Interactions between bumblebees and cyanobacterial blooms

FRIDA BRODIN LARSSON 2017
MVEK02 EXAMINATION PAPER FOR BACHELOR'S DEGREE 15HP
ENVIRONMENTAL SCIENCE| LUNDS UNIVERSITY

Interactions between bumblebees and cyanobacterial blooms

Frida Brodin Larsson

2017

LUNDS UNIVERSITET

Frida Brodin Larsson

MVEK02 Examination paper for Bachelor's degree 15hp, Lund University

Supervisor: Rebecca Stewart, Centre for Environment and Climate Research/Aquatic Ecology, Ecology Building, Lund University

CEC - Centre for Environmental and Climate Research
Lund University
Lund 2017

Abstract

Today pollinators provide an important ecosystem service as they contribute to the pollination of 75 % of the world's most important crop yields. Several bee species are threatened though due to different anthropogenic impacts and have declined in numbers. One overlooked cause of the decline might be the eutrophication of lakes and ponds in agricultural landscapes, which favour toxic algae blooms. Cyanotoxins like Microcystins have been proven to accumulate in higher animals, causing toxic events within the organisms sometime resulting in deaths. This study aimed to experimentally evaluate the interaction between toxic cyanobacterial blooms and pollinators. An experiment was hence conducted with six bumblebee colonies exposed to toxic cyanobacteria through their drinking water and with another six colonies used as controls. The bees were recorded by security cameras during the experiment to evaluate differences in drinking behaviour. The results did not show any significant differences in behaviour, colony status or concentration of Microcystins in the bees. Some tendencies were seen though and the method used for extraction of Microcystins from the bees is believed to need further development before obtaining reliable results. It is believed to be relevant to decide threshold concentration for the bees when evaluating to what extent toxic cyanobacterial blooms might affect pollinators. Further studies are motivated as the loss of pollinators as an ecosystem service would be both extremely hard and costly to replace with artificial pollination.

Contents

Introduction

Pollination

Pollination is vital for plant reproduction but also to ensure human food supply as 35 % of globally important crop types are estimated to depend upon pollination from pollinating animals (Klein et al 2016). Hence pollinators provide an important ecosystem service. Pollinators have been reported to decline in number in recent years though and some species have even been reported extinct (Potts et al 2016).

As the human population increases and people become richer, the global demand for food rises. The world´s population is expected to grow by 34 % before 2050 according to FAO (2009), which means that the global production of food has to increase by 70 %. To meet this demand, cereal production must rise by almost 1 billion tonnes (FAO 2009). Ecosystems services, such as pollination, will hence become even more important in the future. Studies including 24 ecosystem services, such as water supply, indicates that 60 % of the studied services are negatively affected due to human activity (FAO 2009). If action is not taken to stop this negative trend, challenges will rise to feed the world´s population in the near future.

If pollination services are lost, approximately between 5 and 8 % of global crop yield will be lost since pollinators are directly linked to 75 % of the world´s most important crop yields (Klein et al 2016; Aizen et al 2009). How much of the yield that will be lost in a certain region are highly geographically dependent though as developing parts of the word rely more on animal pollinated crops (Aizen et al 2009). Over the last years, pollinators have become more important and a greater majority of agriculture depends on pollination today (Fig. 1). Beyond food, pollinators also provide humans with other important resources from nature, like medicines, biofuels and fibres (Potts et al 2016).

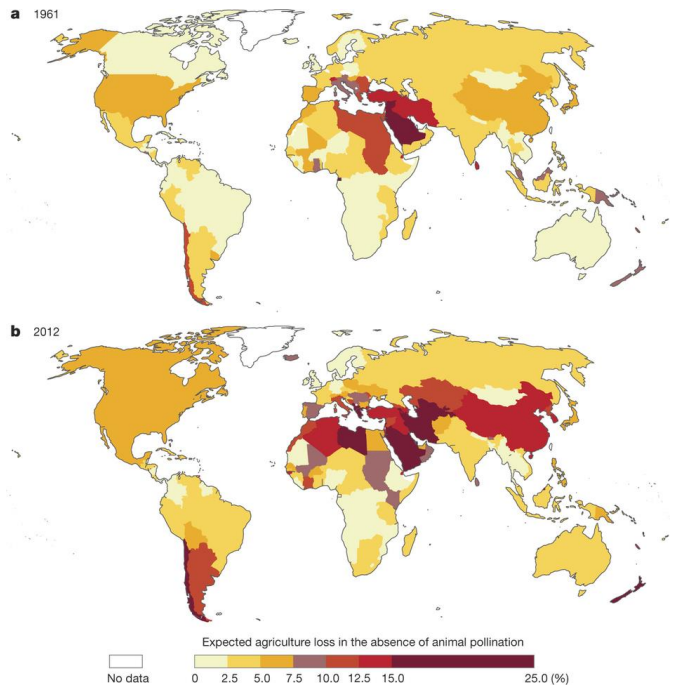

Figure 1. An overview of agricultural dependence of pollinators made by Potts et al (2016). The figure shows the expected agricultural loss due to pollinator decline. Over the last 50 years (1961-2012) pollinators have become twice as important for agricultural output. The large regional differences are explained by crop species, as coffee, almonds, cacao and soybeans are highly dependent of pollinating animas (Potts et al 2016).

According to Potts et al (2016) there are five major anthropogenic reasons behind the decline of pollinating animals; land-use changes and management intensity, climate changes, pesticides and GMOs, pollinator management and invasive alien species. Fertilizers are heavily used as a consequence of intensive land-use which have led to eutrophication of many cropland-associated water bodies. These ponds and lakes have become prone to toxic cyanobacterial blooms (WHO 2013), which are believed to be an additional and overlooked cause of the reduction of pollinators. Studies on European honey bees suggest that the cyanobacterial produced neurotoxin beta-N-methylamino-L-alanine (BMAA) produces adverse effects in honey bees (Okle et al 2013). Okle et al (2013) argue that BMAA cause an increased mortality (Fig. 2), impair learning and short term memory, elevate Ca^{+} concentrations on brain neurons and increases spontaneous brain activity in the bees.

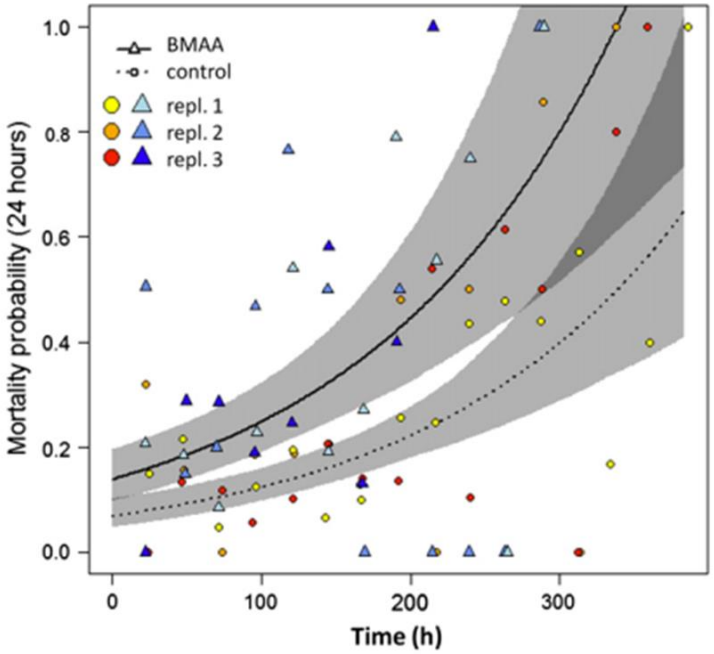

Figure 2. Okle et al (2013) conclude in their report that mortality is significantly higher for bees exposed for the cyanobacteria produced toxin BMAA through their food, compared to control bees over time. O: control bees Δ: bees exposed to 5 mM BMAA.

