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Genetic Transformation of *Acutodesmus obliquus* and *Neochloris oleoabundans* by bacterial conjugation

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i. Abstract

Microalgae are photosynthetic organisms with a vast array of industrial applications, from the production of feed and food ingredients such as lipids and proteins, the synthesis of biofuels, and production of antibodies. However, a cost-effective production of microalgae-derived products is in many cases not achieved due to too low product yields and productivity, and inefficient or costly biorefinery of microalgae. Strain development is therefore required, but has been severely hampered by the lack of optimized transformation techniques for microalgae. One of the novel methods to do so is by bacterial conjugation, a process through which a donor cell is capable of transferring DNA into a recipient cell. It is known to occur abundantly between bacteria, though has recently been implemented for transformation of diatoms as well.

In this study the green microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans* were successfully transformed using bacterial conjugation using *E. coli* as a donor, with the episomal pPtPuc3 plasmid, while genetic transformation with the episomal pTpPuc3 plasmid still requires confirmation. Protocols for the genetic transformation of both microalgae species were developed, but still require further optimization.

The focus of the protocols' optimization efforts was conjugation time, concentration of zeocin or nourseothricin in the medium for transconjugant selection, and the period of culture exposure to light. For *A. obliquus*, a correlation between longer conjugation times and a higher number of colonies was found. Comparatively however, a 60-minute conjugation time showed a better colony ratio between transformants and controls, while longer conjugation of 90 and 120 minute periods showed a small margin between the number of possible transformants and the number of colonies in the controls. Much like *A. obliquus*, the transformation of *N. oleoabundans* shows a relation between longer conjugation time and number of colonies, with a two-hour period generating a greater number of colonies. For *N.oleoabundans* however, no link was found between shorter incubation periods and an advantageous non-confirmed transformant/control colony ratio. In addition, it was found the number of colonies was increased by prolonging the cultures' exposure to light.

This is the first reported instance of green microalgae being genetically transformed by bacterial conjugation, and only the second transformation method used successfully on *A. obliquus* and *N. oleoabundans*.

Keywords: *Neochloris oleoabundans*, *Acutodesmus obliquus*, Green microalgae, Conjugation, Genetic transformation

i. Resumo

As microalgas são organismos fotossintéticos com uma vasta game de aplicações industriais, desde a produção de alimentos e ingredimentes alimentares como lípidos e proteínas, a síntese de biocombustíveis, e produção de anticorpos. A produção custo efectiva de produtos derivados de microalgas não é em muitos casos atingida devido ao baixo rendimento de produção e de biorefinaria de microalgas custosa e ineficiente. O desenvolvimento de estirpes revela-se então necessário, mas tem sido severamente prejudicado pela ausência de técnicas de transformação optimizadas para microalgas. Um dos novos métodos para o fazer é por conjugação bacteriana, um processo pelo qual uma célula doadora é capaz de transferir DNA para um célula receptora. É conhecido por ocorrer abundantemente entre bactérias, e foi recentemente implementada na transformação de diatomáceas também.

Neste ensaio as microalgas verdes *Acutodesmus obliquus* e *Neochloris oleoabundans* foram transformadas com sucesso usando *Escherichia coli* como doador, com o plasmídeo episssomal pPtPuc3, enquanto que a transformação com o plasmídeo episssomal pTpPuc3 ainda necessita de ser confirmada. Foram desenvolvidos protocolos adequados para a transformação genética de microalgas, ainda que careçam de optimização. Foi dada maior atenção à optimização dos parâmetros de tempo de conjugação, concentração de antibiótico no meio de selecção de transformantes e ao período de exposição das culturas a luz.

No caso de *A. obliquus*, foi encontrada uma correlação entre o prolongamento do tempo de conjugação e um maior número de colónias presentes no meio de selecção. No entanto, o tempo de conjugação de 60 minutos demonstrou um melhor rácio entre colónias transformantes e as presentes nos controlos, enquanto que para 90 minutos e 120 minutos de conjugação verificou-se apenas uma pequena margem entre o número de colónias possivelmente transformantes e as colónias presentes nos controlos. Tal como *A. obliquus*, a transformação de *N. oleoabundans* demonstra uma relação entre o prolongamento do período de conjugação e o número de colónias, onde um período de duas horas gerou o maior de número de colónias. *Para N. oleoabundans*, não foi encontrada qualquer relação entre períodos de incubação mais curtos e relação transformantes não confirmados/controlo vantagosa. Adicionalmente, foi visto que o número de cólonias foi aumentado ao prolongar a exposição das culturas a luz.

Este é o primeiro caso de transformação genética de microalgas verdes por conjugação bacteriana, e apenas o segundo método de transformação utilizado com sucesso em *A. Obliquus* e *N. oleoabundans*.

Palavras-chave: *Neochloris oleoabundans*, *Acutodesmus obliquus*, microalgae verdes, Conjugação, Transformação Genética

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ii. Abbreviation list

TAG: triacylglycerides

ATMT: *Agrobacterium tumefaciens* mediated transformation

PEG: Polyethylene glycol

HGT: Horizontal gene transfer

oriT: Origin of transfer

T4SS: Type IV secretion system

L/D: Light/Dark

OD_{600}: Optical density at 600 nm

FW: Freshwater

TE: Transfer efficiency

SDS: Sodium dodecyl sulfate

PCR: Polymerase Chain Reaction

C+: Positive control

C-: Negative Control

DMSO: Dimethyl sulfoxide

bp: base pair

GPAT: Glycerol-3-phosphate acyltransferase

DGAT: Acyl-CoA diacylglycerol acyltransferase

LPAT: Lysophosphatidyl acyltransferase

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1. Introduction

1.1. Microalgae biology

Microalgae are microscopic photosynthetic eukaryotic organisms. Though most are unicellular, like *Chlamydomonas reinhardtii (*figure 1A), several described species are colony forming, namely species of the *Volvox* genus and *Botryococcus braunii* (figure 1B) [1]. Microalgae can inhabit a wide variety of habitats. While they occur mainly in fresh and salt-water environments, several species have been isolated from such inhospitable terrestrial sites as deserts and snowfields [2]. Microalgae have a tremendous variety, and are amongst the oldest living organisms [3]. Thousands of species have already been characterized with recent studies suggesting a number of existing species at around 10.000-500.000 on Earth [3]. This enormous variety also extends to their metabolic and biochemical pathways [2] and properties, as they can grow either autotrophically or heterotrophically, which allows them to obtain carbon from inorganic or organic sources, respectively. Autotrophic microalgae are either photoautotrophic or chemoautotrophic and heterotrophic microalgae can be classified as photoheterotrophs or chemoheterotrophic [3]. Some species can display mixotrophic behaviour, using both autotrophic and heterotrophic strategies [3].

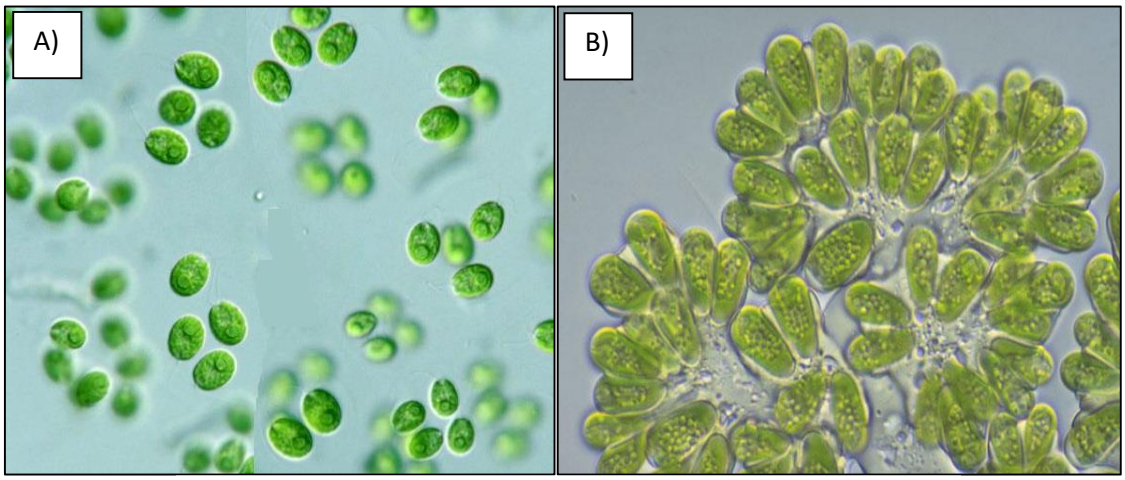


Figure 1: A) *Chlamydomonas reinhardtii* [4] and B) *Botryococcus braunii* [5].

Photosynthetic microalgae are responsible for the production of half of the oxygen available in the atmosphere [2] while simultaneously capturing carbon dioxide and converting it into biomass. This process is carried out much more effectively in microalgae than in higher plants due to the microalgae's unicellular structure [3]. Chlorophyll A is the most prominent light-harvesting pigment present in microalgae, though chlorophyll b and carotenoids can also be found microalga. Chlorophyll b however is not found in all species [2].

1.2. Biotechnological applications of microalgae

Biotechnological research into microalgae has seen a resurgence in in the last decades, a lot of which focused towards the production of compounds of interest to a vast range of industries [2], offering an alternative to conventional methods of production, namely agriculture. Amongst the most sought after applications resulting from microalgae cultivation are the production of bioactive products, used in the pharmacological and cosmetic industries, lipids for the production of feed, food or biofuels such as biodiesel; and treatment of industrial and municipal wastewater mainly through the removal of phosphates, nitrates and heavy metals [2]. Table 1 shows a list of microalgae with the potential to become a source of lipid.

Table 1: Lipid content of several species of microalgae, expressed in terms of the percentage of biomass composition, and the conditions under which they were grown.

Species	Strain	Cultivation method	Lipid content (% Dry weight)	Reference
Botryococcus braunii	UTEX 572	Phototrophic	20.8	[6]
Chrolella vulgaris	#259	Phototrophic	33.0 - 38.0	[7]
		Heterotrophic	23.0 - 36.0	
		Mixotrophic	21.0 - 34.0	
Chrolella protothecoides	-	Heterotrophic	50.3 - 57.8	[8]
Dunaliella tertiolecta	ATCC 30929	Phototrophic	60.6 - 69.8	[9]
***Nannochloris* sp.**	UTEX LB1999	Phototrophic	29.9 - 40.3	[10]
Neochloris oleoabundans	UTEX 1185	Phototrophic	15.9 - 56.0	[11]
Scenedesmus obliquus	-	Phototrophic	12.7	[12]
	-	Mixotrophic	6.6 - 11.8	
Spirulina maxima	LB 2342	Phototrophic	4.1	[13]
Tetraselmis suecica	F&M-M33	Phototrophic	27.0	[14]

Microalgae have a number of advantages over plants that make their use very appealing: higher biomass productivity and growth rate, improved carbon dioxide fixation and oxygen production rates, their production doesn't compete with land use for food-oriented agriculture as they can be grown in liquid medium and on non-arable land [15]. Large-scale production of these organisms does not negatively impact surrounding ecosystems, and reduces stress on water sources as a result of smaller water requirements [3]. Furthermore, it avoids soil desertification and soil chemical contamination since pesticides and herbicides are not necessary for their protection [3].

1.3. *Neochloris oleoabundans*

Neochloris oleoabundans is a fresh water unicellular green microalgal species, which belongs to the *Chlorococcaceae* family [16]. It can also be found in terrestrial locations, as it has been isolated from sand dunes from Saudi Arabia [17].

N. oleoabundans has been shown to produce high levels of lipid accumulation, surpassing 50% of cell dry weight when grown under stress conditions such as nitrogen starvation at high pH [18] and in photoautotrophic mode [19]. About 80% of the total lipids present in the cells of *N. oleoabundans* are triacylglycerides (TAGs) [19]. The majority of its fatty acids are saturated with carbon chains ranging from 16 to 20 carbon atoms [20] which makes *N. oleoabundans* ideal for the production of biodiesel [21]. *N. oleoabundans* also appears to accumulate lipids under saline conditions as demonstrated by Santos *et al*. [19]. In spite of this, a strategy to maximize productivity of lipids and other bioactive products in this species has not yet been devised [22].

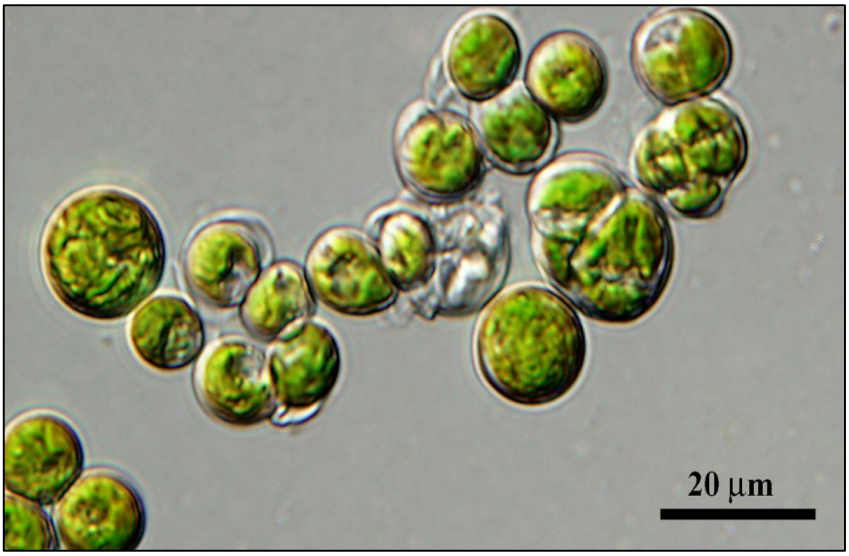


Figure 2: *N. oleoabundans* cells viewed through a microscope [23].

1.4. *Acutodesmus obliquus*

Acutodesmus obliquus (previously classified as *Scenedesmus obliquus*) is a fresh water oleaginous green microalgae that is often proposed as a suitable candidate for the production of biofuel [24, 25], much like *N. oleoabundans*. Additionally, *A. obliquus* contains high protein concentrations, making it an attractive source for the production of animal feed additives [25]. It is further characterized by its robustness, versatility and relatively high biomass productivity [24]. Moreover, while under nutrient rich conditions lipid content does not exceed 30% of its cell dry weight [12], it has been shown to rival *N. oleoabundans'* lipid accumulation potential under specific conditions, with the highest reported value of 58.3% of dry cell weight [12]. It has been the subject of several studies regarding increased lipid content through bioprocess engineering approaches (the most notable

being nitrogen starvation), lipid harvesting, CO_2 mitigation and genetic transformation [25]. Furthermore, it thrives in industrial wastewater from a wide range of sources [26].

