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Master's Thesis

Cultivation of *Tetraselmis suecica* with different light regimes to improve the survival of *Ostrea edulis* larvae in hatcheries

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Statutory Declaration

I hereby declare, that I have authored this thesis independently, that I have not used any other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources, and that this work or a similar version has not yet been part of a study or exam performance.

Helgoland, February 16, 2022 Signature: 

Only after the last tree has been cut down,

Only after the last river has been poisoned,

Only after the last fish has been caught,

Then will you find that money cannot be eaten.

— Slogan of the U.S. and West German environmental movements in the 1980s

Abstract

In the context of the reintroduction project of the European oyster *Ostrea edulis* (Linnaeus, 1758), a hatchery is necessary as the stocks cannot be restored naturally due to the strong decline caused by anthropogenic impacts in the past years. Optimizing the survival rate of the larvae is of great importance in this respect. The increased use of antibiotics is intended to face this challenge. However, this approach cannot be applied in the framework of ecological restoration, since it does more harm than good. Alternatives must be developed.

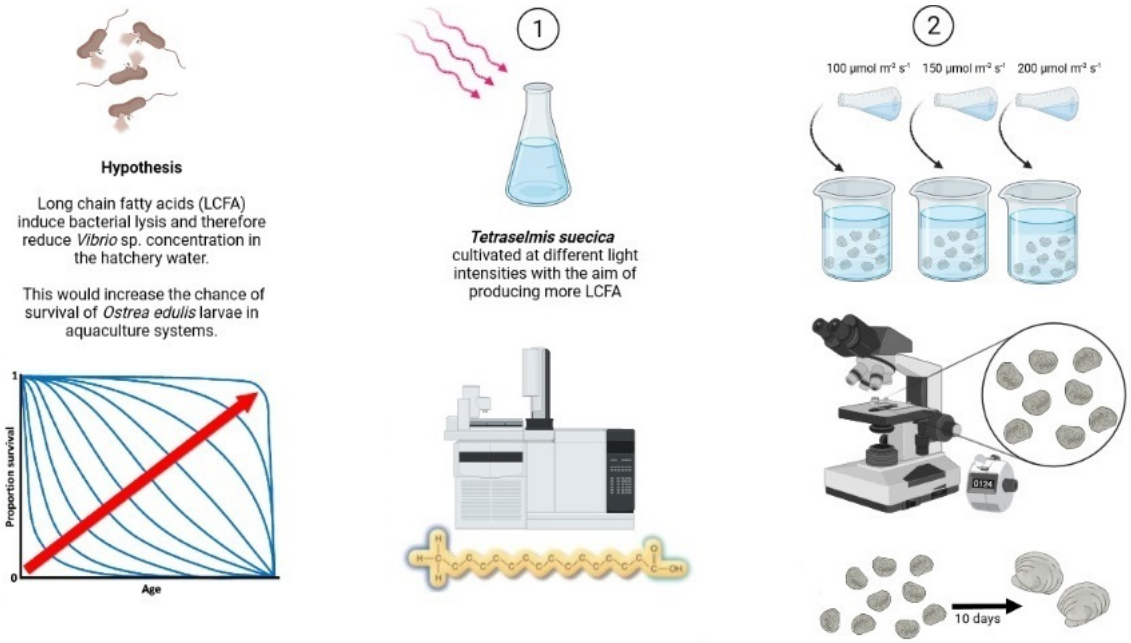
In this work, the long chain fatty acids (LCFA) of *Tetraselmis suecica* exposed to different light intensities (100, 150 and 200 µmol photons m^{-2} s^{-1}) were analyzed. According to various references, light is one of the factors that is supposed to influence LCFA concentration. Additionally, it is hypothesized that with higher LCFA concentrations, also antibacterial activity in microalgae culture is increased. This antibacterial activity can be utilized to reduce bacterial infection of sensitive *Ostrea edulis* larvae grown in large-scale aquaculture. The *T. suecica* cultures maintained at different light intensities were each added to a 3-mixed diet with *Isochrysis galbana* and *Chaetoceros muelleri* in beakers containing 700 mL of 1 µm filtered and UV-treated seawater and 3,500 *O. edulis* larvae. Controls with a 2-mixed-diet addition with only *I. galbana* and *C. muelleri* and a 3-mixed-diet addition with *I. galbana*, *C. muelleri* and *Rhodomonas salina* served as control. The results were used to examine whether the addition of *T. suecica* to the holding water increases the larval survival rates. Thus, in this work, sustainable alternatives to the use of antibiotics in hatcheries were sought.

Light intensity was found to have no impact on the percentage of fatty acids in their complete composition per *T. suecica* cell, but did affect the total LCFA concentration. It was found that the highest LCFA concentration per *T. suecica* cell was found in cultures grown at a light intensity of 150 µmol m^{-2} s^{-1}. The lowest LCFA concentration per *T. suecica* cell was found in cultures maintained at 200 µmol m^{-2} s^{-1}.

Compared to the 2-mixed-diet control, the 3-mixed-diet treatments did not perform as well. For example, in the treatments with the lowest LCFA concentration in the *T. suecica* cells (cultivation at 200 µmol m^{-2} s^{-1}), a 100 % larval mortality rate was observed. Clear results could not be obtained in this sub-trial, but a tendency for better survival of *O. edulis* larvae with increased long chain fatty acid content in *T. suecica* cultures using as feed was evident. However, besides the LCFA concentration of the food, other influencing factors regarding the survival of the sensitive *O. edulis* larvae should be considered and excluded in the future. Here, it is recommended to optimize the experimental set-up to get clearer results.

Graphical abstract

Cultivation of *Tetraselmis suecica* with different light regimes to improve the survival of *Ostrea edulis* larvae in hatcheries



Keywords: *Tetraselmis suecica*, Fatty acids, Antibacterial, Light intensity, *Ostrea edulis*, Oyster, Larvae, Hatchery, Aquaculture

Table of Contents

List of Figures

List of Tables

List of Symbols

Symbol	Unit	Meaning
ARA	–	Arachidonic acid
AS	–	Autoclaved seawater
β	g L^{-1}	Biomass concentration
C1 or C2	–	Control 1 or 2
DHA	–	Docosahexaenoic acid
DMS	–	Dimethyl sulfide
DMSP	–	Dimethyl sulfoniopropionate
DO	mg L^{-1} or %	Dissolved oxygen
EPA	–	Eicosapentaenoic acid
F_{x}	d^{-1}	Feeding amount per day
FA	–	Fatty acids
l	µm	Cell size
LCFA	–	Long chain fatty acids
m_{bio}	mg per 10^6 cells	Dry mass
msl	µm	Mean shell lenght
μ	d^{-1}	Specific growth rate
n	–	Number (of microalgae species)
N_{L}	–	Number of larvae to be fed
N_{m}	d^{-1}	Cells ingested per larva per day
PUFA	–	Polyunsaturated fatty acids
T1 or T2 or T3	–	Treatment 1, 2 or 3
S	PSU	Salinity
t	d	Time
T	°C	Temperature
t_{d}	d^{-1}	Doubling time
TDA	–	Tropodithietic acid
TK	–	Temperature-controlled
v_{bio}	μm^{-3}	Cell volume
VP	10^6 cells mL^{-1} d^{-1}	Volumetric productivity
v/v_{x}	–	*Isochrysis* equivalent factor
x_{max}	10^6 cells mL^{-1}	Cell density

1 Introduction

Once, the European Flat Oyster *Ostrea edulis* (Linnaeus, 1758) was native and widespread in the North Sea (Gercken and Schmidt, 2014). Considering the high demand for seafood as a highly valued product for consumption, oysters were fished for centuries until the 1920s (Key and Davidson, 1981). However, due to intensive fishing, it is now deemed as functionally extinct and is therefore on Germany's Red List (Rachor et al., 1998), which documents species threatened with extinction. In addition, ground-touching fishing methods (trawls) have destroyed their valuable ecosystem: oyster reefs. Over-harvesting also caused harm to oyster populations that could not recover quickly enough and continued to decline (Gercken and Schmidt, 2014; Pogoda, 2019).

Yet, *O. edulis* is recognized as a key ecological species in its typical community of species. This is because their oyster reefs provide important ecosystem functions and services and are considered a hotspot of biodiversity in marine waters. As a biogenic reef builder, this species provides many valuable ecosystem services that positively influence the surrounding flora and fauna. The biogenic reef is a settlement substrate for the flora growing on it and for the fauna associated with it in various ways . Its countless nooks and crannies provide habitat for hundreds of species, from small fish and invertebrates seeking shelter to larger fish searching for food (Haelters and Kerckhof, 2009). Furthermore, their high filtration capacity can also improve water quality in the oceans, for example, by reducing concentrations of locally toxic algal blooms. They also increase benthopelagic coupling and consolidate loose sediments by ingesting and recycling planktonic organisms and suspended organic matter Sawusdee et al. (2015). On account of the latter point, they play an essential role in coastal protection and thus also for humans.

Due to the many ecological services provided by *O. edulis*, it belongs to the specially protected habitat type "reef" according to the Fauna-Flora-Habitat Directive, whose favorable conservation status is to be maintained or restored (Pogoda et al., 2020). Their reintroduction into the North Sea can induce succession of many other plant and animal species, contributing to an immediate increase in ecosystem biodiversity and enhancement of the surrounding ecosystem.

Currently, a research pilot reef of *O. edulis* is being established as a conservation measure in the Natura 2000 site Borkum Reefground as part of the ongoing RESTORE II project, conducted by the Alfred-Wegener-Institute (AWI), Bremerhaven. It is biologically and ecologically investigated as the preliminary investigation has already delivered positive results (RESTORE I). Comprehensive field investigations have shown the basic suitability of *O. edulis* for reintroduction into the German North Sea under the environmental conditions prevailing today. For these experiments, approximately 24,000 certified healthy seed oysters with a shell length of 2 mm from various European hatcheries were released (Merk

et al., 2020; Pogoda et al., 2020).

To counteract the concrete problem of long-term availability of sufficiently suitable seed oysters for marine conservation measures (Pogoda et al., 2019), a technologically innovative breeding facility for the establishment of healthy parent stock and the production of suitable seed oysters was established at AWI's Helgoland site as part of the PROCEED subproject, since stable oyster seed production is a mandatory requirement for the successful reintroduction of *O. edulis*. This project aims to provide and support the long-term restoration program with sufficient seed oysters not only through mere production but also through targeted research, thus ensuring the conservation and enhancement of biodiversity through the oyster bed habitat.

In commercial aquaculture, *O. edulis* production is still largely reliant on natural seed production and is primarily based on empirical concepts (Colsoul et al., 2021). Considering the special requirements in the context of ecological restoration, the absence of pathogens and the maintenance of high genetic diversity is indispensable, but the current supply is insufficient. Many of the problems faced by producers have become the subject of research, but not all of them have been solved yet, as some questions remain unanswered (zu Ermgassen et al., 2020). Also, some problems and issues have not even been included in the research and should be addressed as soon as possible. In this respect, there are still many open questions and relevant research topics for sustainable seed supply that need to be explored in this area.

1.1 Bacteria as the most common cause of larval mortality in oyster aquaculture

Repeatedly, the major issue in shellfish hatcheries is bacteria that may infect the larvae in large-scale systems (Brown, 1981; Lodeiros et al., 1987; Prado et al., 2005, 2014; Petton et al., 2015; Dubert et al., 2016, 2017). Since different ontogenetic stages of marine species can differ in their susceptibility to microbial agents, infectious diseases can be crucial for the mortality of a developing oyster (Guillard, 1959). Especially in the early stages, larvae are susceptible to any interfering factors due to the rudimentary immune system, which has not yet fully developed (Wang et al., 2018). According to Kesarcodi-Watson et al. (2012), larval rearing of the Pacific oyster *Crassostrea gigas* has so far been unproblematic, but survival rates seem to be rather poor for the larval rearing of *O. edulis*. The European oyster is mostly known for its sensitivity to water pollution and diseases, and needs therefore very clean water for its survival within hatcheries. This is exactly why the lethal effect of bacteria on the most sensitive phase of the life cycle is the reason for the biological failure of this species in a hatchery (His et al., 1999).

The bacteria responsible for the occurrence of mass mortalities in oyster aquaculture are mainly within the genus *Vibrio* sp., which may originate from untreated broodstock, algal, and larval cultures (Colsoul et al., 2021). *Vibrio* spp. are ubiquitous in marine waters, and a total of 118 species are recognized in this genus, wherein classification and accurate identification are still problematic (Oliver et al., 2012). Due to their genetic and

ecological variability, they are often implicated in several and severe diseases that could lead to malformations or even high mortalities of oyster larvae (Mardones-Toledo et al., 2015; Travers et al., 2015). The species *Vibrio anguillarum*, for example, is known to produce potent exotoxins, including a low molecular weight ciliostatic toxin that inhibits beating of the cilia of the velum in larvae (Helm et al., 2004). Despite the pathogenic potential of many strains (Wendling et al., 2014), their virulence can vary among hosts and the environment (Wendling and Wegner, 2015; Wendling et al., 2017). In case that one of the opportunistic pathogens enters the hatchery, this incident can result in severe epizootics (Colsoul et al., 2021), which are further favored by rearing at high larval density (Goulden et al., 2013). Even though the number of *Vibrio* sp. in hatchery influent water can be effectively reduced by filtration and UV light, *Vibrio* strains may still be detected as part of the normal flora in the hatchery and within the larval tanks (Lodeiros et al., 1987).

However, when the first symptoms of disease are observed, it may already be too late to avoid contagion with considerable biological and economic losses. Hence, there is a strong incentive to take prophylactic action and discover a way to obtain a stable hatchery that promotes high larval health and survival.

1.2 The antibacterial activity of *Tetraselmis suecica*

Antibiotics are the most commonly used method to control bacterial infection, but they can cause the emergence of resistant strains of bacteria that prolong and complicate the infection (Dubert et al., 2016). Other, more natural methods should therefore be considered and developed.

Microalgae, for example, are not only essential as a food source but also improve the quality of the aquaculture stock (Brown and Blackburn, 2013). Some of them may have non-nutritional factors but stimulate the digestive and immune systems of marine organisms' larvae (Kokou et al., 2012). Different microalgae species have been studied for their antibacterial activity *in-vitro* and in co-culture with pathogenic bacteria, and some studies have also been conducted *in-vivo* with the "green water" technique, and using microalgae as nutritional supplements in recent years (Falaise et al., 2016). It can be inferred that microalgae may have advantages in terms of live-food quality for various marine organisms by reducing the number of associated pathogenic *Vibrio* strains and allowing a lower risk of transmission to oyster larvae.

Even though feeding with *Tetraselmis suecica* (Kylin) Butcher, 1959 for broodstock conditioning is not recommended as a particular feed due to the low ingestion and absorption by the *O. edulis* and poor biochemical compound's transfer (González-Araya et al., 2011), it is still proven, that *T. suecica* can improve larval growth of *O. edulis* when feeding them with a three-species dietary which includes this kind of species (Helm et al., 2004). However, even a diet with a bi-specific combination including *T. suecica* may provide a better-balanced diet and larval growth can be significantly higher (Helm, 1977).

The *Vibrio* inhibitory activity of *T. suecica* has been reported not only *in-vivo* using *T. suecica* as a food supplement for marine organisms such as the Atlantic salmon or

White prawn (Austin and Day, 1990; Regunathan and Wesley, 2004), but also *in-vitro* using the microalgal supernatant (Austin and Day, 1990; Austin et al., 1992). Thus, the use and the potential application of this microalga in hatcheries can be seen as beneficial in suppressing the colonization of *Vibrio* sp., including potentially pathogenic species, like *Vibrio alginolyticus* and *V. anguillarum*.

It is strongly suspected that certain fatty acids inhibit bacterial growth, but the exact active components have not been isolated and characterized yet. Many authors have already found antibacterial activities of microalgae due to fatty acids, which may also apply to *T. suecica*. The bactericidal effects of unsaturated and saturated long chain fatty acids has been described by Nieman (1954) and Galbraith and Miller (1973*a*,*b*,*c*). They have shown that fatty acids with a chain length of more than ten carbon atoms induce lysis of bacterial protoplasts. Only a few compounds with activity against marine bacteria have been accurately characterized. However, Desbois, Mearns-Spragg and Smith, 2009 found that eicosapentaenoic acid (EPA) is one of them. In addition, long chain polyunsaturated fatty acids (PUFA) are, as for most marine organisms, essential for oyster (larval) growth and development (Webb, 1983).

The cosmopolitan, green microalgae *T. suecica* is well known for its high protein PUFA's (Schwenzfeier et al., 2011), especially EPA (Fábregas et al., 2001), and its vitamin E (α-tocopherol) content (Bong and Loh, 2013). The latter was found to have the highest antioxidant activity *in-vivo* (Kamal-Eldin and Appelqvist, 1996) and therefore, is able to support the health of humans and animals. The protein composition of *T. suecica* was further linearly linked to *Artemia* sp. growth and survival rates when semi-continuous cultures were maintained at renewal rates up to 40% (Fábregas et al., 2001). However, Helm et al. (2004) also demonstrated improved growth in larval rearing of *O. edulis* by adding *T. suecica* to the initial dietary components of *Chaetoceros muelleri* (Lemmermann, 1898) and *Isochrysis galbana* (Parke, 1949). The recommended food ratio for feeding *O. edulis* larvae is 0.1 : 1 : 0.75 for *T. suecica*, *I. galbana* and *C. muelleri* (Helm et al., 2004).

1.3 State of the Art and background of applied research

The microalga *T. suecica* can grow under autotrophic, heterotrophic, and mixotrophic conditions (Grabowski, 2017), whereby the biomass concentration under mixotrophic conditions (Fábregas et al., 1997) is higher than under autotrophic conditions (Ulloa et al., 2012). However, the relative cost of the substrate to products in terms of energy balance is more costly for heterotrophic than for autotrophic cultivation (Chen et al., 2011).

Performance productivity has not yet been verified for vertical photobioreactors (PBRs) which are at Helgoland Oyster Hatchery (AWI), where the microalgae are cultivated for oyster feeding. In order to efficiently use the microalgae production unit of the hatchery, these reactors should also be used to cultivate an additional species such as *T. suecica* in the same room, and thus, this species is also to be cultivated under photoautotrophic conditions.

Various cultivation techniques can manipulate the biomass composition of microalgae. These cultivation techniques are mainly related to some of the controllable cultivation

and environmental factors such as nutrients, light, and temperature, which are known to influence algal growth and biomass composition (Markou et al., 2012). Light intensity is one of the most important limiting factors in microalgae cultivation. Thus, light duration and intensity can directly affect the photosynthesis and biochemical composition of microalgae. Guzmán et al. (2010) have already demonstrated in their study that fatty acids composition of microalgae is significantly affected by irradiation. The fact was also substantiated by Go et al. (2012). Here, a scale of 15 L batch cultures was studied. There were no significant differences between the tested treatments by varying the nitrogen concentration, but there were differences found with changing light intensities which were examined separately (Go et al., 2012). In this study however, as in many others, the focus was not placed on looking at essential fatty acids for oysters but more important ones for fuel/oil production (Balloni et al., 1983; Rodolfi et al., 2009; Bondioli et al., 2012). Much of the research done on *T. suecica* focused on finding potential feedstocks for biofuel production.

Studies have also been carried out by investigating the change in the chemical composition of *T. suecica* by different salinities and pH values. Here, it was found that the highest protein, lipid, and carbohydrate contents were detected under the conditions of 30 PSU and pH = 7.5 - 8.5 (Khatoon et al., 2014). Cultivation of *T. suecica* was shown to be most productive at a temperature of approximately 22 °C (Laing and Helm, 1981). However, this study did not provide qualitative values for light intensity, which further complicates the standardized and optimal cultivation of *T. suecica* on a large-scale in this respect.

Many large-scale production works were obtained with Conway medium but not with f/2 medium (Guillards and Ryther, 1962). This cultivation strategy should be optimized in the hatcheries' algal production in which f/2 medium is commonly used for microalgae species to save costs by not using a different kind of nutrient media. Here, 'Cell-Hi F2P' medium from Varicon Aqua is used - a commercial medium with a composition comparable to the f/2 medium, but the exact components are not published. This medium proved to be quite advantageous due to the fast production process and the excellent growth results for all microalgae species cultivated here at AWI, Helgoland. So far, these species are *I. galbana*, *C. muelleri* and *Rhodomonas salina* (Wislouch) D. R. A. Hill and R. Wetherbee, 1989.

1.4 Aim and objectives

In this study, three different light intensities for the cultivation of *T. suecica* were tested. By examining the growth rates, cell volumes, and sizes as well as its biomass at different growth stages of the microalgae cultivated in different light intensities, this study aims to provide answers to the possibility of producing this microalgae species under optimal light conditions for oyster feeding in *O. edulis* hatcheries and improving their survival rate in large-scale aquaculture systems. In addition, fatty acids were measured in the *T. suecica* cultures to determine their composition at different light intensities, as these, especially long-chained ones, could be important for the antibacterial activity of this species.

2 Material and methods

2.1 Source and preparation of culture media

For the cultivation of marine microalgae in the Helgoland Oyster Hatchery (AWI), the 'Cell-Hi F2P' medium from Varicon Aqua (= F2P medium) is used for all species as its production proved to be very time-saving. In the experiements of this study, this medium was also used to represent the microalgae cultivation in aquaculture. The composition of the commercial medium is based on the f/2 medium (Guillards & Ryther, 1962), and thus contains nutrients that are necessary for optimal algae growth, such as carbon, inorganic nitrogen and phosphorus compounds, vitamins, and trace metals (see Tab. 2.1). The required F2P stock solution was prepared at 1000x concentration according to the recommendation of the 'Cell-Hi' product sheet by dissolving 500 g of the powdered F2P medium in 5 L distilled water in an autoclaved bottle. According to the manufacturer, the final product can be autoclaved. So, to ensure the absence of contamination of any kind and thus longer shelf life, the solution was autoclaved and filter-sterilized (Syringe filters Minisart® NML Plus Sterile (ETO-sterilized), pore size: 0.2 µm). Since vitamins are particularly heat-sensitive, vitamins in the medium may be destroyed due to the high temperature occurring during the autoclaving process. To ensure that the loss of vitamins would not be a limiting factor in the microalgae cultivation, they should therefore be added to the medium after this process. The F2P stock solution was stored in a cool, dry, and clean area. To replace the missing vitamins, a vitamin mix was prepared separately consisting of 890 mL MilliQ water, 100 mL of a 2 g L^{-1} thiamine, 10 mL of a 0.1 g L^{-1} biotin (vitamin H) and 1 mL of 1 g L^{-1} cyanocobalamin (vitamin B12) stock solution, which was also filter-sterilized with the use of two filter holder and bottle (Nalgene Thermo Scientific, PSF GL 45, 500 mL) a vacuum pump (sartorius stedim biotech, Microsart® mini.vac, Göttingen, Germany) and a membrane filter (Whatman, GF/D, 47 mm, pore size: 0.2 µm). The filtrate was then divided into 50 mL aliquots in Falcon tubes (Greiner Bio-one, 50 mL polypropylene tube, PP, 30x115 mm, conical bottom, graduated, sterile) and frozen at -20 °C until use. It should be noted that viruses, prions, and DNA fragments can pass through these filters.

Since the experiments conducted in the work did not include diatoms, no sodium silicate solution was needed.

2.2 Upscaling process of *Tetraselmis suecica*

Starter cultures of *T. suecica* (4x 15 mL) were obtained from Ifremer Laboratoire PFOM/L-PI (strain: CCAP 66/4). Initial stock cultures were subcultured in six 50 mL culture flasks containing 40 mL nutrient-enriched seawater (1000x F2P stock solution and vitamins). The stock cultures were maintained by repeated weekly subculturing into 50 mL culture flasks and 100 mL Duran® Erlenmeyer flasks, narrow necks every week. Algal production was then scaled up to 200 mL with an 20 % v/v inoculation of the stock culture and then 1 L and 5 L SCHOTT botlles, and 10 L in carboys following the same procedure.

Tab. 2.1: Chemical components and their final molar concentration [M] in the f/2 medium (Guillard & Ryther, 1962), including the carbon, nitrogen and phosphorus supply. In addition, this table shows also the composition and molar concentration of the trace metal and vitamin solution in the final medium. The respective stock solutions were prepared in sufficient quantity, autoclaved, and stored in the refrigerator at 5 °C until using.

	Components	**Concentration [M]**
Nitrate	$NaNO_3$	8.82×10^{-4}
Phosphate	$NaH_2PO_4 \times H_2O$	3.62×10^{-5}
Carbon	Na_2CO_3	1.06×10^{-4}
Trace metal	$FeCl_3 \times 6\ H_2O$	1.17×10^{-5}
	$Na_2(EDTA) \times 2\ H_2O$	1.17×10^{-5}
	$CuSO_4 \times 5\ H_2O$	3.93×10^{-8}
	$Na_2MoO_4 \times 2\ H_2O$	2.60×10^{-8}
	$ZnSO_4 \times 7\ H_2O$	7.65×10^{-8}
	$CoCl_2 \times 6\ H_2O$	4.20×10^{-8}
	$MnCl_2 \times 4\ H_2O$	9.10×10^{-7}
Vitamin	thiamine HCl	2.96×10^{-7}
	Biotin	2.05×10^{-9}
	Cyanocobalamin	3.69×10^{-10}

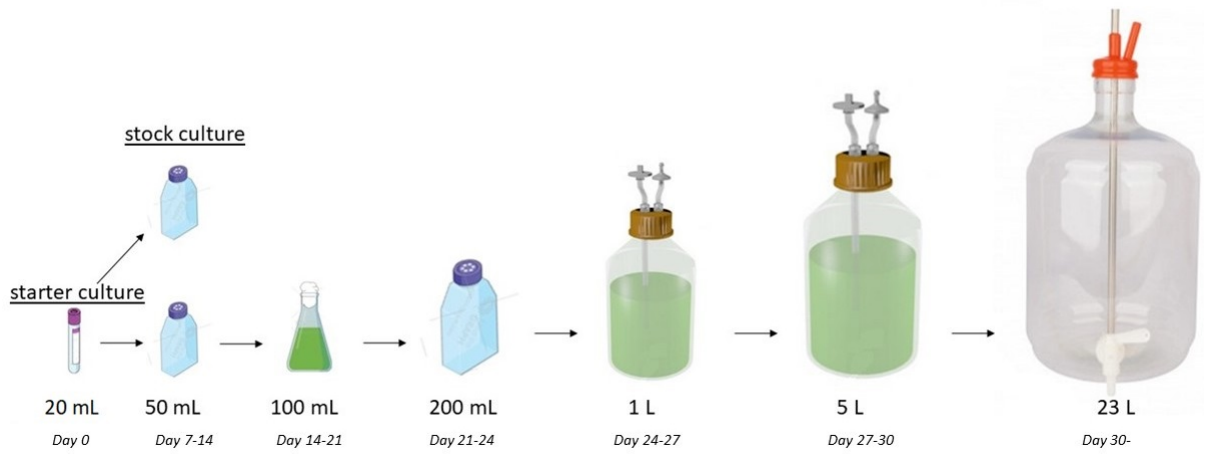


Fig. 2.1: Exemplary routine used for microalgae cultivation at the Helgoland Oyster Hatchery (AWI, Project PROCEED). Sketch was created using an infographic tool called "BioRender"(online version).

2.3 Preliminary experiment

In order to reduce or even eliminate any contamination starting from microalgae cultures that could affect the larval growth and survival rate of *O. edulis*, it was decided to perform a preliminary experiment to select the best option for the cultivation of *T. suecica* in the main experiment. The optimization of the cultivation process in terms of the maximum possible absence of contamination in cultures is essential in terms of the ability to make a statement about the behavior of *O. edulis* larvae with a single factor – namely, the possible improvement of water quality by using *T. suecica* as an antibacterial microalgae species and thus, reducing bacterial contamination of the seawater used in the Helgoland

Oyster Hatchery. These experiments were performed in a batch system with a continuous atmospheric air supply.

