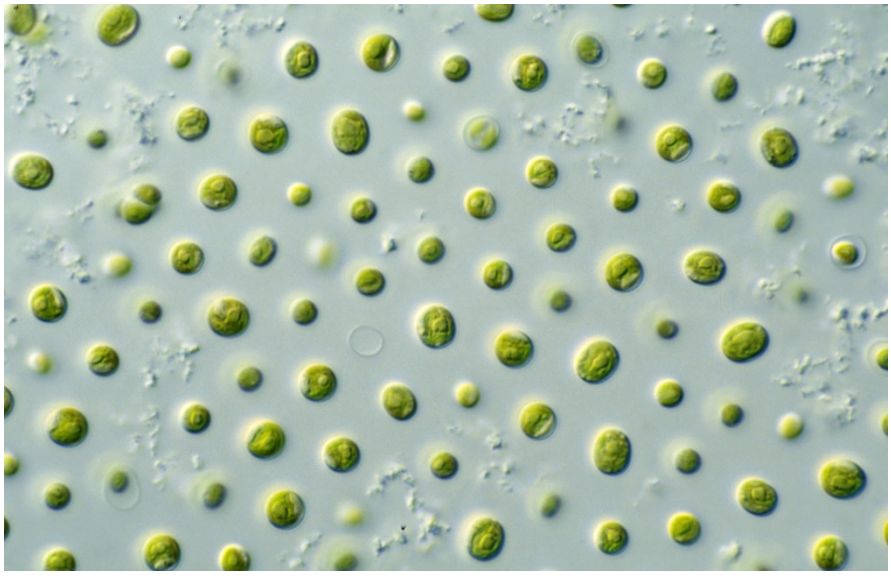
Australian Government

Department of Health

Office of the Gene Technology Regulator

The Biology of *Nannochloropsis oceanica* Suda & Miyashita (a microalga)



Version 1: October 2019

This document provides an overview of baseline biological information relevant to risk analysis of genetically modified forms of the species that may be released into the Australian environment.

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Cover photo: Microalgae – *Nannochloropsis* sp. by CSIRO.

ABBREVIATIONS

APVMA	Australian Pesticides and Veterinary Medicines Authority
Cas9	CRISPR associated protein 9
CO_2	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DHA	Docosahexaenoic acid (22:6*n*-3; 22:6$^{\Delta4,7,10,13,16,19}$)
DPIR	Department of Primary Industries and Resources
EC_{50}	Half-maximal effective concentration
EPA	Eicosapentaenoic acid (20:5*n*-3; 20:5$^{\Delta5,8,11,14,17}$)
GM	Genetically modified
HGT	Horizontal gene transfer
IAA	Indole-3 acetic acid
IC_{50}	Half-maximal inhibitory concentration
IODE	International Oceanographic Data and Information Exchange
kb	Kilobase
LC-PUFAs	Long chain polyunsaturated fatty acids
Mb	Megabase
mg	Milligram
µm	Micrometre
ms	Millisecond
n-3	Omega-3 polyunsaturated fatty acid
n-6	Omega-6 polyunsaturated fatty acid
nm	Nanometre
NHMRC	National Health and Medical Research Council
NSW	New South Wales
NT	Northern Territory
PCB	Polychlorinated biphenyl
ppb	Parts per billion
ppm	Parts per million
PUFAs	Polyunsaturated fatty acids
Qld	Queensland
SARDI	South Australian Research and Development Institute
SD	Standard deviation
TAG	Triacylglycerol
TFA	Total fatty acids
US DOE	United States Department of Energy
UV	Ultraviolet radiation
WA	Western Australia

PREAMBLE

This document describes the biology of *Nannochloropsis oceanica* Suda & Miyashita with particular reference to the Australian environment, cultivation and use. Information included relates to the taxonomy and origins of cultivated *N. oceanica*, general descriptions of morphology, reproductive biology, biochemistry, and biotic and abiotic interactions. This document also addresses the potential for gene transfer to closely related species. The purpose of this document is to provide baseline information about the parent organism for use in the risk analysis of genetically modified (GM) *N. oceanica* that may be released into the Australian environment.

Nannochloropsis oceanica is a single-celled microalga that lives in marine environments around the world. For cultivation, *Nannochloropsis* is grown in ponds or in more intensive bioreactor systems.

Nannochloropsis accumulates high levels of lipids, especially when grown under stress conditions. A high proportion of the lipid content of *N. oceanica* is made up of triacylglycerols, which are easily transesterified to produce biodiesel. Strains of *N. oceanica* are considered good candidates for the development of third generation biofuels. These microalgae are a potentially environmentally sustainable source of lipids, as they can harvest nutrients from wastewater and carbon dioxide from fossil fuel burning power stations.

Nannochloropsis is used in aquaculture as a valuable feed, providing polyunsaturated fatty acids, essential vitamins and amino acids, along with energy. In particular, the omega-3 fatty acid eicosapentaenoic acid (EPA) is produced in relatively high concentrations. Dietary omega-3 fatty acids have been shown to provide health benefits to humans, particularly coronary health. *Nannochloropsis oceanica* is being investigated as a potential source of omega-3 fatty acids for nutritional supplements and animal feed supplements.

As *N. oceanica* was first described in 2002, information about the biology of the species is limited. Therefore, research about other species of *Nannochloropsis* and, sometimes, closely related *Microchloropsis* is provided where information specific to *N. oceanica* is not available. This biology document will be refined as further information about *N. oceanica* is published.

In this document, the general terms ‘microalga’ or ‘microalgae’ will be used to refer to eukaryotic green microalgae, which include *N. oceanica*, unless otherwise specified. Species of microalgae will be referred to by the species name.

SECTION 1 TAXONOMY

The term ‘algae’ encompasses a wide range of organisms within four kingdoms: Chromista, Plantae, Protozoa and Bacteria (Figure 1; Guiry, 2012). Green microalgae, including *Nannochloropsis*, belong to the kingdom Chromista (Table 1), which also includes brown algae and diatoms. The kingdom Plantae includes green, red and glaucophyte algae (Ruggiero et al., 2015). Macroscopic brown, green and red algae are also known as seaweed (Hurd et al., 2014). The kingdom Bacteria includes cyanobacteria, which have previously been known as ‘blue-green algae’ (Guiry, 2012). The higher phylogeny of algae is complicated by a series of endosymbiosis events that resulted in the horizontal movement of genes (Stiller et al., 2014).

The single-celled microalgal species *Nannochloropsis oceanica* was first described in 2002 (Suda et al., 2002). The species was named *oceanica* as it is found in marine environments, with the type specimen collected from the Pacific Ocean, off the coast of Japan.

The *Nannochloropsis* genus was proposed by Hibberd (1981) to include very small, green eustigmatophycean algae that contain chlorophyll *a*, but not chlorophyll *b* or *c. Nannochloropsis* was previously classified as marine *Chlorella* (Pan et al., 2011). All species of *Nannochloropsis* are marine, except *N. limnetica*, which lives in freshwater (Fawley et al., 2015).

Due to limited differences in morphology (Section 3), species of *Nannochloropsis* are characterised by DNA sequencing of the nuclear 18S ribosomal RNA gene (18S rDNA) and the plastid *rbc*L gene (Fawley

et al., 2015). As new strains are discovered and older strains are sequenced, taxonomic rearrangements continue to occur.

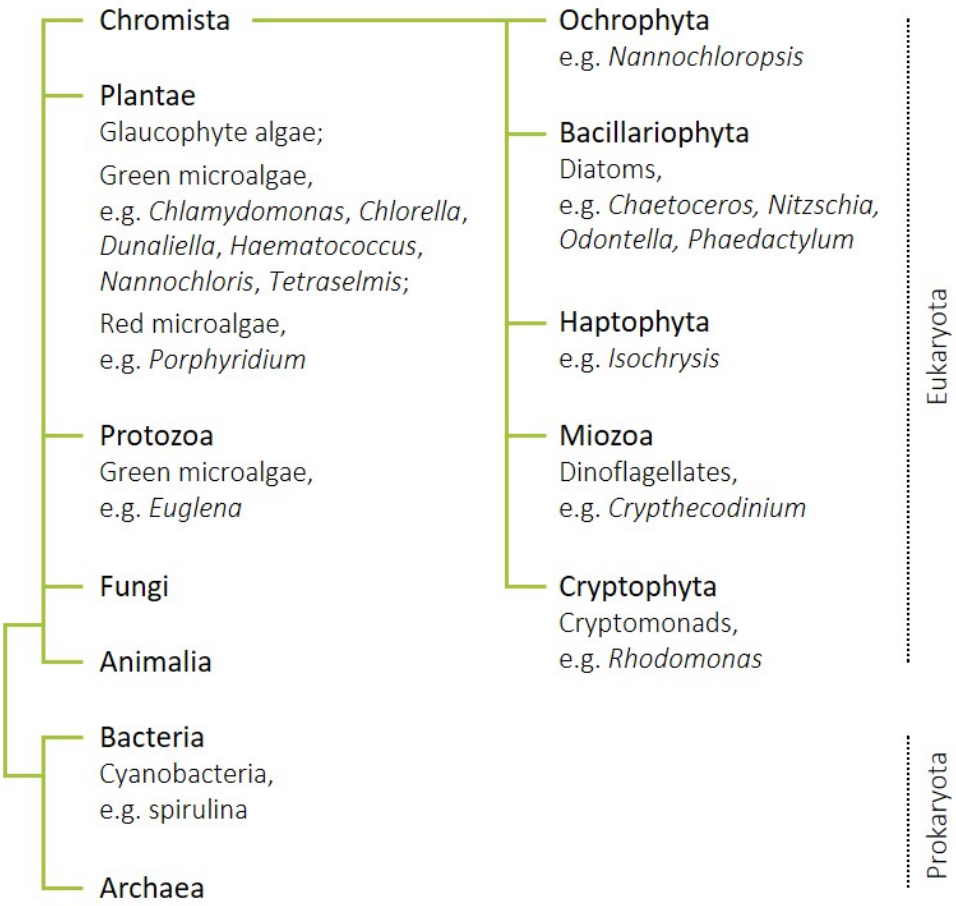

Figure 1 Examples of microalgae across kingdoms and a subset of phyla within Chromista. The term 'microalgae' describes a broad range of microalgal genera in four different kingdoms. Taxonomic classifications according to AlgaeBase (accessed 4 February 2019).

