Phytoplankton and Thiamine

Studying the Growth of Phytoplankton in Thiamine Enriched Water

by

Erika Eriksson

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Supervisor: Sven Burreau High School: Vägga High School

Abstract

Thiamine deficiency has recently been identified as a massive threat to ecosystems in the Northern Hemisphere and in the Baltic region. It has been suggested that phytoplankton might be at the core of the problem and that auxotrophy regarding thiamine might make a difference to the dynamics of ecosystems. The purpose of this work was to examine the growth of phytoplankton in water enriched with thiamine, with the aim to investigate the share of thiamine auxotrophs in the Baltic Sea. In the present study the growth of phytoplankton in thiamine-enriched water has been observed by primary adding plant nutrient to water collected from the Baltic Sea. Half of the water was then enriched with thiamine while the other half was not. The water was divided into 16 Erlenmeyer flasks and left to grow. Three days later the samples were filtered with suction and the filter papers were then placed in acetone for overnight extraction. The acetone was spectrophotometrically analysed and the concentration of chlorophyll *a* in the samples was calculated. The experiment showed that the concentration of chlorophyll *a*, after the period of growth, was significantly higher (ANOVA, p=0.05) in the water enriched with thiamine than in the water that was not. This implicated that the share of thiamine auxotrophs among the phytoplankton was large at the time the experiment was carried out. The composition of phytoplankton-species in the Baltic Sea is still to be examined more closely. In conclusion, this study serves to draw attention to the underlying causes of thiamine deficiency and as an inspiration for further research in this area.

Acknowledgements

The method for filtering with suction and measuring concentrations of chlorophyll *a* used in this project was developed using *Krukprojektet* (Näslund & Rex, 1991) and *Mäta vatten* (Bydén, Larsson & Olsson, 2003), with support from Samuel Hylander. The study was designed, planned and conducted by me during the late summer and early autumn 2019.

All calculations in this project were done by myself. This work has been translated from Swedish and edited by myself as an entry to the Stockholm Junior Water Prize 2020.

I would like to express my deepest appreciation to my supervisor Sven Burreau, who with his patience provided me with guidance and insightful suggestions. I would also like to extend my gratitude to Samuel Hylander from the Linnæus University for giving me invaluable insight into thiamine deficiency and aquatic ecosystems. In addition, I would like to thank Jonathan Jilg for valuable advice and constructive criticism improving this project. Thanks should also go to Mattias Holmquist and Blekinge Archipelago Biosphere Reserve for guidance upon starting this project and for informing the public about thiamine deficiency. Special thanks to Lennart Balk for his indefatigable work to bring the thiamine deficiency to light and to Josefin Engelhardt for her helpful advice and extensive knowledge. I am also grateful to my mother Eva Eriksson for always supporting me and helping me in the best way possible.

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1. Introduction

Among animal species in a large number of food webs in the northern hemisphere an inexplicably high mortality has been observed. In the Baltic Sea region the mortality has primarily been detected in salmon, eider and elk but other species have also been affected. The animals lack a vitamin, vitamin B_1. Yet, no one has been able to ascertain why the animals are in need of vitamin B_1, which is also known as Thiamine (Engelhardt, 2019). The widely spread thiamine deficiency in and around the Baltic Sea should promptly be scrutinised, since it risks to seriously affect the biodiversity to such an extent that it can not recover.

Furthermore, action regarding this topic must be taken for Sweden to attain the 16 national environmental quality objectives. This is owing to the fact that 6 out of the 16 objectives are unreachable because of thiamine deficiency. These are: (8) *Flourishing lakes and streams*, (10) *A balanced marine environment, flourishing coastal areas and archipelagos*, (11) *Thriving wetlands*, (12) *Sustainable forests*, (13) *A varied agricultural landscape* and (16) *A rich diversity of plant and animal life* (Engelhardt, 2019 ; Swedish Environmental Protection Agency, n.d.). From a global perspective, the achievement of several Global Goals for Sustainable development are threatened by thiamine deficiency.

Moreover, the deficiency results in a complicated situation for the counties and municipalities economically dependent of animals and plants in and around the Baltic Sea. An example of this is the county of Blekinge. In Blekinge, the inhabitants does to a great extent earn their living through nature tourism like salmon-fishing and bird-watching. It is inevitable that the influx of tourists to and the profit of the salmon-fishing declines when the number of salmons decline. This entails devastating consequences for municipalities, along with inhabitants, entrepreneurs and employers, who earlier relied on income from visiting tourists (Rigoll, 2019).