For both preliminary experiments described below, samples were taken from each bottle/carboy daily using a disposable 10 mL syringe (B.Braun, Omnifix® Luer Lock Solo), transferred to a 50 mL Falcon tube. Cell concentration and size were measured using the Multisizer™ 4e Coulter Counter® (Beckman Coulter GmbH, Krefeld, Germany). Also, all cultures were microscopically inspected for contamination at 400x magnification every day using a Hemocytometer (Improved Neubauer chamber, Brand GmbH + Co KG, Wertheim, Germany) – three replicates with and three without fixation with Lugol's solution. Photos, sketches and videos of any contamination type were made to facilitate their identification.

2.3.1 Large-scale (10 L) cultivation

For this experiment, four 23 L PET carboys (Øbase: 25 cm, Humlegardens Ekolager AB, Sollentuna, Sweden) were used, which, including their lids, were chemically disinfected prior to use. These were filled with 8 L of 0.2 µm filtered and UV-treated seawater, which was chemically disinfected with 75 mL of 5.2 % sodium hypochlorite (0.013 % v/v) and then neutralized with 13 mL of 2.6 % sodium thiosulfate (0.007 % v/v) after an exposure time of at least 1 hour. The calculation of the exact required amount of sodium hypochlorite and thiosulfate is attached to the work as an appendix (A1).

After filling the carboys with seawater, 10 mL of the prior filter-sterilized F2P medium and 10 mL vitamin solutions were added. Then the carboys were each inoculated with 2 L of dense *T. suecica* culture (20 % v/v). Two of the upscaled and contamination-free 5 L cultures were used as inoculum for four carboys.

All carboys were stored in a temperature-controlled room (20 °C) and connected to an air supply via autoclaved glass tubes and the PVC hoses. The air was filtered using in-line Venting filters (Midisart 2000, PTFE membrane, pore size: 0.2 µm). The carboys had a distance of exactly 10 cm to the LED-light system integrated into the rack (SYLVANIA Start, Twin 1500 IP65, waterproof cool-white LED, 7100 lm). The cultures were cultivated with an approximate light intensity of 360 µmol m^{-2} s^{-1} and a light period of 12:12.

Abiotic factors such as water temperature, pH, and salinity were measured every three days.

2.3.2 Small-scale (1 L) cultivation

Due to the increased contamination in the carboys, another preliminary experiment was performed, which involved growing the microalgae in smaller, more sterile cultures since the 1 L SCHOTT bottles are autoclavable.

This preliminary experiment also aims to provide answers to the necessity of autoclaving seawater after water filtration (pore size: 0.2 µm) and UV treatment. Not autoclaving seawater can lead to time savings and thus, it can increase the efficiency of process runs by allowing culture flasks to be autoclaved along with other solids that need to be sterilized.

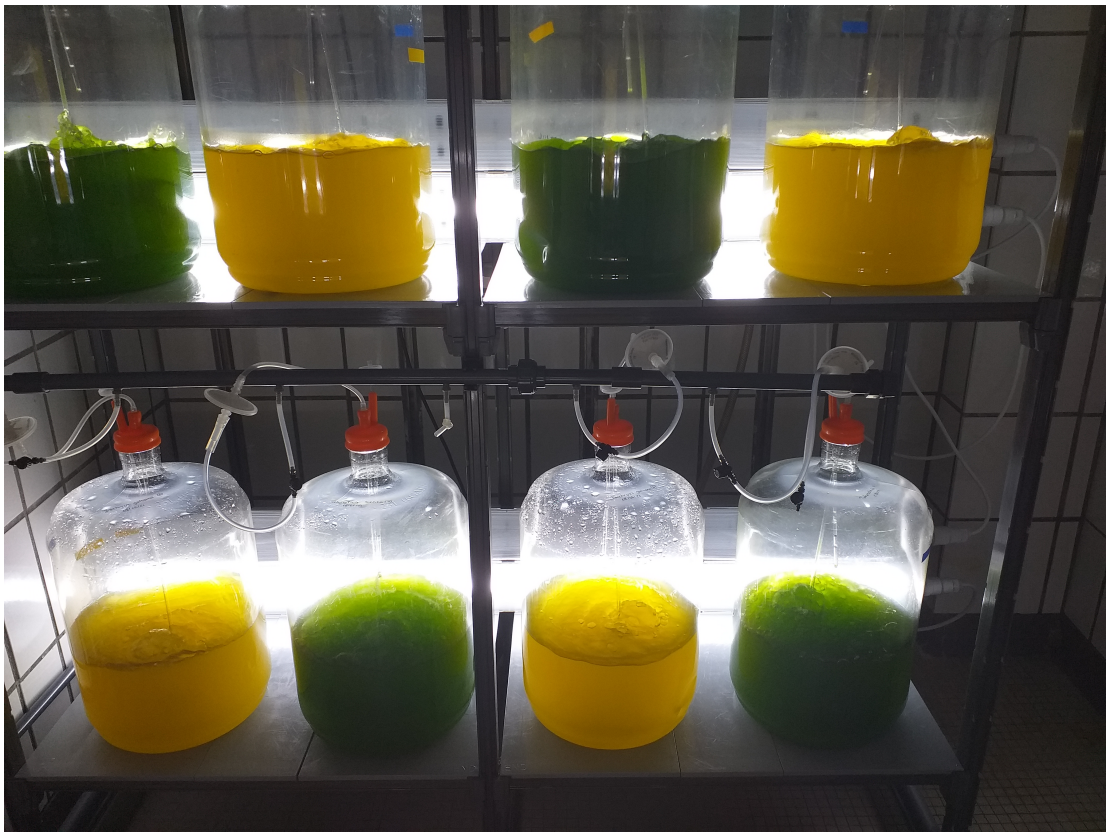


Fig. 2.2: Experimental setup of the contamination trial with *Tetraselmis suecica* in 10 L cultures kept in 23 L PET carboys. Between them are also cultures of *Isochrysis galbana* for the feed production *Ostrea edulis* larvae.

Liquids must go through other autoclaving processes to ultimately reach the same level of sterilization as solids. An autoclave run takes about 2 hours.

A silicon tube, glass tubes including the PVC hoses, and three 1 L SCHOTT bottles were autoclaved using the program for autoclaving solids (Systec VX-150, Systec GmbH, Linden, Germany). After disinfecting the water outlet with ethanol, the silicone tube was attached to it. The autoclaved bottles were each filled with 700 mL of 0.2 µm filtered and UV-treated seawater, but not autoclaved again. During the filling, the bottle openings were kept as concealed as possible by the lid to minimize the degree of contamination that could enter the bottles. These replicates will be referred to as "US" (= unautoclaved seawater) from now on.

Further, three bottles were filled with 200 mL of 1 µm filtered seawater and autoclaved together with the glass tubes and PVC hoses, which were already integrated with the lid, using the program for autoclaving liquids. These replicates will be referred to as "AS" (= autoclaved seawater) from now on.

All bottles were inoculated with 300 mL of *T. suecica* under the clean bench after enrichment with 1 mL of F2P medium and 1 mL of vitamin solution each (starting cell concentration approximately 500,000 cells per mL). Cultures were maintained in a temperature-controlled room (TK room 25) at 22 °C, supplied with 0.2 µm filtered

atmospheric air. Light intensities were 100, 150, 200 µmol m^{-2} s^{-1} for each US and AS bottle with a dark-to-light period of 12:12.

2.4 Main experiment

The main experiment was divided into two sub-experiments, which should build on and be based on each other. First, the cultivation of *T. suecica* took place under three different light intensities to manipulate the fatty acid composition in the culture, and second, different feeding strategies, including the different *T. suecica* cultures exposed to different light intensities were performed on *O. edulis* larvae to determine with which strategy the larvae grow and survive best. Here, this experiment should provide answers to the possible extent of *T. suecica*'s influence on the survival rate of susceptible *O. edulis* larvae under consideration of the fatty acid composition. It is hypothesized that the higher the concentration of long chain fatty acids in *T. suecica* cells, the more likely bacteria can be eliminated due to the inducing lysis of their protoplast. This implies that *T. suecica* might contribute to a better survival rate of *O. edulis* larvae in aquaculture systems.

2.4.1 Cultivation of *T. suecica* under different light conditions and discontinuous operation

Four replicates per light treatment were planned for the trial. The upscaled 1 L bottles were mixed and used for inoculating twelve 1 L bottles after adding 1 mL of each culture medium stock solutions (F2P and vitamins), which were aerated by atmospheric 0.2 µm filtered air using a glass tube, PVC hose, and in-line Venting filters.
All cultures were grown in a TK room at 22 °C using artificial light with a photoperiod of 12:12 and an illumination of 100, 150, and 200 µmol m^{-2} s^{-1} (SYLVANIA Start, Twin 1500 IP65, waterproof cool-white LED, 4276 lm) by varying the distance from the bottle edges to the LED-lamps. The bottles with the respective light treatments were distributed randomly in the TK room (Fig. 2.3) to minimize statistically random mistakes in the evaluation. An opaque tarp was hung in front of the algae cultures to prevent external light influences that could manipulate the experiment.
Samples were taken from each bottle daily, using a 10 mL syringe and transferred to a 50 mL Falcon tubes to measure the cell density (x), size (l), and volume (V_{bio}) by using the Multisizer 4e. The samples were also microscopically inspected every day (Kern Optics, Balingen, Germany; 400x zoom) to monitor possible contamination. Abiotic factors such as water temperature (T), pH, and salinity (S) were measured daily to ensure standardized experimental conditions. A growth curve was created in the end. Organic biomass as dry mass per cell (m_{bio}) was also measured every day by filtering 5 mL of the samples through muffled and in HCl acidified circular glass fiber filters (Whatman, GF/D, 25 mm, 2.7 µm), subsequently washing with distilled water, and drying those at 105 °C in a drying cabinet (Drying and heating cabinet UL 50 with natural air circulation, Memmert GmbH + Co. KG, Schwabach, Germany) for at least 24 hours (= until dry mass was constant). Then, biomass concentrations (β) in g L^{-1} were calculated.

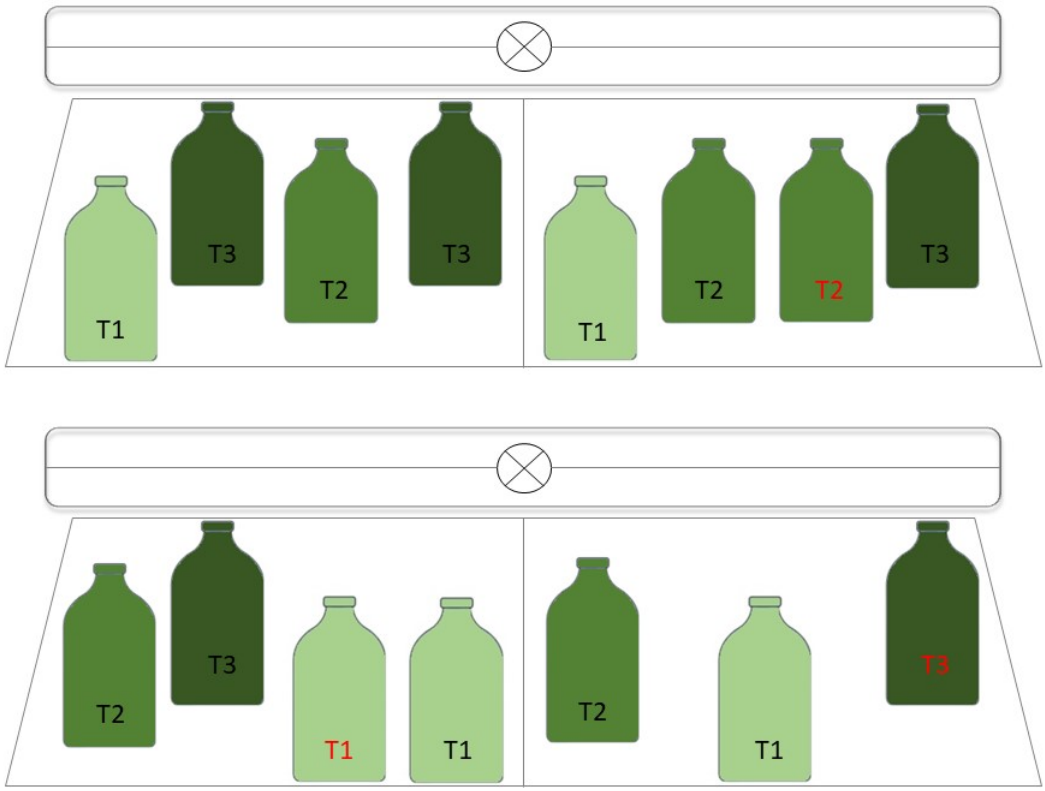


Fig. 2.3: Experimental design of the main experiment with *Tetraselmis suecica* cultured in three different light intensities by varying the distance to the lamp (X). T1 = 100 µmol photons m^{-2} s^{-1}, T2 = 150 µmol m^{-2} s^{-1}, T3 = 200 µmol m^{-2} s^{-1}. Bottles with red labels represent cultures where abiotic factors were measured. This experiment was performed in 1 L Schott bottles linked to an atmospheric air supply and took place at a temperature-controlled room at 22 °C.

After all cultures have reached the stationary phase, the cultivation of the microalgae changes to semi-continuous by mixing all cultures maintained at the same light intensity in a 5 L SCHOTT bottle (total volume of 4 L). Daily, 600 mL of culture were replaced with fresh seawater, enriched with medium and vitamins (15 % renewal rate). The light conditions remained the same (one culture each at 100 = T1, 150 = T2 and 200 µmol m^{-2} s^{-1} = T3). The semi-continuous cultivation ensures that the cultures have a stable cell concentration until the time of feeding and have sufficient nutrients to grow and survive after it was also verified that the fatty acid concentrations showed barely any change in the course of growth.

2.4.2 Fatty acids analysis

Samples were taken at the beginning, middle, and end of the exponential growth phase of *T. suecica* cultures for fatty acid (FA) analysis. For this purpose, samples were taken from each culture using a syringe, and 5 mL of them were transferred by filtration onto a 0.2 µm Whatman filter at a maximum pressure of 400 bar. Filters were stored in glass tubes (Duran®, culture and screwed thread tube GL 14 with DIN-thread, outer diameter 12 mm)

at -80 °C until definitive analysis, which were previously rinsed with a solution consisting of dichloromethane (DCM)/methanol (ratio of 2:1) and 200 mg butylhydroxytoluene (BHT)/L (= DCM/metnhanol+BHT) and with n-hexane and dried under the fume hood. Other materials needed for FA analysis, such as additional Duran® glass tubes, Pasteur pipettes, and conical glass vials, were rinsed and dried accordingly.

For FA extraction, 4 mL of the DCM/methanol+BHT was added to the glass tubes containing the respective filters. For the internal standard, 10 µL of C23 solution (= methyl tricosanoate dissolved in 250 µg mL^{-1} n-hexane) was added. Samples were frozen at -80 °C for another 72 hours.

After freeze-drying, the samples were defrosted again and the liquid was transferred to new Duran® glass tubes using a Pasteur pipette, rinsed twice each with 1 mL DCM/-methanol+BHT solution, and transferred again. Then, 2 mL of KCl (concentration of 0.88 %) were added to the transferred liquid for dehydration. The samples were then centrifuged (Table centrifuge, incl. three rotors, Maschinenfabrik Berthold Hermle AG, Gosheim, Germany) for 10 minutes at a temperature of 4 °C and a rotation of 750 rpm (Rcf 1500 g). The upper phase was then removed and discarded. The lower phase was transferred to a new Duran® glass tube using a Pasteur pipette. The transferred samples in the glass tube were then blown out with elemental nitrogen using an evaporator (N-EVAP™ 11645 Nitrogen Evaporator, Organomation Associates, Inc., River Road West Berlin, USA) until no liquid was found in the tube. For esterification of fatty acids to fatty acid methyl esters (abbreviated as FAME), 3 mL of methanol/sulfuric acid (97:3 ratio) were added to the Duran® glass tubes after blowing out and sampled were placed in a block heater (Stuart® SBH130D/3/120v Block Heater, 3 Block, Digital, 130 deg C, 120v, Cole Parmer, Staffordshire, UK) at 70 °C for 1 h and 15 min. After the glass tubes cooled, they were refilled with 2 mL each of n-hexane and centrifuged for 10 min at 4 °C and 750 rpm. The upper phase was transferred to a conical glass vial, which was again blown out with elemental nitrogen. Then, 50 µL of n-hexane were added, and the vials were sealed using crimping tongs. Storage was at -5 °C until measurement in a gas chromatograph (GC).

The measurement was performed using the GC machine (Gas Chromatograph system CP-3800 with FID & Splitless Capillary Inlet, Varian Medical Systems, Inc., Palo Alto, California) and the Galaxie™ software (Varian Inc. 2002-2005, Chromatogram Data System Photodiode Array Software, version 1.9). The chromatograms were “cleaned“ after the measurement. The “cleaning“ involves comparing peaks, eliminating fluctuations in the baseline that were detected as peaks, and integrating peaks adequately.

2.4.3 Feeding experiment design: experimental setup and feeding calculation

The *O. edulis* larvae were taken with a collector from the Helgoland Oyster Hatchery in section 1. During the incubation through the parental animals and at the time of larval collection, the water temperature was 20 °C, the pH value 8, and the salinity 32 PSU in the (collection) tank. The dissolved oxygen content in the holding water was DO = 9 mg L^{-1} or about 100 %. The parent stock were fed with two types of microalgae: *R. salina* and *C. muelleri*. However, their larvae generally need to be fed with other microalgae species.

In most hatcheries, *I. galbana* is used for larval feeding instead of *R. salina* because this combination showed the best satisfactory larval development in the species of *O. edulis* (González-Araya et al., 2012; Robert et al., 2017).

For the experiment, 20 glass beakers were filled with a volume of 700 mL 1 µm filtered seawater and 3,500 larvae added (= 5 larvae mL^{-1}). These beakers were supplied with atmospheric air and stored opaque in a TK room (T = 24 °C). The beakers with the different feeding strategies were placed randomly (Fig.2.4). Water was changed daily, and the water was always maintained at a constant temperature for at least 24 hours by storing it in the same room to ensure a constant water temperature for the larvae.

Larval survival and growth were determined by sampling exactly 1 mL into 6-well plates (determination in triplicates of each beaker). Thorough but careful mixing of each beaker was ensured prior to sampling. Larval monitoring and analyses were performed under a binocular (Digital stereo microscope SZX16 with camera adapter U-TV0.63XL, Olympus Europa SE & Co. KG, Hamburg, Germany). Photos were taken and analyzed using an image analysis software (Olympus Cooperation 2008 - 2016, cellSense, version: 1.12) in which the mean shell length of each larva was measured. After each water exchange, the larvae were fed with different microalgae compositions. It was considered that approximately the same biovolume of microalgae was fed on each larva. Calculations were made according to Helm et al. (2004), referring to cell volume equivalences of different microalgae species (Tab. 2.2) and known feeding rates of larvae with different mean shell length (Tab. 2.3).

Tab. 2.2: *Isochrysis galbana* cell volume equivalences, where the biovolume of one cell *I. galbana* corresponds to 40 $µm^3$. Here, the equivalence factor v/v_x is derived from the biovolume ratio between one *I. galbana* cell and the respective other microalgal species cell. The table shows only the *I. galbana* equivalences, of the microalgae species used in the work.

Microalgae species	Microalgae abbreviation = x	Biovolume [µm³]	*Isochrysis galbana* equiv. = v/v_x	Reference*
Isochrysis galbana	IG	40	1.00	A
Chaetoceros muelleri	CM	53	0.75	A
Rhodomonas salina	RS	160	0.25	B
Tetraselmis suecica	TS	308	0.13	A

* A = Helm et al., 2004, B = Gonzalez Araya et al., 2012

The required feeding amounts (= F_x) of the individual microalgae species per beaker and day were calculated using this self-established formula based on example calculations according to Helm et al. (2004):

$$F_x = \frac{1}{n} \cdot N_m \cdot N_L \cdot \frac{v}{v_x} \tag{2.1}$$

Since $v/v_{IG} = 1.00$ (see Tab. 2.2), the abbreviated formula 2.1 is derived for the *I. galbana* cell number to be fed to *O. edulis* larvae. Following, this means for the four microalgal species listed in Table 2.2 in general:

$$F_{IG} = \frac{1}{n} \cdot N_m \cdot N_L \tag{2.2}$$

$$F_{\mathrm{CM}} = \frac{1}{n} \cdot N_{\mathrm{m}} \cdot N_{\mathrm{L}} \cdot \frac{v}{v_{\mathrm{CM}}} \tag{2.3}$$

$$F_{\mathrm{RS}} = \frac{1}{n} \cdot N_{\mathrm{m}} \cdot N_{\mathrm{L}} \cdot \frac{v}{v_{\mathrm{RS}}} \tag{2.4}$$

$$F_{\mathrm{TS}} = \frac{1}{n} \cdot N_{\mathrm{m}} \cdot N_{\mathrm{L}} \cdot \frac{v}{v_{\mathrm{TS}}} \tag{2.5}$$

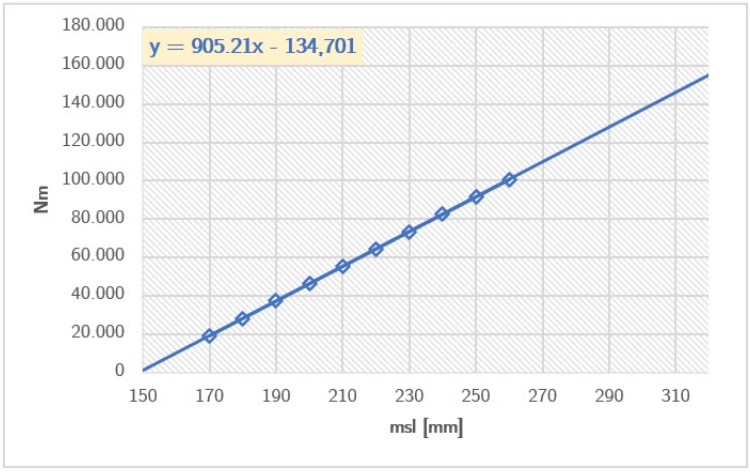
From which the following formula for the entire feed is ultimately derived:

$$F_{\mathrm{total}} = \frac{1}{n} \cdot N_{\mathrm{m}} \cdot N_{\mathrm{L}} \cdot \sum_{i=1}^{n} \frac{v}{v_{\mathrm{x}}} \tag{2.6}$$

with n = Number of microalgae species intended for the feeding strategy
N_{m} = Cells (*Isochrysis* equiv.) ingested per larva per day (Tab. 2.3)
N_{L} = Number of larvae to be fed
v/v_{x} = *Isochrysis* equivalent factor, where x = microalgae species (Tab. 2.2)
F_{x} = Total cell number of algae that is to be fed, where x = microalgae species.

Tab. 2.3: Number of microalgae cells ingested per *Ostrea edulis* larva per day (= N_{m}) relative to its mean shell length (= msl). Values are shown as cells equivalent in size to *Isochrysis galbana*, where the right graph should illustrate the impression of a linear relationship between msl and N_{m}.

msl [µm]	N_{m}
170	19,200
180	28,200
190	37,300
200	46,300
210	55,400
220	64,500
230	73,500
240	82,600
250	91,600
260	100,600



Following feeding strategies (= treatment) were tested in this work (4 replicates each):

- *I. galbana* + *C. muelleri* (control 1 = **C1**)
- *I. galbana* + *C. muelleri* + *R. salina* (control 2 = **C2**)
- *I. galbana* + *C. muelleri* + *T. suecica* (light treatment 1 = 100 µmol m^{-2} $^{-1}$ = **T1**)
- *I. galbana* + *C. muelleri* + *T. suecica* (light treatment 2 = 150 µmol m^{-2} $^{-1}$ = **T2**)
- *I. galbana* + *C. muelleri* + *T. suecica* (light treatment 3 = 200 µmol m^{-2} $^{-1}$ = **T3**)

Thereby, *I. galbana* was the strain SAG 13/92, *C. muelleri* CCAP 1010/3 and *R. salina* CCAP 978/27.

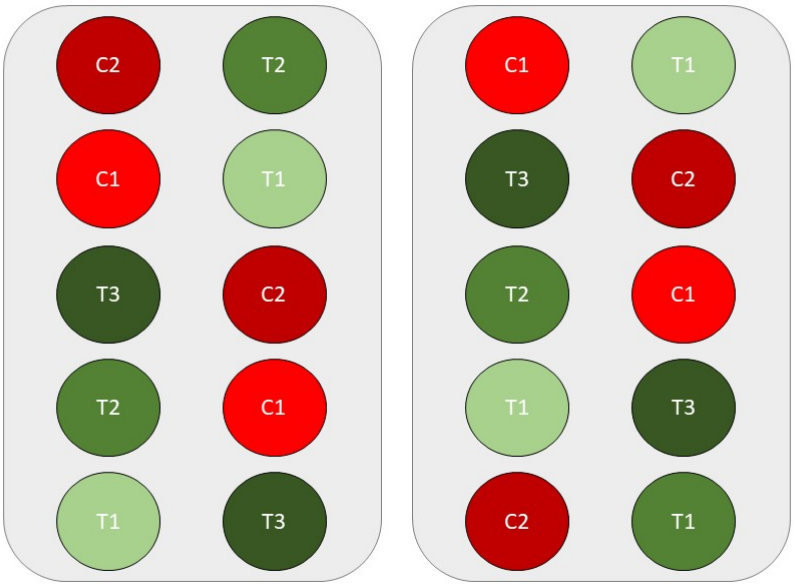


Fig. 2.4: Experimental design of the feeding experiment. There were 3,500 larvae per beaker filled with 700 mL seawater (1 µm filtered and UV treated). **C1** = control 1 = 2-mixed-diet with *Isochrysis galbana* and *Chaetoceros muelleri*, **C2** = control 2 = 3-mixed-diet with further addition of *Rhodomonas salina*, **T1** = 3-mixed diet with further addition of *Tetraselmis suecica* cultured at a light intensity of 100 µmol m^{-2} s^{-1}, **T2** = same but addition of *T. suecica* cultured at 150 µmol m^{-2} s^{-1}, **T3** = addition of *T. suecica* cultured at 200 µmol m^{-2} s^{-1}.

Calculations according to formulas 2.2-2.5 were performed for each mean shell length listed in Table 2.3 and tabulated below (Tab. 2.4).

Tab. 2.4: Calculated number of respective microalgae species to be fed per day, divided into 2-mixed and 3-mixed-diet. This table is based on the mean shell length of *Ostrea edulis* larvae, which were not only examined for survival but also for size before feeding.

2-DIET	Number of cells to feed if the oyster larvae mean shell length is ... [10^6 cells/day]									
[mm]	**170**	**180**	**190**	**200**	**210**	**220**	**230**	**240**	**250**	**260**
I. galbana	33.60	49.35	65.28	81.03	96.95	112.88	128.63	144.55	160.30	176.05
C. muelleri	25.20	37.01	48.96	60.77	72.71	84.66	96.47	108.41	120.23	132.04

3-DIET	Number of cells to feed if the oyster larvae mean shell length is ... [10^6 cells/day]									
[mm]	**170**	**180**	**190**	**200**	**210**	**220**	**230**	**240**	**250**	**260**
I. galbana	22.40	32.90	43.52	54.02	64.63	75.25	85.75	96.37	106.87	117.37
C. muelleri	16.80	24.68	32.64	40.51	48.48	56.44	64.31	72.28	80.15	88.03
T. suecica	2.91	4.28	5.66	7.02	8.40	9.78	11.15	12.53	13.89	15.26
OR										
R. salina	5.60	8.23	10.88	13.51	16.16	18.81	21.44	24.09	26.72	29.34

At the end of this sub-trial, all larvae from one treatment were mixed, counted and measured again to compare the previously collected data and verify the absolute accuracy of sampling.