Several methods have already been attempted in order to increase lipid content in *A. obliquus*, specifically the creation of starchless mutants (*slm1* mutants) in association with nitrogen starvation approaches [27]. This method resulted in strains with negligible production of starch (which competes with lipid synthesis for the same precursors), and a TAG content increase from 45% to 57%. [27]

Figure 3: *A. obliquus* cells viewed under a microscope [28].

1.5. Microalgae strain-improvement by genetic engineering

The improvement of microalgae has been recognized as an essential requirement to make low-value compound production economically sustainable. This is a consequence of none of the microalgae species characterized so far having the necessary traits to make them economically suitable in an industrial setting for their production. Though genetic optimization of algal strains has received little attention [29] and few species have been the focus of this kind of research [30], it is theorized that several aspects of microalgae have the potential to be improved through genetic modification. These include photosynthetic efficiency, growth rate, oil content, increased resistance to abiotic factors such as temperature, the elimination of the light saturation phenomenon, reduction of photo-inhibition, and decreased susceptibility to photo-oxidation [29]. The development of a microalgae strain with these characteristics could substantially decrease the current economic constraints placed on the industrial production of microalgal compounds.

Genome information of many microalgae of interest in general is still lacking, with only approximately 30 genomes sequenced so far (annotated and not-annotated) [31]. This significantly limits the current understanding of microalgal biology and metabolism, which is essential when attempting to enhance metabolic pathways for the improved production of lipids (or any other bioactive compound) on a large scale. In order to

understand key aspects of microalgae biology, comparative genomics is often used. Well annotated genomes provide a great deal of information on the metabolic pathways present in the organism as well as the enzymes involved in the system. This is crucial in the identification of genes and regulatory elements of interest [31].

The main challenge regarding the genetic transformation of microalgae resides in the fact that due to their immense diversity no single transformation method or protocol can be used for all species. This means that a thorough study of each organism is needed before an optimal transformation toolkit is devised for a given alga [30]. Other reported complications include the silencing of transgenes in several microalgae species resulting in very low or immeasurable levels of gene expression, and type of selection marker used for the identification of the successfully transformed cells. The selection of most species is reliant on the use of antibiotic resistance markers but in some cases, like *Chlamydomonas reinhardtii*, auxotrophic markers can also be used [30]. *C. reinhardtii* is often used as a model organism for higher plants and algae alike and because of this, is often used as a basis for the development of metabolic and genetic engineering of other species of microalgae, as well as methods of transformation used. Conventional methods to transfer DNA into microalgae include electroporation, microparticle bombardment, *Agrobacterium tumefaciens*-mediated transformation (ATMT) and the glass bead transformation method.

1.5.1. Electroporation

Electroporation is widely used for transformation of competent and non-competent bacteria, yeast, mammalian and microalgae cells [32]. This technique involves the use of high-intensity electric pulses to induce the permeabilization of the cell membrane, thus allowing the uptake of DNA directly from the surrounding environment. The effectiveness of transformation through this method is mediated by length of the electric pulses, the composition of the medium, presence or absence of a cell wall, concentration of the heterogeneous DNA and strength of the electric field [32]. The medium often demonstrates an increase in temperature after the procedure and as a result, a portion of the cells present in the suspension will be lost. Electroporation has already been successfully used for the transformation of many microalgae species such as *C. reinhardtii*, *Chlorella* sp., *Nannochloropsis sp.* and *Dunaliella sp.* [32].

1.5.2. Microparticle bombardment

The transformation of microorganisms through particle bombardment allows delivering exogenous nucleic acid into cells through the cell wall by shooting microparticles coated with nucleic acids against the immobilized target cells. This method requires sophisticated equipment, called a gene gun, and recombinant DNA applied to the surface of microparticles (like tungsten or gold), which are accelerated by a helium-driven pistol (though gunpowder or compressed air may also be used) driving the DNA-coated particles into the target cells [33, 34]. This method is extremely useful when transforming plant cells as well as bacteria and yeast. In the case of microalgae, whose cells contain three different genomes, this technique has allowed the efficient transformation of the nucleus, chloroplast and mitochondria. It is also notable for being able to deliver multiple

copies of the recombinant DNA into the target chloroplast making it highly efficient, and leading to very stable transformation of the nuclear genome and that of the chloroplast [35].

In addition to the high level of transformants resulting from the bombardment of cells with metal microparticles, other advantages related to biolistic transformation includes its simplicity and reproducibility, with the only notable drawback being the cost of the equipment itself and the possibility of the insertion of several copies of DNA into the cell's genome [35].

1.5.3. *Agrobacterium tumefaciens*-mediated transformation

Agrobacterium tumefaciens is a gram-negative bacterium species found in soil, more commonly known for infecting a wide variety of dicotyledonous plants, causing the growth of tumors on their roots (a disease referred to as crown gall disease). The infection is triggered by the release of phenolic compounds from wounds in plant roots and is controlled by the "*vir* regulon" present in the Ti plasmid. This leads to the transfer of the T-DNA from the bacterium's Ti plasmid (tumour inducing plasmid) into the plant cells where it permanently integrates into the host genome causing the aforementioned tumours [36]. It was later discovered that disarmed Ti plasmids could be used to transform plant cells in order to produce genetically engineered plants with improved features [37]. More recently it was demonstrated that this same system could also be used to transform microalgae [36, 38]. This technique holds several advantages over other transformation methods. Not only does it allow the transfer of large DNA molecules with little rearrangements, but also demonstrates a high rate of integration into potentially transcribed regions and usually leads to the integration of a singular transgene in the target's genome [36-39]. In contrast with other transformation procedures, transformation frequency and gene stability do not appear to be frequent complications and can also result in homologous recombination [39] facilitating the localization of the integrated DNA segments [39]. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a form of conjugation as it also involves the physical contact between the organisms involved in the process.

Kumar *et al.* (2004) [38] were able to implement this method for the first time in an algal system through the nuclear transformation of *C. reinhardtii*. When compared with other approaches like the glass bead method, transformation mediated by *Agrobacterium* was shown to have an increase of transformation frequency of 50-fold when transforming *C. reinhardtii* [38]. In addition, it allows for the nuclear transformation of microalgae without the need for the removal of the cell wall, a trait only shared with particle bombardment [38]. An optimized protocol for the *Agrobacterium*-mediated transformation of *C. reinhardtii* was devised in 2013 [36].

1.5.4. Glass bead method

The glass bead transformation method was originally developed for the genetic transformation of yeast, but was later adapted to be used with microalgae. In this approach, permeabilization of the cell wall is obtained by vortexing the cells with glass beads and PEG, in the presence of DNA. [40] The cells are impacted by the beads, making it possible for the DNA to penetrate them [34].

This technique has been successfully used for the transformation of cell-wall deficient mutants and wild-type *C. reinhardtii* treated with cell wall degrading enzymes [40]. This can be seen as drawback, as it has been observed that the efficiency of this method drastically decreases without the removal of the cell wall beforehand [41]. Additionally, because of the stress imposed on the cells there is a 25% decrease in viability; and furthermore the copy number of integrated DNA is much lower when compared with other methods like microparticle bombardment [34]. In spite of these disadvantages, it holds the benefit of not requiring specialized equipment [40] and, in the case of *C. reinhardtii*, a transformation efficiency of 103 transformants/µg DNA was achieved following agitation [42].

1.5.5. Conjugation

Conjugation is a DNA transfer process from one cell to another, requiring physical contact between donor and recipient cells [43] (Figure 4). It is a major contributor to the horizontal gene transfer (HGT) between bacteria, and as such it is a major contributor for evolution in these microorganisms [44]. Consequentially, it is a significant cause for the plasticity of the bacterial genome and has played, in no small measure, a part in the spread of multidrug resistance across many kinds of bacteria [43].

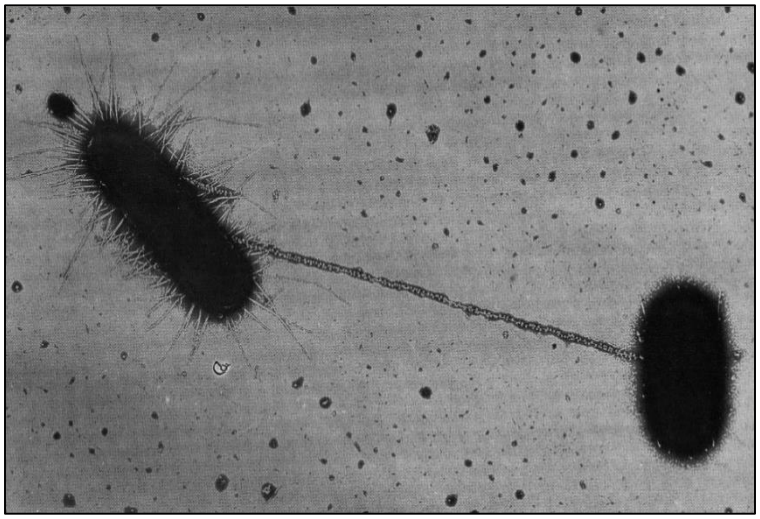


Figure 4: Bacterial donor cell establishing physical contact with recipient cell to transfer DNA through conjugation [45].

Though conjugation is more closely associated with the DNA transfer between bacteria, it is possible that any type of cell can serve as a recipient, since all events that occur during conjugation are wholly dependent on the donor cell and much of the necessary machinery is generally plasmid-encoded [43]. Considerable evidence has been accumulated for conjugation between organisms of large taxonomic distances, and even trans-kingdom conjugation between prokaryotes and eukaryotes [43]. The genome analysis of the red algae *Cyanidioschizon melolae*, for example, revealed that even though most photosynthetic genes have a cyanobacterial origin, the alga's genome contains genes encoding the large and small subunits of the Rubisco enzyme which are more similar to proteobacterial genes [44].

Theoretically, the process of DNA transfer only requires a viable recipient candidate and for that cell to have an extracellular DNA uptake mechanism. As such, the transfer efficiency depends on the recipient [4]. The environment may also benefit the occurrence of conjugation. Microbial biofilms, for example, promote high densities of bacteria making them a DNA transfer-rich environment [43]. Another factor to consider is the size of the transferring DNA in question. Smaller DNA molecules are transported with a greater efficiency, whilst large molecules are transferred at lower rates [46].

Though plasmids are usually the genetic material in question, this phenomenon has been shown to allow the transfer of entire chromosomes and other DNA molecules up to 4.7 Mb [46]. Mobilizable plasmids carry only the relaxosomal components *oriT* (a short DNA sequence which is the only sequence required in *cis* for a plasmid to be conjugally transmissible), a relaxase gene, and one or more auxiliary nicking proteins. On the other hand, conjugative plasmids carry all the machinery needed for self-transfer. This normally includes, aside the aforementioned relaxosome components, the type IV coupling protein (T4SS) and the components of the mating channel that assemble a T4SS [47]. Conjugative plasmids are relatively large, on the account of additional genes encoding the conjugation machinery (like the *mob* and *tra* genes - which encode the mobilization proteins and the capacity to carry out the transfer functions, respectively, which promote DNA transfer by interaction with the *oriT*). This can prove advantageous; as larger sizes can provide much needed plasmid stabilization [43]. Conjugative plasmids are usually low copy plasmids, while smaller plasmids maybe be found in excess of hundreds of copies [43].

The most significant feature of conjugation plasmids is their ability to inhabit the host cell separately from the chromosome, as well as replicating independently from the same. Secondly, conjugative plasmids are able to be transferred horizontally between cells by conjugation, in turn avoiding the risk of extinction within a given population. This property may, depending on the plasmid, give the hosts selective advantages over the rest of the population. Lastly, they are dispensable, in that they are not necessary for the cell to remain viable [43].

Structurally the plasmids are modular in nature, and often contain specific gene clusters organized as functional groups responsible for the propagation and maintenance of the plasmid. They consist of backbone and plasmid-selfish modules which may provide the host with beneficial traits [43]. Backbone modules are generally made up of compactly arranged genes [43] and are usually associated with the origin of replication and transfer (*oriT*) and centromere sites for plasmid segregation [43].

For maintenance the plasmid must also contain replication modules. These must ensure that the replication occurs in line with the host cell growth cycle without the plasmid copy number being a metabolic burden on the cell. On the other hand, the copy number should be high enough that the host does not become plasmid-free. This means that replication modules are subject to both intracellular and extracellular pressures [43]. Lastly, plasmid propagation modules are responsible for the mating pair formation and the relaxosome formation [43].

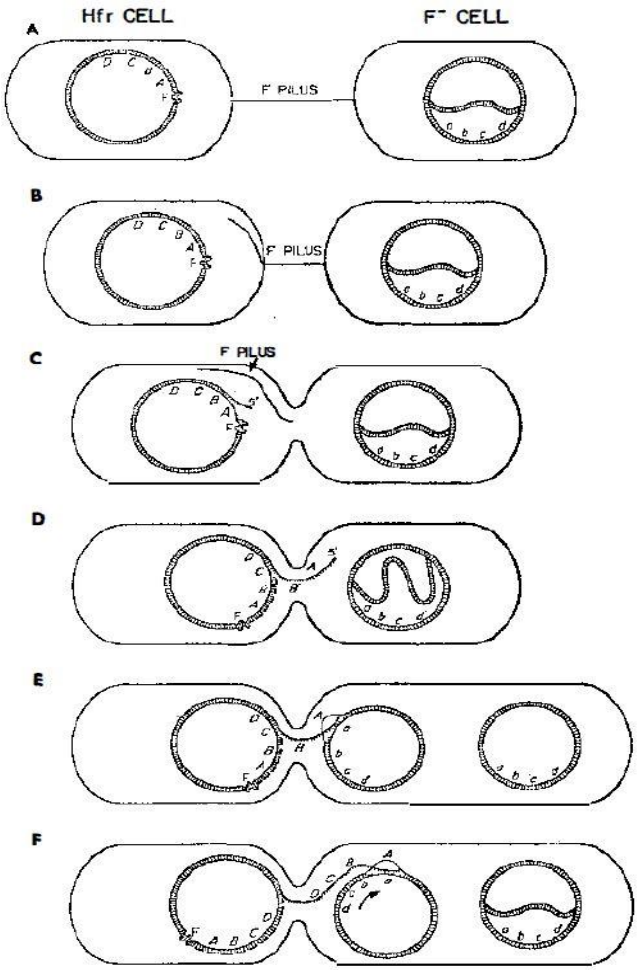


Figure 5: Proposed mechanism of chromosomal DNA transfer from an *E. coli* donor (Hfr) to a recipient cell (F^-) by conjugation [48]. **Legend: A-** Specific pair formation, **B-** Effective pair formation, **C-** Chromossome mobilization, **D, E-** Chromossome transfer, **F-** Recombinant formation.

