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Developing Molecular Tools To Genetically Engineer The Microalga Nannochloropsis

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Abstract

The main objective of this thesis was to develop an efficient transformation protocol for *Nannochloropsis oceanica* CCMP1779 by biolistic transformation using the linearized pSELECT100 plasmid which confers hygromycin B resistance. In addition the toxicity of several antibiotics for different *Nannochloropsis* species was determined.

Nannochloropsis is the genus of unicellular photosynthetic microalgae in the class of Eustigmatophyceae, which have industrial significance due to their production of high amounts of lipids.

The biolistic genetic transformation system of *Nannochloropsis oceanica* CCMP1779 is still not well established. In the current study, transformation of *N. oceanica* CCMP1779 was achieved by particle bombardment with the PDS-1000/He instrument using plasmid coated onto gold and tungsten microparticles. Results show that the type of microparticle is an important factor for the efficiency of transformation. *N. oceanica* is transformed more efficiently with 0.6 μm gold particles than with 0.7 μm tungsten particles. Using the optimized transformation condition, the transformation efficiency was 5-25 transformants per 1.3×10^9 recipient cells, which is more efficient comparing with those reported in this species transformation studies.

Antibiotics are chemical substances that have antagonistic effects on the growth of other microorganisms. This study investigated the growth inhibiting effects of eleven antibiotics on three *Nannochloropsis* species: *Nannochloropsis gaditana*, *Nannochloropsis limnetica* and *Nannochloropsis oceanica*. Ampicillin and Kanamycin were not toxic at the tested concentrations (20 to 200 $\mu\text{g}/\text{mL}$) to the three species, whereas Zeocin and Cycloheximide were the most toxic to the three *Nannochloropsis* species (Resulted more than 65% growth reduction at 1 $\mu\text{g}/\text{mL}$). Chloramphenicol, Zeocin, Hygromycin B, Cycloheximide, and G418 neomycin had a promise as selective reagent for the three *Nannochloropsis* species.

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Chapter One: Introduction to *Nannochloropsis*

Nannochloropsis is a small, simple, photosynthetic and world-widely distributed (marine, fresh and blackish water) genus of microalgae (Wang et al., 2014), which belongs to class Eustigmatophyceae of Heterokontophyta, a diverse algal group that includes brown algae and diatoms (Hibberd DJ., 1981). *Nannochloropsis* cells reproduce asexually, dividing to yield two daughter cells that then shed their mother cell wall (Scholz MJ. et al., 2014).

Eustigmatophytes are apparently unicellular coccoid algae with a yellow-green appearance. Since the species exhibit little morphological diversity, cells found in field samples are difficult or impossible to identify using light microscopy; this has restricted the description of new species and hampered efforts to estimate the biodiversity of the group (Andersen et al., 1998); and therefore the species have been delimited primarily by DNA sequence analysis (Fawley KP. et al., 2007).

The genus *Nannochloropsis* includes six species which are phylogenetically grouped into *Nannochloropsis granulata*, *Nannochloropsis oceanica*, *Nannochloropsis limnetica*, *Nannochloropsis gaditana*, *Nannochloropsis oculata* and *Nannochloropsis salina* (Vieler A. et al., 2012). Of the six species, *N. limnetica* are the only fresh water isolates. The species in genus *Nannochloropsis* are often found offshore, which are usually 2-5µm in size (Li S. et al., 2011). It is difficult to identify species in this genus through traditional morphological observations, so DNA sequences such as 18S rDNA has been used in the phylogenetic analysis of class Eustigmatophyceae (Andersen et al., 1998). The plastid of these algae is surrounded by four membranes derived from a secondary endosymbiosis event which explains the diversity of photosynthetic eukaryotes (Adrian R. et al., 2007).

Nannochloropsis is an emerging model for photosynthetic production of oil (triacylglycerol) because of its ability to grow fast rapidly and tolerate a wide range of environmental conditions (Wang et al., 2014). Although the growth of microalgae *Nannochloropsis* depends of characteristics of the species, they mostly double their biomass within 24 hrs. and biomass doubling times during exponential growth are commonly as short as 3.5 h (Moazami N. et al., 2012).

Table 1.1: Taxonomic classifications of microalgae *Nannochloropsis* (Copied from Chernyavskaya O., 2014)

Domain	Eukaryota
kingdom	Chromalveolata
phylum	Hetrokontophyta
class	Estigmatophyceae
Family	Estigmataceae
Genus	<i>Nannochloropsis</i>

1.1 Genomic Engineering

Genomes are fundamental for genetic manipulation and further genetic engineering, which not only provide the location and the distribution of metabolic pathways and enzymes but also aid in the identification of elements that can improve genetic engineering (Qin et al., 2012).

Access to microalgal genome sequences that are of interest for academic or industrial applications greatly facilitates genetic manipulation, and the availability of rapid large-scale sequencing technology represents a revolution in microalga research (Gong Y. et al., 2011). Several nuclear genome sequencing projects have now been completed, and this leads to improve the levels of certain products in microalgae (Simas R. et al., 2015).

Genetic engineering has been implemented for the development of molecular tools and optimization of lipid production capacities of microalgae that show potential for biodiesel fuel production (Daboussi F. et al., 2014). Due to the potential of *Nannochloropsis* algae as an industrial feedstock and the progress made in developing homologous gene replacement, several research groups have set out to sequence the genome of different *Nannochloropsis* strains (Vieler A. et al., 2012).

1.2 Why Microalgae - as a reservoir of new natural products (biofuels)

Microalgae are characterized by higher solar energy yield, the potential for year-round cultivation in many locations, the ability to grow in brackish and saline water as well as water produced from oil and gas extraction, higher areal productivities than oil seed crops (Quinn et al., 2012).

Microalgae represent a promising source of biomass feedstock for fuels and chemicals because many species possess the ability to grow rapidly and synthesis large amounts of storage neutral lipids in a form of triacylglycerol (TAG) from sunlight and carbon dioxide (Wang et al., 2014). The combustion of the biomass coming from microalgal cultures can be exploited as an interesting alternative for biodiesel production (Antonio M. et al., 2008).

Finding clean and sustainable energy (for example biodiesel) for future life is getting one of the most vital research area all over the world (Radakovits R. et al., 2010). Biodiesel is usually produced from oleaginous crops, such as rapeseed, soybean, sunflower and from palm, through a chemical transesterification process of their oils with short chain alcohols. However, average biodiesel production yield from microalgae can be 10 to 20 times higher than the yield obtained from oleaginous seed and vegetable oils (Gouveia L. et al., 2009).

Microalgae have been identified as a possible source of new generation biofuels since they do not compete with food and feed crops, attain higher oil yields than currently available agricultural crops, and can be cultivated in seawater on non-arable land (Bondioli P. et al., 2012). In particular, species like *Chlorella vulgaris*, *Chlorella emersoni*, *Nannochloris* sp., *Nannochloropsis* sp., *Neochloris oleoabundans*, *Phaeodactylum tricornutum* and *Tetraselmis sueica* have been reported as a promising alternatives of fossil fuels for producing biodiesel, because they accumulate important lipid amounts (Carerero A. et al., 2011).

The ability of microalgae to fix CO₂ can also be an interesting method of removing gases from power plants, and thus can be used to reduce greenhouse gases with a higher production microalgal biomass and consequently higher biodiesel yield (Radakovits R. et al., 2010). Different scientific reports have been delivering information regarding the capability of different groups of microalgae for producing biofuel based on their biomass oil content (Gouveia L. et al.,

2009). As it is shown in the table below, genus *Nannochloropsis* is proved to be suitable as raw materials for biofuel production, due to their high oil content (Gouveia L. et al., 2009). *Nannochloropsis* species (Eustigmatophyceae) show total lipid contents from 10 to 60 wt% in dry matter (Carrero A. et al., 2011)

Table 1.2: comparison of microalga biomass oil content (%) (Adapted from Gouveia L. et al., 2009)

Microalga species	Oil content (%)
<i>Scenedesmus obliquus</i>	17.7
<i>Chlorella vulgaris</i>	5.1
<i>Dunaliella tertiolecta</i>	16.7
<i>Spirulina maxima</i>	4.1
<i>Nannochloropsis</i> sp.	28.7

Generally, some of the advantages of microalgae for being suitable alternation for biodiesel production include

- ✓ They are the most efficient biological producer of oil (Gouveia L. et al., 2009)
- ✓ they have high photosynthetic efficiency and higher biomass productivities
- ✓ they have faster growth rate than higher plants (Rozan; Tan., 2011)
- ✓ they don't have seasonal production and can be harvested daily (Quinn et al., 2012)

Chapter Two: Antibiotic susceptibility tests on the growth of *Nannochloropsis* sp.

2.1 Introduction

The American microbiologist Selman Waksman and his colleagues were the first scientists who used the term “antibiotics” to describe chemical substances produced by microorganisms and having antagonistic effects on the growth of other microorganisms (Sengupta S. et al., 2013). Then, a number of scientists have been defined the term in different ways based on sparse knowledge of the ecology and biology of naturally occurring low- weight organic compounds (Davies J., 2006).

The discovery and development of penicillin produced by a fungus, along with streptomycin, produced by soil bacteria over 50 years ago, initiated an era of unusually rapid advances in studies on the antibiotics (Clardy J., 2009). The majorities of antibiotics discovered to date are produced by *Actinomycetes*; includes Tetracycline, Streptomycin, Chloromycetin and the macrolide family (Korybski T. et al., 2013). Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious disease in humans, animals and plants (Kummerer K., 2009). However, it may result in a potential risk of non-target microorganisms that provide important ecosystem services, such as nutrient cycling, organic matter mineralization and degradation of pollutants (Nåslund et al., 2008). Each antibiotic have their own features and characteristics that dictate how and which cellular process is inhibited; Yim G et al (2006) summarizes the diversity effects of antibiotics on cellular functions as follows:

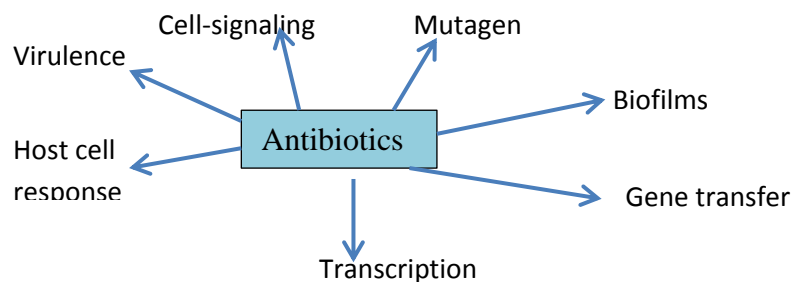


Figure 2.1: some effects of antibiotics on cellular functions

2.2 Mode of actions of antibiotics used in this experiment

Each antibiotic has their own mode of action when they are introduced into cells. Some antibiotics are characterized by a wide spectrum, others by a very narrow spectrum of antimicrobial activities (Grollman AP., 1966). It has been reported that ribosome is a vital target for a wide variety of antibiotics and most ribosomal antibiotics interfere with its function by binding to specific sites on the ribosome (Brodersen DE. et al., 2000; Moore D., 2013).

Hygromycin B is produced by *Streptomyces hygroscopicus* and known to inhibit preferentially translocation of mRNA and tRNAs on the ribosome in both bacteria and eukaryotes; and exert multiple effects on protein synthesis (Borovinskaya MA. et al., 2008). Gentamycin, paromomycin, and neomycin are structurally related aminoglycosides which target mitochondrial and bacterial ribosomes and eukaryote ribosomes to a lesser extent (Manuvakhova M. et al., 2000).

Chloramphenicol as a bacteriostatic agent, concentrations from 1 to 10 µg per mL completely inhibited all species of bacteria, with no or little effect on eukaryotic cells at concentrations which completely inhibit bacterial growth (Marchant R. et al., 1968). As noted by Morris I (1966), not only protein synthesis, but also the synthesis of polysaccharide and nucleic acid is also inhibited by chloramphenicol.

Ampicillin and Streptomycin are antibiotics commonly used to eliminate prokaryotes from the cultures of eukaryotic algae (Kviderova J. et al., 2005). However, they have different mode of actions; Ampicillin inhibits peptidoglycan synthesis of the prokaryotic cell wall and is not expected to influence metabolism and growth of eukaryotes (Kviderova J. et al., 2005) while Streptomycin interacts with the 30s ribosomal subunits and inhibits the growth of eukaryotic organisms by binding to the chloroplast ribosome, resulting in selective inhibition of plastid protein synthesis (Qian H. et al., 2012).

Cycloheximide inhibits protein synthesis and has been found to be toxic for fungi, protozoa, and mammalian cells but not for bacteria (Goldberg HI., 1965). Cycloheximide inhibits the transfer reaction in protein synthesis and has no effect on amino acid activation or the synthesis of aminoacyl-sRNA (Grollman AP., 1966).

Streptothricin-class of antibiotics, Nourseothricin inhibits protein synthesis by induced miscoding, has been used as a selection marker for a wide range of organisms including bacteria, plant cells and mammalian cells (Kochupurakkal BS. et al., 2013). The other antibiotic which is effective at low concentration against bacteria and a wide variety of eukaryotic organisms is Zeocin; causes cell death involves binding to and cleavage of DNA (Moore D., 2013).

In general, DNA replication, transcription, translation by 70-S ribosomes, cell membranes, transcription by 70-S and 80-S ribosomes and cell wall synthesis have been reported as a main site of antibiotic mode of actions (Demain AL., 2000). This date, biotechnology uses the advantage of antibiotics that kill or inhibit the growth of bacteria and other microorganisms to clean contaminants from different algal cultures and to utilize antibiotics as a selective reagent for the genetic manipulation of algae.

So far, successful attempts to obtain bacterial free cultures of algae by using different antibiotics have been reported. For instance, Provasoli et al. (1948) were among the first investigators to show interest in the use of antibiotics to obtain axenic algae cultures; they also found the use of appropriate combinations of the antibiotics resulted in the elimination of bacterial contaminants from the algal and protozoan cultures (Hunter EO. et al., 1961). Recently, Vieler A. et al. (2012) strengthen the idea of antibiotic essentiality for eliminating contaminants from cultures and genes conferring resistance to antibiotics are frequently used as markers for the introduction of foreign DNA into the algae genome.

Recent studies have demonstrated the effect of different antibiotics on the growth of *Nannochloropsis* sp. on agar-solidified medium (Vieler A et al., 2012; Chernyavskaya O., 2014). Results which came from the reported experiment shows that Zeocin (5 µg/mL) and Hygromycin B (25 µg/mL) were chosen for use in subsequent selection marker studies, while higher concentrations of Ampicillin (200 µg/mL) and Chloramphenicol (100 µg/mL) could be useful for selecting against bacterial contaminants in *Nannochloropsis* culture.

In this study, three *Nannochloropsis* sp. growing in liquid culture have tested for their sensitivity to a range of different antibiotics. In the previous studies, cells were plated out on agar-solidified medium containing antibiotics for detection, but here antibiotics were supplemented into liquid cell cultures and placed on a rotational shaker. Eleven antibiotics with a range of concentration

were used and apparent results of each growth inhibition effects were investigated in this study. Effect was assessed by measuring of absorbance at 750 nm (OD₇₅₀). This work also identified a selection reagent for a stable transformation system in three *Nannochloropsis* species by re-examining the sensitivity to the antibiotics used in the previous study. There are not many studies in the literature that report the effect of antibiotics on the growth of liquid cultures of *Nannochloropsis* species, so this study could help as a starting point to discover new selection marker for the genetic engineering of *Nannochloropsis* species.

2.3 Objectives of the study

The main objectives of this study were:

- ✓ To compare the effect of different antibiotics with a range of concentration on the growth of three *Nannochloropsis* sp.
- ✓ To figure out the lethal antibiotic doses on each species and to find new selection agents for designing of new plasmids with different resistance genes.
- ✓ To identify possible antibiotics suitable for cleaning *Nannochloropsis* cultures from contaminations.

2.4 Materials and Methods

2.4.1 Test compounds

Test compounds were chosen to represent different classes of antibiotics. The availability of antibiotics, the essentiality of results for selection marker and the degree of inhibition were the criteria for selection. The following antibiotics were selected: Ampicillin, Chloramphenicol, Cycloheximide, Gentamycin, G418 Neomycin, Hygromycin B, Kanamycin, Nourseothricin, Paromomycin, Streptomycin and Zeocin (see Table 2.1 for working concentration).

Stock solutions of each antibiotic were made in high purity water (sterile distilled water). The antibiotics were stored in 4°C and in the dark (for light sensitive antibiotics; for example, Hygromycin B). Only Chloramphenicol dilutions were freshly prepared before each experiment was taken place. The concentrations of each antibiotic in the inhibition test were adjusted from each stock solution. The table below shows the antibiotics used for the growth test and how their respective working concentration was prepared.

Table 2.1: list of antibiotics used and their final working concentrations. The dilution was done by mixing stock antibiotic solutions with sterile distilled water and kept frozen except Hygromycin B and Paromomycin.

