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Mixotrophy in diatoms: Molecular mechanism and industrial potential

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Abstract

Diatoms are microalgae well known for their high variability and high primary productivity, being responsible for about 20% of the annual global carbon fixation. Moreover, they are interesting as potential feedstocks for the production of biofuels and high-value lipids and carotenoids. Diatoms exhibit trophic flexibility and, under certain conditions, they can grow mixotrophically by combing photosynthesis and respiration. So far, only a few species of diatoms have been tested for their mixotrophic metabolism; in some cases, they produced more biomass and with higher lipid content when grown under this condition. *Phaeodactylum tricornutum* is the most studied diatom species for its mixotrophic metabolism due to available genome sequence and molecular tools. However, studies in additional species are needed to better understand the conservation of this process in diatoms and its potential in industrial applications. Here, we describe the photosynthetic and respiratory pathways involved in mixotrophy and provide an overview of the trophic variability in diatoms. This review also highlights promising areas of industrial applications for diatoms when cultivated under mixotrophy.

1 | INTRODUCTION

Microalgae are unicellular microscopic photosynthetic eukaryotes that populate both marine and fresh waters. Among microalgae, diatoms are responsible for about 20% of the annual global carbon dioxide fixation via photosynthesis. They are the most heterogeneous group of phytoplankton, counting about 20,000 different species that range in diameter from 5 μm to few millimetres. They are surrounded by a silica skeleton, also known as the frustule (Round et al., 1990). Based on the symmetry of the cell, diatoms can be pennate (with bilateral symmetry) or centric (with radial symmetry).

A peculiarity of diatoms is their pigment profile that is quite different from that found in land plants and green algae. In fact, while diatoms possess chlorophyll *a* and *c*, green algae and land plants possess chlorophyll *a* and *b* (Green, 2011). They also have a large amount of a xanthophyll-type of carotenoid named fucoxanthin (Fx), which

gives the golden-brown colour. Trimeric and oligomeric complexes of fucoxanthin-chlorophyll-proteins in the thylakoid membrane are involved in light harvesting. The light-harvesting complexes in land plants and green algae instead contain the carotenoid lutein as auxiliary pigment. Due to this difference in pigment composition, diatoms are able to harvest light in the blue-green region as compared to the red-blue region preferred by land plant and green algae (Baldisserotto et al., 2019). Diatoms also possess other xanthophylls, such as diadinoxanthin, diatoxanthin, violaxanthin, antheraxanthin and zeaxanthin, with the main function in photoprotection against excess light (reviewed in Kuczynska et al., 2015).

Due to their high complexity and ecological success, diatoms are used as a model to study photosynthesis and photoprotective mechanisms. Furthermore, their ability to accumulate lipids for energy storage (20%–80% of dry weight) makes them industrially attractive for the production of biofuels and high-value compounds. Due to their

biological and industrial relevance as well as relatively small genomes, the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* were the first and the third microalgae species to be sequenced, respectively (Bowler et al., 2008). The sequencing projects revealed the conservation of many genes between the two species, which can be reconducted to their common complex evolutionary history, that is, they originated from secondary endosymbiosis between a heterotrophic ancestor and a red alga (Green, 2011). In addition to hundreds of genes derived from red algae and the animal-derived ornithine-urea cycle, hundreds of bacterial genes were discovered as proof of horizontal gene transfer (Bowler et al., 2008). The genome of the oleaginous diatom *Cyclotella cryptica* was more recently sequenced and compared with that of *T. pseudonana* (Traller et al., 2016). The authors revealed substantial differences between the two species in the pyruvate, carbohydrate and triacylglycerols (TAGs) metabolisms that could explain the higher lipid productivity in *C. cryptica*. In addition, thanks to the development of genetic transformation methods, it has become possible to modify specific pathways in *Fistulifera solaris*, *P. tricornutum* and *T. pseudonana* leading to an increase in the production of polyunsaturated fatty acids (PUFAs) and TAGs (Cui et al., 2018; Manandhar-Shrestha & Hildebrand, 2015; Muto et al., 2015). Another way to stimulate lipid metabolism is the assimilation of carbon from an organic source thanks to the activation of both photosynthesis and respiration, a process known as mixotrophy (Kitano et al., 1997, Villanova et al., 2017). The mechanism and the consequences of mixotrophy have been investigated in detail only in *P. tricornutum*, where it was possible to elucidate the main route of glycerol metabolism thanks to -omics and isotope labelling experiments (Huang et al., 2015, Villanova et al., 2017). However, to better understand the environmental adaptation and other conserved pathways in diatoms, it is necessary to sequence the genomes of other ecologically relevant diatom species.

A few previous reviews focused on mixotrophy in microorganisms such as bacteria and dinoflagellates (Matantseva & Skarlato, 2013), plankton from the Polar Seas and other microalgae (Pang et al., 2019). This minireview aims to give an overview of the trophic variability in diatoms with focus on mixotrophy and to highlight promising areas of industrial applications when cultivated under this trophic mode, such as biofuel production and wastewater treatment.

