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Microalgae of the genus *Nannochloropsis*: Chemical composition and functional implications for human nutrition

Lorenzo Zanella, Fabio Vianello*

Department of Comparative Biomedicine and Food Science, University of Padua, Viale dell'Università 16, Legnaro 35020, Italy

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ABSTRACT

The richness of bioactive compounds in microalgae has long inspired their exploitation for human nutrition. However, very few species are authorized for this use to date. Microalgae of the genus *Nannochloropsis* are already exploited in aquaculture for their high content in PUFAs, carotenoids, polyphenols and vitamins. These molecules can contribute to delay aging processes and prevent the onset of many metabolic disorders triggered by unhealthy lifestyles and diets with an excess of saturated fatty acids. Moreover, *Nannochloropsis* is efficiently cultivated at an industrial scale in photobioreactors, without the use of pesticides and preventing biological or chemical contaminations. Here, information on its chemical composition, safety and toxicology is discussed. *Nannochloropsis* can be an eco-sustainable alternative to fishery products as source of bioactive compounds. Importantly, the daily consumption of few grams of biomass would result in a regular dietary intake of essential molecules for the prevention of cardiovascular pathologies and other disorders.

1. Introduction

Microalgae have long been considered alternative unconventional protein sources and food supplements for animal and human nutrition, but their commercial large-scale production started only few decades ago (Becker, 2004; Christaki, Florou-Paneri, & Bonos, 2011). Their exploitation as source of proteins and lipids is generally limited to traditional uses of cyanobacteria by native populations. As examples, spirulina (*Arthrospira* sp.) was historically harvested by Aztecs living close to Mexican Texcoco lake and, nowadays, by local populations inhabiting the region of the Kossorom lake in Chad; *Nostoc commune* is consumed in some Asian regions and *Nostoc punctiforme* is a traditional food in China, Mongolia and South America (Gantar & Svirčev, 2008). All these microalgae are filamentous cyanobacteria occurring in peculiar natural environments and forming dense suspended biomasses, benthonic mats or globular/gelatinous colonies, easily harvested by sieving, or other simple methods, and then consumed raw, dried, in soups or as food thickeners.

Traditional dietary uses of microalgae 'sensu stricto' (i.e. belonging to Eukaryota domain) are not known, but some of them have been exploited for decades as food supplements, raw ingredients or extracts: e.g. *Hematococcus pluvialis*, *Chlorella* spp., *Dunaliella salina* and others (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). In fact, the interest of advanced economies for microalgae is focused on their secondary metabolites with high biological activity (Pulz & Gross, 2004; Spolaore et al., 2006), exploitable for producing nutritional supplements and functional food ingredients (Chacón-Lee & González-Mariño, 2010; Gouveia, Batista, Sousa, Raymundo, & Bandarra, 2008; Sathasivam, Radhakrishnan, Hashem, Abd, & Allah, 2019). Polyunsaturated fatty acids (PUFAs) were certainly the first compounds attracting the attention of nutritionists for their antithrombotic activity (Geill, Hansen, & Lund, 1960). This discovery arose from the observation that the Inuit populations enjoyed complete protection from cardiovascular diseases thanks to their abundant intake of eicosapentaenoic acid (EPA, C20:5n-3), due to a diet based on raw fish (Dyerberg & Bang, 1978). During the last three decades, however, other active compounds comprised in microalgae composition have been identified as important for human health. Among these, attention was devoted to powerful antioxidants, such as carotenoids and polyphenols. These compounds, in addition to the scavenging of reactive oxygen and nitrogen species, have shown to regulate gene expression of enzymes protecting against oxidative stress (Berthon et al., 2017; Sathasivam & Ki, 2018).

Despite microalgae are a source of many beneficial compounds recommended for the prevention of metabolic problems related to aging, only few species or derivatives are approved for being included in the

Abbreviations: DHA, docosahexaenoic acid; DW, dry weight; EPA, eicosapentaenoic acid; PBRs, photobioreactors; PUFAs, polyunsaturated fatty acids

* Corresponding author.
E-mail address: fabio.vianello@unipd.it (F. Vianello).

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human diet. The short list includes the cyanobacterium "spirulina" (*Arthrospira platensis*.), the microalgae *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Chlorella luteoviridis*, *Odontella aurita* and, since 2014, also *Tetraselmis chui*. Among the strains whose inclusion is recommendable, a priority should be given to species of the genus *Nannochloropsis*, due to their suitability for intensive culture and high content of PUFAs (in particular EPA), antioxidants and some vitamins.

The present contribution is aimed at reviewing the information currently available on the composition and biological properties of *Nannochloropsis*, which are discussed with regard to recommended daily allowances and manufacturing cautions aimed to preserve bioactivities. Potential health benefits are outlined, with special attention to the positive effects on the preservation of marine natural resources. Besides, safety data available for this microalga are discussed, proposing quality and microbiological parameters to be considered in the case of authorization for human nutrition.

2. Systematics of the genus *Nannochloropsis*

Hibberd (1981) described the genus *Nannochloropsis*, family *Monodopsidaceae*, order *Eustigmatales*, class *Eustigmatophyceae*, that later was intensively exploited for its rich PUFA composition, favourable growth rate and high cell density under culture conditions. Apart from applications in aquaculture and development of biofuels, the use of *Nannochloropsis* as food for humans has not been pursued yet.

Systematics of this genus is extremely difficult due to its simple and little variable morphology (Fig. 1). The non-motile spherical cells show diameters ranging from 2 to 8 μm, characterised by a physical 'continuum' between the external plastid membrane and the external nuclear envelope, whereby these organelles are duplicated and segregated in a coordinated way through cell replication (Guo et al., 2019; Ma, Chen, Yang, Liu, & Chen, 2016). Recent genetic studies based on the plastid *rbcL* gene (barcode analysis) led to a revised interpretation of the phylogenesis of this genus, with the description of a new species and the shifting of *Nannochloropsis gaditana* and *N. salina* into the new genus *Microchloropsis* (Fawley, Jameson, & Fawley, 2015; Starkenburg et al., 2014). To date, seven "*Nannochloropsis* group" species have been formally described (Guiry & Guiry, 2019), comprising species now transferred to the sister genus *Microchloropsis*: *Nannochlorospis australis* Fawley, Jameson & Fawley, 2015; *N. granulata* Karlson & Potter, 1996; *N. limnetica* Krienitz, Hepperle, Stich & Weiler, 2000; *N. oceanica* Suda & Miyashita, 2002; *N. oculata* (Droop) Hibberd, 1981; *Microchloropsis gaditana* (Lubián) Fawley, Jameson & Fawley, 2015; *M. salina* (Hibberd) Fawley, Jameson & Fawley, 2015. Due to their homogeneous morphological traits, genetic analysis is required for a reliable taxonomic diagnosis of these taxa. For this reason and for historical continuity, the *Microchloropsis* species will be here cited with their updated names but considered as comprised in *Nannochloropsis* 'sensu lato', i.e. the genus name hereinafter used for indicating the whole species-group. Furthermore, all these taxa show similar chemical composition and nutritional properties, at least in qualitative terms, and this makes reasonable the evaluation of their suitability for human consumption jointly. Importantly, isolated and classified strains are currently available for commercial use in official and private collections, which allow to trace the origin of the microorganisms destined to food production.

Fig. 1. *Nannochloropsis oculata*. Image from Malakootian, Hatami, Dowlatshahi, and Rajabizadeh (2016) licensed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0).

3. Use history of *Nannochloropsis*

The exploitation of microalgae for industrial and zootechnical processes mainly derives from marine aquaculture, which developed the fundamental applications concerning their cultivation. Species-specific cultures of microalgae were introduced at the beginning of the twentieth century for the nutrition of micro-invertebrates (Allen & Nelson, 1910). It assumed commercial perspectives few decades later, with applications for breeding of bivalve molluscs (Bruce, Knight, & Parke, 1940; Rhyter & Goldman, 1975) and for feeding phytophagous larval phases of shrimps (De Pauw, Morales, & Persoone, 1984). Indeed, bivalves and some shrimp larvae are micro-phytophagous and can be feed directly with alive microalgal cells released in the breeding tanks.