Antibiotics	Stock solution (mg/ml)	Dilution from the stock solution (ratio)	Concentrations utilized for the test (µg/mL)
Ampicillin	100	1/20	5
Kanamycin	50	1/10	5
Chloramphenicol	34	1/6.7	5
Zeocin	100	1/100	1
Streptomycin	20	1/20	1
Hygromycin B	50	1/10	5
Cycloheximide	50	1/50	1
Gentamycin	20	1/4	5
Paromomycin	50	1/50	1
G418 Neomycin	50	1/50	1
Nourseothricin	20	1/4	5

2.4.2 *Nannochloropsis* sp. and growth conditions

Three *Nannochloropsis* species (*Nannochloropsis oceanica* CCMP1779, *Nannochloropsis gaditana* CCMP526 and *Nannochloropsis limnetica* KRIENITZ 1998/3) were tested for their sensitivity to a range of antibiotics. *N. oceanica* CCMP1779 cells were obtained from National Center for Marine Algae and Microbiota (NCMB) former CCMP (<https://ncma.bigelow.org/>). *N. gaditana* CCMP1779 cells were provided by the Posewitz lab (Colorado School of Mines); for more information (<https://ncma.bigelow.org/node/1/strain/CCMP526/printInfo>) while *N. limnetica* KRIENITZ 1998/3 cells were kindly provided by Postdoc student who came to do antibiotic test experiment at Department of Biology, NTNU.

N. oceanica CCMP1779 and *N. gaditana* CCMP526 cells were cultivated in a media where 50% seawater was filtered, autoclaved and finally enriched with Cell-Hi NC solution (1mL for 1L of 50 % seawater) which was obtained commercially from Varicon Aqua Solutions. Both cells were grown in liquid culture under photoautotrophic conditions; under constant light intensity of 100 $\mu\text{E m}^{-2}\text{s}^{-1}$ at a temperature of 23°C and constant rotational shaker at Heidolph® Rotamax 120 orbital shaker.

Cell cultures of *N. limnetica* KRIENITZ 1998/3 were also grown in liquid culture under photoautotrophic conditions; under constant light of 100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ but in Bourrelli modified medium at 20 °C and 150 rpm shaking. At the start of growth tests, each algal culture was diluted with fresh medium to achieve the lowest initial cell densities (Esther V. et al., 2010).

Cell culture plates with 24 wells were used for cultivation and assessing the effect of different antibiotics in liquid cultures. 1 mL of cell culture supplemented with appropriate concentration of antibiotics were transferred into each wells in the plate. All species were spotted three times for the same degree of antibiotics (biological triplicate method). The liquid medium itself and cell cultures without antibiotics (called wild type) were used as blank and control respectively. After 48hrs exposure, OD values of the cells were measured by Tecan Infinite® 200 Pro Multimode Reader (Plate reader, Switzerland) with absorbance value of 750 nm. The incubation time chosen to investigate the effect of antibiotics on the growth of the three *Nannochloropsis* sp. was 8 days. Culture densities were monitored by means of OD₇₅₀ measured at day 0, 2, 4, 6 and

day 8. Culture densities were determined by means of OD₇₅₀ measurement using a predetermined regression based on direct cell counts (Chernyavskaya O., 2014). Duplicates of untreated samples (wild types) were used as controls.

All the samples, media (blank) and cells without antibiotics (wild type) were measured 16 times (4*4) per well. Since each sample was spotted three times in different well, there were 16 *3 OD_{750nm} results for one sample. Finally, four values ((1, 2), (1, 1), (2, 1) and (2, 2)) was collected and added from each well and divided by 12 to calculate the average OD_{750nm} of the sample. Standard deviation was calculated to determine the discrepancy between generated OD_{750nm} values. In addition to that, to make sure that the media didn't affect the growth of cells, the value of blank was subtracted from all values of sample.

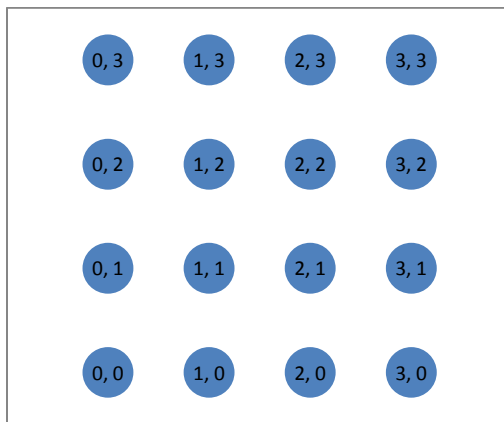


Figure 2.2: Multiple Reads Per- well Alignment.

2.4.3. Determining lethal doses of Antibiotics

For the determination of lethal antibiotic concentration on each species, OD_{750nm} values of wild type cells (cells growing without antibiotics) were assumed to be 100 % unaffected. All results from last measurement (192 hours of exposure to respective antibiotic concentrations or after 8 days) were changed to percentage based on the value of wild type cells. 30 % growth difference between wild type cells and cells growing with a range of concentration was taken as lethal antibiotic dose (if 30 % of target cells are killed). Column graph was drawn for easily determination of lethal doses through observing the graph.

2.5 Results and Discussions

2.5.1 Ampicillin - to determine the effect of ampicillin on the growth of *Nannochloropsis* species, three ranges of concentrations including 20, 100 and 200 $\mu\text{g/mL}$ were used.

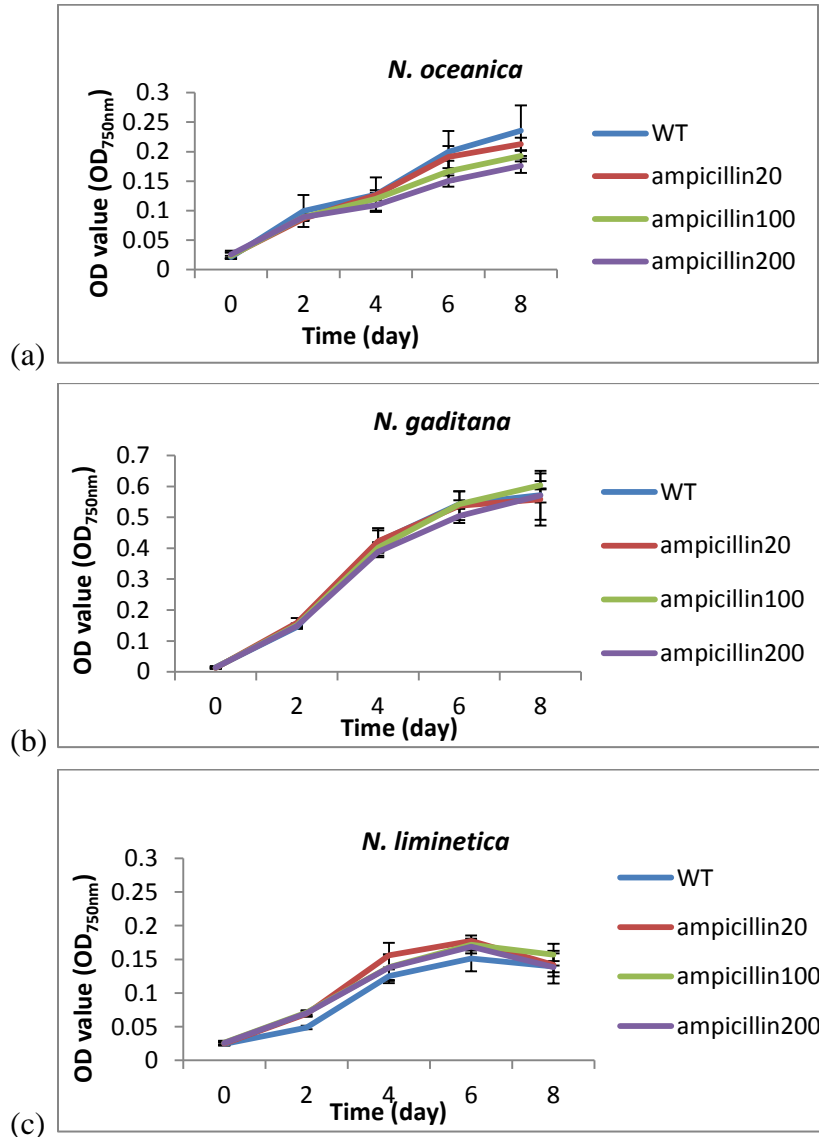


Figure 2.3: inhibition Effect of Ampicillin on the growth of *N. oceanica* (a), *N. gaditana* (b) and *N. limnetica* (c). WT (wild type) are cells grown without antibiotics. Shown antibiotic concentrations are in $\mu\text{g/mL}$. OD_{750nm} values are the mean (three replicates) of each treatment.

From the growth curves, it was observed that, neither of the species of *Nannochloropsis* investigated was sensitive to ampicillin even at higher concentrations (200 $\mu\text{g/mL}$). This indicates that the effect of ampicillin on growth of *Nannochloropsis* sp. was not very significant.

2.5.2 Kanamycin – concentration of 100 µg/mL of kanamycin produced detectable inhibitory effects only on one of the species tested, *Nannochloropsis limnetica*. During the last day of incubation (day 8), *N. limnetica* failed to grow noticeably. However, after incubation for 8 days, no effects of kanamycin were not evident on the growth of *N. oceanica* and *N. gaditana* (Fig. 2.4a and c).

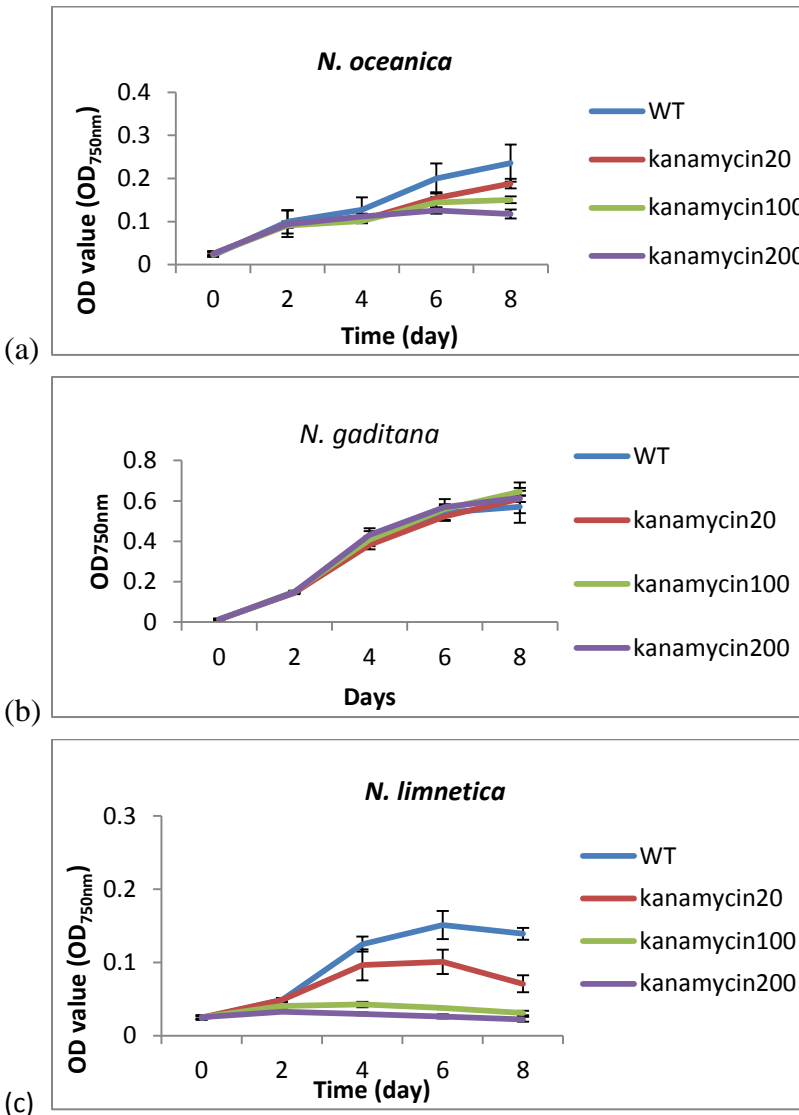


Figure 2.4: growth inhibition effect of kanamycin on *N. oceanica* (a), *N. gaditana* (b) and *N. limnetica* (c). WT (wild type) are cells grown without antibiotics. Shown antibiotic concentrations are in µg/mL. OD_{750nm} values are the mean (three replicates) of each treatment.

2.5.3 Chloramphenicol - In this test, 4 (10, 20, 100 and 200 $\mu\text{g}/\text{mL}$) concentrations of chloramphenicol were tested in the culture medium to assess the effect on the growth of all three *Nannochloropsis* species. The inhibitory growth curves are shown below during 8 days of exposure.

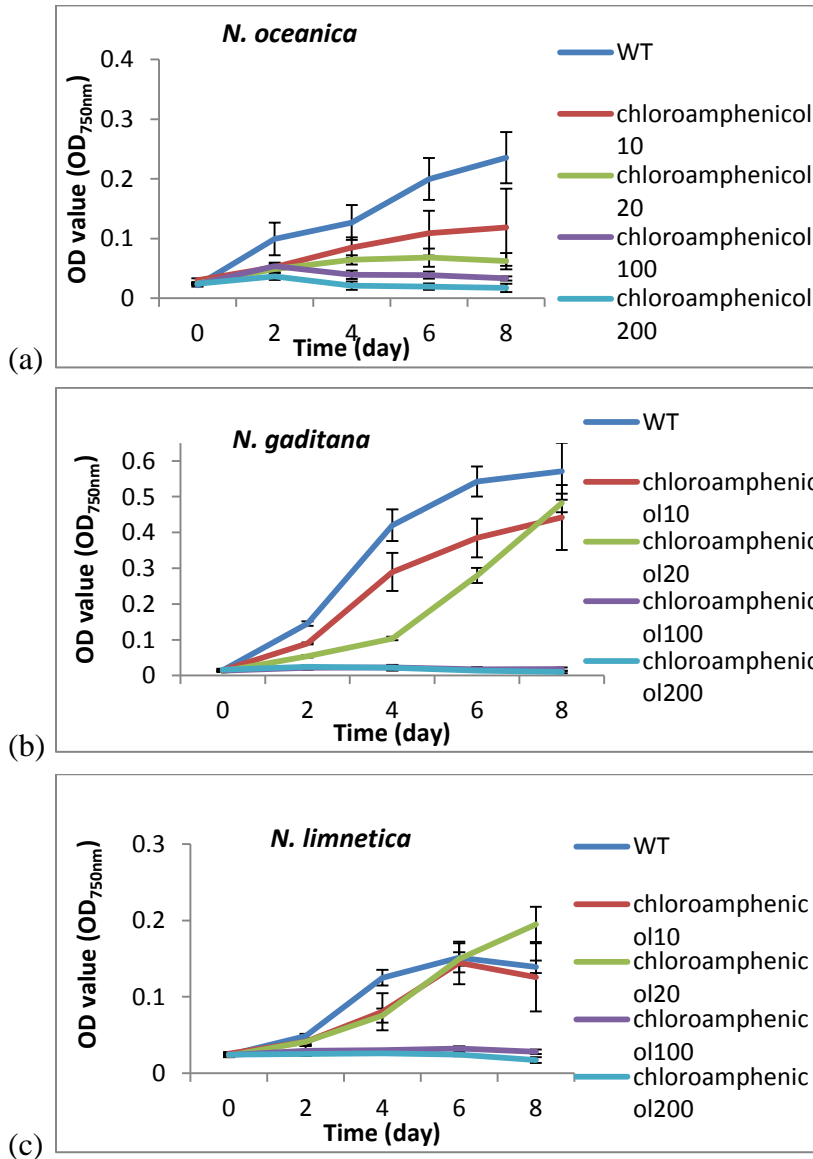


Figure 2.5: cell growth curve of *N. oceanica* (a), *N. gaditana* (b) and *N. limnetica* (c) in four concentrations of Chloramphenicol. WT (wild type) are cells grown without antibiotics. Shown antibiotic concentrations are in $\mu\text{g}/\text{mL}$. OD_{750nm} values are the mean (three replicates) of each treatment.

The figure shows strong growth inhibition ability of chloramphenicol concentrations of 100 and 200 $\mu\text{g}/\text{mL}$ for all tested species. *N. oceanica* cells were found to be sensitive to low

chloramphenicol concentrations of 10 $\mu\text{g/mL}$ (resulted 50% growth reduction) as compared to other species based on the growth curve (Fig. 2.5a).

2.5.4 Hygromycin B - Like chloramphenicol, four hygromycin B concentrations were tested for their sensitivity on the growth of *Nannochloropsis* species. The cells grown in liquid culture both with and without hygromycin B are shown below.