2 | PHOTOAUTOTROPHY, HETEROTROPHY AND MIXOTROPHY: PRINCIPLES AND MECHANISMS

Photoautotrophy is the major trophic mode among photosynthetic organisms, including diatoms, where sunlight is used as an energy source to fix inorganic carbon (CO_2) into organic compounds (carbohydrates). The underlying process, photosynthesis, occurs in two steps: (1) sunlight energy is captured and converted into ATP (the so-called light-dependent reactions), and (2) ATP is used to fix CO_2 into carbohydrates (the so-called carbon fixation reactions). The photosynthetic reactions are well conserved, but some of the involved proteins are different in diatoms from the rest of the photoautotrophs.

The light-dependent reactions begin with the absorption of sunlight energy through the antenna complex and proceed with the transfer of energy to reaction centres. In diatoms, the complex is represented by the fucoxanthin chlorophyll *a/c*-binding proteins (Lang & Kroth, 2001). Here, this energy is used for charge separation and electron flow from water to $NADP^+$ through a photosynthetic chain that, in diatoms, is comprised by Photosystem II (PSII), plastoquinone (PQ), cytochrome b_6f (cyt b_6f), cytochrome c_6 (cyt c), Photosystem I (PSI), ferredoxin (Fd) and Fd-$NADP^+$-oxidoreductase (FNR). This process represents the linear electron flow (LEF), but electron transport can also occur in a cyclic mode known as the cyclic electron flow (CEF). CEF involves the electron transfer either from Fd or from NADPH and the PQ pool back to the cyt b_6f, cyt c and then again to PSI (Figure 1A, Allen, 2003). In both green algae and land plants, the CEF is the main alternative electron flow used to balance the ATP/NADPH ratio, while this is regulated by the energetic exchanges between the chloroplast and the mitochondrion in diatoms (Bailleul et al., 2015). Both LEF and CEF generate a proton-motive force across the thylakoid membrane, composed of an electric field ($\Delta\Psi$) and a pH gradient (ΔpH), both used to build ATP through the H^+-pumping ATP-synthase.
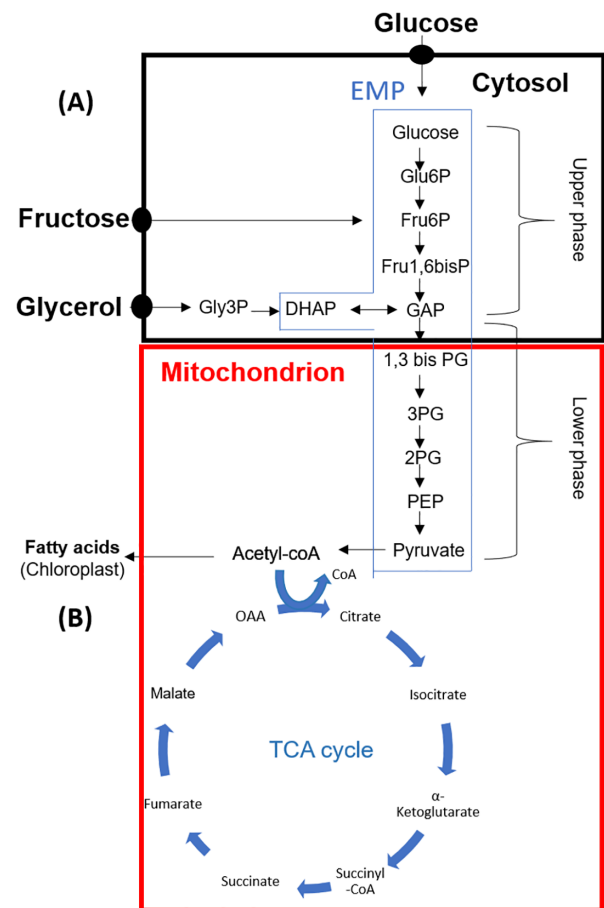
The NADPH and ATP generated during the light-dependent reactions are then used for carbon fixation into carbohydrates. Photoautotrophs can fix CO_2 by a C3 pathway (Calvin-Benson-Bassham cycle, CBB) with the formation of the 3-carbon compound phosphoglycerate (PGA) and a C4 pathway with the formation of the 4-carbon compound oxaloacetate (OAA). Both C3- and C4-types of compounds were found in diatoms, suggesting that both pathways are functional (Roberts et al., 2007). The key enzyme in the C3 pathway is Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (RuBisCO), which catalyses a reaction between CO_2 and D-ribulose-1,5-bisphosphate (RuBP). However, RuBisCO is also capable of fixing O_2 into RuBP during a process known as photorespiration. The two reactions are competitive since, at a high CO_2 concentration (and low O_2 concentration), the enzyme performs carbon fixation instead of photorespiration, and vice versa (Parker et al., 2004). Reinfelder et al. (2000) found that the C3- and C4-pathways can occur simultaneously in diatoms thanks to their different cellular location, namely the chloroplast and cytosol, respectively. During the C4 pathway, bicarbonate (HCO_3^-) is first fixed in the cytosol into OAA and then into malate that is transported into the chloroplast. The reactions of C3- and C4-types of carbon fixation and photorespiration in diatoms are shown in Figure 1B.

