}=19.86 ˚C, t_{av3}=23.89 ˚C. Thus, temperature was most favorable for algal and bacterial growth during the third batch experiment and least favorable during the second. Temperatures in test containers were always slightly higher than in control containers; it probably occurred due to the fact that test containers had volume of water twice higher than controls, and therefore water was cooling down slower in test containers.

Dissolved oxygen values were decreasing in test containers of all three experiments after the beginning of ultrasonication. It was concluded that under the effect of ultrasound, air bubbles present in water connect and form bigger volumes of air. Bubbles of bigger volume tend to approach water surface faster and leave the liquid. Thus, oxygen removal from water is faster when USD is on.

Specific conductivity showed similar development during first and third experiments; it was steadily growing in both experiments. However, in the second experiment the picture was disrupted by the addition of extra algae culture on the second day. In all three experiments conductivity was a little higher in test containers. As conductivity is directly dependent on temperature, warmer water in test containers is the reason for this occurrence.

Turbidity developed in different ways in the experiments. However, there was one thing in common: turbidity was almost always higher in control containers than in test containers. The reason for that should be the mixing procedure. Even though test containers had a volume of 44l versus 22l in control containers, the same mixing procedure lasting 30 seconds was implemented before the measurements. Thus, the particles in control containers were more evenly distributed. It is also confirmed by the first USD operation day measurements: turbidity is growing for the first fifteen minutes of USD operation in second and third experiments. As algae, which is the main contributor to turbidity value, cannot multiply this fast, the reason for growing turbidity is most probably a more thorough mixing.

Water pH was basic in all three batches. The water used for testing was obtained from a shallow lake in July-August, the time of summer stratification when upper water layer has a higher pH than the bottom layer, thus exceeding the pH of water in the mixing period. pH in control containers after two days of measurements was always rising higher than that of test batch. It could be linked to the decrease of algae population in test containers due to the USD operation; however, the pH change had been beginning before the device activation.

Nutrients concentrations were always sufficient to ensure algal growth. However, it should be noted that phosphate values in all three cases were lower than would be expected for a eutrophied lake (0.005-0.015mg/l on the first measurement after water delivery).

USDs with frequencies of 40kHz, 37-45kHz and 29-37kHz and power output 8W were used in tanks containing 40l of tested water. Thus, the power density is estimated 0.2W/l.

Growth of both algae and cyanobacteria in test containers exceeded the growth in control containers during the first batch experiment. Moreover, algal and cyanobacterial concentrations in test containers

were not decreasing after USD activation, while control values showed slight decrease between the USD activation day and the last day of the experiment. The microscopy analysis showed that all algal cells detected except for *Desmodesmus* were damaged before USD activation. In the sample taken 24 hours after the device activation *Desmodesmus* cells were damaged as well. However, both 24 and 48 hours after USD activation some healthy cells of *Pseudanabaena, Nitzschia* and *Desmodesmus* were found. From all the data obtained it can be concluded that the ultrasonic device with frequency 40kHz and output power of 8W did not show satisfactory performance and can be marked as ineffective in algae and cyanobacteria growth control.

Within the second batch experiment total algae concentration in test containers showed clear decline after six days of ultrasonication, while control group was multiplying for the first two days of USD operation and stayed at the same concentration later. Cyanobacterial Chl a concentration in test containers was the same on first and last days of ultrasonication, while in control group it grew more than twice. Microscopic cell count showed the decline of cells concentration during the first six days of USD operation from 457000cells/ml right before USD activation to 187000cells/ml. Microscopy analysis showed the culture to be healthy before the device activation; 24 hours into ultrasonic treatment damaged members of all the detected algae species were found, with the exception of *Chlorella vulgaris.* Cells with both disrupted and whole cell wall were detected. Everything said above indicates the effectiveness of ultrasonic treatment with frequency 37-45kHz and output power 8W in algae and cyanobacteria inactivation; however, treatment needs to be 24 hours or longer.