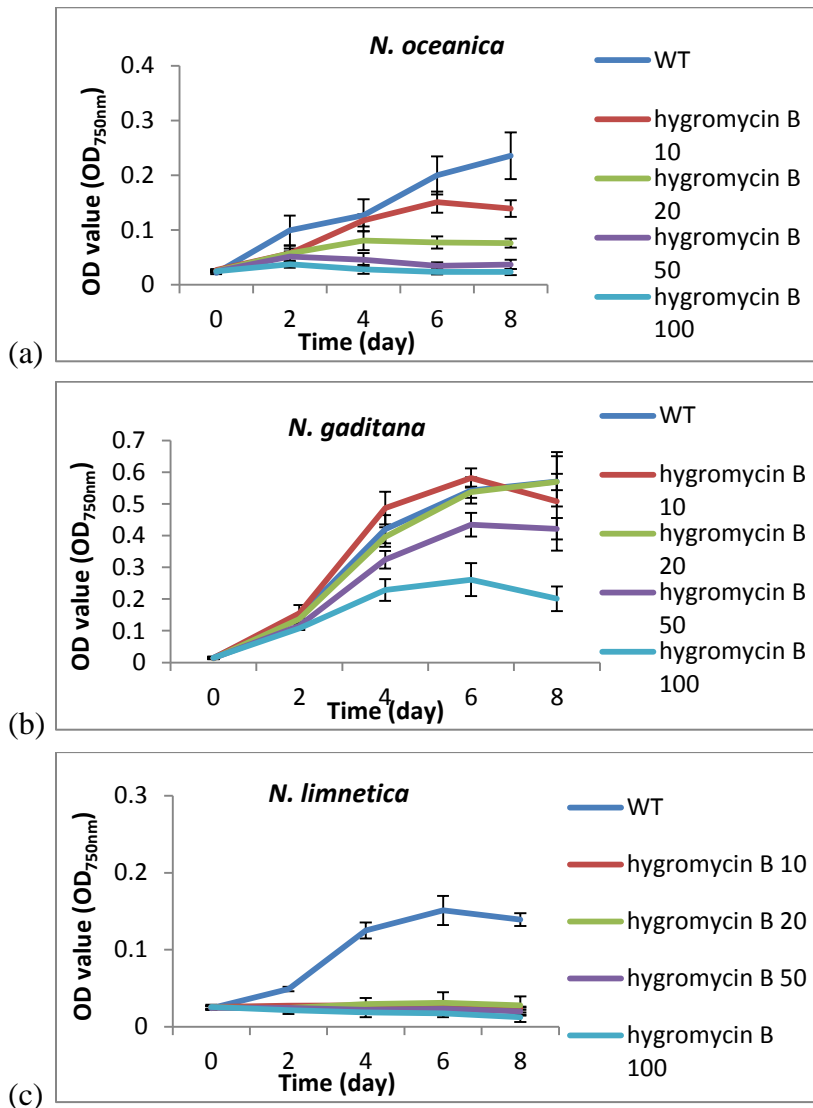


Figure 2.6: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (c) *N. limnetica*, in the absence (WT) and in the presence of different Hygromycin B concentrations ($\mu\text{g/mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

The cell growth and sensitivity results were obtained by continuous OD_{750nm} measurement during 8 days of Hygromycin B exposure. In this experiment, *N. limnetica* found to be much

more sensitive even in low Hygromycin B concentration (10 $\mu\text{g}/\text{mL}$) (Fig 2.6c). As shown above in the graph, the growth of *N. limnetica* cells were completely inhibited by all tested Hygromycin B concentrations while both *N. oceanica* and *N. gaditana* appeared to be tolerable to the lowest tested Hygromycin B concentrations, especially *N. gaditana* seemed to be more resistant.

2.5.5 Gentamycin - Growth of *N. limnetica* was reduced at 20, 100 and 200 $\mu\text{g}/\text{mL}$ of Gentamycin over a 8 days period of exposure (Fig. 2.7c).

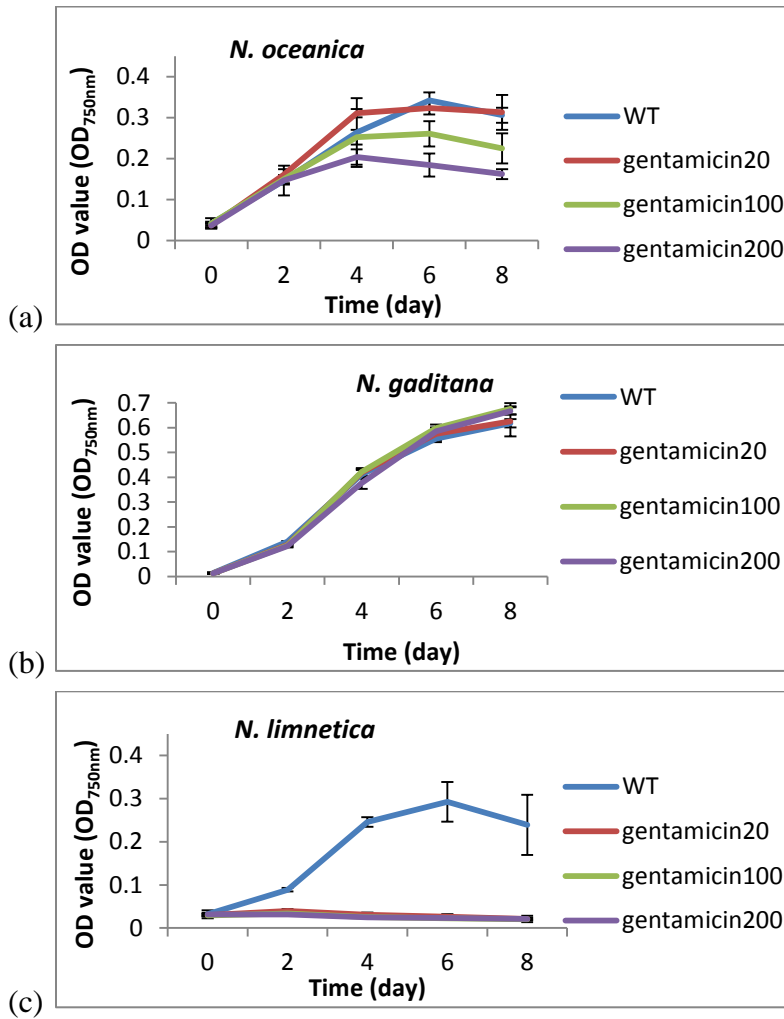


Figure 2.7: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (c) *N. limnetica*, in the absence (WT) and in the presence of different Gentamycin concentrations ($\mu\text{g}/\text{mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

20 μg of Gentamycin per milliliter of *N. limnetica* liquid culture resulted in 91% lower cell density compared to the control culture (WT). On the other hand, exposure of *N. gaditana* cultures to Gentamycin at 20, 100 and 200 $\mu\text{g}/\text{mL}$ resulted no detectable negative effects

compared to the controls over the 8 days period of the test (Fig. 2.7b). In *N. oceanica* a consistent pattern of growth inhibition was observed based on the exposed concentration

2.5.6 Nourseothricin – of the *Nannochloropsis* sp. investigated, *Nannochloropsis limnetica* was the only species extremely inhibited even at the lowest concentrations (10 $\mu\text{g/mL}$) of Nourseothricin used (Fig. 2.8c). The concentrations of Nourseothricin resulted partial or strong inhibition of *Nannochloropsis* species during 8 days incubation are shown in the figure below.

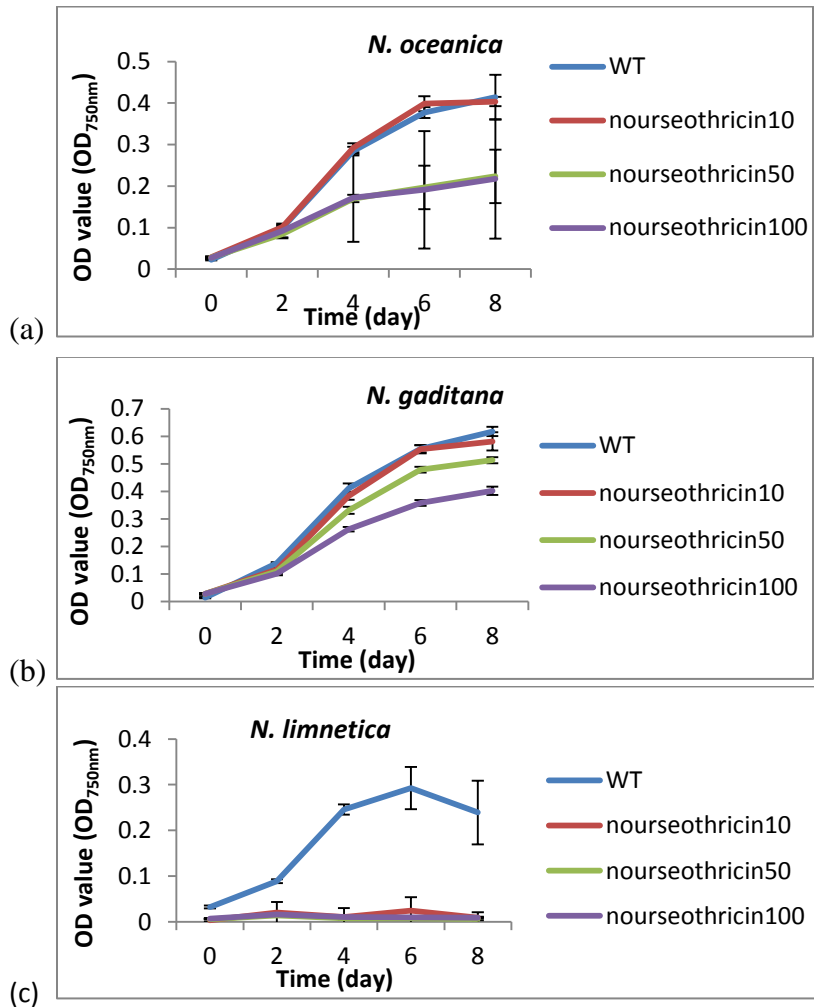


Figure 2.8: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (c) *N. limnetica*, in the absence (WT) and in the presence of different Nourseothricin concentrations ($\mu\text{g/mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

2.5.7 G418 neomycin – this antibiotic showed inhibition effects on the three *Nannochloropsis* species. G418 neomycin inhibited the growth of *N. limnetica* at all treatment concentrations, including 10, 20, 50 and 100 $\mu\text{g/mL}$ (Fig. 2.9c). But exposure of *N. oceanica* and *N. gaditana* to lower tested concentrations did not affect growth. However, G418 neomycin significantly affected the growth of *N. oceanica* at doses of 20 $\mu\text{g/mL}$ and higher concentrations including 50 and 100 $\mu\text{g/mL}$ (Fig. 2.9a). G418 neomycin inhibited the growth of *N. gaditana* at a concentration of 100 $\mu\text{g/mL}$ (Fig. 2.9b).

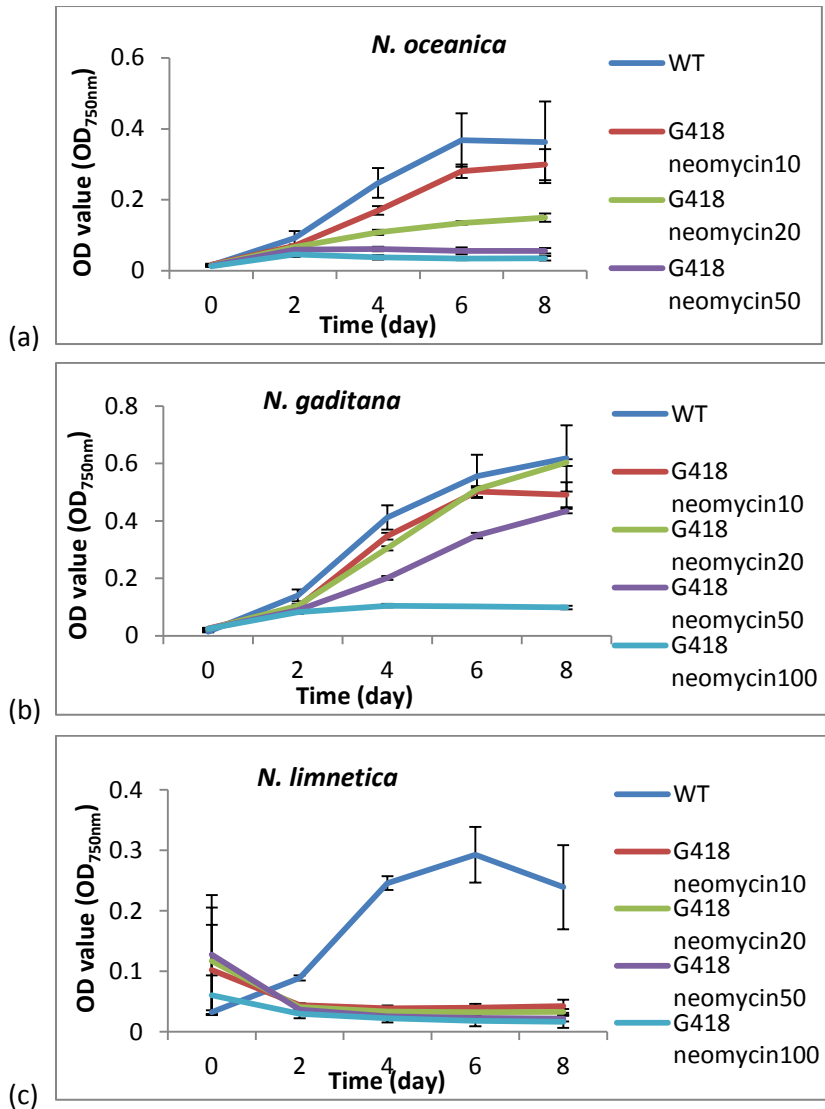


Figure 2.9: effects of G418 neomycin ($\mu\text{g/mL}$) on the growth of (a) *N. oceanica*, (b) *N. gaditana* and (C) *N. limnetica*. WT (wild type) are cells grown without antibiotics. Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

2.5.8 Cycloheximide – concentrations of 1 – 20 $\mu\text{g/mL}$ of Cycloheximide produced significant detectable inhibitory effects on all the three tested *Nannochloropsis* species. Complete inhibition of growth in all the species occurred within 8 days in liquid cultures containing 1 $\mu\text{g/mL}$ of cycloheximide. This antibiotic was found to be one of the most sensitive for all the three *Nannochloropsis* species tested here.

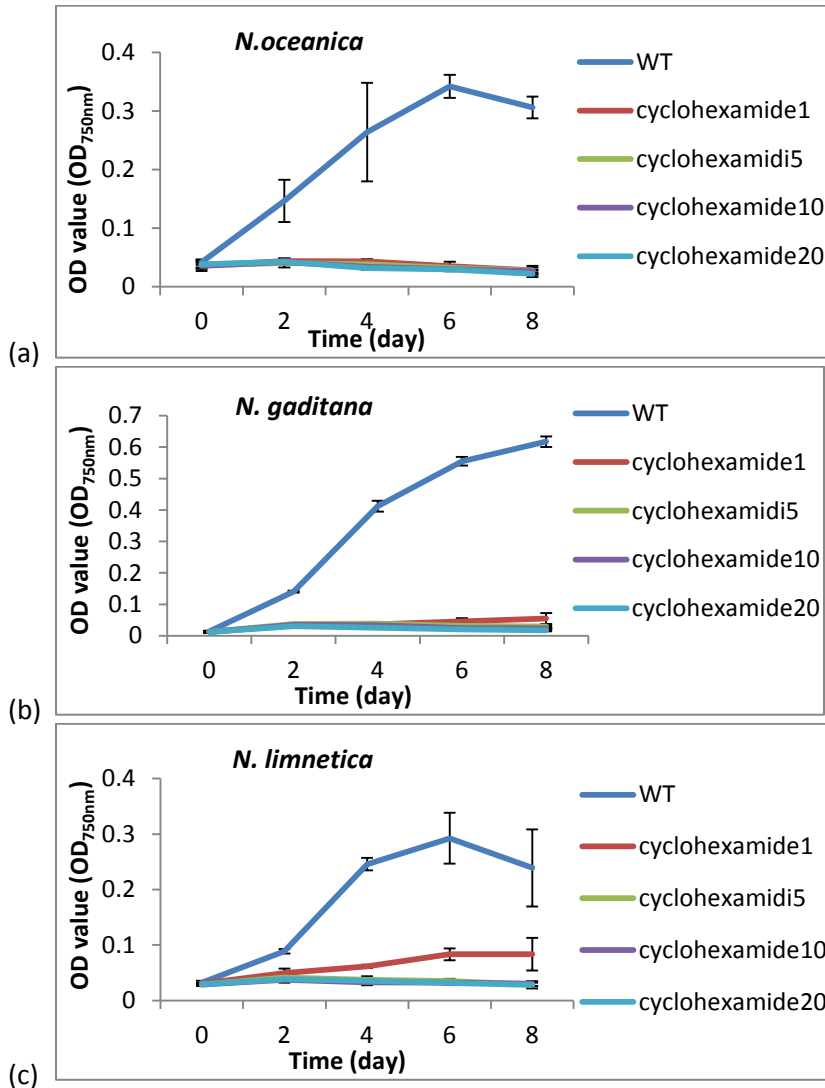


Figure 2.10: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (C) *N. limnetica*, in the absence (WT) and in the presence of different Cycloheximide concentrations ($\mu\text{g/mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

2.5.9 Paromomycin – at the concentrations employed (1, 5, 10 and 40 $\mu\text{g/mL}$), paromomycin caused no detectable effects on the growth of *N. gaditana* (Fig. 2.11b) even at the higher tested concentrations. However, both species of *Nannochloropsis*, *N. oceanica* and *N. limnetica* were sensitive to higher and lower concentrations of paromomycin respectively (Fig. 2.11a and c).