Diatoms are among the most productive organisms in the ocean thanks to their high flexibility to environmental changes and their ability to grow under low CO_2 concentration. Indeed, they have developed a CO_2 concentrating mechanism (CCM) relying on the transport of HCO_3^- into the chloroplast and the activity of the enzyme carbonic anhydrase. This enzyme catalyses the conversion of HCO_3^- into CO_2, and so increases the concentration of the latter near the catalytic site of RuBisCO. The C4 pathway also increases the efficiency of RuBisCO, particularly when there is a problem with the CCM (Reinfelder et al., 2000).
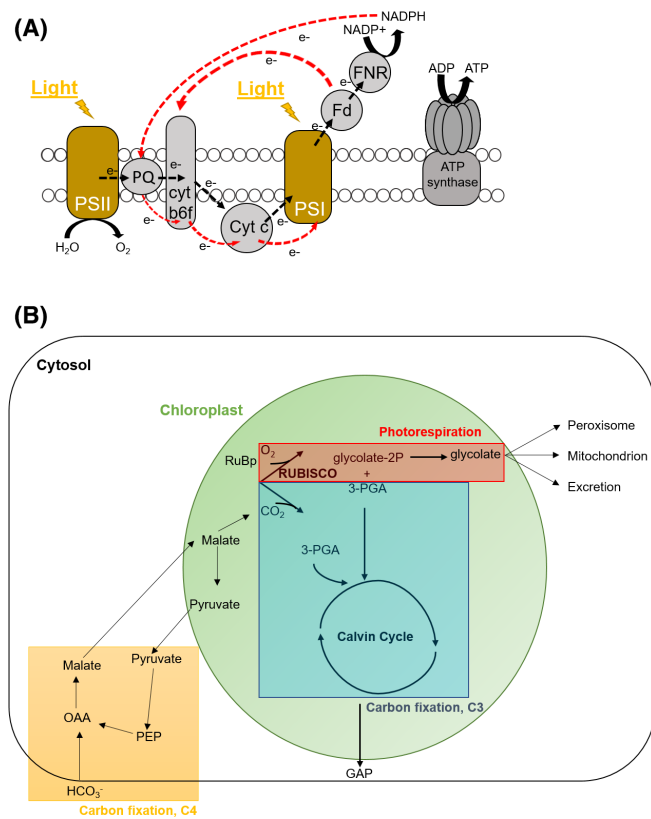
FIGURE 1 Schematic representation of the photosynthetic reactions in diatoms. (A) Linear (LEF) and cyclic electron transfer (CEF). The LEF goes from water to $NADP^+$ through photosystem II (PSII), cytochrome b_6f (cyt b_6f) and PSI (black dotted lines); the CEF represents the electron transfer around PSI (dotted red lines). (B) Carbon fixation C3, C4 and photorespiration. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) can catalyse both the carboxylation (carbon fixation C3) and the oxygenation (photorespiration) of the ribulose-1,5-biphosphate (RuBP). The photorespiratory pathway (red box) forms glycolate that can be directed to other cellular compartments or is excreted outside the cell. The carbon fixation C3 (blue box) brings to the formation of 3-phospho-D-glycerate (3-PGA) that enters the Calvin-Benson-Bassham cycle (CBB) and forms glyceraldehyde-3-phosphate (GAP). The latter can take part in various metabolic pathways leading to the formation of other organic compounds. During the C4-type of carbon fixation (yellow box) HCO_3^- is fixed into oxaloacetate (OAA) and then into malate that can be transported to the chloroplast supplying CO_2 to CBB

FIGURE 2 Main pathways involved in sugar metabolism in diatoms. (A) The Embden–Meyerhof-Parnas pathway is divided into upper phase in the cytosol and lower phase in the mitochondrion. The end product pyruvate is then converted to acetyl-CoA that can enter either the TCA cycle or the fatty acid synthesis. (B) The TCA cycle takes place in the mitochondrion where the oxidation of the acetyl-CoA leads to the formation of CO_2, NADH and ATP. EMP: Embden-Meyerhof-Parnas, Glu6P, glucose 6-phosphate; Fru6P, fructose 6-phosphate; Fru1,6bisP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; Gly3P, glycerol 3-phosphate; 1,3 bis PG, 1,3 bisphosphoglycerate; 3PG, 3-phosphoglycerate, 2PG: 2-Phosphoglycerate, PEP, phosphoenolpyruvate; TCA, tricarboxylic acid cycle