Both total algae and cyanobacteria Chl a concentrations in the end of third batch experiment were lower in test containers. However, the difference between algae content in test and control groups is not very big; also the difference in total algae Chl a between day 3 and day 6 in both groups is approximately the same. On the other hand, cell count shows a clear decline of algae concentration throughout the ultrasonication. Cyanobacteria in test and control batches showed a similar development up to day 3 of ultrasonication, and after that cyanobacteria concentration in test containers rapidly decreased by day 6. Microscopy cell count showed a steady decline in the number of algal cells from 246000cells/ml at the moment of the device activation to 63000 cells/ml on day 6 of ultrasonication. Microscopy analysis showed no damage to cyanobacteria 48 hours after USD activation, however, many green algal cells were damaged. Some sells had only damaged organelles, while other had disrupted cell wall. Again, all the *CV* cells detected were healthy before and after sonication. There are no microscopy data available for later dates of the experiment. The results of microscopy analysis comply with AlgaeTorch measurements. Thus, the ultrasonic device with frequency 29-37kHz and output power 8W is found to be effective for cyanobacteria given a long radiation time (6 days +). It is also potentially effective for the reduction of green algae population.

USD with frequency 37-45kHz is a confident leader among the tested devices. It was highly effective in elimination of green algae and suppressed the growth of cyanobacteria. 29-37kHz USD is coming up next, showing itself effective in cyanobacteria elimination given radiation time 6 days and longer; however, its ability to reduce green algae population is doubtful. Ultrasonication at 40kHz frequency showed itself ineffective in both green algae and cyanobacteria inactivation. The summary of these results can be seen in Table 19.

It should be noted that total algae and cyanobacteria population development graphs in this experiment were built based on AlgaeTorch measurements. According to bbe Moldaenke GmbH, “AlgaeTorch uses

in vivo fluorescence of algae cells: the algae pigments are selectively excited by colored LEDs and emit red fluorescence light as a natural phenomenon at high sensitivity; the intensity of the chlorophyll fluorescence is used to calculate the different algae as chlorophyll-a" (bbe Moldaenke GmbH (a), 2015). However, the presence of chlorophyll in water does not mean all of it is inside healthy algae cells. It can be released from a damaged cell, or be contained within a dead cell. Dead cells cannot multiply, however, if they still contain Chl a, they will be counted by the device. As chlorophyll takes time to decay, AlgaeTorch will be showing higher values when doing measurements in water with dying culture until Chl a is decayed. The average conversion factor used by the producing company is 1000000 cells/µg Chl a (bbe Moldaenke GmbH, 2014). Below a table presenting the comparison of cell count, calculated Chl a/l and measured with AlgaeTorch Chl a/ml values can be found. It can be seen that during the second batch experiment measured Chl a is much higher than calculated 24 and 48 hours after USD activation, however, it approaches the calculated value closely by day 6. If we look at the third batch values, cell count shows clear population decline, while AlgaeTorch show high variability. Although both methods are not completely accurate, it should be noted that in this experiment the use of microscopy in population control rather than AlgaeTorch would be favorable.

	Cell Count, cells/ml	**Calculated Chl a concentration*, µg/l**		**Measured Chl a concentration, µg/l**
Second batch experiment (37-45kHz)				
28 July, 2015 (USD on)	457000	457	>	421
29 July, 2015	281000	281	<	363
30 July, 2015	250000	250	<	469
3 August, 2015	187000	187	<	203
Third batch experiment (29-37kHz)				
4 August, 2015 (USD on)	246000	246	>	105
5 August, 2015	188000	188	>	161
6 August, 2015	105000	105	<	136
10 August, 2015	62500	62.5	<	84

*Table 18. The comparison of cell count (cells/ml), calculated Chl a (µg/l) and measured with AlgaeTorch Chl a (µg/l) values. *The calculation is based on bbe Moldaenke GmbH suggested equation: 1 µg Chl a =1000000 cells.*

Data obtained using AlgaeTorch within the first USD operation day procedure was not useful in terms of determining population development: chlorophyll a concentration and turbidity did not show a drastic change during the first 3 hours of ultrasonication. However, cell count showed the decline from 457000cells/ml to 356000cells/ml within the first three hours of USD operation in second batch experiment, and from 250000cells/ml to 125000cells/ml in the third.