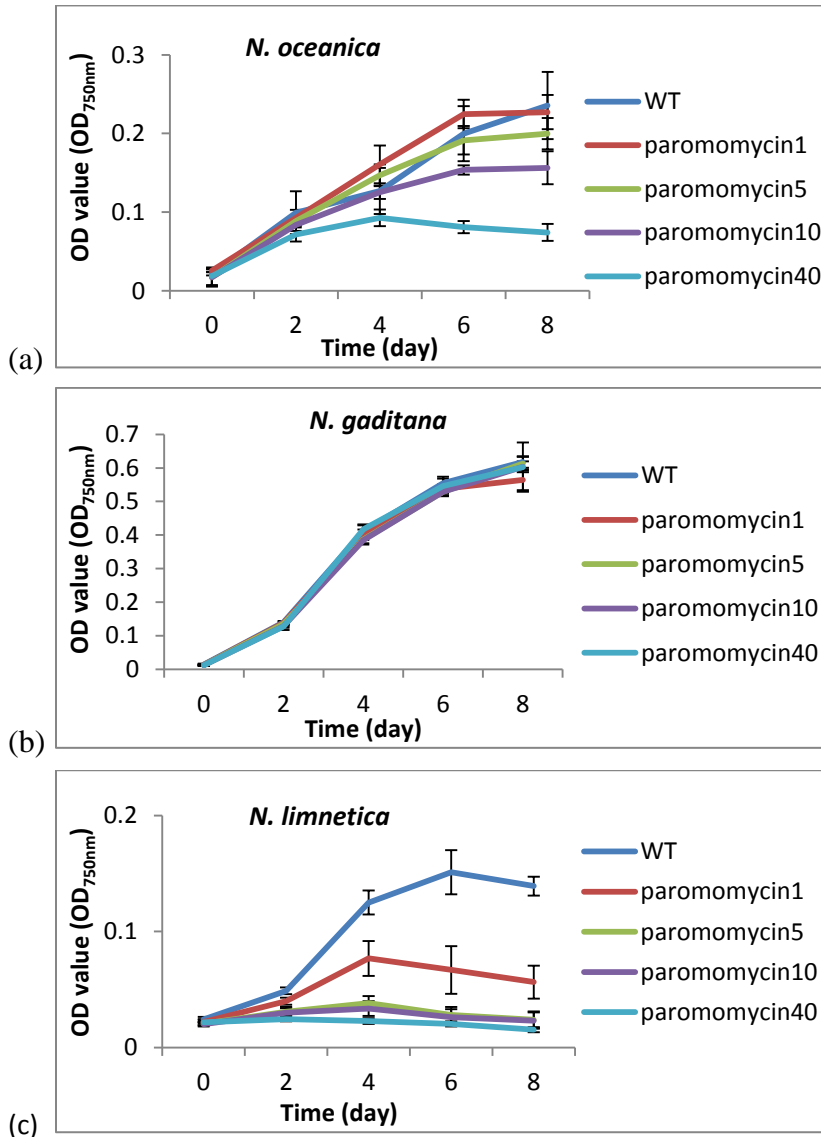


Figure 2.11: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (c) *N. limnetica*, in the absence (WT) and in the presence of different Gentamicin concentrations ($\mu\text{g/mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

2.5.10 Streptomycin - four (1, 5, 10 and 40 $\mu\text{g/mL}$) concentrations of streptomycin in the culture medium were added to investigate its effect on the growth of *N. oceanica*, *N. gaditana* and *N. limnetica*. The inhibitory effects of streptomycin on these cells were assessed after 8 days of exposure and the effect was very significant, especially on the growth of *N. limnetica* even at the lower streptomycin concentrations. Both *N. oceanica* and *N. gaditana* were relatively resistant to the lower streptomycin concentrations.

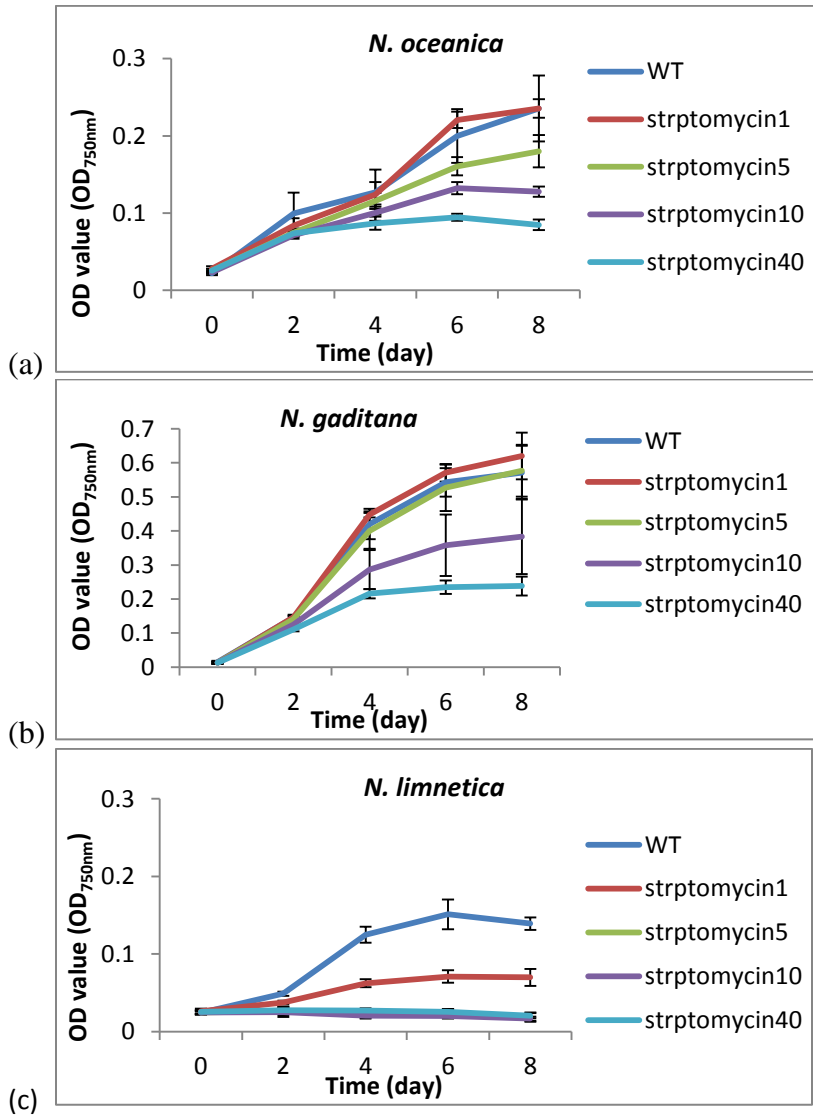


Figure 2.12: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (c) *N. limnetica*, in the absence (WT) and in the presence of different Streptomycin concentrations ($\mu\text{g/mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

2.5.11 Zeocin – to determine the sensitivity of *Nannochloropsis* species to zeocin, the following concentration gradient of zeocin was prepared (1, 5, 10 and 40 $\mu\text{g/mL}$). It was found that all tested *Nannochloropsis* species were highly sensitive to zeocin. 1 $\mu\text{g/mL}$ of zeocin completely inhibited the growth of *N. oceanica*, *N. gaditana* and *N. limnetica*, and produced more than 70% growth reduction compared to untreated cells (WT).

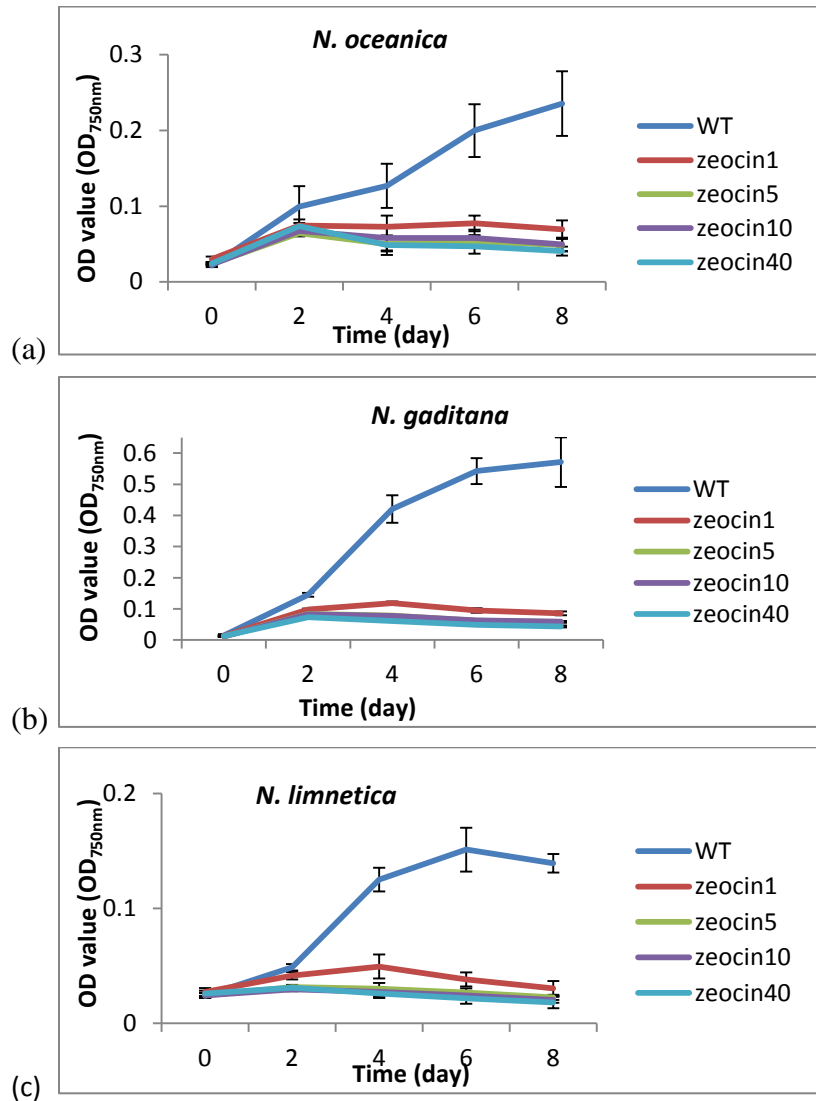


Figure 2.13: Growth of three *Nannochloropsis* species: (a) *N. oceanica*, (b) *N. gaditana* and (c) *N. limnetica*, in the absence (WT) and in the presence of different Zeocin concentrations ($\mu\text{g/mL}$). Values are the mean (three replicates) of OD₇₅₀ values in each treatment.

2.5.12 Lethal doses of antibiotics

The growth inhibition data, collected for all antibiotics with their respective concentrations at day 8 of the experiment, were employed to determine the lethal antibiotic doses on the growth of all three *Nannochloropsis* species. Significant growth differences between treatments and controls were taken as a lethal dose (if the tested concentration lowered the growth by 30% or can be labeled as EC₃₀: effective concentration that inhibits 30% of *Nannochloropsis* growth).

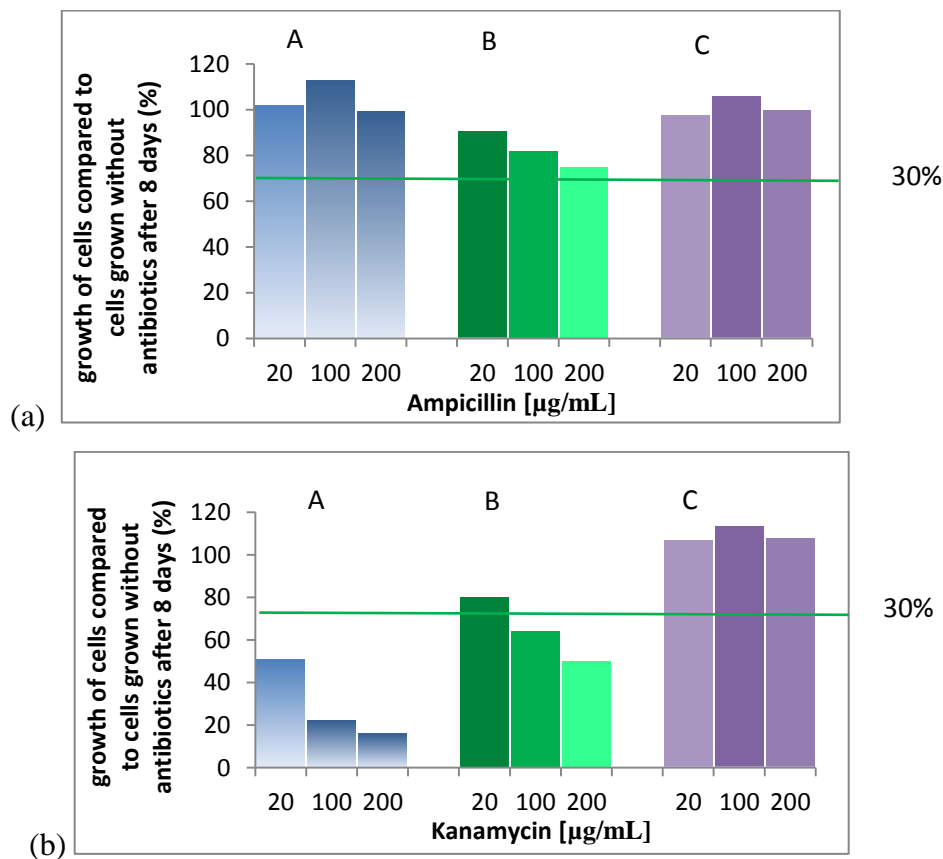


Figure 2. 14. Growth of *N. limnetica* (blue), *N. oceanica* (green) and *N. gaditana* (violet) supplemented with Ampicillin (a) and Kanamycin (b) compared to cells grown without antibiotics after 8 days (%). The green horizontal line indicates 70% growth (30% growth inhibition) and values (%) below this line are lethal for the cells.

As shown in the graph, exposure to Ampicillin revealed no effect for all tested cells at concentrations up to 200 μg/mL, whereas all tested Kanamycin concentrations were lethal for *N. limnetica* (greater than 30% growth inhibition) (Fig. 2.14b). 100 μg/mL Kanamycin concentration was toxic for growth of *N. oceanica* (produced 34% growth inhibition). *N. gaditana* was not affected by both antibiotics.

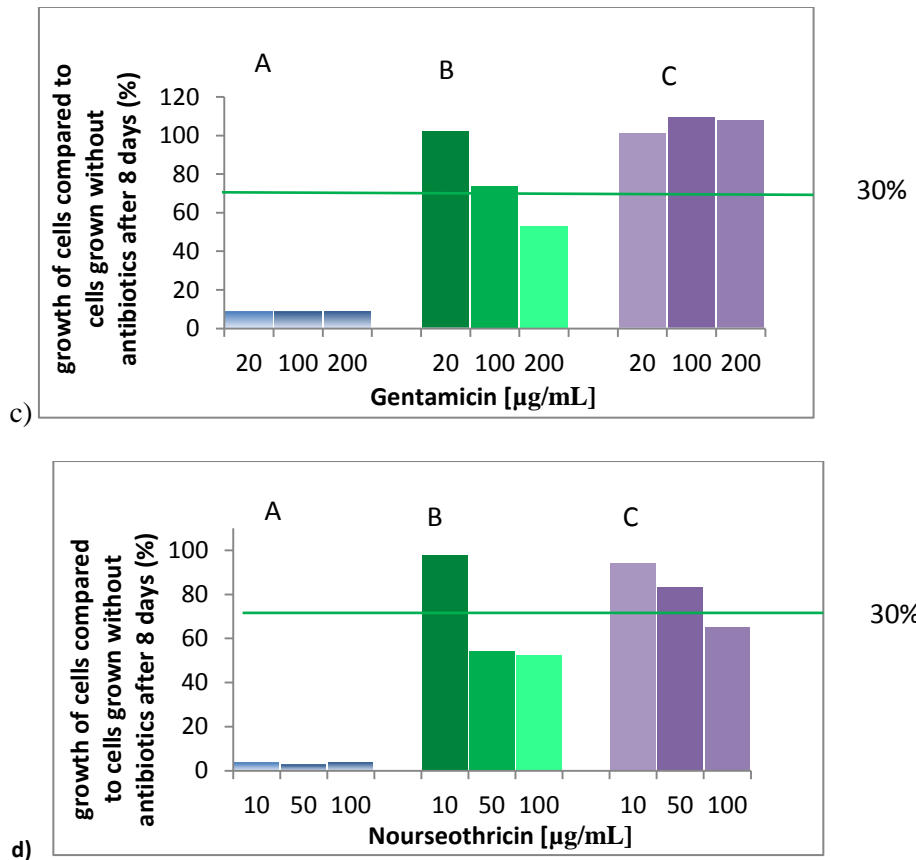


Figure 2.15. Growth of *N. limnetica* (blue), *N. oceanica* (green) and *N. gaditana* (violet) supplemented with Gentamicin (c) and Nourseothricin (d) compared to cells grown without antibiotics after 8 days (%). The green horizontal line indicates 70% growth (30% growth inhibition) and values (%) below this line are lethal for the cells.

As can be seen in the graph, it was found that exposure of *N. limnetica* to Gentamicin and Nourseothricin can result more than 90% growth inhibition even at the lower tested concentrations. While *N. gaditana* seemed to be more resistant for both antibiotics, growth of *N. oceanica* was inhibited at 200 and 50 $\mu\text{g/mL}$ concentrations of Gentamicin and Nourseothricin respectively. This indicated that *N. limnetica* cells were more sensitive than *N. gaditana* and *N. oceanica* for both antibiotics.