Okle et al (2013) explain the increased mortality over time for honey bees due to an accumulation of events that negatively affect the bees. Long-time exposure to toxic cyanobacteria might hence be a threat against whole bee populations. High levels of cyanobacteria are, as previously described, common in ponds close to agricultural land due to run off. If the bees use these ponds as drinking resources, then there is a risk the bees might accumulate toxins produced by cyanobacteria through their drinking water. According to Abou-Shaara (2012) water collection is of high importance for bees, not only for drinking but to regulate the body temperature and humidity of the nest and for the dilution of honey.

Cyanobacteria

Today 150 different genera of cyanobacteria are known (Sarma 2012), commonly referred to as blue-green algae. Cyanobacteria have properties found both among bacteria and algae but in contrast to algae some of the cyanobacteria sometimes

accumulate in fertilized waters and form toxic bacteria blooms (WHO 2003). Today 60 % of all tested cyanobacteria samples have been proven to contain toxins but the result might change depending on when the sample is taken (WHO 2003). It is a challenge to identify the toxic species though cyanobacteria have the properties of producing both toxic and non-toxic strains. One of the most common toxins produced by cyanobacteria are microcystins, a cyclic heptapeptide (WHO 2003). Like Okle et al (2013) demonstrated that the mortality probability for honey bees increases over time, Fitzgeorge et al (1994) conclude that liver damage due to microcystins is cumulative in higher animals. Reports have documented deaths among both animals and humans due to cyanobacteria toxins in drinking water (Codd et al 1989, Jochim et al 1998). Hence it is likely that cyanobacteria toxins even might have negative effects on pollinating animals like bumblebees.

Depending on the toxic symptoms, cyanobacteria are divided into three groups, hepatotoxins, neurotoxins and dermatoxins (Sarma 2012). Among hepatotoxins, *Microcystis aeruginosa* is the most common one found in freshwaters, producing the toxin Microcystin (Sarma 2012). The toxin interferes with liver functions, causing weakness, reduced appetite, vomiting, diarrhea and cancer among humans and animals (Sarma 2012). According to WHO (2013) it is hard to give guidelines of safe cyanobacteria levels in water for humans since individual sensitivity varies as do the properties for different toxic cyanobacteria. Attempts have been made but only a provisional guideline value are yet set for the most toxic microcystins congener, Microcystins-LR, 1 µg/l (WHO 2011). Even though all cyanobacteria have not been proven to produce toxins that harm other organism or threshold concentrations have not been set, WHO (2013) conclude that *"…it is prudent to presume a toxic potential in any cyanobacterial population."*

Objectives

The aim of this project is to understand the interaction between pollinators and toxic cyanobacterial blooms. Since cyanobacterial blooms occur in waters, it requires that bees drink water to be exposed for the toxins produced by the bacteria. Hence this paper would experimentally investigate whether bees were exposed to cyanobacteria through their drinking water or not. If the bees were consuming the cyanobacteria, what were then the effects in the individuals and the colonies? The hypothesis is that bumblebees will be negatively affected by toxic cyanobacterial blooms.

Bombus terrestris and *Microcystis aeruginosa*

Between the 29th of July and the 16th of September 2016, an experiment to investigate the interaction between the bumblebee *Bombus terrestris* and the cyanobacteria *Microcystis aeruginosa* was carried out in Lund, Sweden. Twelve bumblebee colonies were included in the study, where six of the colonies were exposed to cyanobacteria through their drinking water. The exposed bumblebees had 50 mg *M. aeruginosa* added to their drinking water while the control colonies had no cyanobacteria added to their water. Each colony was placed in a flight cage (Fig. 1) together with a drinking pot, security camera and some oil-seed rape plants and monitored for 40 days (Fig. 2 and 3). The oil-seed rape plants were placed in the cages to serve as an additional food source for the colonies.

Figure 1. Flight cages used in the experiment.

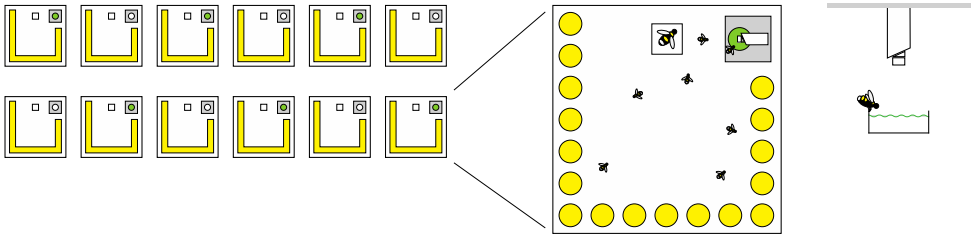

Figure 2. Sketch made by Björn Klatt of the experiment where 12 bumblebee colonies were placed in flight cages next to each other together with a water pot, a security camera and

some oil-seed rape plants. The big bee indicates the colony nest, the yellow points the oil-seed rapes and the green point indicates if the water has been treated with the cyanobacteria *M. aeruginosa* or not.

Figure 3. A bumblebee colony (inside the white box) was placed inside each flight cage next to a garden table with a security camera fixed under it. The water pot is placed directly underneath the camera with a stone inside. The oil-seed rape plants can be seen along the flight cage walls.

The colonies were held in plastic boxes inside Styrofoam boxes with a sugar solution under the nest. The sugar solution served as an important food source for the bumblebees as the oilseed rape plants were considered not to be a sufficient food source. Once a week 50 g of pollen was sprinkled over the colony to add some extra energy and to make sure that the bees hade enough nutrients. The purpose of the camera was to record the activity of the bumblebees to see if there were any differences in behaviour, depending on water quality. *M. aeruginosa* were cultivated for the experiment which started on the 4th of August and the water pots were filled with new water five times; 16/8, 23/8, 29/8, 4/9 and 9/9. A water sample was taken each time the pots were filled with new water, both from the old water in the pot and from the new fresh water poured into the pot. The water samples were taken to later analyse that the control and cyanobacteria treated water had different concentrations. In total 14 water samples were collected during the experiment by empty the pots in to two bigger pots, one for the clean water and one for the *M. aeruginosa* treated water. When the experiment ended all colonies were frozen to evaluate potential effects on the cyanobacteria exposed bumblebees.

Methods

Video data

To find out about the bumblebees drinking behaviour, all videos have been watched between 5 am and 6 pm. The videos have been watched using Windows Media Player and data collected every 15 minute by fast-forwarding, e.g. 15 minutes have been watched, 15 minutes have been skipped to efficiently go through all recorded data. Observations were made of whether the bees flew around the outside of the water pot, if they were sitting/crawling on the stone placed in the water pot or if they flew over the water. Bees was observed and their behaviour recorded, up to a maximum of 15 minutes since the next 15 minutes were skipped. If a bee disappeared under the stone but came back in the picture after one or two minutes, still on the stone, it was presumed to be the same bee.