2.4.4 Preparatory measures for the evaluation and statistical analyses

Based on the regularly recorded microalgae cell counts, a growth curve was created at the end for every partial experiment. The growth evolution curves were generated using the statistical program R (R core team 1995-2020, version 4.3). The formula for a bounded exponential growth curve (Kuhn et al., 2005) was adapted and created according to the respective plotted points (means of cell densities) to render a realistic approximation of a growth curve. This formula describes the microalgae growth in batch cultivation best and was therefore used for the curve construction:

$$f(x) = x_{\max} - a \cdot e^{-b \cdot t} \tag{2.7}$$

with $x_{\max}$ = maximal achievable cell density
a = constant $\in \mathbb{R}$
e = Euler's number
b = constant > 0, and t = time.

Cell density peak (x_{max}), volumetric productivity (VP), specific growth rate (μ), and doubling time (t_d) were parameters to estimate the growth of cells in the *T. suecica* culture with different light treatments. Therefore, calculations of these variables were conducted. The following formulas were used to calculate the variables mentioned above (Lee and Shen, 2004).

specific growth rate

$$\mu = \frac{ln(x_1/x_0)}{t_1 - t_0} \tag{2.8}$$

volumetric productivity

$$VP = \frac{x_1 - x_0}{t_1 - t_0} \tag{2.9}$$

doubling time

$$t_\mathrm{d} = \frac{ln(2)}{\mu} \tag{2.10}$$

A simple linear regression analysis was performed to plot the larval growth trend of the different treatments, including the two controls in terms of the increase in their mean shell length.

Statistical analyses were accomplished by also using the program R. The aforementioned collected and/or calculated data were analyzed using variance analysis. The two-sample *t*-test was used for the comparison of two groups. If the data were not normally distributed, the Mann-Whitney U test was performed instead. The significance level was set to $p < 0.05$ in all cases mentioned above.

Depending on the meeting of the conditions, the normal-model based ANOVA, Welch-ANOVA, or Kruskal-Wallis test was used for the analysis of variance for the comparison of more than two groups among each other. The significance level was set to $p < 0.05$.

Afterward, the posthoc test was performed with the TukeyHSD command for normally distributed and homogeneous variances, otherwise with the Dunn test for non-normally distributed data. For simultaneously inhomogeneous variances, the Games-Howell test was used. The significance level was set to $p < 0.017$ (after Bonferroni corrections of $n = 3$ to counteract the problem of multiple comparisons).

Since large variations in fatty acids measurements are quite common (Di Pane & Ecker, 2021, personnel communication), the significance level for the actual fatty acids content per microalgal cell with respect to the different treatments was increased to $p < 0.1$.

For testing the stochastic independence of different variables of the main experiment (e.g., light treatment of the *T. suecica* culture, its fatty acid composition, and related larval survival rate), the Chi-square test was used (null hypothesis = H_0 = data are independent of each other, $p < 0.05$), whereby the data were first transferred to a contingency table. If H_0 was rejected, the tested variables were dependent on each other, and an additional correlation test was performed. These hypothesis and significance tests were also performed with R.

3 Results

No irregularities were recognizable in the recorded data of the abiotic factors, neither in the preliminary nor in the main experiments (see Appendix: A2). The variance of ambient cultivation and water temperatures of all experiments was a maximum of ± 1 °C. Fluctuations in pH values in the algal cultures occurred but are not unusual in batch cultivation systems. Salinity in the respective cultures increased slightly by one unit over a period of one week.

3.1 Preliminary experiment

3.1.1 Contamination level in carboys

Considering the growth curve of the different replicates, the highest cell concentration of $x_{max} = 1.4 \cdot 10^6$ cells mL^{-1} on average was reached after 14 days (Fig. 3.1). The variances were within limits so that an arithmetic formation of the mean value can be permitted concerning the generation of the growth curve.

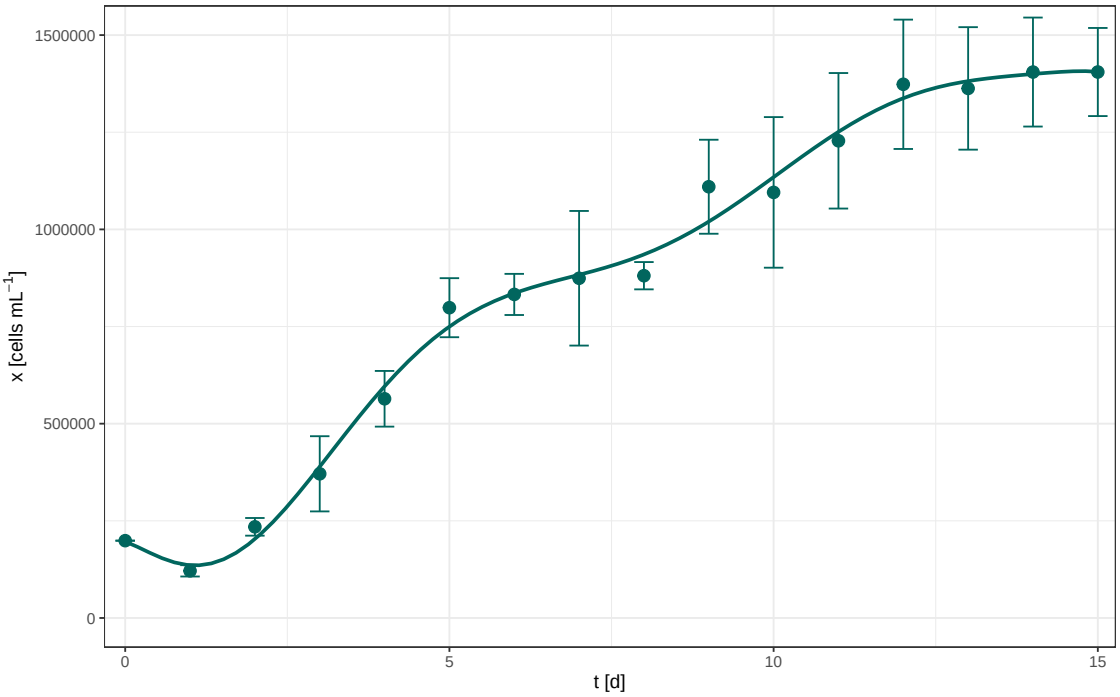


Fig. 3.1: Growth curve of *Tetraselmis suecica* cultured in carboys over a 15-day period. Plotted points show the mean of four measured carboys. No regression was performed here, the points were only connected to visualize the unstable course.

When looking at the individual carboys, biofilm formations on the carboy walls became visible already after a few hours in *T. suecica* cultures. Also, under the microscope at a magnification of 400x, some ciliates could be detected from day 4 onwards (Fig. 3.2, Fig. 3.3). An average of 2 ciliates per mL were detected per carboy; most were detected

with 10 per mL in the 3rd replicate on day 6. These included the species *Uronema* cf. *marinum* (Fig. 3.2), whose cell size is estimated to be 8 - 10 µm. The identification was made with the help of the redescription according to Thompson (1964).

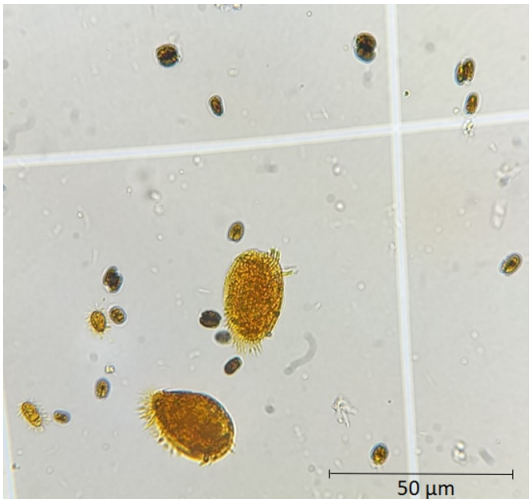


Fig. 3.2: Detail from the view through the microscope. This is a *Tetraselmis suecica* culture grown in carboys. The sample was fixed with Lugol's solution. Left: two *Uronema* cf. *marinum*, right: two *Euplotes vannus*.

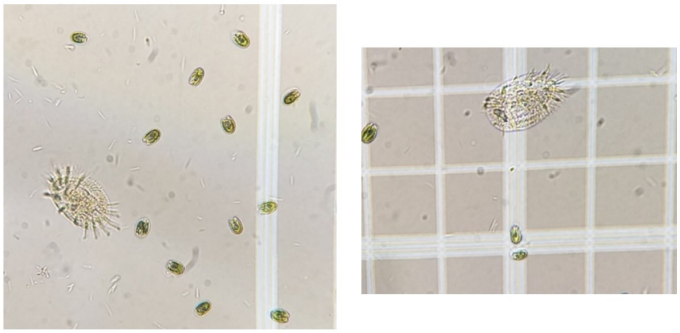


Fig. 3.3: Detail from the view through the microscope. This is a *Tetraselmis suecica* culture grown in carboys. No fixation of the sample taken. Left: Top view of *Euplotes vannus*. Right: Lateral view of *E. vannus*.

Another species was also identified, of which individuals were primarily found in a "non-activated sludge". Fronto-ventral cirri, four to five transverse cirri, two caudal cirri, two left marginal cirri were clearly visible under the microscope at a magnification of 400x (see Appendix: A3, A4). Sometimes a lateral ciliary row was visible on either side of the ventral surface, sometimes just on one side. The body shape could be described as ellipsoidal and dorsoventrally flattened about 3:2 in cross-section. The size of this species was estimated to be 50 µm with a food vacuole of about 10 µm. Nuclei were not recognizable under the microscope with the highest possible magnification. Those ciliates were identified as *Euplotes vannus* as redescribed by Jiang et al. (2019).

3.1.2 Contamination level in small-scale cultivation

Based on the visual assessment, a biofilm has occurred in the US culture bottles. It remains to be determined what type of contamination this is (Fig. 3.4, Fig. 3.5).

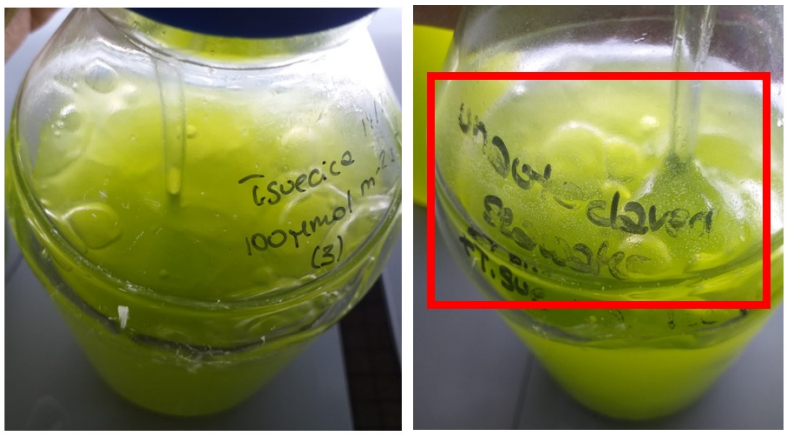


Fig. 3.4: In the cultivation bottle with unautoclaved seawater, a biofilm can be seen near the bottle neck after four days (right picture, see red rectangular border), which in turn cannot be found in the cultivation bottle with autoclaved water (left picture).

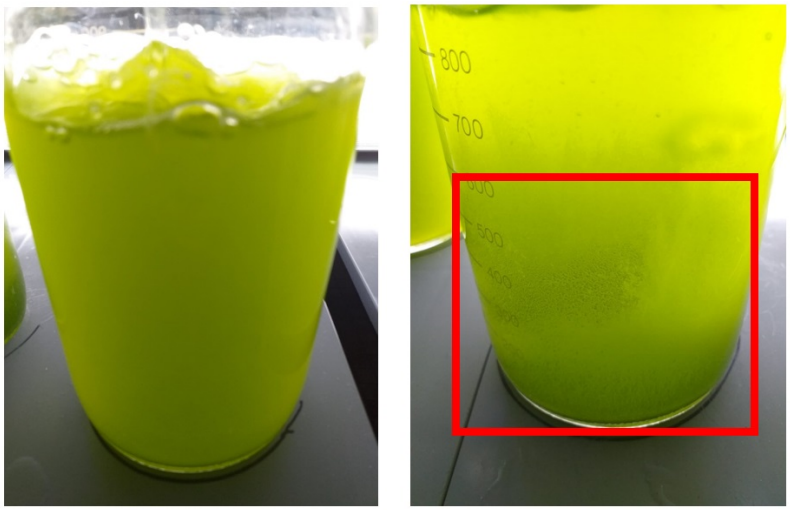


Fig. 3.5: Likewise, in the bottle with unautoclaved seawater, a biofilm was able to form in the lower part of the bottle wall as well as at the base (picture on the right, see red rectangular border). In the cultivation bottle with autoclaved seawater, no deposition of any kind can be seen (left picture).

Contamination with two other protists per mL in the US culture was detected from the 2nd day of growth. Those protists could not be identified to species level and are included here for further discussion (Fig. 3.6).

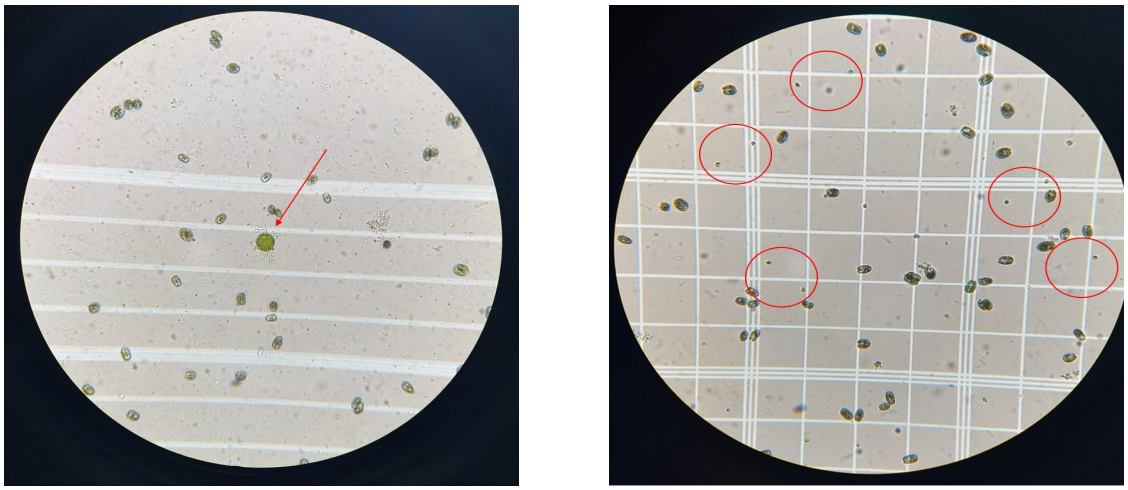


Fig. 3.6: Left: Unidentified protist in *Tetraselmis suecica* culture with unautoclaved seawater (US) (400x magnification). Recorded on the second day of growth. Right: *Tetraselmis suecica* in US culture (400x magnification). Within the red circles, we find either cell fragments, other microalgae species or bacteria. An exact identification has not been possible due to the given equipment. Recorded on the sixth day of growth.

Similarly, some green microorganisms were detected in the US cultures after about six days. While these could potentially represent other types of microalgae and thus another type of contamination, they could also be cell fragments of *T. suecica* (Fig. 3.6). However, these were not detectable in the AS culture. Also, the bacterial concentrations were striking, which were comparatively higher towards the end in the US cultures.

When considering the evolution of the microalgal growth (Fig. 3.7), small differences between US and AS cultures can be seen. While a growth maximum of about $x_{max} = 1.4 \cdot 10^6$ cells mL^{-1} was reached after seven days in the AS culture, *T. suecica* in the US culture reached a cell density of $x_{max} = 1.2 \cdot 10^6$ cells mL^{-1} after only five days. Although the US culture grew faster in the initial exponential phase (t_d [d] = 4.24), it reached the stationary phase after five days, whereas the AS culture appeared to continue growing after eight days. The growth curve of the AS culture has a flatter shape and indicates a doubling time of t_d [d] = 4.67. The growth rate of the US culture is μ [d^{-1}] = 0.163, that of the AS culture μ [d^{-1}] = 0.148.

Tab. 3.1: Arithmetic values of the growth-describing variables of VP (volumetric productivity), μ (specific growth rate), and t_d (doubling time) of AS (autoclaved seawater) and US (unautoclaved seawater) cultures, as well as the P-values.

	VP [cells mL^{-1} d^{-1}]		**μ [d^{-1}]**		**t_d [d]**	
	mn	sd	mn	sd	mn	sd
US	203,944	20,830	0.20	0.02	3.46	0.30
AS	132,873	17,991	0.14	0.01	4.84	0.26
p-value	**0.0230**		**0.005**		**0.005**	

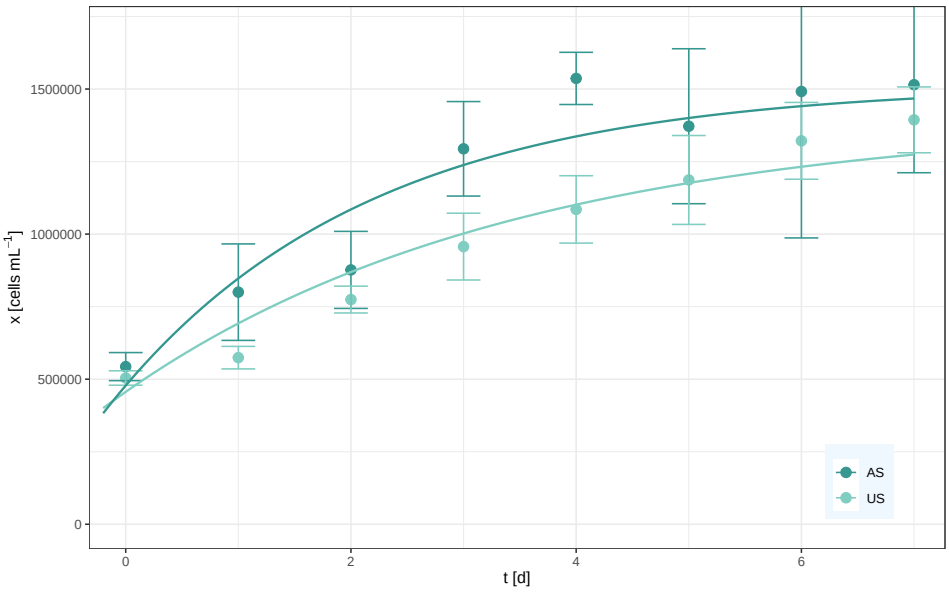


Fig. 3.7: Growth curves of *Tetraselmis suecica* 1 L cultureswith autoclaved (AS) and unautoclaved seawater (US), over a seven-day period.

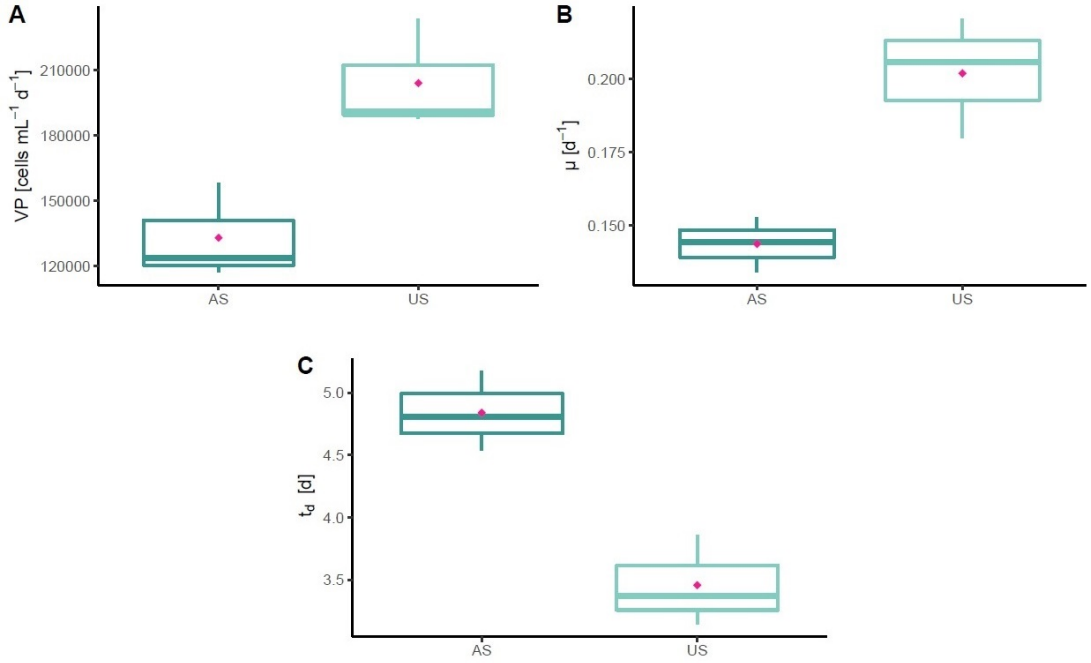


Fig. 3.8: Boxplot of three growth-descriptive variables of AS (autoclaved seawater) and US (unautoclaved seawater) cultures: **A** = VP (volumetric productivity), **B** = μ (specific growth rate), and **C** = t_d (doubling time). The rhombs filled in pink are meant to represent the mean values in the data sets.

The *t*-test showed significant differences for every growth describing variable (VP, μ, and t_d) (Fig. 3.8, Tab. 3.1).

Based on this result, it can be said with certainty that an autoclaving process is essential for producing the cleanest microalgae culture in this case.

3.2 Main experiment

3.2.1 Growth curve and relevant variables in comparison

No contamination was detected under the microscope during the entire period of the experiment, but algal cells were observed rapidly circling around themselves in the cultures, which was exposed to the highest light intensity. Based on the growth curves of the *T. suecica* cultures kept under three different light intensities (100 µmol m^{-2} s^{-1} = T1, 150 µmol m^{-2} s^{-1} = T2, 200 µmol m^{-2} s^{-1} = T3), some clear differences were visually visible. For example, T3 grew much better and faster compared to the other cultures T1 and T2 showing a maximum cell density of $x_{max} = 1.79 \cdot 10^6$ cells mL^{-1} after eight days, while T1 and T2 ranged between x_{max} (T1) = 1.29 and x_{max} (T2) = $1.52 \cdot 10^6$ cells mL^{-1}. However, these differences were not statistically significant (Tab. 3.2).

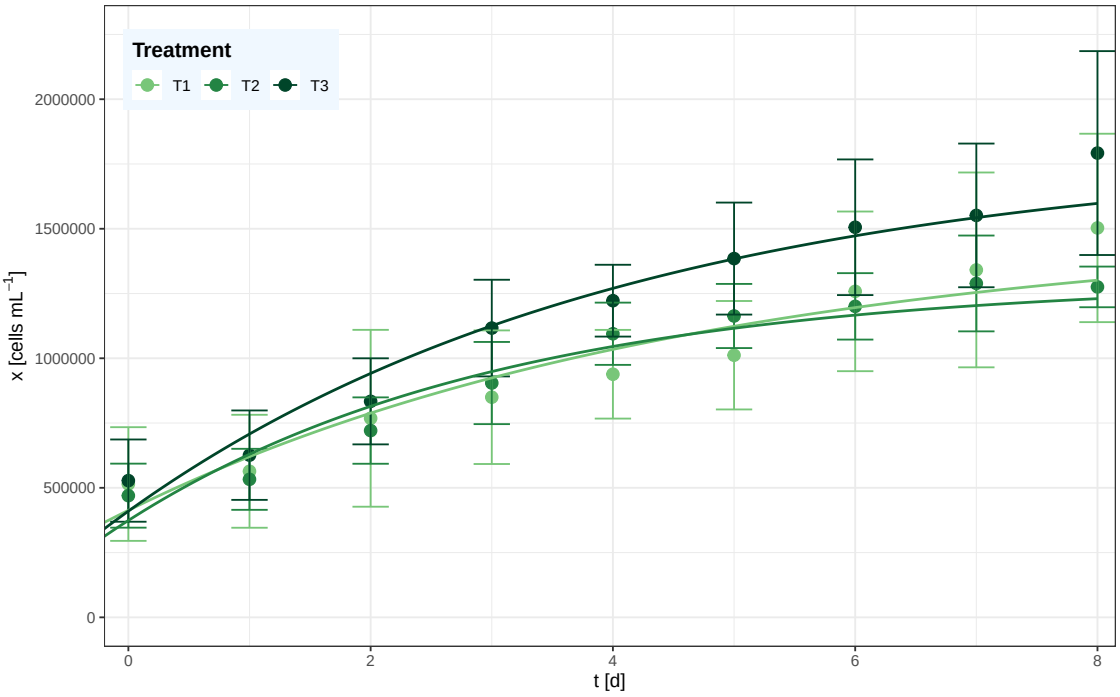


Fig. 3.9: Growth curves of *Tetraselmis suecica* cultures over the eight-day observation period approximated using a bounded exponential growth curve formula. T1 = 100 µmol photons m^{-2} s^{-1}, T2 = 150 µmol m^{-2} s^{-1}, T3 = 200 µmol m^{-2} s^{-1}.

Nevertheless, significant differences in cell size (l) and cell volume (v_{bio}) as well as volumetric productivity (VP) were detected in the different treatments (Fig. 3.10, Tab. 3.2, Tab. 3.3). The differences were significant between treatments T1 and T2 as well as between T2 and T3, which in turn implies that T1 and T3 had similar cell properties. These were significantly lower than the values observed in culture T2.

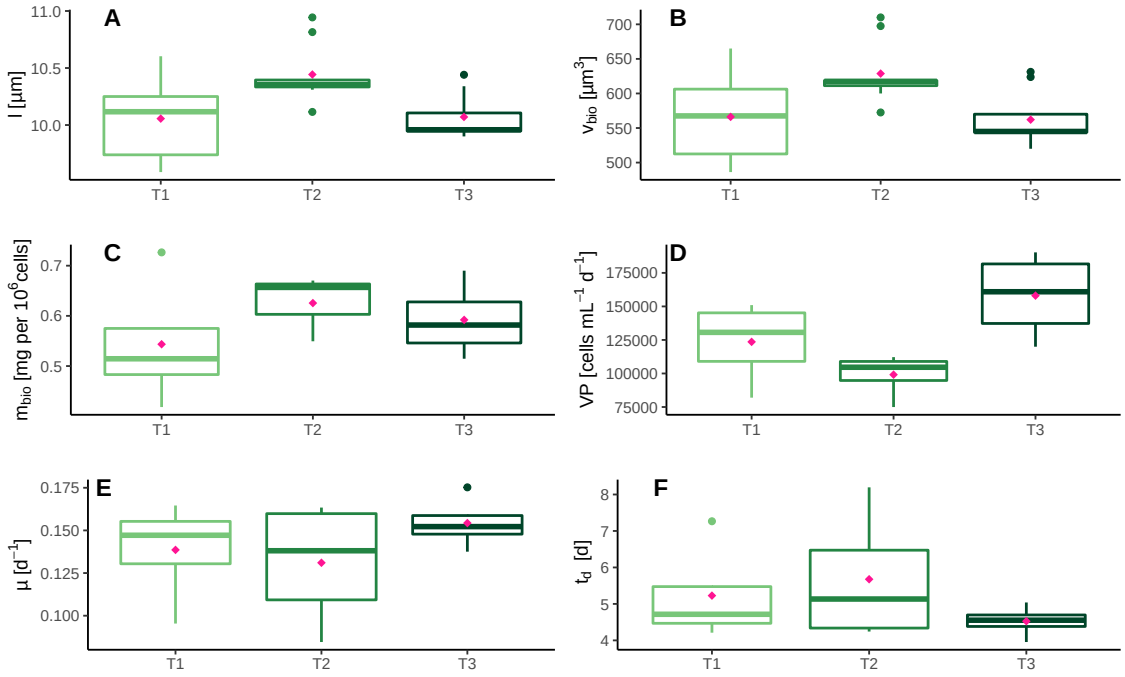


Fig. 3.10: Boxplot of biological parameters of microalgae cells and growth-descriptive variables of *Tetraselmis suecica* cultures, which were maintained at different light intensities. The rhombs filled in pink are meant to represent the mean values in the data sets.