Certain species of diatoms can also grow in darkness by assimilating organic carbon substrates during respiration (Armstrong et al., 2000; Kamikawa et al., 2015; Pahl et al., 2010), a process known as heterotrophy. Heterotrophs can be divided into two categories, namely obligate heterotrophs that lack photosynthetic pigments and are not able to perform photosynthesis, and facultative heterotrophs that can separately perform photosynthetic and respiratory metabolisms. The heterotrophic organisms are able to take energy from the assimilation and oxidation of an external carbon source (e.g., glucose, fructose and glycerol). Once the organic carbon is taken up by the cell through membrane transporters, it is first phosphorylated before entering glycolysis. The degradation of these phospho-compounds produces ATP and pyruvate that can enter various metabolic pathways. In particular, diatoms possess an atypical glycolytic pathway known as the Embden–Meyerhof-Parnas pathway (EMP). The upper phase of the EMP pathway is located in the cytosol, while the lower phase is located in the mitochondrion (Figure 2A) (Kroth et al., 2008). The glycolytic end-product, namely pyruvate, is converted into acetyl-CoA by the pyruvate dehydrogenase that can enter either the tricarboxylic acid cycle (TCA cycle) or the fatty acids synthesis (Figure 2A). The TCA cycle takes place in the mitochondrion and involves the oxidation of acetyl-CoA to CO_2, ATP and NADH (Figure 2B). This pathway provides intermediates useful for another pathway, for example, malate can enter the lipid metabolism.

Mixotrophy is the trophic mode in the presence of both light and organic carbon, thanks to the simultaneous activation of both respiration and photosynthesis. Molecular mechanisms of photosynthesis and respiration in diatoms have been widely investigated, but only a few works have been focused on the communication between these processes during mixotrophy (Bailleul et al., 2015; Villanova et al., 2017). In *P. tricornutum*, the transport activities between the chloroplast and mitochondrion as well as the physical connection between the two organelles suggest an intense energetic exchange. Bailleul et al. (2015) showed that NADPH generated in the chloroplast is exported to mitochondria to generate additional ATP that is then imported into the chloroplast, where it is used for carbon fixation. The authors proposed that this process may be conserved in other diatoms (*T. pseudonana*, *T. weissflogii*, *Fragilaria pinnata* and *Ditylum brightwellii*). As a corollary, the simultaneous use of carbon and light energy sources (mixotrophic metabolism) could stimulate growth, and thus enhance biomass production of diatoms while reducing energy costs, making them attractive and competitive for biotechnological applications. In particular, when the organic carbon enters the cells, it can take part in different pathways that stimulate mitochondrial respiration and lipid production, including fatty acid synthesis in the chloroplast and de novo TAGs synthesis in lipid bodies (Liu, Duan, Li, & Sun, 2009; Villanova et al., 2017). In addition, the mixotrophic mode can increase the carbohydrate content, as shown by mathematical modelling and metabolomic analysis in *P. tricornutum* (Villanova et al., 2017). In particular, the authors showed an increase in chrysolaminaran, the most abundant storage carbohydrate in diatoms, but also in trehalose and mannitol, which are red algae and prokaryote-derived carbohydrates, respectively (Michel et al., 2010). The communication between the chloroplast and mitochondrion during the mixotrophy and how this increases the biomass and lipid production are summarised in Figure 3.

3 | TROPHIC VARIABILITY AMONG DIATOM SPECIES AND INDUSTRIAL APPLICATIONS

Even if the majority remains photosynthetic, many diatom species are facultative mixotrophs, allowing them to use external organic carbon sources for growth in fluctuating light conditions (Tuchman et al., 2006). Finally, a much smaller group is represented by heterotrophs, able to grow in darkness using organic carbon sources. The trophic variability in diatoms is summarised in Table 1. As explained above, diatoms are able to grow under low CO_2 concentration thanks to the CCM mechanism. However, under this condition, the microalgal growth and product accumulation are limited. In *P. tricornutum*, it was shown that it is possible to enhance both biomass and lipid production by increasing the CO_2 concentration (Wu et al., 2015). Similarly, Gardner et al. (2012) demonstrated that adding HCO_3^- can stimulate the accumulation of TAGs in both *Scenedesmus* and *P. tricornutum*. Most recently, it was shown that the mixotrophic performance of *P. tricornutum* can be improved by adding both HCO_3^- and glycerol (Villanova et al., 2021).
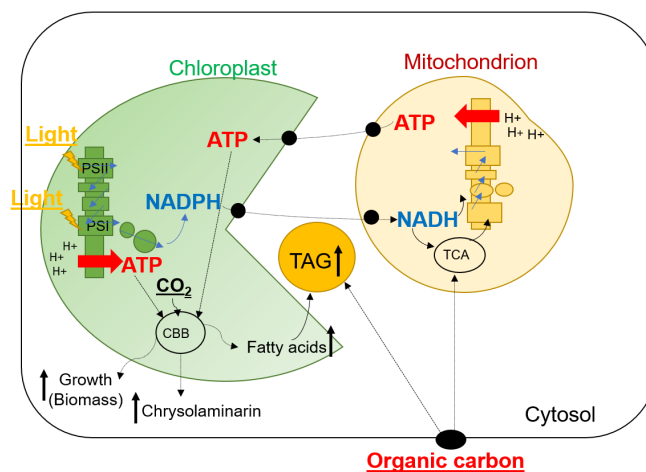
FIGURE 3 Communication between the chloroplast and mitochondrion in diatoms. ATP is produced during mitochondrial respiration, and part of it can be transported to the chloroplast, where it is used in the CBB cycle. At the same time, NADPH is produced during photosynthetic light reactions, and part of it is transported to the mitochondrion, where it is reduced to NADH and used in the TCA cycle. This communication stimulates growth, carbohydrate (chrysolaminarin) and lipid (fatty acids and TAG) production. CBB, Calvin-Benson-Bassham cycle; TCA, tricarboxylic acid cycle; TAG, triacylglycerol