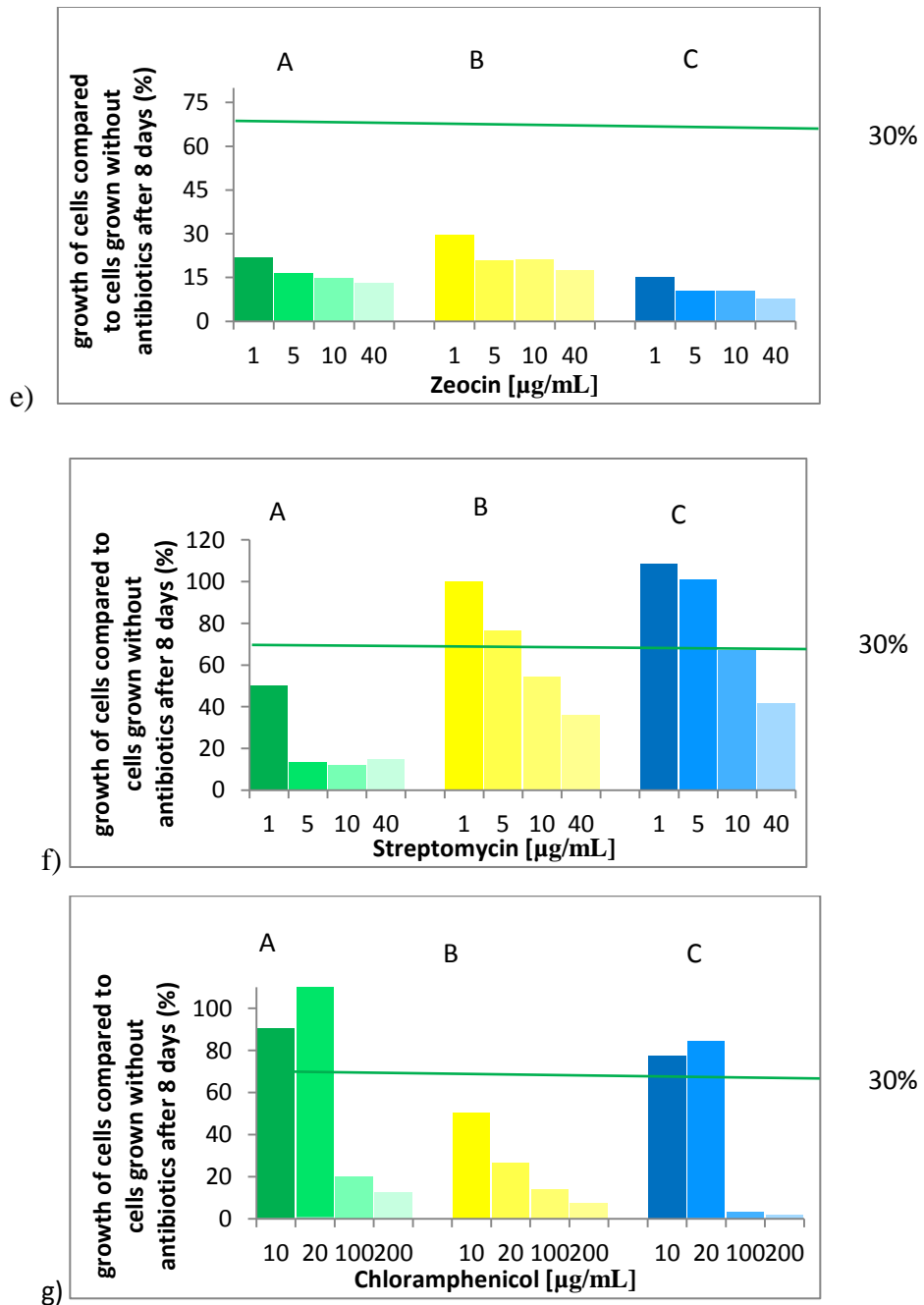


Figure 2.16. Growth of *N. limnetica* (green), *N. oceanica* (yellow) and *N. gaditana* (blue) supplemented with Zeocin (e), Streptomycin (f) and Chloramphenicol (g) compared to cells grown without antibiotics after 8 days (%). The green horizontal line indicates 70% growth (30% growth inhibition) and values (%) below this line are lethal for the cells.

Zeocin had the most negative effect, with more than 80% growth inhibition in all tested species. Streptomycin also had significant effect particularly on growth of *N. limnetica* (more than 50% growth inhibition in the lowest tested concentrations). In the case of Chloramphenicol, only *N.*

oceanica was found to be sensitive in lower concentrations, 10 µg/mL of Chloramphenicol caused approximately 50% growth inhibition. The highest tested concentration of both Streptomycin and Chloramphenicol had significant effect on the growth of *N. gaditana*.

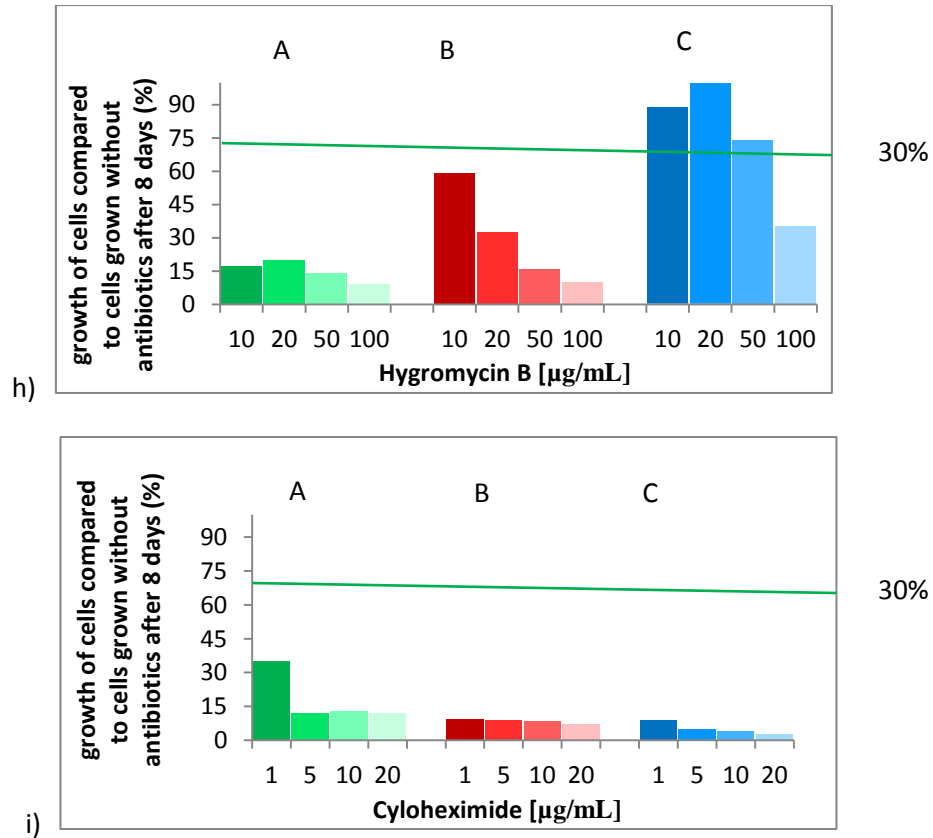


Figure 2.17. Growth of *N. limnetica* (green), *N. oceanica* (red) and *N. gaditana* (blue) supplemented with Hygromycin B (h) and Cycloheximide (i) compared to cells grown without antibiotics after 8 days (%). The green horizontal line indicates 70% growth (30% growth inhibition) and values (%) below this line are lethal for the cells.

Among the three *Nannochloropsis* sp. tested for Hygromycin B, *N. limnetica* showed the highest sensitivity while *N. gaditana* looked unaffected at a concentrations where both *N. limnetica* and *N. oceanica* were affected. 10 µg/mL of Hygromycin B caused 82%, 41% and 11% growth inhibition in *N. limnetica*, *N. oceanica* and *N. gaditana* respectively. on the other hand, Cycloheximide was very toxic in all tested species and produced more than 70% growth inhibition at a concentration of 1 µg/mL.

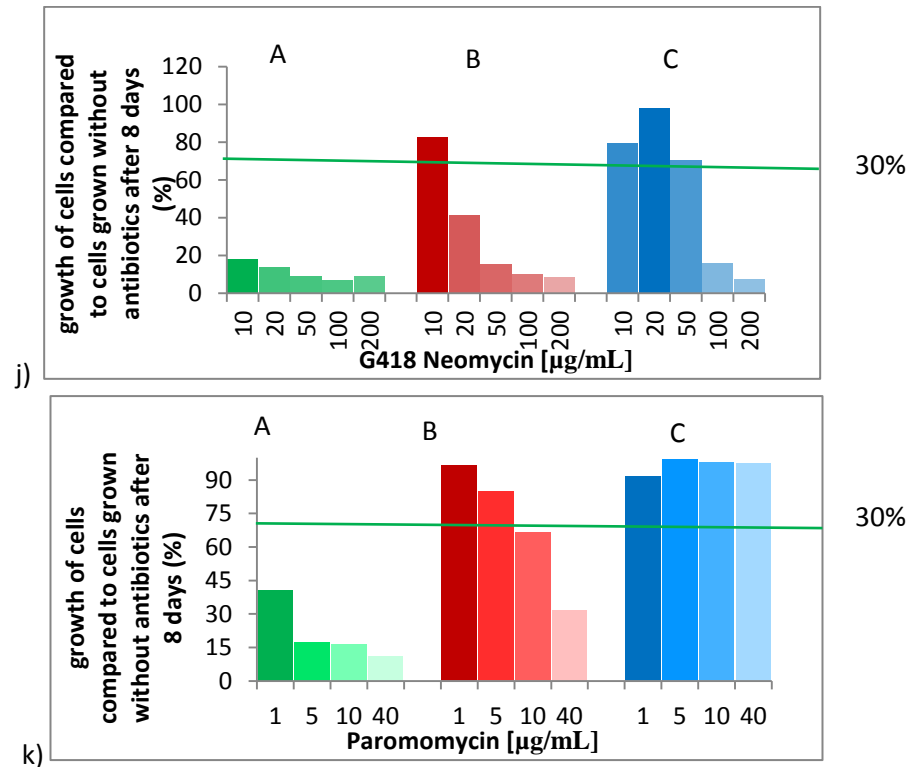


Figure 2.18. Growth of *N. limnetica* (green), *N. oceanica* (red) and *N. gaditana* (blue) supplemented with G418 neomycin (j) and Paromomycin (k) compared to cells grown without antibiotics after 8 days (%). The green horizontal line indicates 70% growth (30% growth inhibition) and values (%) below this line are lethal for the cells

Both G418 neomycin and Paromomycin showed significant inhibition effects on *N. limnetica*. The former antibiotic inhibited the growth of *N. limnetica* at concentrations of 10 μg/mL, while the latter inhibited at concentrations of 1 μg/mL. These low concentrations of G418 neomycin and Paromomycin caused more than 80 and 60% growth inhibition in *N. limnetica* as compared to cells grown without antibiotics respectively. G418 neomycin and Paromomycin inhibited the growth of *N. oceanica* in comparison with unexposed cultures for concentrations of 20 and 10 μg/mL (Fig. 2.18j and k, red graph), giving an average percentage reduction in cell density of 59 and 34% for the two concentrations, respectively. Exposure of *N. gaditana* to 40 μg/mL of Paromomycin showed no detectable effect, causing an average inhibition in cell density of less than 3%. In the case of G418 neomycin, the strongest effect started at 100 μg/mL which is equal to 84% growth reduction over the control level.

Table 2.2: Comparison of the effect of antibiotics on different *Nannochloropsis* species. Shown are the lethal antibiotic doses in $\mu\text{g/mL}$. Values given in parenthesis indicate percentage of growth reduction after 8 days caused by the antibiotics (%)

	Antibiotics	Lethal antibiotic doses ($\mu\text{g/mL}$)		
		<i>N. limnetica</i>	<i>N. oceanica</i>	<i>N. gaditana</i>
Prokaryotic antibiotics	Ampicillin	Not detected at 200	Not detected at 200	Not detected at 200
	Kanamycin	20 (34)	200 (51)	Not detected at 200
	Gentamycin	20 (91)	200 (44)	Not detected at 200
	Chloramphenicol	100 (80)	10 (50)	100 (96)
Eukaryotic antibiotics	Hygromycin B	10 (83)	20 (41)	100 (65)
	zeocin	1 (78)	1 (70)	1 (85)
	Cycloheximide	1 (65)	1 (91)	1 (92)
	Streptomycin	1 (50)	10 (46)	40 (59)
	G418 neomycin	10 (81)	20 (59)	100 (84)
	Nourseothricin	10 (96)	100 (47)	Not detected at 100
	Paromomycin	1 (60)	40 (69)	Not detected at 40

Table 2.2 presented lethal dose values of the eleven antibiotics examined in this experiment. After an exposure of 8 days to Ampicillin and Kanamycin, all tested species of *Nannochloropsis* showed no effect at concentrations up to 200 $\mu\text{g/mL}$, indicating that these antibiotics were not toxic to the *Nannochloropsis* species, except *N. limnetica* (more than 50 % growth inhibition at 100 $\mu\text{g/mL}$ of Kanamycin). Both Ampicillin and Kanamycin work only against bacteria by the inhibition of cell wall synthesis and disruption of protein synthesis respectively. Chloramphenicol inhibited the growth of the three *Nannochloropsis* species in different concentrations (10 $\mu\text{g/mL}$ for *N. oceanica* and 100 $\mu\text{g/mL}$ for both *N. gaditana* and *N. limnetica*). But, all of the *Nannochloropsis* strains examined in the previous report (Vieler A. et al., 2012) were found to be resistant to 100 $\mu\text{g/mL}$ of Chloramphenicol. However, it should be noted that both the form of medium (liquid for this study vs. solid) and may be the exposure time (8 days for this study vs. unknown time) were different. These antibiotics have been reported as antibiotics essential for eliminating contaminants from algal cultures (Hunter EO. et al., 1961, Lai HT. et al., 2009; Vieler A. et al., 2012).

Results showed that growth of *Nannochloropsis limnetica* was inhibited by low concentrations of most tested antibiotics, Zeocin (1 µg/mL), Cycloheximide (1 µg/mL), Paromomycin (1 µg/mL), Streptomycin (1 µg/mL), Hygromycin B (10 µg/mL), Nourseothricin (10 µg/mL), G418 neomycin (10 µg/mL) and Gentamycin (20 µg/mL). With regard to *N. oceanica* and *N. gaditana*, complete growth inhibition was recorded only at low concentrations of Zeocin and Cycloheximide.

Interestingly, growth stimulatory effects were observed for some antibiotics. For instance, the effect of Kanamycin, Gentamycin and Streptomycin on the growth of *N. gaditana* was markedly stimulatory for exposures to levels of 200, 100 and 1 µg/mL (Fig. 2.14b, 2.15a and 2.16a) respectively, resulting up to 10% increase compared to untreated cells.

This study also proved that, different *Nannochloropsis* species have different sensitivities to the same gradient of antibiotics. Levels of sensitivity of the three *Nannochloropsis* species to the antibiotics were evaluated. Results demonstrated that *N. limnetica* was more sensitive to all tested antibiotics than *N. oceanica* and *N. gaditana* (Table 2.2). As reported by Vieler A. et al (2012), and confirmed in this investigations, *N. oceanica* was more susceptible to antibiotics than *N. gaditana*. The possible explanation for this discrepancy could be that the differences in cell wall composition or thickness allowing more efficient uptake of antibiotics.

Generally, in the inhibition effect of the growth of *N. limnetica*, the sensitivities of the antibiotics rank in increasing order as follows: Ampicillin < Chloramphenicol < Kanamycin < Gentamycin < G418 neomycin < Hygromycin B, < Nourseothricin < Streptomycin < Paromomycin < Cycloheximide < Zeocin. In the inhibition effect of the growth of *N. oceanica*, the sensitivities of the antibiotics rank in increasing order as follows: Ampicillin < Gentamycin < Kanamycin < Nourseothricin < Paromomycin < Hygromycin B < G418 neomycin < Streptomycin < Chloramphenicol < Zeocin < Cycloheximide. But, most of antibiotics used in this experiment were less sensitive to *N. gaditana* except Zeocin, Cycloheximide and Streptomycin.

The results also provide important data in developing new selectable markers in genetic engineering of *Nannochloropsis* species. Based on the findings Chloramphenicol, Zeocin, Hygromycin B, Cycloheximide, and G418 neomycin had a promise as selective reagent for the three *Nannochloropsis* species (*N. limnetica*, *N. oceanica* and *N. gaditana*). Paromomycin and

Nourseothricin could also serve as a selection reagent in the genetic transformation of *N. limnetica*. Because antibiotic like Nourseothricin is much less effective in salt-containing media and is not ideal for use as selection marker with marine algae (Radakovits R. et al., 2010). Of these antibiotics, plasmids with genes confer resistance to Chloramphenicol and Hygromycin B are commonly used for transformation of *N. oceanica* as reported in Chernyavskaya O. (2014).

2.6 Conclusions

In the present study, growth inhibition tests were performed using eleven antibiotics to evaluate the growth effects on three *Nannochloropsis* species; *N. limnetica*, *N. oceanica*, and *N. gaditana*. Growth of *N. limnetica* was adversely affected by all of the antibiotics assayed except ampicillin, while *N. gaditana* was found to be the most tolerant species. The most toxic antibiotics for the three species were Zeocin and Cycloheximide. Ampicillin was not toxic to the *Nannochloropsis* species, whereas Kanamycin and Gentamycin showed very low growth inhibition in *N. limnetica* and *N. gaditana*. Hence these antibiotics could be used as an agent to clean contaminants from these species. Other antibiotics including, Zeocin, Cycloheximide, Hygromycin B, Chloramphenicol, G418 neomycin, Nourseothricin (for *N. limnetica*), Paromomycin (for *N. limnetica*) and Streptomycin can be chosen for use in selection marker studies in genetic engineering of *Nannochloropsis* species. *N. limnetica* was in general more sensitive than *N. oceanica*. On the other hand, *N. oceanica* was more sensitive than *N. gaditana*. Moreover, It was also interesting to note that there was growth stimulatory effect for some antibiotics.