The DNA transfer process can be broken down into three distinct biochemical subprocesses: DNA processing, DNA recruitment and, finally, DNA transfer [47]. Conjugation begins with the contact between donor and recipient cells by producing a mating bridge, otherwise known as a pilus (Figure 5A and 5B). At this stage a nick will be made at the site of the *oriT* of the DNA sequence to be mobilized, followed by the extension of the 3' end of *oriT*, thus leading to the displacement of one of the strands. Once this step is complete the strand is transferred to the recipient cell.

The formation of the mating complex begins with the synthesis of T4SS which generates the pilus that will extend from the donor to the recipient cell. The morphology of the pilus also plays a determining role in whether conjugation proceeds optimally in liquid media [43]. Once cell contact is established a signal in the donor triggers the mating bridge formation and the transfer of the vector. A nick within the plasmid *oriT* sequence followed by extension of 3’ end displaces the single strand that is then transferred into the recipient (Figure 5E).

For large conjugative plasmids the conjugative transfer genes are usually encoded within the plasmid itself, but this may also be achieved by a helper plasmid for smaller conjugative plasmids. The expression of conjugative pili can be induced by environmental factors like change in temperature but also by the donor coming into contact with plasmid-free cells, which in turn results in the spread of the conjugative plasmid through the recipient population [43].

Processing and transfer is the second step of the process. It involves the nicking of the plasmid at the origin of transfer and the formation of the relaxosome (Figure 5C). The relaxase is a key protein in conjugation, since it recognises the *oriT* and catalyses the initial and final stages in conjugation. In other words, it catalyses the initial cleavage of *oriT* while still in the donor, to ultimately produce the DNA strand that will be transferred, and the final ligation of the transported DNA in the recipient cell that reconstitutes the conjugated plasmid [43]. Relaxosome activity is aided by a coupling protein that helps docking with the T4SS after which it is transported to the recipient cell [43].

The entry of the plasmid stimulates the expression of backbone genes encoding several proteins that help establish the plasmid in the new host. If a plasmid is too slow to establish itself, it might lead to a forced integration of operative elements into the new chromosome, either by homologous recombination or transposition events [43]. Hosts transformed by conjugation are named transconjugants (Figure 5F).

Conjugation has yet to be shown to be an appropriate method for the genetic manipulation of green microalgae, however it has been successfully employed to genetically modify other eukaryotic organisms like yeast [49], mammalian [46] and diatoms [47]. Karas *et al.* (2015) [50] reported the transformation of the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* using bacterial conjugation. Given the phylogenetic proximity of diatoms to green microalgae it would seem feasible to use the same approach for these same organisms.

1.6. Metabolic Engineering of microalgae

As previously discussed, there has been a great deal of research conducted related to the understanding of how culture conditions can maximize growth rate and the lipid content present in microalgal cells. However, this has long been known not to be sufficient. While a nutrient starvation approach to lipid accumulation is more readily available, it negatively impacts growth rate of the cultures [51], ultimately decreasing the total biomass produced had the cultures been cultivated under more nutrient rich conditions, while simultaneously leading to a decrease in the amount of lipids extracted from the cultures. For products derived from low-value compounds extracted from microalgae to become affordable, sustainable and competitive, it is necessary to gain a deeper understanding of the lipid biosynthesis pathways so that they can be manipulated [52]. This will allow for the development of overproducing strains that could produce and store a greater amount of lipids within the cells without sacrificing growth yield.

This process is not without challenges however, as there is very little experimental data on the biosynthesis of fatty acids and other lipid compounds in microalgae. It is known that their production occurs in the chloroplast, and while several enzymes that regulate lipid synthesis have been identified, gene expression and the metabolic pathways they belong to are poorly understood. This lack of understanding is largely due to the small amount of information on microalgae genomics. As of 2016 only 30 microalgae genomes have been sequenced, and fewer still have been adequately annotated. Most of the information that is available, has as its source the model organism *C. reinhardtii* [53].

Although the use of these genomic approaches in microalgae is still very much in its early stages, metabolic engineering of several microalgae has led to successful alteration of their physiology towards overproduction of lipids. The first recorded success in this regard came in 1994, when Dunahay *et al.* [54] were able to overexpress acetyl-CoA carboxylase (the enzyme responsible for the initial step in lipid biosynthesis) in a strain of the diatom *Cyclotella cryptica*. The authors' findings however indicate that in this case at least this metabolic disruption, by itself, did not significantly alter the quantity of lipids produced, in spite of a 3-fold increase in enzymatic activity [54].

As an example of the downregulation of competing metabolic pathways, we can look to *C. reinhardtii* mutant strains lacking the *sta6-10* genes (involved in the synthesis of starch) [55]. It was demonstrated that remodelling the metabolic pathway of starch, by effectively blocking it, led to a significant increase in the production of TAG. This increase was further augmented once the starchless mutants were maintained in an N-deficient environment. Consequently, the already 10-fold increase was further boosted to a 30-fold production of lipid bodies [2]. Trentacoste *et al.* [56] proposed in their research that the disruption of lipid catabolism, rather than the modification of biosynthesis pathways, could allow for the accumulation of lipids without affecting growth. This hypothesis was tested on the diatom *Thalassiosira pseudonana,* using plasmids containing antisense RNA and RNAi methods to knockdown a gene encoding a lipase, known to degrade lipid droplets. The resulting mutant strains showed increased lipid accumulation while maintaining growth yield comparable to that of the wild type strain. In addition, accumulation occurred sooner in the cultivation period, and at an accelerated rate [56]. Since growth was not affected, the cultures contained a greater total amount of TAG during both exponential and stationary phase [56].

1.7. Objective and Approach

In this project we sought to use bacterial conjugation to, for the first time, demonstrate it as a viable method of microalgae transformation. The objectives of this study are twofold:

(i) The first and main aim is the genetic transformation of the green microalgae *Neochloris oleoabundans* UTEX 1185 and *Acutodesmus obliquus* SAG 276-6 through bacterial conjugation.

(ii) The secondary objective of this project is the optimization of the transformation procedure, through the assessment of multiple growth and selection parameters.

A conjugation protocol based on that of Karas *et al.* (2015) will be developed for the green microalgae *Neochloris oleoabundans* UTEX 1185 and *Acutodesmus obliquus* SAG 276-6, using the yeast-derived episomes pPtPuc3 and pTpPuc3 and altering relevant parameters as needed. These parameters include: (i) the amount of microalgae and *E. coli* cells in the conjugation reaction, (ii) conjugation time, (iii) antibiotic sensitivity to nourseothricin and zeocin and (iv) light exposure under different day/night cycles.

2. Materials & Methods

2.1. Organisms and growth conditions

Stock cultures of *Neochloris oleoabundans* UTEX 1185 and *Acutodesmus obliquus* SAG 276-6 were maintained on fresh water (FW) solid media (appendix table 7), while the stock cultures of the diatom *Phaedactylum tricornutum* SAG 1090-1b were maintained on solid L1 media (appendix 2). All three organisms were maintained under a 16:8 light/dark cycle (L/D cycle) at a light intensity of 50-60 µmol m^{-2} s^{-1} and 25°C. Liquid cultures of both green microalgae and diatoms were grown and maintained under a 16:8 L/D cycle at a light intensity of 50-60 µmol m^{-2} s^{-1}, 25°C and 100 rpm.

Stock cultures of *Escherichia coli* DH10B containing the cargo plasmids pPtPuc3 (appendix figure 1) and pTpPuc3 (appendix figure 2), were grown overnight in LB media complemented with kanamycin (50 and 100 µg/ml) at 37°C, after which the cultures were maintained at 5°C. Both *E. coli* strains also contained the conjugative plasmid pTA-Mob, necessary for the cargo plasmids to be transferred.

Table 2: List of microalgae and bacterial species used along with their notable characteristics.

Species	Characteristics	Source/Reference
Neochloris oleoabundans **UTEX 1185**	-	UTEX Culture Collection in Texas [57]
Acutodesmus obliquus **SAG 276-6**	-	SAG Culture Collection in Göttingen [58]
Phaelodactylum tricornutum **SAG 1090-1b**	-	SAG Culture Collection in Göttingen [59]
Escherichia Coli **DH10B**	(1) Houses two plasmids: Cargo pPtPuc3 (Kan^R, Zeo^R), Conjugative pTA-Mob (Gm^R) (2) Houses two plasmids: cargo pTpPuc3 (Kan^R, Nsr^R), conjugative pTA-Mob (Gm^R)	(Strains without conjugative plasmid) [50]

2.2. Growth monitoring

Growth of green algae and diatoms in liquid cultures was monitored by measuring optical density at 750 nm (OD_{750}) using a spectrophotometer (Hach Langer DR-600) and cell concentration (X) using a Coulter Counter (Beckman Coulter Multisizer™ 3). The optical density of *N. oleoabundans* and *A. obliquus* samples was measured using FW medium as blank solution, while the optical density of the diatom *P. tricornutum* was obtained using L1 medium as blank solution.

For monitoring of cell concentration, *N. oleoabundans* and *A. obliquus* samples were prepared using an adequate amount of Isoton buffer (Coulter® Isoton® II diluent), while *P. tricornutum* samples were diluted in 1 M CoulterCounter marine buffer solution (appendix), for a total volume of 20 ml.

2.3. DNA transfer to *P. tricornutum* through bacterial-mediated conjugation

The DNA transfer procedure carried out in this project was based on that devised by Karas *et al*. (2015). From an exponential phase liquid grown *P. tricornutum* culture 250 µL adjusted to 1.0 x 10^8 cells/ml was plated on ½ strength L1, 1% agar plates and grown for 4 days at 18°C under constant light exposure (50 µmol m^{-2} s^{-1}). After this period, 500 µl of L1 medium was added to the plates and the cells scraped off, collected and counted, after which cell concentration was adjusted to 5.0 x 10^8 cells/ml.

Both *E. coli* strains were grown overnight in 50 ml LB cultures (37°C, 150 rpm) and their optical density at 600 nm (OD_{600}) adjusted to 0.8 – 1.0. These cultures were then spun down in 50 ml sterile tubes (10 minutes at 3000 x *g*) and the resulting pellets were resuspended in 500 µl of SOC medium.

For DNA transfer, 200 µl of both *P. tricornutum* and *E. coli* culture (pPtPuc3 to obtain zeocin resistant transconjugants, and pTpPuc3 used for negative controls) were added to 1.5 ml Eppendorf tubes and mixed by pipetting up and down. The mixtures were subsequently plated on ½ strength L1, 5% LB, 1% agar plates and incubated in the dark at 30°C for 90 minutes, then moved to 18°C under constant light exposure (50 µmol m^{-2} s^{-1}). After 2 days, the cells were scraped off after adding 1 ml of L1 medium to the plates, and 200 µl of this was plated on ½ L1, 1% agar supplemented with different concentrations of zeocin: 15, 20, 25 and 30 µg/ml. Plates were then incubated for 16 days, under the same conditions.

To confirm that the colonies formed after selection were positive transformants, several colonies were replicated on plates containing the same concentration of zeocin. These cultures were maintained at 18°C and 50-60 µmol m^{-2} s^{-1}.

2.4. Development and optimization of a bacterial-mediated conjugation protocol for *A. obliquus* and *N. oleoabundans*

The procedure to transform the green microalgae *A. obliquus* and *N. oleoabundans* through bacterial-mediated conjugation followed the same work-flow used to produce *P. tricornutum* transconjugants, with the necessary modifications to the protocol in order to favour the growth of the green microalgae.

From an exponentially grown microalgae culture 250 µl was plated on 1.6% agar FW plates and incubated at 25°C, under a 16:8 L/D cycle (50-60 µmol m^{-2} s^{-1}), for 6 to 10 days (table 2). After the incubation stage, the cells were scraped from the plates with 500 µl of FW media, collected, counted and the concentration of the cell suspension adjusted (table 2).

Both *E. coli* strains were prepared in the same manner as in the *P. tricornutum* transformation, as was the mixing of microalgae with *E. coli* cells. The mixtures were plated on 1.6% agar FW plates with 5% LB and incubated in the dark, at 30^{o}C, for three different conjugation times: 60, 90 and 120 minutes. This was followed by recovery incubation at 25^{o}C, under 16:8 L/D cycle (50-60 µmol m^{-2} s^{-1}) for 2 days. Following the recovery period, 1 ml of FW medium was added to the plates for the cells to be scraped, and 200 µl of the retrieved cells were plated on FW selection plates supplemented with different concentrations of zeocin or nourseothricin; and 1 µg/ml of gentamycin, for the transconjugant selection. These cultures were maintained at 25^{o}C and 20 µmol m^{-2} s^{-1} until isolated colonies were observed. The parameters tested in this procedure were (i) cell concentration, (ii) conjugation time, (iii) antibiotic concentration and (iv) light condition. Table 3 compiles the different combinations of parameters tested.

Once isolated colonies of sufficient size were observed, several were picked (from both transconjugant plates and their respective controls) and subsequently replated in solid FW medium supplemented with the same concentration of zeocin. These cultures were then maintained at 25°C and 20-30 µmol m^{-2} s^{-1}, until enough biomass was obtained for DNA extraction.

The calculation of the transformation efficiency (T.E.) was performed using the equation below:

$$T.E. = \frac{Number\ of\ non{-}confirmed\ transformant\ colonies}{Number\ of\ possible\ recipient\ green\ microalgae\ cells} \quad \text{(equation 1)}$$

Where the number of possible transformant colonies is the average of colonies found in the selection plates, while the number of possible recipient cells is the number of green microalgae cells mixed with the *E. coli* cells.

Table 3: Parameters tested for transformation of *A. obliquus* and *N. oleoabundans* through *E. coli* - mediated conjugation

Organism	Initial cell concentration (cells/ml)	Cell concentration mixed with *E. coli* (cells/ml)	Conjugation time (min)	Antibiotic	Antibiotic Concentration (µg/ml)	L/D cycle during selection
A. obliquus	5.5×10^7	2.0×10^8	60	Zeocin	35	16:8
			90		40	
			120		50	
					60	
			60	Nourseothricin	40	
			90			
			120			
N. oleoabundans	1.0×10^8	5.0×10^8	90	Zeocin	35	16:8
			120		40	
			60	Nourseothricin	20	
			90			24:0
			120			

2.5. Total DNA and plasmid extraction

Total DNA extraction of selected colonies was conducted to confirm the presence of the transferred plasmid molecule. The biomass from the selected colonies was dissolved in 300 µl of SDS in bead beater tubes. Immediately after, 150 µl of vortexed Chloroform: Phenol: Isoamyl alcohol (25:24:1) solution was added to the tubes. The collected cells were then lysed using a bead beater (Bertin, Precellys 24) at 4000 rpm and 2x 60s periods (with 20 seconds between them). After lysis, the samples were spun down for 5 min at 13400 rpm.