Different species of *Nitzschia* are colourless pennate diatoms that probably originated from one or multiple mutations of a photosynthetic progenitor able to use extracellular carbon for growth (Kamikawa et al., 2015). These species have non-photosynthetic chloroplasts and, hence, they are not able to perform photosynthesis, carbon fixation and chlorophyll synthesis, but still have ATP synthase genes. They are, therefore, obligate heterotrophs, often associated with seaweed and mangroves, and capable of growing in darkness using complex carbohydrates (Armstrong et al., 2000; Kamikawa et al., 2015).

The centric diatom *C. cryptica* possesses a high content of eicosapentaenoic acid (EPA) and is therefore used in aquaculture, but it also has a great potential in biofuel production (Traller et al., 2016). *Cyclotella cryptica* is a facultative heterotroph, able to grow in darkness in the presence of glucose with similar productivity and lipid content (including EPA and DHA) as compared to the photoautotrophic control (Pahl et al., 2010). Finally, *C. cryptica* showed the highest fucoxanthin level when grown in heterotrophy as compared to other diatom species (Guo et al., 2016). Some microalgae are obligate photoautotrophs because they possess an inefficient uptake of the organic carbon source or because they lack some essential metabolic pathways. On the other hand, the development of genetic tools represents a potential approach for the modification and utilisation of these organisms in biotechnology. As an example, the introduction of the gene coding for the human glucose transporter (GLUT1) in *P. tricornutum* allowed for the uptake of glucose and growth in darkness (Zaslavskaia et al., 2001).

Examples of facultative mixotrophs among diatom species are *P. tricornutum* (Villanova et al., 2017), *Navicula saprophila*, some

TABLE 1 Trophic variability in diatoms and industrial applications

	Centric diatoms			Pennate diatoms			
Species	*Skeletonema costatum*	*Cyclotella cryptica*	*Thalassiosira pseudonana*	*Navicula saprophila*	*Nitzschia sp.*	*Phaeodactylum tricornutum*	*Fistulifera solaris*
Trophic mode	Photoautotrophy, mixotrophy	Photoautotrophy, heterotrophy	Photoautotrophy	Photoautotrophy, mixotrophy, heterotrophy	Mixotrophy, heterotrophy	Photoautotrophy, mixotrophy	Phototrophy, mixotrophy
Organic carbon source	Acetate	Glucose	n.a.	Acetate	Complex carbohydrates	Glucose, glycerol, acetate, fructose	Glycerol
Application	Aquaculture	Aquaculture, biofuels	Aquaculture	Aquaculture	Aquaculture	Aquaculture, biofuels	Biofuels
Molecule of interests	EPA	EPA, Fx, TAGs	EPA	EPA	EPA	EPA, Fx, TAGs	TAGs

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Fx, fucoxanthin; n.a., not available; TAG, triacylglycerols.

species of *Nitzschia* (Kitano et al., 1997) and *Skeletonema costatum* (Guihéneuf et al., 2008). Some species of green algae exhibit similar growth in photoautotrophy and in mixotrophy due to the reduction of photosynthetic efficiency in the latter trophic mode (Sforza et al., 2012). In the case of diatoms, there are contrasting results about the way they perform mixotrophic growth. For instance, the pennate *N. saprophila* is able to grow on acetic acid in either heterotrophy or mixotrophy as well as in the phototrophic mode. The highest growth rate of *N. saprophila* was reported in mixotrophic culture and corresponded to about the sum of the growth rates obtained in heterotrophy and phototrophy. In addition, the mixotrophic growth yielded the highest EPA productivity among all three trophic modes (Kitano et al., 1997). *Skeletonema costatum* is a centric diatom that commonly dominates coastal waters and its mixotrophic growth was tested using acetate as organic carbon (Guihéneuf et al., 2008). In this trophic mode, it was able to grow at low irradiance yielding more biomass and EPA than the phototrophic control. The oleaginous pennate diatom *F. solaris* is rich in TAGs that can be used for the production of biofuel (Matsumoto et al., 2014). When glycerol kinase was overexpressed in this species in order to enhance the organic carbon assimilation, it also resulted in a 12% increase in lipid production (Muto et al., 2015).