Chapter Three: Biolistic Transformation

3.1 Introduction to genetic transformation

Transformation, the introduction of exogenous DNA from an external source for changing the genetic makeup of an organism, was discovered in *Streptococcus pneumonia* by Frederick Griffith in 1928 (Griffiths AJF. et al., 2000). Research on genetic transfer of eukaryotic algae started in the 1980`s at a time bioengineers realized the importance of the development of marine resources (Ying C. et al., 1998). At present, the genetic transformation of microalgae has been established and stably transformed microalgae are employed to produce vaccines, recombinant antibodies and to study gene functions, as well as being targeted for genetic engineering (Mikami K. et al., 2011). Genetic transformation of microalgae is also considered a key to yielding high lipid productivity (Leon-Banares R. et al., 2004; Qin S. et al 2012).

To date, successful genetic transformation of more than 20 algal species has been demonstrated with various transformation methods; and most of these were achieved by nuclear transformation (Hallmann A., 2007). The fresh water alga *Chlamydomonas reinhardtii* is the first and best studied alga with an established transformation system, as its complete genome sequencing has made it an excellent model system for diverse biotechnological research areas (Leon-Banares R. et al., 2004). Different methods of algal transformation have been introduced. The methods used so far for the introduction of genes include, agitation with glass beads, *Agrobacterium tumefaciens*-mediated transformation, particle-gun method (also known as particle bombardment or biolistic transformation), microinjection, and electroporation (Coll JM., 2006). Artificial transposon methods and recombinant eukaryotic algal viruses have also shown promise for genetic manipulation of algae (Qin S. et al., 2012). Recently, the first nuclear episomal vector for diatoms and a plasmid delivery method via conjugation from *Escherchia coli* to the diatoms *Phaeodactylum tricorutum* and *Thalassiosira pseudonana* have described (Karas BJ. et al., 2015). An overview of techniques for the genetic manipulation of algae is presented in Table 3.1.

Genetic transformation approaches have been targeted toward oleaginous microalgae; and reproducible genetic transformation systems have been developed in order to optimize the lipid production capabilities and to increase potential for biodiesel fuel production (Dunahay TG. et

al., 1996; Schenk PM. et al., 2008). Most of the eukaryotic microalgae successfully transformed belong to green algae, diatoms and red algae.

Recently, stable gene transfer has been reported in *Nannochloropsis* sp. (Zhang C. et al., 2014) that have shown significant potential for biodiesel production. Also, *Nannochloropsis* species show a benefit compared to diatoms or other marine algae due to the fact that they have a haploid genome (Radakovits R. et al., 2010). In *Nannochloropsis* it has been reported that electroporation has a high efficiency to transform nuclear targets (Kilian O. et al., 2012; Vieler A. et al., 2012). During the applied method the plasmid pSELECT100 and PL90 vector which confer resistance to Hygromycin B and Zeocin respectively were successfully introduced. Kilian O. et al (2012) reported 9000 transformants using 3 µg linearized PL90 transformation vector through electroporation (the amount of electroporated cells were 10^9 in 100 µl), while Vieler A. et al (2012) obtained 125 resistant colonies per µg of linearized pSELECT100 transformation plasmid from 5×10^8 /mL using the same transformation method.

Table 3.1. Summarized comparisons of the glass bead, electroporation and bombardment methods for transforming eukaryotic microalgae (adapted from Coll JM., 2006)

Criteria	Glass beads	Electroporation*	Bombardment*
Required equipment	simple	complex	complex
Predominant type of transformation	nucleus	nucleus	chloroplast
Number of transformants reported $\times 10^{-6}$	~ 100	~ 1000	~100
Removal of cell wall required	Yes	Yes (but not now)	No
Cotransformation rates	6-50%		20-70%
Demonstrated presence of exogenous DNA	yes	yes	yes
Stability of transformants in the absence of selection	66.6%	33.3%	90.9%

* Bombardment penetrates both the plasma and organelle membranes whereas electroporation and glass bead are unable to penetrate the latter membrane. Therefore, the removal of cell wall is required for efficient introduction of exogenous genes by both methods.

3.2 Biolistic transformation - Introduction

Biolistic transformation (also known as particle bombardment or “gene gun”), is a gene transfer technique, that uses small particles that are coated with genetic material projected with high velocities into target cells. This technique was developed and first described by Sanford and coworkers and utilized tungsten particles as a microcarrier (Finer JJ. et al., 2000). The physical nature of this technology makes it extremely versatile and easy to use (Kikkert JR., 1993). This method has been used to transfer exogenous DNA to fungi, bacteria, algae, mammals and insects regardless of the thickness or rigidity of cell wall and life cycle (Gong Y. et al., 2011). Organelles such as chloroplasts and mitochondria have also been transformed using biolistic techniques. Qin S. et al., (2012) stated that biolistic transformation as the only efficient gene transfer tool to genetically manipulate marine diatoms.

Physical parameters used in bombardment can be optimized for the transformation of different cell types. Some of the parameters of the particle bombardment process include the concentration of plasmid DNA to be transferred, vacuum pressure applied during bombardment, and particle travel distance before striking the target cells (Taylor NJ. et al., 2002). In addition to these parameters, the cell concentration to be transformed also determines the transformation efficiency.

As biolistic transformation uses DNA-coated gold or tungsten microprojectiles that are delivered into target cells by a helium-driven gun, the choice of microparticle type and size is important and will determine the area and depth of penetration (Gong Y. et al., 2011). Even if it is very expensive relative to tungsten, the denser nature and more even surface of the gold particles allows better control of penetration (Finer JJ. et al., 2000). It has been shown that tungsten, which was widely utilized in the early experiments, has toxic effects on target cells or tissues (Anderson BR. et al., 2015). The microcarrier/DNA mixture must be delivered into the target tissue without causing excessive injury in order to get successful transgene integration. Wang K. and Frame B. (2009) analyze in depth the relationship between the velocity at which the microcarriers strike the target cells and other parameters, and summarized as follows: the velocity can be adjusted by changing the helium pressure, the microcarrier travel distance to the stopping screens and the distance between stopping screen and target cells. The number of bombardment/shooting has several effects on the transformation efficiency, and each sample can

be bombarded more than once to increase the number of transformed cells. However, multiple bombardments increase the level of damage and stress to the target cells (Qin S. et al., 2012).

The biological system being targeted for transformation determines the level of the vacuum required in the bombardment chamber. Commonly used protocols require a vacuum level within the bombardment chamber between 15-29 inches of mercury (www.bio-rad.com: The Biolistic® PDS-1000/He device). Different rupture disks (450 psi to 2,200 psi) can be used based on the particle velocity required and the types of target cells to be bombarded. All these parameters can be adjusted to different algal materials, and this makes biolistic transformation a very flexible method (Finer JJ. et al., 2000).

To date, transformation methods with particle bombardment have been established for several species of microalgae including, *Phaeodactylum tricornutum* (Apt KE. et al., 1996), *Chlamydomonas reinhardtii* (Debuchy R. et al., 1989), *Volvox carteri* (Hallmann A. et al., 1997), *Nannochloropsis* sp. (Kilian O. and Vick B., 2012; Chernyavskaya O., 2014). All of the above organisms were transformed under different parameters of biolistic transformation such as amount of DNA, pressure (psi), microcarriers, transferred cell amount before bombardment, the number of shoots, distance between microcarriers and target cells etc.

This chapter describes the successful effort to develop an efficient biolistic transformation system for the oleaginous algal *Nannochloropsis oceanica* CCMP1779. Both gold and tungsten microcarriers have been used for the introduction of the transforming plasmid pSELECT100 carrying a selective marker (genes conferring resistance to Hygromycin B). This study describes a detailed evaluation and refinement of parameters for the biolistic transformation of *Nannochloropsis oceanica* CCMP1779, based on previously reported work (Kilian O. et al., 2012; Chernyavskaya O., 2014)

3.3 Objective of the study

To our knowledge, so far biolistic transformation was not used successfully or with low transformation efficiency for the species *Nannochloropsis oceanica* CCMP1779 (Kilian O. et al., 2012; Chernyavskaya O., 2014). Therefore, the main objective of this experiment was to develop an efficient transformation protocol for *Nannochloropsis oceanica* CCMP1779 by biolistic

transformation using the linearized pSELECT100 plasmid which confers hygromycin B resistance.

3.4 Material and Methods

3.4.1 Strain and Growth condition

Nannochloropsis oceanica CCMP1779 cells obtained from National Center for Marine Algae and Microbiota (NCMA, former CCMP, <https://ncma.bigelow.org/>) was used as host strain. The strain was cultured in 50 % seawater enriched with Cell-Hi NC under constant light intensity of $100 \mu\text{E m}^{-2}\text{s}^{-1}$ at 23°C on continuous rotator for shaking (see Chapter 2- growth conditions).

N. oceanica CCMP1779 cells were grown to a density of 8.1×10^7 cells/mL. After cells reached an exponential growth phase (a week after first inoculation), 5 mL of *N. oceanica* cells (1.19×10^9 cells/mL) were collected by centrifugation at $4200 \times g$ for 15 minutes at room temperature (experiment 1). Aliquots of 1 mL of centrifuged cells in suspension were spread on agar solidified 50 % sea water medium (Cell-Hi NC added) one night prior to biolistic transformation. Three plates contained 1.3×10^9 cells/mL were prepared for both gold and tungsten microcarrier experiments, and incubated in the same growth conditions. For experiment 2, cells were grown in the same medium but with addition of 50 $\mu\text{g/mL}$ ampicillin to avoid contamination.

The *Escherichia coli* strain used for *in vivo* amplification of transforming plasmid was DH5 α , cultured in LB medium at 37°C . LB medium (1L) was prepared from 5 g yeast extract, 10 g Bacto-Tryptone, and 10 g sodium chloride (NaCl) and agar (for agar plate). Ampicillin (50 $\mu\text{g/mL}$) was added for selecting and propagating resistant bacteria (QIAGEN).

3.4.2 Antibiotic sensitivity of *N. oceanica*

The sensitivity of *N. oceanica* to a number of antibiotics (Ampicillin, Hygromycin B, Streptomycin, Zeocin etc.) was investigated in Chapter 2. Cells were incubated in the 50 % sea water medium supplemented with various types of antibiotics in the concentration ranges from 1 to 200 $\mu\text{g/mL}$ and the growth of *N. oceanica* was monitored for 8 days.

3.4.3 Plasmid

The plasmid pSELECT100 (Figure 3.1), utilized for biolistic transformation was obtained from the Benning laboratory at Michigan State University and includes of 790-bp long lipid droplet surface protein (LDSP) promoter that was amplified from *N. oceanica* CCMP1779 genomic DNA and 35S terminator region. The promoter and terminator mediate the expression of a hygromycin B resistance gene that can be used as a selection marker. The pSELECT100 backbone also include a gene conferring resistance to ampicillin and pBR2 origin (Vieler A. et al., 2012).

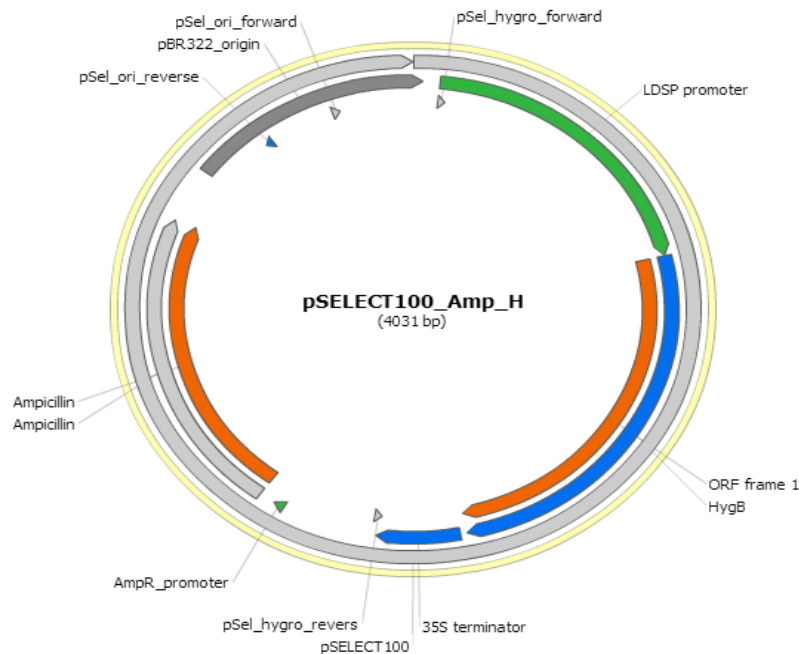


Figure 3.1: The plasmid map of pSELECT100 which was utilized in the biolistic transformation of *N.oceanica* CCMP1779 using both gold and tungsten particles.

3.4.4 Plasmid verification and amplification methods

To identify and confirm the received pSELECT100 plasmid, the plasmid was digested by *Sma*I restriction enzyme. The digestion should result in three different fragment sizes, because the plasmid pSELECT100 has three cutting sites for the *Sma*I restriction enzyme. The gel electrophoresis was prepared

from the mixture of 0.8% agarose and GelGreen nucleic acid stain. To verify plasmid band patterns, 1 μ L of pSELECT100 plasmid cut with *Sma*I, 1 μ L 10x DNA loading dye was added to 7 μ L sterile distilled water. The gel electrophoresis was carried out at 85 volts for 60 minutes. The images of fragments on the gel were visualized by using Molecular Imager ChemiDoc

XRS+ (Bio-Rad). In addition to *SmaI* restriction enzyme, *KpnI* and *BamHI* were utilized to make the pSELECT100 plasmid linearized for biolistic transformation.

DNA sequencing of pSELECT100 transformation plasmid was performed (GATC Biotech) to verify its identity, particularly the presence of the hygromycin resistance cassette. The resulted sequences were aligned with the available plasmid map of pSELECT100 provided by the Benning lab resulting in perfect match with one exception. Two extra nucleotides were detected on the pSELECT100 plasmid at the LDSP promoter region in front of the hygromycin resistance gene (68 – 855bp), which are not present in the supplied DNA sequence of pSELECT100 (Chernyavskaya O., 2014).

These extra nucleotides were positioned at 831-bp and 832-bp. In order to eliminate these two nucleotides, four different primers were designed to amplify pSELECT100 transformation plasmid without the two extra inserted nucleotides. This resulted in two different sizes of the pSELECT100 plasmid fragments which were joined by Gibson Assembly.

The following primers were designed for the amplification of the plasmid.

Mist_rev_set1 5`- CTC CTG TGT TGA TGC GGG CTG AGA -3`

Mist_fw_set1 5`- ATG GAG TGG ATG GAG GAG GAG G - 3`

Mist_fw_set2 5`- GCA TCA ACA CAG GAG GGC CC - 3`

Mist_rev_set2 5`- CCT CCT CCA TCC ACT CCA TCT TA – 3`

Two methods (1) heat shock transformation of plasmid into *E.coli* competent cells (Froger A. et al., 2007; Yoshida et al., 2009) and (2) touch-down (TD) PCR (Table 3.2) were used for the amplification of the pSELECT100 transformation plasmid for biolistic transformation of *N. oceanica* CCMP1779 cells. During heat-shock transformation of *E.coli* competent cells (DH5 α), 2 μ L of pSELECT100 plasmid was added into 100 μ L of competent *E. coli* cells and kept on ice for 30 minutes. Afterwards, the mixture was incubated at 42 $^{\circ}$ c for 45 seconds (heat shock) in the water bath and placed in ice for 3 minutes again. From the incubated mixture, 500 μ L was transferred into 1.5 mL micro centrifuge Eppendorf tube and 1 mL of normal LB medium was added in a sterile bench. After an hour of incubation with agitation at 37 $^{\circ}$ C, 200 μ L of the culture

was plated out on solidified LB agar medium containing 50 µg/mL ampicillin. Then, the plate was incubated in the incubator for overnight at 37°C. Single colonies were picked to grow in liquid cultures overnight and plasmids were extracted using WIZARD Midi DNA purification kit.

The table below shows the TD PCR mix with phusion polymerase to amplify pSELECT100 plasmid. The primers used to amplify is also listed below,

pSEL_ori_fwd 5`- cgacctacaccgaactgagata-3` tm^o - 63.0
 pSEL_ori_rev 5`- tctagtgtagccgtagttaggc-3` tm^o - 59.2

Table 3.2. PCR components for pSELECT100 plasmid amplification (left), and protocol parameters for PCR amplification of pSELECT100 by touchdown PCR (right).

component	reaction volume (µl)	Step	Temperature (°C)	Time
Nuclease-free water	33	Initial denaturation	98	2 min.
5×phusion GC buffer	10	Denaturation	98	30 s
10 mM dNTPs	1	Annealing	55-65	30 s
10 mM forward primer	2.5	Extension	72	2 min.
10 mM reverse primer	2.5	Denaturation	98	30 s
Template DNA (30-40ng/µl)	0.5	Annealing	57	30 s
DNA phusion polymerase	0.5	Extension	72	2 min.
Total	50	Hold	4	

The touch down PCR was performed in a reaction volume of 50 µl using Bio-Rad iCycler (Thermo Cycler). Amplification results were resolved by electrophoresis in 0.8% agarose gel and observed under UV light after gel green staining.

After amplification of pSELECT100 plasmid using the above methods, the plasmid was analyzed by gel electrophoresis (expected size 4031 bp). Enzyme digested samples were also run on 0.8% agarose gel electrophoresis for identification and characterization of fragments. At the end of purification process, total volume of 11 μL with 1589 ng/ μL final concentration of pSELECT100 plasmid (15.89 μg) was collected to be bombarded into *N. oceanica* CCMP1779 cells via Biolistic[®] PDS-1000/He particle delivery system (Bio-Rad).