Following cell lysis, 200 µl from the top phase of the samples was recovered (without touching the beads), and added to a 1.5 ml Eppendorf, as well as 20 µl of sodium acetate (1/10th of total volume of the collected sample). This was followed by the addition of 400 µl of cold ethanol (100%) and placing the tubes in the cold (-23^{o}C) for 1 hour. Subsequently the samples were centrifuged for 15 min at 14000 rpm and 4^{o}C. The supernatant was carefully removed without disturbing the pellet and 250 µl of cold ethanol (70%) was added. The samples were spun down one final time for 5 min, 14000 rpm and 4^{o}C. The entirety of the supernatant was discarded and the remaining ethanol was removed by placing the open tubes at 37^{o}C.

The extracted DNA was resuspended by adding 15 µl of water (RNase and DNase free) and pipetting up and down several times and a quick spin down to precipitate all of the extracted DNA to the bottom of the tubes was performed. The DNA was quantified using a Nanodrop reader.

Plasmid DNA from *E. coli* cultures was extracted using a standard plasmid extraction kit protocol (complete protocol can be found in the appendix). The biomass from which the DNA as extracted was collected from 50 ml LB cultures of three strains of *E. coli* carrying three different plasmids (pPtPuc3, pTpPuc3 and pHR16) grown overnight at 37°C.

2.6. Colony PCR confirmation of transformants

Polymerase Chain Reaction (PCR) was used to confirm the transformants resulting from conjugation from *E. coli* to the green algae. The confirmation was based on the amplification of a fragment of the zeocin resistance marker (370 bp) using primers Forward-Zeo (5'- ATG GCC AAG TTG ACC AGT GC -3') and Reverse-Zeo (5'- TCA GTC CTG CTC CTC GGC -3'). In addition to the samples, a blank sample (containing all of the PCR reagents with the exception of the DNA template), a negative control (C-, DNA extracted from colonies replicated from control plates or wild-type strain) and a positive control (C+, pPtPuc3 plasmid) were also tested for the presence of the *shble* gene (responsible for providing zeocin resistance to transformants).

The PCR reactions were run with an initial denaturation for 5 minutes at 95^{o}C and 40 cycles of 30 seconds at 95^{o}C, an annealing step for 30 seconds at 59^{o}C for the *shble* gene sequence and extension for 80 seconds at 72^{o}C. A final extension step, took place for 5 minutes at 72^{o}C. The PCR products were then indefinitely kept at 4^{o}C. The PCR products were separated by agarose electrophoresis gel at 80 V, using GelRed in order to stain the amplified DNA.

2.7. Antibiotic sensitivity assays

In order to further evaluate the sensitivity of the strains used in this work to selective antibiotics, each organism was plated on solid media supplemented with increasing concentrations of either zeocin or nourseothricin: 0, 5, 10, 15, 20, 30, 40 and 50 µg/ml.

N. oleoabundans and *A. obliquus* were first plated on FW solid media at a concentration of 1.0 x 10^8 and 5.5 x 10^7 cells/ml, respectively, and incubated for 6 days at 25^{o}C under the light intensity of 50-60 µmol m^{-2} s^{-1}. After this period, the cells were scraped, after adding 500 µl of FW liquid medium to the plates, counted and the cell concentration adjusted to 5.0 x 10^8 and 2.0 x 10^8, respectively. 100 µl of the adjusted cell suspensions were then plated on FW supplemented with the aforementioned concentrations of nourseothricin.

Likewise, *P. tricornutum* was plated on ½ L1 medium at a concentration of 1.0 x 10^8 cells/ml and incubated for 4 days at 18^{o}C and 50-60 µg/ml (constant illumination). Afterwards 500 µl of L1 medium was added to the plates and the cells scraped. The cell suspensions were adjusted to 2.0 x 10^8 and plated on selection plates with the desired antibiotic, under the same concentrations as those used for the sensitivity assays of green microalgae.

2.8. Transformation of *A. obliquus* and *N. oleoabundans* via electroporation

The electroporation protocol as developed by Camilo Muñoz (Muñoz, 2016) was performed on *A. obliquus* and *N. oleoabundans*.

Cells were harvested from 100 ml liquid cultures and by centrifugation at 2500 g for 10 minutes at 4°C. After discarding the supernatant, the pellet was washed twice by resuspending the cells in equal volume of cold washing/suspension buffer (0.1 M sorbitol, 0.1 M mannitol, 0.01 M HEPES, 0.005 M KCl, 0.005 M $CaCl_2$) and then once more spun down at 2500 rpm for 10 minutes at 4°C. Following this step, the cells were resuspended in the same solution, adjusting the cell concentration to 1.0 x 10^8 cells/ml. The suspension was then transferred to 15 ml falcon tubes after which 10 µg/ml of linearized DNA, 1% DMSO and 25 µg/ml of salmon sperm DNA carrier (previously boiled at 95°C for 5 minutes) were added. The mixtures were then incubated for 10 minutes on ice. After the incubation period, the mixture was transferred to 2 mm electroporation cuvettes and subjected to 1300 V (*N. oleoabundans*) or 1600 V (*A. obliquus*) for 3 ms. After being exposed to the electric pulse, the cuvettes were placed on ice in the dark for 10 minutes, after which the electroporated cells were transferred to 10 ml FW medium in 15 ml tubes and incubated in the dark for 24h at 25°C.

The resulting algae cultures were centrifuged at room temperature at 2500 g for 10 minutes. The supernatant was discarded and the pellet resuspended in 200 µl of FW medium. The cell suspensions were finally plated in FW solid medium supplemented with the corresponding antibiotic (zeocin: 50 µg/ml; nourseothricin: 50 µg/ml).

3. Results

3.1. Transformation of *P. tricornutum* through bacterial-mediated conjugation

In an effort to increase confidence in the designed approach to the transformation of the green microalgae *A. obliquus* and *N. oleoabundans*, the protocol devised by Karas *et al.* (2015) was followed to firstly transform *P. tricornutum* using *E. coli*.

As it can be seen from figure 6 the number of isolated colonies decreases as the concentration of zeocin in the selection medium increases from 15 to 25 µg/ml. It is also possible to see that the number of isolated colonies present in the controls is higher than those where the transfer of plasmid conferring resistance to zeocin would have taken place. In the wake of these results, two more sets of cultures were prepared, following the same protocol and using the same conditions, where the transconjugant selection took place in ½ L1 plates supplemented with 20, 25 and 30 µg/ml of zeocin. No *P. tricornutum* colonies were observed on any plate in either attempt, regardless of zeocin concentration. In their place however, several bacterial colonies could be found (not shown).

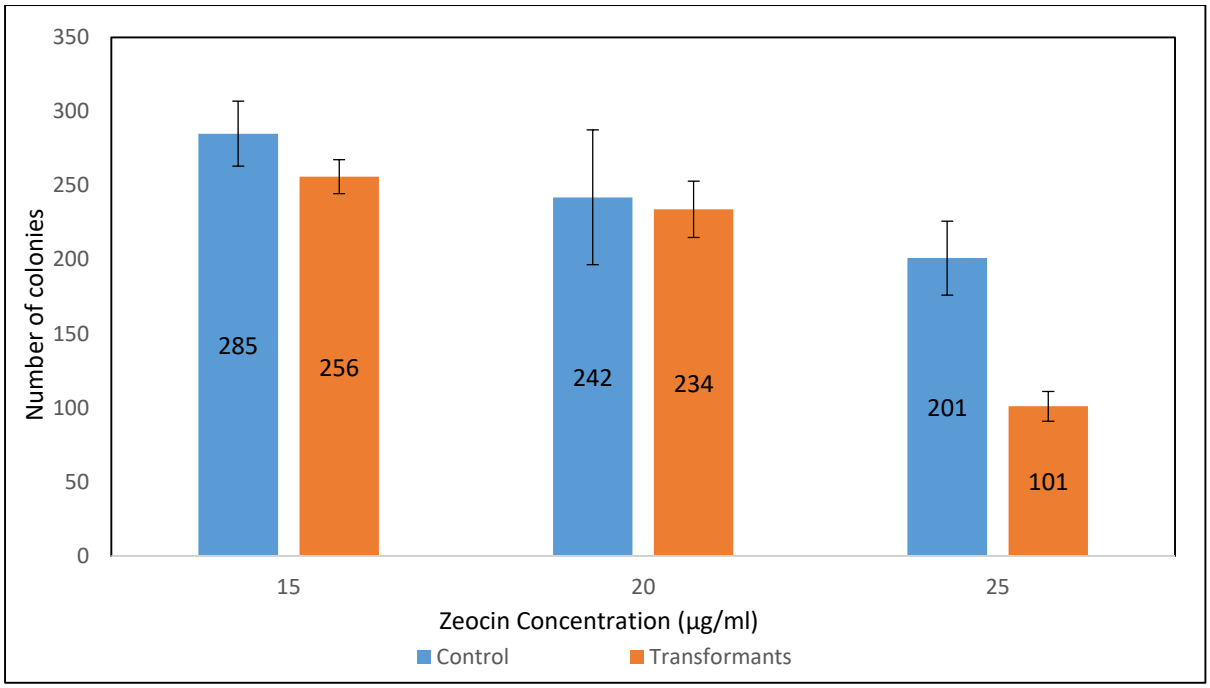


Figure 6: Number of *P. tricornutum* colonies counted in the selection plates supplemented with different concentrations of zeocin.

3.2. Antibiotic sensitivity assays

In concurrence with the first transformation experiment, the sensitivity of *P. tricornutum* to several concentrations of zeocin and nourseothricin was gauged in order to collect further information on the transformant selection potential of each antibiotic and the chosen concentrations.

When growing *P. tricornutum* in media supplemented in zeocin, the diatom showed the highest amount of growth when exposed to concentrations of 30 µg/ml and higher of the antibiotic. In contrast, cultures maintained in media containing 20 µg/ml of less displayed much lower colony growth. Surprisingly, the *P. tricornutum* cultures grown in the complete absence of zeocin did not show the highest amount of growth (table 4).

Table 4: Observed growth of P. tricornutum on ½ L1 solid medium supplemented with increasing concentrations of zeocin and nourseothricin. Legend: (+++) high growth, (++) slightly inhibited growth, (+) highly inhibited growth, (-) complete inhibition.

		Antibiotic Concentration (µg/ml)							
Antibiotic	**Replicates**	0	5	10	15	20	30	40	50
Zeocin	1	++	++	+	+	+	+++	+++	+++
	2	+	+	+	+	+	+++	+++	-
Nourseothricin	1	+	++	+	-	-	-	-	-
	2	+	+	+	-	-	-	-	-

Regarding the sensitivity to nourseothricin, from table 3 we can see that 10 µg/ml appears to be the maximum antibiotic tolerance for *P. tricornutum*. Cultures grown at higher concentrations showed complete growth inhibition. In spite of this, growth on non-supplemented plates showed lower growth than anticipated, as well as lower than one the replicates grown on medium containing 5 µg/ml of nourseothricin.

In order to optimize the transformation procedure for conjugation derived genetic transformation of *N. oleoabundans* and *A. obliquus*, the sensitivity to the antibiotic nourseothricin was tested. From figure 7 and 8 below, it is possible to notice that the increase in antibiotic concentration resulted in a consistent decrease in algal growth. Figure 7 and table 4 show that, on average, there is a consistent decrease in *N. oleoabundans* isolated colonies as the concentration of nourseothricin increases. Additionally, once the antibiotic

concentration reaches 20 µg/ml, complete growth inhibition for this microalgae occurs, with all subsequent increases in concentration showing the same behaviour.

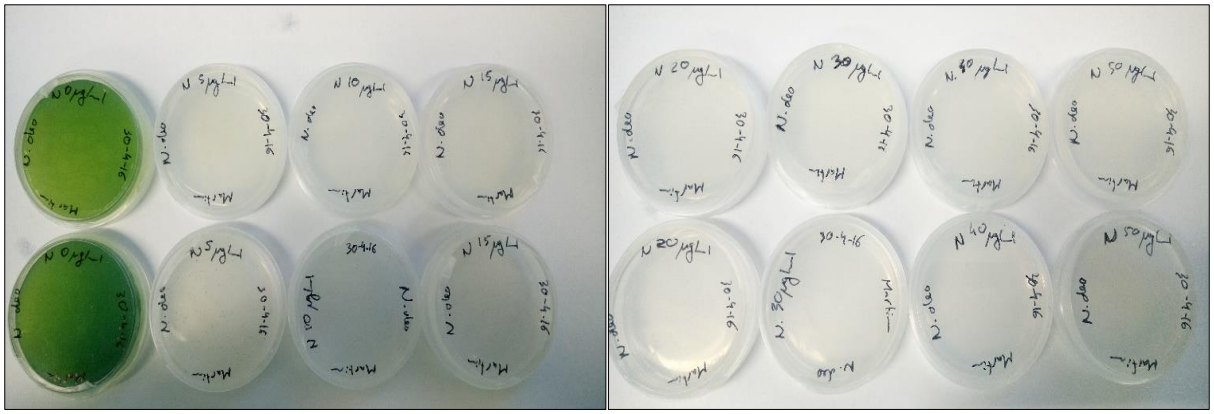


Figure 7: *N. oleoabundans* cultures under increasing concentrations of nourseothricin, after 13 days of growth. Nourseothricin concentrations (in duplicate) from left to right: 0, 5, 10, 15, 20, 30, 40, 50 µg/ml.

Likewise, without nourseothricin present in the media, *A. obliquus* displays confluent growth (figure 8). Once nourseothricin is introduced into the growth media there is a visible inhibition of algal growth, with concentration greater than 5 µg/ml of nourseothricin causing a significant decrease in the *A. obliquus* population. In spite of this, none of the nourseothricin concentrations used led to the complete inhibition of *A. obliquus.*

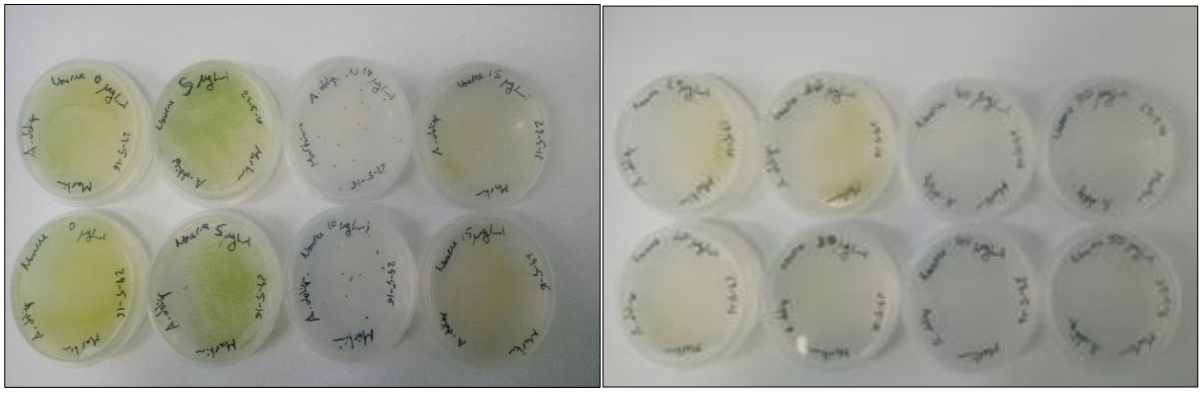


Figure 8: *A. obliquus* cultures under increasing concentrations of nourseothricin, after 15 days of growth. Nourseothricin concentrations (in duplicate) from left to right: 0, 5, 10, 15, 20, 30, 40, 50 µg/ml.