Finally, the pennate *P. tricornutum* is a facultative mixotroph able to use various carbon sources such as glycerol, acetate, glucose and fructose (Liu, Duan, Li, Xu, et al., 2009). So far, glycerol has been shown to be the best substrate for enhancing biomass and lipid production and its role in central carbon and lipid metabolism has been deeply investigated (Villanova et al., 2017). *Phaeodactylum tricornutum*, similarly to *C. cryptica*, is also a promising source of Fx; however, contrasting results have been reported on the production of this carotenoid in mixotrophy. Different studies showed that *P. triconutum* decreases its pigments' content, including Fx, during mixotrophic growth, resulting in a decrease of photosynthetic activity (Liu, Duan, Li, Xu, et al., 2009; Liu, Duan, Li, Sun, 2009). However, the authors did not consider the nutrient concentration in photoautotrophy and mixotrophy. Indeed, *Phaeodactylum* grown in an optimised medium can enhance both respiration and photosynthetic activity, hence boosting the biomass, lipid and fucoxanthin production in mixotrophy (Villanova et al., 2021). Finally, Yang and Wei (2020) showed that the Fx level raises when blue light is used in mixotrophy on a repleted medium. Like *P. tricornutum*, *T. pseudonana* is one of the most studied diatoms for lipid production due to its high EPA content. It is a model-centric species commonly used as live feed in aquaculture (Cong et al., 2018). However, no studies have been dedicated to its mixotrophic growth.

4 | LIPID METABOLISM DURING MIXOTROPHY IN DIATOMS

As described above, photoautotrophic organisms are able to use sunlight energy to convert CO_2 into various metabolites, many of them

TABLE 2 Biomass productivity and composition for high-lipid producing diatoms

Species	Productivity (g DW L^{-1} day^{-1})	FA, % DW	Protein, % DW	EPA, % DW
Cylindrotheca fusiformis	0.029 ± 0.003	24.5 ± 3.1	10.5 ± 1.5	2.3 ± 0.3
Cyclotella cryptica	0.017 ± 0.000	23.5 ± 3.2	13.8 ± 2.7	3.8 ± 0.3
Phaeodactylum tricornutum	0.080 ± 0.020[a]	23.1 ± 0.4[a]	n.a.	3.7 ± 0.5[a]
Nitzschia ovalis	0.013 ± 0.004	20.4 ± 3.2	20.3 ± 2.8	3.1 ± 0.4
Thalassiosira pseudonana	0.010 ± 0.003	21.4 ± 6.3	13.5 ± 4.1	2.2 ± 0.8

Note: Data were derived from Slocombe et al. (2015) unless otherwise stated.

Abbreviations: DW, dry weight; EPA, eicosapentaenoic acid; FA, fatty acid; Fx, fucoxanthin; n.a., not available.

[a]Villanova et al. (2021).

having an interest in biotechnological applications. Microalgae, in particular diatoms, are a promising source of TAGs and PUFAs. Slocombe et al. (2015) screened hundreds of microalgae species grown in phototrophy and selected four species of diatoms for their high fatty acids (20%–25% of dry weight) and EPA content (2%–4% of dry weight). This analysis revealed *C. fusiformis* and *C. cryptica* as the best diatom for biomass and EPA, respectively (Table 2). In particular, *C. cryptica* showed similar fatty acids and EPA content to *P. tricornutum*, which had, however, fourfold higher biomass productivity (Villanova et al., 2021). The biomass composition of microalgae can change dramatically due to the environment. Indeed, under optimal conditions, photoautotrophs use almost all the energy derived from carbon fixation for the biosynthesis of carbohydrates and growth (Figure 2, Melis et al., 2013). In contrast, under unfavourable conditions, the cells stop growing and start accumulating energy-storing molecules such as lipids (Cheng & He, 2014). Obviously, the biomass production of microalgae is highly reduced under these conditions. If organic carbon substrates are available, the mixotrophic mode is activated and stimulates the production of biomass and lipids in certain species (Figure 3). The biosynthetic metabolism of lipids is similar to the one in land plants and consists of both eukaryotic and prokaryotic pathways (Hu et al., 2008). These pathways take place in three compartments of microalgae cells, namely the chloroplast, cytosol and the endoplasmic reticulum, and will be discussed below.

4.1 | PUFAs biosynthesis

PUFAs are fatty acids having a chain with at least 16 carbon atoms and two or more double bonds. Among them, docosahexaenoic acid (DHA) and EPA are essential for health and nutrition. Humans are unable to synthesise them, and therefore DHA and EPA have to be obtained from the diet. The primary source for these PUFAs are fish and algae, the latter being the natural producers. EPA is the main PUFA in diatoms, hence the increasing demand for industrial cultivation of these microalgae. Diatoms greatly vary in the fatty acid composition as compared to land plants and green algae, which cannot accumulate large amounts of the long chain (LC) PUFAs (Guschina & Harwood, 2006).