Table 1 Taxonomic hierarchy of *Nannochloropsis oceanica.*

Taxon	Nomenclature	Comment
Superkingdom	Eukaryota	
Kingdom	Chromista	
Superphylum	Heterokonta	[= "Supergroup Stramenopiles"]
Phylum	Ochrophyta	[= Heterokontophyta]
Class	Eustigmatophyceae	Unicellular algae; photosynthetic autotrophs
Order	Eustigmatales	
Family	Monodopsidaceae	Cells without mucilage layers; no zoospores
Genus	*Nannochloropsis*	Cells <5 µm maximum diameter
Species	*oceanica*	

Higher level classifications according to Ruggiero et al. (2015). Lower level classifications according to Hibberd (1981) and Eliáš et al. (2017).

Based on sequence analysis, Fawley et al. (2015) proposed that two species previously classified as *Nannochloropsis*, *N. gaditana* and *N. salina*, be renamed at genus level and classified within a new genus *Microchloropsis.* Indeed, comparing genome sequences between *N. oceanica* and *N. gaditana*,

Vieler et al. (2012) noted that the differences between these species was of a similar magnitude to the differences between monocotyledonous and dicotyledonous plant species. These species will be henceforth referred to as *M. gaditana* and *M. salina* in this document.

Following this rearrangement, there are five named species of *Nannochloropsis*: *N. australis, N. granulata, N. limnetica, N. oceanica* and *N. oculata* (Suda et al., 2002; Fawley et al., 2015). In addition, there are references in the literature to "*N. maritima*", which is phylogenetically closely related to *N. oceanica*, but has not been formally described (Fawley et al., 2015; Zhang et al., 2015; Eliáš et al., 2017).

1.1 Methods of identification and differentiation

Light microscopy and scanning electron microscopy are used to examine the morphology of microalgae, including cell wall ornamentation and cellular inclusions (Eliáš et al., 2017). Morphology is discussed in Section 3.

Due to a lack of consistent morphological features for differentiating species of *Nannochloropsis*, identification requires gene sequencing (Eliáš et al., 2017). Sequences of the nuclear 18S ribosomal RNA gene (18S rDNA) are often used to bar-code microorganisms; however there is not enough variation in 18S rDNA sequences to identify strains of *Nannochloropsis* at species level (Fawley et al., 2015). A combination of sequence data from 18S rDNA and the plastid Rubisco *rbc*L gene is generally required to identify the phylogeny of *Nannochloropsis* species (Suda et al., 2002; Fawley et al., 2015).

Li et al. (2015) suggest that lipid profiles may be used to distinguish between different strains of *N. oceanica* by using, e.g. ultra-performance liquid chromatography coupled with electrospray ionization – quadrupole – time of flight mass spectrometry (UPLC-Q-TOF-MS).

1.2 Whole genome sequencing

The *N. oceanica* genome was first sequenced by Pan et al. (2011), who reported a genome size of approximately 30 Mb with 11,129 predicted genes. Vieler et al. (2012) sequenced *N. oceanica* CCMP1779, noting a genome size of 28.7 Mb and 11,973 predicted genes.

Several *Nannochloropsis* genomes, including two *N. oceanica* strains, were sequenced by Wang et al. (2014). The whole genome size of *N. oceanica* IMET1 and *N. oceanica* CCMP531 was 30.1 Mb and 35.5 Mb, respectively, with mitochondrial genomes of 38.1 kb and chloroplast genomes of approximately 118 kb. *Nannochloropsis* appear to have 22 chromosomes.

The *Nannochloropsis* genome appears to contain relatively few mobile elements (long terminal repeat retrotransposons), compared with other microalgae and plants (Vieler et al., 2012; Wang et al., 2014). Vieler et al. (2012) found a low abundance of transposable elements in the *N. oceanica* genome, but noted that the identified transposons were diverse, with similarity to transposons in other algae, plants and animals.

The *Nannochloropsis* photosynthetic plastid (chloroplast) was acquired from red algae by endosymbiosis. Stiller et al. (2014) argued that the red algal plastid was first acquired by cryptophyte algae in a secondary endosymbiosis event; this plastid was subsequently acquired by an ochrophyte ancestor of *Nannochloropsis* from cryptophytes via tertiary endosymbiosis around 500 million years ago. However, the evolutionary history of the plastid transfer continues to be elucidated. The *Nannochloropsis* plastid genome encodes genes involved in carbon assimilation and photosynthesis, while the mitochondrial genome encodes genes involved in the respiratory electron transport chain and ribosome biosynthesis (Wei et al., 2013). Genes involved in photosynthesis and respiration are also found encoded in the nuclear genome (Vieler et al., 2012).

In the *N. oceanica* IMET1 genome, Wang et al. (2014) identified 99 genes that had been putatively introduced by horizontal gene transfer (HGT). This represents approximately 1% of nuclear genes. A relatively high number of HGT candidates were involved in lipid biosynthesis. Fatty acid synthase genes originating in bacteria appear to have been introduced into an ancestor of *Nannochloropsis* by HGT. In addition, *N. oceanica* has a relatively high number of type II diacylglycerol acyltransferase

(DGAT-2) genes compared with known organisms, including other heterokonts, plants, algae, fungi and animals. These genes are expressed during triacylglycerol synthesis. Based on sequence comparisons, DGAT-2 genes were likely incorporated into the *Nannochloropsis* genome by ancient endosymbiotic gene transfer events from green and red algae, and a eukaryotic secondary host (Wang et al., 2014).

SECTION 2 ORIGIN AND CULTIVATION

2.1 Natural habitat and geographic distribution

Nannochloropsis oceanica has been collected from oceans around the world. Currently described strains mainly originate in subtropical Northern Hemisphere waters; however, *N. oceanica* has also been found close to the Arctic Circle and in the Southern Hemisphere.

Most of the studied strains of *N. oceanica* originate near China, Japan and Taiwan (Chen et al., 2013; Fawley et al., 2015). The species has also been isolated from waters off western Norway (Sandnes et al., 2005), from the Mediterranean Sea and Red Sea near Israel (Suda et al., 2002), the Mediterranean Sea near Egypt (Ashour et al., 2019), Kuwait (Jinkerson et al., 2013), the west coast of India (Ashour et al., 2019), Argentina (Bongiovani et al., 2014), and Deception Bay, Qld, Australia (Fawley et al., 2015). It is expected that further exploration of Southern Hemisphere waters will reveal more diversity within the genus (Fawley et al., 2015).

Nannochloropsis-like species were found in Antarctic lakes. These are likely to be more closely related to the freshwater *N. limnetica* (Bielewicz et al., 2011; Karlov et al., 2017).

2.2 Commercial uses

Nannochloropsis is currently used in the production of microalgal concentrates for fish hatcheries. Researchers are hoping to realise the potential for microalgae to provide a sustainable source of biofuels and health foods (Figure 2); however biological properties of individual strains, and culturing and extraction techniques need to be improved to reduce costs before production can become commercially viable (Al-Hoqani et al., 2017).

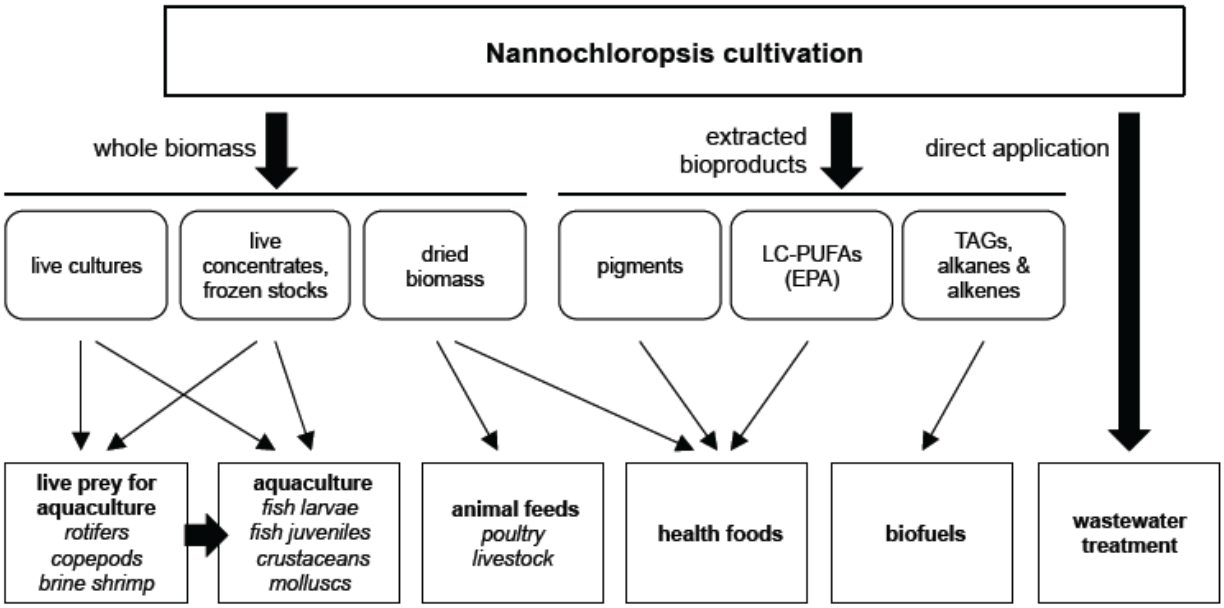

Figure 2 Current and potential applications of *Nannochloropsis*.
Reproduced from Al-Hoqani et al. (2017) under Creative Commons.

2.2.1 *Nutritional supplements*

Microalgae are used to produce nutritional supplements containing omega-3 long chain polyunsaturated fatty acids (*n*-3 LC-PUFAs). The value of *n*-3 LC-PUFAs in the human diet is

well-documented. In particular, health benefits are attributed to two fatty acids present in seafood: eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3; Stark et al., 2016). Fish are unable to synthesize *n*-3 LC-PUFAs, but accumulate these fatty acids through their diet (Al-Hoqani et al., 2017).

The major source of human dietary *n*-3 LC-PUFAs is from fish, fish oil supplements, and functional foods that have been enriched with fish oil (Martins et al., 2013). The sustainability of fishing industries is of concern, as is the contamination of larger fish with environmental pollutants, so alternative sources of *n*-3 LC-PUFAs are sought.