3.4.5 Gold and Tungsten particle preparation

The microcarrier preparation was performed based on previously reported papers or manuals (Wang K. and Frame B. 2009). In this protocol, 15 mg gold (0.6 μm) particles were weighed and transferred into sterile, 1.5 mL microcentrifuge tubes and washed with 500 μL 100% ethanol. The suspension was sonicated in an ultrasonic water bath for 15s and centrifuged for 60 s at 3000 rpm right before removing ethanol supernatant. Then, the gold particles were rinsed three times with 1 mL sterile distilled water by dribbling the water down the side of the microcentrifuge tube. Finally the pellet was suspended in 500 μL sterile water and the suspension was ultrasonicated for 15 s. Aliquots of 50 μL of gold particles (sufficient for 5 bombardments) were prepared in sterile microcentrifuge tubes and preserved at -20°C until use.

3.4.6 Coating the microcarriers with DNA.

Experiment 1. 50 μL Aliquot of gold (0.6 μm) or tungsten (0.7 μm) particles were thawed and resuspended by carefully vortexing for 5 minutes. 5 μL of the transformation plasmid pSELECT100 (1589 ng/ μL), 50 μL of 2.5 M CaCl_2 and 20 μL of 0.1 M sperimidine were added to an aliquot gold particles while vortexing at low speed. The mixtures were centrifuged for 2 seconds at $2000 \times g$. The supernatant was carefully removed by a pipette. 150 μL of 70% ethanol was added to resuspend and vortex the micro particles at low speed for washing. The supernatant was discarded after microparticle centrifugation at $2000 \times g$ for 2 minutes. The micro particles were resuspended in 170 μL of 96% ethanol and vortexed for 1 minute at low speed, and centrifuged at $2000 \times g$ for two seconds. The supernatant was discarded. Finally, micro particles were resuspended in 55 μL of 96% ethanol for five biolistic shots in one hour. The same procedure was applied for **experiment 2** as well, however the latter experiment was done a week after the first one.

Control. For biolistic transformation control was used as follows; agar plate spread with $1.3E+09$ *N. oceanica* CCMP1779 were bombarded with particles prepared and coated with sterile distilled water instead of the transformation plasmid (pSELECT100). All other parameters (cell concentrations, the place where plate placed, number of shoots per plate etc.) were similar between experiment 1 and 2 (see Table 3.4).

3.4.7 Loading the macro-carriers for bombardment

While shaking the tube thoroughly, 10 μ L of homogenous bead solution (DNA-coated gold or tungsten particles) was transferred to the center of each sterile macro-carrier and left for the complete evaporation of ethanol before bombardment.

In these experiments, cells were bombarded using the Bio-Rad biolistic PDS-1000/HE particle delivery system (Figure 3.3) fitted with 1700 psi rupture discs (Bio-Rad). This device was operated as previously described (Kikkert JR., 1993). Briefly, the interior part of Bio Rad® PSD-1000/He particle Delivery System was wiped with 70% ethanol to make sure that the environment is sterile. The macrocarriers, metallic stopping screens, and macrocarriers holders were also sterilized by soaking in 96% ethanol for 5-10 minutes and left in a sterile petridish to allow complete evaporation of ethanol before shooting. The desired rupture discs were dipped in isopropanol and placed in the rupture disk retaining cap. The macrocarrier was installed on the top of stopping screen, while the rupture disk was fitted in the disk retaining cap. For the bombardment, the target plates with *N. oceanica* CCMP1779 cells were placed at 6 cm of travel distance for DNA-coated microcarriers from the launch site (Figure 3.3.). In each plate with different microcarriers, five bombardments were performed using the mixture of DNA and water for control.

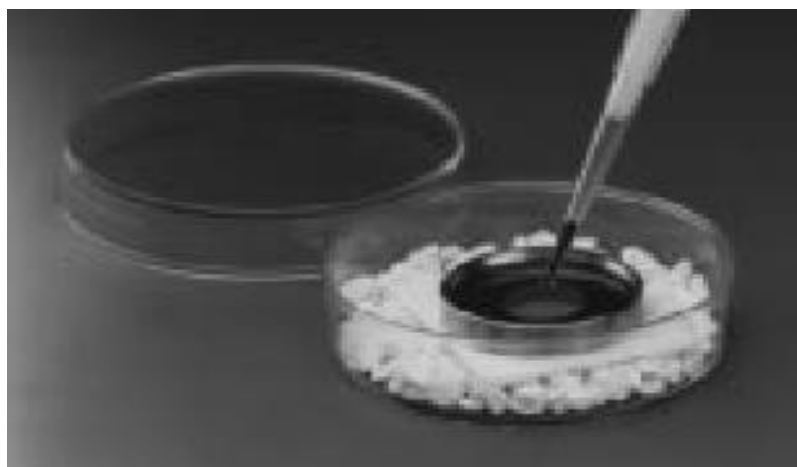


Figure 3.2. Macrocarrier holder placed on the desiccant (CaCl_2) which is covered with a piece of filter paper. The loaded DNA coated particle is used within an hour.

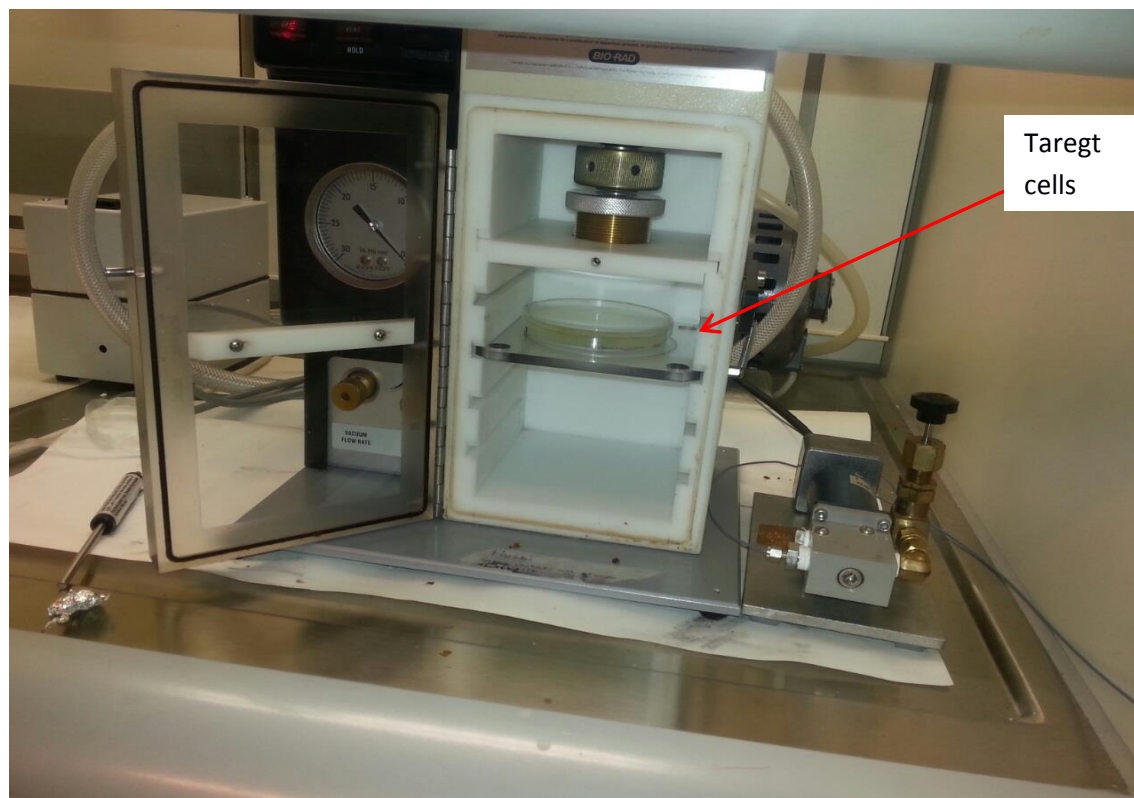


Figure 3.3. Partial view of Biolistic[®] PDS-1000/He particle delivery system. The target cells were placed on the second shelf level (from the top). The picture was taken during the experiment.

3.4.8 Post bombardment cell handling

After bombardment, cells were incubated on 50% seawater solidified agar medium, (containing no selection marker) overnight at 23°C with constant light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 18-20 hours (cells divided once during this period) (Apt KE. et al., 1996).

The 50% seawater solidified agar plates containing 150 $\mu\text{g/mL}$ hygromycin B as a selective marker for successful transformants, were prepared before particle bombardment took place. 3ml of Hygromycin B with 50mg/mL as a stock solution was transferred into 1000 mL of liquid 50% seawater with agar to reach 150 $\mu\text{g/mL}$ of final concentration.

The bombarded cells were washed off from the plate with 50% seawater medium by repeatedly pipetting on the spot of algae or by using a spatula to remove cells sticking to the surface of agar plates after 20 hours of incubation. After washing the cells two or three times, the liquid was transferred into sterile falcon tube. 5mL of total cell suspension was collected and 0.5mL of cells were distributed on the agar plate containing 150 $\mu\text{g/mL}$ hygromycin B for both gold and tungsten coated plasmid bombarded cells. The plates were sealed with parafilm and incubated on selective media for 2-3 weeks at 23°C under constant light.

3.4.9 Selection of transformants

Selection of transformants was conducted after 13 days of incubation on selective medium containing Hygromycin B. Colonies appeared on plates containing alga bombarded with tungsten or gold particles. The number of the colonies that grew on the medium with 150 $\mu\text{g/mL}$ hygromycin B were counted, and colonies were collected on two master plates with the same selective marker. The transformation efficiency was determined based on colony numbers.

Confirmation of successful transformant was performed by applying an efficient colony PCR procedure (Wan M. et al., 2011). The extracted genomic DNA of transformed *N. oceanica* CCMP1779 cells were utilized as a template during colony PCR methods. Previously different techniques have been used to release genomic DNA from transformed cells (Chernyavskaya O., 2014).

In this experiment the extraction of *Nannochloropsis* genomic DNA was conducted as follows. Potentially transformed colonies were picked with a toothpick or sterile yellow pipette tips from

the master plate and resuspended in 50 µL DNA extraction buffer (10 mM EDTA) in eppendrof tubes. The tubes were incubated at 100°C for 10 minutes and kept for 2 minutes in ice for cooling. The mixture was vortexed and centrifuged for 1 minute at 10,000 x g. 1µL of the supernatant was used as the template for a colony PCR procedure in a 25 µL volume PCR reaction. The other parts of the supernatant was preserved at -20°C for subsequent PCR reactions as a template. PCR products were examined using 0.8% agarose gel electrophoresis.

Two oligonucleotide primers were used for the detection of the existence of the transferred plasmid in successful transformants of *N. oceanica* cells by colony PCR analysis:

hygro_fwd 5`- aagatggagtgatggagga-3` (forward) and

hygro_rev 5`- catgcctgcaggtcactgga -3` (reverse primer)

The primers for the detection of the endogenous 18S rRNA gene, which served as an internal control, were:

18SF: 5`- CAGAGGTGAAATTCTTGGA-3` (forward primer) and

18SR: 5`- TCACCTACGGAAACCTTGTTACGAC -3 (reverse primer`).

Each PCR sample consisted of 25 µL of solutions containing template, primers, dNTPs and enzyme in PCR buffer.

Sixteen *N. oceanica* cell samples including the transformation plasmid pSELECT100 as a control were examined for the first round of colony PCR. The PCR mix which was used to amplify the hygromycin resistance cassette of released genomic DNA was prepared as follows. 100 µL dNTP mixes was prepared from 10 µL of each nucleotide solution of adenosine, thymidine, guanosine and cytosidine mixed with 60 µL dH₂O. The genomic DNA of transformants that grew on selective medium containing 150 µg/mL was used as a PCR template. Each PCR sample consisted of 25 µL of solution containing 10 mM dNTPs, 10 µM of each primer, genomic DNA as a template and DyNAzyme II DNA polymerase in a 10× PCR buffer (Optimized DyNAzyme buffer, Thermo Scientific). Amplification products were analyzed on agarose gels.

Table 3.3. The setting used for hygromycin resistance cassette amplification in colony PCR methods (25 total cycles)

steps	Temperature (°C)	Time (min)
Initial denaturation	94	2
Denaturation	94	0.30
Annealing	50	0.30
Extension	72	1.2
Hold	4	5.0

The parameters used for biolistic transformation applied for this experiment are summarized in the table below. Controlled transformation condition include cell concentration, plasmid amount, number of bombardments, distance between microcarrier and target cells as well as type and size of microcarriers (see Table 3.4).

Table 3.4. Summary of important parameters for the biolistic transformation of *N.oceanica* CCMP1779 cells using linearized pSELECT100 transformation plasmid containing hygromycin B resistance gene. Cells of same concentrations were transformed with different microcarrirs coated with plasmid and sterile distilled water as a negative control.

Transformation condition	Experiment 1	Experiment 2	control
Cell concentration before centrifugation	8.1E+07 cells/mL	4.1E+07 cells/mL	4.5E+07 cells/mL
Centrifugation condition	4000 rpm for 10 minutes at room T ^o	4000 rpm for 10 minutes at room T ^o	4000 rpm for 10 minutes at room T ^o
Cell concentration after centrifugation	1.19E+09 cells/mL	0.5E+09 cells/mL	0.53E+09cells/mL
Cell concentration for bombardment	1.3E+09 cells	1.3E+09 cells	1.3E+09 cells
Incubation of cells on plate before bombardment (hr)	18-20	18	6 (cells were plated freshly)
Distance between microcarreries and cells	6 cm (second level from the top)	6 cm	6 cm
microcarriers	Both gold (0.6 μm) and tungsten (0.7 μm)	Both gold (0.6 μm) and tungsten (0.7 μm)	Gold (0.6 μm)
Pressure (psi)	1700	1700	1800
Number of bombardment/plate	5	5	5
Total plasmid/plate (μg)	8	10	No plasmid (10 μL of sterile distilled water instead of plasmid)
Incubation of cells after bombardment on agar plate without antibiotic (hr)	21	19	20
Cell amount plated out on one agar plate with selective marker	1.3E+08 cells (in 10 plates)	1.3E+08 cells (in 10 plates)	1.3E+08 cells (in 10 plates)
Incubation period of cells on selective media (day)	13-16	16	16

3.5 Results

3.5.1 Selective marker for genetic engineering of *N. oceanica*

Cell growth of *N. oceanica* in 50 % sea water liquid medium with antibiotics was investigated. *N. oceanica* showed different sensitivities to the eleven antibiotics. Among these, Ampicillin, Kanamycin and Gentamycin had no effect on growth, even at the highest concentrations tested. Low concentrations of Nourseothricin had no impact on the growth of *N. oceanica*, however, inhibition of growth became clear at a concentration higher than 50 µg/mL. High sensitivity of *N. oceanica* to Zeocin, Streptomycin, Cycloheximide, Hygromycin B, Chloramphenicol and Paromomycin was observed. Cells growth was completely inhibited by Zeocin and Cycloheximide at the lowest test concentration (1 µg/mL). Inhibition of cell growth by Chloramphenicol, Hygromycin B and Paromomycin appeared at a concentration of 10µg/mL and at 5µg/mL for Streptomycin. Therefore, it can be concluded that Zeocin, Cycloheximide, Hygromycin B, Chloramphenicol and Streptomycin may be suitable selection markers for *N. oceanica*. Since Hygromycin B was used as selective marker for microalgal genetic engineering (Vieler A. et al., 2012) the plasmid pSELECT100 (constructed and provided by the Benning laboratory at Michigan State University (Figure 3.1) was used to establish biolistic transformation protocols.

3.5.2 Plasmid Confirmation

Endonuclease digestion of received transforming pSELECT100 plasmid by *Sma*I and *Bam*HI resulted three fragments and one fragment respectively (Figure 3.4). The gel images revealed that the plasmid which was digested by *Sma*I has three cutting sites approximately at 2650 bp, 1333 bp and 113 bp as shown below in Figure 3.4.

As demonstrated in the figure 3.4., *Bam*HI cuts pSELECT100 plasmid at one site (4031 bp) and result in a linearized plasmid that can be used for transformation. Fragment lengths obtained from *Sma*I and *Bam*HI digestions provided a reasonable confirmation that the plasmid was pSELECT100.