Colony status

After the experiment ended in September, the colonies have been preserved frozen. To enable an organised and consequent analysis of the nests, a protocol has been constructed (App. 1) involving ten steps.

All bees and cocoons have been counted and measured in each nest after being separated into different size classes; workers, drones and queens. In some of the colonies, four size classes had to be used since the difference between the bees were too large for only using three size classes.

First all visible bees on top of the nest and the ones lying close to the nest were collected with tweezers and placed on different petri dishes depending on size class. Some bees were found in the corners of the plastic box (the nests were placed in plastic boxes) but left out since the bees uses the corners for storages of waste, e.g. dead bees in the corners have been placed there by other bees while the colony still was alive (Fig. 4). To evaluate the status of the nests, only bees that died when the colonies were frozen have been included in the study. Once all bees had been collected, the wax cover was removed from the nest. Under the wax cover most of the bees were found since they crawl in under it for protection when they are

exposed for cold (Fig. 5). The cocoons were then removed from the nest by hand and placed on a petri dish. Some non-bee larvae were found next to or on the cocoons and placed on a separate petri dish.

Once the bees and cocoons where taken out from the nest, counted and sorted by size, 10 samples from each size class were measured using a digital calliper. All bees from each size class where lined up, from the smallest to the biggest and then the two first and the two last bees where picked out from each line to be measured. The remaining six bees where randomly picked out from each line. The same method was used for the cocoons. If a size class had less than ten samples, then all bees or cocoons were measured in that group. The intertidial distance (distance between wing buds) was measured on the bees and the length of the cocoons were measured.

Present/absence of mites were also noted, in a first step on the queen and if present, the rest of the colony were checked for mites.

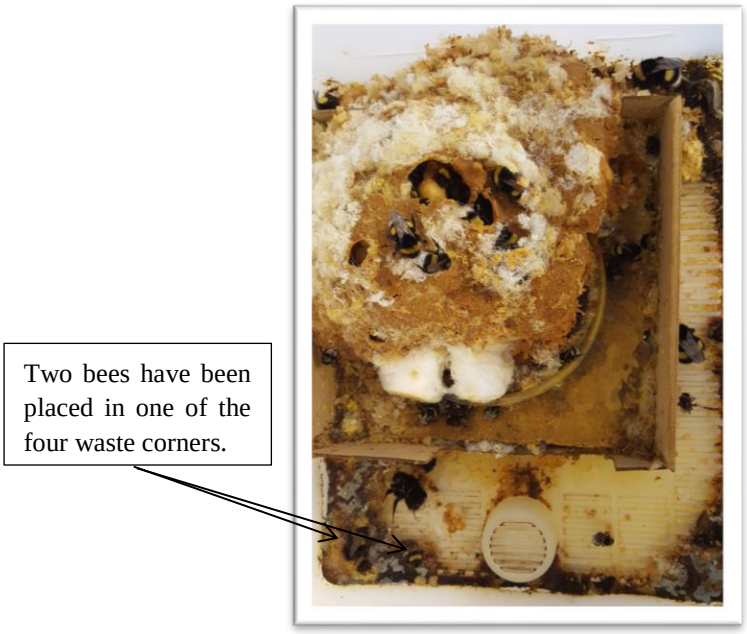

Figure 4. Picture of a bumblebee colony before the wax cover is removed from the nest. Each one of the four corners in the plastic box have been used by the bees to store waste. Apparently, something has disrupted this cyanobacteria exposed colony, there is a lot of cotton left and mould can be seen growing in the pot.

Figure 5. Picture over a bumblebee colony after the wax cover has been removed from the nest. The cocoons are yellow and in the lower part of the picture, wax cocoons containing non-bee larvae can be seen.

Waters samples

Water samples have been analysed using an Abraxis Microcystins-ADDA ELISA (Enzyme-Linked Immunosorbent Assay) kit. Microcystins are cyclic toxin peptides produced by cyanobacteria during toxic cyanobacteria blooms.

First the water samples were freeze-thawed three times in order to damage the cell walls. One millilitre of each sample was then transferred into Eppendorf tubes and centrifuged for 20 minutes at 6900g (standard acceleration due to gravity). After the centrifugation, 50 µL from six standard solutions (0-5) and a control solution provided by the ELISA kit, were added in duplicate to the wells of a microtiter plate. 50 µL from each water samples were then added, in duplicate, to the wells of the microtiter plate (Tab. 1). An antibody solution (50 µL) was then added to each well, using a multi-channel pipette since it is important to fill all wells with the solution within two minutes (the reaction starts as soon as the antibody solution is added). The microtiter plate was covered with parafilm, moved in a circular motion over the bench for 30 seconds, in order to mix the sample and

reagent and then covered with foil (to keep it dark as light can interfere with the reaction) and left for incubation for 90 minutes.

Table 1. Working scheme over the microtiter plate. Number 0-5 are the standard solutions, C is the control and S1, 2, 3 etc. are the water samples without any cyanobacteria. Cy indicates that the water sample has been treated with cyanobacteria.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0	0	S1 cy	S1 cy	S5 cy	S5 cy	S9 cy	S9 cy	S13 cy	S13 cy		
B	1	1	S2	S2	S6	S6	S10	S10	S14	S14		
C	2	2	S2 cy	S2 cy	S6 cy	S6 cy	S10 cy	S10 cy	S14 cy	S14 cy		
D	3	3	S3	S3	S7	S7	S11	S11				
E	4	4	S3 cy	S3 cy	S7 cy	S7 cy	S11 cy	S11 cy				
F	5	5	S4	S4	S8	S8	S12	S12				
G	C	C	S4 cy	S4 cy	S8 cy	S8 cy	S12 cy	S12 cy				
H	S1	S1	S5	S5	S9	S9	S13	S13				

After the incubation, the foil and parafilm were removed from the microtiter plate and the contents of the wells were decanted into a sink. The wells were washed three times with a wash buffer solution. The ELISA kit provided a wash solution which has to be diluted with distilled water to a ratio of 1:5. After the plate had been washed it was dried by patting it on top of some paper placed on the bench three times. The wells were filled with 100 µL of an enzyme conjugate solution by using the multi-channel pipette to make sure all wells were filled within two minutes and the plate was covered with parafilm. After the plate had been circulated on top of the bench for 30 seconds, it was covered with foil and incubated for 30 minutes.

After the incubation, the plate was washed with wash buffer solution and patted on to some paper three times. The wells were filled with 100 µL of a substrate (colour) solution by using a multi-channel pipette to be able to add the substrate to all wells within two minutes. Parafilm were used to cover the plate before it was

circulated on top of the bench and covered with foil for protection from sunlight and incubated for 30 minutes.

The parafilm were removed after 30 minutes of incubation and 50 µL of a stop solution were added to the wells by using the multi-channel pipet. The stop solution was added to the wells within two minutes in the same sequence as the colour solution. A microplate ELISA photometer was then used to read the absorbance from the plate at 450 nm. The reading has to be done within 15 minutes after the stop solution has been added to the wells.