The relatively high concentration of EPA present in *Nannochloropsis* has led to the development of vegetarian dietary supplements. Capsules containing EPA-rich oil are produced from *N. oculata* (Al-Hoqani et al., 2017).

2.2.2 *Animal feed supplements*

Microalgal concentrates are used in commercial aquaculture to feed zooplankton, such as rotifers, which are then eaten by fish hatchlings (Al-Hoqani et al., 2017). *Nannochloropsis* provides a source of highly unsaturated fatty acids, which are taken up by fish, either directly or indirectly, when they consume zooplankton that have fed on the microalga. The green water technique for rearing fish larvae uses microalgae to stabilize water quality and control microbes (FAO, 1996). Several companies offer *Nannochloropsis* for sale as live, frozen or freeze-dried cells (Al-Hoqani et al., 2017). For example, *N. oculata* is used in a commercial green water product (Reed Mariculture website, accessed 26 February 2019).

Supplementation of chicken feed with 5–10% spray dried *M. gaditana* can increase the abundance of DHA and carotenoids in egg yolks (Bruneel et al., 2013). Microalgal EPA is converted to DHA in the chicken prior to being incorporated into yolk lipids.

Supplementation of ruminant diets with whole dried *N. oceanica* biomass is being investigated as a means of increasing *n*-3 LC-PUFAs in meat and milk (Alves et al., 2018). The cell wall of *Nannochloropsis* appears to slow the hydrogenation of *n*-3 LC-PUFAs by bacteria in the rumen.

2.2.3 *Biofuels*

Microalgae are considered a promising source of third-generation biofuel (Maity et al., 2014). First generation biofuels are produced from traditional food crops and animal fats, while second generation biofuels are produced from plants such as jatropha (*Jatropha curcas*) and lignocellulosic plant materials. These sources of biomass require large areas of land for cultivation and, therefore, might not be produced in large enough quantities to replace fossil fuels. A high photosynthetic rate allows microalgae to rapidly accumulate biomass, with the potential to accumulate much greater amounts of biomass per land area compared with traditional biofuel plant crops. Under laboratory conditions *N. oceanica* strain W2J3B[1] has a doubling time of around 14 hours (Kilian et al., 2011). Thus, third generation microalgal biofuels have the potential to sustainably replace fossil fuels (Maity et al., 2014).

Nannochloropsis is being investigated for biofuel production as it accumulates high concentrations of lipids, particularly during the stationary phase of growth (Beacham et al., 2014). Lipid content of the *N. oceanica* IMET1 strain reached approximately 53% after 22 days of cultivation, with lipid productivity of 159 mg L^{-1} day^{-1} (Ma et al., 2014).

Close to half of the lipid content of the IMET1 strain is made up of triacylglycerols (TAGs), which are easily transesterified to produce biodiesel (Ma et al., 2014). In the presence of excess methanol and a catalyst, TAGs are broken down into methyl esters (biodiesel) and glycerol (Chisti, 2007). Lipids are typically extracted by organic solvents, but alternative methods have been trialled, including ultrasonic- and microwave-assisted extraction, and supercritical fluid extraction (Lee et al., 2015).

[1] *Nannochloropsis* sp. (W2J3B) identified as *N. oceanica* in Li et al. (2014).

After lipid extraction, the remaining biomass may be anaerobically digested to produce methane (Chisti, 2007). Hydrogen gas may be produced using *Enterobacter aerogenes* via dark fermentation[2], carried out under anaerobic, axenic conditions (Nobre et al., 2013).

Following saccharification[3], residual biomass may also be fermented to produce bioethanol (Lee et al., 2015); however, due to inadequate carbohydrate content, Schneider et al. (2012) did not consider *Nannochloropsis* to be amongst the most suitable genera for bioethanol production.

Bio-oil can be produced from wet microalgae by hydrothermal liquefaction at 300–374 °C or from dried microalgae by pyrolysis at 450–500 °C (Lee et al., 2015). Pyrolysis converts TAGs, carbohydrates, proteins and fats into a black liquid bio-oil with high heating value and high viscosity.

2.3 Cultivation

Historically, microalgae have been harvested from natural ponds and lakes (Hamed, 2016).

Microalgae are now usually cultivated in commercial quantities in two systems: 'open' raceway ponds and 'closed' photobioreactors (Borowitzka and Vonshak, 2017). These systems can use saline water, recycle nutrients in wastewater and use carbon dioxide emissions from power stations that burn fossil fuels (Chisti, 2007; Maity et al., 2014; Hulatt et al., 2017).

Raceway ponds are open-air closed loop recirculation channels in which the algal solution is kept in constant motion using a paddlewheel (Figure 3a).

Photobioreactors are made up of an array of narrow transparent tubes or plates containing a recirculating algal solution (Figure 3b). These systems maximise the amount of light that can be captured and allow single-species cultures to be maintained for longer periods. The closed system requires degassing zones to remove dissolved oxygen and bubbles, and to replenish carbon dioxide (Chisti, 2007). There are several different photobioreactor designs, including tubular, flat plate, column and biofilm systems (Hulatt et al., 2017).

There are various types of culture techniques, ranging from batch to continuous cultures (FAO, 1996). In batch culture, microalgal cells are increased from a single inoculation, e.g. in a test tube, sequentially through increasing volumes to the final culture, e.g. an open pond. The entire culture is harvested just prior to reaching stationary phase. Continuous cultures are maintained at close to the maximum growth rate by regularly harvesting a portion of the culture and replacing the lost volume with fresh medium.

Nannochloropsis cultures often reach densities of approximately 3×10^7 cells mL^{-1}, e.g. Dunstan et al. (1993); but may reach up to 1900×10^7 cells mL^{-1} (or 67.3 g L^{-1}) in flat plate reactors (Zou et al., 2000).

[2] Dark fermentation is the process of converting an organic substrate to biohydrogen in the absence of light.

[3] Saccharification is a process by which carbohydrates are broken down into simple sugars.

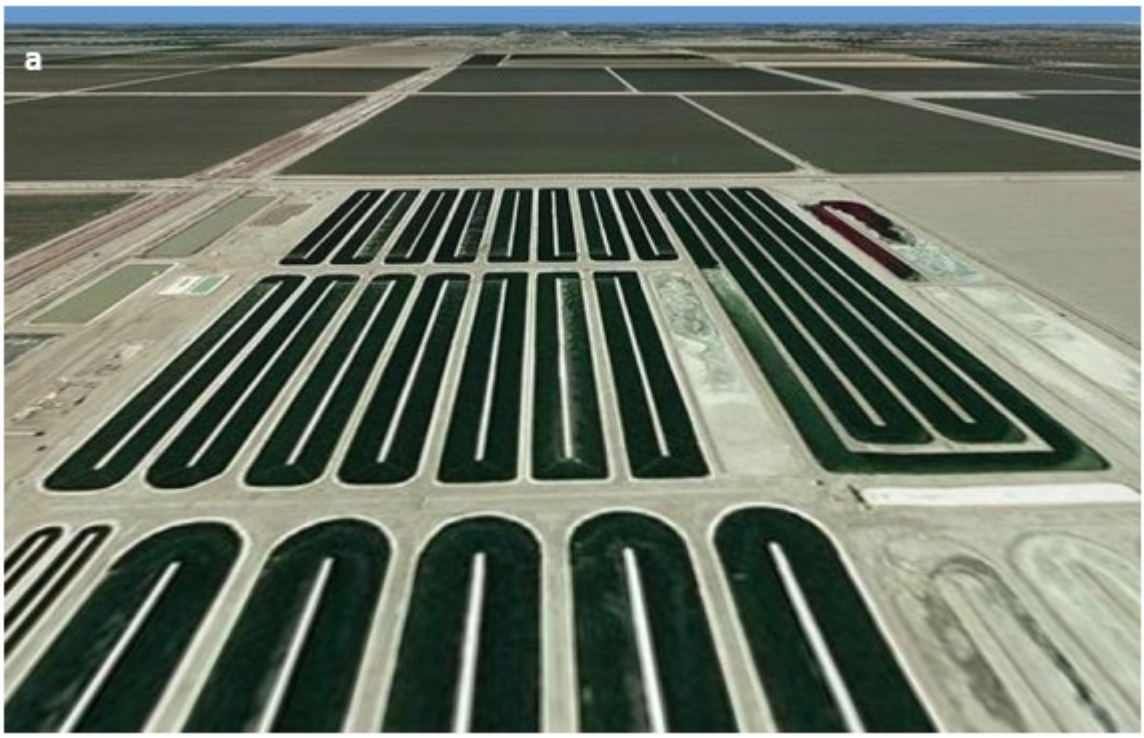

Figure 3 Production of microalgae in a) open raceway ponds, and b) a closed photo bioreactor. Image credits: Courtesy of US DOE/Pacific Northwest National Laboratory (via Flickr); IGV Biotech (via Wikipedia).

2.3.1 *Microalgal production in Australia*

Nannochloropsis has been grown for biofuel production in a pilot facility near Karratha, WA (Murphy, 2013). *Nannochloropsis oculata* is grown as a food source for aquaculture organisms (Renaud et al., 1991). The South Australian Research and Development Institute (SARDI) has cultivated microalgae, including *N. oceanica*, in outdoor raceway ponds and photo bioreactors in Adelaide, South Australia (SARDI, 2015).

Several microalgal production facilities have been built in Australia and research into microalgal fuel production is being carried out at universities around the country (Borowitzka et al., 2012). Microalgae are being grown for astaxanthin[4] production in Qld (*Haematococcus pluvialis*; Pacific Bio website, accessed 3 September 2019), for β-carotene production in WA (*Dunaliella salina*; Boruff et al., 2015), and for nutritional supplements in the NT (spirulina; NT DPIR website, accessed 26 February 2019).

Factors that need to be considered for the selection of sites for commercial microalgal pond systems include:

- water availability
- lipid productivity, e.g. high solar irradiance and temperature
- availability of flat lands
- proximity to main transport networks, e.g. main roads, railroads or ports
- gross national income per capita (as a substitute for the availability of low labour costs)
- proximity to known industrial carbon dioxide sources (Correa et al., 2019, and references therein).

In additional to these criteria for increased profitability, the impact of microalgal cultivation on food production and biodiversity should be minimised (Correa et al., 2019).