An important turning point came from the use of microalgae in the phyto-zooplankton chain for the nutrition of marine fish larvae. In fact, in the 1970s, the reproduction and breeding of marine fish had little success, due to both low survival of the early larval phases and high malformation rate showed by the survived fries. These problems were overcome only when scientists guessed that larvae of several marine fish required the administration of rotifers enriched with microalgae characterised by a high content of polyunsaturated fatty acids (PUFAs) and other essential nutritional factors (Watanabe, Kitajima, & Fujita, 1983). Indeed, the nutritional value of rotifers depends on the microalgae in their gut and on the composition of lipids they have stored from previously digested microalgae. The biological activity of highly unsaturated long chain fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and probably some carotenoids, traditionally not included among essential vitamins, highlighted their importance as nutritional factors.

It should be emphasized that the application of microalgae for fish feeding can be considered a severe toxicological bioassay, continued for decades, since the larval stages of these aquatic animals are very sensitive to toxic compounds and even unfavourable nutritional factors. *Nannochloropsis* has never produced negative effects on reared marine organisms whereas, on the contrary, it has provided essential nutrients for their correct development and well-being. Several studies focused on the nutritional value of *Nannochloropsis* for different aquatic animals, e.g. seabass (Haas et al., 2016), gilthead seabream (Cerezuela, Guardiola, Meseguer, & Esteban, 2012; Navarro & Sarasquete, 1998), red sea bream and Japanese flounder (Sakamoto, Okimasu, & Amemura, 1998), short-neck clam (Gireesh & Gopinathan, 2008), Manila clam (Yamasaki, Ishii, Taga, & Kishioka, 2018), brine shrimps (Salarzadeh & Nahidi, 2016) and rotifers (Lubzens, Gibson, Zmora, & Sukenik, 1995).

4. Novel foods derived from microalgae already authorized in the European Community

There is a high interest about the use of microalgae, or their derivatives, as novel foods or food ingredients. Nevertheless, few species have been authorized for this use, possibly due to the difficulty of proving the absence of toxicity and complying all strict safety criteria imposed by the current legislation. In Europe, novel foods are regulated by the Regulation (EU) n. 2015/2283, which amends the previous Regulation (EU) n. 1169/2011 and repealing the Regulations (EC) n.

258/1997 and n. 1852/2001. The use of microalgae falls within the application field as follows:

Article 3

[…]

2. The following definitions also apply:

(a) 'novel food' means any food that was not used for human consumption to a significant degree within the Union before 15 May 1997, irrespective of the dates of accession of Member States to the Union, and that falls under at least one of the following categories:

[…]

(ii) food consisting of, isolated from or produced from microorganisms, fungi or algae; 11.12.2015 L 327/7 Official Journal of the European Union EN

[…]

(vi) food consisting of, isolated from or produced from cell culture or tissue culture derived from animals, plants, micro-organisms, fungi or algae;

(vii) food resulting from a production process not used for food production within the Union before 15 May 1997, which gives rise to significant changes in the composition or structure of a food, affecting its nutritional value, metabolism or level of undesirable substances;"

The cited art. 3 of the EU regulation establishes that microorganisms and algae have to be treated as novel foods but, importantly, the paragraph '2a) vii' clarifies that also the related production practices need to be described and approved.

In 2012, *Nannochloropsis gaditana* (now *Microchloropsis*) was cited as a case of novel food pending application in the Petition n. 0287, notified to the European Community Members by Silvia Beltràn Pallarès (Spanish), on behalf of Plataforma Europea de los Consumidores y del Medio Ambiente (Beltràn Pallarès, 2012). In fact, in 2011, Fitoplancton Marino S.L. (Spain) filed an application to the Agencia Española de Seguridad Alimentaria y Nutrición (ES) for the authorization of *M. gaditana* as a novel food, in compliance with the regulation (EC) n. 258/97 then in force. It was not possible to find any further information on this case.

Enzig, Ploeg, Barbosa, and Sijtsma (2014) published a report for the Joint Research Centre (Institute for Prospective Technological Studies) of the European Commission, entirely dedicated to the state of knowledge on the use of microalgae and their derivatives for animal and human nutrition. *Nannochloropsis* was classified as a good source of EPA, extracted and marketed as food supplements (producer Ocean's Alive, USA) for human diet (Enzig, Ploeg, Barbosa, & Sijtsma, 2014). The same document reported that no toxins are known for the genus *Nannochlorospis* (Enzig, Ploeg, Barbosa, & Sijtsma, 2014).

It is worth summarising the microalgal strains currently authorized for human consumption in the European Community, in order to point out the impact deriving from the introduction of *Nannochloropsis*. Importantly, its authorization process could be facilitated by the principle of equivalence with species already authorised (see the case of *Odontella aurita* below) or concerning active compounds shared with approved extracts:

- cyanobacteria "spirulina" (*Arthrospira platensis*) and *Aphanizomenon flos-aquae*, already used as a food in the EU before 1997 and not subject to the novel food regulation;
- *Chlorella pyrenoidosa*, *C. vulgaris*, *C. luteoviridis* already used as food in the EU before 1997 and not subject to the novel food regulation;
- *Odontella aurita*, authorized with notification issued to the applicant (Innovalg SARL, France) by the "Agence Française de Sécurité Sanitaire des Aliments" on December 9, 2002, in acceptance of the equivalence with other already authorized microalgae;
- *Tetraselmis chui*, authorized for use as food ingredient on March 4, 2014 to the applicant (Fitoplancton Marino S.L., Spain) by the "Ministry of Health, Social Services and Equality AECOSAN – Spanish Consumer Affairs, Food Safety and Nutrition Agency", then listed among the authorised novel foods in the Commission Implementing Regulation (EU) 2017/2470 on December 20, 2017.

The following products derived from microalgae have also been authorized:

- β-carotene dispersion extracted from *Dunaliella salina*. Use as food colour, maximum dose about 50 ppm (equivalent to about 10 ppm β-carotene). European Commission on June 13, 1997 ;
- mixture of carotenoids extracted from *Dunaliella salina* harvested in the large salt lakes in the Whyalla region (Southern Australia). Major component is β-carotene, but α-carotene, lutein, zeaxanthin and β-cryptoxanthin may also be present. Commission Directive 2008/128/EC on December 22, 2008;
- DHA and EPA extracted from *Schizochytrium*. Commission Decision 2003/427/EC at the request by Martek Biosciences Corporation (USA) (formerly OmegaTech GmbH) presented on February 13, 2001. Related decisions were: Commission Decision 2009/778/EC at the request by Martek Biosciences Corporation (USA) presented on January 14, 2008; Commission Implementing Decision 2015/546/EU at the request by DSM Nutritional Products presented on November 19, 2012;
- oil rich in PUFA, and in particular of DHA, obtained from *Ulkenia* sp. Commission Decision 2009/777/EC at the request by Nutrinova (Germany) presented on November 15, 2004;
- astaxanthin-rich oleoresin extracted from *Haematococcus* (maximum 4 mg/capsule). Favourable opinions of equivalence released to US Nutra (USA) on June 28, 2004; AstaReal AB (Sweden) on May 17, 2006; Cyanotech Corporation (USA) on March 7, 2007; Alga Technologies (Israel) on April 14, 2008; Beijing Gingko Group Biological Technology Co., Ltd. (China) on December 22, 2016; Algalif Iceland ehf (Iceland) on April 19, 2017; Algamo s.r.o. (Czech Republic) August 9, 2017, Yunnan Alphy Biotech Co., Ltd (China) August 21, 2017;
- oil with high EPA content extracted from *Phaeodactylum tricornutum*. Favourable opinion by the Food Safety Authority of Ireland (August 1, 2017) at the request presented by Simris Alg AB, Hammenhög (Sweden).