3.2.2 Larval growth and survival at different feeding strategies

Based on the regression of the larval growth in terms of mean shell length (msl) per day, the larvae in the two control treatments C1 and C2 showed a similar average increase. The greatest increase was recorded in T2, followed by T1 and T3, although from the 9th day no larvae survived in T3 so that on the 10th day, no more larvae could be measured in that treatment. Nevertheless, the differences were considered as substantial.

The created regression lines can be expressed with the following equations:

For **C1**: $f(t) = 5.38\ t + 150.44$ with $R^2 = 0.935$,
C2: $f(t) = 5.46\ t + 150.11$ with $R^2 = 0.949$,
T1: $f(t) = 6.21\ t + 151.06$ with $R^2 = 0.932$,
T2: $f(t) = 7.12\ t + 146.07$ with $R^2 = 0.828$,
and **T3**: $f(t) = 4.15\ t + 154.97$ with $R^2 = 0.946$.

Comparing the larvae's mean shell length increment of the respective treatments after ten days directly, C1 and T2 showed the highest values. In T3 the lowest growth was recorded, followed by C2. T1 performed moderately in this comparison, as the larval growth could be classified in the middle range among the others (Fig. 3.11).

As a percentage, no clear progression can be seen in the survival rate over time. From day 4, the number of living larvae in treatment T3 strongly decreased, resulting in 0 % survival rate at day 9 of the experiment. Larvae in the respective other treatments also show decreases. T1, T2, and C2 show survival rates between 5 and 8 % after ten days. C1 shows the highest survival rate of 15 %.

Tab. 3.2: Summarized results of *Tetraselmis suecica* 1 L cultures grown at different light intensities. Here, biological parameters as well as growth-describing variables are presented in the MN = arithmetic mean and their SD = standard deviation. Based on the F- and P-values, statements can be made about how close it is to assume that the tested variables are significantly different.

variable		**T1**		**T2**		**T3**		**F-value**	**P-value**
symbol	unit	MN	SD	MN	SD	MN	SD		
l	µm	10.06	0.34	10.44	0.25	10.07	0.19	5.491	0.011
v_{bio}	μm^3	566	76	628	57	562	78	5.096	0.014
m_{bio}	mg per 10^6 cells	0.54	0.11	0.63	0.05	0.59	0.07	1.315	0.316
β	g L^{-1}	0.69	0.05	0.77	0.06	0.91	0.11	1.101	0.373
x_{max}	10^6 cells mL^{-1}	1.52	0.33	1.29	0.16	1.79	0.34	2.290	0.157
VP	10^6 cells mL^{-1} d^{-1}	0.12	0.03	0.10	0.01	0.16	0.03	4.585	0.042
µ	d^{-1}	0.14	0.03	0.13	0.03	0.15	0.01	0.666	0.537
t_d	d	5.23	1.20	5.68	1.59	4.53	0.38	0.733	0.507

No significant differences between the treatments in following variables: l, µ and t_d.

Tab. 3.3: Comparison of *Tetraselmis suecica* cultures to determine the level of significance. Thus, the table shows the results of the posthoc test.

	T1-T2	**T1-T3**	**T2-T3**
l	0.019*	0.918	0.004**
v_{bio}	0.026*	0.871	0.004**
VP	0.455	0.236	0.035*

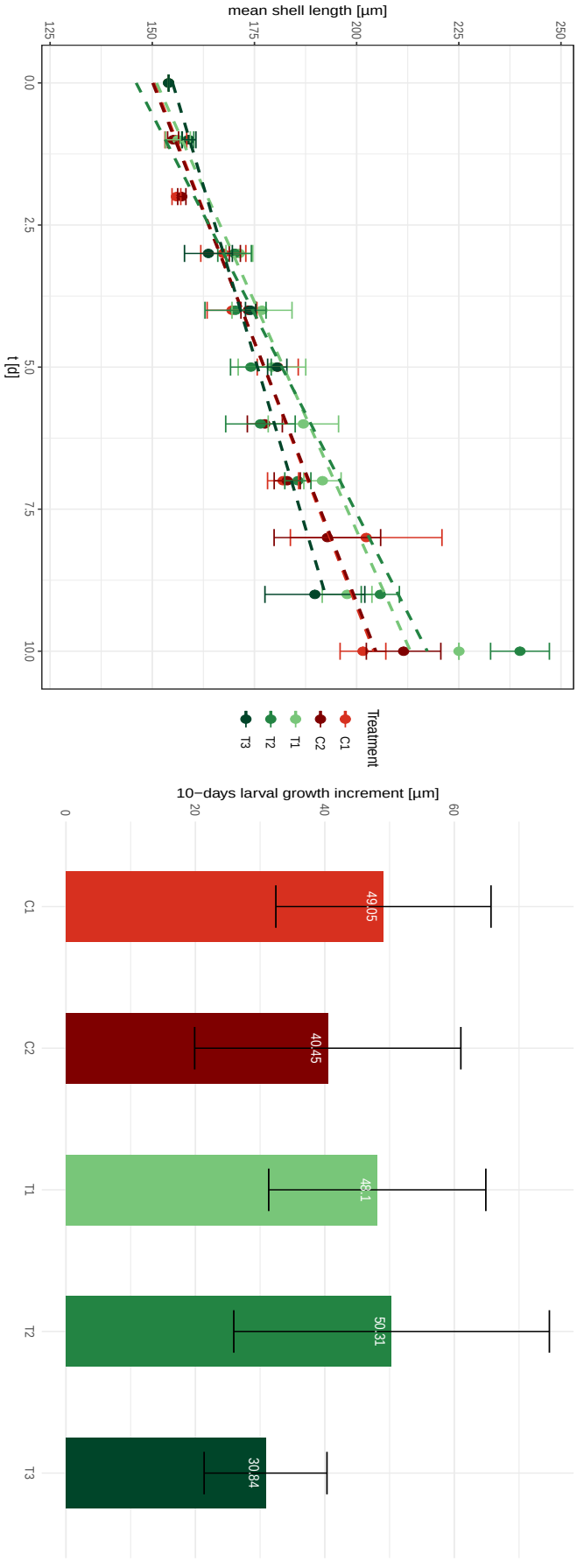


Fig. 3.11: Left: Plot of increment in mean shell length of *Ostrea edulis* larvae per day treated with different feeding strategies. Right: Summary plot of growth in mean shell length over ten days of *O. edulis*, with respective treatments contrasted in bars for comparison.

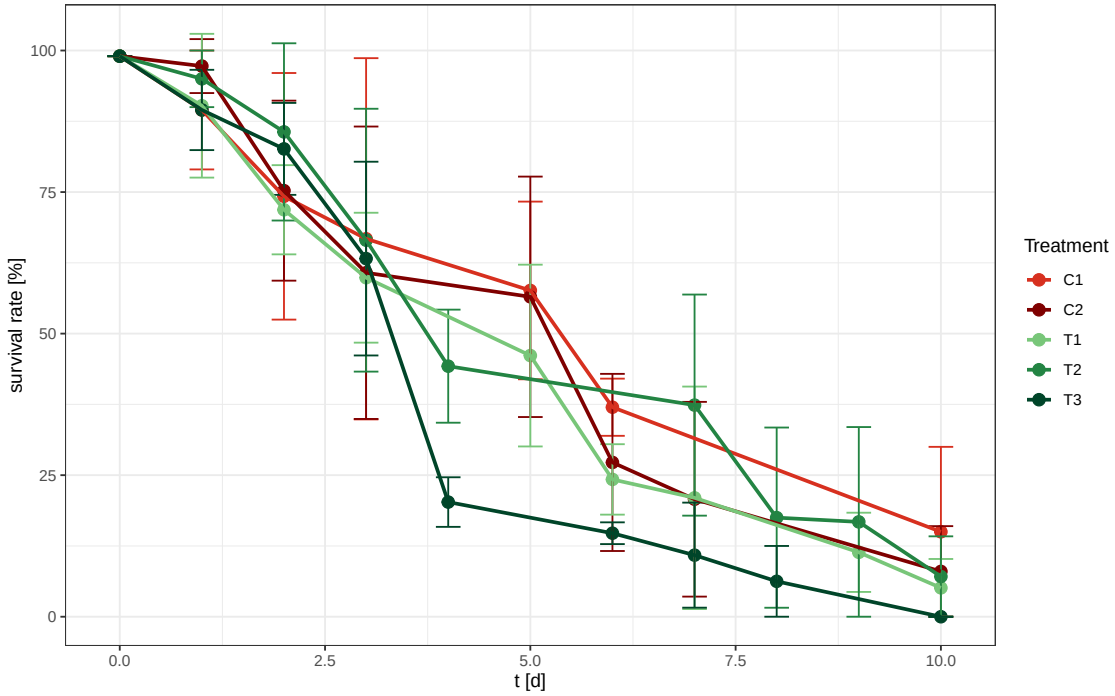


Fig. 3.12: Survival evolution curve of *Ostrea edulis* larvae fed and cultivated at different treatments over ten days.

Nevertheless, significant differences were not found due to the large variances within the individual treatments (Fig. 19). The P-value was 0.129 for the Kruskal-Wallis test ($p > 0.05$).

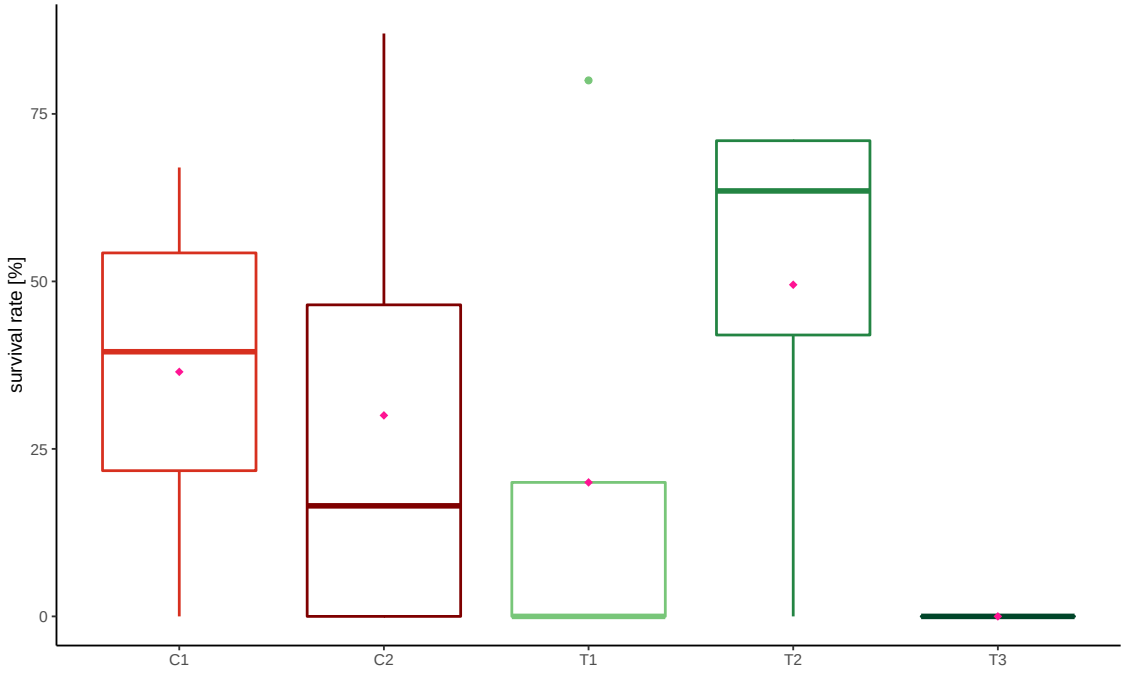


Fig. 3.13: Boxplot of survival rate of *Ostrea edulis* larvae, which were fed with different strategies.

3.2.3 Fatty acid content and composition in relation to larval growth and survival

Although no significant differences in the percentage composition of the respective fatty acids (FA) per cell were detected, there were differences in the actual total amount of FA per cell (Fig. 3.15). Thus, while the FA quantity between T1 and T2 did not differ substantially (94 % agreement), significant differences were found between T1 and T3 and between T2 and T3 (Fig. 3.14), respectively.

However, analyzing the FA composition per light treatment in particular, no dependency between treatments and FA composition was observed. Hence, the percentage composition of long chain fatty acids (LCFA) per cell is independent of the light intensity at which the cultivation took place.

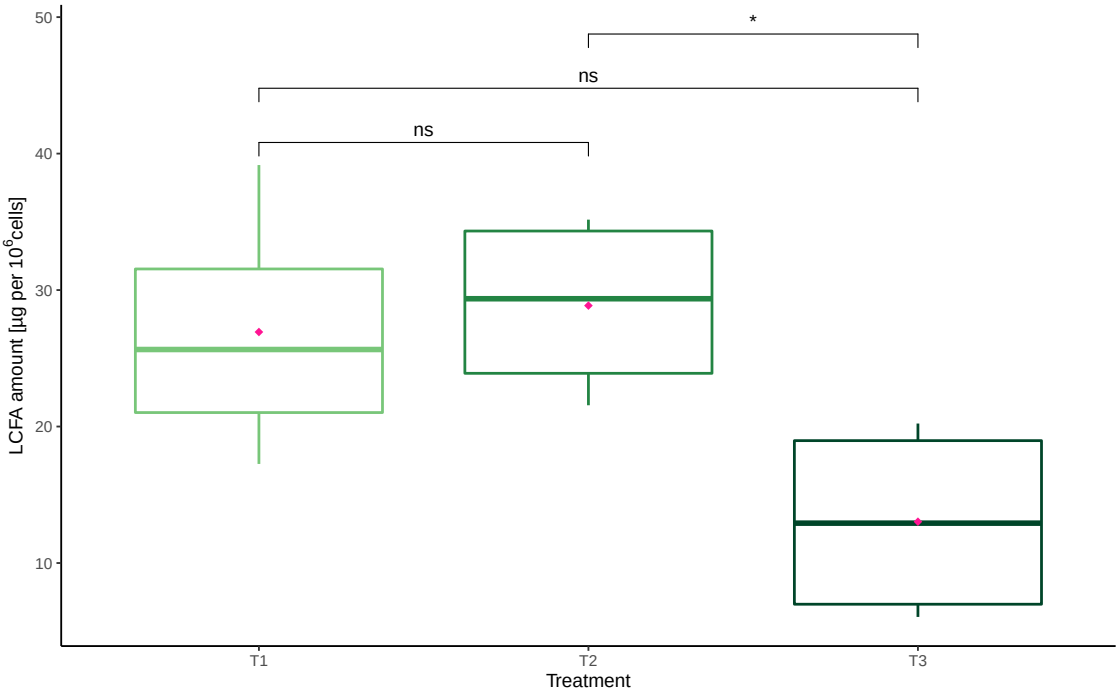


Fig. 3.14: Boxplot of long chain fatty acids (LCFA) concentration per 10^6 *Tetraselmis* suecica cell treated with different light intensity.

There is a positive correlation between FA quantity and larval survival rate (cor = 0.53), but it is not significant (p = 0.08). There is neither a dependency between FA quantity and larval growth, nor a significant correlation (p = 0.07; cor = 0.48).

Considering physiological and nutritional effects, FAs with high nutritional value are listed separately (Fig. 3.16): linoleic acid (18:2 n-6), α-linolenic acid (18:3 n-3), eicosatrienoic acid (20:4 n-3), arachidonic acid (ARA; 20:4 n-6) and eicosapentaenoic acid (EPA; 20:5 n-3). Docosahexaenoic acid (DHA; 22:6 n-3) is high of importance for oyster larvae but does not occur in *T. suecica* cells. The demand is sufficiently covered by other microalgae species supplementing the diet, such as *C. muelleri*, and especially *I. galbana*.

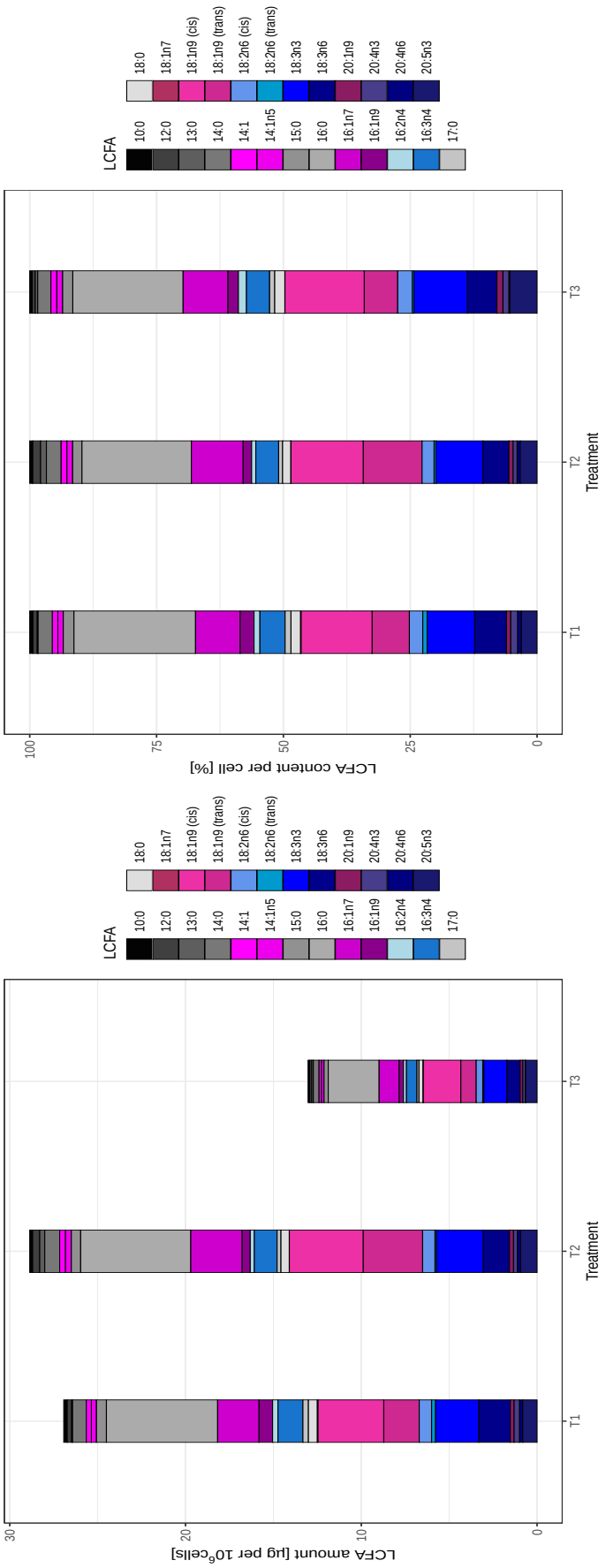


Fig. 3.15: Comparison of absolute and percent LCFA composition in *Tetraselmis suecica* cells maintained at different light intensities.

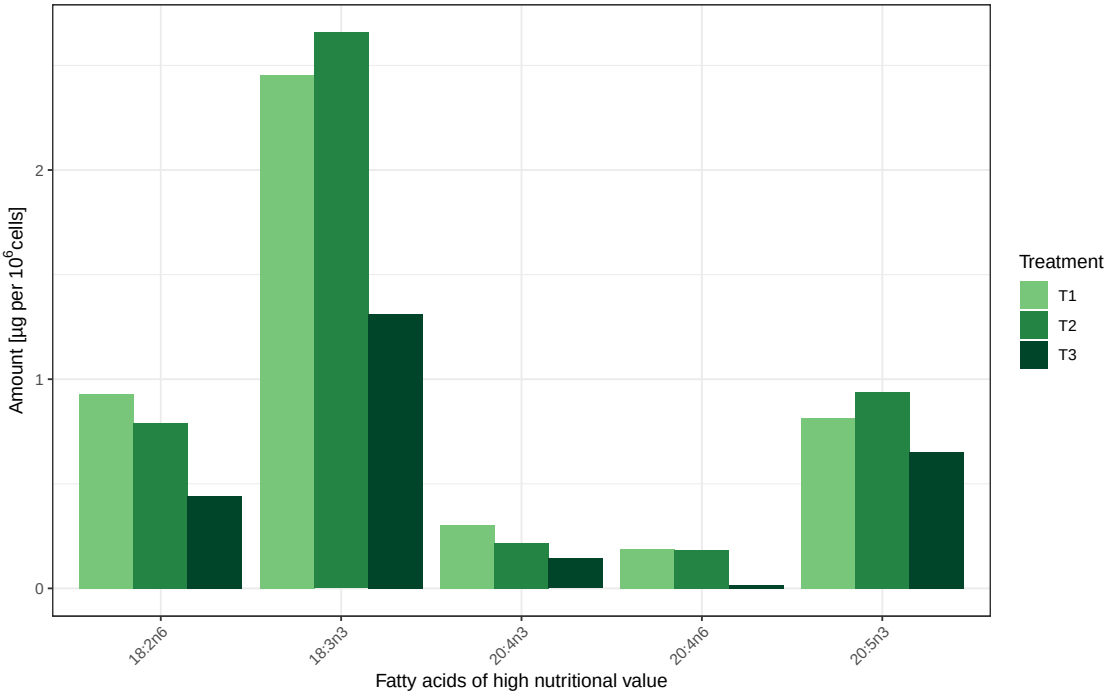


Fig. 3.16: Boxplot of long chain fatty acids (LCFA) concentration per 10^6 *Tetraselmis suecica* cell treated with different light intensity.

Although Fig. 3.16 suggests the contrary, hardly any significant differences could be detected within the three different treatments concerning FA of high nutritional value due to high variances (Tab. 3.4). Thus, only a significant difference in the amount of FA 18:3 n-3 was detected. The significant difference was between T1 and T3 and between T2 and T3. The data from T1 and T2 were in 72 % agreement with each other.

Tab. 3.4: Absolute fatty acid concentration of the most relevant FAs for *Ostrea edulis* larvae. F- and P-values are listed, for a visual representation of the statistical evaluation. FA = fatty acid, T = treatment, MN = arithmetic means, SD = standard deviation.

High value FA [µg per 10^6 cells]	T1		T2		T3		F-value	P-value
	MN	SD	MN	SD	MN	SD		
18:2n6	0.93	0.33	0.79	0.22	0.44	0.25	2.65	0.12
18:3n3	2.46	0.54	2.66	0.76	1.31	0.63	3.79	0.06
20:4n3	0.30	0.38	0.22	0.02	0.14	0.08	0.38	0.69
20:4n6	0.19	0.25	0.18	0.16	0.02	0.03	1.00	0.41
20:5n3	0.82	0.21	0.94	0.18	0.65	0.32	1.02	0.40

4 Discussion

4.1 The occurrence and interaction of ciliates and microalgae

In general, many biological contaminants have been found to affect microalgal growth (Croft et al., 2005; Di Caprio, 2020).

Large-scale cultures are susceptible to grazing by zooplankton which can reduce algal concentrations and microalgae production to low levels within a few days (Benemann, 2008). In the past, zooplanktonic organisms as efficient predators of microalgae were thought to be the primary cause of cultivation failure. But the most common predatory taxa include ciliates (Rosetta and McManus, 2003). Ciliates are a common but understudied group of grazers that can invade microalgal cultures. Ciliates occur in most aquatic habitats and function in mariculture ecosystems as bacterial consumers (Xu et al., 2004). Many species also feed on flagellates, amoebae, algae, or fungi, still others live predatorily and eat other ciliates. The concentration of contaminants at which the microalgae culture perishes is still an open question (Wang et al., 2013).

Infections and contaminations caused by biological contaminants can be controlled by various approaches such as filtration, chemical and biological drug additions, and adapted growth conditions. However, water and air cannot be sterilized entirely by filtration because biological contaminants such as viruses can inevitably enter cultures through microporous membranes (Wang et al., 2013).

Moreover, most ciliates produce resting cysts that persist in the air or in marine water (Nguyen et al., 2020). Therefore, it is possible that resting cysts enter the cultures through the air. In this case, the contamination could have passed the elastic lid of the carboys (used for the large-scale cultivation steps in the Helgoland Oyster Hatchery), which is less tight than a screw cap, and entered the culture (Chapter 3.1.1). As the experiments were carried out during the season of increased ciliates occurrence in the Helgoland ambient water (Fig. 4.1), the probability is high that all cultures, which were not tightly sealed, were also contaminated.

Bacteriophagous ciliates, which appear to be ubiquitous, respond to growing bacterial populations and can thereby enter an exponential growth phase. For example, Plunket and Hidu (1978) found that low *Uronema marinum* concentration corresponded with high bacteria concentration and vice versa. This study defined a specific trophic niche for *U. marinum* as a bacteriophage and thus, this species was not identified as being general responsible for the mortality of *O. edulis* larvae in aquaculture, for example, when introduced into the larval rearing system via algal cultures. However, they can infect unhealthy, i.e. intact and/or weakened oysters that have already been infested with bacteria.

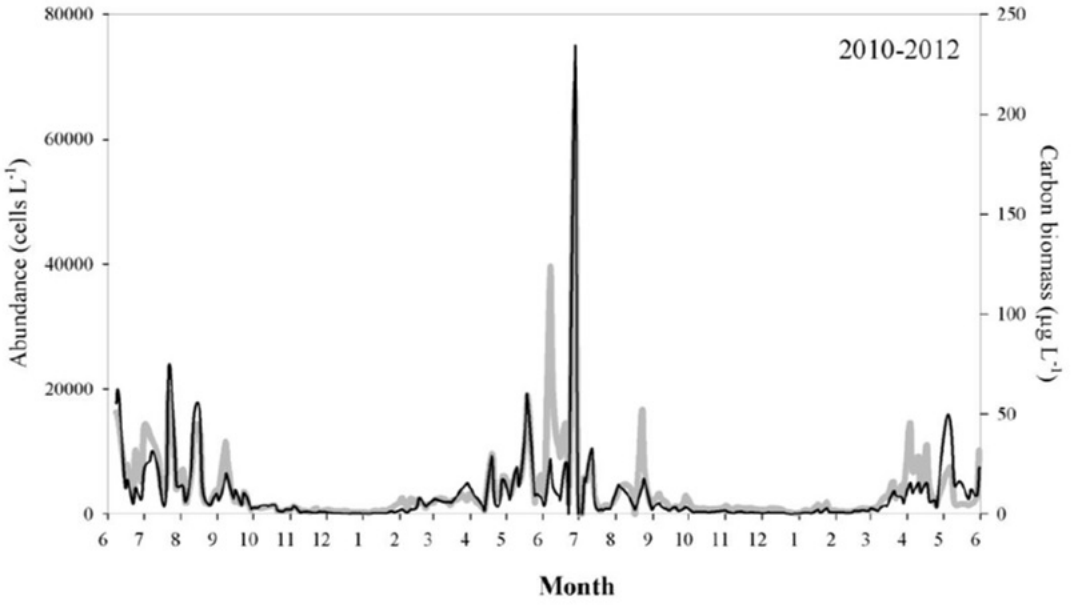


Fig. 4.1: Total abundance and carbon biomass of the ciliate community at Helgoland's Roads from 2010 - 2012 (Yang, 2014). Gray line represents total ciliates abundance, black represents total carbon biomass.