Bumblebee analysis

Two bees from each colony (approximately 100 mg of material per sample) have been analysed using the ELISA method. In order to perform the analysis, the bees had to be broken down to extract microcystins. The bees were freeze-thawed three times before being homogenised for 20 minutes in 2 mL sample tubes. Each tube contained 1 mL of 75 % aqueous methanol and a metal bead (cleaned by UV light) to help break down the bees. The tubes were then bath sonicated for 30 minutes in room temperature for further degradation of the cells and extraction of microcystins. Since methanol might interfere with the ELISA test kit, the tubes were evaporated over night at 45 ºC to remove the methanol from the samples.

The methanol had not evaporated fully from the samples the next morning and sample tubes were hence centrifuged for 10 minutes at 10 000g (20 ºC). Some of the tubes contained quite a high amount of methanol which could be pipetted out after the centrifugation. The tubes were then evaporated at 50 ºC for 70 minutes for further removal of remaining methanol.

An additional sample of lake water (Krankesjön, July 2015) which had been taken during a known cyanobacterial bloom were included in the analysis to evaluate the impact of the methanol. 1 mL of lake water were transferred into two sample tubes. Methanol were added to one of the tubes and treated in the same way as the tubes containing a bee. An ELISA was then preformed in the same way as described for the water samples (see “Water samples”).

Data analysis and literature

T-tests and ANOVAs were performed to analyse the obtained data. A t-test was performed when checking for differences between the two treatments (control and cyanobacteria exposed colonies) while a two-way ANOVA was performed when checking for differences within the colonies. A t-test was performed when

analysing the water samples and a two-way ANOVA when analysing the video observations. Parameters used were size (mm), length (mm), numbers (counts) and concentration (μg/L) and the significance level was set to < 0.05. Excel was also used for making figures. A reference research has been made to support the hypothesis of toxic cyanobacterial blooms being harmful to pollinating animals (App. 2).

Result

Video observations

No significant differences (ANOVA: p = 0.65) could be found between the control colonies and the cyanobacteria exposed colonies regarding total number of visits to the water pot when including all three observed behaviours (flying around the outside of the water pot, sitting/crawling on the stone in the water pot and flying over the water surface). Neither could a significant difference be found when separating the three different behaviours. A tendency (not significant p = 0.16) for more bees spending time over the cyanobacteria treated water could be seen when visualising number of bees observed (Fig. 6). According to the result, 60 % more bees were observed over the cyanobacteria treated water. Differences could also be visualised considering the colonies. While only two of the six control colonies had bees visiting the water pot, the colonies with cyanobacteria treated water had five of six colonies visiting the water pot.

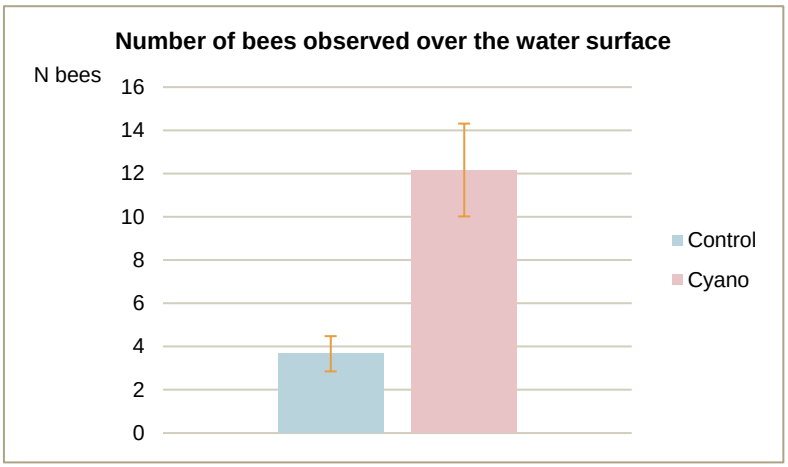

Figure 6. The number of bees observed over the cyanobacteria exposed water shows a tendency to be higher, even though the result is not significant.

Status of the colonies

Number of bees

The number of bees in control colonies and cyanobacteria exposed colonies did not differ significantly ($p = 0.3$) (Fig. 7). Neither did the number of bees differ within the three size classes (small, medium and large) for control colonies and cyanobacteria exposed colonies (Fig. 8).

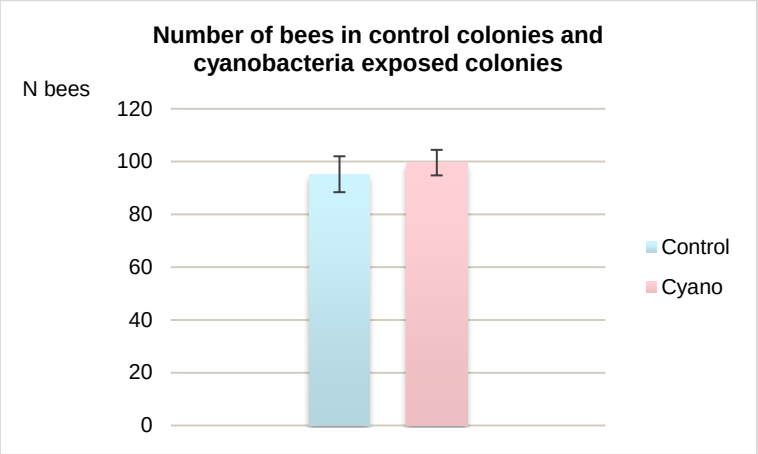

Figure 7. The number of bees in control colonies and colonies exposed for cyanobacteria did not differ significantly.

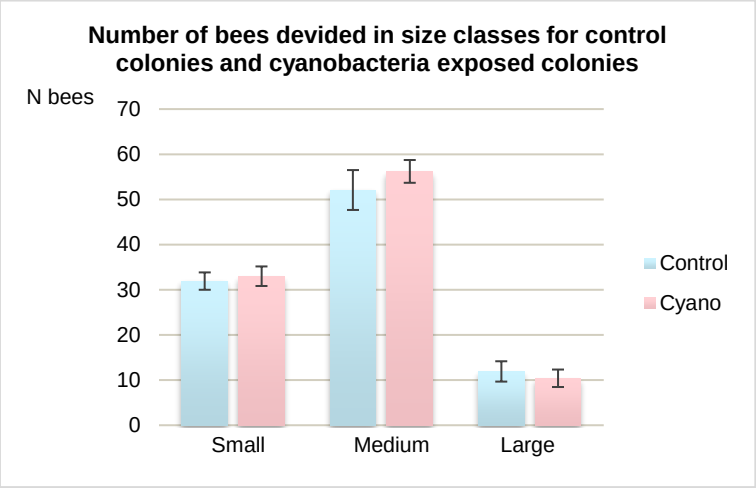

Figure 8. The number of bees within each size class are not affected by toxic cyanobacteria. A significant difference (ANOVA p = 0.005) is found between the tree size groups though, which are to be expected in a healthy colony, the number of workers (small) and drones (medium) are higher.

Colonies containing a queen did show a tendency (not significant, p = 0.18) to differ (compared to workers, p = 0.5 and drones, p = 0.5) between the treatments though, queens were only found in two of the cyanobacteria exposed colonies while a queen was found in five of the control colonies. Of the five queens found in the control colonies, two were considered dead before the colonies were frozen.

Intertidial distance

No significant difference between intertidial distance was found between the control colonies and cyanobacteria exposed colonies (Fig. 9). A control colony tends to have a 6 % longer intertidal distance compared to a cyanobacteria exposed colony though. Neither was a significant difference showed within the different size classes (Fig. 10).