Table 5 summarizes the observed growth behaviour of *N. oleoabundans* and *A. obliquus* in FW medium complemented with increasing concentration of nourseothricin. As the concentration of the antibiotic media increased, it leads to a consistent decrease in *N. oleoabundans* colony growth. The information gathered from experiment allowed for the decision to further attempt testing of conjugation on selective media containing nourseothricin for both microalgae, with 20 µg/ml and 40 µg/ml for *N. oleoabundans* and *A. obliquus*, respectively.

Table 5: Observed growth of *N. oleoabundans* and *A. obliquus* on plates of increasing nourseothricin concentrations. Legend: (+++) high growth, (++) slightly inhibited growth, (+) highly inhibited growth, (-) complete inhibition. Number of isolated colonies observed in each plate is indicated between brackets.

		Nourseothricin Concentration (µg/ml)							
Organism	**Replicates**	0	5	10	15	20	30	40	50
A. obliquus	1	+++	++	+ (21)	+	+	+ (42)	+ (23)	+ (27)
	2	+++	++	+ (23)	+	+	+ (46)	+ (32)	+ (25)
N. oleoabundans	1	+++	+ (125)	+ (41)	-	-	-	-	-
	2	+++	-	+ (45)	+ (9)	-	-	-	-

3.3. Transformation of green microalgae by bacterial conjugation

The transformation of the green microalgae *N. oleoabundans* and *A. obliquus* was attempted using *E. coli* as a means to transfer the plasmids of interest into the algal cells. This was carried out using the protocol described in the Materials and Methods, based on that of Karas *et al*. Simultaneously, several conditions were tested in an attempt to optimize several parameters of the aforementioned protocol, with antibiotic selection, conjugation time and L/D cycle receiving the most attention. The results for the transformation assays of both green microalgae are described in the following sections.

3.3.1. *N. oleoabundans* transformation through bacterial-mediated conjugation

The first transformation trial of N. oleoabundans through bacterial conjugation with the pPtPuc3 plasmid, was performed using zeocin concentrations of 35 and 40 µg/ml for the selection of transconjugants. Very few colonies were obtained in this trial, with only as many as 3 colonies per culture for both conditions and their respective controls, and with substantial inconsistency between replicates.

The few colonies obtained from these selection conditions, were replicated onto FW media with the same zeocin concentration, so that enough biomass could be collected for DNA extraction and confirmation of conjugation. Part of the biomass was also replicated onto LB plates so that DNA of the bacteria potentially present in the media could also be extracted.

The second transformation attempt of *N. oleoabundans* was conducted using nourseothricin as selective antibiotic, for different times of conjugation and L/D cycles (figure 9). From the information displayed in figure 9, it is possible to see that in both cases the longer conjugation period (120 minutes) produced the highest average number of colonies. An exception to this trend is the 90-minute conjugation assay in figure 9b, where the number is lower than that of 60-minute incubation. While in figure 9a there is a steady increase in the average number of colonies as the conjugation period increases, the same is not observed when the plates were exposed to constant illumination (figure 9b).

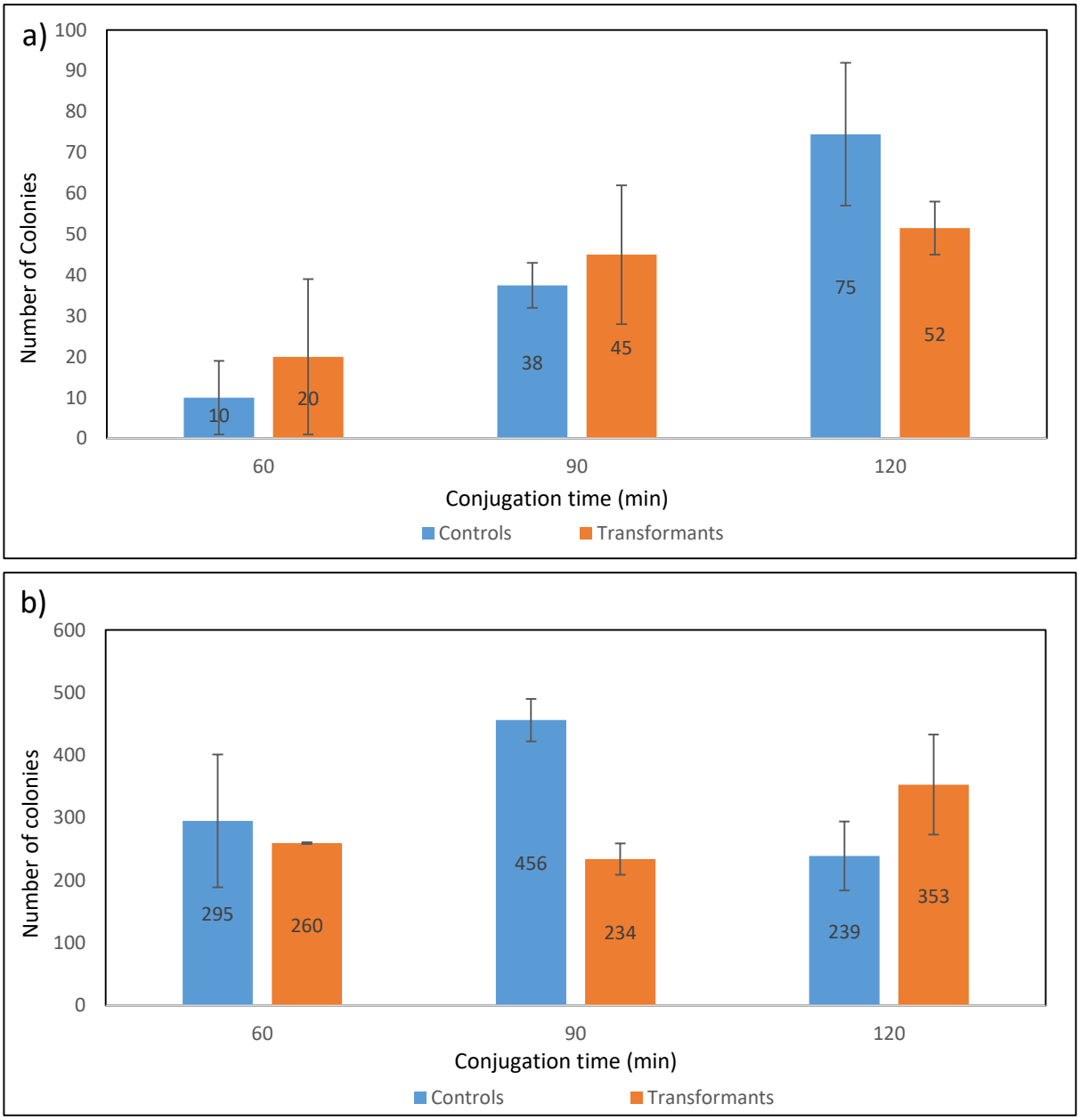


Figure 9a: Number of *N. oleoabundans* colonies, after 14 days of growth, in the selection plates supplemented with 20 µg/ml of nouseothricin, different conjugation periods (60, 90 and 120 minutes) and under a 16:8 L/D cycle. **Figure 9b:** Number of *N. oleoabundans* colonies in the selection plates supplemented with 20 µg/ml of nouseothricin, and different conjugation periods under continuous light. Error bars denote the standard deviation of the mean from two biological replicates per condition.

In these trials, the number of colonies present in the control plates was expected to be lower than their corresponding transformant plates. However, that was not the case in the control plates of 120-minute conjugation assays (figure 9a) as well those assigned 60 and 90 minutes of conjugation in the third trial (figure 9b).

While the number of potentially transformant colonies to choose from, containing the pPtPuc3 plasmid, was extremely limited the few colonies that could be found were replated onto FW media with the same zeocin concentration, so that enough biomass could be collected for DNA extraction for confirmation that conjugation took place, and that false positives could be eliminated. Once colonies had grown sufficiently their DNA was extracted and used as templates for amplification of the *shble* gene (responsible for the zeocin resistance), only present in the pPtPuc3 plasmid. The same was carried out for the extracted bacterial DNA.

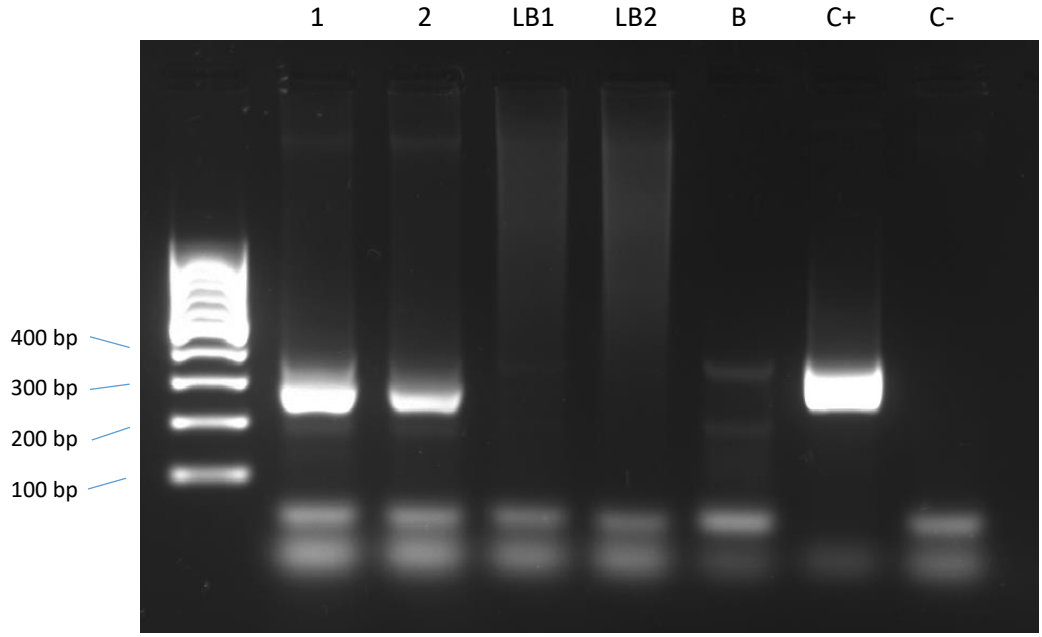
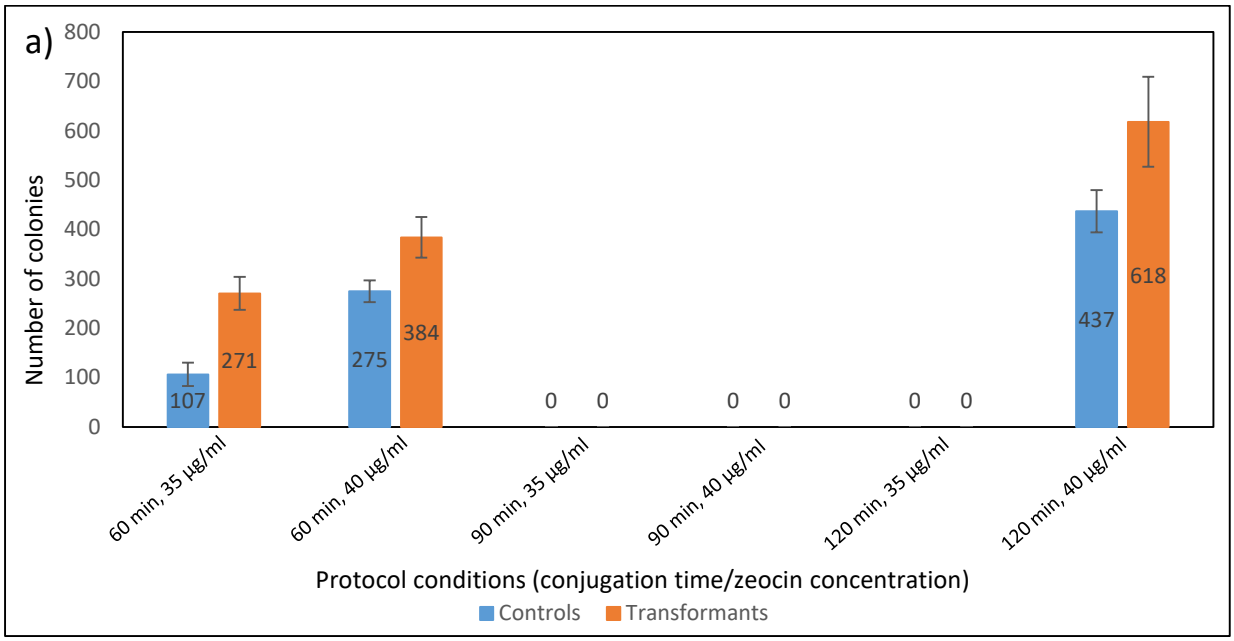


Figure 10: Amplification of the zeocin resistance gene (*shble* gene) present in the pPtPuc3 plasmid, after DNA extraction from *N. oleoabundans* conjugation colonies. Legend: (1,2) Microalgae DNA samples, (LB1, LB2) Bacterial DNA samples, (B) blank, (C+) positive control, (C-) negative control (wild-type strain).

Figure 10 shows both the results for the amplification of *the shble* sequence from both algal colonies present in FW media (lanes 1 and 2) and the corresponding bacterial colonies found in LB plates (lanes LB1 and LB2). Both DNA samples from *N. oleoabundans* colonies showed a strong band of comparable size to that of the positive control (pPtPuc3 plasmid) and thus showed positive amplification of the *shble* gene, while neither of the corresponding samples from the LB plates any such bands. Two weak bands were found in the blank sample; however, neither was of the same size as that of the positive control or any of the other amplified DNA sequences. All the bands seen in figure 10 show lengths smaller (close to 250 bp) than the size for the amplified gene, which was expected to be 370 bp long. Additionally two weak fragments of approximately 300 and 200 bp can be seen in the blank sample, neither of which matches the amplified sequence in the positive control in size.

3.3.2. *A. obliquus* transformation through bacterial-mediated conjugation

The first genetic transformation trials of *A. obliquus* through conjugation, was tested using the antibiotic zeocin for the selection of transformants and three different times of conjugation (60, 90, 120 minutes). The results for these first steps can be seen in figure 11 below.