Based on the experiment results presented in Figures 7, 16, 26 and Table 18, it can be assumed that Chl a decay takes approximately 1 to 6 days after the cell inactivation. Unfortunately, no research on arbitrary chlorophyll decay was found to support the theory.

Pernet and Tremblay, (2003), used ultrasonication as a tool for lipid extraction through the destruction of diatom *Chetoceros gracilis* cell wall. One of the treatments tested was ultrasonication of samples three times for 10 minutes in 1.5ml dichlormethane/methanol with power output of 50W (33333W/l), frequency was not mentioned. The result must have been the destruction of cells' organelles and cell wall followed by the release of needed substances to the solution. The treatment expectedly appeared to be effective given a very high power density. Similar ultrasonic cell disruption technique is described

in the paper of Furuki et al., (2003): *Spirulina platensis* was suspended in buffer and after undergoing a number of centrifugations and dilutions it was exposed to ultrasonication with power input 80W (80000W/l) and frequency 20, 28 or 40kHz. The study discovered that the purity of the desired substance was decreasing with the time of irradiation due to cell disruption to an excess extent when divided particles are too fine to separate. Thus, treatment with these parameters for less than 1 minute is enough for the destruction of the chosen cyanobacteria cell wall dispuption.

LaLiberte and Haber, (2014), imply that the main mechanism of ultrasoinic cyanobacteria inactivation is a rupture of gas vacuoles that control cyanobacterial buoyancy. They also say that some cyanobacteria have weaker gas vacuoles than the others, therefore their cell inactivation requires different power input. It was found that at 20kHz with power inputs from 43 to 320W/l sonication caused increase in extracellular cyanotoxins; the concentration depended on power intencity and exposure time. Some field investigations of ultrasound applications revised in the work showed that mixing and flushing implementation along with ultrasonication helps to prevent sinked cyanobacteria's vacuole regeneration. It was also mentioned that treatment in ponds has to be continuous, otherwise algae/cyanobacteria quickly regrow. Moreover, the population of species more resistant to ultrasound treatment prevails, and therefore further ultrasonication shows poorer results.

The data obtained within this research together with information obtained through literature lead to a conclusion that the suggested treatment can be effective for algal and cyanobacteria inactivation given high power density (30000W/l and higher) and/or long enough irradiation time (24 hours and longer). The same parameters however provide a risk of cyanotoxins release: the higher the power and the longer the time, the higher the risk. It is mentioned in the work of LaLiberte and Haber, (2014), that at certain conditions cyanobacteria with destroyed gas vacuoles can regrow them and reinitiate multiplication. Therefore, given that the used water containers are quite shallow and the light could reach the bottom, it seems possible that in this experiment cyanobacterial cells even with ruptured gas vacuoles could still stay viable.

HPC showed drastic decrease in bacteria concentration between day 1 and day 4 of ultrasonication: it reduced from more than $300x10^5$ CFU/ml to less than 10^3 CFU/ml. The decrease was gradual, and the effect could already be seen 30 minutes after device activation. However, TC and *E. coli* selective analysis showed that these bacteria had no response to ultrasound treatment. The overall picture shows that the device used in the first batch experiment was highly effective in the reduction of bacterial population, although had little to no effect on coliforms.

Highest and lowest concentrations of bacteria found in test containers the day before the device activation were $56x10^5$CFU/ml and 10^5CFU/ml versus $26x10^5$CFU/ml and 10^3CFU/ml right before the USD activation respectively. Thus, before any external influence had been introduced, bacterial population in test containers was declining. On the following days the value was fluctuating showing the lowest max/min concentrations 48 hours after the device activation ($4x10^5$CFU/ml and 10^3CFU/ml) and highest max/min on the last day of ultrasonication ($106x10^5$CFU/ml and 10^3CFU/ml). However, a different set of colonies was present on the plates at different stages of ultrasonication. Before USD activation pink colonies could be found in almost all the d_0 samples; 30mins – 3h after the initiation of the treatment pink colonies disappeared, yellow and white colonies were found on the plates, many of them matte; after 48h of ultrasonication shiny white colonies were prevailing. It can be suggested that certain strains of bacteria have been killed by ultrasonication, and thus another strains more resistant to

USD were given opportunity to multiply. Selective analysis showed the reduction of *E. coli* from 160CFU/100ml to >1CFU/100ml and of *P. aeruginosa* from 5CFU/ml to >1CFU/ml. Although the effectiveness of second batch device for general bacterial concentration reduction cannot be explicitly defined based on HPC, it can be recognized as effective for *E. coli* and *P. aeruginosa* elimination.