Concerning the above background, it is worth noting that, apart from cyanobacteria and microalgal extraction derivatives, the only eukaryotic microalgae authorized as foods are *Chlorella* spp., *Odontella aurita* and *Tetraselmis chui*. Approved intake dosages of compounds shared with *Nannochloropsis* may facilitate the approval process of the latter, with a significant expansion of the market offer for microalgae foods.

5. Production process of *Nannochloropsis* and implications for biomass quality and safety

Nannochloropsis is suitable for cultivation in photobioreactors (PBRs), i.e. closed bioreactors in which a light source (natural or artificial) is used for the autotrophic culture of this species. PBRs are technological systems suitable to produce algal biomass in continuous, conceptually derived from traditional batch production techniques adopted in aquaculture as a starting point for the phyto-zooplankton chain intended for feeding marine fish larvae. PBRs are more efficient than traditional polyethylene bags and suitable to produce high density mono-specific cultures in liquid medium, kept under agitation by insufflating filtered air enriched with CO_2 to support photosynthesis. The culture medium consists of sterile sea water supplemented with mineral micronutrients, whereas hazardous chemicals, such as antibiotics or pesticides, are avoided.

PBRs can widely vary for volumetric capacity, ranging from a few hundred litres to few cubic meters of algal culture, as well as in terms of yield, construction and management costs. In spite of technical and

Table 1
Composition of *Nannochloropsis* sp. and other strains expressed as fraction dry weight (% DW) biomass.

Strain	*Nannochloropsis sp.p.*					*N. oceanica*	*N. oculata* CS 216	*N. granulata*	*N. limnetica*	*M. salina*	*M. gaditana*
Reference	(1)	(2)	(3)	(4)	(5)	(6–7)	(8)	(9)	(10–11)	(12)	(13–14)
Compound	%	%	%	%	%	%	%	%	%	%	%
Protein	28.8	30.29	26.7	43		14.5	22.6	45.8	~37	18.1–36.2	47.0
Lipid	18.36	21.78	15.3	30	28.4	30.7	8.2	28.5	24	6.2–26.0	16.5
Carbohydrates	35.9	9.62	32.1	35		8.3	6.4	14.9	10	17.8–36.2	21.7
Fiber	2.41		17.7								4.0
Ash	9.44	11.32	8.31					7.8			10.1
Moisture	3.1	1.84									4.7
Nitrates	0.074										
Energy (kJ)	1760							2340			
Saturated fatty acids											
C14:0	0.63				0.57	1.69	0.27		0.63		0.9–2.3
C16:0	5.05		0.03		3.10	1.72	1.46		3.61		4.0–8.8
Monoinsaturated fatty acids											
C16:1-n7	4.72		2.6		4.0	1.82	2.18		3.71		4.4–11.9
C18:1-n9	3.79		n.d.		0.27	0.41	0.63		3.01		0.1–1.5
Polyunsaturated fatty acids											
C18:2-n6	0.36	0.411	n.d.		0.34	0.97	0.24		0.38		1.6–3.5
C18:3-n3		0.023				0.50			0.04		0.3–1.1
C20:4-n6	0.69	0.745	n.d.			0.37	0.58		0.53		0.4–3.4
C20:5-n3 (EPA)	2.24	3.676	3.65		3.44	2.34	2.33		2.81		4.4–11.0

Whenever possible, values referred to standard cultures were chosen rather than data from experimental treatments. (1) Rebolloso-Fuentes et al., 2001; (2) Kent et al., 2015; (3) Molino et al. (2018); (4) Chua & Schenk, 2017; (5) Bernaerts et al., 2020; (6) Ashour & El-Wahab, 2017; (7) Patil, Kallqvist, Olsen, Vogt, & Gislerød, 2007; (8) Volkman, Brown, Dunstan, & Jeffrey, 1993; (9) Tibbets, Bjornsson, & McGinn, 2015; (10) Freire et al., 2016; (11) Krienitz & Wirth, 2006; (12) Safafar et al., 2016; (13) Molino et al., 2019; (14) Mitra, Patidar, George, Shah, & Mishra, 2015.

engineering manifold variants, all PBRs are designed to maintain cell culture under optimal conditions and isolated from uncontrolled exchanges with the external environment, thus providing biomasses characterised by high quality and safety standards on an industrial scale (for a comprehensive review see also Acién et al., 2017; Tredici, 2004). In this respect, they represent a more expensive but definitely safer technology than open pond systems, used for most of the massive cultivations of microalgae worldwide, e.g. *Chlorella*, *Haematococcus* and *Arthrospira*.

Briefly, PBR chambers are progressively filled with a sterilised liquid medium and inoculated with a suitable high-density culture, obtained under strictly controlled laboratory conditions. When the PBR culture reaches a suitable density, is partially harvested and diluted with fresh culture medium (daily or after proper timing), obtaining a continuous production of microalgal biomass throughout the production cycle of the PBR unit. These systems prevent biological contaminations by microorganisms from the external environment. However, in the event of a significant contamination, the crop must be eliminated and PBRs need to be thoroughly disinfected before reusing. Concerning the cultured biomass, it should be clarified that generally microalgal cells are not cultivated under axenic conditions, since commensal or symbiont bacteria play a useful role for the healthy and rapid development of the crop (Fuentes et al., 2016). For instance, Brown and Miller (1992) suggested that symbiont bacteria could be responsible for the high content of ascorbic acid in *N. oculata*. Microalgal cultures lacking the specific associated bacterial community, namely axenic, are feasible, but this condition requires special PBRs, quite expensive and hard to be scaled up to commercial production (Tredici, 2004).

At harvest, the crop appears as a green liquid, whose cell density can greatly vary according to cultured strain, PBR model and environmental variables. For instance, culture densities ranging from 0.74 to 0.90 g/l dry weight (DW) have been reported for *N. oceanica*, 1.2–2.2 g/l DW for *N. oculata* and 1.18–2.67 g/l DW for *M. gaditana* (Ashour & El-Wahab, 2017). Tredici (2004), discussing the relationships between light intensity and PBR panel thickness, cited 2.6 g/l DW as an optimal density for summer cultures of a generic *Nannochloropis*, whereas Zou, Zhang, Cohen, and Richmond (2000) obtained ultrahigh cell density cultures (40.6 – 67.3 g/l DW), in extreme experimental conditions (1000–3000 μmol photons/m^2/s).

The cell material is then separated from the culture medium and concentrated by centrifugation, filtration or sedimentation, sometimes requiring an additional flocculation step (Molina Grima, Acién Fernández, & Robles Medina, 2004). All these procedures can be adopted with good results in terms of preservation and integrity of the biomass, depending on the processed species and on the product quality required. The obtained wet biomass must be rapidly dehydrated to result in a product suitable to be preserved and used as a food as such or as food ingredient. Dehydration by freeze-drying is particularly suitable in order to preserve all nutritional and antioxidant properties of *Nannochloropsis*, since it occurs at low temperature and in the absence of oxygen, i.e. minimising oxidative reactions. The material results in a green flour that can be easily mixed with other food ingredients, encapsulated or compacted for tablets.

6. Chemical composition of *Nannochloropsis* biomass

The chemical composition of *Nannochloropsis* biomass can show quantitative variations depending on the microalga strain and environmental conditions. Type of PBR, quality and quantity of light, temperature and composition of the culture medium are among the main factors affecting the chemical composition of the algal biomass.

Rebolloso-Fuentes, Navarro-Pérez, García-Camacho, Ramos-Miras, and Guil-Guerrero (2001) characterised some biomasses of *Nannochloropsis* sp. grown in Guillard's F/2 medium (a standard medium for marine microalgae), using a cylindrical column PBR irradiated with continuous artificial light. The comprehensive chemical composition is showed in Table 1 in comparison with data reported for different strains and culture conditions by other authors. Interestingly, lipid profile includes mono- and poly-unsaturated fatty acids of high nutritional value, among which an EPA amount higher than 2% is remarkable. Other dominant fatty acids are palmitic acid, palmitoleic acid and, with less regularity, oleic acid.