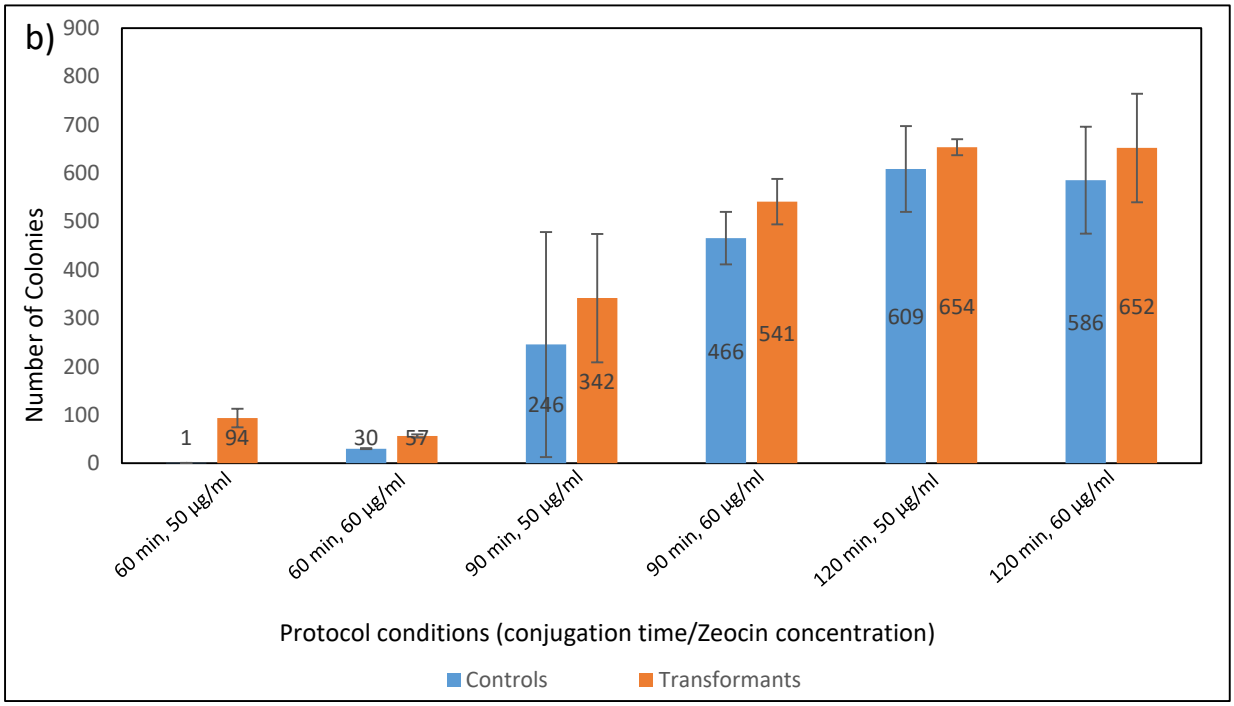


Figure 11a: First trial of *A. obliquus* transformation through conjugation. Number of *A. obliquus* colonies in the selection plates supplemented with different concentrations of zeocin, and different conjugation periods. **Figure 11b:** Second trial of *A. obliquus* transformation through conjugation. Number of *A. obliquus* colonies in the selection plates supplemented with different concentrations of zeocin (50, 60 µg/ml), and different conjugations periods. Error bars denote the standard deviation of the mean from two biological replicates per condition

In figure 11a we can see that as the time of conjugation increases, so does the number of isolated colonies observed in growth medium, with 120 minutes leading to the highest number of colonies, while allowing conjugation to occur for only 60 minutes produces a much lower number. Due to the high amount of residual growth it was not possible to properly count the number of colonies. This is seen for both transformation assays and the control plates. As a result, all trials assigned to 90 minutes of conjugation and one assigned to 120 minutes of conjugation, no colonies could be identified.

Following the first transformation trial for *A. obliquus*, a second trial was undergone where the zeocin concentration was increased to 50 and 60 µg/ml. The results are compiled in figure 11b. Like in figure 11a, when conjugation period is prolonged from 60 to 120 minutes up to 6-fold increase in colony forming cells can be seen. From figure 11, it is possible to see that in all cases where isolated colonies were possible to be counted, that the number of colonies present in the transconjugant plates is greater than those for the controls.

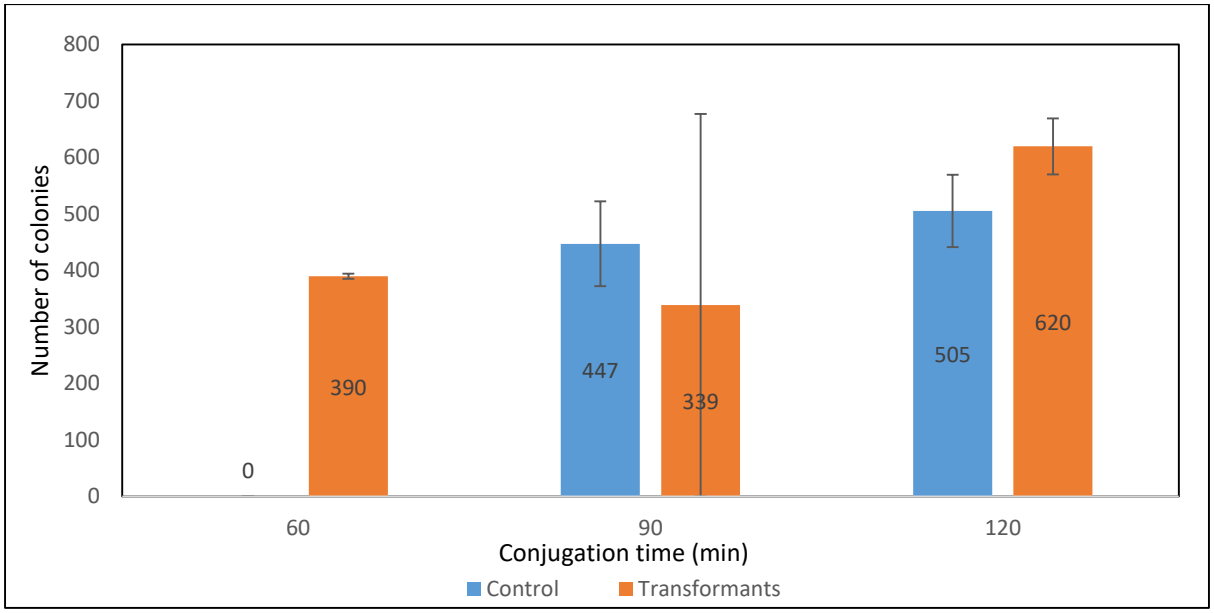


Figure 12: Number of *A. obliquus* colonies counted in selection plates supplemented with 40 µg/ml of nourseothricin, and different conjugation periods (60, 90 and 120 min) under a 16:8 L/D cycle. Error bars denote the standard deviation of the mean from two biological replicates per condition.

Figure 12 above shows the results from the selection of possible transformation using media supplemented with 40 µg/ml of nourseothricin. Much like in the previous trials, the average total colony number for 120 minutes of conjugation showed the highest colony count. As a result of the complete growth inhibition in one of the 90-minute conjugation replicates, it wasn't possible to determine if the average number of colonies for this condition would be in relation to the other to conjugation periods. Unlike what had previously been seen, 60 minutes of conjugation showed a remarkably high number of isolated colonies when transformed with pTpPuc3, without the presence of colonies in its respective control, being the sole case in which there was no colonies found for the controls.

In order to confirm that conjugation had occurred PCR amplification for the *shble* gene was performed on both algal and bacterial DNA. The first PCR was meant to amplify the *shble* gene from microalgal colony DNA samples (present in the pPtPuc3 plasmid); and a second PCR where the *shble* gene would also be amplified from bacterial DNA samples collected from LB plates. Amplified DNA sequences of around 370 bp would serve as preliminary confirmation of successful transformation.

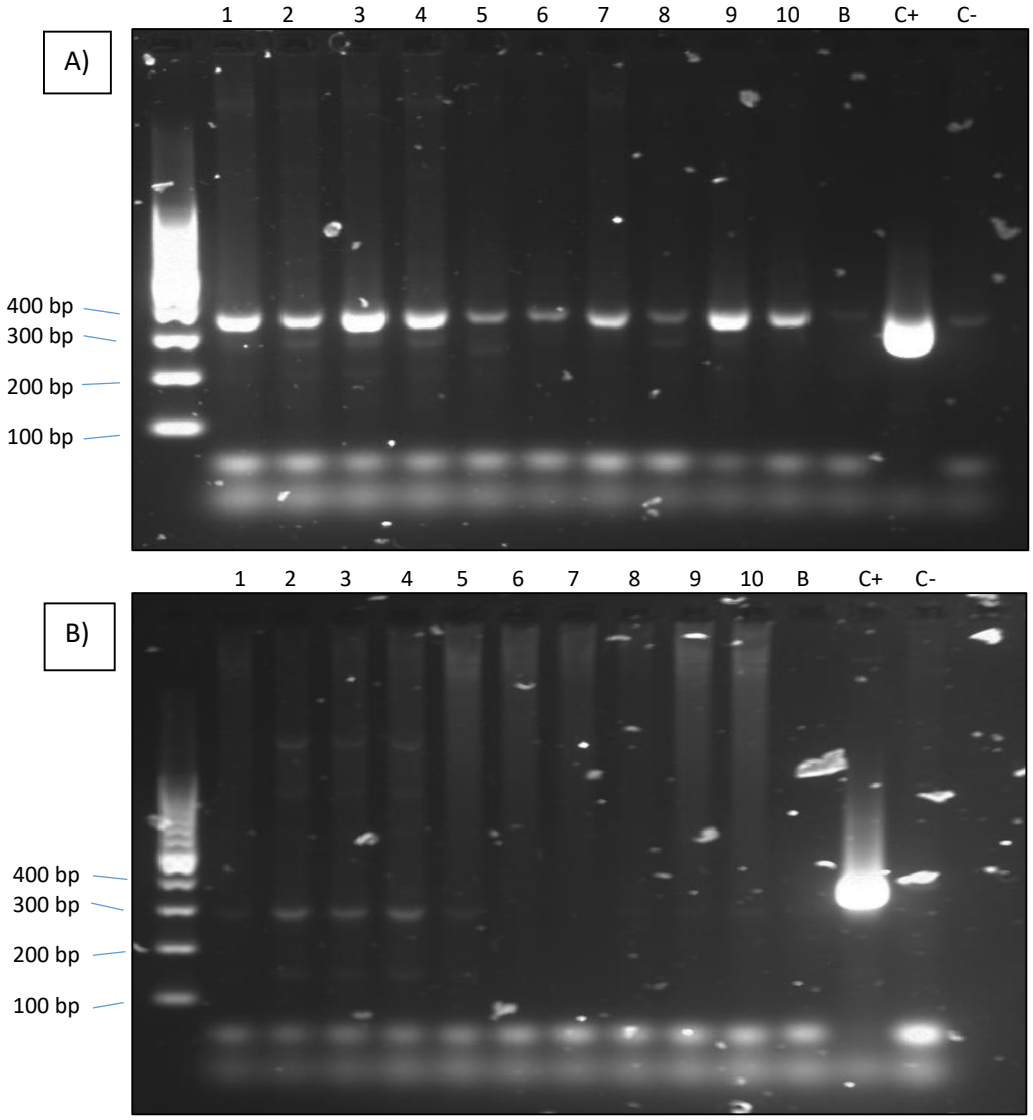


Figure 13a: Amplification of the *shble* gene from DNA extracted from 10 random *A. obliquus* colonies. **Figure 13b:** Amplification of the *shble* gene from DNA samples extracted from bacterial colonies found in the corresponding LB plates. **Legend:** (1-10) DNA samples, (B) blank, (C+) positive control, (C-) negative control (wild-type strain).

After the extraction of DNA from the chosen algal and bacterial colonies, the DNA samples were adjusted to the same concentrations in order to obtain comparable results. From figure 13a we can see that the DNA samples from all ten microalgae samples showed the presence of an amplified DNA sequences with comparable size to that of the positive control, of about 370 bp. Figure 13b shows the separation of the

fragments amplified from bacterial DNA samples. This was run concurrently with the gel from figure 13a. In this case no fragments of the same size to that of the positive control were produced, though several slightly shorter bands, close to 300 bp, can be seen in three of the bacterial DNA samples. Unexpectedly, similar sized (but much weaker) bands can be seen for the blank and the negative control in figure 13a, which are absent in figure 13b.

3.4. Transformation of *A. obliquus* and *N. oleoabundans* through electroporation

A. obliquus and *N. oleoabundans* were also subjected to genetic transformation using electroporation with the conjugation plasmids as to compare efficiency of transformation with these plasmids using either electroporation or conjugation. This could indicate if conjugation plasmids could be introduced in another or more effective way as well. For *A. obliquus*, transformant selection was conducted on two different media: FW supplemented with 50 µg/ml of zeocin; and FW supplemented with 40 µg/ml of nourseothricin. These selection conditions had been previously used for the selection of transformants obtained by bacterial conjugation.

The results regarding the transformation of *A. obliquus* by electroporation are shown in figures 14a and 14b below. Selection of *A. obliquus* transformants in media supplemented with zeocin produced a greater number of possible transformant colonies (217±40) in comparison with nourseothricin (8±0.5). In both cases the number of colonies on the controls outnumbered the possible transformants.

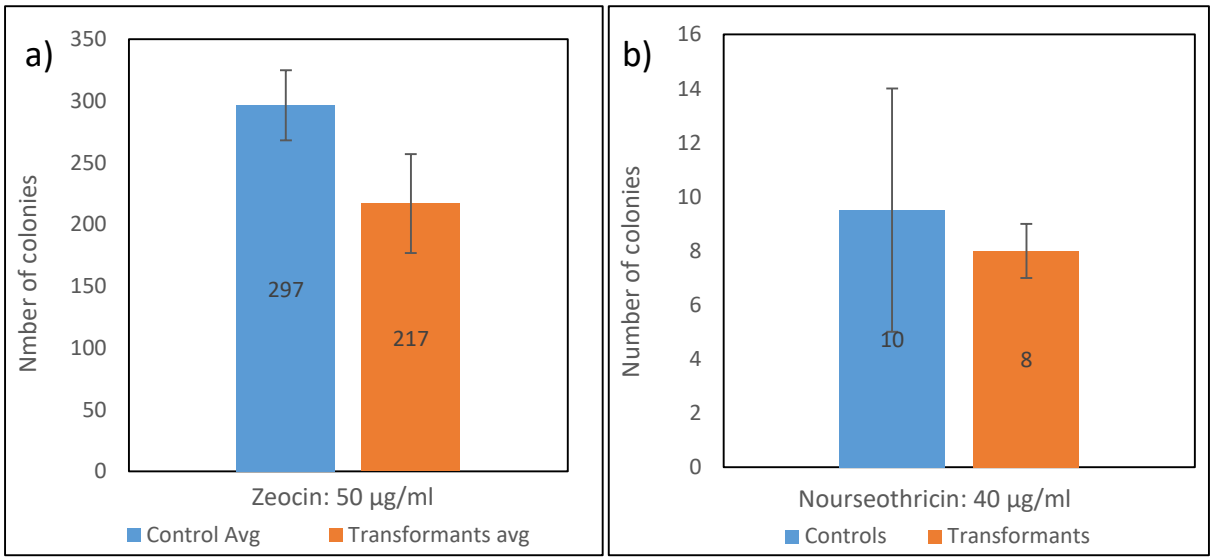


Figure 14a: Number of *A. obliquus* colonies obtained by electroporation and in selection medium supplemented with 50 µg/ml of zeocin. **Figure 14b:** Number of *A. obliquus* colonies obtained by electroporation and in selection medium supplemented with 40 µg/ml of nourseothricin. Error bars denote the standard deviation of the mean from two biological replicates per condition.