HPC before the device activation was between $2x10^5$CFU/ml and more than $300x10^5$CFU/ml. During the next 24 hours it showed slight reduction, but was fluctuating around the same value. Later bacterial concentration range reduced to 10^3CFU/ml - $6x10^5$CFU/ml 48 hours after USD activation and 10^3CFU/ml – $60x10^3$CFU/ml on day 6 of ultrasonication. *E. coli* concentration reduced from 40CFU/100ml to >1CFU/100ml, however, the same happened in control containers. Thus, it can be said that USD used in the third batch experiment is potentially effective for the reduction of bacteria in water.

Out of the three tested ultrasonic devices the one with frequency of 40kHz (first batch) showed best performance in bacteria elimination, while 29-37kHz device showed lower efficiency, and 37-45kHz USD was found to be ineffective for this purpose. It should be noted that no selective bacteria analysis was conducted in the first experiment as well as no control group HPCs were performed in these studies. Thus the decline in bacterial population could still happen independently from the treatment. Also the inoculation of plates was performed in semi-sterile conditions, so contamination was possible. It was earlier suggested that 37-45kHz device is effective for *E. coli*; however, in the third experiment both test and control groups were tested for TC and *E. coli*, and the results showed the elimination of *E. coli* in both groups. Therefore, independent *E. coli* extinction in both cases can be suspected.

	Total algae elimination	**Cyanobacteria elimination**	**Bacteria elimination**	***E. coli* elimination**
1. 40kHz	Ineffective	Ineffective	Effective	No data
2. 39-47kHz	Effective	Potentially effective	Ineffective	Potentially effective
3. 29-37kHz	Potentially effective	Effective	Potentially effective	Potentially effective

Table 19. Summary of USDs effectiveness based on algal and bacterial populations' analysis.

According to research presented in "Ultrasonic processes and devices in biology and medicine" (LLC "Matrix-production Plus" (b), 2014), 100% disinfection has never been achieved with ultrasonication alone. It is also noted that while some microorganisms are destructed under the effect of ultrasound, the agglomerations of bacteria can be separated by the wave, and each separated bacterium will start a new colony. However, ultrasonication combined with chemical and heat treatment was found to give much better results.

Similarly, Naddeo et al., (2014), in their revision of main ultrasound disinfection studies imply that sonication can provide powerful disinfection at the cost of high energy comsumption, and therefore coupling with another treatment is preferred. The study implies that the the best results in water desinfection with relation to TC and *E. coli* were obtained at 20-40kHz frequencies with less than 120W/l power input and irradiation time 3 to 15 minutes. It was also found proven that ozonation combined with ultrasonication and elyctrolysis allowed 100% microorganism inactivation in as little as 1 minute; USD parameteres not specified. It was noted, however, that further studies on ultrasonic desinfection need to be done.

Neis and Blume, (2002), in their work on ultrasonic treatment of wastewaters say that ultrasonication is used on some of the German sewage treatment plants to break agglomerations of bacteria so that the further treatment such as ultraviolet irradiation or chlorination is more effective. In this research ultrasonication at 20kHz frequencies with power densities of 10 to 400W/l is analyzed; it is concluded that such power input is insufficient for microbial cell wall destruction.

Some research stating that ultrasound can enhance bacterial growth can be found as well. It was found that low frequency ultrasound of low intensity increases the growth rate of S*taphylococcus epidermidis*, *P. aeruginosa* and *E.coli.* The experiment was conducted with frequencies lower than 100kHz and power density less than 2W/cm^2; the conclusions stated that the power of cavitation created by the devices with such parameters is not enough to destroy bacterial cell wall, therefore the wave only breaks the bacterial agglomerations allowing the cells to multiply faster (Pitt, Ross, 2003).