1.1. Purpose and Research Question

The purpose with this project is to investigate if the share of thiamine auxotrophic phytoplankton in the Hanö Bight of the Baltic Sea in September 2019 is large. This is achieved by examining the growth of phytoplankton in thiamine enriched water from the Baltic Sea, collected in *Näsviken,* Karlshamn in the Hanö Bight.

This study will investigate the share of thiamine auxotrophic phytoplankton qualitatively based on quantitative measurements of chlorophyll *a* in water, which indicates the share of thiamine auxotrophic phytoplankton.

The above has resulted in the following research question

- How does the concentration of phytoplankton change upon addition of thiamine?

2. Background

Thiamine, vitamin B_1, is a water-soluble vitamin. Water-soluble vitamins are, with the exception of vitamin B_{12}, secreted with the urine when the demand of the body has been met Accordingly, water-soluble vitamins must consequently be continuously supplied to meet the demand. Thiamine acts, in its phosphorylated forms, especially as thiamine pyrophosphate (TPF), as a cofactor to multiple of the enzymes active in the metabolism. Therefore, the metabolism of saccharides, proteins and lipids are broken down by thiamine deficiency. This results in death (Balk, 2019).

In the late 1980s high mortality among sac fry in multiple Swedish rivers was observed. Also amid migrating salmon in North America similar mortality was found. In 1994, the Canadian researcher John D. Fitzsimons discovered that this was due to the North American salmon eggs and larvae suffering from thiamine deficiency. Later, researchers from multiple universities surrounding the Baltic Sea established that also Salmon and Sea Trout in the Baltic Sea suffered from thiamine deficiency (Balk & Hansson, 2017 ; Fridolfsson, n.d.).

When investigating the sickness closer, it appeared that the mortality with its many symptoms had emerged as early as during 1974. This gave the sickness its name, M74, a combination of the swedish word miljöfaktor (meaning environmental factor) and the year during which it was first discovered. Accordingly, it was suspected that the illness was due to environmental problems. In relation to M74, the share of females, whose offspring perishes as yolk-sac fry or embryo, is counted (Balk & Hansson, 2017 ; Fridolfsson, n.d.). Visually, ill sac fry can be identified from their pale colour, depending on low concentration of astaxanthin. Young salmons that are afflicted have impaired ability to swim and coordination. Simultaneously, they are pale, growing slow and retain their yolk sac longer than healthy fish.

A considerable problem with the sickness is that it is episodic. In *Figure 1* it can be read that, in average, almost 80% of the atlantic salmons in Swedish rivers in the middle of the 1990s suffered from thiamine deficiency. Five years earlier the number was roughly 10%.