In Australia, sites of indigenous significance and environmentally sensitive areas are also excluded (Borowitzka et al., 2012; Boruff et al., 2015). Using relevant factors to model algal biofuel production potential for WA, Boruff et al. (2015) identified a large area of coastline around Karratha and Port Hedland as most suitable for commercial scale ponds, noting that there is a high frequency of cyclones during summer months in this region.

2.3.2 *Harvesting and processing microalgae*

Harvesting of microalgae involves the removal of microalgal biomass from the cultivation solution to allow downstream processing (Molina Grima et al., 2004; Christenson and Sims, 2011; Alhattab et al., 2015). There are many different techniques, and combinations of techniques, that can be used to harvest microalgae depending on the scale of production and microalgal species. Research into optimal harvesting techniques is ongoing, as dewatering is a large cost in microalgal production.

The most common harvesting techniques used in larger scale microalgal production are:

- Flocculation. This process is generally used as a pre-treatment in combination with other harvesting techniques (Christenson and Sims, 2011). Flocculation causes microalgal cells to aggregate, increasing particle sizes. Metal salts are often used for flocculation, although this depends on the intended final uses of the microalgae. Flocculation is discussed further in Section 4.4.
- Centrifugation. This method separates microalgal cells from the solution due to differences in density (Molina Grima et al., 2004; Alhattab et al., 2015).
- Settling/sedimentation. Gravity causes microalgae to settle to the bottom of sedimentation tanks. Sedimentation is a relatively slow process (Christenson and Sims, 2011; Alhattab et al., 2015).

[4] Astaxanthin is marketed as an antioxidant nutritional supplement for humans and used as a natural pigment supplement in salmon feed.

- Dissolved air flotation. This method is often used in combination with flocculation. Gas bubbles passing through the microalgal suspension adhere to particles, causing them to float to the surface and allow collection (Alhattab et al., 2015).
- Filtration. Solid particles are retained as the microalgal solution is forced through a filter membrane using vacuum, pressure or gravity (Alhattab et al., 2015).

Following harvest, the microalgal biomass may be further dried or processed to extract desired substances (Molina Grima et al., 2004).

Recycling the water remaining after harvest reduces the water footprint of microalgal cultivation systems; however the growth of subsequent cultures may be affected (Farooq et al., 2015). Recycling of the culture medium can result in improved or reduced growth of subsequent *Nannochloropsis* cultures, depending on various factors, e.g. the addition of harvesting agents.

2.3.3 *Potential negative environmental impacts of microalgal cultivation systems*

A comprehensive review of potential environmental impacts of large-scale microalgal cultivation is provided by Usher et al. (2014). Key points that have not been addressed elsewhere are summarised below.

Cultivation of microalgae requires enrichment with nutrients and carbon dioxide. The accidental release of a microalgal culture into waterways can upset the nutrient balance and result in eutrophication, leading to a loss of aquatic biodiversity and the release of greenhouse gases.

If open ponds are not well designed or constructed, the microalgal culture may leach and contaminate groundwater. Leaching is more likely to go undetected in ponds, compared with enclosed photobioreactors. Leaching may be particularly problematic for wastewater treatment facilities or if microalgae are grown in saline water.

While autotrophic *Nannochloropsis* fixes atmospheric carbon dioxide, microalgal cultivation may lead to the release of other potent greenhouse gases. Under optimal aerobic production conditions, these emissions are at low concentrations. Examples of potential greenhouse gas emissions include:

- aerobic production of methane
- production of nitrous oxide (N_2O) by bacterial contaminants, particularly if anoxic conditions develop
- emission of organohalogens by microalgae cultivated in saline water.

2.4 Development of improved strains

2.4.1 *Mutagenesis*

As an asexual haploid[5] (see Section 4.1), *Nannochloropsis* is readily 'bred' by chemical mutation, when combined with an efficient mutant screening method. After mutating *N. oceanica* with nitrosoguanidine, Wang et al. (2016b) selected three mutants with increased lipid content. Mutant phenotypes were found to be stable for over 200 generations grown over two years.

Heavy-ion irradiation with carbon ions was used to create *N. oceanica* mutants with increased growth rate and lipid productivity (Ma et al., 2013).

2.4.2 *Genetic modification*

Techniques have been developed that allow efficient nuclear transformation of *Nannochloropsis* (Kilian et al., 2011; Weeks, 2011). Transformation of the *N. oceanica* chloroplast genome has also been demonstrated (Gan et al., 2018).

[5] Also known as monoploid.

Unlike *Chlamydomonas*, the cell walls of *Nannochloropsis* do not need to be removed prior to electroporation, although higher electroporation voltages are required. Transformation of microalgae is, nevertheless, generally more difficult than transformation of bacteria or yeast (D. Frampton[6], personal communication, 2019). The sequencing of nuclear genomes of several strains of *N. oceanica*, along with the development of transformation techniques, allows genetically modified strains of *N. oceanica* to be readily developed (Eliáš et al., 2017).

Loss-of-function mutants of *N. oceanica*, unable to use nitrate as a nitrogen source, were generated using a CRISPR/Cas9 gene editing system (Poliner et al., 2018b). The CRISPR/Cas9 system was expressed from an episome. Following transformation the episome was gradually lost from cells when selection pressure was removed, leaving modified cells with no remaining markers or introduced DNA.

Overexpression of fatty acid desaturase genes in *N. oceanica* increased the concentration of EPA (Poliner et al., 2018a).

Nannochloropsis oculata was transformed to express a fish growth hormone (Chen et al., 2008). The GM microalga was first fed to brine shrimp, which digested cell walls, making the growth hormone protein available in the fish diet. This significantly increased weight gain in red tilapia fish larvae. The inserted DNA was unstable in most transformants, but was inherited for 50 generations in a small number of clones.

Regardless of the desired GM trait, is it sensible that a control trait that provides a fitness disadvantage under natural environmental conditions is also inserted into modified *Nannochloropsis*, e.g. extreme sensitivity to UV, or growth only in the presence of a chemical that does not occur naturally and can be applied at commercial scale (I. Jameson[7], personal communication, 2019). The risks of open pond cultivation of GM microalgae are discussed in reviews by Henley et al. (2013) and Beacham et al. (2017). They raise concerns that dispersal of GM microalgae during cultivation and harvest in open pond mass production systems would be inevitable. Depending on the microalgal strain selected for modification and the type of modification, harms could occur through horizontal gene transfer, or increased competitive fitness of the GM microalgae against native phytoplankton. Potential harms include altered species composition and food web dynamics in the natural environment, and the formation of harmful algal blooms.

SECTION 3 MORPHOLOGY

Nannochloropsis is a single-celled pear-shaped, spherical or oval organism with dimensions 2–4 x 3–5 µm (Suda et al., 2002). Cells are solitary. The cell wall is smooth, with a small papilla (a plug-like structure). Cell dry weight is approximately 3–6 pg $cell^{-1}$ (FAO, 1996; Zou et al., 2000).

Nannochloropsis has relatively thick, rigid cell walls, approximately 85–113 nm thick (Skrede et al., 2011; Beacham et al., 2014). Cell wall structure was investigated by Scholz et al. (2014) in *M. gaditana*. The cell wall is composed of approximately 75% cellulose, with the remainder comprised of algaenan biopolymers, proteins, other carbohydrates and minerals. An inner plasma membrane is connected by struts to the cell wall bilayer. The inner cell wall layer is cellulose-based, while a thinner outer layer is algaenan-based. Extensions < 100 nm in length protrude from the algaenan outer wall.

The structure of *Nannochloropsis* algaenan has not been fully elucidated, but appears to be composed of long, straight, saturated aliphatic chains with ether cross-links, which may be derived from C_{18} fatty acids (Scholz et al., 2014). It is speculated that *Nannochloropsis* algaenans are similar to the cutan found in plants such as *Agave* and *Clivia*.

A single yellow-green chloroplast lies adjacent to the cell wall (Figure 4). *Nannochloropsis* chloroplasts have several bands of lamellae, with three thylakoids per band. The plastid is surrounded by four

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membranes, with the outermost endoplasmic reticulum membrane being continuous with the nuclear envelope membrane (Suda et al., 2002; Murakami and Hashimoto, 2009).

The major light-harvesting accessory pigments are the carotenoids violaxanthin and vaucheriaxanthin esters (Vieler et al., 2012). These carotenoids transfer light energy to chlorophyll *a* and protect the photosynthetic apparatus from excess light, through fluorescence quenching (Masojídek et al., 2004; Eliáš et al., 2017).

Nannochloropsis cells contain immobile granular inclusions and vesicles in the cytoplasm along with, usually, a mitochondrion and a Golgi body (Suda et al., 2002). Cells of *N. oceanica* may also contain refractile bodies and a red body (reddish globule). These lipidic red bodies are characteristic of the eustigmatophytes, becoming larger and darker as cells age (Eliáš et al., 2017). During reproduction the red body is inherited completely by one of the daughter cells. The function of red bodies is unclear. Some authors (e.g. Cao et al., 2013; Bongiovani et al., 2014) describe red bodies as 'eyespots'; however eyespots are distinct organelles present in certain flagellate algae, including eustigmatophyte zoospores, and are thought to be involved in photoperception (Kreimer, 2009; Eliáš et al., 2017). The single red body also appears to be different from lipid droplets (Figure 4), which may be multiple in number (Vieler et al., 2012; Eliáš et al., 2017).

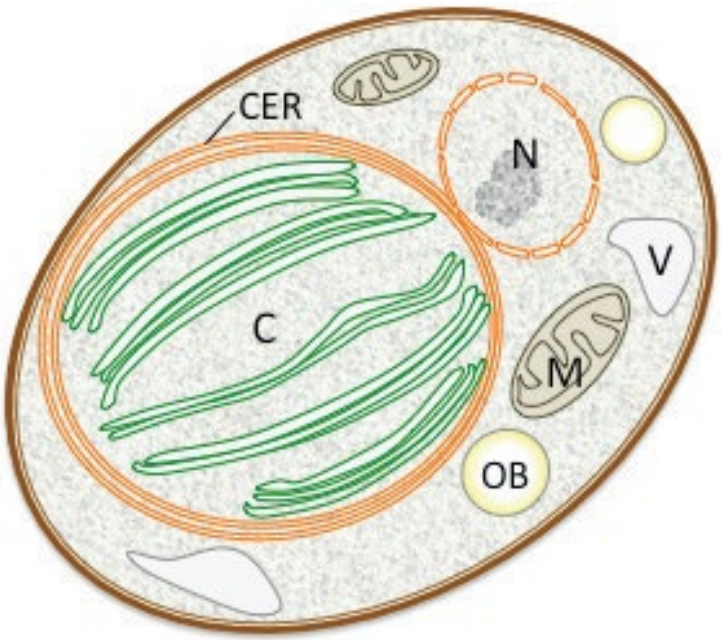

Figure 4 Cell structure of *Nannochloropsis* under nutrient replete conditions.
C, chloroplast (plastid); CER, chloroplast endoplasmic reticulum; M, mitochondrion; N, nucleus; OB, oil body (lipid droplet); V, vesicle. Reproduced from Al-Hoqani et al. (2017) under Creative Commons.