Notably, all the research mentioned above used devices with much higher power density comparing to the one used in this research. When ultrasound is applied in water treatment setups, its aim is often not to destroy the cells but to break agglomerations and improve the efficiency of another treatment method. In many reports the power input is expressed through the intensity of the emitted wave or W/cm^2, which makes it difficult to compare the results. Also, the devices used in research always have one permanent frequency; research on efficiency of devices with fluctuating frequencies was not found.

Based on this work as well as the findings of other research, it can be stated that ultrasonication alone with power density of 0.2W/l is not suitable for water disinfection. Another issue with power input concerns the intention to use the tested devices in water tanks. According to WHO, the standard for minimum amount of high quality water required per capita per day is 20 liters. Therefore, it would be logical to assume that a typical household would like to maintain a water tank with a volume higher than 40l containers used in this experiment. If the same USD is used in higher volume of water, power density will be even lower than 0.2W/l. The study proved that this intensity is already quite low, so lower number would give even poorer results.

The device is intended for the use in African countries. Therefore, it should be taken into account that African natural waters have essentially different microbial composition than that of a north German lake water. Thus, the results of identical experiment using water from, for example, Kenya can be absolutely different. Also, the temperature of water stored in tanks depending on the region of African continent can be much higher during daytime and/or much lower during the night. This condition can affect the behavior of algal and bacterial populations.

According to Tables 7, 12 and 17, in all three experiments water after treatment did not meet WHO requirements for drinking water quality. The required HPC and turbidity values were never approached; cyanobacterial concentration did not decline to 0 in any of the setups. The only values that complied with GDWQ were pH and nitrate concentration, however, these parameters are not affected by ultrasonication. It indicates that none of the tested devices should be used as a single treatment for water tanks; it should either be combined with other treatment techniques or replaced by another method.

The 40kHz USD showed the best performance regarding bacterial content; however, the final HPC was twice higher than maximum HPC set by WHO for drinking water. The device with frequency 39-47kHz was admitted a leader in algae inactivation among the ones tested. It reduced algae population by 59%

in six days. It also suspended the growth of cyanobacteria. Thus, water quality after the treatment required extra measures aimed at bacteria elimination, organic matter, cyanobacteria, cyanotoxins and other dissolved substances removal.

Attenuation of ultrasound and solar power implementation were not part of this study and, therefore, should be explored. For further improvement of ultrasonic treatment protocol, distance that USD wave travels should be discovered. Also the dependence of the ultrasound frequency and power on distance needs consideration. In similar experiment it should be calculated whether devices operating in test containers can affect a control batch. Such parameters as the absorption of sound by various substances and reflection coefficient must be taken into account. The use of solar power to operate USDs needs research as well. Future studies can be aimed at discovering the parameters of solar panel required for the device support, finding the solution for ensuring USD nighttime operation and calculating the total cost of the installation.

Dead algae cells settle quite fast. Also, cyanobacteria may be viable in water tanks even when their gas vacuoles are damaged. Therefore, removal of sediment during ultrasonication would probably increase water quality. Using a device with higher power output in combination with circulation system could reduce the treatment time and enhance the effect. Also a usage of device with broader variation of frequencies could show a better performance in similar research. The combination of ultrasonic treatment with chlorination, heat treatment and/or filtration can be a topic of further studies.

For further attempts to finding a cost effective implementation of USD in household water treatment in Africa, I would suggest testing an installation that would include USD and circulation system, combined with other types of water treatment. First of all, water should be collected from the top layers of lake/pond/other source as it would probably be less contaminated with various substances. Second, filtration should occur to remove agglomerations of algae and bacteria, organic matter and other relatively big particles. In household conditions a simple filtration through multiple layers of cloth can be performed. Third, ultrasonication can follow. I would suggest testing a device with fluctuating frequency in the range from 29kHz to 47kHz; USD and a pump should be combined so that the power density is 400W/l. For example, if a USD has the power output 8W, the irradiation should occur in a 50ml camber through which water would be pumped at a slow pace. Such power input should allow gas vacuoles' disruption without damaging the cell wall, and therefore without releasing intracellular cyanotoxins. This treatment should notably reduce algal and cyanobacterial content. Next, in order to rid water of remaining microorganisms and to approach drinking water quality, heat treatment or chlorination should follow. Solar water disinfection is already being used in some areas of Africa and Asia and is proven to be effective in elimination of biological contaminants. It is also an easy and cheap practice, however, time-consuming (Wikipedia, 2012). Likewise, chlorination is easy to perform and is considered a cheap practice, however, it might not be accessible for every African household (36 Ready, 2013).