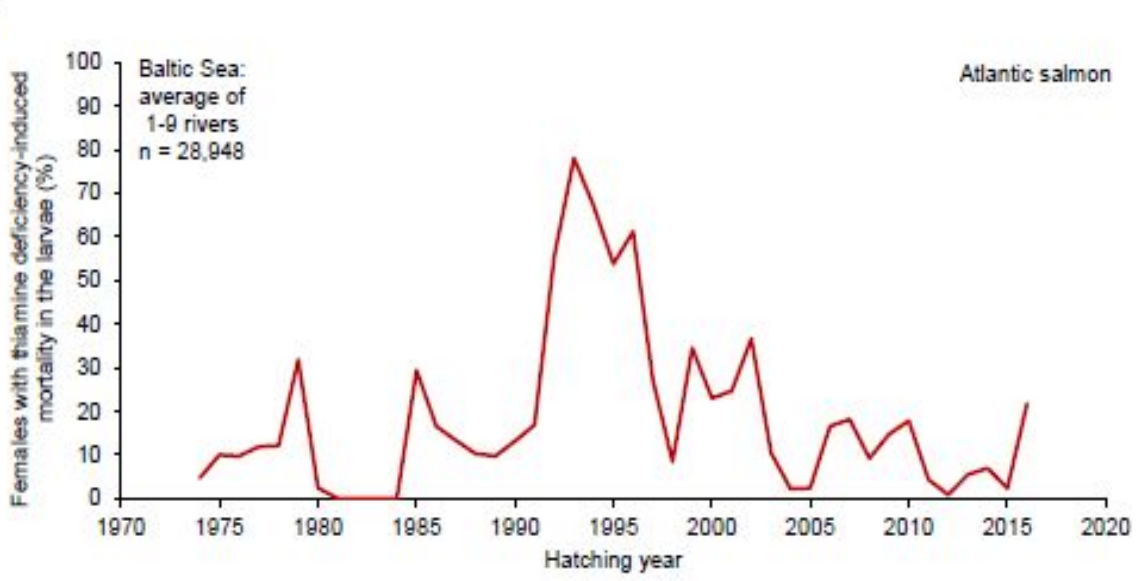

***Figure 1*:** Average occurrence of M74 in Atlantic Salmon in Swedish rivers 1970-2016 (Source: Balk, L.*et al*.,2016)

In 2009, the report *Wild birds of declining European species are dying from a thiamine deficiency syndrome* was published, in which Balk, L. *et al.* reported on finding that many of the declining bird species in northern Europe to a great extent suffered from thiamine deficiency. Moreover, during the first half of the 2010s the "elk mortality" in Blekinge, Sweden, was of great immediate interest. Only during 2012 close to 80 dead elk were found in the county. These are suspected to have suffered from thiamine deficiency, although studies proving it are yet to be published (Papadopoulou, 2013).

To identify thiamine deficiency in an individual a chemical method as well as a biochemical method can be used. However, the both are often combined to get a confident result (Engelhardt, 2019). Thiamine deficiency can also be detected by treating the individual with thiamine. Treatment with thiamine on affected individuals improves health and implies recovery but has no effect on healthy animals. In these, the superfluous thiamine is secreted with the urine (Balk & Hansson, 2018).

One matter suspected to cause the widespread thiamine deficiency is phytoplankton. Phytoplankton are planktonic algae and other autotrophs. The dynamics of a phytoplankton community varies and changes depending on water temperature, salinity, wind and weather (The County Administrative Board, n.d. ; Stockholm University Baltic Sea Centre, 2015). This implies that different species of phytoplankton are favoured by different seasons.

Phytoplankton is the foundation in aquatic food chains. Out in open masses of water the phytoplankton is responsible for the whole primary production. Together with prokaryotes such as cyanobacteria, they constitute the diet for organisms on higher trophic levels (Bernes, 2011 ; Castro & Huber , 1996).

Auxotrophic organisms, auxotrophs, are organisms who depend on supplying of a specific organic substance for their growth and are not able to synthesise it *de novo* (Auxotrof, n.d.). Out of the 332 species of phytoplankton who have been studied hitherto 27% are thiamine auxotrophs. Hence, they are, unlike most phytoplankton, unable to synthesise thiamine. Since the thiamine auxotrophs are in need of an continuous supply of thiamine, they are simultaneously affected by the solubility of thiamine.

Thiamine auxotrophs does generally not miss the whole biosynthetic path needed for producing thiamine, nor one or more vital genes. Therefore, many thiamine auxotrophs are able to synthesize thiamine if the right constituents are available (Sylvander, 2013). The concentration of dissolved thiamine, its components and degradation products in the sea is however unknown, since it in studies have shown to be non-measurable or of picomolar amount (Andersson et al., 2019).

How thiamine auxotrophic phytoplankton acquire thiamin is still unknown. However, symbiotic relationships with thiamine producing bacteria has been suggested as a possible way (Sylvander, 2013).

The biomass of planktonic algae, phytoplankton, can be obtained by measuring the content of chlorophyll *a* in water. Chlorophyll *a* serves as the most frequently used pigment when photosynthesizing. Accordingly, it is, with the concentration of chlorophyll *a* in water, possible to conclude if the water contains a lot of planktonic algae or not (SMHI, 2010).

The size of a population and its total production can be regulated either from lower trophic levels or from higher trophic levels. When an ecosystem is regulated from lower trophic levels it is called bottom-up regulation (Arvanitis, Hamza & Sundberg, 2015). If a problem, such as thiamine deficiency, exists on lower trophic levels it will consequently exist on higher ones. This is called a bottom-up effect. With thiamine deficiency the problem was originally observed among the top consumers and then among organisms lower in the food chain.