Several investigations have been carried out in order to enhance the biomass lipid content (Ma et al., 2016), especially EPA, mainly by limiting macronutrients in the culture medium, such as N and P. The

biomass total lipids resulted highly increased under particular experimental conditions (such as depletion of some macronutrients, extreme temperatures, etc.) becoming, in some cases, the prevalent part of the total biomass (Bondioli et al., 2012; Rodolfi et al., 2009).

Ashour and El-Wahab (2017) cultured *N. oceanica* in culture media with different nitrogen to phosphorus ratio (N:P), finding that the biomass lipid content increased along with the decrease of this ratio. At the same time, the primary productivity of the crop decreased. Olofsson et al. (2014) studied the same issue in *N. oculata*, showing an increase of the biomass lipid content following the lowering of the N:P ratio, with a very modest loss of the primary productivity. However, this enhanced lipid production was mainly due to the contribution of saturated and monounsaturated fatty acids. Bondioli et al. (2012) cultivated *Nannochloropsis* sp. (F&M − M24 strain) under N deprivation conditions, obtaining biomasses with a maximum lipid content of 68.5% compared to 39.1% of the control. Even in this case, however, the increase of total lipids was mostly due to neutral lipid fraction, i.e. the poorest in PUFAs and EPA content. Hulatt, Wijffels, Bolla, and Kiron (2017) studied the productivity and composition of *Nannochloropsis* sp. CCAP 211/78 in response to macronutrients deprivation at constant N:P ratio. Under nutrients abundance, the dry biomass reached 50–55% protein content, while under nutrient deficiency the protein content decreased to 25–30%. Besides, in this last case total lipids were two times higher than controls, even if the content of EPA remained substantially unchanged (about 4.2–4.9% DW). Van Wagenen et al. (2012) subjected *M. salina* CCPM1770 to different light intensities (5–850 μmol photons/m^2/s) and temperatures (13–40 °C), observing that total fatty acids increased under photo-oxidative stress or extreme (i.e., high or low) temperatures, whereas EPA increased at the lowest light intensities and temperatures. *M. gaditana* expressed a similar trend in winter, i.e. under low temperature and radiation exposure conditions, but performances were affected by the type of PBR used (Camacho-Rodríguez et al., 2014). However, in another study on *Nannochloropsis* sp. cultivated outdoor and exposed to seasonal temperature and irradiance variations, EPA showed modest variations around 4% DW in spite of significant seasonal variations of the total lipid content (Chini Zittelli et al., 1999). Pal, Khozin-Goldberg, Cohen, and Boussiba (2011) showed that in *Nannochloropsis* sp. the EPA fraction with respect to total fatty acids was negatively correlated to NaCl concentration and light intensity.

Table 2 summarises results reported in literature on the lipid abundance of different strains of *Nannochloropsis* cultured under different conditions. In spite of a considerable variation in composition, PUFAs ranged from 3 to 6% DW in most cases, mainly for the contribution of EPA, which generally varied between 2.0 and 5.5% DW with an estimated average of 4% DW. Importantly, the EPA is not strictly proportional to the amount of total lipids.

Overall, these data suggest that *Nannochloropsis* is a valuable source of PUFAs, of which EPA is the dominant compound, and a biomass optimised for food uses could reasonably be characterised by PUFAs set around the highest part of the variation range. According to Safafar, Hass, Møller, Holdt, and Jacobsen (2016), EPA can represent up to 44% of the total fatty acids in the biomass.

Operatively, cultivation techniques intended to increase the total lipid content, with potential advantages for specific aims, such as biodiesel production, do not meet the composition desired for nutritional purposes.

It appears that *Nannochloropsis* cultures tend to accumulate total lipids in slow cell proliferation phases, when most of the energy is available for storing in reserve molecules, whereas EPA and PUFAs increase with the cell density because of their role as cytoplasmic membrane components (Rebolloso-Fuentes et al., 2001). This is quite consistent with the findings of Dunstan, Volkman, Barrett, and Garland (1993), who reported a maximal concentration of PUFAs and EPA (pg/cell) during the logarithmic phase of *N. oculata* batch cultures, when the cell replication is intense and the polar/neutral lipids ratio favourable.

Furthermore, it seems that the amount of PUFAs and EPA are inversely proportional to temperature, as physiological adaptations aimed to maintain the cell membrane fluidity (Hoffmann, Marxen, Schulz, & Vanselow, 2010; Van Wagenen et al., 2012).

However, since factors affecting EPA biosynthesis have not been fully elucidated yet (Chini Zittelli et al., 1999), the optimisation of *Nannochloropsis* production for nutrition aims probably requires a fine tuning depending on strain, production site and cultivation system adopted.

To note that, protein composition is obviously relevant for the nutritional quality of these microalgae, but relatively uninteresting due to the high cost of these microorganism biomasses compared to other protein sources.

6.1. Carotenoids

Carotenoids are among the most active compounds of nutritional interest in *Nannochloropsis*. They contribute to light harvesting as secondary pigments in the photosystem II of the photosynthetic apparatus of the microalga, and provide an important protection of the thylakoid membranes from photo-oxidative processes (Zakar, Laczko-Dobos,

Table 2
Minimum-maximum total contents (% DW) of lipid, PUFAs and EPA in biomasses obtained from different *Nannochloropsis* strains and cultivation conditions.

Species	Total lipids	PUFAs	EPA	References
N. oceanica NIVA-2/03	–	3.8	2.3	Patil, Källqvist, Olsen, Vogt, and Gislerød (2007)
N. oceanica CY2			3.8–5.6	Chen, Chen, Huang, Ho, and Chang (2015)
N. oceanica IMET1	28–59		2.7–5.2	Meng et al. (2015).
N. sp.		5.1–5.9	3.6–4.3	Chini Zittelli et al. (1999)
N. sp F&M − M24	28–60			Rodolfi et al. (2009)
N. sp.	–	–	2.3–5.7	Zou et al. (2000)
N. sp.	15.3	5.0	3.7	Molino et al. (2018)
N. sp.	21.8	4.5	3.7	Kent et al. (2015)
N. sp.	22–31	4.6–5.8	3.8–5.1	Xu, Cai, Cong, and Ouyang (2004)
N. sp. F&M − M24 (N starved)	68.5	~9.3*	~4.1*	Bondioli et al. (2012)
N. sp. CCAP211/78		4.4–6.1	4.2–4,9	Hulat, Wijffels, Bolla, & Kiron (2017)
N. oculata ST-6 (wild type)		3.0	2.4	Chaturvedi and Fujita (2006)
N. limnetica SAG18.99		0.84–12.2	0.22–5.6	Krienitz and Wirth (2006)
M. salina		2.5–4.5	1.1–3.5	Hoffmann et al. (2010)
M. gaditana	22.3–38.6	7.7–18.5	4.4–11.0	Mitra et al., 2015
M. gaditana	24–28	2.8–5.8	2.1–4.3	Camacho-Rodríguez et al. (2014)
estimated average EPA (mg/g DW)			**40.0**	

In order to show the maximum range of variation, each value has been reported as independent datum, without regard for the consistency of culture conditions, harvest timing, etc. The average content of EPA is estimated in the last row and used as representative of a generic biomass. *approximate values estimated from graphs or variation ranges.

Table 3
Methanol-solubilized carotenoids (expressed as % total carotenoids) in samples of *M. salina* and *N. oculata* collected at different cultivation times between day 14 and day 98.
modified adapted from Antia & Cheng, 1982

Carotenoids (exceeding 1% of total)	*M. salina* (%)	*N. oculata* (%)
β-carotene	2.6–3.0	9.2–10.3
Canthaxanthin	2.1–6.6	1.4–7.7
Cryptoxanthin epoxides	0	0–4.4
Astaxanthin (free & esters) + Astacene	1.5–9.4	2.5–13.0
Vaucheriaxanthin (free & esters)	33.5–34.9	25.7–30.3
Zeax. + Antherax. + Violax. + Luteox.	47.1–58.5	43.1–53.2

Toth, & Gombos, 2016).