Lipid droplets store fatty acids. Lipid synthesis is likely to occur by two separate pathways: the prokaryotic pathway associated with the chloroplast and the eukaryotic pathway associated with the endoplasmic reticulum (Vieler et al., 2012). The first fatty acid synthesis steps for TAG synthesis occur in the chloroplast (Liu and Benning, 2013). Following recent work in *Chlamydomonas*, it is unclear whether subsequent TAG synthesis steps occur in the plastid envelope membranes, the endoplasmic reticulum, or both. Synthesis of EPA in *Nannochloropsis* is expected to occur in the endoplasmic reticulum (Poliner et al., 2018a).

The presence of a pyrenoid-like structure in *Nannochloropsis* was noted by Suda et al. (2002) and others. Pyrenoids are CO_2-concentrating structures associated with the chloroplast (Gee and Niyogi, 2017); however Suda et al. (2002) did not see a clear connection between pyrenoid-like structures and

chloroplasts. Further studies have not confirmed the presence of a pyrenoid (Eliáš et al., 2017). Pyrenoids were not observed in *N. oceanica* CCMP1779 (Gee and Niyogi, 2017) or *N. oceanica* CCALA978 (Cao et al., 2013; Bongiovani et al., 2014).

SECTION 4 DEVELOPMENT

4.1 Reproduction

Based on the lack of genes associated with meiosis, Pan et al. (2011) concluded that *N. oceanica* is not capable of performing meiosis and, therefore, is unable to reproduce sexually.

Along with the other members of the Monodopsidaceae family, *Nannochloropsis* does not produce zoospores (Hibberd, 1981).

Evidence shows that *N. oceanica* is haploid (Kilian et al., 2011; Pan et al., 2011). Mitosis in *N. oculata* was described by Murakami and Hashimoto (2009) as 'peculiar', with the inner nuclear envelope dividing before the outer nuclear envelope. The nucleus and plastid remain connected by the outer nuclear envelope membrane during division. During replication either the nucleus may divide before the plastid, or vice versa.

4.1.1 *Doubling time*

Doubling time is dependent on growth conditions. Under experimental conditions, observed average doubling times for *N. oceanica* range from approximately 14 hours (Kilian et al., 2011), 25 hours (Poliner et al., 2018b), 27–40 hours (Silkina et al., 2019), to 41 hours (Beacham et al., 2014).

4.2 Ability to form survival structures

Survival structures allow organisms to survive for long periods of time, to withstand conditions that are not favourable for growth, and to survive passage through the gut of predators (Hargraves and French, 1983; Anderson et al., 2004).

A diverse range of microalgae, including diatoms (Phylum Bacillariophyta), dinoflagellates (Phylum Miozoa), raphidophytes (Phylum Ochrophyta), green microalgae and cyanobacteria contain representative species that form temporary cysts, resting cysts or resting spores (Anderson et al., 2004; Tomaselli, 2004; Smayda and Trainer, 2010).

In a review of the literature, one report was found to suggest that *Nannochloropsis* can form survival structures. Palanichamy and Rani (2004) investigated the survival of *N. oculata* stored at 3–5 °C over 18 months. Cultures remained viable, although with a reduction in cell size. The authors reported a change in colour of the culture, which they attributed to the formation of cysts, although it is unclear whether cysts were observed.

4.3 Dispersal

Microalgae are readily dispersed via aerosol formation. *Microchloropsis* and other ochrophytes have been detected in air samples (Tesson et al., 2016; Lewandowska et al., 2017). Microalgae enter the atmosphere by processes such as sea spray forming through wind friction or breaking waves (Tesson et al., 2016). Bubbles bursting in sea foam or in aerated aquariums eject microalgae into the air (Schlichting Jr, 1974). Their small size allows microalgae to be transported potentially over thousands of kilometres, remaining airborne for many days, before being deposited by particle settling or by removal via precipitation (Wilkinson et al., 2012; Tesson et al., 2016). Microalgae of the size of *Nannochloropsis* can cause nucleation of ice in the atmosphere, initiating their own deposition. The survival of aerially dispersed microorganisms depends on their ability to both withstand the abiotic stresses encountered during transit and to become established in the environment in which they are deposited.

A study on the short-range dispersal of GM *Scenedesmus dimorphus*, a freshwater microalgae in kingdom Plantae, from open cultivation ponds was conducted by Szyjka et al. (2017). At approximately 10 µm in length, these microalgae are slightly larger than *N. oceanica* (AlgaeBase, accessed 17 July

2019). The GM microalgae first reached dispersal traps at 5, 20 or 50 m distance approximately 15, 18 and 25 days after initial inoculation of the open ponds, respectively. Prevailing wind direction appears to have had some effect on the timing and frequency of detection of the GM microalgae in the traps at 5 m distance. Many other algae and fungi from the local environment, including *Nannochloropsis*, also colonised the dispersal traps during this time.

Leaks and spills are considered almost inevitable during the harvest of large scale cultures of microalgae, and can be a means of dispersal if not adequately contained or controlled (Beacham et al., 2017).

Microalgae may also be dispersed by animals, including birds, insects and humans (Beacham et al., 2017).

Marine micro-organisms, including microalgae, can be dispersed over long distances by ship transportation. Ships can carry microalgae in ballast water, in ballast tank sediments and within biofilms formed on tank surfaces (Drake et al., 2007). *Nannochloropsis* has the ability to survive in storage for many months (Section 4.2) and can tolerate a wide range of environmental conditions (Section 6). These properties allow the organism to withstand conditions during transport, and to establish and persist in a range of new environments. This is supported by the distribution of *N. oceanica* across a wide range of latitudes and marine water bodies (Section 2.1).

4.4 Flocculation

Flocculation is thought to occur when the normally negatively charged microalgal surface is neutralized or reduced by chemical or biological means, or with electrical impulses (Wrede et al., 2014). The aggregation of microalgal cells into clumps facilitates biomass harvesting in commercial cultures (Molina Grima et al., 2004). Aggregation of microalgal cells can also occur naturally, e.g. during blooms, causing a 'marine snow' of sinking cells (Wang et al., 2016a).

Nannochloropsis flocculation can be achieved using chemicals such as multivalent cations, e.g. aluminium sulfate, ferric chloride, or cationic polymers (Shen et al., 2013). The effectiveness of chemical flocculants depends on solution pH and ionic strength, flocculant dose, and microalgal strain.

Bacteria can create microalgal aggregates, which contribute to nutrient cycling in natural systems. A bacterium isolated from a groundwater aquifer was found to aggregate *N. oceanica* (Wang et al., 2012). Bacterial flocculation is being investigated as a means of harvesting microalgae in commercial cultures to avoid the use of chemical agents.

A *Bacillus* sp. bacterium was able to aggregate *N. oceanica* in the presence of calcium and magnesium ions within 30 seconds (Powell and Hill, 2013). Aggregation required a pH of 8 or above and was reversible. As aggregation occurred whether or not bacteria and/or algae were alive, it was concluded that cell surface properties were responsible.

The bacterium *Solibacillus silvestris* produces a proteoglycan molecule, which is able to aggregate *N. oceanica* (Wan et al., 2013). Maximum flocculation efficiency occurred at pH 8.7, was stable from 4–65 °C and did not require the addition of metal ions.

The fungus *Aspergillus fumigatus* is able to tightly bind a range of microalgal species, including *N. oculata* (Wrede et al., 2014). The authors hypothesized that binding occurs through the interaction between negatively charged microalgal cell surfaces and positively charged cell walls of fungal hyphae. The algal-fungal mixture pelletizes and flocculates, dropping out of solution.

Diatoms of an *Amphora* sp. contaminated a *Nannochloropsis* culture and via polysaccharide excretion caused the microalgal cells to stick together (Zmora and Richmond, 2004).

Autoinhibitory substances secreted by *Nannochloropsis* have been implicated in the aggregation of *Nannochloropsis* cells cultured in recycled growth medium (Rodolfi et al., 2003).

SECTION 5 BIOCHEMISTRY

Nannochloropsis oceanica is not a pathogen and not capable of causing disease in humans, animals or plants. Rather, *Nannochloropsis* is considered a beneficial component of food chains. Few studies have investigated the potential for *Nannochloropsis* to contain toxins, allergens, or substances that may have a detrimental effect on other organisms. *Nannochloropsis* is not reported to have been involved in any harmful algal events (IODE Harmful Algal Event Database, accessed 30 July 2019).

5.1 Toxins

Nannochloropsis is not known to produce toxins. Some studies, described below, have shown that the digestibility of *N. oceanica* is lower than fish meal, and that replacement of fish meal with *N. oceanica* may reduce animal growth rate.

No toxicological effects were observed in mice when 5–25% of the diet was supplemented with milled, freeze-dried *N. oceanica* for 14 days (Neumann et al., 2018). Liver concentrations of EPA increased, compared with the control diet. Protein quality and availability parameters did not differ significantly between diets incorporating processed *N. oceanica* and the control diet.

The half-maximal inhibitory concentration (IC_{50}) for *N. oceanica* strain F&M-M24 towards brine shrimp (*Artemia salina*) and human dermal fibroblasts was measured by Niccolai et al. (2017). IC_{50} values were calculated based on viability of brine shrimp and fibroblasts. An IC_{50} could not be calculated for *N. oceanica* for water extracts or methanolic extracts fed to brine shrimp, as these were not toxic at the highest concentration tested. The *N. oceanica* IC_{50} value for fibroblasts was 11.2 ± 2.1 g L^{-1}, which is considered low toxicity. The authors concluded that *N. oceanica* is substantially non-toxic.