The first step in fatty acid biosynthesis is the conversion of acetyl-CoA into malonyl-CoA catalysed by acetyl-CoA carboxylase

(ACCase) in the chloroplast. The main fatty acid classes found in *P. tricornutum* are C14:0, C16:0, C16:1, C20:5, C22:6 and C24:0, but these can differ among diatom species and under different culture conditions (Abida et al., 2015; Yi et al., 2017). In the chloroplast, fatty acids are then extended to a maximum length of 18 carbon atoms by enzymes called fatty acid synthases. Fatty acids are further extended by other enzymes in the cytosol with the help of fatty acid elongases (Elo$_s$), resulting in long-chain (C20–C24) saturated or monounsaturated fatty acids. The PUFAs production ends with the action of a series of desaturases (FADs) that are responsible for the formation of double bonds in fatty acids. Specifically, PUFAs of the C16 and C18 classes are synthesised in the chloroplast, while the synthesis of LC-PUFAs occurs between the endoplasmic reticulum and the cytosol (Figure 4A). Increased content of both EPA and DHA and the upregulation of one of the FADs were reported in *P. tricornutum* when cultivated in mixotrophy using glycerol; this was attributed to membrane lipid turnover (Villanova et al., 2017). An elevated EPA content in mixotrophy was also reported in other diatoms, as mentioned above (Guihéneuf et al., 2008; Kitano et al., 1997). However, how organic carbon takes part in the biosynthetic pathway has not yet been investigated.

4.2 | TAGs biosynthetic pathways

TAGs, also known as oils, are esters of glycerol with fatty acids and can be used for biodiesel production. Biodiesel contains methyl- or ethyl esters derived from the transesterification of animal or vegetal oil. Microalgae-derived oil is the most competitive alternative to petroleum since microalgae possess higher productivity and require less land than vegetable crops (Yusuf, 2007).

TAGs biosynthesis in photoautotrophs can take place by two pathways: (1) de novo synthesis, also known as the Kennedy pathway, and (2) remodelling of membrane lipids (Abida et al., 2015). The Kennedy pathway involves the transfer of acyl groups onto glycerol-3-phosphate by endoplasmic reticulum membrane acyltransferases (Hu et al., 2008). This reaction leads to the formation of phosphatidic acid, which is then dephosphorylated by a phosphatase to form&lsquodiacylglycerol (DAG). The DAG represents the starting point for the biogenesis of membrane lipids (phosphatidylcholine, monogalatosyldiacylglycerol, etc.) as well as TAGs. The biosynthesis
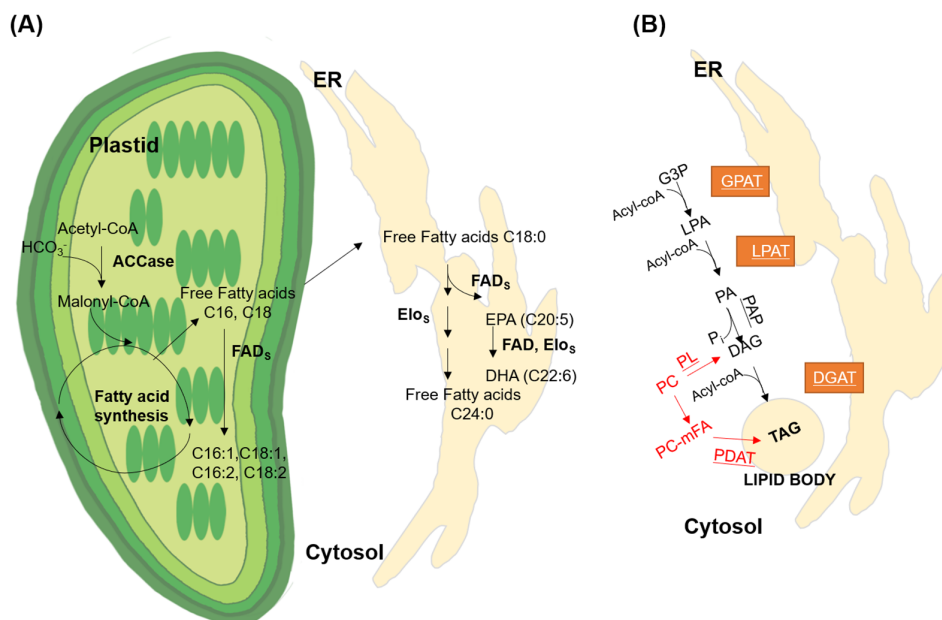
FIGURE 4 (A) Fatty acid biosynthetic pathways in diatoms. The fatty acid biosynthesis starts with the conversion of acetyl-CoA into malonyl-CoA in the chloroplast with the help of ACCase. Here short chain fatty acids (C16–C18) are synthesised by a serial of fatty acid synthases. Fatty acids are then extended by Elos in the cytosol. Short PUFAs are formed by the action of FADs in the chloroplast, whereas long chain PUFAs (EPA, DHA) are synthesised between the cytosol and the endoplasmic reticulum (ER). ACCase, acetyl-CoA carboxylase; FADs, fatty acid desaturases; ER, endoplasmic reticulum; Elos, fatty acid elongases; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid. (B) Triacylglycerol biosynthetic pathways in diatoms. Black and red arrows indicate de novo (Kennedy pathway) and PC-derived TAG synthesis, respectively. Kennedy pathway is catalysed by a series of membrane acetyl transferases (GPAT, LPAT, DAGAT) and one phosphatase (PAP) between the cytosol and ER. PC can be degraded via PL or incorporated directly into FA and TAGs. TAGs are accumulated in lipid bodies. Acyl-CoA, acyl coenzyme a; DAG, diacylglycerol; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; TAG, triacylglycerol. Names of the enzymes are underlined: DGAT, acyl-CoA:DAG acyltransferase; GPAT, acyl-CoA:G3P acyltransferase; LPAT, acyl-CoA:LPA acyltransferase; PAP, PA phosphatase; PL, phospholipase; PDAT, phospholipid:diacylglycerol acyltransferase