Carotenoids can be distinguished in two main groups: carotenes (e.g. β-carotene and lycopene), which do not possess oxygen in their structures, and xanthophylls, which exhibit a very similar structure but are oxygen-containing molecules (Sathasivam & Ki, 2018).

Rebolloso-Fuentes et al. (2001) reported on a total carotenoid content in *Nannochloropsis* of 0.6 mg/g DW. Antia and Cheng (1982) showed that astaxanthin and canthaxanthin increased with aging of *N. oculata* and *M. salina* cultures (Table 3), but the prevailing carotenoids were vaucheriaxanthin, violaxanthin and xanthophyll cycle associated compounds, namely zeaxanthin, antheraxanthin and luteoxanthin. The prevalence of vaucheriaxanthin and violaxanthin in *Nannochloropsis* 'sensu stricto' was also confirmed by other studies (Faé Neto, Borges Mendes, & Abreu, 2018; Forján Lozano, Garbayo Nores, Casal Bejarano, & Vílchez Lobato, 2007; Lubián et al., 2000). As observed for proteins and lipids, the synthesis of carotenoids can be modulated as a response to different culture conditions. For instance, they can increase under low light irradiation or low light penetration due to high culture density (Häubner, Sylvander, Vuori, & Snoeijs, 2014; Rebolloso-Fuentes et al., 2001), restriction of P and S (Forján Lozano, Garbayo Nores, Casal Bejarano, & Vílchez Lobato, 2007) and changes of N-source ingredients in the culture media (Faé Neto et al., 2018). Differences can be observed not only in the relative concentration, but also in terms of qualitative composition. For instance, studies showed that a stressing increase of temperature and light irradiation affected the xanthophyll cycle promoting a rapid conversion of a significant amount of violaxanthin into antheraxanthin and zeaxanthin (Gentile & Blanch, 2001; Lee, Min, Chang, & Jin, 2006; Lubián et al., 2000; Wang & Jia, 2020).

Faé Neto et al. (2018) obtained *N. oculata* biomasses with a total carotenoid content ranging from about 0.4 to 2.9 mg/g DW (approximate values estimated from graphical representation) in cultures grown 17 days in F/2 medium or experimental low cost media. Goiris et al. (2012) characterised two biomasses of *N. oculata* and *N.* sp. with a total carotenoids content of 1.65 and 2.17 mg/g DW, respectively. Safafar, Van Wagenen, Møller, and Jacobsen (2015) reported on a carotenoid content between 3 and 5 mg/g DW biomass for *N. limnetica* and *M. salina* (Table 4), respectively, with a prevalence of violaxanthin, neoxanthin and antheraxanthin in the former and violaxanthin, zeaxanthin and β-carotene in the latter. Kent, Welladsen, Mangott, and Li (2015) found a total carotenoid content of 0.86% DW in an Australian *Nannochloropsis* sp., not better characterised, of which 0.67% due to astaxanthin (Table 4). Notably, astaxanthin seems to be synthesised in significant amounts only by strains belonging to the genus *Microchloropsis*. Lubian et al. (2000) showed that *M. salina* and *M. gaditana* can accumulate astaxanthin and canthaxanthin, whereas the same does not occur in *N. oculata* and related species. They estimated up to 0.7% DW astaxanthin in *M. gaditana*, a value very close to that reported by Kent et al. (2015), suggesting that the Australian *Nannochloropsis* studied by these latter authors could belong to the genus *Microchloropsis*.

6.2. Phenolic compounds

Phenolic compounds are secondary plant metabolites, generally involved in protective functions, such as defense against ultraviolet radiation or aggressions by pathogens, deterrence to animal grazing because of their astringent/toxic nature (Beckman, 2000; Pandey & Rizvi, 2009). When introduced in the diet, they provide significant protection against many human chronic pathological conditions (Pandey & Rizvi, 2009).

Table 5 shows the amounts of total phenols and flavonoids recorded in biomasses of some *Nannochloropsis* 'sensu lato', which indicatively range from 1.4 to 6 mg/g DW. These quantities are not particularly high if compared to other available plant foods, but may significantly contribute to the antioxidant activity derived from the combined action of other bioactive compounds, such as carotenoids and tocopherols. For descriptive cases of phenolic composition concerning some strains of *Nannochloropsis* see Safafar et al. (2015) and Tessmer Scaglioni et al. (2018).

6.3. Other active compounds

Microalgae are good sources of vitamins (Table 5), among which vitamin E (α-tocopherol) is particularly important for the protection of cell membranes against lipid peroxidation processes (Häubner et al., 2014). Safafar et al. (2016) recorded α-tocopherol contents in *M. salina* up to 109.2 μg/g DW in lab cultures, which increased to 431 μg/g DW in large-scale cultures in PBR. Lee, Chuang, Su, and Wu (2013) extracted up to 836.6 μg/g DW of α-tocopherol from *N. oculata* (strain CCAP 849/1), whereas, in biomasses of the same strain, Durmaz (2007) quantified vit. E ranging between 483 and 2325 μg/g DW, depending on the culture condition and phase of growth. In the latter study, N starvation led to the highest α-tocopherol content, but this effect was impaired by the reduction of primary productivity of the culture. According to this author, α-tocopherol synthesis and accumulation is strongly affected by microalgal strain, temperature, light intensity and periodicity. For food use, the vitamin concentration should be considered as a priority with respect to the amount of produced biomass, which instead becomes more relevant for cultivations aimed at the extraction and purification of specific compounds.

Finally, among the bioactives still insufficiently investigated are polyamines, which play key roles in multiple cellular functions and were detected as putrescine and spermidine in *N. oculata* (Lin & Lin, 2019).

7. Biomass preservation

The preservation of nutritional and beneficial properties of *Nannochloropsis* depends on the post-harvest processes. It is commonly acknowledged that decomposition and oxidation reactions can be influenced by environmental factors (temperature, humidity, light, atmospheric gases, pH and microbial contamination), which potentially directly interfere with the stability of compounds.

Few studies were dedicated to preparation and packaging of algal biomasses and only one, at the best of our knowledge, dedicated to the preservation of *Nannochloropsis* group-species. Safafar, Langvad, Møller, and Jacobsen (2017) studied the effects of temperature (5, 20 and 40 °C) and air exposition (under vacuum vs. ambient pressure) on *M. salina* stored for 56 days, observing that lipid deterioration (carotenoids, tocopherols and EPA) occurred both due to enzyme-induced lipolysis and non-enzymatic autoxidation. Low temperature was more effective in preserving the compounds of interest than vacuum packaging.

Case studies referring to other microalgae species can provide useful indications valid also for *Nannochloropsis*. Colla et al. (2017) studied the impact of thermal and photo-oxidative stresses on the antioxidant potential of the cyanobacterium *Spirulina platensis* (junior synonym of

Table 4
Carotenoid content measured in some strains of *Nannochloropsis*.

Pigment (mg/g DW)	*N. limnetica* (1)	*M. salina* (1)	*N. oceanica.* (2)	*N.* sp. (3)	*N.* sp. (4)	Average value
Vaucheriaxanthin	0.165	0.085				*0.13*
Fucoxanthin	0.183	0.013				*0.10*
Neoxanthin	0.423	0.053				*0.24*
Violaxanthin	1.228	1.679	0.5–5.8		4.25	*2.69*
Antheraxanthin	0.344	n. d.			1.87	*0.74*
Astaxanthin	n. d.	n. d.	0–0.38	6.40		*1.36*
Canthaxanthin	0.003	0.136	0–0.14			*0.07*
Diadinoxanthin	n.d.	0.140				*0.07*
Alloxanthin	0.056	0.130				*0.09*
Diatoxanthin	0.136	n. d.				*0.07*
Lutein	n. d.	n. d.				*0.00*
Zeaxanthin	0.137	0.584	0.19–0.53		0.42	*0.37*
β-carotene	0.284	2.223	0.1–1.7	0.67	2.87	*1.31*
α-carotene	n. d.	0.084				*0.04*
Total carotenoids	**2.961**	**5.130**		**8.57**		***5.55***

From (1) Safafar et al., 2015; (2) Wang & Jia, 2020; (3) Kent et al., 2015; (4) Bernaerts et al., 2020.