Some species of marine and freshwater microalgae that cause algal blooms produce paralytic shellfish toxins, which are toxic to humans and animals. In a survey of 73 strains of Australian cultured microalgae across 11 taxonomic classes for paralytic shellfish toxin production, extracts of *N. oceanica*[8] did not inhibit binding in sodium channel and saxiphilin assays. This indicates that the species does not produce paralytic shellfish toxins (Negri et al., 2003).

Safety assessments have been carried out with *N. oculata.* In a feeding study, Kafaie et al. (2012) fed rats a single dose of dried *N. oculata* at 12 g kg^{-1} body weight and found no adverse effects after 14 days. Likewise, there were no adverse effects on weight gain, liver or kidney weight, plasma chemistry or mortality after rats were fed *N. oculata* at 3 or 6 g kg^{-1} body weight for 60 days. Similarly, Kagan and Matulka (2015) found no signs of toxicity in rats fed at least 10^8 viable cells of *N. oculata* daily for 14 days. This is equivalent to approximately 7 mg dry weight kg^{-1} body weight (FAO, 1996).

The digestibility of crude protein and lipids was reduced when 24–49% of the fish meal component of mink (*Mustela vison*) diets was substituted with *N. oceanica* (Skrede et al., 2011). The authors speculated that using a processing technique to rupture the microalgal cell wall could have provided better access to the cell contents by digestive enzymes. It is also possible that *N. oceanica* contains a lipase protein inhibitor, as do some distantly related algae (Bitou et al., 1999; Skrede et al., 2011; Ben Gara et al., 2017).

Reduced weight gain has also been noted when more than 10% of fish meal was replaced by defatted *N. oceanica* meal in the diet of Atlantic salmon, even though feed intake increased (Sørensen et al., 2017). Reduced digestibility of proteins and cell wall carbohydrates, compared with fish meal, was suggested to be the reason for the reduction in growth rate.

5.1.1 *Bioaccumulation of toxicants*

Although *Nannochloropsis* does not produce toxins, it may accumulate heavy metals and harmful compounds present in its environment.

[8] *Nannochloropsis* sp. (CS-246) identified as *N. oceanica* in Fawley et al. (2015).

Microalgal cells can adsorb metals, thereby altering metal bioavailability in water (Debelius et al., 2009). Metals are adsorbed either to the cell surface or taken up into the cell (Torres et al., 2017). Depending on the concentration of each metal in solution, *M. gaditana* accumulates up to 6.8 pg cell^{-1} copper and 1.5 pg cell^{-1} lead (Debelius et al., 2009).

Microchloropsis salina was shown to readily adsorb the water contaminants cadmium, lead, cobalt, zinc and copper, with adsorption increasing with increasing concentration of each metal in solution (Torres et al., 2017). Selenium, arsenic, chromium and nickel were also adsorbed, but at lower efficiencies. Biomass growth was reduced most by high concentrations of nickel and copper, followed by arsenic, zinc and chromium.

A species of *Nannochloropsis* isolated from an arsenic contaminated region in India was shown to ameliorate arsenic accumulation and toxicity in rice, when added as an inoculum in a hydroponic system (Upadhyay et al., 2016). The authors did not measure adsorption or accumulation of arsenic in the microalgae, but speculated that these may be mechanisms for reducing toxicity, along with algal-mediated transformation of arsenic.

Polychlorinated biphenyls (PCBs) are a group of compounds closely related to dioxins (FSANZ, 2004). Long-term exposure to dioxins leads to adverse effects, including developmental delays, thyroid hormone alterations and cancer. These compounds are persistent and widespread in the environment, and increase in concentration up food chains by accumulating in the fatty tissues of animals. Fish, crustaceans and molluscs are the major dietary contributors to PCB accumulation in Australian adults. PCBs are readily accumulated by *Nannochloropsis* in solution. Wang et al. (1998) demonstrated that PCBs accumulated in *N. oculata.* In a co-culture experiment, they further showed that brine shrimp larvae *(Artemia* sp.) also accumulate PCBs, ostensibly through ingestion of the contaminated microalgae, causing dose-related reductions in growth and larval concentration. This biomagnification of lipophilic organic compounds to higher trophic levels is supported by previous studies cited in Wang et al. (1998).

Azo dyes used by the textile industry can be harmful to human health and the environment if released in effluent water. Non-living cells of *N. oceanica* had a similar azo dye adsorption capacity as *Chlorella vulgaris* and *Spirulina* spp. (Zuorro et al., 2017).

5.2 Allergens

Allergic reactions have been reported towards some common airborne freshwater microalgae, such as *Chlorella* sp. (Tiberg et al., 1995; Tesson et al., 2016). Exposure to these microalgae is often via inhalation. Sensitization to microalgae may occur in a similar manner to sensitization to moulds.

A comprehensive literature search has not yielded any evidence of allergic reactions to *Nannochloropsis*.

5.3 Nutritional composition

5.3.1 *Fatty acids*

Nannochloropsis produces high levels of fatty acids. In one study, fatty acid profiles differed between species and strains of *Nannochloropsis*, with *N. oceanica* strains ranging from 2.90–8.35% EPA (Ma et al., 2014).

Relative abundances of lipids vary over time and under different nutrient conditions. Hulatt et al. (2017) grew *N. oceanica*[9] under nutrient-replete and nutrient-starved conditions. Polyunsaturated omega-3 and omega-6 fatty acid concentrations did not change markedly under different nutrient conditions; however saturated and monounsaturated fatty acids increased under low nutrient conditions (Table 2).

[9] *Nannochloropsis* sp. (CCAP 211/78) identified as *N. oceanica* in Fawley et al. (2015).

Under low nutrient conditions, fatty acid production reached 280.8 mg g^{-1} (or 28% of dry weight) after 16 days (Table 2). Studying the effect of varying light levels, Xiao et al. (2015) measured maximum fatty acid production of 408.2 mg g^{-1} (or 41% of dry weight) under high light conditions after 20 days of cultivation.

Nannochloropsis lipids also include cholesterol and phytosterols (Lu et al., 2014).

Table 2 Fatty acid concentrations (mg g^{-1} dry weight) in *N. oceanica* strain CCAP 211/78 under different nutrient conditions, according to Hulatt et al. (2017).

	High nitrate and phosphate			Low nitrate and phosphate		
Day	**8**	**12**	**16**	**8**	**12**	**16**
Saturated fatty acids	24.0 ± 0.7	27.0 ± 1.7	38.8 ± 4.2	29.9 ± 1.6	74.6 ± 2.1	122.4 ± 6.0
Monounsaturated fatty acids	22.5 ± 0.6	23.9 ± 3.0	32.7 ± 2.5	24.6 ± 3.3	55.9 ± 0.8	97.2 ± 3.1
Polyunsaturated fatty acids	44.4 ± 1.4	46.4 ± 5.7	53.9 ± 6.0	45.2 ± 2.1	55.9 ± 7.4	61.2 ± 3.0
Omega-3 fatty acids	41.5 ± 2.7	43.2 ± 3.6	49.3 ± 3.9	43.2 ± 1.1	45.0 ± 5.1	46.8 ± 2.4
Omega-6 fatty acids	2.8 ± 1.5	3.2 ± 2.6	4.7 ± 2.1	2.0 ± 2.1	10.9 ± 2.5	14.5 ± 0.6
Total fatty acids	90.8 ± 0.7	97.3 ± 10.1	125 ± 10.9	99.6 ± 6.4	186.3 ± 4.6	280.8 ± 6.0

Mean ± *SD*, number of samples = 3.
Saturated fatty acids: C14:0, C16:0, C18:0; Monounsaturated fatty acids: C16:1*n*-7, C18:1*n*-9;
Polyunsaturated fatty acids: C18:2*n*-6, C20:4*n*-6, C20:5*n*-3 (EPA).
Omega-3 fatty acids: C20:5*n*-3 (EPA); Omega-6 fatty acids: C18:2*n*-6, C20:4*n*-6.

5.3.2 *Proximate components and amino acids*

As mentioned in Section 5.3.1, the chemical composition of *N. oceanica* biomass can vary significantly under different growth conditions. In the experiments of Hulatt et al. (2017), protein concentration remained steady under replete nutrient conditions, but halved between days 8 and 16 under low nutrients.

The proximate composition and amino acid content for a *N. oceanica* product that is used as a replacement for fish meal is given in Table 3. Average mineral content for *Nannochloropsis* is given in Table 4.

Table 3 Proximate composition and amino acid content of *Nannochloropsis oceanica*, according to Skrede et al. (2011).

Proximate composition		**Essential amino acids** (g 16 g^{-1} nitrogen)		**Non-essential amino acids** (g 16 g^{-1} nitrogen)	
Dry matter (DM; g kg^{-1})	949	Lysine	4.8	Aspartic acid + asparagine	7.0
Crude protein (g kg^{-1} DM)	477	Threonine	3.6	Serine	3.3
Fat (g kg^{-1} DM)	84.1	Methionine	1.8	Glutamic acid + glutamine	9.7
Starch (g kg^{-1} DM)	0.3	Tryptophan	1.7	Proline	9.8
Ash (g kg^{-1} DM)	74.8	Valine	4.6	Glycine	3.8
Organic matter (g kg^{-1} DM)	874	Isoleucine	3.5	Alanine	5.2
		Leucine	6.7	Tyrosine	2.4
		Phenylalanine	3.9	Cysteine + cystine	0.7
		Histidine	1.5		
		Arginine	4.9		

Table 4 Mean mineral content of *Nannochloropsis* grown in seawater with F/2 medium nutrients, according to Rebolloso-Fuentes et al. (2001).
Components of F/2 medium are given in Table 5.

Mineral	mg 100 g^{-1} dry biomass
Sodium	659 ± 50
Potassium	533 ± 182
Calcium	972 ± 60
Magnesium	316 ± 22
Iron	136 ± 14
Zinc	103 ± 3
Copper	35.0 ± 1.4
Manganese	3.4 ± 0.1
Lead	0.38 ± 0.08
Cadmium	0.028 ± 0.02
Chromium	0.37 ± 0.07
Nickel	0.22 ± 0.09
Cobalt	<0.1
Sulfur	529 ± 119

5.3.3 *Bioactive compounds*

Nannochloropsis produces compounds that may be beneficial in mitigating inflammatory diseases and cancers (Talero et al., 2015). The fatty acids EPA and docosapentaenoic acid have anti-inflammatory properties. The anti-inflammatory and antioxidant properties of the carotenoid zeaxanthin are associated with protection against macular degeneration and colorectal cancer, and beneficial effects are ascribed to the carotenoid astaxanthin (Pan et al., 2011; Talero et al., 2015). *Nannochloropsis* phenolic compounds may have antioxidant activity (Talero et al., 2015).