Until day 5, mainly *U. marinum* were detected in all carboys, but gradually disappeared, "being replaced" by a larger ciliated species called *E. vannus*. Based on this, we suggest that *E. vannus* were feeding on *U. marinum*. The ciliate species *E. vannus* feed selectively, but their diet definitely includes bacteria and unicellular microalgae as well as other protozoa (Dolan and Coats, 2008).

Tubiash et al. (1965) noted the presence of *Euplotes* sp. in moribund larval and juvenile mollusks and suspected that ciliates function as necrophages in oysters weakened by the bacterial attack. However, in general, they were not harmful to cultured animals.

Euplotes spp. can capture and ingest food between 0.57 and 10 µm in diameter, but require high food densities (Wilks and Sleigh, 1998; Drillet and Dutz, 2014). The *T. suecica* cells measured in the described experiments had a cell size of 8 - 10 µm. Based on this, it can be presumed that *E. vannus* could cause low microalgal concentrations in the respective cultures.

However, Ratti et al. (2013) found that *T. suecica* cultures grown in the presence of *Euplotes* sp. showed an increased growth rate, probably due to the rapid nutrient turnover associated with ciliate metabolism. The dinoflagellates likewise exhibited an avoidance strategy and enveloped themselves in the presence of *Euplotes* sp..

This work lacked a comparison to uncontaminated cultures in the large-scale batch system to confirm this statement. According to the literature, *T. suecica* cultures at such scales should have an average maximum cell density of $x_{max} = 3.8 \cdot 10^6$ cells mL^{-1} (Cid et al., 1992), but only a cell density of $x_{max} = 1.8 \cdot 10^6$ cells mL^{-1} could be achieved in this work,

which represents a deviation of almost 50 %.

Thus, it could be conceivable that in this case, *E. vannus* resorted to these microalgal cells due to the low food supply and took up more microalgal cells than they were able to proliferate. This activity specifically could not be observed under the microscope within this work. Here, *E. vannus* tended to feed on undefinable "non-activated sludge", but this does not preclude their ingestion of *T. suecica* cells either.

A continuous CO_2 supply should be considered to kill or get rid of zooplankton. It is strongly suspected that CO_2-enriched air could harm animal protozoa but not microalgae (own data, not published). Due to the building structure and safety precautions in the TK rooms, this is not possible, but worth considering to cultivate at least parts of cultures in rooms where CO_2 supply is feasible to reduce possible contaminations and thus also to provide for faster growth.

In addition, an alternative for carboys should be considered because although contamination can be reduced initially by chemical disinfection, contamination can still enter the cultures through the rubber lids, which are not as airtight as screwed caps.

4.2 The occurrence and interaction of bacteria and microalgae

Unlike ciliates, some bacterial species provide growth-promoting substances for phytoplankton, such as vitamins (Haines and Guillard, 1974; Croft et al., 2005). This means that aquaculturists can improve the cultivation of microalgae by promoting the growth of certain bacterial species in mass cultures (Fig. 4.2), which in turn could help microalgae cultures to grow better.

The bacteria species *Roseobacter*, for example, play a beneficial role in increasing the productivity and stability of algal mass cultures (Fuentes et al., 2016). The cells of the microalgae *T. suecica* are known to produce large amounts of the organic sulfur compound dimethyl sulfoniopropionate (DMSP), which can be converted to dimethyl sulfide (DMS) by marine bacteria (Malin, 2006). The *Roseobacter* can synthesize tropodithietic acid (TDA) as a biologically active secondary metabolite from this, which has antibacterial properties and prevents algicidal bacterial species from damaging phytoplankton (Brinkhoff et al., 2004). Under this condition, the healthy algal host provides DMSP as an attachment surface for *Roseobacter*, while the bacterial symbiont delivers the antibacterial compound to the microalgae, creating a mutualistic symbiotic relationship between the partners (Seyedsayamdost et al., 2011).

The symbiotic cultures of microalgae and bacteria can also result in the complete elimination of other harmful bacteria in aquaculture systems. This can be explained by the competitive exclusion principle, which is usually prominent in such ecological communities. This principle, also known as Gause's law, states that two species cannot co-exist within the same ecological niche, or within the same habitat at the same time (Gause, 1934).

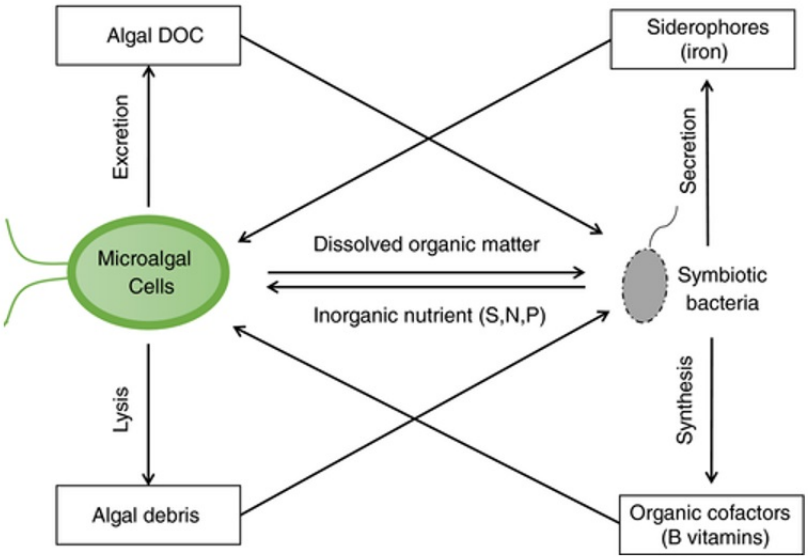


Fig. 4.2: A simplified diagram of general and specific interactions between microalgae and symbiotic bacteria (Yao et al., 2019), indicating some bacterial species' providing growth-promoting substances for microalgae.

Based on this principle, it is possible that bacteria associated with microalgae in aquaculture prevent the proliferation of other microorganisms potentially pathogenic to oyster larvae, for example, by producing antimicrobial substances.

In addition to the positive effects on algal growth, adverse effects of bacteria on biomass yield of microalgal cultures can also be expected in some cases. Non-axenic batch cultures of microalgae with little or no microbial control could result in lower algal cell density compared to axenic algal cultures. In cultures with high cell density, the presence of bacteria is expected to reduce the availability of light to the microalgal cells (Solimeno et al., 2017). As a result, the yield of algal biomass in non-axenic photoautotrophic cultures could decrease. However, some bacterial species, so-called phytoplankton-lytic bacteria, also have the potential to inhibit microalgal growth (Imai et al., 2001; Mu et al., 2007; Wang et al., 2010, 2012) and thus, may actually have negative effects on microalgal growth and hinder microalgal mass cultivation.

Considering the results of this study (Chapter 3.1.2), we observed that additional autoclaving of the already 0.2 µm filtered seawater increases the growth of *T. suecica* cultures. We noticed a statistically significant differcence to with non-autoclaved seawater (US). Thus, it can be surmised that autoclaving eliminated growth-inhibiting bacteria, as no other contaminants were detected under the microscope (such as ciliates). Also, this may explain the "dots" in Figure 3.6, which may indicate bacterial species in the US cultures.

A more precise identification of the bacterial species was not possible within the framework of the Helgoland Oyster Hatchery. In general, the elimination of biological contaminants should be the priority so that additional autoclaving of the already 0.2 µm filtered seawater

in up to 5 L cultures is recommended. While this eliminates growth-promoting bacteria, it also eliminates species that are harmful to the microalgae. This ensures that the microalgae cultures do not die due to growth-inhibiting bacterial contamination.

On this basis, the argument that the *T. suecica* cultures did not grow as well in carboys and exhibited an unstable growth curve because of *E. vannus* can be revised. Thus, it can be equally surmised that the ciliates fed on the symbiotic bacteria in the culture and thus used up additional nutrients for enhanced growth such as demineralized sulfur, nitrogen, and phosphorus from the microalgae (Fig. 4.2). The low performance of *T. suecica* can therefore be explained by the loss of the symbiotic bacteria rather than by the predation by *E. vannus* on the microalgae cells.

A better understanding of how these biological contaminants, whether intentional or not, interact with the host microalgae and appropriate cultivation technology could lead to the development of effective control methods. Unfortunately, these approaches also have their weaknesses. Consideration should be given to strain selection as a possible most viable approach, with non-susceptibility/resistance to biological contamination being an important factor in selecting production strains.

4.3 The effect of light intensities on accumulation of long chain fatty acids in *Tetraselmis suecica*

The major genes involved in the biosynthesis of some polyunsaturated fatty acids (PUFA's) are known, but the effects of light and other environmental factors on the transcription of such genes are, so far, only poorly understood (Conceição et al., 2020).

Light plays a central role for photosynthetic organisms. Therefore, in this work, the microalgae species *T. suecica* was studied for its physiological response to different light intensities (100, 150, and 200 µmol photons m^{-2} s^{-1}) and analyzed for the biosynthesis of long chain fatty acids (LCFA's), which is important because of its high potential as a nutritional supplement for oyster larvae, but mainly because of its importance in antibacterial activity against *Vibrio* sp..

The results show that a higher light intensity of 200 µmol m^{-2} s^{-1} facilitated rapid growth, but only a low LCFA content per microalgal cell was detected. Comparatively, cultures exposed to light intensities of 100 or 150 µmol m^{-2} s^{-1} were limiting algal growth, but these intensities appear to be beneficial for LCFA accumulation.

Thus, the faster growth of *T. suecica* and higher cell densities do not necessarily mean better live feed for larvae (Fig. 3.9, 3.14, 3.15). Based on these results, the *T. suecica* strain had the highest fatty acids content when the microalgae were cultured at 150 µmol m^{-2} s^{-1}. However, these cultures showed the lowest growth compared to the others. We postulated that the slower the microalgae cells proliferate, the more nutritious they are.

More replicates need to be measured in the fatty acid analysis to make more accurate

conclusions about fatty acid content concerning light intensity, which could not be done in this work due to some technical problems. It is suggested for further meaningful interpretations in terms of fatty acid composition and content to repeat the experiment with several series of measurements (at least n = 10). However, the described experiments already provide a reasonable basis for further research.

4.4 Faster growth with a 3-mixed-diet but lower survival rates of *Ostrea edulis* larvae

Not all microalgae species of suitable size have good nutritional value for oyster larvae (Helm et al., 2004). The nutritional value of a particular alga is determined not only by its biochemical composition but also by its digestibility. For example, diatoms with long, siliceous spines can be challenging to uptake and can be perceived as irritating by larvae and would be expelled by them (Guedes and Malcata, 2012). Thick cell walls can also be a hindrance, making them almost indigestible (Guedes and Malcata, 2012). Still others, including *T. suecica*, lack certain essential PUFA's that are required for larval development and, although digestible, have little or no nutritional value (Helm et al., 2004). A diet deficient in PUFA's known to be important, including EPA (20:5 n-3) and DHA (22:6 n-3), may thus be reflected in the survival rate of *O. edulis* larvae, as they have limited or no ability to synthesize these essential fatty acids from less highly unsaturated precursors then (Khoeyi et al., 2012). For this reason, the ratio for the demanding larvae should include nutritionally valuable flagellates such as *I. galbana* and diatoms such as *C. muelleri*, all of which are rich in one or another of these PUFA's. Thus, for many of the demanding species, feeding a combination of microalgae rich in either EPA or DHA (or both) should provide the best results. The microalgae cells of *T. suecica* is conspicuously lacking in C22 fatty acids (Oostlander et al., 2020) (see also Chapter 3.2.3) which are considered essential for bivalve larval nutrition (Waldock and Holland, 1984), but were replaced by high levels in *I. galbana* cells. Other literature also discovered this deficiency (Helm et al., 2004).

However, the main focus in this work was not on the nutritional value of *T. suecica* itself but on the reduction of bacterial contamination concerning *Vibrio* sp. species. Thus, after finalizing the experiment, 2 L water samples were taken from each of the treatments and stored at -80 °C for later *Vibrio* sp. analyses. Despite the still pending results, this work can already make some statements, which will be further highlighted in the following.

The survival rate of larvae from the D-stage to metamorphosis varies among species. In some oyster and clam species, it averages 50 to 70 %, while it averages only 15 to 30 % in scallops (Helm et al., 2004). However, the proportion of larvae that reach metamorphosis is highly dependent on rearing conditions, including, as mentioned earlier, food quality and quantity, temperature, and salinity, as well as relatively uncontrollable factors such as seawater quality and disease (Helm et al., 2004). The survival rate recorded in the control (C1) was 15 % for *O. edulis* larvae used in the experiments. Treatments T1 and T2 (*T. suecica* cultured at 100 and 150 µmol m^{-2} s^{-1}) did not show such a good survival rate compared to C1, but faster larval growth. The highest mortality rate of 100 % was

recorded in treatment T3, where *T. suecica*, maintained at the highest light intensity of 200 µmol m^{-2} s^{-1}, was also added. It should be noted that the high variances within the treatments do not allow definitive interpretations or further statements. The following causes could have led to high variances and discontinuity of the results: Due to the one-sided aeration, the microalgae with a larger cell diameter and weight settled at the bottom of the vessel. Hence, the cells were not accessible for *O. edulis* larvae and therefore, they received less feed. Increased aeration would not have been beneficial at this point, as the larvae, which were already stressed by the daily water exchange, would have been further stressed. The *O. edulis* larvae are susceptible to external influences and may have also died from the inconsistent water quality and water exchange action. Thus, the death of *O. edulis* larvae in this experiment cannot be fully excluded based on the mentioned factors.

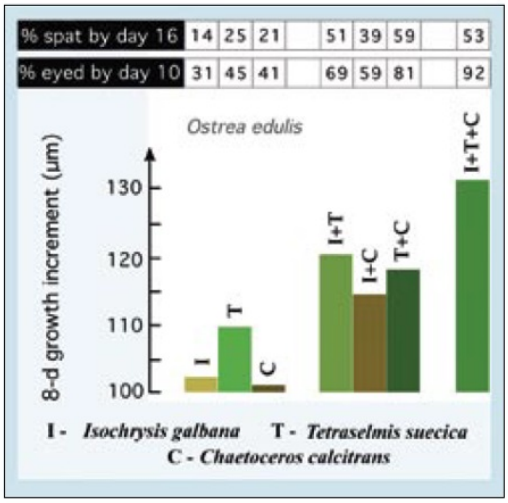


Fig. 4.3: "Growth (in an 8-day period), development (% eyed larvae by Day 10) and settlement (percentage spat of the initial larval number at Day 0) of *Ostrea edulis* larvae fed various single and mixed diets of the three algal species [I = *Isochrysis galbana*, T = *Tetraselmis suecica*, C = *Chaetoceros muelleri*] indicated. Values are the means of a large number of trials." (Helm et al., 2004)

Many studies have observed faster growth rates of oyster larvae when more microalgae were added to their diet (Gabbott and Holland, 1973; Millican and Helm, 1994; Rico-Villa et al., 2006; González-Araya et al., 2012). Helm et al. (2004) also showed that *O. edulis* larvae grow much faster with a 3-mixed diet than with a 2-mixed or monospecific diet (Fig. 4.3). However, the described experiments show, at least to some extent, that a faster growth does not mean that the larvae are subsequently healthier or would survive during the whole rearing period. This cannot be validated by literature, so this represents a novelty of this study. It is hypothesized that faster growth of larvae is not necessarily an exclusion criterion for the healthiness and thus higher survival rates of larvae in aquaculture. However, more research is required here.

4.5 Optimization of the experimental set-up using a flow-through system

Finally, it is pointed out that the scope of the experiments is limited technically and in time. In addition, however, it is emphasized that their implications justify further research. Much remains to be done, but at this stage of understanding, it seems that the debate on whether adding *T. suecica* with increased LCFA content (culturing at 150 µmol m^{-2} s^{-1}) as other live microalgae feed increases the survival of *O. edulis* larvae should be continued by optimizing the experimental set-up.

For further research, the experimental set-up can be optimized to reduce the stress level of the larvae. On the one hand, this can be achieved by not exchanging the water every day and by building a continuous system for the constant water supply instead, ensuring a permanent good water quality (Fig. 4.4). With this approach, it can also be ensured that microalgae cells with a larger diameter can no longer settle since they are in constant motion in the water due to the system technology and are thus constantly accessible to the larvae. Such a system was initially foreseen for this study. Due to logistic challenges, resulting from the Corona pandemic, the set-up has been replaced by the current designs. It is recommended that this experiment should be conducted again, using the larval rearing system as explained in Figure 4.4.

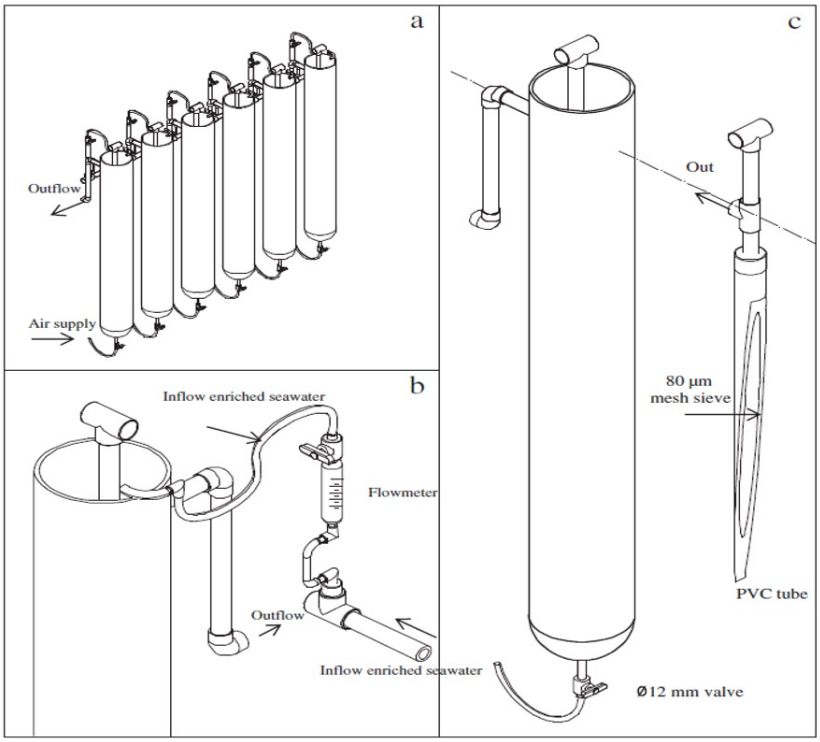


Fig. 4.4: "(a) Overview of 5 L cylinder for *Ostrea edulis* larval rearing, (b) details of inlet and outlet of phytoplankton enriched seawater and (c) overview of mesh sieve in larval tank." (González-Araya et al., 2012)

In conclusion, the fatty acid production of the microalgae species *Tetraselmis suecica* (strain: CCAP 66/4) was highest at 150 µmol photons m^{-2} s^{-1} with 28.86 µg per 10^6 cells. It is assumed that higher light intensities led to increased stress of the microalgal cells (Chapter 3.2.1: "[...] algal cells were observed rapidly circling around themselves." = sign of stress), so that fatty acid production dropped significantly when cultivating *T. suecica* with a light intensity of 200 m^{-2} s^{-1} and above. Other factors affecting the production, such as nutrient additions to the culture, were not considered in this work due to the fact of sustainable production. The reason for this is that the commercial 'Cell-Hi F2P' medium from Varicon Aqua will also be used at the Helgoland Oyster Hatchery in the future, because a change to a different medium for one microalgae species is not subject to economic but also not to ecological thinking. Hence, switching to another medium is not currently planned. Still, it is recommended to continue doing research on fatty acid production of this microalgae species with light intensity and duration especially in combination with other influencing factors. For this purpose, this work provides a fundamental basis along with other scientific works (Balloni et al., 1983; Guzmán et al., 2010; Rodolfi et al., 2009; Bondioli et al., 2012; Go et al., 2012). However, it should be noted that there can be a high variability of marine microalgae lipid content during the seasons. (Pernet et al., 2003)

According to previous results of scientific works, a feeding strategy with a multiple-combination diet promises better growth of *Ostrea edulis* larvae (Gabbott and Holland, 1973; Millican and Helm, 1994; Rico-Villa et al., 2006; González-Araya et al., 2012), but no good statistics concerning their survival rate, if the data of this master thesis can be trusted. In the experiment conducted in this work, there were also many factors linked together that could lead to the oyster larvae's death (Chapter 4.4). To minimize errors in this respect, these factors should be included and an optimized larval rearing system according to González-Araya et al. (2012) should be used for future experiments. Clear results could not be obtained, but a tendency for better survival of *O. edulis* larvae with increased long chain fatty acid content in *T. suecica* cultures using as feed was evident. Further research is needed in this field as well. This work is the first step for further investigation in exploiting the antibacterial activity of *T. suecica* in oyster hatcheries producing *O. edulis* and provides a broad foundation for further experiments in this aspect. Laboratory results regarding the *Vibrio* sp. concentration at the end of the feeding experiment also remain to be seen.

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Appendix

Appendix A1:
Calculation of chemical water disinfection and its neutralization

	M [g mol^{-1}]	C [%]	β [g L^{-1}]	n [mol]	V [L]
NaOCl	74,44	2,6	26	0,026	0,075
$Na_2S_2O_3$	158,11	5,2	52	0,007	0,020

Equations:

$$n = \frac{\beta \times V}{M} \quad \text{OR} \quad V = \frac{n \times M}{\beta}$$

with
- n = amount of substance in carboy *
- β = mass concentration of stock solution
- V = volume (need to be) added in carboy
- M = molar mass
- C = concentration of stock solution in percent

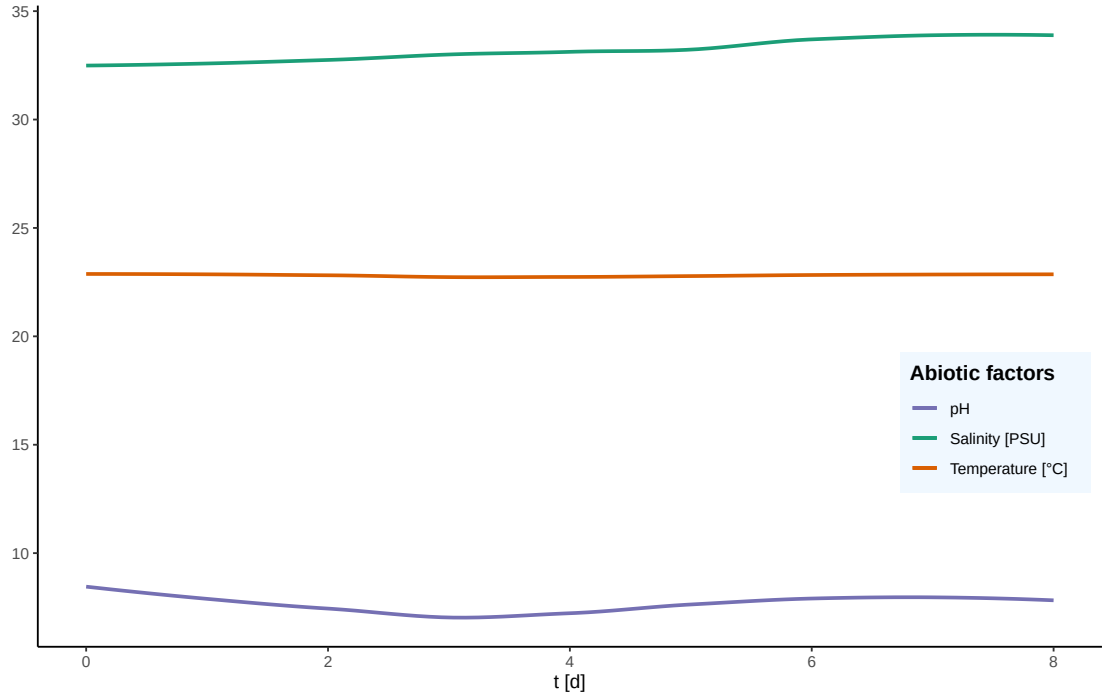
* following applies for neutralization:

$$n(Na_2S_2O_3) = \frac{n(NaOCl)}{4}$$

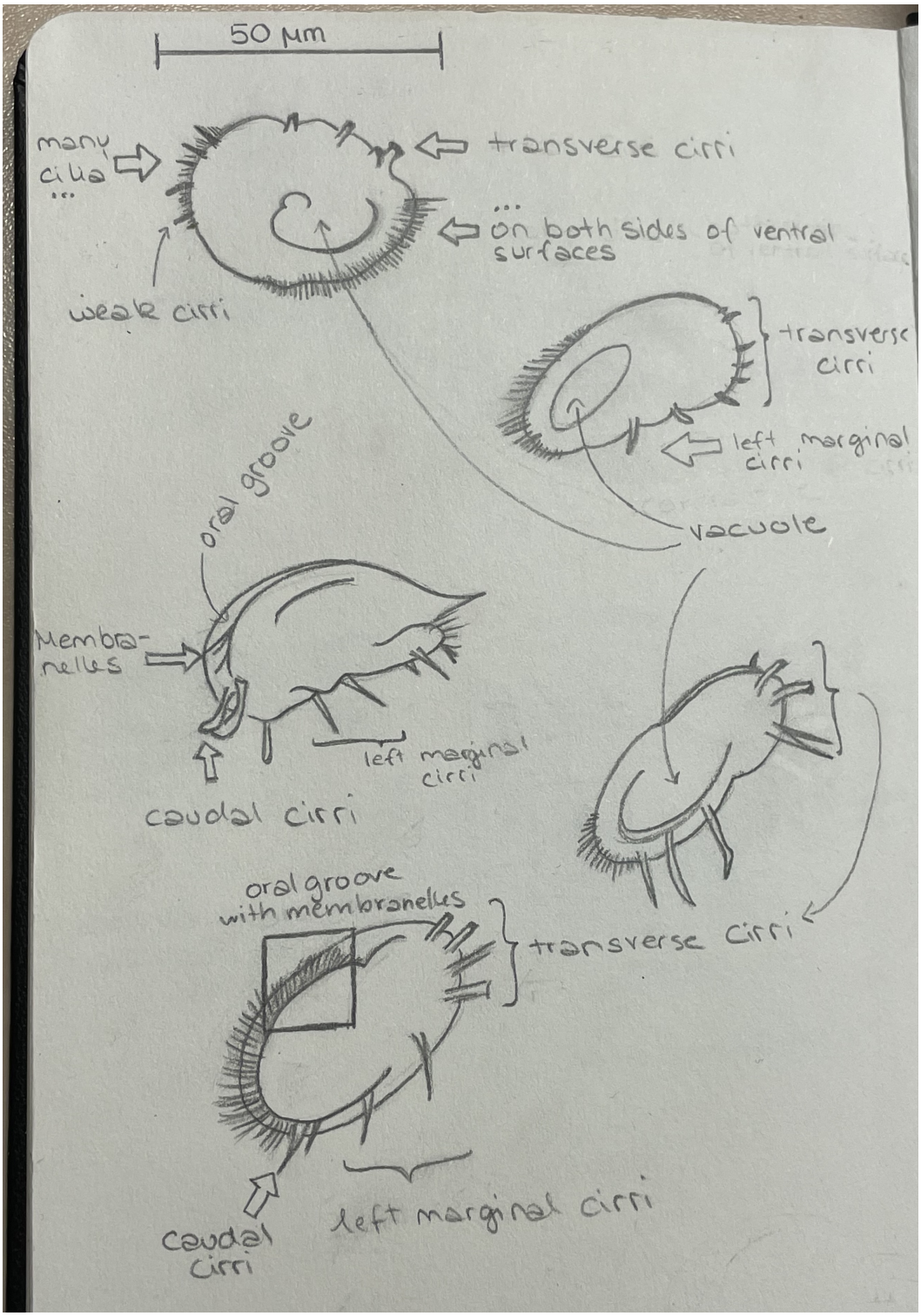
... because:

$$4\ NaOCl + 1\ Na_2S_2O_3 + H_2O \rightarrow 2\ Na_2SO_4 + 2\ HCl + 2\ NaCl$$

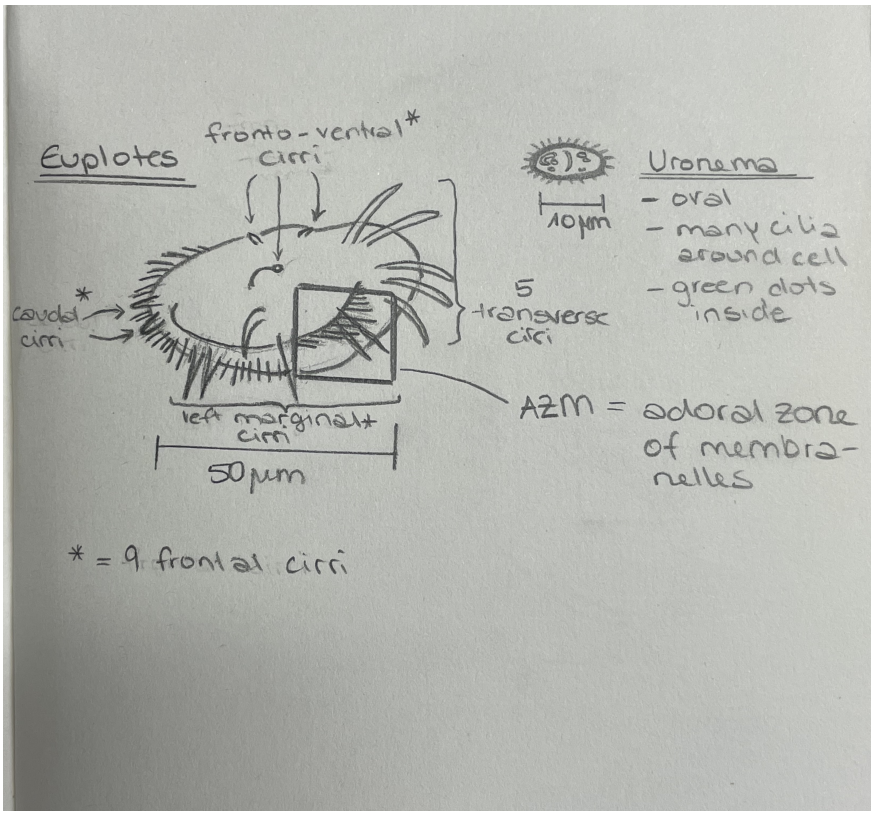
Appendix A2:
Evolution of different abiotic factors in the *Tetraselmis suecica* 1 L culture