Table 5
Compounds of nutritional interest in the biomass of *Nannochloropsis* species.

Compound	*N. oceanica* (CS 246)	*N. oculata*	*M. salina*	*N. limnetica*	N. spp.	Average value
Ascorbic acid mg/g	1–3.2[1]	6[7] – 11.8[10]	–	–		5.5
β-carotene mg/g	0.3–1.1[1]	0.07[9] – 0.14[10]	2.22[4]	0.284[4]	0.3[11]	0.63
retinol (A) μg/g	< 0.25[1]	–	–	–		0
thiamine (B1) μg/g	51–70[1]	–	–	–		60.5
riboflavin (B2) μg/g	25–62[1]	37 – 39[8]	–	–		40.75
pyridoxine (B6) μg/g	3.6–9.5[1]	–	–	–		6.55
biotin (B7) μg/g	0.95–1.1[1]	–	–	–		1.025
folate (B9) μg/g	17–26[1]	–	–	–		21.5
cobalamin (B12) μg/g	0.85–1.7[1]	–	–	–		1.275
ergocalciferol (D2) μg/g	< 0.35–0.45[1]	–	–	–		< 0.35
colecalciferol (D3) μg/g	< 0.35[1]	–	–	–		< 0.35
α-tocopherol (E) mg/g *	0.18–0.35[1]	0.77[3] – 2.33[2]	0.044[4] – 0.43[6]	0.021[4]	1.7[11]	0.73
Total phenols mg/g **		2.04[5]	6.5[4]	5.8[4]	1.39[5]	3.93
Total flavonoids mg/g ***			3.2[4]	2.6[4]		2.9

* In some cases the value is referred to total tocopherols;
** As gallic acid equivalent;
*** As quercetine equivalent;
[1] Brown, Mular, Miller, Farmer, and Trenerry (1999);
[2] Durmaz (2007);
[3] Bong and Loh (2013);
[4] Safafar, Van Wagenen, Møller, and Jacobsen (2015);
[5] Goiris et al. (2012);
[6] Safafar, Hass, Møller, Holdt, & Jacobsen (2016);
[7] Brown and Miller (1992), data log. growth phase;
[8] Brown and Farmer (1994), axenic strain;
[9] Fae Neto et al. 2018;
[10] El-Sheekh, Khairy, Gheda, & El-Shenody, 2016;
[11] Santiago-Morales, Trujillo-Valle, Márquez-Rocha, & Hernández, 2018.

Arthrospira platensis; Guiry & Guiry, 2019). Their findings obtained under atmospheric oxygen, showed that the antioxidant potential decreased significantly even at 25 °C (−60% in 60 days) and can be completely degraded by UV irradiation. Freeze-drying or spray-drying and vacuum packaging in opaque bags are the preferable downstream processes (Cerón-García et al., 2010; Ryckebosch, Muylaert, Eeckhout, Ruyssen, & Foubert, 2011). Air drying is also used for processing biomasses of *Chlorella* with good results, but preservation of lipids and color are less effective in comparison to freeze-drying treatments, which should be considered the gold standard (Hosseinizand, Sokhansanj, & Lim, 2018). Tests on the preservation of spray-dried *Haematococcus* biomasses showed that astaxanthin undergoes relevant losses also under nitrogen if stored at 21 °C, whereas the lowest degradation occurred under nitrogen at −21 °C (Raposo, Morais, & Morais, 2012). Molina Grima et al. (1994) showed that the lipid profile of lyophilized and frozen biomasses of *Isochrysis* did not change significantly after 1 month. Cerón-García, Campos-Pérez, Macías-Sánchez, Bermejo-Román, Fernández-Sevilla, Molina-Grima (2010) tested the stability of frozen, freeze-dried, and spray-dried biomasses of *Scenedesmus* after 6 weeks storage under inert atmosphere at −18 °C, finding that freeze-drying allowed the best preservation of lutein, followed by freezing, whereas spray-drying produced 30% loss in the period. In the same study, lutein, violaxanthin, ß-carotene and fatty acid profile of the freeze-dried biomass, under nitrogen, were affected at increasing temperature with different impact depending on the compound considered (ß-carotene was the most stable).

In other cases, the main factor responsible for the carotenoids degradation was the exposition to oxygen. Gouveia and Empis (2003) studied the stability of carotenoids in biomasses and extracts of *Chlorella* and *Haematococcus* stored under different conditions (temperature, light exposure, addition of antioxidants, exposure to air), showing that for both microalgae the most protective condition was under vacuum,

followed by nitrogen atmosphere, which preserved 94 and 80% carotenoids, respectively, even after 18 months. The same study evidenced that light exposure was particularly harmful for carotenoids, and that these compounds were more labile in extracts than in raw dried biomasses.

Overall, it seems that light, temperature and oxygen can impair the microalgae preservation according to an impact prevalence that depends on the biomass and the compound considered. An optimal manufacturing process might include the freeze-drying and vacuum packaging in opaque bags of the biomass, finally stored at −20 °C until the time of shipment for retail sale.

In analogy with other microalgae supplements, a typical retail product intended for human diet could be prepared in tablets for oral administration. These formulations are obtained by a simple pressing process that expels the atmospheric gas included among dehydrated cells, possibly carried out at low temperature and under inert atmosphere. For increasing product stability, the tablet surface can be coated with a gas-impermeable polymer film. Alternatively, the microalgae powder could be dosed in bio-absorbable capsules, preferably under a nitrogen atmosphere during the preparation. In any case, all materials and ingredients involved in the manufacturing processes for food supplements should be selected taking into account possible interferences with digestive processes and other factors affecting the bioavailability of the active compounds of interest (Augustin & Sanguansri, 2012; Gonçalves, Martins, Duarte, Vicente, & Pinheiro, 2018; Vidhyalakshmi, Bhakyaraj, & Subhasree, 2009).

8. Technical specifications and recommendations

In order to be authorised as novel food, *Nannochloropsis* must meet all the chemical and biological safety requirements imposed by the European regulations. On this regard, maximum levels of biological and chemical contamination could be established according to equivalences between microalgae and traditional food categories regulated by legislation, which are not always easy. In this section, an interpretative evaluation of this issue is proposed.

As general recommendation, a biomass intended for human consumption should preferably be cultivated in closed PBRs, adopting culture media prepared from drinking water to prevent the introduction of any chemical and microbiological contaminations. However, in order to reduce production costs, cultivations in open systems might be proposed, if managed according to the related best practices in use for other microalgae intended for human diet (e.g. *Haematococcus*, *Chlorella* etc.).

Regardless the cultivation technology, it is advisable that the final dry biomass respects the physical, chemical and microbiological characteristics reported in Table 6. Since it was not always possible to find reference values for food categories comparable to microalgae in the European regulations, some data have been taken from technical protocols issued for foods by the Italian Health Authority and Research Organization for Animal Health and Food Safety (Turin, Italy) (Istituto Zooprofilattico Sperimentale di Torino, 2016).

The novel food must obviously complies with the current legislation regarding mycotoxins, dioxins, dioxin-like PCBs (polychlorinated biphenyls) and polycyclic aromatic hydrocarbons. These latter aspects should not require specific controls if the culture criteria described for productions in bioreactor are adopted, provided that the materials used to build the plant meet the criteria prescribed for food purposes.

9. Toxicology

Microalgae are potential sources of dangerous toxins to human health and about 2% of the 4000 known algae produce neuro- or hepato-toxins that can accumulate in some fish products through the food chain (Katırcıoğlu, Akin, & Atici, 2004; Van der Spiegel, Noordam, & Van der Fels-Klerx, 2013). No toxins produced by *Nannochloropsis* are known (Enzig, Ploeg, Barbosa, & Sijtsma, 2014) and, as already pointed out, the toxicological safety of this microalga was proved by its long-term use as food for marine fish and shellfish larvae.