SECTION 6 ABIOTIC INTERACTIONS

6.1 Light and energy requirements

Nannochloropsis oceanica is an obligate photoautotroph with facultative heterotrophy. As a photoautotroph, *Nannochloropsis* fixes carbon dioxide via photosynthesis.

In mixotrophic growth, the microalgae use both an organic carbon source along with fixed carbon dioxide (Grobbelaar, 2004; Lee, 2004; Cheirsilp and Torpee, 2012). A culture supplied with organic carbon may alternate between heterotrophic growth at night and mixotrophic growth during the day (Grobbelaar, 2004). Mixotrophic growth, with the addition of glucose, was reported by Pagnanelli et al. (2014) to increase the growth rate of *N. oculata*.

The ability of *Nannochloropsis* to grow under heterotrophic conditions, i.e. in the dark, is unclear. In heterotrophic culture *Nannochloropsis* uses organic carbon as an energy source and does not photosynthesise. Cheirsilp and Torpee (2012) found that *Nannochloropsis* grew equally well in photoautotrophic and heterotrophic systems; in mixotrophic culture the strain produced double the biomass of strains grown in the other systems. Conversely, Vazhappilly and Chen (1998) reported a strain of *N. oculata*, which was unable to grow at all heterotrophically. Similarly, Gladue and Maxey (1994) found that only four of twelve *Nannochloropsis* strains tested were able to grow heterotrophically, and that heterotrophic growth was very slow in these four strains.

The biomass growth rate of microalgae increases with increasing light intensity up to a maximum point, after which the growth rate declines due to photoinhibition[10] (Chisti, 2007). This maximum point can vary with temperature, nutrient status and growth conditions of the cells, making inter-study comparisons difficult. For example, using the same model *N. oceanica* IMET1 strain, Chi and Takiguchi (2015) obtained maximum and inhibited growth rates at 53 and 132 µmol photons m^{-2} s^{-1}, respectively, when grown in microplates; whereas Xiao et al. (2015) obtained maximum productivity at 331 µmol photons m^{-2} s^{-1} when grown in culture tubes[11].

In dense cultures of microalgae, such as in photobioreactors, individual microalgal cells experience a range of light intensities due to the constant circulation of the culture solution. Light loses intensity as it penetrates the culture, so cells may travel from unsaturated light zones to saturated zones over the course of several seconds (Molina Grima et al., 2000). When cells move rapidly between dark and light zones, e.g. every 10 ms, productivity can be greater than if cells are exposed to the same amount of light without fluctuation (Chisti, 2007). Flashing light-emitting diodes (LEDs) make use of this effect to optimize growth in photobioreactors (Schulze et al., 2017). However, prolonged exposure to high intensity light at the upper surface of the culture may lead to photoinhibition (Masojídek et al., 2004).

In sunlit systems, light intensity varies over the course of the day and may result in photoinhibition during maximum irradiance around noon (Richmond, 2004). When light intensity changes over a longer period of time, microalgae adjust the concentration of light-harvesting pigments in the process of photoacclimation (Vonshak and Torzillo, 2004).

Microalgae are also able to acclimate to variations in light spectra. Photosynthetic parameters vary depending on the wavelengths to which *Nannochloropsis* is exposed (Vadiveloo et al., 2017). When grown under blue wavelengths *Nannochloropsis* has greater biomass productivity than under red wavelengths.

6.2 Nutrient requirements

On average, the ratio of carbon, nitrogen and phosphorus in marine plankton is very similar to the ratio of these elements in seawater, at approximately 100:16.7:1.85, respectively (Redfield, 1934). To allow growth of microalgal biomass, these nutrients must be available in the culture solution at roughly this ratio (Chisti, 2007).

In large-scale microalgal aquaculture, seawater is supplemented with nitrogen, phosphorus and micronutrients using commercial fertilisers, along with carbon dioxide during daylight hours (Chisti, 2007). In the laboratory *Nannochloropsis* is generally cultured in Guillard's F/2 medium, with components given in Table 5 (Guillard and Ryther, 1962; Guillard, 1975).

[10] Photoinhibition is the result of excessive or prolonged exposure to light temporarily damaging the reaction centre of photosystem II (Vonshak and Torzillo, 2004; Nikolaou et al., 2015).

[11] Full sunlight corresponds to a light intensity of 2000 µmol photons m^{-2} s^{-1} (Demmig-Adams and Adams III, 2000).

Table 5 Components of F/2 medium, according to Guillard (1975).

Nutrient	mg L^{-1}
Sodium nitrate ($NaNO_3$)	75
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	5
Sodium metasilicate ($Na_2SiO_3.9H_2O$)[a]	15–30
EDTA disodium (Na_2.EDTA+)	4.36
Ferric chloride ($FeCl_3.6H_2O$+)	3.15
Copper(II) sulfate ($CuSO_4.5H_2O$)	0.01
Zinc sulfate ($ZnSO_4.7H_2O$)	0.022
Cobalt chloride ($CoCl_2.6H_2O$)	0.01
Manganese chloride ($MnCl_2.4H_2O$)	0.18
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.006
Thiamine.HCl	0.1
Biotin	0.5 µg L^{-1}
Vitamin B_{12}	0.5 µg L^{-1}
Seawater	to 1 litre

[a] may be omitted for non-diatom cultures

Manipulating the concentration of nutrients in solution changes the growth rate and lipid accumulation of *Nannochloropsis.* Under low nitrogen and phosphorus conditions, biomass and protein content decrease, while fatty acid concentrations increase (Hulatt et al., 2017). The fatty acid profile of *N. oceanica* also changes when nutrients are limited (see Section 5.3.1, Table 2).

A similar increase in lipid production in nitrogen-starved *N. oceanica* was noted by Dong et al. (2013). Consistent with the results of Hulatt et al. (2017), nitrogen depletion significantly increased the concentration of C14:0, C16:0, C16:1*n*-9 and C18:1*n*-9 fatty acids, with no significant change in EPA. Chlorophyll *a*, violaxanthin and carotenoids decreased under low nitrogen, but zeaxanthin concentration increased, causing cells to change colour from green through yellow to light yellow (Dong et al., 2013). This increase in zeaxanthin was suggested to provide photoprotection from excess light not able to be used for photosynthesis. Upon reintroduction of nitrate into the system, cells recovered quickly and regained their green colour.

The addition of indole-3 acetic acid (IAA), a natural auxin, to *N. oceanica* cultures results in changes to the growth rate and fatty acid profile (Udayan and Arumugam, 2017). After an initial lag phase, cell concentration doubled in IAA treated cultures. In *N. oceanica* CASA CC201 the production of EPA increased from 1.87% of TFA (total fatty acids) to 10.76% when IAA was added at 40 ppm.

6.3 Temperature requirements and tolerances

Nannochloropsis oceanica grows well up to approximately 30 °C. Sandnes et al. (2005) noted that the growth rate rapidly declines to zero between 30–35 °C; however Lai (2015) recorded substantial growth at 35 °C. The optimum growth temperature for *N. oceanica* in laboratory cultures is 25–29 °C, with optimal temperature increasing with increasing light intensity.

At the other end of the scale, cultures of *N. oculata* are able to remain viable for 18 months when stored at 3–5 °C in a laboratory refrigerator (Palanichamy and Rani, 2004). The freshwater species *N. limnetica* appears to be well adapted to cold water temperatures approaching 0 °C, with blooms occurring in spring in Germany (Fawley and Fawley, 2007).

Low temperature limits carbon metabolism in microalgae and may lead to photoinhibition, even under low light conditions (Vonshak and Torzillo, 2004). This can limit productivity in outdoor cultures when there is a lag in water warming at the beginning of the day.

Total fatty acid production increases with increasing temperature from 14–30 °C under elevated carbon dioxide; however EPA as a percent of TFA decreases (Hu and Gao, 2006).

6.4 Salinity

Rapid changes in salinity result in osmotic stress; however microalgae can acclimate to large changes in salinity in gradual increments (Vonshak and Torzillo, 2004). Reducing the salinity of a culture of *M. salina* from 90% seawater to 10% seawater in 20% increments over the course of two months, Beacham et al. (2014) noted that greater dilution increments would cause cultures to die. The reduction in salinity resulted in a 20% increase in cell wall thickness.

Nannochloropsis oceanica[12] grows well at salt concentrations of 22–49 g L^{-1}, equivalent to 63–140% seawater, with best growth under elevated carbon dioxide at 31 g L^{-1}, i.e. 89% seawater (Hu and Gao, 2006). When grown in water at the lower end of this salinity range, TFA and EPA as a percentage of TFA, increase.

It is possible to develop strains of *N. oceanica* that are able to grow in both seawater and freshwater media. Adaptation of *N. oceanica* to freshwater medium was achieved by Guo et al. (2018) through incremental changes from f/2 medium to BG11 medium every month over a period of approximately four years. The resulting freshwater-adapted strain of *N. oceanica* grew better in both freshwater and seawater culture than the wild type strain.

6.5 Other abiotic stresses and tolerances

High oxygen concentration inhibits photosynthesis in microalgae, but may provide a level of photoprotection during high light stress (Vonshak and Torzillo, 2004).

The pH of cultivated *Nannochloropsis* is usually in the range of pH 7–10 (Fulbright et al., 2016), but cultures can tolerate large decreases in pH for several hours (see Section 7.4).

Strains of *N. oceanica* are sensitive to the antibiotics zeocin, paromomycin and hygromycin B (Vieler et al., 2012). All *Nannochloropsis* strains tested were resistant to low concentrations of the antibiotics rifampicin, benomyl and nystatin, and higher concentrations of spectinomycin, ampicillin and chloramphenicol.

Growth inhibitory substances may accumulate over time in *Nannochloropsis* cultures, requiring regular replacement of growth medium (Richmond, 2004).

SECTION 7 BIOTIC INTERACTIONS

7.1 Weeds

Large-scale cultures of *Nannochloropsis* may become contaminated with other microalgae. Common weedy microalgae include *Tetraselmis* and *Chlorella* (Fulbright et al., 2016).