3. Method and Material

The study consisted of 16 samples of Baltic water containing phytoplankton, 8 of which that were enriched with a thiamine solution and 8 without enrichment. Thiamine enriched water is distinguished by T and non-thiamine enriched water with V. (For a simplified and lucid version of the method, see *Figure 2*.) After a 3 day period of growth the water was filtered with suction and the filter papers were left with acetone for extraction of chlorophyll from the plankton. The acetone was then analysed spectrophotometrically and the concentration of chlorophyll in the samples was calculated from the absorbances.

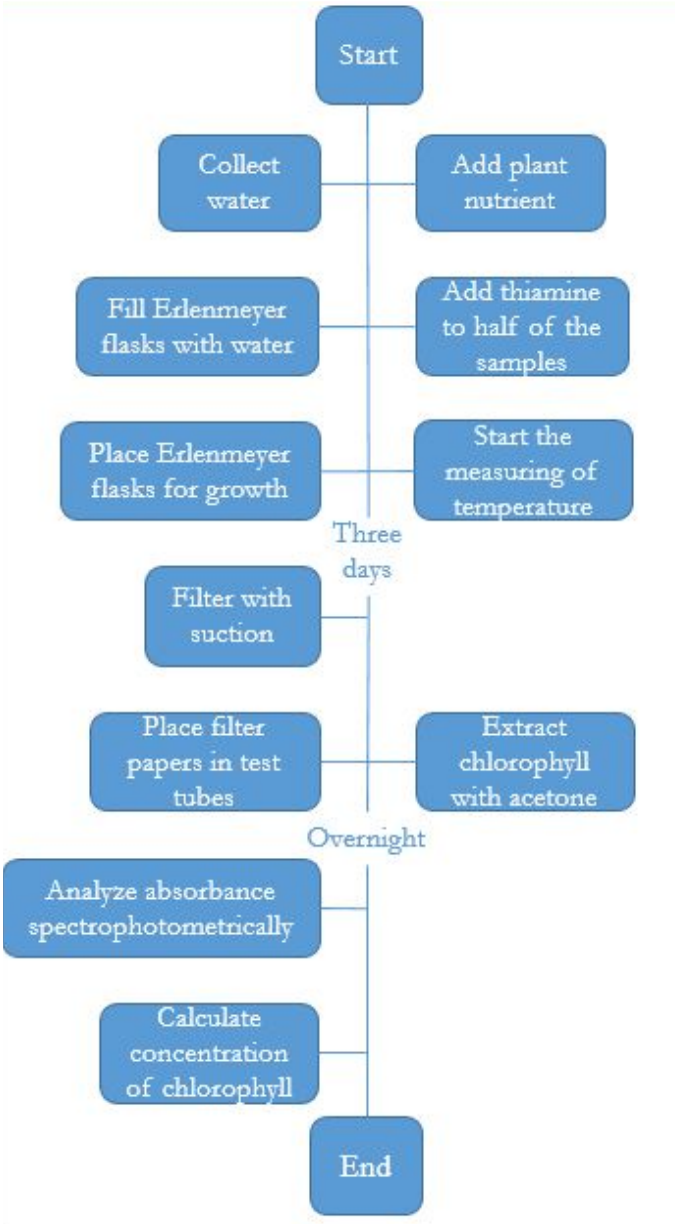

Figure 2: Method simplified in flowchart. (Source: own picture)

3.1. Material

Material for collection of water

2 Buckets (10 L) were used.

Material for preparation of water and growth of phytoplankton

A Container (> 16 L), a glass rod, a funnel, an automatic pipette (10 µL), 16 Erlenmeyer flasks (> 1 L), parafilm and a Labquest with thermometer-device were used.

Material for filtering with suction and extraction

Filter paper (Pore size 1-2 µm, Manufacturer: Grycksbo pappersbruk AB, class 00H), a Büchner funnel, a Büchner flask, a Rubber bung, Rubber tubing, a Pincette, Paper towels, 16 hermetic test tubes, a tube rack and a refrigerator (4°C) were needed.

Material for spectrophotometric analysis

A Spectrophotometer (VWR, V-1200), a cuvette, a pincette and a cloth were used.

Speciality chemicals

Blomstra plant nutrient, thiamine solution (1 g/L) and acetone (90%) were needed.

3.2. Method

Method for collection of water

Two 10-litre buckets were filled with 16 litres of Baltic water containing phytoplankton. This took place just under the surface to avoid inconvenient surface debris. The water was then transported in the buckets.