Tests have been carried out on mammals in order to verify the safety of *Nannochloropsis*. Andrés, Raúl, Luis, and Mariane (1992) fed rats with a diet consisting of 5 and 10% *Nannochloropsis* sp. for up to 4 weeks without detecting metabolic abnormalities. Kafaie, Loh, and Mohtarrudin (2012) confirmed the absence of toxicity of *N. oculata* by performing an acute intoxication test (12 g/kg body weight) and a 60 days sub-chronic test (6 g/kg/day) on Sprague-Dawley rats. No abnormalities or negative metabolic effects were observed, except for a significant reduction in blood creatinine that was deemed as not

Table 6
Quality and safety parameters suggested for *Nannochloropsis* products intended for human consumption.

Parameter	Specification	Reference method
Physical appearance	Powder	visual inspection
Moisture	Max. 10%	ISO 712
Ash	Max. 10%	ISO 5984:2002
Protein	20–55%	ISO 1871:2009
Lipid	12–60%	ISO 11085:2015
Carbohydrates	25–60%	AOAC 986.25
Fibers	Max 20%	ISO 5498:1981

Substance	Limit	Reference method
Arsenic	< 0,1 mg/kg[(1)]	EN 15763:2009, ICP-MS
Lead	< 3 mg/kg[(2)]	EN 15763:2009, ICP-MS
Cadmium	< 3 mg/kg[(2)]	EN 15763:2009, ICP-MS
Mercury	< 0,1 mg/kg[(2)]	EN 15763:2009, ICP-MS

Microorganism	Limit	Reference method
Yeasts	< 1000 cfu /g[(3)]	ISO 21,527
Moulds	< 1000 cfu /g[(3)]	ISO 21527–1/2
Listeria monocytogenes	< 100 cfu /g[(4)]	ISO 11290–1/2
Cronobacter spp. (*Enterobacter sakazakii)* only if verified as a potential risk	Absence in 10 g[(5)]	ISO/TS 22,964
Enterobacteriacee	Absence in 10 g[(6)]	ISO 21528–1
Escherichia coli	< 10 cfu /g[(3)]	ISO 16649–1o 2
Salmonella	Absence in 25 g[(7)]	ISO 6579
presumptive Bacillus cereus	m 50 – M 500 cfu/g[(8)]	ISO 7932

(1) Commission Regulation (EU) n. 2015/1006 of 25 June 2015;
(2) Commission Regulation (EU) n. 629/2008 of 2 July 2008;
(3) Istituto Zooprofilattico Sperimentale di Torino (2016) (Flours and mixed flours for further preparations);
(4) Commission Regulation (EU) n. 2073/2005 of 15 November 2005 (1.3. Ready-to-eat foods unable to support the growth of *Listeria monocytogenes*, other than those intended for infants and for special medical purposes);
(5) Commission Regulation (EU) n. 2073/2005 of 15 November 2005 (1.23. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age);
(6) Commission Regulation (EU) n. 2073/2005 of 15 November 2005 (2.2.10. Follow-on formulae in powder form);
(7) Commission Regulation (EU) n. 2073/2005 of 15 November 2005 (1.22. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age).
(8) These values are managed as food safety criteria if they exceed the limits established by Regulation (EC) 2073/2005 and subsequent amendments or the limits indicated in Annex 2 to the Technical Protocol in RTE foodstuffs at the marketing stage. Quality and safety parameters to be detected in at least 5 sample-batches, of which 1 can give values between m and M and the others < m.

relevant for toxicological evaluation.

Kagan and Matulka (2015) administered to rats 10 mL/kg /day of a suspension of alive *N. oculata* cells (10^8 cells/mL) for 14 days without the occurrence of abnormalities or undesirable effects on animal weight, appetite, urine and blood analyses, histology and organs weight. An extract of the polar lipid fraction obtained from *N. oculata*, rich in EPA, was evaluated in a 14- and 90-day study on Sprague-Dawley rats treated by oral gavage at dose levels of 0, 250, 500, and 2500 mg/kg/day and 0, 200, 400, and 2000 mg/kg /day, respectively, without any undesirable effect (Kagan, Sullivan Jr, Gad, & Ballou, 2014). Notably, this kind of extract is already marketed in the United States as a human food supplement with the Almega PL™ brand.

In contrast with the robust indications of non-toxicity above reported, Nuño et al. (2013) observed intestinal damage and reduction of acid-lactic bacteria in the gut of 4 over 5 healthy rats treated with 50 mg/day *N. oculata* for 8 weeks. The experimental design included the treatment of healthy and diabetic animals with diets comprising *N. oculata* or *Isochrysis galbana*, respectively. Omitting the effects on diabetic animals, which are not relevant here, healthy animals fed with *N. oculata* showed intestinal lesions and a significant lower weight increase compared to control subjects. These authors reported that treated rats showed an initial average weight of about 218 g, which means that a 50 mg/day ration corresponded to a maximum dosage of about 230 mg/kg/day. This dosage is far lower than 6 g/kg/day tested by Kafaie et al. (2012) and probably even than the 10% microalgae diet tested by Andrés et al. (1992), which did not describe any negative effect. Overall, the results of Nuño et al. (2013) do not find support in other studies and plausibly they could be ascribed to an undetected quality problem of the biomass. The absence of toxicity of *Nannochloropsis* is also in agreement with the findings reported by Neumann et al. (2018), who treated C57BL/6 mice with isocaloric and isoproteic experimental diets enriched with different microalgae for 14 days, one of which composed of 25% *N. oceanica*. Despite this high dosage, treated mice showed neither histological nor metabolic damage, whereas they achieved a significantly higher weight gain than the control group.

10. Nutritional value

The introduction of *Nannochloropsis* as novel food is justified by the high protein and lipid content, both of appreciable nutritional value and bioavailability. Neumann et al. (2018) tested diets containing 5, 15 or 25% *N. oceanica* in mice, in comparison to an isoenergetic and isoproteic control diet containing casein and soybean oil as reference compounds. The experimental *Nannochloropsis* containing diets showed an apparent 84–89% protein digestibility, higher than 83% of the control diet, whereas the apparent protein biological value was 59–61%, lower than 66% of the control diet. The apparent fatty acid absorption was lower for the *Nannochloropsis* supplemented diet in comparison to the control diet, but a clear increase of liver EPA levels and a higher n-3/n-6 fatty acid ratio was observed in animals treated with the algae-richest diet. The importance of a fine grinding for the complete breaking of the microalgae cell walls in order to improve the bioavailability of nutritional compounds was also reported (Neumann et al., 2018). This is consistent with the findings of Bernaerts et al. (2020), who showed that the disruption of *Nannochloropsis* cells strongly enhances bioaccessibility of carotenoids and ω3-LC-PUFA. Similar conclusions were also reported by Goh, Loh, Fatimah, and Perumal (2009), comparing the bioaccessibility of carotenoids and tocopherols in raw biomass and extracts of *N. oculata*.

In fact, cell wall of *Nannochloropsis* can interfere with the solubilization and digestion of the cell compounds. Brown (1991) found that the cell wall polysaccharides of *Nannochloropsis oculata* contained ~68% glucose along with about 4 to 8% rhamnose, mannose, ribose, xylose, fucose, and galactose. Vieler et al. (2012) studied the cell wall of *N. oceanica* CCMP1779 describing a similar composition, with the addition of arabinose. Scholz et al. (2014) characterised a large part (86.5%) of the cell wall of *M. gaditana*, and resulted composed of carbohydrates (79.2%), proteins (6.2%) and minerals (1.1%). The wall architecture showed an outer algaenan layer (lipid-related polymers highly resistant to alkali/acid hydrolysis and aqueous/organic solubilisation) protecting an inner cellulose layer. A fine mechanical milling, high pressure homogenisation or treatment with cell wall degrading enzymes could be determinant for improving the biomass digestibility (Bernaerts et al., 2020; Zuorro, Miglietta, Familiari, & Lavecchia, 2016).