Popular alternatives for cost effective water purification include a combination of cloth filtration and solar water disinfection (only plastic bottles are needed), slow sand filtration (estimated 0.000685$ per liter water) and a combination of cloth filtration and chlorination with liquid bleach (approximately 0.01$ per liter water). Slow sand filtration and chlorination can as well remove up to 80% of present cyanotoxins (Centers for Disease Control and Prevention, 2014; 36 Ready, 2013; Wikipedia, 2012; World Health organization (f), 2015).

6 REFERENCES

Antusheva, T. (2013). Some Features of the Effect of Ultrasound on Microorganisms. *JBKS, 4*, p. 1-13. Retrieved September 2015, from http://www.jbks.ru/archive/issue-4/article-11

bbe Moldaenke GmbH (a). (2015). *AlgaeTorch*. Retrieved November 2015, from bbe moldaenke: http://www.bbe-moldaenke.de/en/products/chlorophyll/details/algaetorch.html

bbe Moldaenke GmbH. (2014, February 26). Questions made while bbe Webinar „AlgaeTorch". Schwentinental, Germany. Retrieved November 2015, from https://www.google.de/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0ahUKEwj6sNbAi5_JAhUFi3IKHcpOCtsQFgggMAA&url=http%3A%2F%2Fwww.bbe-moldaenke.de%2Fde%2Fwissen-medien%2Fwebinare.html%3Ffile%3Dfiles%2Fproducts%2Fchlorophyll%2Falgaetorch%2Fdo

Carmichael, W. A. (2001, July). Human Fatalities from Cyanobacteria: Chemical and Biological Evidence for Cyanotoxins. *Environmental Health Perspectives, vol. 109*(no. 7), pp. 663-668. Retrieved November 2015, from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1240368/pdf/ehp0109-000663.pdf

Fondriest Environmental Inc. (2015). *pH of Water*. Retrieved October 2015, from Fundamentals of Environmental Measurements: http://www.fondriest.com/environmental-measurements/parameters/water-quality/ph/#p11

Goma Epidemiology Group. (1995, February 11). Public health impact of Rwandan refugee crisis: what happened in Goma, Zaire, in July, 1994? *The Lancet, vol. 345*, pp. 344-349. Retrieved October 2015, from National Center for Biotechnology Information: http://www.ircwash.org/sites/default/files/824-RW95-13332.PDF

Hielscher Ultrasonics GmbH. (2015, October 22). *Ultrasonic Extraction and Preservation*. Retrieved October 2015, from Hielscher – Ultrasound Technology: http://www.hielscher.com/extraction_01.htm

Hielscher, T. (2012, January). Ultrasonic production of nano-size dispertions and emulsions. *IPTonline – The Pharmaceutical Technology Journal*, pp. 1-3. Retrieved September 2015, from http://www.iptonline.com/pdf_viewarticle.asp?cat=3&article=825

Lavens, P., & Sorgeloos, P. (1996). *Manual on the production and use of live food for aquaculture.* Rome: FAO Fisheries Technical Paper. Retrieved October 2015, from http://www.fao.org/docrep/003/w3732e/w3732e01.htm#PREPARATION%20OF%20THIS%20DOCUMENT

LLC "Matrix-production Plus" (a). (2014, August 10). *What is cavitation*. Retrieved October 2015, from Theory of Ultrasound: http://www.uzo.matrixplus.ru/kavitac.htm