7.2 Pests and diseases

7.2.1 *Predators*

Being at the bottom of the food web, *Nannochloropsis* is a source of energy and nutrients for many organisms. The main consumers of marine primary producers are microzooplankton (Calbet and Landry, 2004). Further up the food chain are copepods, planktivorous fish and primary predatory fish (Sommer et al., 2002).

Large-scale microalgal culture systems are prone to contamination by predatory protozoa, fungi and bacteria (Borowitzka and Vonshak, 2017). The flagellate grazer *Paraphysomonas imperforata* can cause a culture of *Nannochloropsis* to crash within 24 hours (Zmora and Richmond, 2004). *Euplotes* sp. is a protozoon grazer that predates *Nannochloropsis* and can cause cell aggregation. A flagellated

[12] *Nannochloropsis* sp. (PP983) identified as *N. oceanica* in Li et al. (2014).

heterokont, *Pseudobodo* sp., exhibited predatory activity towards several microalgal species, including *Chlorella vulgaris*, and weak predation towards *N. oceanica* (Chen et al., 2014).

7.2.2 *Pathogens*

Nannochloropsis can be infected with unspecified marine viruses (Suttle et al., 1991).

7.3 Interactions with other microorganisms

Microalgae often interact closely with bacteria, with bacteria being more abundant in the phycosphere around microalgae than in the surrounding solution (Wang et al., 2016a). In an open *Nannochloropsis* culture system, the concentration of bacteria has been measured at between 7.72 x 10^5 and 2.39 x 10^6 cells mL^{-1} (Nakase and Eguchi, 2007).

Interactions between microalgae and bacteria are many and varied. Bacteria can inhibit or stimulate the growth of microalgae, just as microalgae influence the growth of associated bacteria (Natrah et al., 2014).

Bacteria may provide beneficial services to microalgae, such as consuming oxygen to produce carbon dioxide, fixing nitrogen into ammonia that can be used by microalgae, producing nutrients such as vitamin B_{12}, and producing hormones, e.g. IAA, to stimulate growth. Microalgae may benefit bacteria by producing oxygen and organic compounds (Natrah et al., 2014). Cell-to-cell communication between microalgae and bacteria may be positive or negative.

Negative impacts occur through competition for nutrients and through bacterial production of algicidal compounds or microalgal production of antibacterial compounds. Some bacteria, fungi and diatoms are able to cause aggregation of *Nannochloropsis*, causing cells to flocculate and precipitate (Section 4.4).

The production of algicidal molecules has been reported by bacteria, cyanobacteria and eukaryotic microalgae (Demuez et al., 2015). Algicidal molecules have different modes of action, such as toxicity to microalgae or by causing damage to cells. Cellulolytic bacteria degrade microalgae by secreting enzymes that break down cell wall molecules.

A bacterial contaminant of an *M. salina* culture, *Bacillus pumilus*, inhibited growth of *M. gaditana* once the ratio of bacterial to microalgal cells reached 100:1 (Fulbright et al., 2016). The inhibitory effect was likely due to molecules secreted by stationary phase *B. pumilus*. The presence of *B. pumilus* in a co-culture of *M. gaditana* and the weedy microalgae *Tetraselmis striata* allowed *T. striata* to rapidly take over the culture, as the molecules secreted by the bacteria did not affect *T. striata*.

Bacterial cellulolytic activity may be species-specific. Cellulolytic activity was observed in six species of bacteria isolated from mussels and clams, with two strains of *Raoultella ornithinolytica* causing significant degradation of *M. gaditana* in co-culture experiments (Muñoz et al., 2014). It was suggested that these bacteria may act in symbiosis with bivalve molluscs to increase nutrient bioavailability of microalgae.

The bacterium *Sagittula stellata* exhibits strong algicidal activity towards *N. oculata* (Wang and Yuan, 2014).

7.4 Controlling contaminants of *Nannochloropsis* cultures

The contamination of microalgal cultures with other microorganisms is a major challenge, particularly in open pond cultivation systems (McBride et al., 2014). Maintaining optimum growth conditions and monitoring physical parameters of cultures helps to reduce the likelihood of contamination (Borowitzka and Vonshak, 2017).

Various methods have been proposed to maintain the dominance of *Nannochloropsis* in cultivation systems. These include adding sodium hypochlorite, and adjusting salinity and temperature (Weissman and Radaelli, 2015). *Nannochloropsis* is able to tolerate high concentrations of disinfectants, including chlorine, chlorine gas, chloride salts, iodine, other halogens and ozone,

allowing these chemicals to potentially be used to reduce contaminants in cultures (Weissman and Radaelli, 2015). Contaminants of *Nannochloropsis* cultures may be controlled by lowering the culture pH for several hours, before returning the pH to 8.0–8.5 (Zmora and Richmond, 2004). Diatoms are removed at pH 6, while *Paraphysomonas* control requires a pH of 2.5.

According to McBride et al. (2014), however, the strategies listed in the previous paragraph have not been successful in open pond systems. The authors describe a management strategy that includes monitoring and identifying the contaminating organisms, and applying a pesticide targeted at the particular contaminant when pest numbers reach a predetermined threshold.

In laboratory cultures, antibiotics can be used to control contaminants. *Nannochloropsis* is resistant to several antibiotics, including spectinomycin, ampicillin and chloramphenicol (Vieler et al., 2012).

SECTION 8 ALGAL BLOOMS

Algal blooms occur when there is a rapid increase in the population of a single species of phytoplankton (Shumway, 1990; Irigoien et al., 2004). Blooms of toxic algae, such as some dinoflagellates, can lead to accumulation of toxins in shellfish; however blooms of non-toxic algae can also have severe impacts on ecosystems (Shumway, 1990). Decaying algae remove oxygen from water, leading to anoxia, killing marine plants and animals. Microalgal cells can clog the gills of filter-feeding organisms. Under anaerobic conditions methane and other greenhouse gases can be produced (Usher et al., 2014).

Shumway (1990) lists factors thought to promote blooms:

- nutrient enrichment (eutrophication)
- decreased grazing pressure
- large scale hydrometeorological changes
- upwelling of nutrient rich bottom water
- heavy precipitation and fresh water run off
- the presence of previous blooms of other phytoplankton species.

Blooms occur when nutrient conditions do not limit the growth of algae. Nitrogen is the major limiting nutrient in tropical and subtropical marine waters (Beman et al., 2005). Water running off from fertilized crops provides a source of nitrogen that can stimulate phytoplankton bloom formation.

8.1 Overgrowth of *Nannochloropsis* spp.

No algal blooms caused by *N. oceanica* have been reported to date; however other species of *Nannochloropsis* have been implicated in blooms.

The first report of a large-scale algal bloom caused by *N. granulata* occurred off the coast of China in 2011 (Zhang et al., 2015). The bloom area covered approximately 180 km^2, with algal cell densities at $10^9 – 10^{10}$ cells per litre.

Nannochloropsis limnetica has been isolated from freshwater blooms in Germany (Fawley and Fawley, 2007).

These blooms are not recorded in the IODE Harmful Algal Event Database (accessed 30 July 2019).

8.2 Control measures

Algal blooms occurring naturally are terminated by various means. Nutrient depletion limits further biomass production, while strong tidal activity and winds may dissipate blooms (Cloern, 1996). In several cases, viruses have been associated with the decline of natural phytoplankton blooms (Brussaard, 2004). Algicidal bacteria can kill and lyse microalgae, and are implicated in controlling blooms (Mayali and Azam, 2004).

Many algicides are registered for use in Australia (APVMA PubCRIS database, accessed 28 September 2017). As discussed in Section 1, the term ‘algae’ covers a broad range of organisms. Algicidal ingredients include chlorine (e.g. as sodium hypochlorite), copper, bromine, acetic acid, nonanoic acid,

hydrogen peroxide, the phenylurea herbicide diuron, the triazine herbicide simazine, and the dithiocarbamate fungicide mancozeb. Some products are registered for use in pools and dams.

Half-maximal effective concentrations (EC_{50}) for growth inhibition of *M. gaditana* by copper solutions is 137 ± 20 ppb (Debelius et al., 2009). *Microchloropsis gaditana* is relatively tolerant to copper and other metals in solution, compared with other microalgae. This may be due to comparatively lower cell membrane permeability and higher adsorption capacity.

Nannochloropsis is sensitive to some antibiotics (see Section 6.5). Flocculants may be used to cause microalgal cells to aggregate and precipitate from solution (see Section 4.4).

SECTION 9 POTENTIAL FOR GENE TRANSFER

9.1 Vertical gene transfer

Nannochloropsis appears to be incapable of meiosis, as it lacks many of the necessary genes (Kilian et al., 2011). Thus, the microalga does not reproduce sexually and vertical gene transfer only occurs via clonal reproduction.

9.2 Potential for intra- and interspecific crossing

As sexual reproduction has not been reported in *Nannochloropsis* (Al-Hoqani et al., 2017), intra- or interspecific crossing is improbable.

9.3 Horizontal gene transfer

Horizontal gene transfer (HGT) and multiple-genome pooling through endosymbiosis events has occurred during the evolutionary history of *Nannochloropsis* (see Section 1.2). No evidence for HGT from *Nannochloropsis* to other organisms has been identified.

Eukaryotic microalgae, such as *Nannochloropsis*, are less capable of HGT than cyanobacteria (Henley et al., 2013). Certain species of cyanobacteria and *Escherichia coli* bacteria are able to exchange DNA via conjugation (Flores and Wolk, 1985). Horizontal gene transfer between these organisms can be observed in the laboratory (Lee, 2019). Transfer of DNA from *E. coli* to two eukaryotic diatom species via conjugation was recently achieved in the laboratory (Karas et al., 2015).

SUMMARY

This document provides baseline information about the microalga *Nannochloropsis oceanica* Suda & Miyashita. The information included relates to the taxonomy and origins of strains; cultivation and commercial uses; general descriptions of morphology, reproductive biology, biochemistry, abiotic and biotic interactions, and methods of control; and the potential for gene transfer to closely related species. The purpose of this baseline information is to inform risk analyses of genetically modified forms of the species that may be released into the Australian environment.

The species *N. oceanica* was first described in 2002. Information specific to the biology of *N. oceanica* is limited and, thus, has been supplemented with information from closely related species where necessary. The document will be updated as further information about *N. oceanica* arises.

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