Appendix A3:
Sketches of *Euplotes vannus*



Appendix A4:
Sketches of *Euplotes vannus* and *Uronema* cf. *marinum*



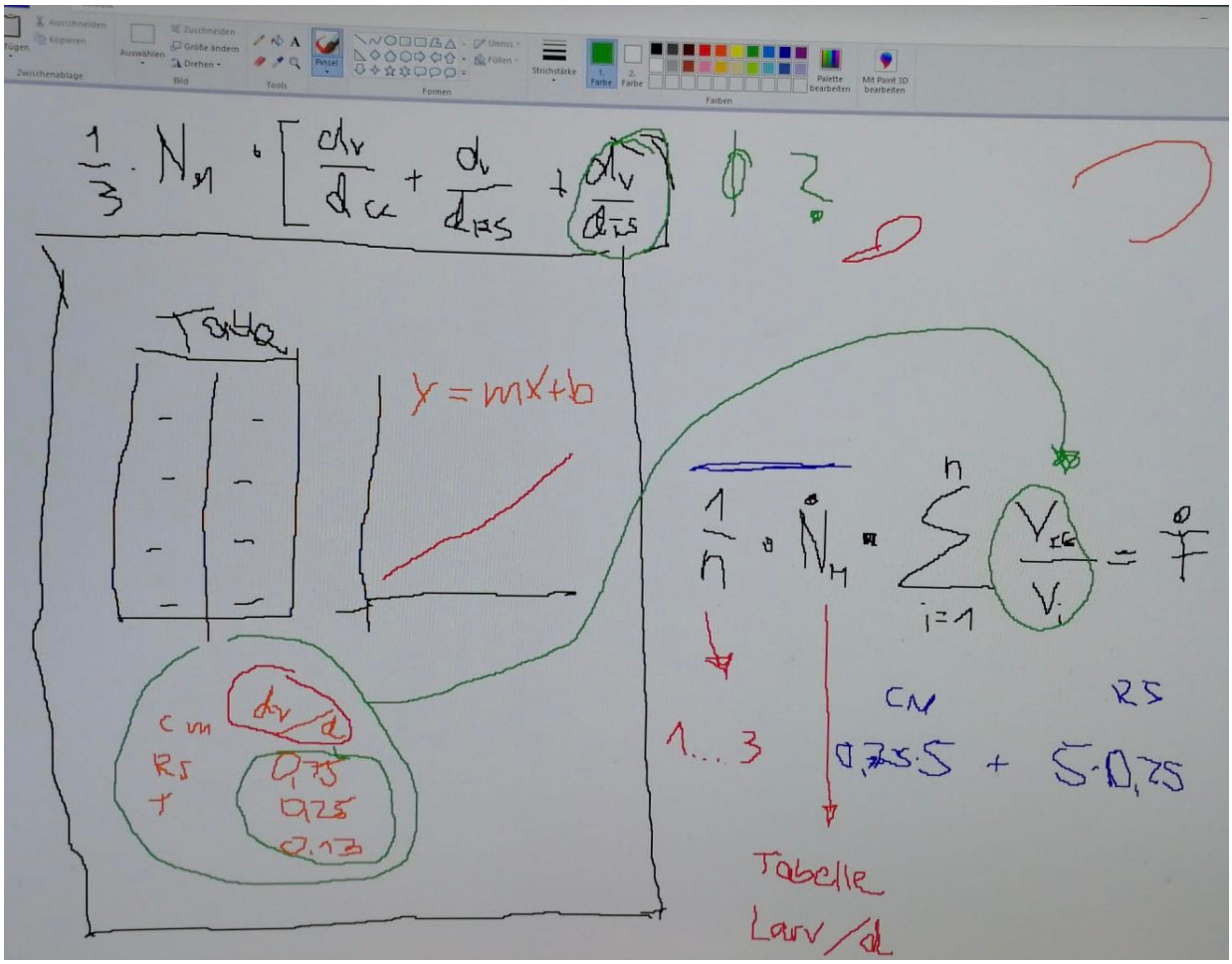
Appendix A5:
Comparison of PUFA's in the microalgae used in the Helgoland Oyster Hatchery

	PUFA content [µg 10^6 cells]	**References**
Isochrysis galbana	4.09	Pernet et al. (2003)
Chaetoceros muelleri	2.07	Pernet et al. (2003)
Rhodomonas salina	13.10	Thoisen et al. (2020)
Tetraselmis suecica	12.04	Phung et al., 2022

Appendix A6:
Raw data - CD

Please refer to the attached CD-ROM to get access to the raw data.

Appendix A7:
Excerpt of a brainstorming process with M.Sc. Niclas Rohde



"Coming together is the beginning. Keeping together is progress. Working together is success." — Henry Ford

Master's Thesis

Cultivation of *Tetraselmis suecica* with different light regimes to improve the survival of *Ostrea edulis* larvae in hatcheries

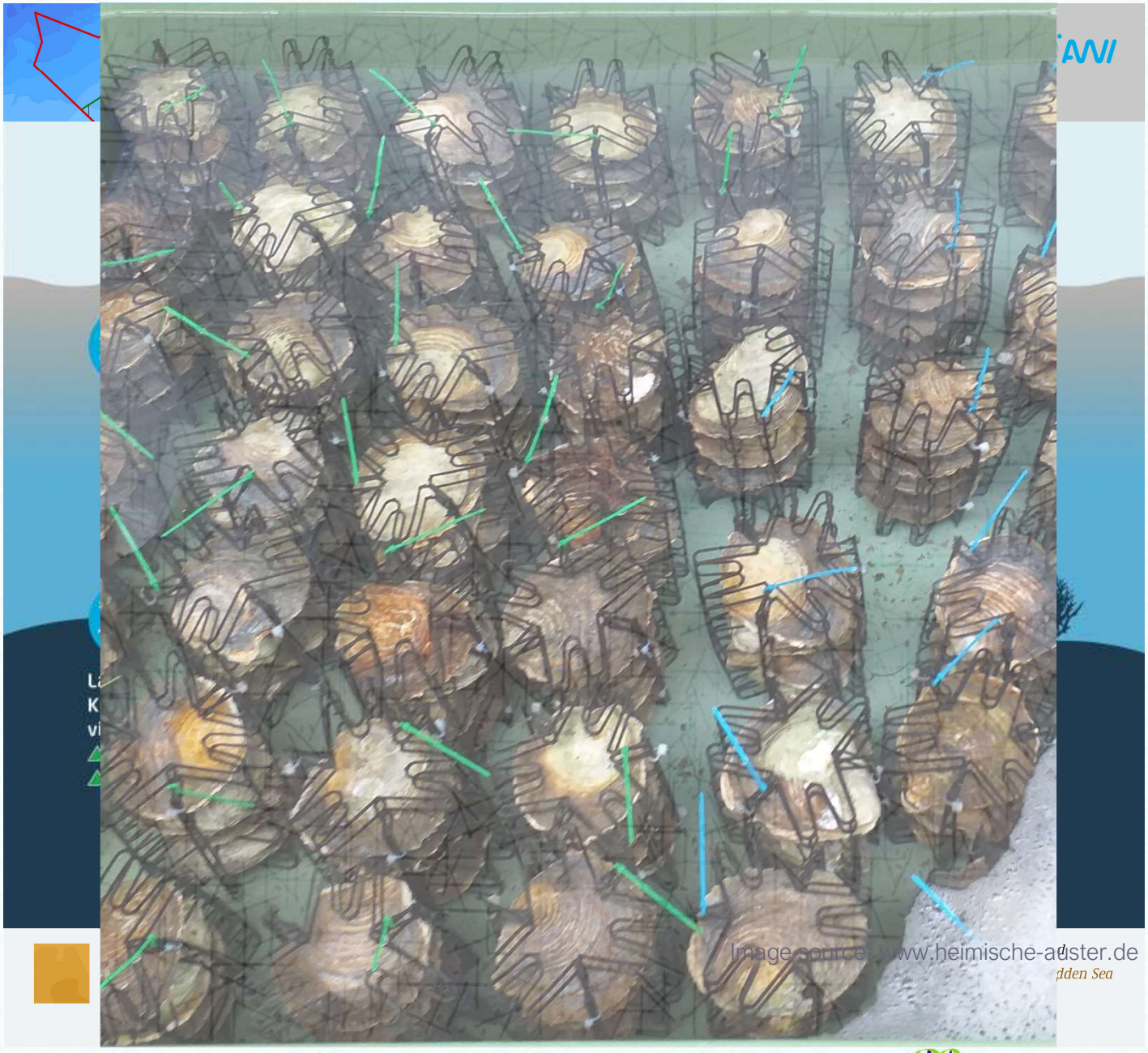
09.03.2022

Thu Thao Phung

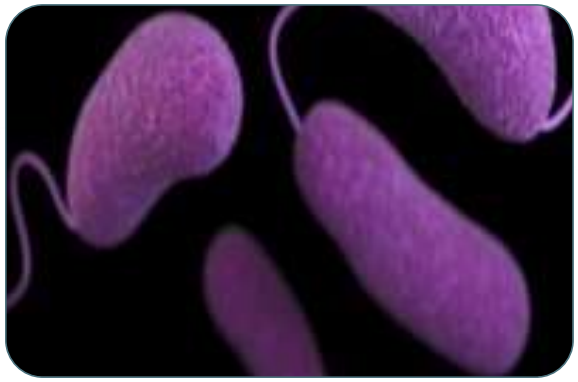
First Examiner:	Prof. Dr. Thomas Klefoth
Second Examiner:	Dr. Bernadette Pogoda
Supervisor:	M.Sc. Bérenger Colsoul

Table of Contents

- The native European flat oyster *Ostrea edulis* (Linnaeus, 1758) was once widespread in the North Sea.
- Due to the fishing methods and overfishing this species is considered to be almost extinct.
- Oyster reefs provide important ecosystem functions and services and are considered a hotspot of biodiversity in marine waters.
- Aquaculture could be a solution for the reintroduction of the oyster species.



Problem and Objective



Vibrio sp.

- Major issue of mass mortalities in oyster aquaculture [1-7]
- Implicated in diseases that could lead to oyster larvae's malformations or mortalities [8,9]

Image source: [20], [21], https://seahorsebreeder.co.uk/store/en/live-phytoplankton-culturing/836-tetraselmis-suecica

Hypotheses

Theory 1

The higher the light intensity, the more > C10 fatty acids (here: LCFA) are produced in the *Tetraselmis suecica* cells.

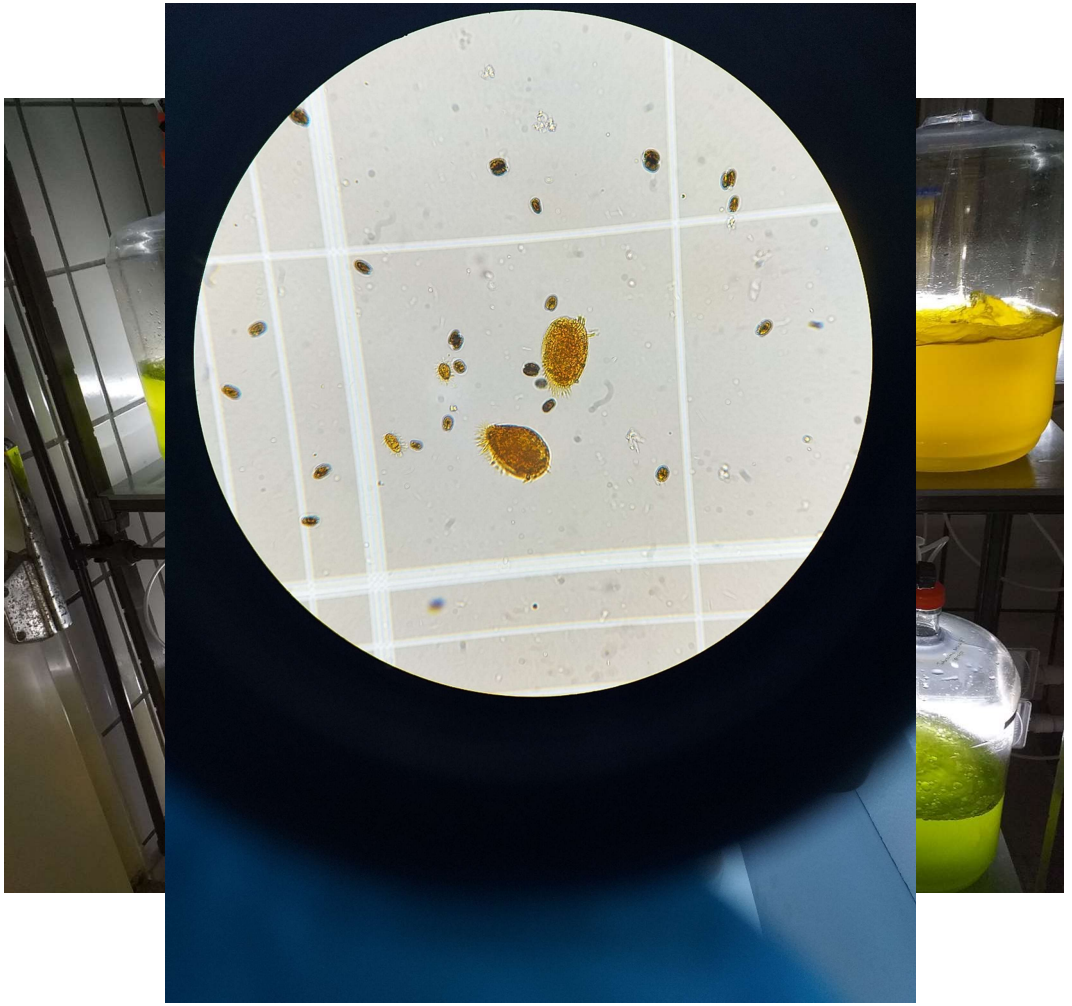
With the mixing of *Tetraselmis suecica* in the feeding of *Ostrea edulis* larvae and thus, adding it to the holding water increases the survival rate of the larvae.

Theory 2

Preliminary experiment 1

- Large-scale (10 L), 4 rep.
- No variances in any factors (e.g. light intensity or nutrient supply)
- Lowest possible light intensity at 360 µmol m^{-2} s^{-1}

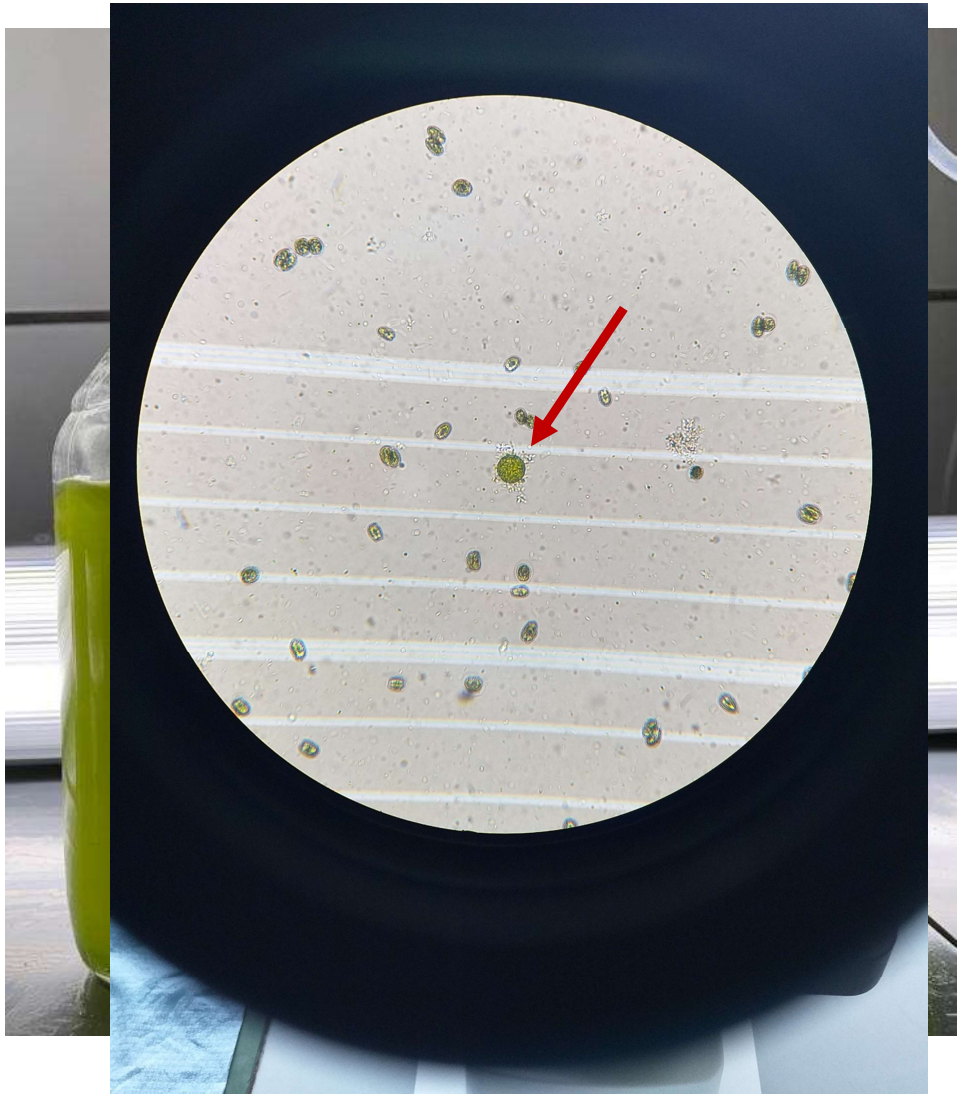
- Contamination with ciliates (*Uronema* cf. *marinum* and *Euplotes vannus*)



Preliminary experiment 2

- Small-scale (1 L), 4 rep.
- No variances in any factors (e.g. light intensity or nutrient supply)
- variation in process of preparation of seawater

- Not identifiable protists in ‚unautoclaved' seawater
- No contaminations in autoclaved seawater



Experimental design 1

- Light treatments:
  - T1 = 100 µmol m^{-2} s^{-1}
  - T2 = 150 µmol m^{-2} s^{-1}
  - T3 = 200 µmol m^{-2} s^{-1}
- 1 L SCHOTT bottles
- T = 22 °C
- 0.2 µm filtered atmospheric air
- No external light influences
- Experiment duration: 8 days

Fig. 1: Experimental design of the main experiment with *Tetraselmis suecica* cultured in three different light intensities by varying the distance to the lamp (X). Photoperiod of 12:12.

Experimental design 1

- Daily measurements
  - Cell density (x)
  - Cell size (l)
  - Cell volume (V_{bio})
  - Dry mass (m_{bio})
  - Abiotic factors (in red labeled bottles): pH, T [°C], S [PSU]
- Fatty acid measurement at the beginning, middle, and end of the exponential growth phase

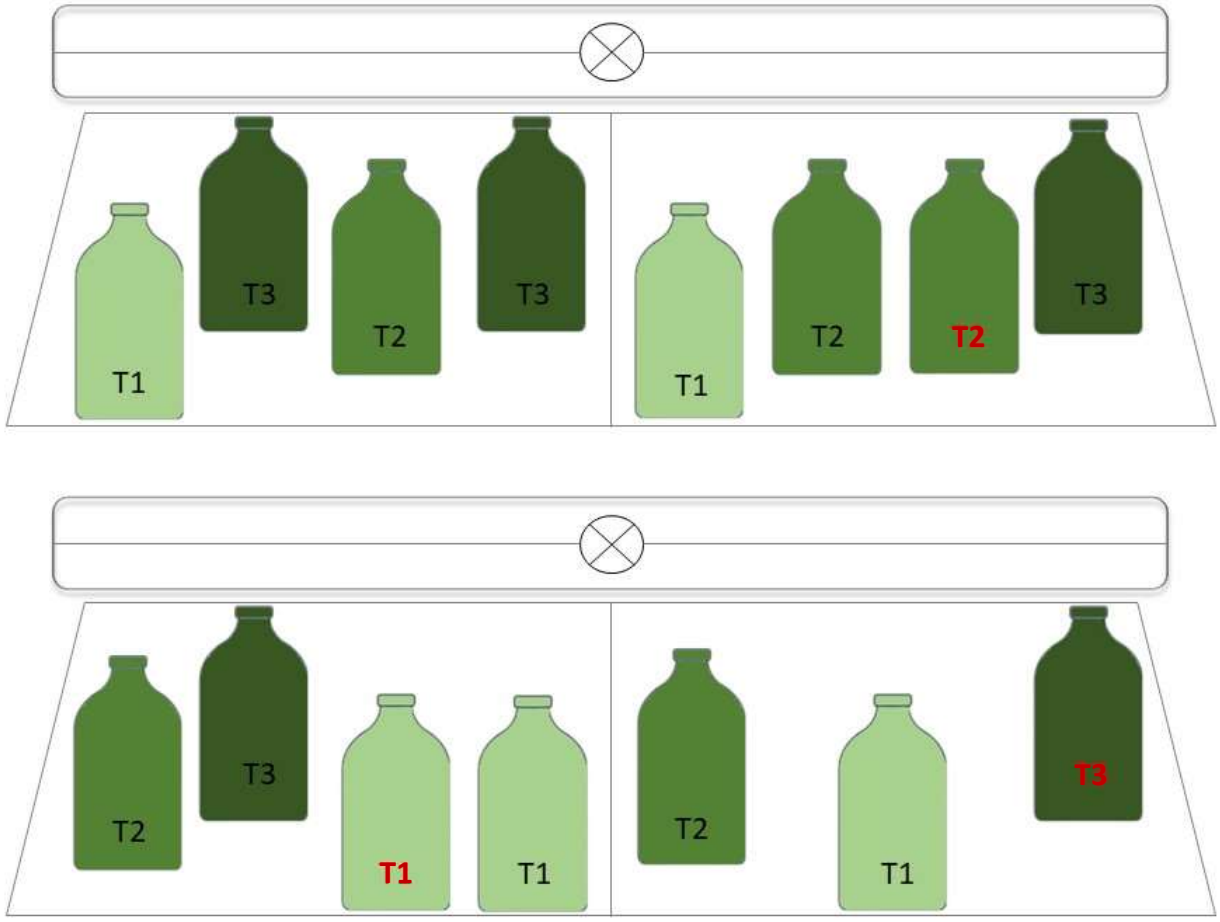
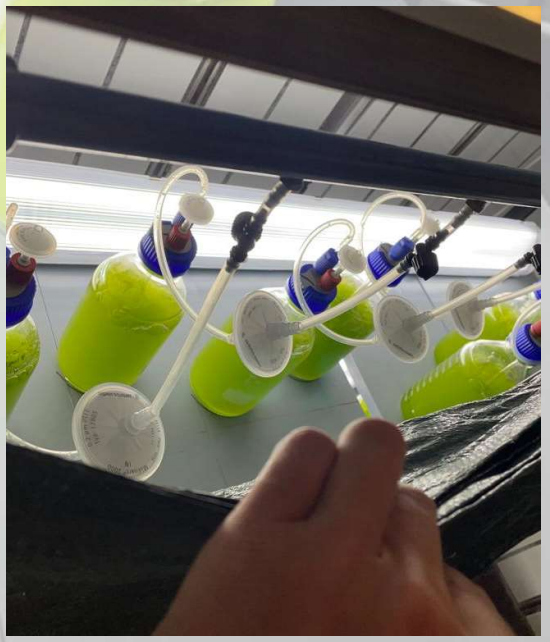


Fig. 1: Experimental design of the main experiment with *Tetraselmis suecica* cultured in three different light intensities by varying the distance to the lamp (X). Photoperiod of 12:12.