LLC "Matrix-production Plus" (b). (2014, August 13). *Ultrasonic processes and devices in biology and medicine*. Retrieved October 2015, from Theory of Ultrasound: http://www.uzo.matrixplus.ru/ultramedbio08.htm

Microsoft. (2015). *Add, change, or remove error bars in a chart*. Retrieved October 2015, from Office Support: https://support.office.com/en-za/article/add-change-or-remove-error-bars-in-a-chart-e6d12c87-8533-4cd6-a3f5-864049a145f0

Pernet, F., & Tremblay, R. (2003). Effect of Ultrasonication and Grinding on the Determination of Lipid Class Content of Microalgae Harvested on Filters. *Lipids, vol. 38*(no. 11), pp. 1191-1195. Retrieved 2015

Plumer, B. (2015, January 1). A toxic algae scare has left 500,000 people in Ohio without drinking water. *Vox, vol. 1.4*. Retrieved November 2015, from http://www.vox.com/2014/8/3/5963645/a-toxic-algae-bloom-has-left-400000-people-in-ohio-without-drinking

SonicSolutions LLC. (2009). *Product Information*. Retrieved 2015, from SonicSolutions, Algae Control without Chemicals: http://www.sonicsolutionsllc.com/products/

United Nations High Commissioner for Refugees. (2000). *State of the World's Refugees, The: Fifty Years of Humanitarian Action* (1st ed.). Geneva, Switzerland: Oxford University Press. Retrieved October 2015, from http://www.unhcr.org/publ/PUBL/3ebf9bb60.pdf

University of Göttingen. (n.d.). *Culture Collection of Algae.* Medium Recipe, Göttingen. Retrieved October 2015, from http://sagdb.uni-goettingen.de/culture_media/01%20Basal%20Medium.pdf

USEPA. (2009, May). *National Primary Drinking Water Regulations*. Retrieved from Water: Drinking Water Contaminants: National Primary Drinking Water Regulations

Van Vuuren, S., Taylor, J., Van Ginkel, C., & Gerber, A. (2006). *Easy identification of the most common freshwater algae.* North-West University, Department of Water Affairs and Forestry, South Africa. Retrieved November 2015, from https://www.google.de/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0ahUKEwjG_L7SvqnJAhXBvQ8KHakxDZoQFggeMAA&url=http%3A%2F%2Fwww.researchgate.net%2Ffile.PostFileLoader.html%3Fid%3D5492e3a7d5a3f2b7598b4689%26assetKey%3DAS%253A273654632386560

World Health Organization (a). (2008). *Guidelines for drinking-water quality: incorporating 1st and 2nd addenda* (3rd ed., Vol. 1). Geneva, Switzerland: WHO press. Retrieved October 2015, from http://www.who.int/water_sanitation_health/dwq/fulltext.pdf

World Health Organization (b). (2008). *Guidelines for drinking-water quality: incorporating 1st and 2nd addenda* (3rd ed., Vol. 1). Geneva, Switzerland: WHO press. Retrieved October 2015, from http://www.who.int/water_sanitation_health/dwq/fulltext.pdf?ua=1

World Health Organization (c). (2011). *Evaluating household water treatment options.* Geneva, Switzerland: WHO press. Retrieved October 2015, from http://apps.who.int/iris/bitstream/10665/44693/1/9789241548229_eng.pdf

World Health Organization (d). (1999). *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management.* (J. Bartram, & I. Chorus, Eds.) Suffolk, Great Britain: St Edmundsbury Press. Retrieved October 2015, from http://www.who.int/water_sanitation_health/resourcesquality/toxicyanbact/en/

World Health Organization (e). (2003). *Guidelines for safe recreational water environments* (Vol. 1: Coastal and fresh waters). Geneva, Switzerland: WHO press. Retrieved October 2015, from http://www.who.int/water_sanitation_health/bathing/srwe1-chap8.pdf

World Health organization (f). (2015, January). Management of cyanobacteria in drinking water supplies: Information for regulators and drinking water suppliers. *Technical brief*. Geneva, Switzerland. Retrieved October 2015, from http://apps.who.int/iris/bitstream/10665/153970/1/WHO_FWC_WSH_15.03_eng.pdf