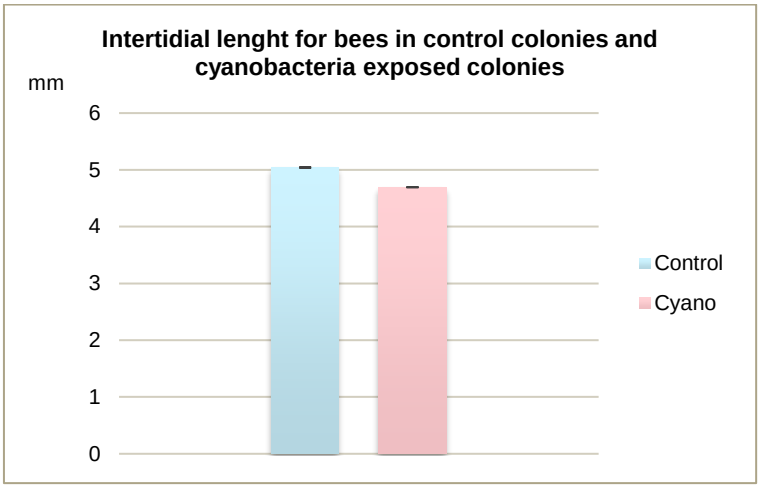

Figure 9. The intertidial length for a cyanobacteria exposed colony do not differ significantly (p = 0.12) from a control colony. The average for a cyanobacteria exposed colony is 4.9 mm while it is 5.1 mm for a control colony.

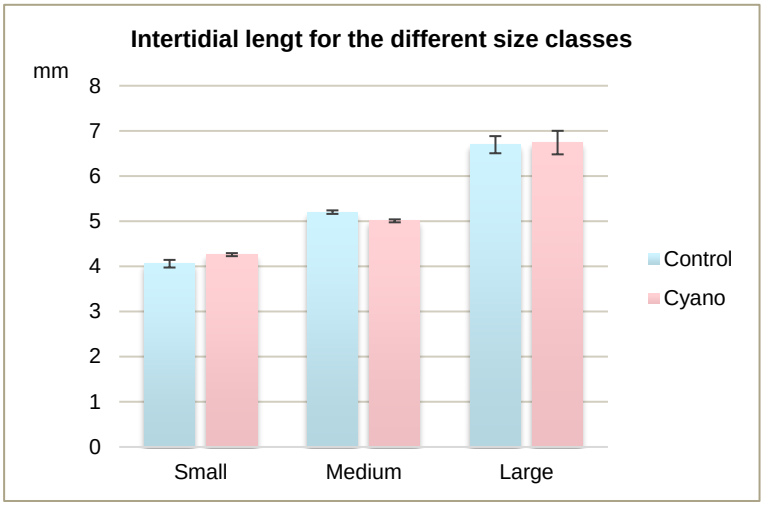

Figure 10. Toxic cyanobacteria do not seem to affect the intertidial distance within the different size classes.

Number of cocoons

Cyanobacteria exposed colonies did not contain significantly fewer cocoons (Fig. 11). No significant difference was found within the two size classes (small and big cocoons) either (Fig. 12). The number of colonies containing big cocoons varies though, only two of the cyanobacteria exposed colonies had big cocoons while four of the control colonies contained big cocoons. The mean value when only including colonies having big cocoons are 10 cocoons for the controls and 5 for the cyanobacteria exposed colonies. Hence there is a difference of 50 % regarding number of big cocoons. If comparing the mean when including all the colonies, then the control colonies have 73 % more cocoons.

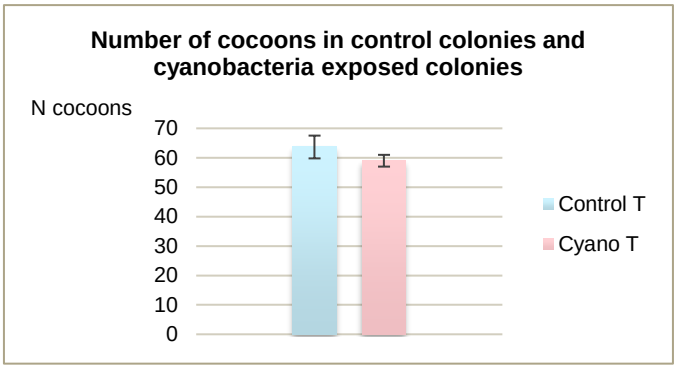

Figure 11. The number of cocoons did not differ significantly between the control colonies and the cyanobacteria exposed colonies.

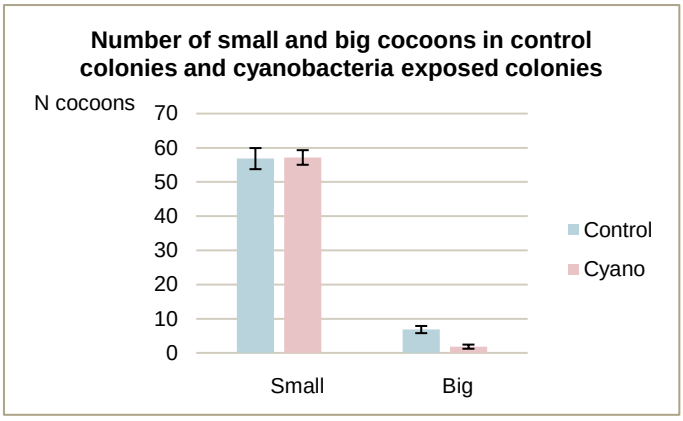

Figure 12. No significant difference was found within the size groups even though the number of big cocoons seem to be higher in the control colonies.

Length of cocoons

The length of the cocoons found in the cyanobacteria exposed colonies did not differ significantly from the once found in the control colonies (Fig. 13). No significant difference was found within the four size groups either (Fig. 14). Even though the results cannot be proved significant, small cocoons seems to be 5 % bigger, medium cocoons 8 % bigger and large cocoons 6 % bigger in the control colonies.

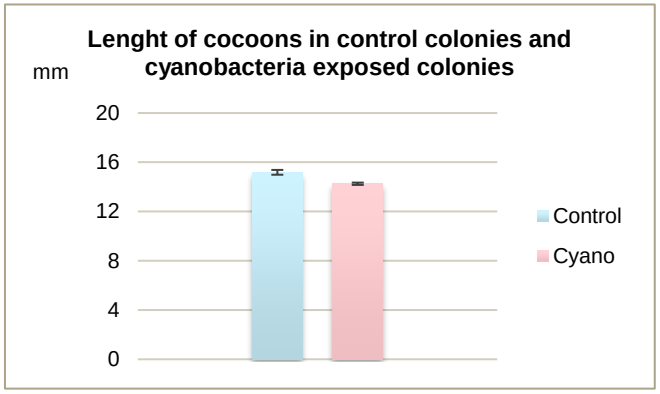

Figure 13. Cyanobacteria exposed colonies did not have significantly (p = 0.3) smaller cocoons compared to control colonies.