Method for preparation of water and growth of phytoplankton

The water was transferred to a large container and plant nutrient was added (1 drop/L →16 drops/16L). The water and nutrient was stirred with a glass rod. Using a funnel each Erlenmeyer flask was then filled with 0.5 L of the water (see *Figure 3*). 8 of the Erlenmeyer flasks were labeled with T (for thiamine) and, with an automatic pipette, 10 µL thiamine solution (conc.1 g/L) was added to each one. After that, all 16 Erlenmeyer flasks were filled with an additional 0.5 L of water. The Erlenmeyer flasks containing thiamine solution was

then stirred with a glass rod to achieve even distribution. The Erlenmeyer flasks were then covered with parafilm to hinder evaporation and placed on a bench by the window in a glasshouse (see *Figure 4*). A Labquest with a thermometer-device was started to measure the temperature in the glasshouse during growth. The Erlenmeyer flasks were then left there for 3 days of growth.

***Figure 3*:** Erlenmeyer flasks during filling with water. (Source: own picture)

***Figure 4*:** The placing of Erlenmeyer flasks. (Source: own picture)

Method for filtering with suction and extraction

After three days the samples were collected and taken to the laboratorium. The equipment for filtering with suction (Büchner funnel, Büchner flask, rubber bung and rubber tubing) was mounted (see *Figures 5 and 6*). A filter paper was placed in the Büchner funnel. The first sample was filtered and the filter paper was then placed, with a pincette, on the paper towel with the plankton mass facing up. There, the filter paper was dried in roughly 4 minutes in room temperature (see *Figure 7*). It was then was folded to a semicircle, with the plankton mass outwards, and rolled into a cylinder using a pincette.

The filter paper and 10 ml 90% acetone were placed in a hermetic test tube (see *Figure 8*). Using a pincette the filter paper was then adjusted to such a manner that the entire area was covered with acetone and the lid was screwed on and carefully tightened. The test tube was then labeled with a letter and number and placed in a tube rack. The Büchner funnel was cleaned and the above steps were repeated for the remaining 15 samples. The tube rack was then placed in a refrigerator for overnight extraction.

***Figure 5*: Assembled equipment for filtration with suction. (Source: own picture)**

***Figure 6*: Assembled equipment for filtration with suction. (Source: own picture)**

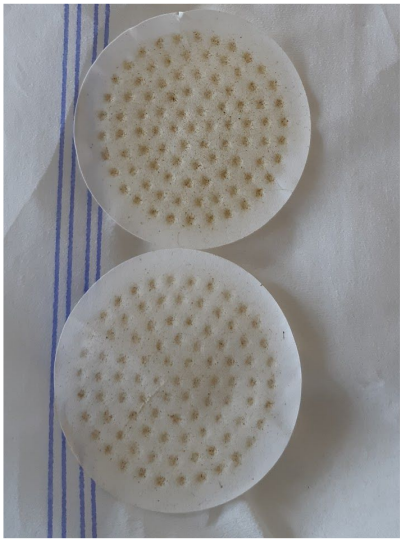

***Figure 7*: Filter paper after filtration. (Source: own picture)**

***Figure 8*: Filter paper and acetone in test tube. (Source: own picture)**

Method for spectrophotometric analysis

The spectrophotometer was started for heating, which took around 30 minutes. The samples were then collected from refrigerator and a blank with 90% acetone was prepared in a cuvette, carefully wiped with a cloth and placed in the spectrophotometer. The samples were shaken and transferred to cuvettes (see *Figure 9*). Then the cuvettes containing samples were carefully wiped with a cloth and placed two at a time in the spectrophotometer together with

the blank. The absorbance was measured and noted for the wavelengths 750 nm and 665 nm for all samples.

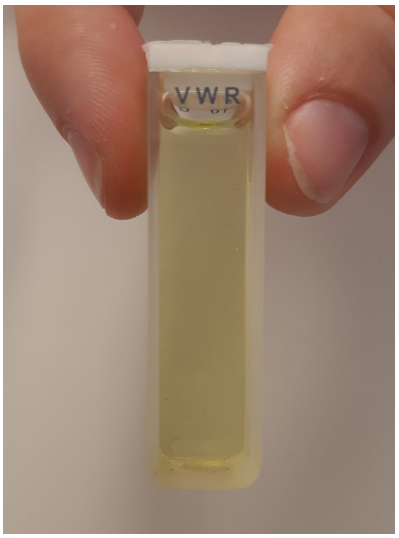

***Figure 9*:** Cuvette containing an extracted sample. (Source: own picture)

Calculation of concentration chlorophyll a

The concentration of chlorophyll *a* in each sample was calculated using the below formula, where *C* is the concentration and *Abs* is the absorbance at different wavelengths. The formula derives from *Mäta vatten* (2003) and 115 is the slope of a known linear regression for known concentrations of chlorophyll as a function of absorbance.