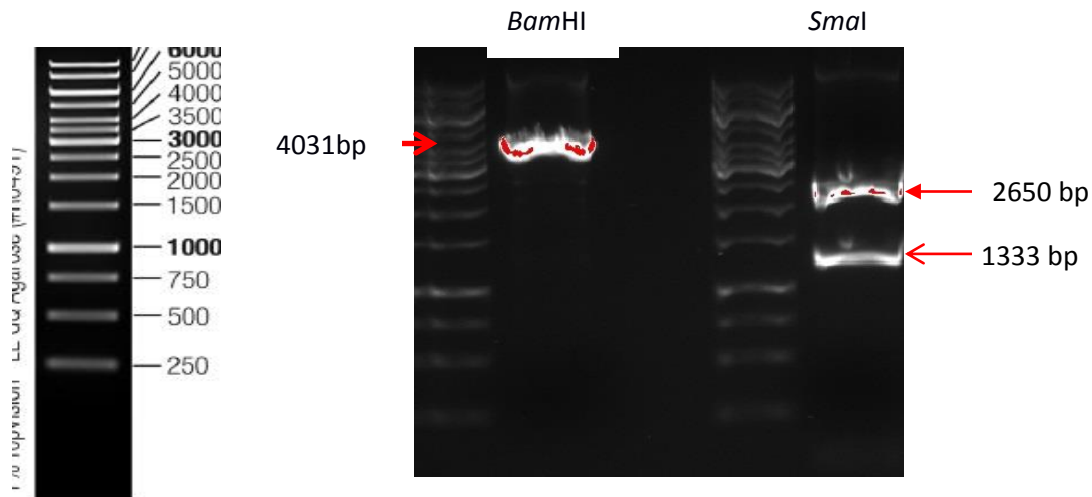


Figure 3.4: the gel image after digesting pSELECT100 plasmid with *Bam*HI and *Sma*I restriction enzymes. The band length of 4031-bp in the second lane is the result of *Bam*HI digestion, and indicates the linearized pSELECT100. The two bands in the last lane are the expected results after *Sma*I digestion. Even if *Sma*I results 3 bands, the third band (113-bp) was not visualized on the gel image, it might gone further down the gel

3.5.3 Biolistic transformation of *N.oceanica* CCMP1779

Chernyavskaya O. (2014) previously reported the transformation of *N.oceanica* CCMP1779 using microparticle bombardment to introduce plasmid pLit_chiL_chlor containing the chloramphenicol resistance gene which confers resistance to chloramphenicol. Based on a protocol similar to that reported previously and with some modifications, microparticle bombardment was used to transform *N.oceanica* CCMP1779 with the plasmid pSELECT100 containing gene which confers resistance to Hygromycin B antibiotic. Experimental conditions tested are summarized in Table 3.4. After 2 weeks of incubation of bombarded cells on agar plates containing 150 μ g/mL hygromycin B as selective marker, the most transformants were obtained using gold (0.6 μ m) in experiment one. Only a small number of resistant colonies were obtained by using tungsten (0.7 μ m) microcarrier (Table 3.5). The size and type of the particles has a significant effect on the number of survived colonies recovered. With the smaller gold particles (0.6 μ m) yielding the most transformants. No hygromycin B-resistant transformants or mutants were detected in controls lacking transformation plasmid.

Table 3.5: Number of resistant colonies achieved by biolistic transformation of *N. oceanica* CCMP1779 cells in the presence of linearized pSELECT100 plasmid coated with two different particles. All transformations took place in the same conditions except type of particles used.

particle	# resistant colonies		# successful transformants	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Gold	31	208	7	25 out of 97 resistant colonies
Tungsten	14	415	5	17 out of 97 resistant colonies

In the course of experiment 2, more than 200 clones on selective agar plates (150 µg/mL Hygromycin B) were obtained using both gold and tungsten in the same conditions as experiment 1 (Table 3.4). To test if these colonies do indeed represent transformants, 97 random colonies were selected from both gold and tungsten bombarded plates. Colony PCR analysis from genomic DNA isolated from these colonies confirmed the presence of hygromycin B resistance gene in 25 and 17 clones of gold and tungsten bombarded cells, respectively (Table 3.5).

3.5.4 Molecular analysis of transformants

To determine whether resistant colonies obtained by bombardment contained DNA sequences derived from plasmid pSELECT100, the genomic DNA from transformants were extracted and analyzed by colony PCR for the existence of region of hygromycin B resistance gene (Figure 3.5). Colony PCR analysis (Wan M. et al., 2011) was performed directly on transformed cells using primers to amplify hygromycin B resistance gene. In successful transformed clones, a 790-bp fragment was amplified, where in wild-type cells, no amplification product was obtained (lane WT in the figure below).

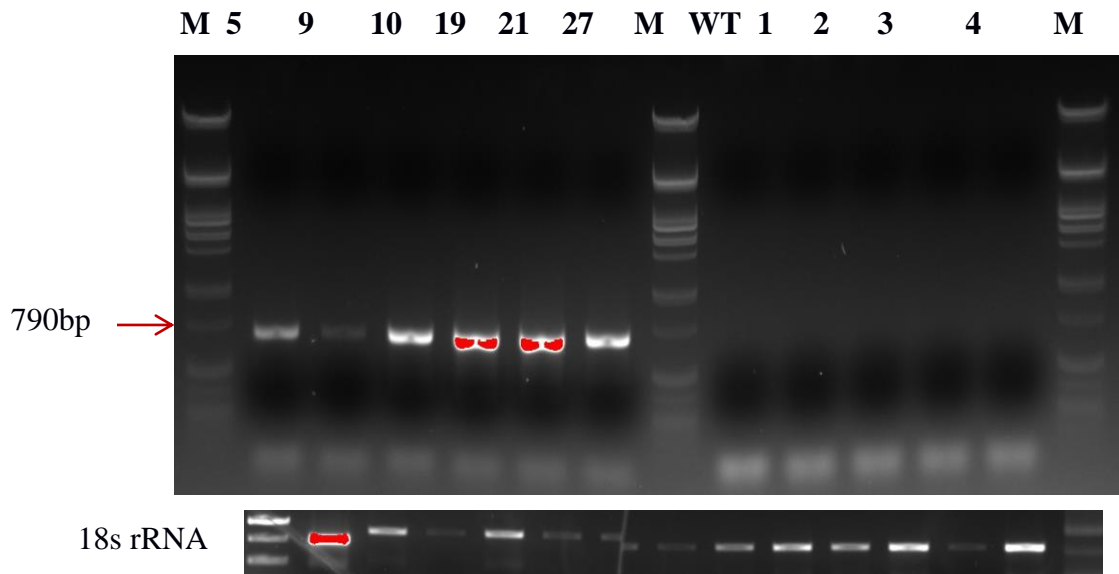


Figure 3.5. Colony PCR analysis of the bombarded *N.oceanica* CCMP1779 cells during experiment 1. Cells were bombarded with **gold** coated pSELECT100 linearized plasmid under appropriate conditions as indicated. After two weeks of incubation on selective media, genomic DNA was extracted from colonies and analyzed by colony PCR. Lane M: molecular marker; lane WT: wild type as control (no plasmid); lanes with numbers: single colony of *N.oceanica* CCMP1779. When primers were used, a 790-bp PCR product was expected to be produced by transformed cells. Amplification of 18S rRNA served as an internal control.

At the same time however, most of transformants obtained after two weeks of incubation (those resistant to 150 $\mu\text{g}/\text{mL}$ Hygromycin B), completely lost their exogenous DNA fragment when examined by colony PCR (Lane 1, 2, 3, 4 and others not shown here). This suggests that the incorporation into the genome was transient.

A single band with the same size (790-bp) was detected in the samples prepared from transformants (lane 5, 9, 10, 19, 21 and 27), whereas no band was observed in the sample from wild type (Lane WT) (Fig. 3.5). These results implied that the plasmid pSELECT100 was successfully introduced in the *N. oceanica* CCMP1779 cells and the hygromycin B resistance gene was integrated into the genome.

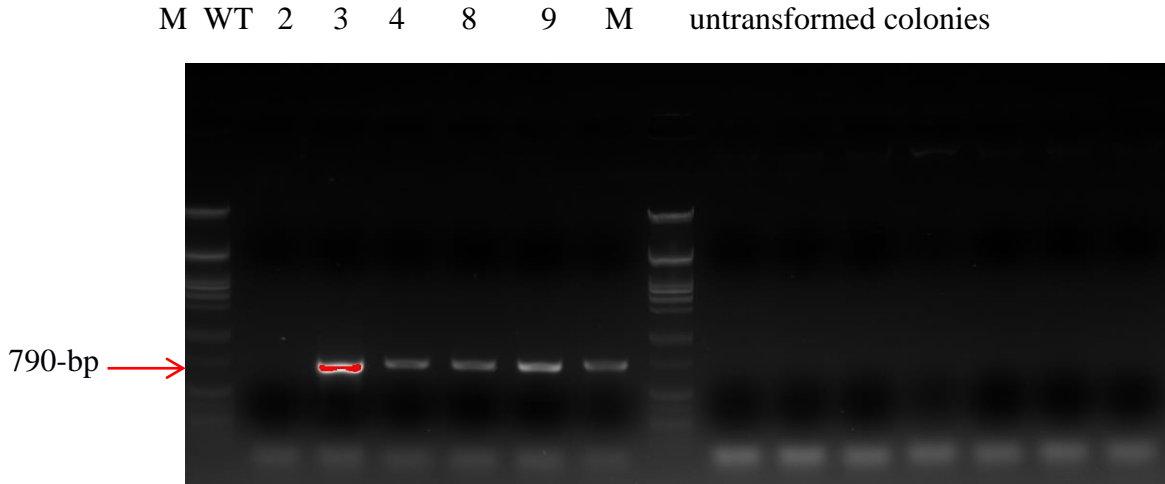


Figure 3.6: colony PCR analysis of twelve hygromycin B-resistant colonies (lane 2, 3, 4, 8, and 9) and untransformed *N. oceanica* CCMP1779 cells. Cells were bombarded with **tungsten** coated linearized pSELECT100 plasmid. Lane M: molecular marker; lane WT: wild type as control; lane 2, 3, 4, 8, and 9 are successfully transformed cells. 790-bp PCR product was expected to be produced by transformed cells.

Based on the results from the above PCR analysis, a 790-bp PCR product was detected in some transformed *N. oceanica* CCMP1770 cells; while most of Hygromycin B resistant colonies which were bombarded by tungsten coated pSELECT100 did not contain the marker gene as demonstrated in Figure 3.6.

The colony PCR analysis of transformants obtained in experiment 2 is summarized in the Figure below. The genomic DNA was extracted from 97 randomly selected transformed *N. oceanica* CCMP1779 colonies. Among these randomly analyzed colonies picked from tungsten bombardment, only 17 were found to be positive for the presence of hygromycin B resistance gene (Fig. 3.7. (d) and (e)), whereas gold resulted in 25 successfully screened transformants among 97 colonies examined (Fig. 3.7 (a), (b) and (c)). This result showed that all of the positive transformants contained a DNA fragment of the correct size (790-bp) and no amplified product was obtained from wild type cells. The presence of this DNA fragment suggested that the exogenous hygromycin B resistance gene has integrated into the genome of *N. oceanica* CCMP1779 cells.

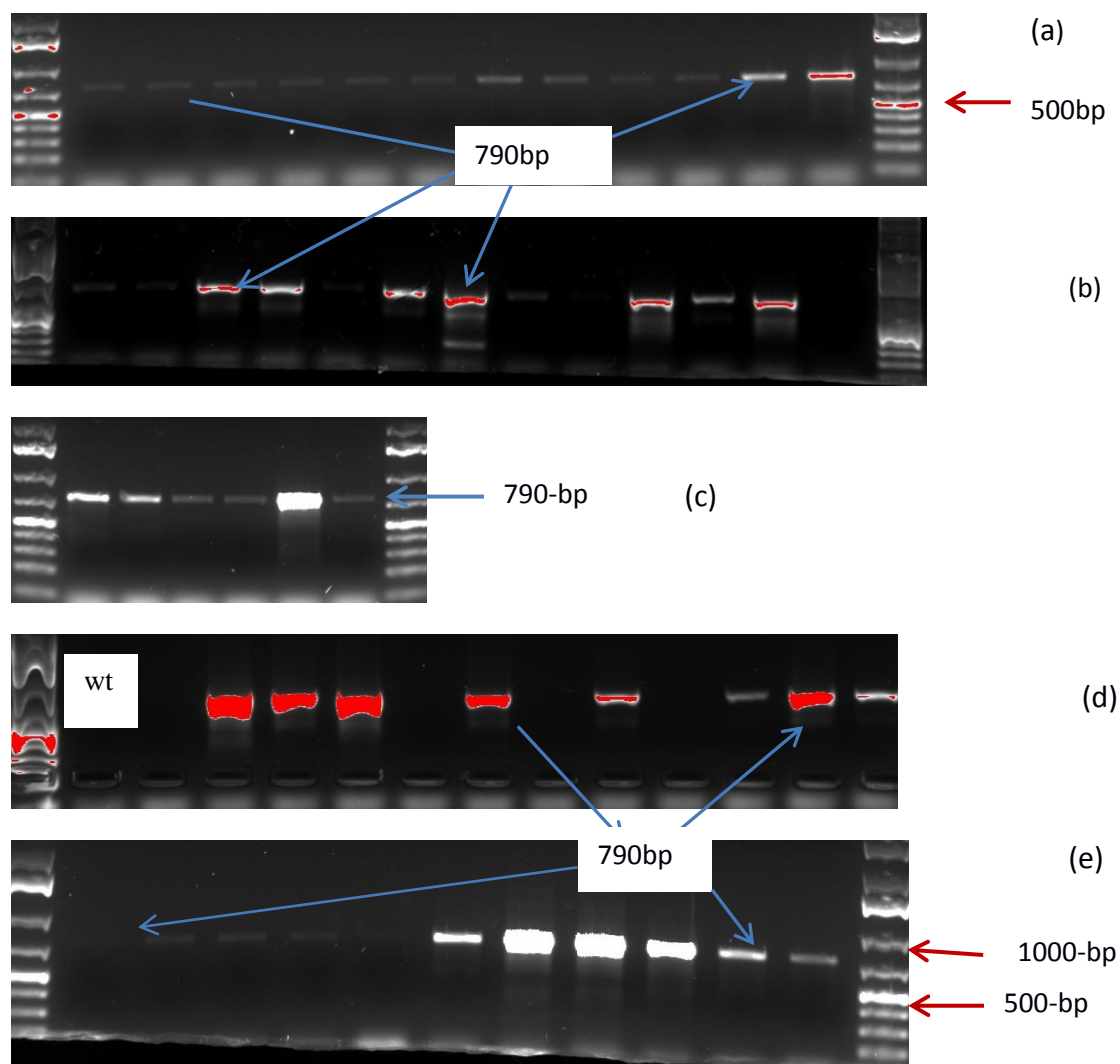


Figure 3.7: colony PCR analysis of transformants resulted gold (a, b and c) and tungsten (d and e) bombardments. PCR were performed using primers specific for the hygromycin B resistance gene. For each particle, 97 randomly selected hygromycin B-resistant transformants obtained from experiment 2 were detected. Transformants harboring plasmid pSELECT100 produced 790-bp PCR product. The 2000-bp DNA ladder plus was utilized.

3.6 Discussions

Nannochloropsis is attracting increasing attention for biofuel production, feed, source of pigments, high quality proteins. But, genetic transformation approach has been in its infancy stage for this microalga, which hampers strain development by genetic engineering and genomics analysis by molecular tools. This means that, a shallow understanding and protocols for nuclear and chloroplast transformation must be available for *Nannochloropsis* to appear as a leading candidate for source of biofuel production.

In this study, efficient transformation protocol for *N. oceanica* CCMP1779, photoautotrophic alga of high importance for biofuels, have established to allow a foreign gene to be transferred into cells. Previously, transformation of *N. oculata* by electroporation has been reported (Chen HL. et al., 2008). Transformation of *N. oceanica* CCMP1779 using biolistic and electroporation has also been reported (Chernyavskaya O., 2014), but antibiotic resistant colonies were not detected further for the harboring of exogenous DNA by colony PCR especially after biolistic transformation and this might make the previous transformation efficiency result uncertain. In contrast, the protocol applied in this study for the genetic transformation of *N. oceanica* CCMP1779 utilized colony PCR for molecular analysis of antibiotic resistant colonies and numbers of false transformants were detected.

To optimize the biolistic transformation conditions for transferring the linearized plasmid pSELECT100 into *N. oceanica* CCMP1779 cells, the effect of gold and tungsten particles were assessed. As depicted in Table 3.5, higher resistant colonies are obtained when gold was employed as a macrocarrier in experiment 1. Particle type and size played an important role for transformation efficiency. Tungsten (0.7 μm) is also required to deliver the plasmid, but it also is harmful to the transformed cells. Therefore, the smaller gold particle used to introduce exogenous gene, the more number of transformants obtained (experiment 1 Table 3.5), this might be due to less cell damage they sustained which could be more easily repaired (Anderson BR. et al., 2015). Although tungsten has been reported as a less efficient microcarrier as compared to gold, more number of visible colonies but less number of transformants with exogenous DNA were obtained in experiment 2 as compared to gold (Table 3.5). In this experiment, the cells were treated with ampicillin before transformation and this might be the reason behind the growth of more colonies and the detection of more transformants. How the Ampicillin treatment increased

the transformation efficiency is unclear but it showed a significant effect on achieving high numbers of successful transformants. It could be that the ampicillin made the cell wall weaker so increasing the possibility of transport of the linearized plasmid into the cell.

Earlier study argued that biolistic transformation is not the right genetic transformation technique for *Nannochloropsis* cells (Chernyavskaya O., 2014). But, results from this study showed that transformation rates of between 5-72 transformants /1.3E+09 cells were obtained for *N. oceanica* CCMP1779 which is comparable with those ever reported in other microalgae. For example, Particle bombardment of *Phaeodactylum tricornutum* and *chlamydomonas reinhardtii* produces between 10-100 transformants/10⁸ cells (Apt KE. et al., 1996) and 100-250 transformants/10⁸ cells (Remacle C. et al., 2006) respectively. Generally, the results in this study indicated that *N. oceanica* CCMP1779 cells can be transformed effectively via biolistic transformation. The detection of hygromycin B resistance genes in transformants by colony PCR analysis strengthen the effectiveness of the protocol used for transformation in this study.

3.7 Conclusions

This work demonstrated the possibility of biolistic transformation of the unicellular marine alga *N. oceanica* CCMP1779 cells. An attempt was made to further enhance the transformation method for *N. oceanica* CCMP1779 cells by testing the effect of type of microcarrier particles used. Results presented here reveal particle type and pre-treatment of the cells with antibiotic is vital parameter for optimizing biolistic transformation. Small gold particles (0.6 μm) appear to be better suited than larger size tungsten (0.7 μm). The smaller particles may cause less damage and therefore better survival of bombarded cells. The overall transformation rate achieved in this experiment represents the highest number of transformants reported so far for this organism.

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