Overall, *Nannochloropsis* can be considered as a good source of proteins and fats, with a very desirable fatty acid profile. The intake of EPA and other PUFAs represents the most relevant element of value for this microalga, enhanced by an important contribution of other antioxidant compounds with high biological activity, i.e. polyphenols, carotenoids and vitamins. The intake of proteins is appreciable for quality and bioavalability, but given the high production costs, it would not be convenient to propose this microalga as a protein source.

The EFSA/EU Menu Guidelines (EFSA, 2014; Appendix 5.2.2) recommended the administration of supplements based on algae or fortifying agents based on essential n-3 fatty acids in a maximum amount of 50 g/day, or 30 g/meal. In another report, EFSA-NDA recommended an intake of DHA + EPA of 250–500 mg/day (EFSA-NDA, 2012).

Table 7
Recommended Dietary Allowance (RDA) of compounds and elements of nutritional interest indicatively present per gram of marine species of *Nannochloropsis* (dry biomass).

	Measure unit	RDA	*Nannochloropsis* Amount/g	% RDA	RDA References
EPA + DHA	mg	250–500	40 (EPA)	8–16	EFSA/EU Menu Guidance, Appendix 5.2.2,
Carotenoids	**mg**	**10**[1]	**5.6**	**56**	Riccioni (2009); Satoh et al. (2009); Ma et al. (2016)
β-carotene	**mg**	**3,6(♀),4,2(♂)**	**1.31**	**31.2–36.4**	LARN (2014)[2]
ascorbic acid (C)	mg	85(♀),105(♂)	5.5	5.2–6.5	LARN (2014)
α-tocoferol (E)	mg	12(♀),13(♂)	0.73	5.6–6.1	LARN (2014)
thiamine (B1)	mg	1.1(♀), 1.2(♂)	0.061	5.0–5.5	LARN (2014)
riboflavin (B2)	mg	1.3(♀),1.6(♂)	0.041	2.6–3.1	LARN (2014)
folate	μg	400 (♀ = ♂)	21.5	5.4	LARN (2014)
pyridoxine (B6)	μg	1300 (♀ = ♂)	6.6	0.5	LARN (2014)
cobalamin (B12)	**μg**	**2.4(♀),2.4(♂)**	**1.28**	**53.3**	LARN (2014)
biotin	μg	30(♀),30(♂)	1.03	3.4	LARN (2014)

Compounds present at high level in the biomass (mid-range value ≥ 10% RDA) are in bold. Values are referred to the recommended daily intake for adults (age 30–59) with a standard weight of 70 kg for men and 60 kg for women (considered values for menstruating women).

[1] There are no recommended doses for xanthophylls, the value has been hypothesized on the basis of safe and effective dosages studied for lycopene, astaxanthin and zeaxanthin;

[2] Value calculated by multiplying × 6 the recommended dose of vit. A (6–700 μg/day).

Table 8
Content of EPA + DHA in fish fillets and conversion ratios from the wild fish used for the production of some feed raw ingredients.

Farmed fish				
	DHA + EPA skinned fillet	skinned fillet yield	wet fish biomass required	Feed required
Seabream	1.25 g (100 g skinned fillet)	42.0%	238 g	405 g (FCR 1.7)
Seabass	1.28 g (100 g skinned fillet)	42.4%	236 g	425 g (FCR 1.8)
Impact of the feed required				
	Seabream (100 g skinned fillet)	Seabass (100 g skinned fillet)	Forage fish consumed	
fish meal (20.2% of feed)	81.8 g	85.9 g	364–382 g (meal yield 22.5%)	
fish oil (14.8% of feed)	59.9 g	62.9 g	1198–1258 g (oil yield 5%)	

Data on farmed fish and on the composition of conventional feeds were calculated according to Di Marco et al. (2017); Fish-meal and fish-oil yield from wet wild fish forage were from Jackson (2009).

Table 7 shows the compounds of nutritional interest and related daily assumptions recommended by the Italian Society of Human Nutrition (LARN, 2014), in comparison with potential intakes provided by a dry biomass of *Nannochloropsis* with composition estimated from average data reported in here reported Tables.

11. Concluding remarks and perspectives

All the available information, i.e. use of *Nannochloropsis* as food for aquaculture species, data on its composition and toxicological tests, supports the conclusion that this microalga shows no contraindication for human nutrition. The quality of proteins and lipids is consistent with this conclusion, but *Nannochloropsis* appears of particular interest for the combination of different active compounds (carotenoids, polyphenols, tocopherols, polyamines) in association with a high content of PUFAs, especially EPA. Among carotenoids, some xanthophylls scarcely available in higher plants occur, with beneficial effects probably not fully elucidated yet. Among these, zeaxanthin is an important component of the retinal macula and can be useful for the prevention of degenerative pathologies affecting the eye (Ma, Liu, Du, Liu, Wu, & Liu, 2016), whereas the whole xanthophyll-complex can contribute to the protection of the cardiovascular system (Riccioni, 2009; Sommerburg et al., 1999). In fact, in addition to an antioxidant activity, they modulate the cell transcriptome and regulate some key points of the enzymatic cascade involved in the inflammatory response (Berthon et al., 2017; Muthuirulappan & Francis, 2013). Furthermore, *Nannochloropsis* is a good source of vit. E, folate and vit. B_{12}.

The phenolic compounds content, indicatively varying between 1.4 and 6.0 mg/g, is not high if compared to other sources, such as fruits (from 5.5 to 40 mg/g DW) (Cieślik, Gręda, & Adamus, 2006), however, this fraction is still insufficiently characterised and its functionality might go beyond the activity as antioxidants. For instance, some anti-inflammatory and antimicrobial activity (gallic acid) and antifungal effect (caffeic acid) have been already disclosed (Tessmer Scaglioni et al., 2018).

The reported data show that the compounds of interest undergo quantitative variation in *Nannochloropsis*, depending on strain and cultivation conditions. As a general assumption, however, the dietary intake of EPA can be considered the key compound for establishing an appropriate daily ration of *Nannochloropsis*. A daily intake of 6 g of microalga, comprising 4% EPA, guarantees the assumption of 240 mg/day EPA, in line with the recommended dietary allowance (RDA) (EFSA-NDA, 2012), aside from about 30% RDA of folate, 35% vit. E, 200% vit. A, 350% vit. B12, respectively (LARN, 2014). Importantly, as showed in Table 8, the intake of long chain PUFAs is comparable with that assured consuming 100 g fillet of seabass or seabream, whose production requires fish-meal and fish-oil obtained from about 400 and 1,200 g wild forage fish, respectively (these amounts neglects losses due to farm mortality). According to Moffat and McGill (1993), some of the fish oils richest in EPA + DHA contain 236–269 mg/g, thus feed containing 60 g fish oil required to produce 100 g fish fillet (Table 8) could vehicle 14–16 g EPA + DHA, of which only about 8% is retained in the final edible product. The advantage of using microalgae in terms of ethicality and sustainability is evident, but also other functional aspects can be considered. For instance, the reported fish fillet composition refers to the raw product, which therefore ignores PUFAs losses and reactions due to cooking. This issue should be considered by nutritionists, since the beneficial effects of EPA described for Inuit populations were related to the consumption of raw fish, while the fate of this compound following different cooking methods has not been sufficiently studied.

Therefore, the administration of microalgal supplements in tablets or capsules can provide an optimal solution to balance the diet of many people, including those who, for various reasons, are reluctant to consume fish or do not find time and opportunity to obtain fish regularly. In this perspective, microalgal supplements represent an opportunity to promote a healthy diet and reduce metabolic and aging pathologies, consistently with an environmentally and economically sustainable approach.

Ethical statement

The proposed work did not include any human subjects and animal experiments

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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