Light experiment: set-up

Experimental design 2

- Feeding treatments
  - C1 = IG + CM
  - C2 = IG + CM + RS
  - T1 = IG + CM + TS (100)
  - T2 = IG + CM + TS (150)
  - T3 = IG + CM + TS (200)

- IG = *Isochrysis galbana* (SAG 13/92)
- CM = *Chaetoceros muelleri* (CCAP 1010/3)
- RS = *Rhodomonas salina* (CCAP 978/27)
- TS = *Tetraselmis suecica* (CCAP 66/4)
- (…) = cultivated at (…) light intensity [µmol m^{-2} s^{-1}]

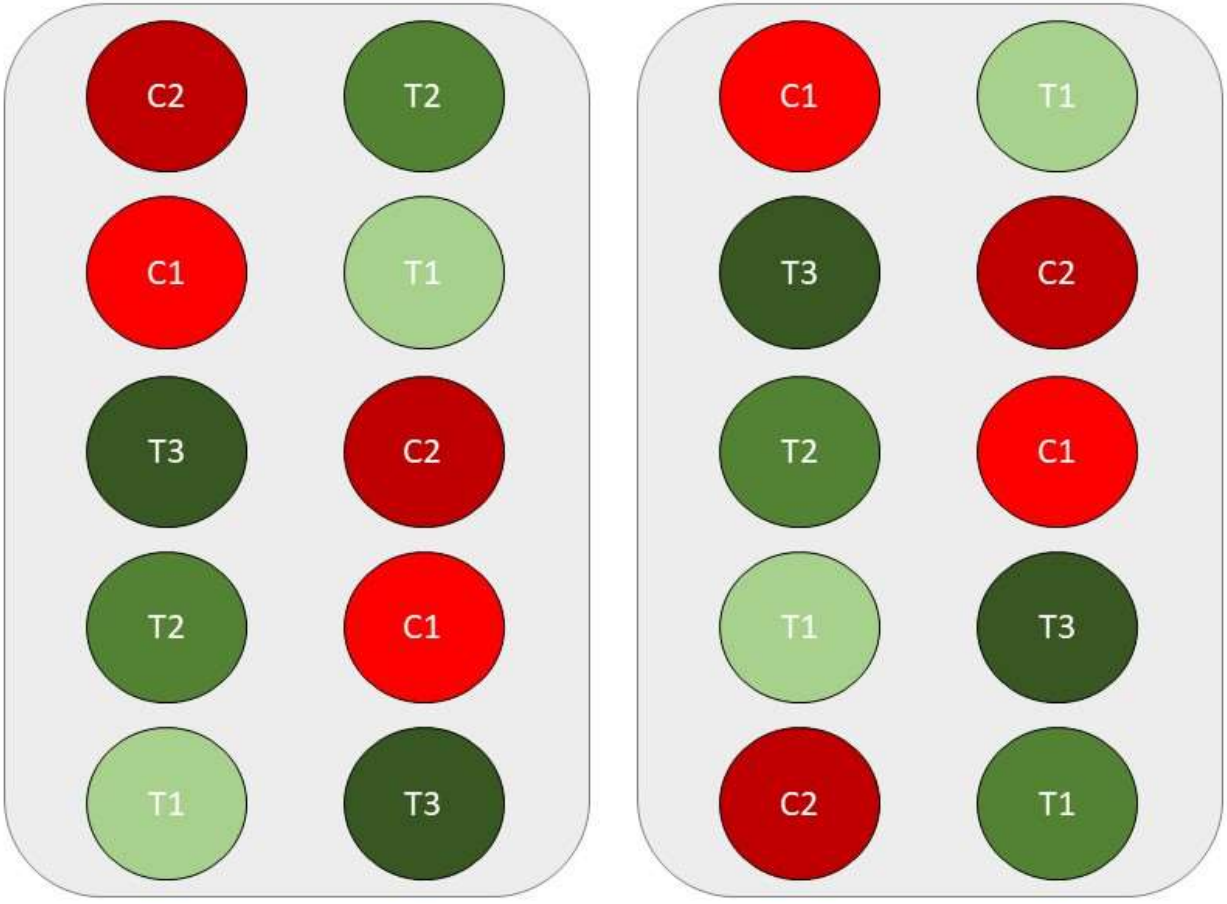
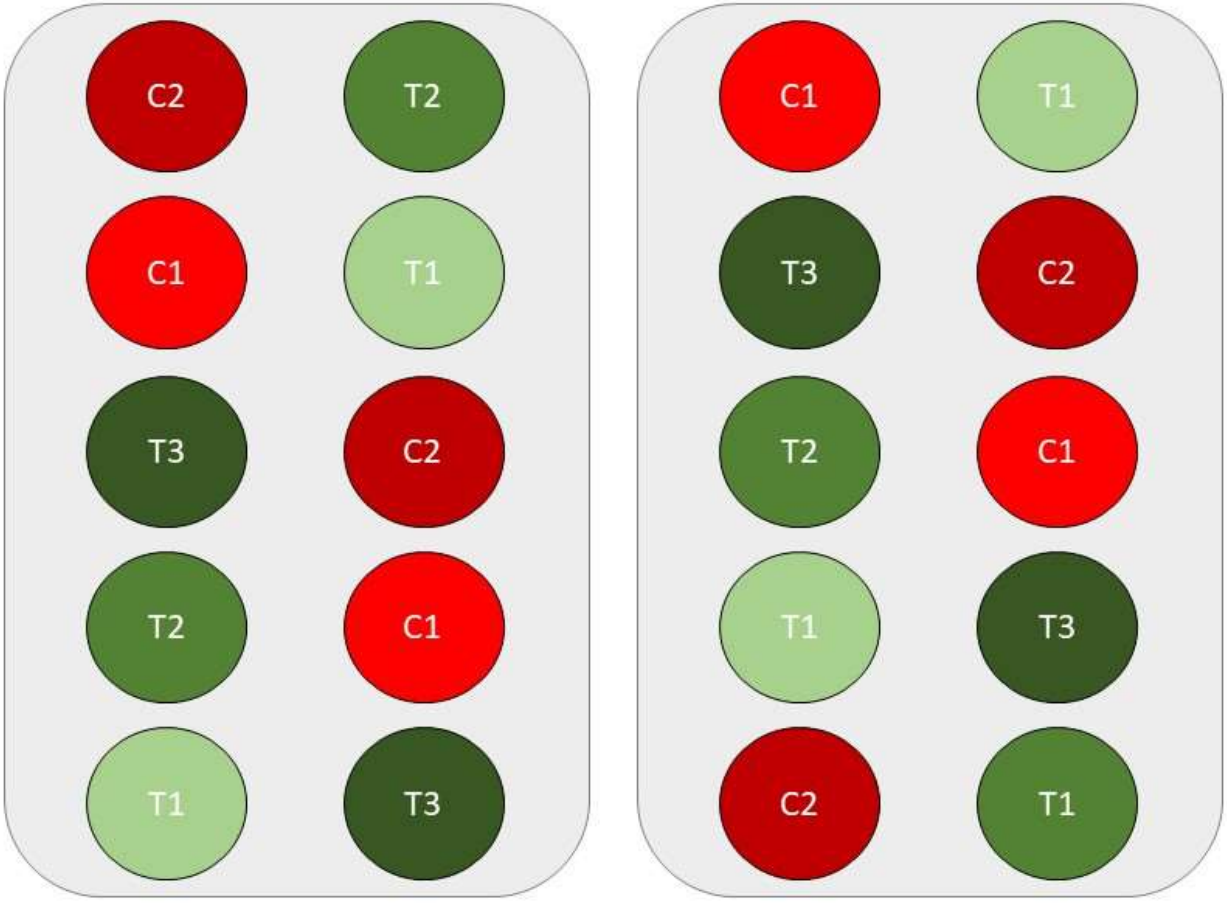


Fig. 2: Experimental design of the feeding experiment in a temperature-controlled room at T = 24 °C. Daily water exchange. Required feeding amount calculated according to Helm et al. (2004) [18]. 3,500 larvae per vessel which contained 700 mL sea water.

Experimental design 2

- Daily measurements
  - Larval growth (mean shell length)
  - Larval survival
- Threefold determination under binocular and image analysis software
- Sampling with 10 mL pipette and 6-well plates
- Experiment duration: 10 days



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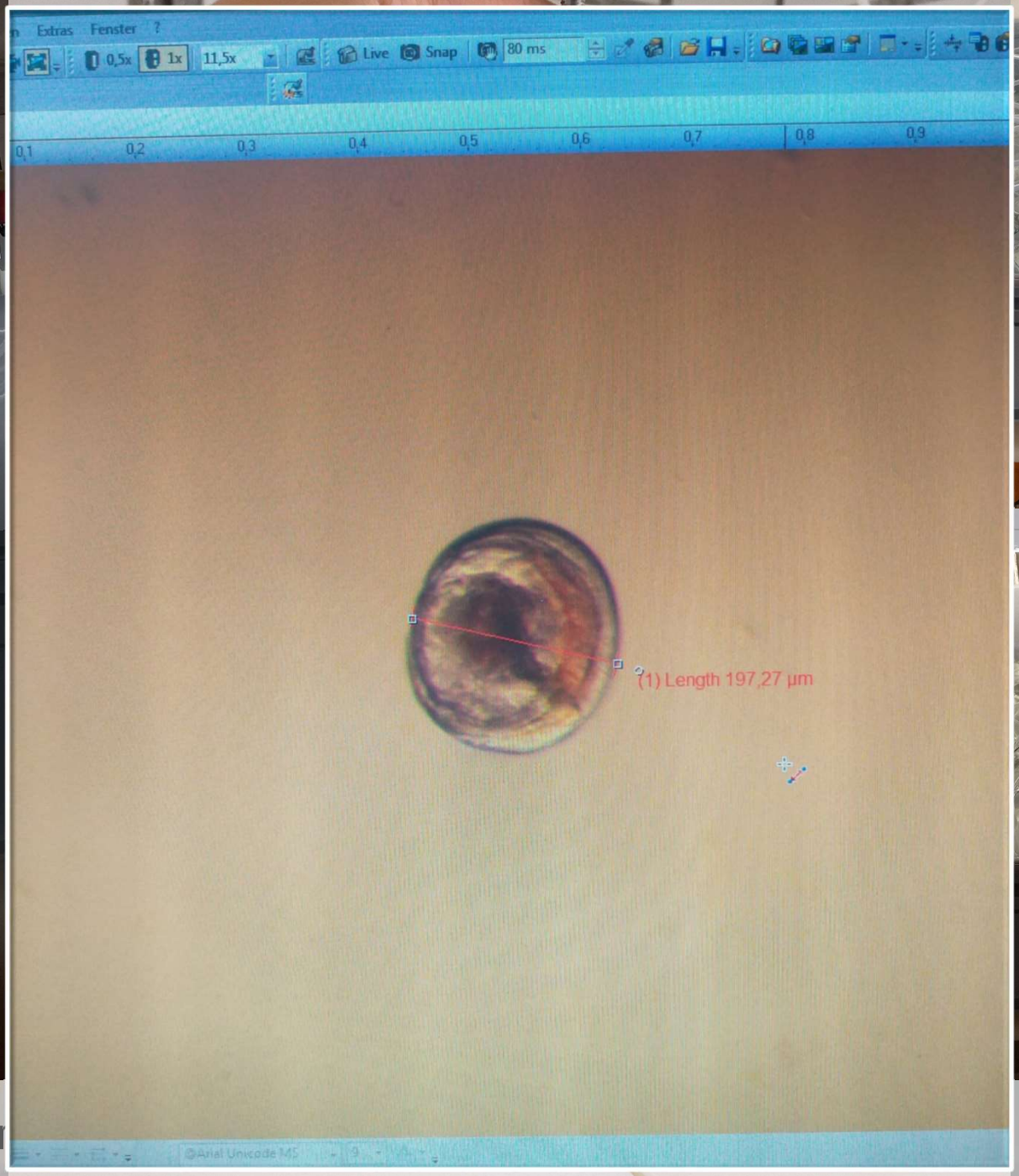
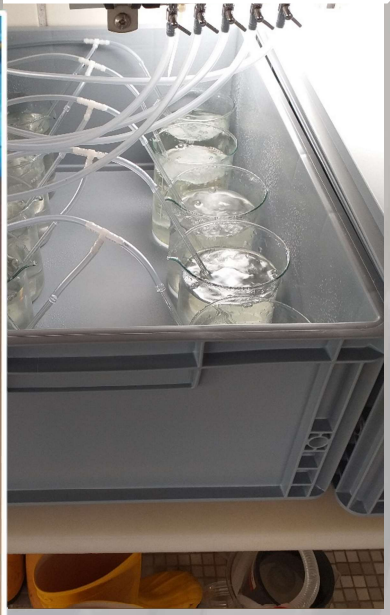
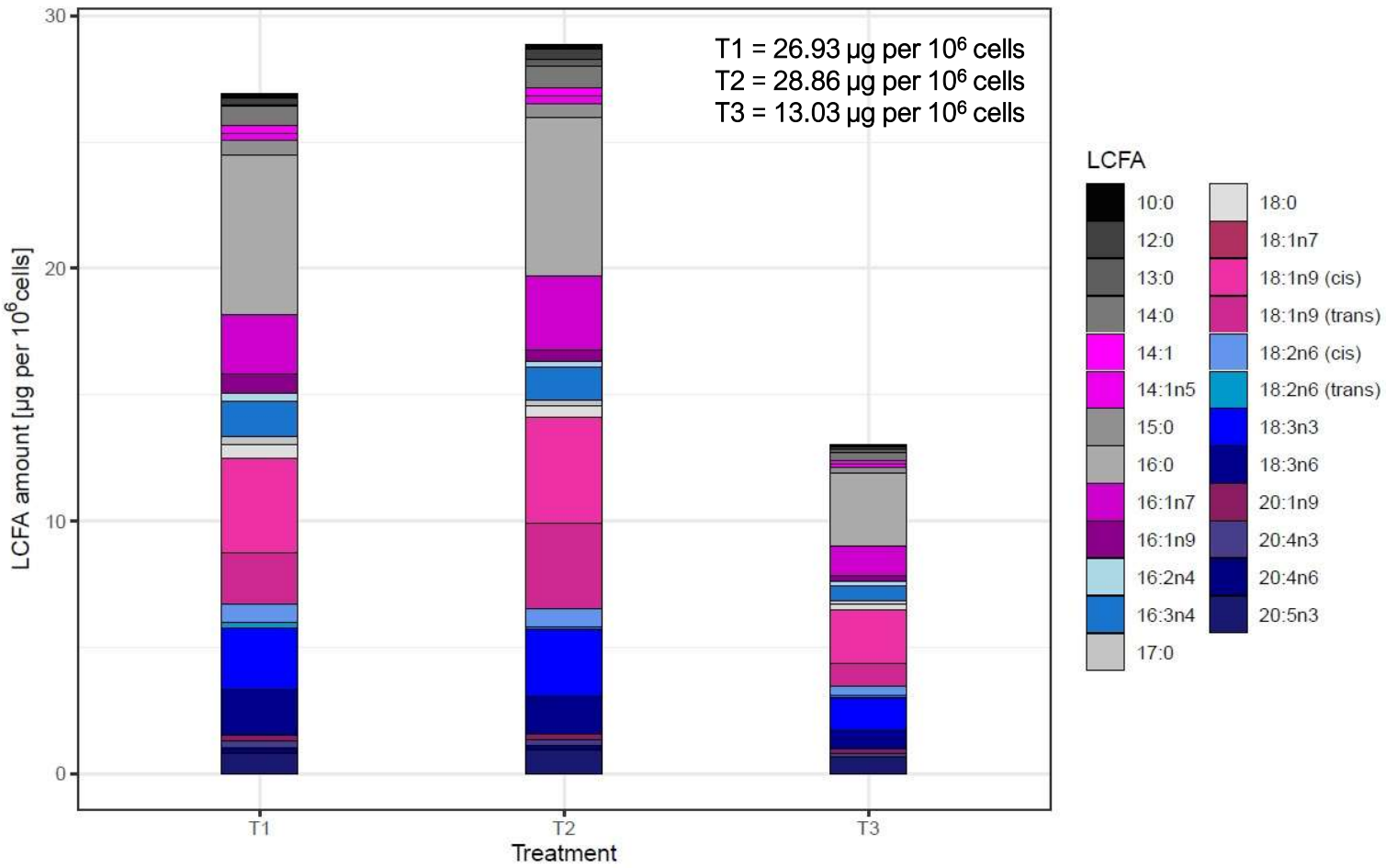




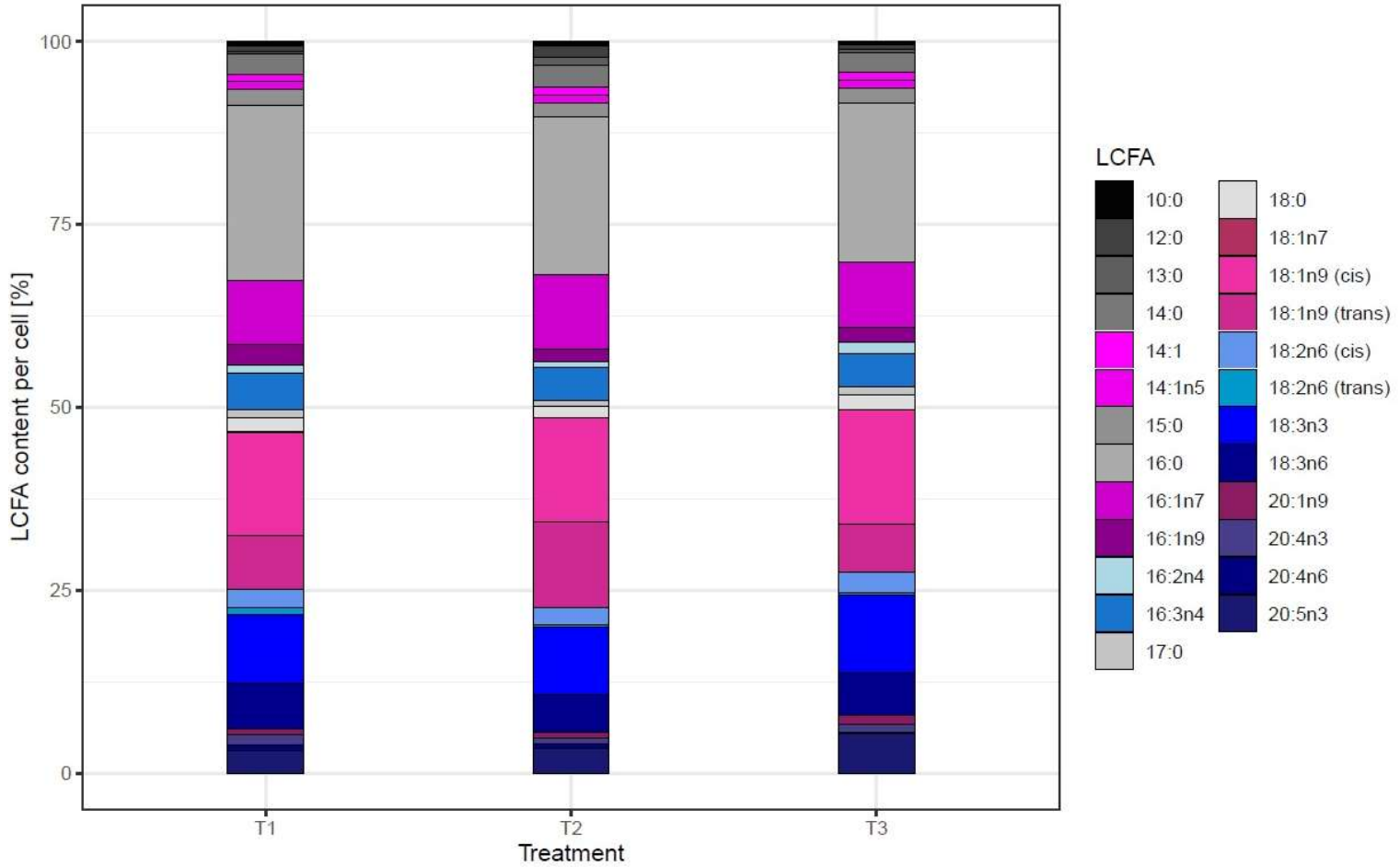
Feeding experi

The effect of light on LCFA in *Tetraselmis suecica* cells



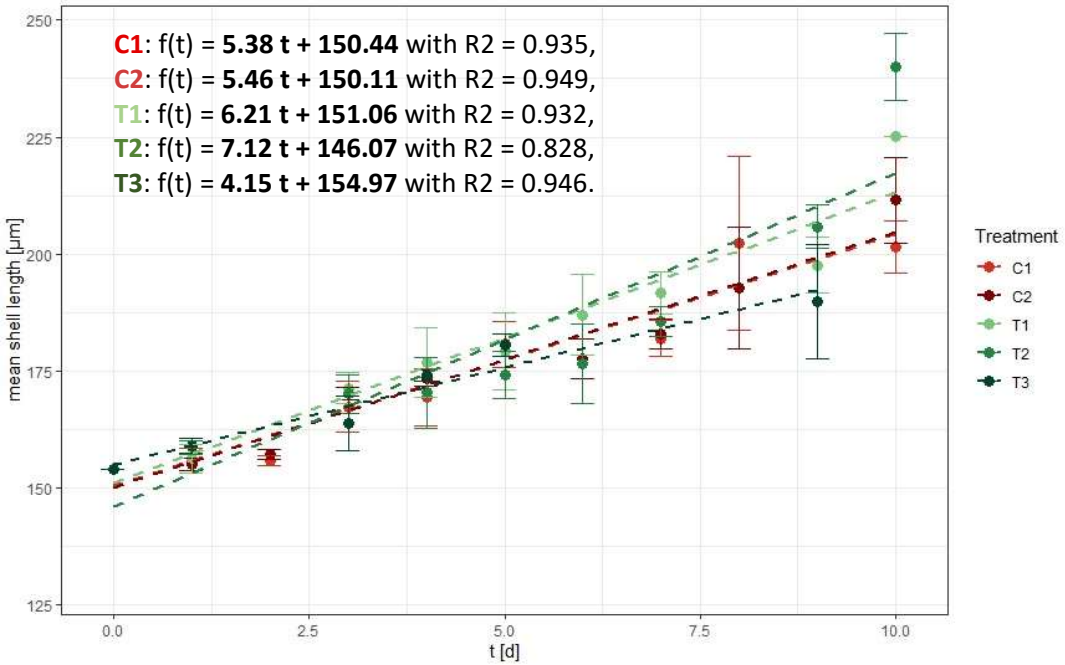
- T3 with lowest amount of total LCFA → significant (p<0.1)
- No significant differences between T1 and T2 (p>0.1)

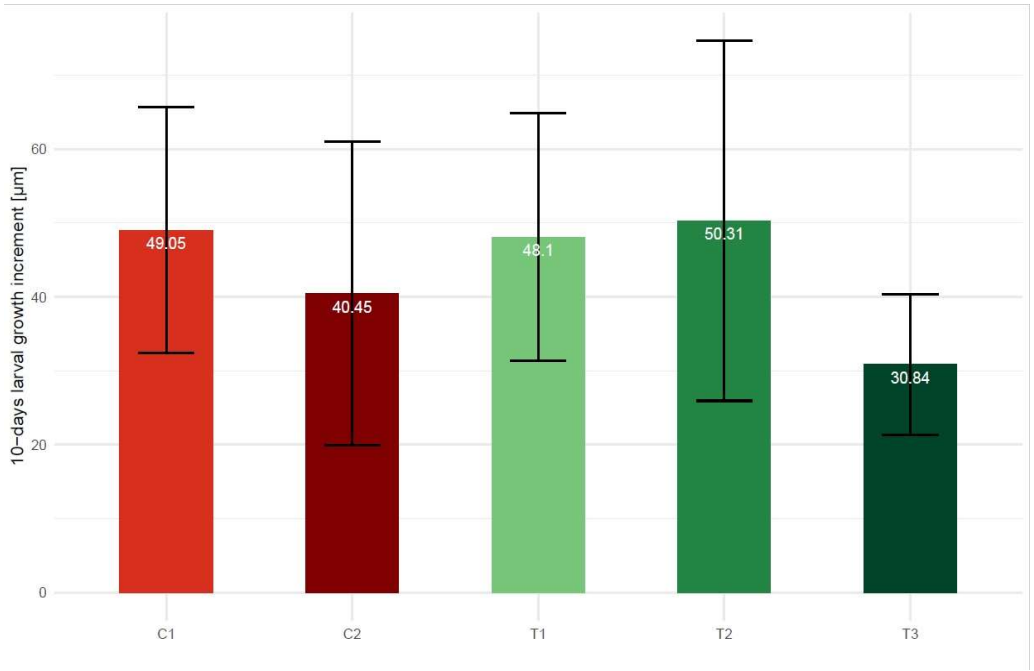
The effect of light on LCFA in *Tetraselmis suecica* cells



- Variations in light intensities did not affect the percent composition of LCFA in *T. suecica* cells (p>0.1)

The effect of 3-mixed diet on *Ostrea edulis* larvae's growth

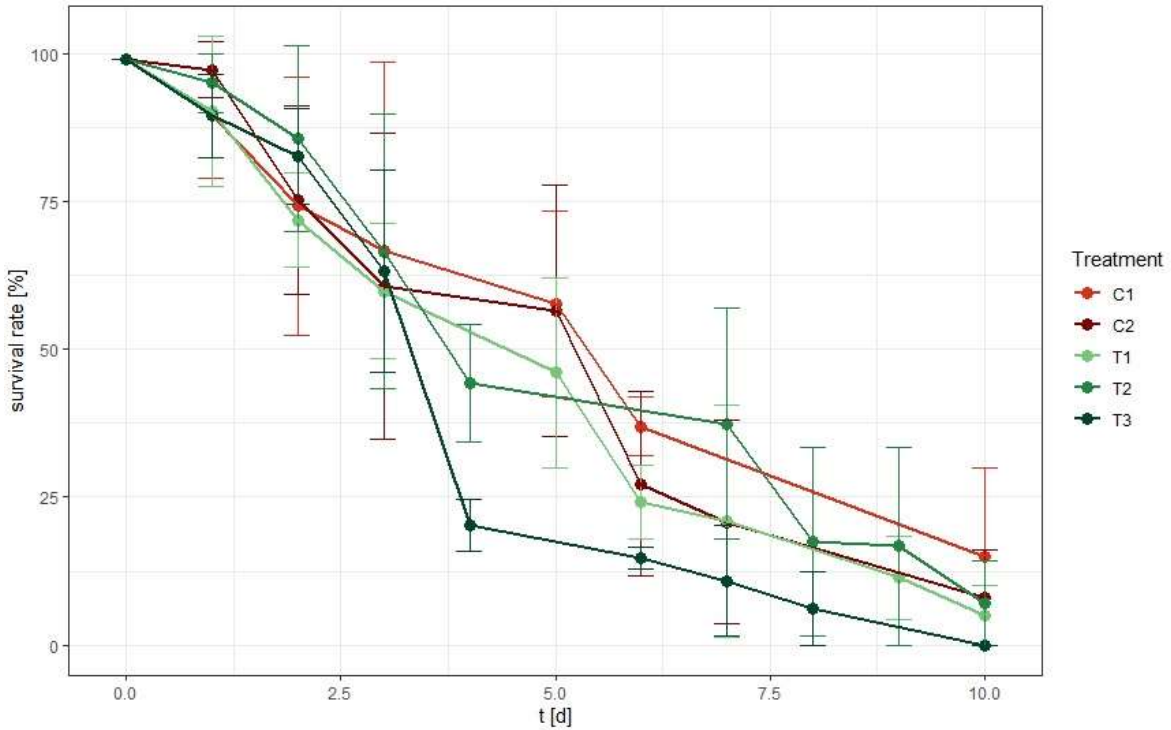




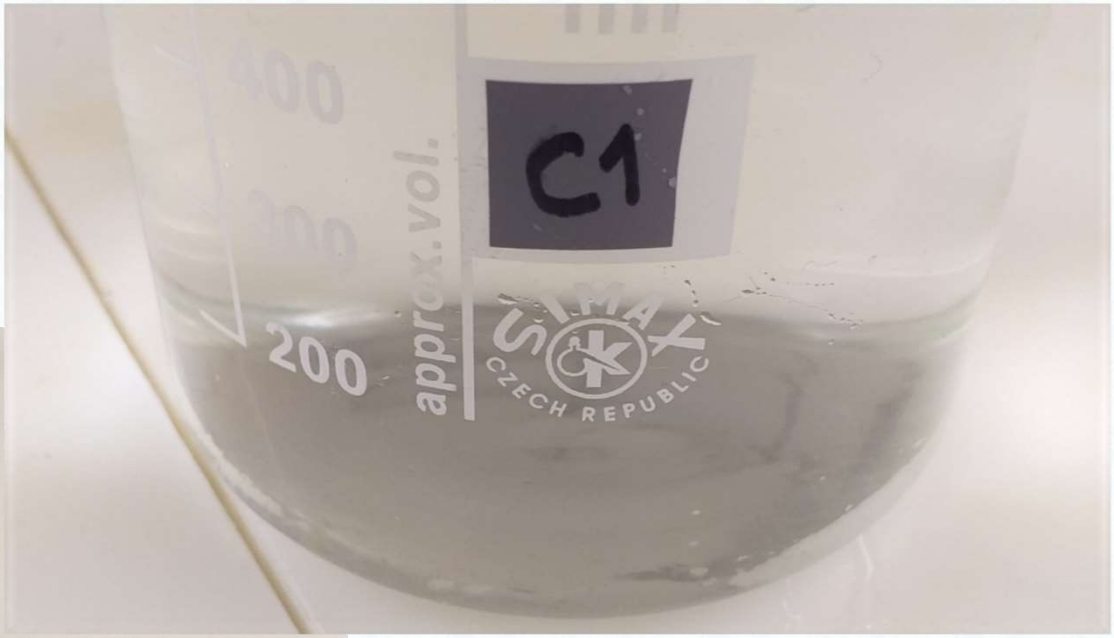
- Fastest growth with T2
- Significant differences between T1 or T2 and T3 (p<0.05)
- Significant differences between T1 or T2 and controls C1 and C2 (p<0.05)