$$C = (Abs_{(665nm)} - Abs_{(750nm)}) \cdot 115$$

3.2.1. Adaptations

In early pilots a net was used to strain of zooplankton from the water. However, the straining had to be excluded from the method since it seemed like the phytoplankton also had been obstructed by the net intended to obstruct zooplankton. This appeared upon spectrophotometrically analysing the samples after growth, filtering and extraction. They all contained little to zero chlorophyll, a lot less than normal levels in the Baltic Sea.

Another adaptation made to the method was the altering of the amount of acetone used to extract chlorophyll. In *Krukprojektet* (Näslund & Rex,1991) and *Mäta vatten* (Bydén, Larsson & Olsson, 2003) 8 ml 90% acetone was used to extract the chlorophyll from the cells. In the method used in this project 10 ml 90% acetone was instead used. This owing to the fact that the test tubes used was so wide that 8 ml of acetone did not cover the filter paper.

4. Results

Table 2 shows absorbances measured with spectrophotometer and concentration of chlorophyll *a* calculated using the absorbances. The results derive in an experiment carried out between the 23rd and the 27th of September 2019. *Figure 10* shows the mean value of chlorophyll *a* in the two groups of samples with error bars representing the area in which the true mean value with 95% certainty lies. The error bars were calculated in Excel using a 95% confidence interval. Moreover, *Figure 11* shows the temperatures measured during the period of growth.

Table 2: shows measured absorbances from the experiment and concentrations of chlorophyll *a* calculated from absorbances.

Sample	Absorbance (750nm)	Absorbance (655nm)	Concentration chlorophyll *a* (mg/m^3)
T_1	0.000	0.117	13.455
T_2	0.010	0.182	19.780
T_3	0.006	0.136	14.950
T_4	0.002	0.108	12.190
T_5	0.006	0.127	13.915
T_6	0.010	0.131	13.915
T_7	0.010	0.164	17.710
T_8	0.002	0.112	12.650
V_1	0.006	0.082	8.740
V_2	0.009	0.071	7.130
V_3	0.008	0.105	11.155
V_4	0.005	0.066	7.015
V_5	0.010	0.112	11.730
V_6	0.003	0.085	9.430
V_7	0.010	0.114	11.960
V_8	0.003	0.061	6.670