of the photosynthetic (thylakoid) membrane occurs inside the chloroplast, whereas the non-photosynthetic (envelope) membrane synthesis occurs in the cytosol and endoplasmic reticulum. The last reaction of the TAG biosynthesis is catalysed by the diacylglycerol acyltransferase (DGAT) that represents the rate-limiting enzyme of the entire pathway (Yen et al., 2008). Transcriptomic analysis of *P. tricornutum* cultivated in mixotrophy with glycerol revealed the upregulation of this enzyme, in line with the enhanced de novo synthesis of TAGs (Villanova et al., 2017). In addition, the overexpression of DGAT resulted in enhanced production of TAGs in *T. pseudonana* (Manandhar-Shrestha & Hildebrand, 2015). Alternatively, DAG can be produced by the degradation of glycerophospholipids catalysed by phospholipases to recycle pre-existent membrane lipids. Finally, TAGs can be produced from acetyl-CoA by an independent mechanism involving the direct incorporation of fatty acids into phosphatidylcholine (PC) and conversion to TAGs by the action of a phospholipid:diacylglycerol acyltransferase (PDAT, Abida et al., 2015) (Figure 4B). The accumulation of TAGs generally occurs in specific structures named lipid bodies that are visible in the microscope.

Another way to enhance lipid accumulation is by preventing lipid catabolism. This is a competing pathway to lipid biosynthesis and can be activated in microalgae under certain environmental conditions to provide acyl groups for cell membrane reorganisation. The

downregulation of a lipase in *T. pseudonana* resulted in a threefold increase in total lipids compared to the wild type (Trentacoste et al., 2013).

5 | CONCLUSIONS AND PERSPECTIVES

Diatoms dominate marine waters under changing environmental conditions thanks to their flexible cell metabolism. They have developed an advanced CCM that allows them to survive at a low CO_2 concentration as well as a better carbon-fixation ability compared to other microalgae. They also dominate coastal waters in the North, normally unfavourable for photosynthesis due to the low temperature, short photoperiod and low irradiation. The industrial potential of local Nordic strains for outdoor cultivation has been reviewed by Cheregi et al. (2019). Most recently, a study dedicated to microalgae from Sweden's west coast revealed two species, including the diatom *Skeletonema marinoi*, being suitable for outdoor cultivation and biomass production for various purposes (Cheregi et al., 2021). Ongoing experiments in our laboratory investigate whether the mixotrophic cultivation could be a strategy to overcome the restricted weather condition in Nordic countries, and thus to increase biomass and lipid productivity.

Even though mixotrophy is documented to stimulate biomass and lipid production in some diatoms, only a handful of research projects have focused on understanding the underlying molecular processes. The sequence of whole genomes and genetic engineering tools are available for several diatom species, and, in some cases, the modification of specific metabolic pathways resulted in an increase in lipid production (Manandhar-Shrestha & Hildebrand, 2015; Trentacoste et al., 2013). Further studies are necessary to reveal the main metabolic pathways involved in mixotrophy and to pinpoint possible targets for metabolic engineering in diatoms. The overexpression of lipid biosynthesis genes or, alternatively, the inhibition of competitive pathways (e.g., lipid catabolism and synthesis of storage carbohydrates) could further enhance the mixotrophic performance in diatoms. Moreover, in order to minimise the additional cost of organic carbon supplementation, wastewater and biodiesel waste (glycerol) can be used as carbon sources in industrial applications. Mixotrophic cultivation of microalgae for wastewater treatment has been reviewed by Pang et al. (2019); however, diatoms have never been tested in this field. The major advantage in using mixotrophic cultures for wastewater treatment is the removal of organic carbon together with inorganic nitrogen and phosphorus. Both marine and freshwater diatom species could be used for nutrient removal from municipal wastewater and aquaculture given the higher rate of nutrient utilisation by diatoms as compared to other algae classes (Litchman & Klausmeier, 2001). In-depth metabolic studies on mixotrophy of highly abundant and ecologically relevant diatoms will help better understand diatoms' global success. In addition, mixotrophic cultivation of diatoms is highly relevant for industrial applications thanks to their ability to produce PUFAs, TAGs and other molecules of interest.

AUTHOR CONTRIBUTIONS
Valeria Villanova wrote the manuscript with input and help from Cornelia Spetea that also provided critical feedback and approved the final version.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analysed in this study.

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