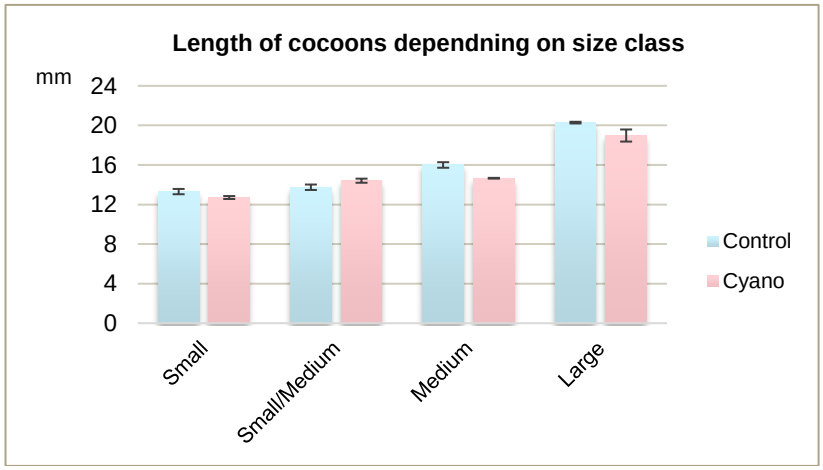

Figure 14. Cocoons found in control colonies tend to be bigger in control colonies compared to cyanobacteria exposed colonies for small, medium and large cocoons. The result is not significant.

ELISA

Water samples

The ELISA showed a significant difference between the control water and cyanobacteria treated water (p=0.0002). In the control the concentration of Microcystins was 0.04 ppm (µg/L) while the cyanobacteria treated water contained 4.55 ppm (µg/L). A significant difference was also find between the in- and out-sample for the cyanobacteria treated water, 6.7 ppm respectively 2.4 ppm (p=0.03).

Bumblebee analysis

Cyanobacteria exposed bees did not contain a significantly higher concentration of Microcystins compared to control bees (Fig. 15). The ELISA showed that cyanobacteria exposed bees contained 5.96 µg/L of Microcystins while the control bees contained 4.30 µg/L Microcystins which gives a difference of 28 %.

Analyse of the water sample from Krankesjön treated with methanol did not differ significantly from the untreated water sample, even though the absorption was a little bit higher.

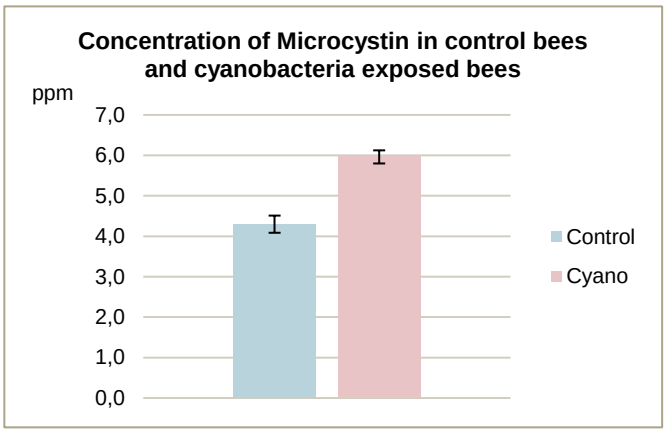

Figure 15. The concertation of Microcystins tend to be higher in the cyanobacteria exposed bees (5.96 respectively 4.30 µg/L), even though the difference is not significant (p = 0.17).

Discussion

To summarize the results, no interactions between toxic cyanobacterial blooms and bumblebees have been possible to prove through this study. Hence the hypothesis that bees will be negatively affected by cyanobacterial blooms cannot be accepted. This study is not enough to reject the hypothesis of a possible negative effect on the bees due to cyanotoxins though. Several studies have shown negative impacts on living organisms due to toxic cyanobacteria blooms (Okle et al 2013; Jochim et al 1998; Fitzgeorge et al 1994; Codd et al 1989). Further studies need to be carried out to evaluate the interaction between pollinators and toxic cyanobacterial blooms. Pollinators are highly important for human food supply and will become even more important in the future as the worlds population increases in number. Until no relationships between toxic cyanobacterial blooms and bees have been proved, it is relevant to apply the precautionary principle as some bee species already are reported extinct (Potts et al 2016).

Prevention of toxic cyanobacterial blooms would not only be a safety precaution against the algae as a possible contributor to the pollinator decline, it would both directly and indirectly benefit human health. Direct benefits in form of a reduced risk of acute poisoning would be the primary advantage of a reduction of toxic algae blooms for humans but indirectly it would rise the possibilities to ensure future human food supply. Other benefits would be a decreased risk for skin irritation and uptake of the toxin in conjunction with fish consumption as cyanotoxins have been reported to accumulate in fish (WHO 2003). Liang et al (2015) proved that fish that consumes toxic cyanobacteria accumulated the toxin. Hence the fish constitute a risk for human health when consumed. Prevention of cyanobacterial blooms would also be beneficial from an economic perspective. Allsopp et al (2008) conclude that wild pollinators have a value between US$49.1–310.9 million for the deciduous fruit industry in South Africa. It would hence be extremely costly to replace a global decline of pollinators with artificial pollination. Regardless to what extent cyanobacteria produced toxins might affect pollinators, it is clear that a prevention of toxic algae blooms would be beneficial from several points of view.

One of the main reasons behind the increased occurrence of algae blooms is the intensification of agricultural land use. The soils become supersaturated with nutrients leaking to surrounding waterbodies (Kuosmanen 2014). Through a more sustainable agricultural management this leakage could be prevented, for example

by the implementation of catch crops. Quemada et al (2012) concluded that catch crops have the potential to reduce the average of nitrate leaching by 50 %. The use of catch crops, like Italian ryegrass (*Lolium multiflorum Lam.*), white clover (*Trifolium repens L.*) and red clover (*Trifolium pratense L.*) (Känkänen et al 2007), would hence have the potential to prevent massive algae blooms and lower the risk for serious poisoning due to cyanotoxins. Furthermore, possible scenarios based on tendencies derived from the results will be discussed and suggestions of improvements when constructing future studies.

Abou-Shaara (2012) argue that water is vital for bees, both for the regulation of own body functions but also to keep the whole colony going. Even though no bees were directly observed drinking form the water pots when watching the videos recorded by the security cameras, bees sitting or crawling on the stones placed in the water were assumed to use the stones as a way to reach the water. This theory is strengthened by the findings of Microcystins in the cyanobacteria-exposed bees from the ELISA. The cyanobacteria-exposed bees tended to have a higher concentration of Microcystins in their body compared to the controls, which they only can have obtained via drinking from the cyanobacteria treated water.

Speculations as to whether bumblebees would avoid water containing toxic cyanobacteria compared to bees offered clean water, can be considered answered by the video observations as no significant differences in bee behaviour between the two treatments were seen. In further studies, it would be interesting to survey whether bees would choose clean water in preference to water containing toxic algae if they were given the opportunity to choose from different water sources. It would also be relevant to offer bees water from different types of water sources, as bees have been proved to show a preference for running water (Abou-Shaara 2012). Abou-Shaara (2012) noticed that running water from a pipe over plant roots were highly popular among honey bees. Perhaps it would be possible to prevent bees from drinking water from still water ponds in agricultural landscape if the bees were offered artificial water sources with clean running water.