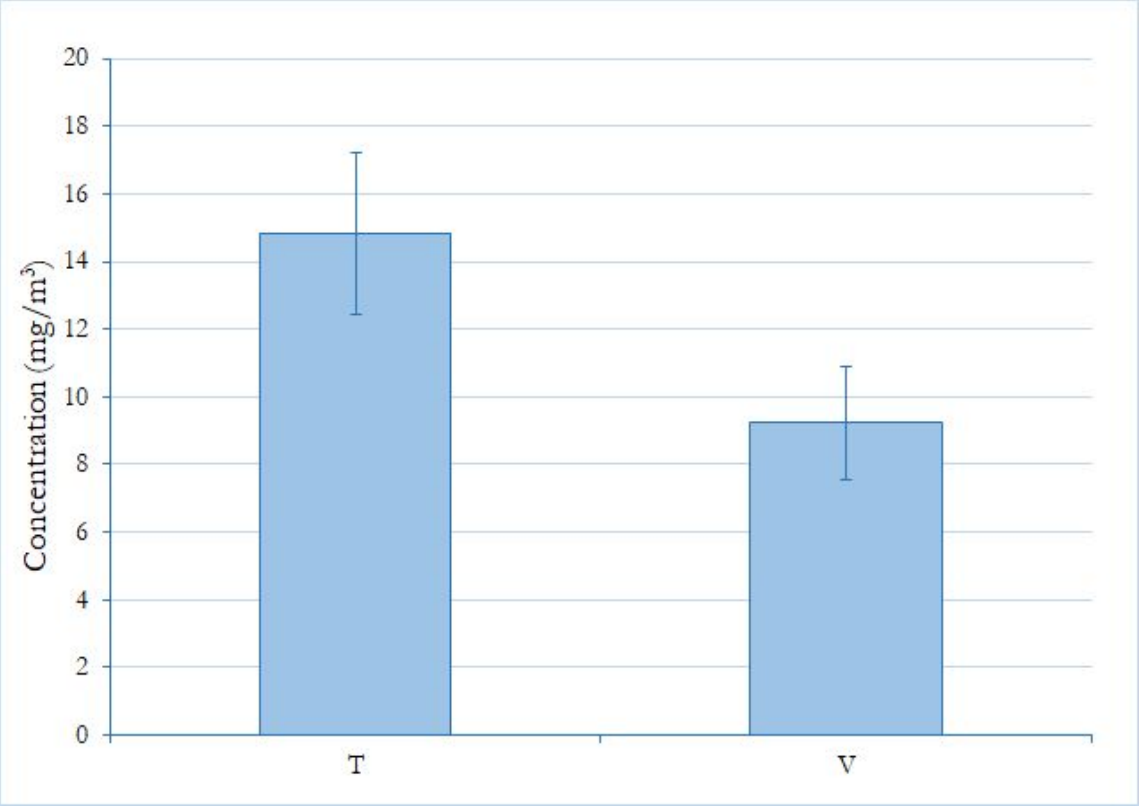

Figure 10: Mean value of concentrations of chlorophyll *a* in group T and group V. The result has been tested with ANOVA and with 95% certainty the true mean value lies within the area of the error bars (calculated using Excel).