The selection of *N. oleoabundans* transformants was done on FW medium supplemented with 20 µg/ml of nourseothricin (figure 15). Much like in the case of *A. obliquus*, the number of colonies in the controls outnumbered on average the colonies found in the transformation trials, with one colony found in each of the replicates.

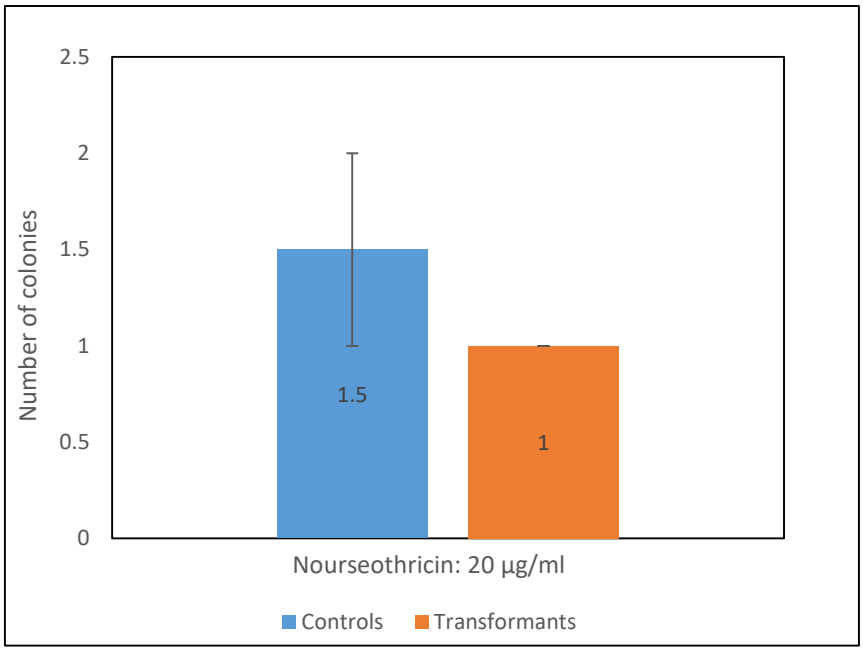


Figure 15: Number of *N. oleoabundans* colonies obtained by electroporation and in selection medium supplemented with 20 µg/ml of nourseothricin. Error bars denote the standard deviation of the mean from two biological replicates per condition.

4. Discussion

The development of genetic transformation techniques for microalgae is still in its infancy. This is evidenced by the fact that transformation has been limited to a limited number of species [60]. Due to their potential use in the industrial production of both high and low value products, it is necessary to explore, investigate and develop efficient transformation techniques in order to increase the production yield of such compounds.

Conjugation is more commonly known to occur between bacteria. However, since this approach has already been shown to work for diatoms (organisms closely related to green microalgae), as well as other eukaryotic organisms, it is feasible that it might also be used to genetically modify green microalgae such as *N. oleoabundans* and *A. obliquus*. In this work we sought to determine whether conjugation was a viable method of microalgal genetic transformation, and secondly tried to optimize as many parameters of the transformation protocol as possible.

To determine if the experimental conditions in the lab would allow for conjugation, we attempted to reproduce the results obtained by Karas *et al.* (2015) when transforming *P. tricornutum*. From figure 6, we can see that in addition to the decrease in colony number as the zeocin concentration increases, the number of colonies present in the control plates shows the same trend while maintain higher values than the corresponding transformation assays. This could be partly explained by the concentration of zeocin used to supplement the media not being high enough to properly select for transformants, or perhaps that as this antibiotic is photosensitive the antibiotic could have been degraded before a proper selection could have occurred. The results seen here diverged greatly from those obtained by Karas *et al.* (2015). Of the observed differences the most notable is the stark difference between the number of colonies obtained. Whereas the optimized protocol used in the paper yielded more than 400 confirmed transconjugant colonies using the same cargo plasmid [50], in our attempts the highest number of non-confirmed transconjugant colonies did not exceed 270 (figure 6).

The transformation of *P. tricornutum* by bacterial-mediated conjugation was attempted two more times, following the same protocol and using the same conditions but no colonies were observed on any plate in either attempt, regardless of zeocin concentration. In addition, the presence of bacterial colonies would go against the findings of Karas *et al.* who reported the complete absence of viable bacterial colonies after the two weeks of *P. tricornutum* incubation on the selective media [50].

It was expected that *P. tricornutum* cultures grown in the absence of any antibiotic would show the highest growth, however from table 4 it is possible to see that this was not the case and the diatom showed much greater growth at higher concentrations of zeocin. When grown in the presence of nourseothricin the cultures showed a more regular behaviour, where after 20 µg/ml of the antibiotic would overwhelm the diatom's resistance threshold. Even still, *P. tricornutum* still displayed low colony growth in the absence of nourseothricin, perhaps as the growth of *P. tricornutum* is not favoured in solid media.

N. oleoabundans showed the expected response to an increase in nourseothricin in the media, with a consistent decrease in microalgal growth (table 5). This ultimately resulted in the complete inhibition of the microalgae in the medium supplemented with 20 µg/ml nourseothricin. *A. obliquus* however did not display a linear response to the increase of nourseothricin concentration evidenced by the fact that after at concentration of 10 µg/ml and higher show similar growth. As it is unlikely that *A. obliquus* would grow similarly over such a wide range of concentration, these results caused have been caused by nourseothricin degradation in the media supplemented with the higher antibiotic concentration. The choice to use 40 µg/ml of nourseothricin for the selection of transconjugants in later trials was made as to ensure that complete inhibition of transformants did not occur.

In spite of not arriving at the same results as those of the paper, we proceeded to transform *N. oleoabundans* and *A. obliquus*, using adapted protocols for each microalgae. The low number of colonies present in the selection plates, along with the time period necessary for them to become visible (25 days), would seem to indicate that the chosen zeocin concentrations used were far beyond the natural resistance of *N. oleoabundans,* as well as above the higher resistance threshold provided by the zeocin resistance gene present in pPtPuc3. The low number of colonies obtained in this first *N. oleoabundans* transformation trial, in conjunction with the high standard deviation between replicates, meant that no statistically relevant information on the efficiency of conjugation as a method of microalgal transformation, or the effect of the different conditions, was produced. Reducing the concentration of zeocin in the selection method would have conceivably led to better results when using this antibiotic in the selection of transformants. The transformation of *N. oleoabundans* using bacterial-mediated conjugation was attempted in two more trials, both using nourseothricin (figure 9). The concentration of antibiotics used in these assays was chosen based on the sensitivity assay performed beforehand.

From figure 9a, we can distinguish a pattern: the longer the conjugation time, the greater the average number of colonies formed. As already mentioned, the number of colonies present in the control plates was expected to be lower than their corresponding transformant plates. However, that was not the case in the control plates of the 120-minute conjugation assays (figure 9b) as well those assigned 60 and 90 minutes of conjugation in the third trial (figure 9a). Considering that the colonies in these controls cannot possibly be composed of resistant cells it is more likely that the reasons for these disparities are inactivation of the antibiotic due to the long incubation period.

The noted relation between the conjugation times was not found for the trial shown in figure 9b, but the fact that two hours of conjugation generates the higher number of colonies remains. That consistency between different experiments seems to indicate that the two-hour period is the optimum conjugation time for *N. oleoabundans*. There is one more important piece of information to be extracted from these last two assays: continuous exposure to light, with the same light intensity, favours the survival of more cells, as evidenced by the higher number of colonies counted in these conditions (figure 9b). Though it is possible that the continuous exposure to light, without a dark period, could stimulate greater cell multiplication and growth, there is also the chance that this same factor could result in a higher deactivation rate of the antibiotic present in the media, in

turn reducing the selective pressure meant to eliminate false positives. To explore these scenarios, it would be necessary to verify the presence of the pTpPuc3 plasmid by PCR in the possible transformant colonies, as previously done for the first transformation experiment.

The first transformation trial for *A. obliquus* was conducted under the same conditions as the first attempt for *N. oleoabundans*. In this case however the zeocin concentrations used were not high enough for *A. obliquus* as it could not completely eliminate the background growth still found in the plates (figure 11a). As a result, in all trials assigned to 90 minutes and another to 120 minutes of conjugation, no colonies could be identified. To overcome this issue zeocin concentration was increased from 30 and 35 µg/ml, to 50 and 60 µg/ml, which led to much clearer colonies on the cultures. Comparing figures 11a to 11b, we can see that the increase in concentration of zeocin in the medium significantly decreased the number of colonies in the plates incubated for 60 minutes. This is in line with an increase in antibiotic concentration. On the other hand, the number of identified colonies in the plates incubated for 90 and 120 minutes using higher zeocin concentrations is greater. This might be that a large number of smaller colonies were still concealed by the background growth in trial 1 (figure 11a). In any case, 120 minutes of conjugation once again produces more colonies, followed by 90 minutes of conjugation than a 60-minute incubation, the same behaviour previously observed in the *N. oleoabundans* transformation trials.

While the longest conjugation period produces the highest number of possible transconjugants, it also leads to the presence of the highest number of non-resistant colonies in the controls. In contrast, 60-minute conjugation assays produced the lowest number of colonies in each of the trials, but it always showed the best transformant to control colony ratio in each of the *A. obliquus* transformation trials, as seen in figures 11 and 12. While unclear as to why this may be the case, the fact that the mixed cultures incubated at 30°C for greater periods of time may have stimulated bacterial growth. This could result in *E. coli* being present in the medium in greater concentration once part of the culture was transferred to the selective media. Once in the selective medium, the greater number of cells in the culture could have reduced the selective effect of the antibiotic. This would account for the increase in colony number for both controls and transformant cultures. The increase in colony numbers as the antibiotic concentration increases (figure 11) however, could be a consequence of the decrease in background growth. The amount of background growth decreases with higher concentrations of zeocin, making colonies that would otherwise be hidden, visible.

Based on these data, when developing a transformation protocol for *A. obliquus* it might prove more beneficial to use a shorter conjugation time as the chance of false positives is lower. Nevertheless, the fact remains that a confirmation of the presence of the plasmid in these would still have to be obtained.

Lastly, an attempt to transform *A. obliquus* using *E. coli* containing the pTpPuc3 was carried out, with the resulting transformants being selected on FW media supplemented with 40 µg/ml of nourseothricin (figure 12). Much like in the last assays, the plates incubated for 120 minutes were found to have more colonies, on average. In this case, however the incubation period of 60 minute showed a greater average number of colonies

than the 90-minute conjugation period. This was a consequence of only one of the 90-minute conjugation plates displaying colonies. The cause of this unexpected result is unclear.

Because both *A. obliquus* and *N. oleoabundans* are known to form associations with several other bacterial species [61], it also became necessary to replate part of the algal biomass onto LB plates to collect DNA from bacteria present in the FW media. As such there could always be the possibility that *E. coli* could serve as a DNA donor to more than one organism in the same culture (even if the number of microalgal cells should far outnumber bacteria). It would also be conceivable that *E. coli* cells could still be present in the media as the growth conditions could allow for the growth of this bacterium in spite of the lack of carbon source in the medium (though this could be obtained from dead cells). Replating several colonies onto LB plates revealed that there were indeed bacteria present in association with the microalgae, but since no colonies could be seen in the FW selection media, most of the extracted DNA from the latter media should likely be from microalgal origin. In any case, the DNA from both algal and bacterial biomass was extracted and the presence of the sh*ble* gene (only present in the pPtPUc3 plasmid) in the samples tested through PCR and electrophoresis.

PCR was performed to amplify the *shble* gene, which had an expected band size of approximately 370 bp. The PCR products were subsequently analysed on agarose gel by electrophoresis (Figure 10 and 13). The bands diverged slightly from the expected size for *N. oleoabundans* (figure 10) as the bands appear shorter by approximately 120 bp. Since this also seems to have affected the amplified fragments in the positive controls (plasmid pPtPUc3) it would be feasible that these inconsistences could be caused by heterogeneity of the electrophoresis gel or due to the interaction with gelred [62]. As an alternative to the use of GelRed, using a post-staining method rather than pre-staining could potentially correct these size inconsistences [62]. The presence of amplified DNA sequences in the *N. oleoabundans* DNA samples of the same size as the positive control, in conjunction with the absence of the same from the bacterial samples and the negative controls, confirms that microalgae transformants were successfully produced.

By contrast, the amplified DNA sequences from the *A. obliquus* samples show the expected 370 bp length. It is uncertain whether the smaller weak bands in several of the bacterial DNA samples (close to 300 bp) in figure 13b were visible due to the presence of residual microalgae DNA or if some of the bacterial cells also contained the conjugative plasmid. It is worth noting however that unlike the bands seen in figure 13a, that none of the weak 300 bp bands seen in figure 13b have the same length as that of the positive control, and so it could also be indicative that this a result of the amplification of non-specific sequences. The presence of faint fragments in blank and negative control in figure 13a, should in all likelihood be explained by contamination of the samples as these same bands were not present in figure 13b. Additionally, the large size of the positive control band was the result of using too large a DNA sample. This problem could be easily remedied by using a sample of a lower DNA concentration.

We should further consider the fact that the presence of colonies in the control plates likely indicates that not all of the colonies observed are actual transformants. On the other hand, positive confirmation for 10

random colonies were obtained through PCR and could imply that still a large of number of these colonies could be transformants. This assertion needs still requires further confirmation.

Given the self-replicating and high copy number properties of the pPtPuc3 plasmid it seems likely that even without the presence of a selective pressure in the LB media, that a number of cells could still possess the plasmid had they received it by conjugation from the *E. coli* donor cells. It would then stand to reason from these results, that the *A. obliquus* colonies from which the DNA was collected were in fact zeocin resistant transformants, as the DNA for the negative control had the *A. obliquus* wild type strain as its source and showed an absence of any fragment of similar length.