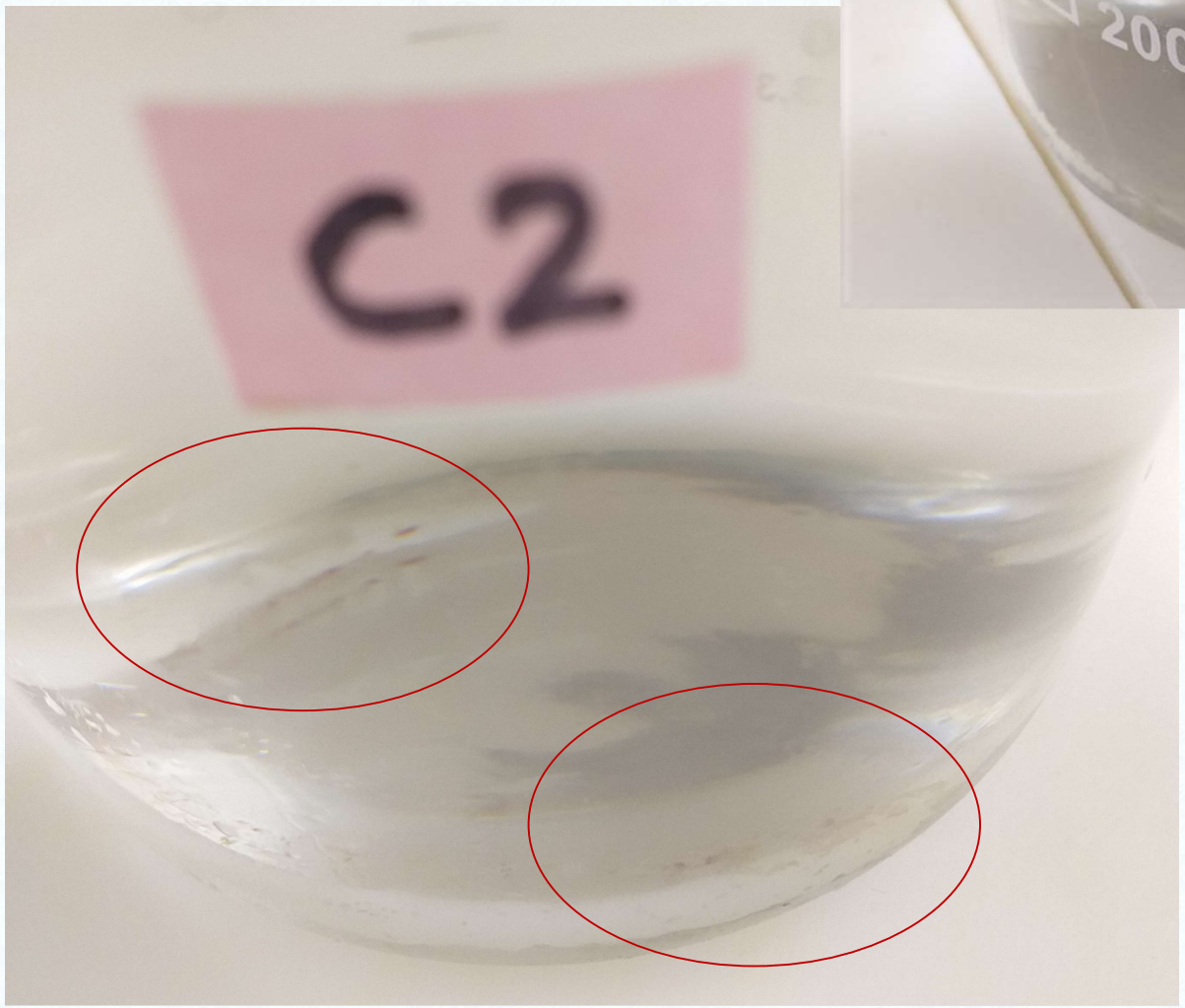
- Largest growth increment in T2
- Significant differences between T1 or T2 and T3 (p<0.05)
- No significant differences between T1 or T2 and others (p>0.05)

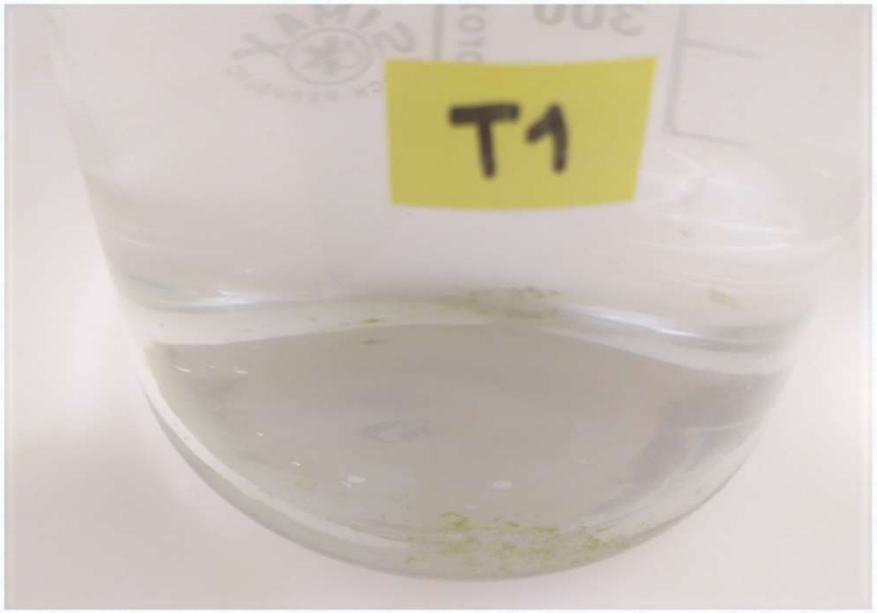
The effect of 3-mixed diet on *Ostrea edulis* larvae's survival

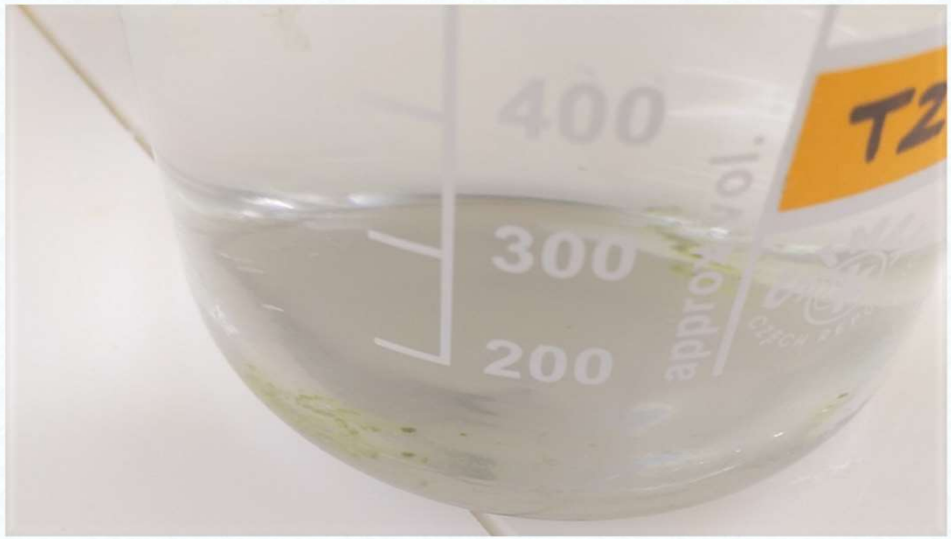


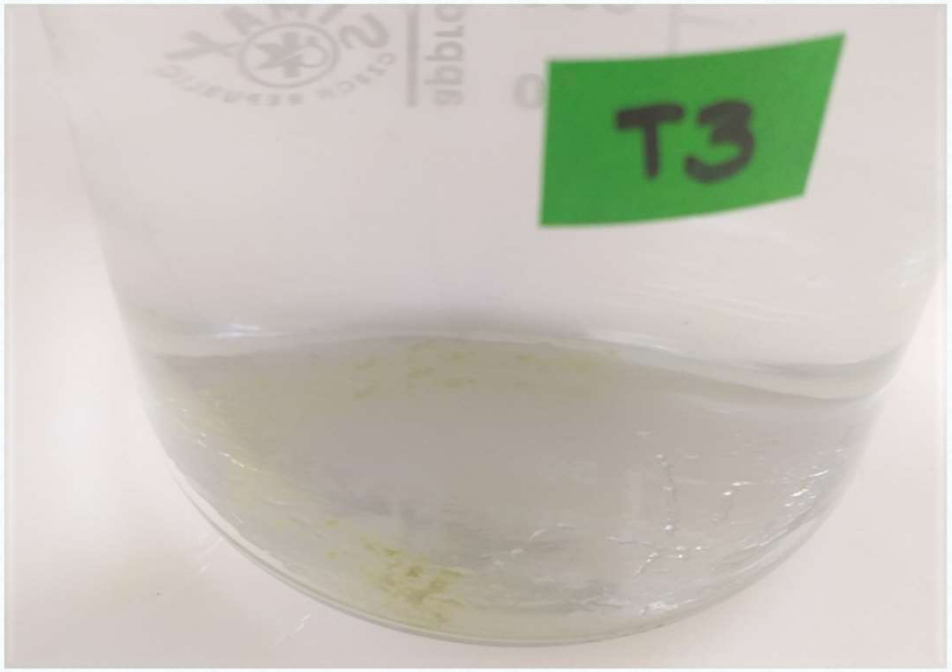
- No survival of the oyster larvae with treatment T3 after 9 days
- Constant mortality rates in the other cases
- Best chances of survival was recorded in C1 (15 %), C2 (8 %), and T2 (7 %)


C1
200
approx.vol.
SIMAX
CZECH REPUBLIC


C2


T1


T2
400
300
200


T3

One-sided aeration resulted in the settlement of microalgae with a larger cell diameter and weight at the bottom of the vessel.

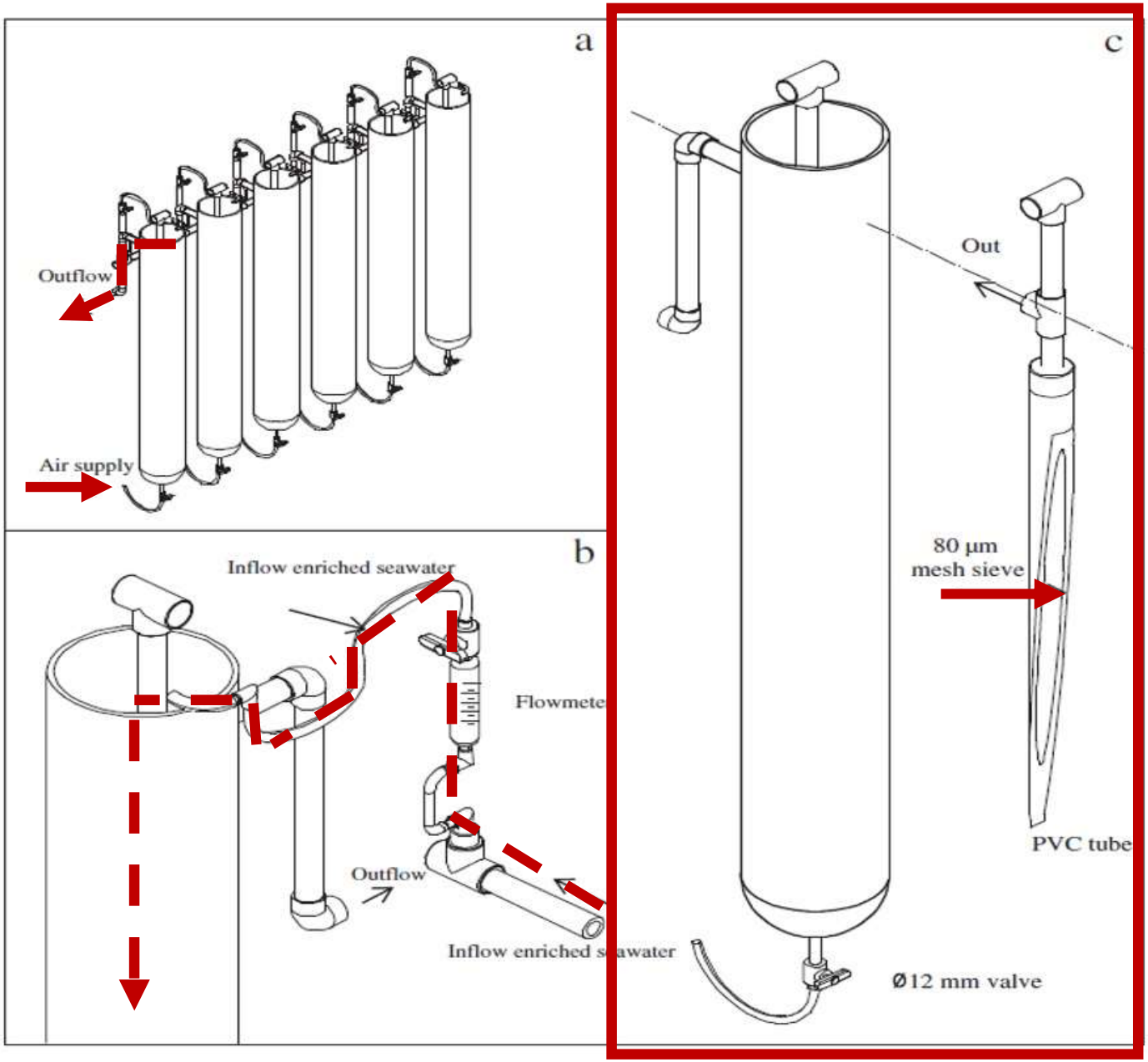
New findings and fundamental basis for further research

1 **Optimal light conditions** for *Tetraselmis suecica* for >C10 fatty acids production = 150 µmol m^{-2} s^{-1}

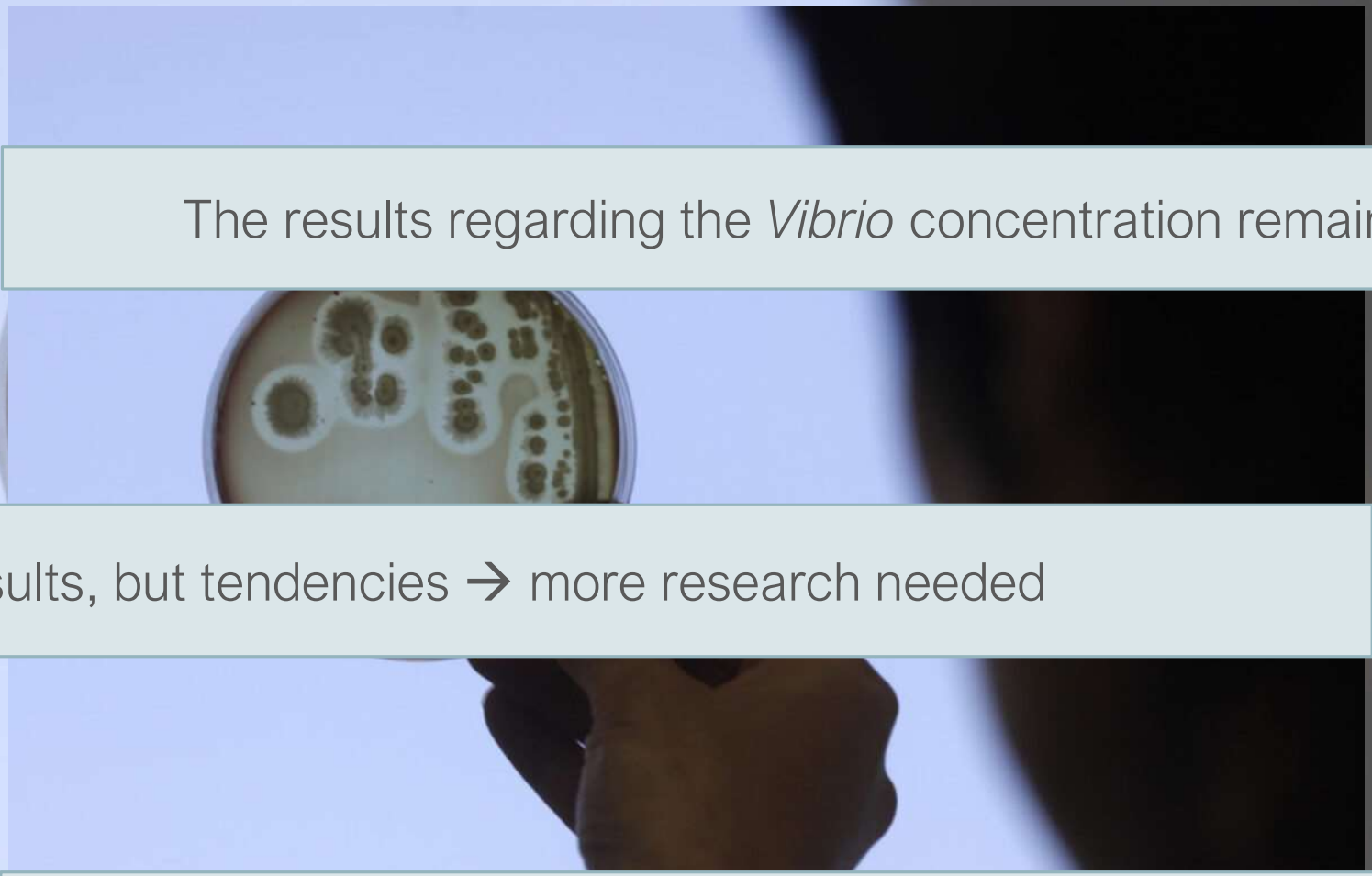
Better growth of *Ostrea edulis* larvae with 3-mixed diet with *T. suecica* but not with *Rhodomonas salina* (***T. suecica*** = **more ‚nutritious'**) 2

3 Better growth does not necessarily mean higher larval survival rates → **representation of novelty of this study**

In order to make a more clearly defined statement about whether increased >C10 fatty acids content increase *O. edulis* larvae's survival rate in hatcheries → **optimization of experimental set-up recommended** (flow-through system)[19] 4


a
Outflow
Air supply
b
Inflow enriched seawater
Flowmete
Outflow
Inflow enriched seawater
c
Out
80 µm
mesh sieve
PVC tube
Ø12 mm valve

[19]



The results regarding the *Vibrio* concentration remain to be seen

No clear results, but tendencies → more research needed

This work is the first step for further investigation and provides a broad foundation for further experiments.

Image Source: https://english.aawsat.com//home/article/1675791/new-type-bacteria-feeds-only-air

Thanks for your attention!

Does anyone have any question?

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Thanks for your attention!

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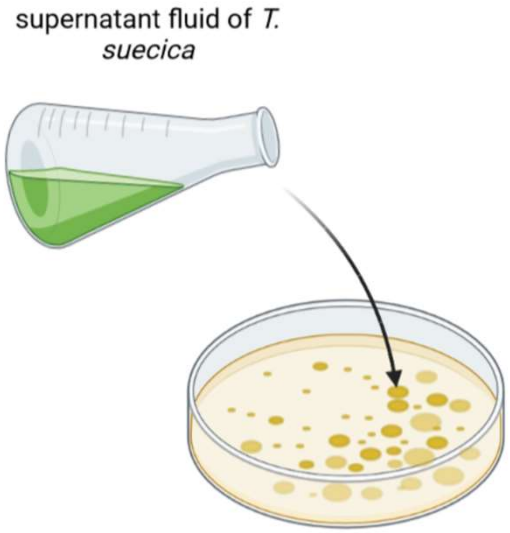
- Backup slides -

Backup – Previous studies

- In-vitro

Austin and Day, 1990

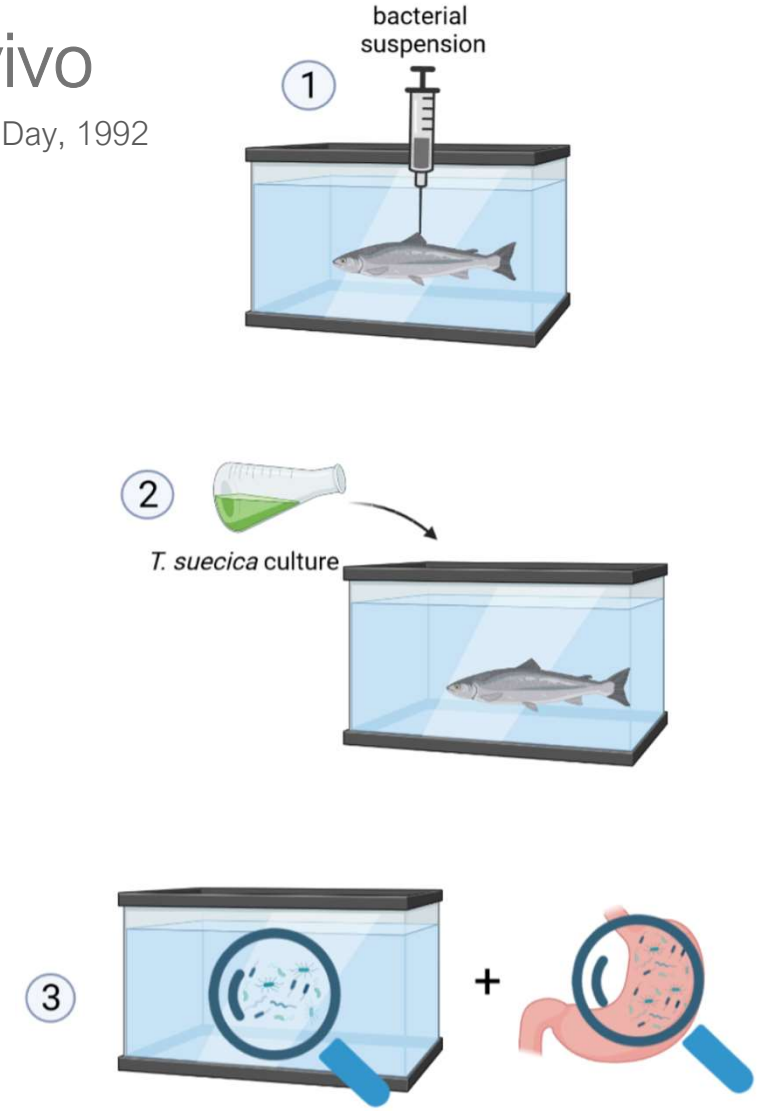


Sketch was created using an infographic tool called "Bio-Render"(online version).

Backup – Previous studies

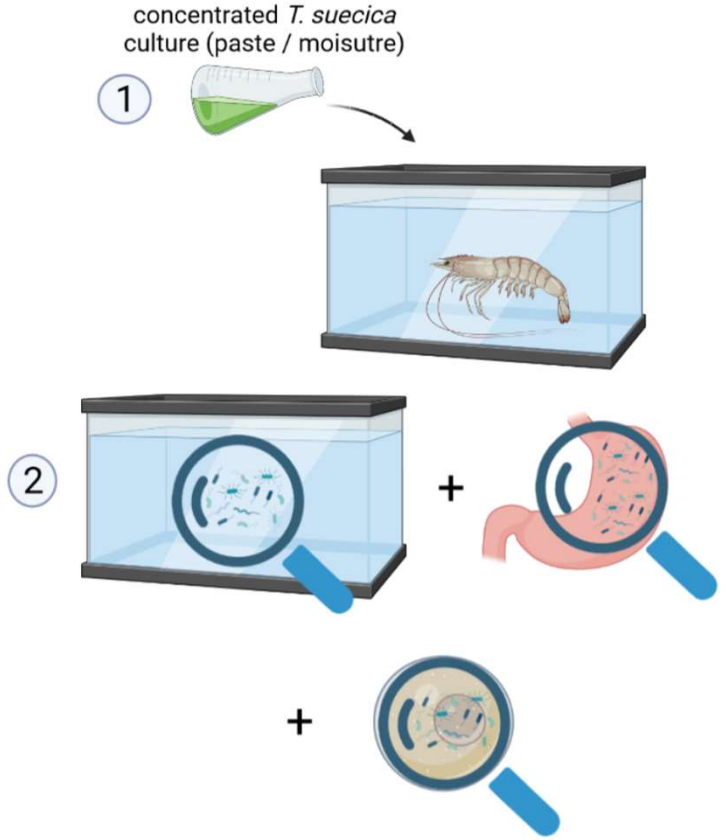
- In-vivo

Austin and Day, 1992



- In-vivo

Regunathan and Wesley, 2004



Sketches were created using an infographic tool called "Bio-Render"(online version).

Backup – Comparison of the different microalgae used in the Helgoland hatchery

- Cell size and biovolume (own data, 2021)

Species	Taxon	Strain	Cell size [µm]	Biovolume [µm³]
Chaetoceros muelleri	diatom	CCAP 1010/3	>4.5	62.5
Isochrysis galbana	flagellate	SAG 13/92	4	37.5
Rhodomonas salina	flagellate	CCAP 978/27	6.7	165
Tetraselmis suecica	flagellate	CCAP 66/4	9	435

- PUFA content

	PUFA content [µg 10^6 cells]	References
Isochrysis galbana	4.09	Pernet et al. (2003)
Chaetoceros muelleri	2.07	Pernet et al. (2003)
Rhodomonas salina	13.10	Thoisen et al. (2020)
Tetraselmis suecica	12.04	Phung et al., 2022

Pernet, F., Tremblay, R., Demers, E. and Roussy, M. (2003), 'Variation of lipid class and fatty acid composition of chaetoceros muelleri and isochrysis sp. grown in a semicontinuous system', *Aquaculture* **221**(1), 393–406.

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Backup – Statistics

- Chi-squared test – Test of Independence

H_0 = The characteristics X and Y are stochastically independent.

If H_0 is rejected, X and Y are dependent.

Correlation test should be done.

Backup - Statistics

- Example 1: (source: https://www.statology.org/chi-square-test-real-life-examples/)

	SPD	CDU	Die Grünen
Male	120	90	40
Female	110	95	45

P-value = 0.65

Since the $p>0.05$, there is not sufficient evidence to say that there is an association between gender and political party preference.

- Example 2: (source: https://www.statology.org/chi-square-test-real-life-examples/)

	High School	Bachelor's	Master's
Married	20	100	35
Single	50	80	15

P-value = 0.000011

Since the $p<0.05$, there is sufficient evidence to say that there is an association between marital status and education level.

- Why is a correlation test necessary after this?

Backup - Statistics

- It is clear:
  - Independent variables are always uncorrelated.
  - Correlation implies stochastic dependence.
- BUT dependent variables do not have to correlate with each other either
- BECAUSE in statistics, correlation implies linearity.

If there is a correlation between data sets, but it is not monotonic (linear), the correlation coefficient can become zero even though the data are stochastically dependent but not correlated.

- CAREFUL: Correlation does not mean causality!!!