It is noteworthy, even though the result is not significant, that cyanobacteria treated water had 60 % more bees hovering over the water surface as did the control water (Fig. 6). Bees are known to hover over water bodies to regulate their body temperature (Abou-Shaara 2012). Questions arise though why bees exposed for cyanotoxins tend to express this behaviour in a lager extent than control bees. As the colonies were close to each other in the same area, temperature differences should not have affected the water demand for regulating the body temperature more for cyanobacteria exposed colonies than for the controls. Neither can the tendency be explained by the cyanobacteria-exposed colonies having more bees than the controls and due to that increase the possibility to observe a bee hovering over the water. Individual differences between the colonies could be a possible explanation as some of the colonies were bigger than others, hence needed more

water. To evaluate this tendency further, a bigger experiment with more replicates would probably be sufficient.

The result did show that only two of six control colonies had bees hovering over the water while the cyanobacteria treated water had bees from five colonies hovering over the water. This observation would rather indicate some kind of attraction to the cyanobacteria containing water but this thought is just based on own speculation and has not been formally tested in an experiment.

Koreivienė et al (2014) summarize reported symptoms in humans after poisoning with Microcystins. Low-level poisoning might cause severe thirst already after 4 hours and if same symptoms are to be found in bees, then it might contribute to more bees seeking water to quench their thirst. This would then be a possible explanation to why more bees are found over the cyanobacteria treated water if the tendency is to be proved significant in future studies. A feed-back loop would then start as the bees drink more of the toxic water and accumulate more of the toxin. Like Okel et al (2013) argue, accumulation of cyanotoxins might lead to an increased mortality among bees.

When preforming the ELISA for evaluation of the concentration of Microcystins in the bees, the controls showed a surprisingly high concentration (4.30 μg/L) as the water only contained 0.04 μg/L Microcystins. It should be stated that the method used in this experiment is new as no earlier studies regarding extraction of Microsystins from bees have been performed. Improvements of the method might hence be necessary before reliable results will be received.

The most likely explanation for the high concentration of Microcystins in the control bees are the use of methanol for extraction. High concentrations of methanol have the potential to cause matrix effects when using the Abraxis Microcystins-ADDA ELISA kit. Evaporation of methanol from the samples were believed to be sufficient enough but the method might need to be developed further. Possible evaporation using a nitrogen evaporator would be more efficient (Keith 2012).

According to the control sample from Krankesjön, which were known to contain Microscystins, there was no significant difference in absorbance after methanol had been added and evaporated from the sample. The high concentration of methanol in the bees would then be an indication of that the methanol might have evaporated more efficiently from the water (Krankesjön sample) compared to the bee mass. This would motivate running another ELISA using a different extraction solution. A potential solvent for the extraction of Microcystins from the bees might be acetic acid. A solution of 5 % acetic acid in 0.2 % trifluoroacetic acid (TFA)–methanol has been proved to be the best way to extract organic material from sediment (Babica et al 2006). An advantage of trying to use this solvent when extracting Microcystins from the bees are that Barbica et al (2006) used ELISA when evaluating their results. What potential the solvent has to extract Microcystins

from bees are yet to be proved but the solvent should not interfere with the reading of the samples.

A low concentration of Microcystins in bees not directly exposed for toxic cyanobacteria through their drinking water would not be completely unreasonable though. Lorraine et al (2014) proved that inhalation might be an additional way of uptake of cyanotoxins due to aerosolized Microcystins. Bees would hence necessarily not need to be in direct contact with cyanobacteria contaminated water to risk accumulation of the cyanotoxin. It could also be that the aerosolized cyanotoxin have contaminated the water in the control cages. This would motivate to keep the control colonies and cyanobacteria exposed colonies separated from each other.

The total number of bees between the treatments were almost even (Fig. 8), indicating that the concentration of cyanotoxin in the exposed bees was not high enough to cause an increased mortality. The treated water contained 4.55 μg/L Microcystins in average, which according to WHO (2003) are to be considered as a low risk level concentration when obtained in recreational waters. High risk levels of Microcystins are reached first when concentrations between 20 and 2000 μg/L are to be measured (WHO 2003). Hence higher concentrations than used in this study might be relevant to use when evaluate the interactions between toxic cyanobacterial blooms and pollinators. Especially when considering that the cyanobacteria did not do very well in the water pots as the concentration of Microcystins in the cyanobacteria treated water significantly differed between the in- and out-sample. Which concentrations that are most suitable to use when evaluating the interaction between pollinators and toxic cyanobacterial blooms need to be answered by future studies.

Even though the concentration of cyanotoxin did not prove to increase the mortality among the bees, a tendency of a reduced intertidial distance were seen. The intertidial distance tend to be 6 % shorter for cyanobacteria exposed bees compared to the controls (Fig. 10). Whether a reduced size will affect the bumblebees function as pollinators is hard to tell but it indicates that some kind of adverse effect might be going on inside the bees. Goulson (2010) discuss the importance of size when looking at competition and niche differentiations in bumblebee communities. He primarily focuses on the tongue length as different bee species are adapted to collect nectar from different flowers. Small bee species cannot reach the nectar in flowers with deep corollas. Differences in tongue length allow species with very similar niches to coexist. If cyanotoxins have the potential to reduce the size of the bees, then there is a risk of disrupting the existing balance between different spices, hence increase the competition for nectar.

The reproduction in the form of number of cocoons was not significantly affected by the concentration of microcystins. When studying the number of colonies containing big cocoons, some differences were possible to distinguish though. Only two of the cyanobacteria exposed colonies did have big cocoons while

four of the control colonies contained big cocoons. Differences were also seen when comparing the number of cocoons between the treatments. The control colonies that contained cocoons had an average of 10 cocoons, while the cyanobacteria exposed colonies had an average of only 5 cocoons. Hence there is a 50 % difference between the cyanobacteria exposed colonies and the controls. If the number of big cocoons are reduced by the present of cyanobacteria, then there could be a decline in number of new queens. As the queens are the ones overwintering and build up new colonies in the spring, a reduced number of queens would result in fewer colonies the coming year.

Conclusion

This study was a first try to understand the interaction between toxic cyanobacterial blooms and pollinators. It is of high relevance to evaluate this interaction as pollinators serves as an important ecosystem service which is direct linked to 75 % of the world´s most important crop yields and since 35 % of these crops are estimate to be dependent of pollinating animals. A lot of tendencies could be distinguished through this study even though no significant results were observed. This has resulted in several new questions that need to be answered by further studies since a decline of pollinators not only would be a threat against species diversity. The loss of pollinators as an ecosystem service would both be extremely hard and costly to replace with artificial pollination.

It is clear that the method used for extraction of microcystins from bees need to be developed further, as a suggestion by the use of acetic acid. Different risk levels are set for human safety but it would be relevant to survey whether these are the same for bees. A more sufficient way to go when evaluate to what extent cyanotoxins might affect pollinating animals like bumblebees could hence be to decide the lowest effect concentration (LOAEL) and the concentration that will kill half of the population (LC_{50}). These values would then be possible to put in relation to concentrations of microcystins measured in natural ponds and lakes. It would also be recommended to us water sources with moving water when constructing similar experiments. This might contribute to more bees showing an interest for the water, hence increase the colonies exposure to toxic cyanobacteria.

Thanks

A big thank you to Rebecca Stewart, Pablo Urrutia Cordero and Björn Klatt for letting me take part of your research and a special thank you to Rebecca for being such a good supervisor. Without your help, this study would not have been possible. I wish you the very best in your continuing work trying to evaluate the interaction between toxic cyanobacterial blooms and pollinators.