Utilizing the data collected from the electroporation transformation assays (figures 14 and 15) we can perform a comparison with the conjugation trials where the transformants were selected under the same conditions (figures 9a, 11b and 12). Under the tested conditions it would seem that conjugation is a more efficient method of DNA transfer for green microalgae, based on the number of colonies present on the plates. This however does not hold true for an optimized electroporation protocol. Using this technique, Guo *et al.* (2012) [63] reported having obtained 494±48 positive *A. obliquus* transgenic clones out of 10^6 recipient cells [63]. That would correspond to a transformation efficiency of 4.94×10^{-4}. When attempting to transform the same algae with the plasmid pPtPuc3, through another electroporation protocol, a mere 217 ± 28.5 colonies of unconfirmed transformants were obtained, out of 2.5×10^7 recipient cells. This would mean a transformation efficiency of 8.68×10^{-6} should all of the observed colonies be transformants. However, this is very unlikely given that the number of false positives present on the control plates exceed the number of colonies obtained in the transformant plates.

We can further compare the electroporation efficiency with that of conjugation. In the case of one of the trials with highest number of colonies (40 µg/ml of zeocin, 120 minutes of conjugation time – figure 11a) where 618 ± 91 colonies out of a possible 4×10^7 recipient cells were obtained, we can arrive at a transformation efficiency of 1.55×10^{-5} (should all of the colonies be transconjugants). On the other hand, if the number of false positives in the controls were to be subtracted from the number of colonies in the transformant plates (181 colonies), a transformation efficiency of 4.53×10^{-6} would result. Ultimately resulting in a transformation efficiency two orders of magnitude lower than that obtained by Guo *el al*.

In addition to the parameters already discussed, it would be important to investigate the possible effects of CO_2 concentration, rich growth medium, as well as methods to better eliminate bacterial presence from the selection medium. The most common approach to eliminate bacteria from microalgae cultures is the use of a combination of antibiotics, namely cefotaxime, carbenicillin, kanamycin and augmentin [64]. The greater presence of antibiotic in the medium could however in turn hinder microalgae growth. A possible measure to counter the negative effect of the antibiotics could be to increase the concentration of carbon dioxide in the incubator, as a greater availability of the carbon source should stimulate the microalgae's growth. Increasing light intensity could also lead to an improved growth. This however holds the risk of accelerated antibiotic degradation since they are highly photosensitive.

5. Conclusions and Recommendations

Green microalgae show a great potential in the industrial production of a vast range of products, from feed, food and biofuels derived from lipids to proteins and antibodies. Many of these applications however are highly dependent on low value products and so, in order to make them industrially and economically viable genetic modification is required. While several microalgae have been reported to have been genetically altered by different methods, *A. obliquus* and *N. oleoabundans* have yet to be transformed by any technique other than electroporation. Moreover, no algae have yet been reported of having been genetically modified by conjugation.

This is the first reported case of genetic transformation of green microalgae using bacterial conjugation, and the second reported transformation method for both *A. obliquus* and *N. oleoabundans*. While a complete optimization of the transformation protocol was not concluded, it was determined that for *N. oleoabundans* a conjugation period of 120 min produces the highest number of colonies. For *A. obliquus* however a shorter conjugation time of 60 minutes appears to be best, as it reduced or even eliminates colonies from the controls which could suggest the presence of fewer false positives.

With this in mind it is clear that in its current state, conjugation is not as efficient as an optimized electroporation approach. Should the protocol be properly optimized is it possible that bacterial-mediated conjugation could be a reliable transformation method for *A. obliquus* and *N. oleoabundans*, though it would hold the disadvantage of being a more time consuming procedure.

Future research into the transformation of microalgae *N. oloeabundans* and *A. obliquus* using bacterial mediated conjugation should focus on the optimization of the transformation protocol for these two species. Parameters to be optimized include antibiotic selective, microalgae/bacteria ratio, light intensity, L/D cycle and CO_2 concentration. Furthermore, other transconjugant identification methods like fluorescent proteins encoded by the cargo plasmids could be employed to confirm that conjugation was successful. Additionally, the amplified DNA fragments should be sequenced to further demonstrate the amplification of the correct *shble* gene sequence.

Once the bacterial conjugation protocol has been optimized, it would be interesting to implement this technique to conduct metabolic engineering by overexpressing enzymes involved in the biosynthesis of TAGs, namely GPAT, LPAD and DGAT. Using this approach, it is possible that the overexpression of these enzymes could lead to a significant accumulation of TAG within the microalgae cells.

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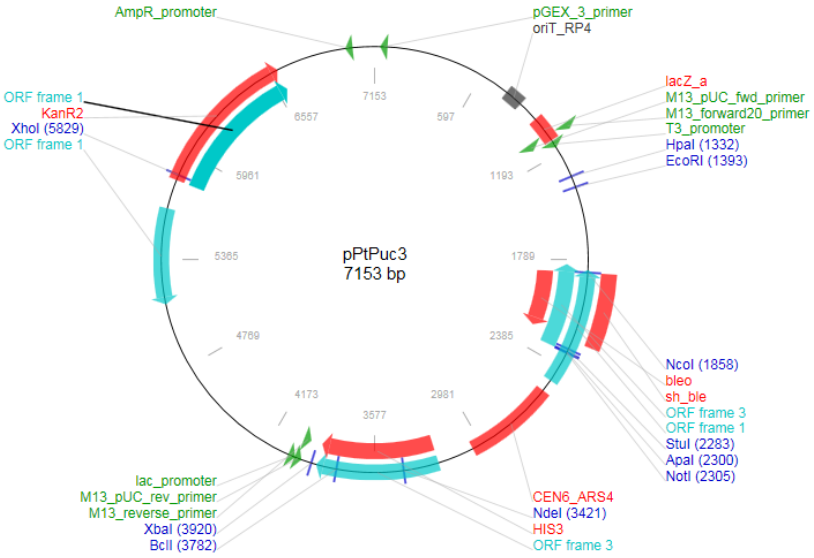
7. Appendix

7.1. Plasmids

Supplementary figure 1: Sequence and structure of the episomal pPtPuc3 plasmid, used to confer zeocin resistance [65]. **Legend**: *OriT* (Yellow), zeocin resistance marker (Red), CEN6_ARS backbone sequence (Green), *His3* gene (blue), Kanamycin resistance marker (Purple), *shble* primer sites (bold).



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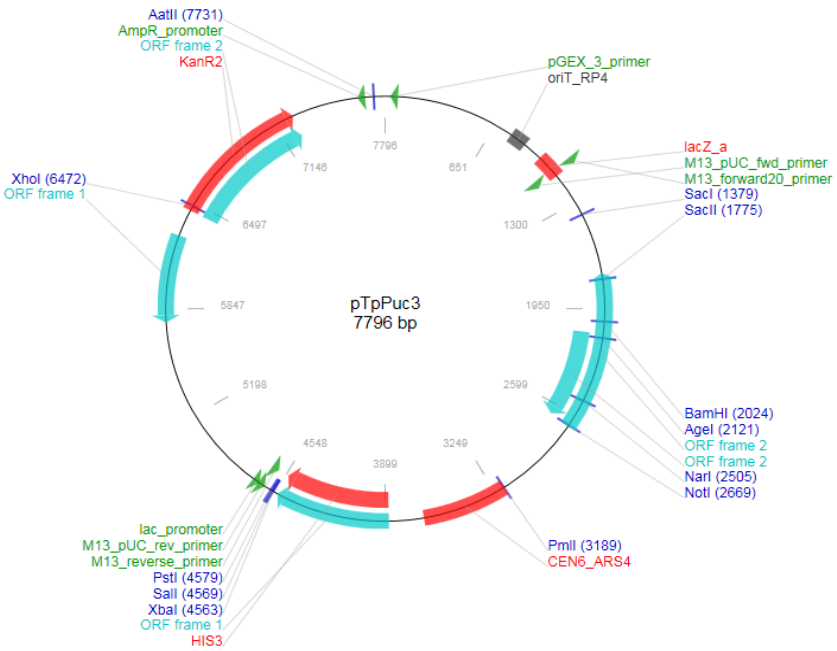
Supplementary figure 2: Content and structure of the plasmid pTpPuc3, used to confer nourseothricin resistance [66].

Legend: *OriT* (Yellow), Nourseothricin resistance marker (Red), CEN6_ARS backbone sequence (Green), *His3* gene (blue), Kanamycin resistance marker (Purple)



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7.2. Plasmid extraction protocol – *E. coli*

1. Centrifuge of 20 ml of an *E. coli* culture grown overnight at 14000 rpm at room temperature.
2. Resuspend the pellet in 250 µl of resuspension solution (stock maintained at 5°C).
3. Tranfer the cell suspension to 2 ml microcentrifuge tube.
4. Add 250 µl of lysis solution and mix by inverting the tube 6 times until the solution becomes viscous and slightly clear.
5. Add 250 µl of Neutralization solution and immediately mix by inverting the tubes 6 times.
6. Centrifuge for 5 minutes at 14000 rpm to pellet cell debris and chromosomal DNA.
7. Transfer the supernadant to a GeneJET spin column, while avoiding to disturb the pellet.
8. Centrifuge for 1 minute. Discard the flow-through amd place the column back into the same tube.
9. Add 500 µl of washing buffer to the GeneJET spin column.
10. Centrifuge for 1 minute at 14000 rpm and discard the flow-through and place the column in the same tube.
11. Repeat step 9. Tranfer the spin column to a 1.5 ml Eppendorf.
12. Add 50 µl of sterile DNAse free water.
13. Incubate at room temperature for 2 minutes.
14. Centrifuge for 2 minutes at 14000 rpm.

15. Discard the column and measure pDNA concentration.
16. Store DNA samples at -20°C.

7.3. Media composition

Supplementary Table 1: Chemical composition of 1 M CoulterCounter marine buffer solution (1 L).

Compound	Supplier	Concentration (M)	Amount (g)
NaCl	Merck	1.17	68.443
Na_2SO_4	Merck	2.25×10^{-2}	3.195
K_2SO_4	-	4.97×10^{-3}	0.848
Hepes Salts	Sigma Aldrich	1.00×10^{-2}	2.383
$MgCl_2.6H_2O$	Merck	7.35×10^{-5}	10 ml
$CaCl_2.2H_2O$	Merck	3.78×10^{-6}	

Supplementary Table 2: Composition of L1 medium, pH adjusted to 7.

Components	Volume (mL)
Aquil Salts	1000
NP stock	2
Trace metal stock	1
F/2 vitamin solution	0.5

Supplementary Tables 3: Composition of anhydrous salts (500 mL) and hydrous salts (500 mL) solutions necessary for Aquil Salts solution.

Component (Anhydrous salts)	Supplier	Amount (g)
NaCl	Merck	24.5
Na_2SO_4	Merck	4.09
KCl	Sigma Aldrich	0.7
$NaHCO_3$	Sigma Aldrich	0.2
KBr	Sigma Aldrich	0.1
H_3BO_3	Merck	0.03
NaF	Sigma Aldrich	0.003

Components (Hydrous salts)	Supplier	Amount (g)
$MgCl_2.6H_2O$	Acros Organics	11.1
$CaCl_2.2H_2O$	Merck	1.54

Supplementary Table 4: Composition of NP stock solution (500 mL).

Components	Supplier	Amount (g)
$NaNO_3$	Sigma Aldrich	37.5
$NaH_2PO_4.H_20$	Merck	2.5

Supplementary Table 5: Composition of Trace metal stock solution (1 L).

Components	Amount (g)
$FeCl_3.6H_2O$	3.15
$Na_2EDTA.2H_2O$	4.36
$CuSO_4.5H_2O$	0.25 mL (9.8 g L^{-1} dH_2O)
$Na_2MoO_4.2H_2O$	3.0 mL (6.3 g L^{-1} dH_2O)
$ZnSO_4.7H_2O$	1.0 mL (22.0 g L^{-1} dH_2O)
$CoCl_2.6H_2O$	1.0 mL (10.0 g L^{-1} dH_2O)
$MnCl_2.4H_2O$	1.0 mL (180.0 g L^{-1} dH_2O)
H_2SeO_3	1.0 mL (1.3 g L^{-1} dH_2O)
$NiSO_4.6H_2O$	1.0 mL (2.7 g L^{-1} dH_2O)
Na_3VO_4	1.0 mL (1.84 g L^{-1} dH_2O)
K_2CrO_4	1.0 mL (1.94 g L^{-1} dH_2O)

Supplementary Table 6: Composition F/2 vitamin solution (500 mL).

Composition	Amount (g)
Thiamine-HCl (Vitamin B1)	0.1
Biotin	5 mL L^{-1} (0.1 g L^{-1} stock)
Cyanocobalamin (Vitamin B12)	0.5 mL L^{-1} (1 g L^{-1} stock)

Supplementary Table 7: Chemical composition of freshwater (FW) medium (1 L), pH adjusted to 7.

Composition	Supplier	Amount (g)
KNO_3	Sigma Aldrich	3.4
Na_2SO_4	Merck	0.1
K_2HPO_4	-	10 ml (43 g/L)
$MgSO_4.7H_2O$	-	10 mL (14.6 g/L)
$CaCl_2.2H_2O$	-	10 ml (7.2 g/L)
HEPES salts	Sigma Aldrich	23.83
Stock 1	-	3 mL
Stock 2	-	3 mL
Vitamins	-	1 mL

Supplementary Table 8: Chemical composition of stock solution 1.

Composition	Amount (g/L)
$Na_2EDTA.2H_2O$	9.92
$MnCl_2.4H_2O$	1.27
$ZnSO_4.7H_2O$	0.4
$CoCl_2.6H_2O$	0.094
$CuSO_4.5H_2O$	0.11
$Na_2MoO_4.2H_2O$	0.0084

Supplementary Table 9: Chemical composition of stock solution 2.

Composition	Amount (g/L)
NaFeEDTA	3.4

Supplementary Table 10: Chemical composition of vitamin solution.

Composition	Amount (g)
Biotin	0.025
Vitamin B1	1.1
Vitamin B12	0.135

7.4. Equipment used

Supplementary Table 11: List of equipment used during this project.

Equipment	Manufacturer/Model
Centrifuge	Beckman Coulter™ AllegraX-3012
Centrifuge	Eppendorf 5430
Coulter Counter	Beckman Coulter Multisizer™ 3
Spectrophotometer	Hach Langer DR-600
Balance/Scale	Sartorius CP4202 S
Incubator (30ºC)	Sanyo
Incubator (25ºC)	-
Incubator (37ºC)	Binder
Incubator (18ºC): I	Snijder Scientific
Incubator (18ºC): II	-