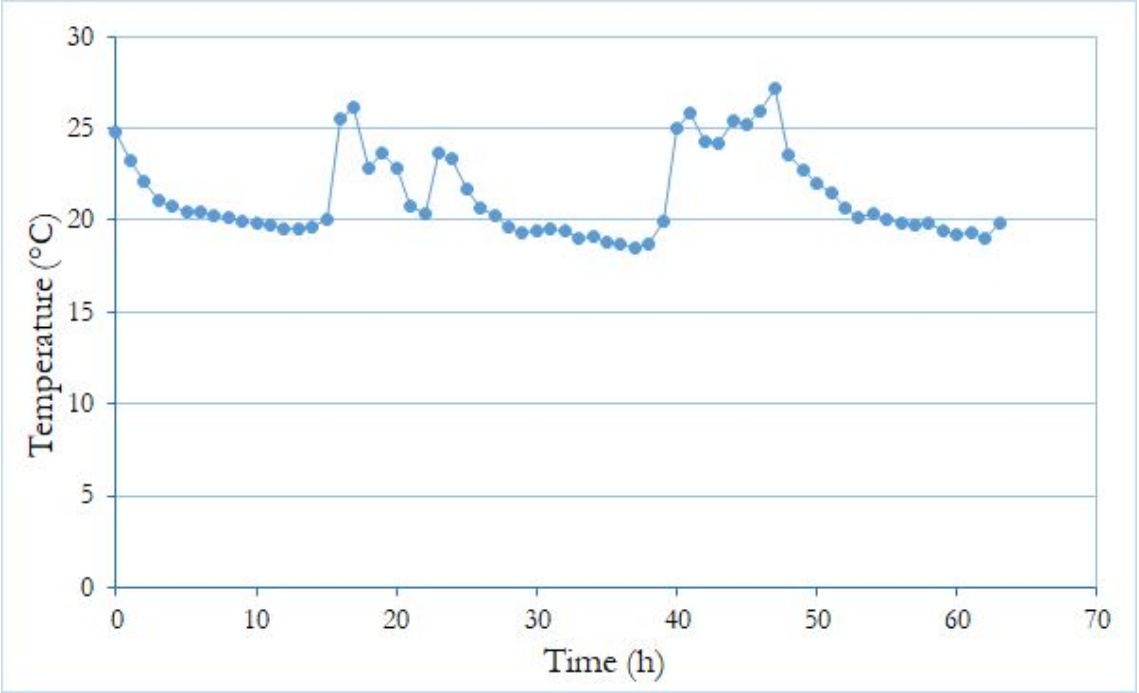

***Figure 11*:** Measured temperatures in greenhouse during growth of phytoplankton.

5. Discussion

In *Figure 10* it can be read that the concentration of chlorophyll *a* after the period of growth was higher in the thiamine enriched water than in the water without addition of the vitamin. Moreover, it was a significant difference between the two groups of samples, over 5 mg/m^3, meaning the mean of the thiamine enriched samples was about 60% higher chlorophyll concentration than the mean of the non-thiamine samples. The significance of the result has been tested with ANOVA with the *p*-value 0.05. This result implies that the growth of phytoplankton was larger in the group of thiamine enriched samples than in the non-thiamine samples. This can be interpreted as the species composition among phytoplankton at the time and place of collection of water being such, that a great share individuals of thiamine auxotrophic species existed. This owing to the fact that the amount of phytoplankton increased significantly. In turn, this could indicate that there is a possibility that the share of thiamine auxotrophs was large in the rest of the Baltic Sea at the time.

5.1. Answering the Research Question

- How does the concentration of phytoplankton change upon addition of thiamine?

The study that has been conducted indicates that the concentration of phytoplankton in water from the Baltic Sea increases upon addition of thiamine. Yet, this conclusion can only be drawn for the specific thiamine concentration tested in this study. Other amounts of added thiamine could give contrasting results. For instance, thiamine could affect the phytoplankton growth differently for large amounts, potentially preventing growth. Still, a significant difference could be identified in this study indicating that the concentration of phytoplankton increases upon addition of the specific amount of thiamine used in this study. With the limitations of this study taken into consideration, the conclusion that the study came to a result indicating that the concentration of phytoplankton increases when adding thiamine can therefore be made.

5.2. Method Discussion

A factor that perchance had an influence on the final result was the decision to remove the net, that in early pilots was used to strain of zooplankton (see 3.2.1.). The zooplankton was removed to prevent them from consuming phytoplankton an thereby affecting the result. The

results in theses pilots were however implausible, since it seemed like the phytoplankton also had been obstructed by the net intended to obstruct zooplankton. This showed by the samples containing extremely low concentrations of chlorophyll *a* when spectrophotometrically analysed, lower than before the period of growth. As a result of this, the step with the straining of water was excluded from the used method.

Likewise, the temperature during the period of growth could be interesting for the result. In *Figure 11* it can be read that the difference between temperatures during night and day were big. This considerable difference does not resemble the small differences in temperature in the water of the Baltic Sea. This has most likely affected the total growth of phytoplankton but can also have had a particular effect on certain species of phytoplankton.

To improve this study, the concentration of chlorophyll *a* in the water should be measured without growth, to ascertain how much phytoplankton that were there to begin with. This should be done to get a value of reference for the concentration of chlorophyll *a* before growth to use as a comparison with the result.

In addition, to continue the project and further map the phytoplankton communities of the Baltic Sea out the study should be done multiple times in the course of a year, since the species composition in the Baltic Sea varies a lot during the different seasons. This is decisive, since the share of thiamine auxotrophs could be changing a lot in the course of a year. It is possible that the share of thiamine auxotrophic phytoplankton was appreciably large or small when the study was carried out and if that is the case, the result would presumably have looked different if the study had been carried out during another season.

Furthermore, the results of studies like this one on different locations and depths in the Baltic Sea would have been interesting to compare. This owing to the fact that the living conditions also vary depending on location and depth.

5.3. Conclusion

From this study, the conclusion that thiamine addition increases growth of phytoplankton in water from the Baltic Sea, is made. This conclusion indicates that the water collected and tested contained a large share of thiamine auxotrophs, which could affect the ongoing thiamine deficiency in and around the Baltic Sea.

It is possible that humans with their ways of life have altered the environment in the Baltic Sea in such a way that thiamine auxotrophic phytoplankton is favoured. This could be problematic, since such species could outcompete other, thiamine producing species of phytoplankton. This could in turn lead to a disequilibrium in thiamine levels, where the amount of thiamine produced in the ecosystems of the Baltic Sea is smaller than the amount needed to assimilate the need of thiamine among organisms on higher trophic levels. This would result in a widespread thiamine deficiency.

Lastly, it is important to stress the urgency and importance in learning which the underlying causes of thiamine deficiency are and acting against it. The acute and widespread thiamine deficiency undermines the biodiversity and threatens both economy and welfare. In addition, to attain both 6 of the Swedish national environmental quality objectives and multiple of the Global Goals for Sustainable development action must be taken.

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