References

Aizen, M., Garibalddi, L. A., Cunningham, S. A & Klein, A. M. (2009). How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. *Annals of Botany,* 103(9), pp. 1579-1588.

Allsopp, M. H., de Lange, W. J & Veldtman, R. (2008). Valuing insect pollination services with cost of replacement. *Public Library of Science,* 3(9), e3128.

Babica, P., Kohoutek, J., Bláha, L., Adamovský, O. & Maršálek, B. (2006). Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents. *Analytical & Bioanalytical Chemistry,* 385(8), pp. 1545-1551.

Codd, G. A., Bell, S. G & Brooks, W. P. (1989). Cyanobacterial toxins in water. *Water Science and Technology*. 21 (3), pp 1-13.

FAO. (2009). How to Feed the World in 2050. http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf Attached 2017-02-28.

Fitzgeorge, R. C. (1994). Routes of Intoxication. *Detection Methods for Cynobacterial toxins.* Royal Society of Chemestry, pp 69-74.

Goulson, D. (2010). *Bumblebees: behaviour, ecology, and conservation.* 2. ed., Oxford; New York: Oxford University Press.

Hossam, F. Abou-Shaara (2012). Notes On Water Collection By Honey Bees. Bee World, 89(4), pp. 86-87.

Hualei, L., Zhou, W., Zhang, Y., Qiao, Q. & Zhang, X. (2015). Are fish fed with cyanobacteria safe, nutritious and delicious? A laboratory study. *Scientific Reports,* 5(15166).

Jochimsen, E. M., Carmichael, W. W., An, J., Cardo, D. M., Cookson, S. T., Holmes, C. E. M., Antunes, M. B., de Melo Filho, D. A., Lyra, T. M., Barreto, V. S. T., Azevedo, S. M. F. O., & William R. Jarvis, W. R. (1998). *New England journal of Medicine*, 338, pp. 873-878.

Klein, A. M., Vaissière, B. E., Cane, J. H., Dewenter, I. S., Cunningham, S. A., Kremen, C. & Tscharntek, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B*, 274, pp 303–313.

Koreivienė, J., Anne, O., Kasperovičienė, J. & Burškytė, V. (2014). Cyanotoxin management and human health risk mitigation in recreational waters. *Environmetal Monitoring and Assessment,* 186(7), pp. 4443-4459.

Kuosmanen, N. (2014). Estimating stocks and flows of nitrogen: Application of dynamic nutrient balance to European agriculture. *Ecological Economics,* 108, pp. 68-78.

Känkänen, H. & Eriksson, C. (2007). Effects of undersown crops on soil mineral N and grain yield of spring barley. *European Journal of Agronomy,* 27(1), pp. 25-34.

Lorraine, C. B., McNeel, S. V., Barber, T., Kirkpatrick, B., Williams, C., Irvin, M., Zhou, Y., Johnson, T. B., Nierenberg, K., Abule, M., LePrell, R., Chapman, A., Foss, A., Corum, S., Corum, S., Hill, V. R., Kieszak, S. M. & Cheng, Y. S. (2010). Recreational exposure to microcystins during algal blooms in two California lakes. *Toxicon,* 55(5), pp. 909-921.

Okle, O., Rath, L., Galizia, C. G. & Dietrich, D. R. (2013). The cyanobacterial neurotoxin-beta-N-methylamino-L-alanine (BMAA) induces neuronal and behavioural changes in honeybees. *Toxicology and Applied Pharmacology*, 207(1), pp. 9-15.

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H. T., Aizen, M. A., Beismeijer, J. C., Breeze, T. D., Dicks, L. V., Garibaldi, L. A., Hill, R., Settele, J. & Vanbergen, A. J. (2016). Safeguarding pollinators and their values to human well-being. *NATURE PUBLISHING*, 540(7632), pp. 220-229.

Quemada, M., Baranski, M., Noble-de Lange, M. N. J., Vallejo, A. & Cooper J. M. (2013). Meta-analysis of strategies to control nitrate leaching in irrigated agricultural systems and their effects on crop yield. *Agriculture, Ecosystems and Environment,* 174 pp. 1–10.

Sarma, T. A. (2013). *Handbook of cyanobacteria.* Boca Raton, Fla.: CRC Press.

WHO (2003). Guidelines for safe recreational water environments, 1, pp. 136-154.

Appendix 1

Bumblebee colony – measurements

Material

- Plastic bags
- Tweezers
- Callipers
- Petri dishes
- Gloves

1. **Carefully collect workers from the colony in a petri dish**
   - Only collect bees that are looking ”fresh”, not rotten ones

2. **Remove the wax cover carefully from the nest**
   - Conserve the wax in a plastic bag

3. **Carefully remove bees found under the wax**

4. **Look if mites are present/absent on the queen**
   - If there are mites on the queen, then have a look at the workers

5. **Measure the intertidial distance (distance between wing buds) on the bees, using callipers**
   - Arrange the bees into different size groups (super small, small, medium etc.) and use the calliper to measure the intertidial distance on the bees, 10 bees from each size group within each colony
   - Put the bees into labelled plastic bags (Colony number and size class)

6. **Carefully collect and count the cocoons**
   - Note what type of cocoon it is:
     Small = worker

     Medium = drone

     Big = queen

7. **Measure the length of the cocoons**
   - Use a calliper to measure the length of the cocoons, randomly collect 10 from each size group

8. **Record the presence of non-bee larvae**
   - If there are any non-bee larvae percent in the colony, then count the number of larvae and how they were (e.g. 4 large fly larvae inside 1 cell).

9. **Sample nectar from the colony**

10. **Place all the plastic bags containing wax, bees and cocoons back in the nest box they came from and then back in the freezer for storage.**

Appendix 2

Literature survey

Reference research have been made in LUBSearch and Web of Science (Tab. 2). Search words have been chosen based on the title "Interactions between bumblebees and cyanobacterial bloom" resulting in; *bumblebees*, *bees*, *water*, *collection*, *cyanobacteria*, *pollinators* and *ecosystem service.* These words have then been combined in different ways in the databases. Relevant publications from FAO and WHO have also been included in the study using the search engine Google. Some reference research had to be added after the experiment had been evaluated, resulting in new search words: *nitrate leaching*, *extraction*, *acetic acid*, *fish* and *valuing pollination.*

Table 2. Summary over used search words and search results.

Database	Search word	Search results	Date
LUBSearch	Bumblebees	6 996	2/2–17
	Bees water	2 022	2/2–17
	Water collection bees	141	2/2–17
	Cyanobacteria	163	4/2–17
	Cyanobacteria bees	14	4/2–17
	Control nitrate leaching	1 441	5/3-17
	Agriculture nutrients	77 407	5/3-17
	Extraction microcystins acetic acid	21	6/3-17
	Cyanobacteria fish	2 353	8/3-17
	Clover nitrate leaching	409	8/3-17
	Valuing Pollination	23	9/3-17
Web of Science	Pollinators ecosystem service	613	6/2–17
	Bumblebees	3 147	2/2–17
	Bees water	1 260	2/2–17
	Water collection bees	54	4/2–17

LUNDS UNIVERSITET

WWW.CEC.LU.SE
WWW.LU.SE

Lunds universitet

Miljövetenskaplig utbildning
Centrum för miljö- och klimatforskning
Ekologihuset
223 62 Lund