Worldwide Research Trends on Microalgae and Recent Work in Cytologia

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Summary The latest issue of Cytologia (Vol. 85, Issue 2) includes a regular paper on the long-awaited karyotype analysis of the primitive red alga, *Cyanidioschyzon merolae* 10D, by Kuroiwa *et al.* (2020) and a technical note by Miyagishima and Fujiwara (2020) introducing a method to induce a controllable gene expression system in *C. merolae*. In recent years, Cytologia has focused on introducing the latest research on microalgae and has published excellent papers, such as the two mentioned above. An analysis of worldwide trends in microalgae research based on a bibliometric study found that the number of published papers has increased exponentially since 1990 and 2008. The most relevant journals in this field are “Bioresource Technology” and “Algal Research.” Similarly, the number of papers in Cytologia is increasing steadily. Much interest is focused on *Chlorella* and *Chlamydomonas*. The major keywords appearing in more than 1,000 articles are those related to microalgae culture, such as “biomass,” “biofuels,” “lipids,” and their applications, or methodologies such as “bioreactor.” Today, microalgae are attracting attention (i) as sources of high-value compounds such as carotenoids, polyunsaturated fatty acids and phycobiliproteins, (ii) as whole biomass used as ingredients for food and feed, (iii) as extracts or processed biomass to produce biofuel and biofertilizers, and (iv) for bioremediation to ameliorate wastewater, soil, and polluted smoke. In this paper, we focus on how algae and microalgae are treated in Cytologia.

Keywords Microalgae, Bibliometrics, *Cyanidioschyzon*, *Chlorella*, Biomass, Biofuel.

To describe the evolution and latest trends in microalgae research, a bibliometric study of worldwide trends reviewed the numbers of papers, their citations, and the most relevant journals and keywords in this field (Garrido-Cardenas *et al.* 2018). This confirmed that the most relevant journals in this area are “Bioresource Technology” and “Algal Research,” and the numbers of papers published in both journals have increased rapidly in the last decade. Although most of the papers are published by American researchers, many come from research institutions in China, France, and Spain. The most frequently cited species are members of *Chlorella* and *Chlamydomonas*. The major keywords appearing in more than 1,000 articles are those related to microalgae culture such as “biomass,” “biofuels,” “lipids” and their applications or methodologies such as “bioreactor.” “Biomass” appears in approximately 20% of the papers.

Bibliometric analysis of publications on microalgae

Garrido-Cardenas *et al.* (2018) confirmed that micro-

algae biotechnology has become a very active area, and research productivity has increased exponentially in recent years, keeping pace with industrial production. The remarkable growth of this relatively new research field over the last few years has been strongly supported by the development of culture technology and the demand for microalgae-based products. Microalgae biotechnology includes both eukaryotic microalgae and prokaryotic cyanobacteria. Although they are distinct groups, the final industrial products/applications overlap, due to the chemical similarity of their principal cell components. Today, these micro-organisms are widely used (i) as sources of high-value compounds such as carotenoids, polyunsaturated fatty acids, and phycobiliproteins, (ii) as whole biomass used as ingredients for food and feed, (iii) as extracts or processed biomass to produce biofuel and biofertilizers, and (iv) for bioremediation to ameliorate wastewater, soil, and polluted smoke.

Although there has been interest in microalgae for decades, the first studies of microalgae culture under controlled conditions began in the 1950s (Fig. 1). The same trend can be seen in Cytologia; the first progress began with research on the unicellular microalgae *Euglena* (Ueda 1958), while a pioneering study of *Spirogyra*, a

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Fig. 1. The number of microalgae papers published each year in Cytologia (bar graph, inner vertical-axis). Comparison with the worldwide evolution of papers on representative microalgae strains (line graph, outer vertical-axis). The horizontal axis starts in 1929, when Cytologia was first published. These analyzes depend on the databases of “Web of Science” and “J-STAGE,” which is an electronic journal platform for science and technology information in Japan.

filamentous charophyte green algae in the order Zygnematales, appeared in the 1930s (Shinke and Shigenaga 1933). Over the years, various photobioreactors have been proposed, such as raceways and tubes (Golueke and Oswald 1963), and are still frequently used today. Globally, the earliest species studied were *Chlamydomonas*, *Chlorella*, and *Scenedesmus*, they are still the most widely cultivated today. There have been extensive physiological and genetic studies of *Chlamydomonas*, which is a model microorganism for photosynthesis and molecular biology studies. *Chlamydomonas* was first reported in Cytologia only in 1979 (Gruber and Rosario 1979).

Early microalgae products were limited to whole biomass, such as for human food or aquaculture feed. Subsequent microalgae biotechnology research had four goals: (i) to search for new strains producing novel, valuable compounds that grew rapidly and easily; (ii) to gain knowledge of the biology of strains and elucidate the mechanisms regulating cell performance; (iii) to improve production systems in terms of both efficiency and capacity; and (iv) to develop new markets and products (Richmond 2000). This was revealed in the bibliometric analysis by Garrido-Cardenas *et al.* (2018).

The most studied microalgae in international publications

Thousands of microalgae strains are now available from culture collections, but only a few have been studied in detail. New strains that have achieved commercial success as sources of valuable compound include *Haematococcus pluvialis* for astaxanthin and *Dunaliella salina* for beta-carotene (Leu and Boussiba 2014). However, hundreds of other strains are reported sources of carotenoids. The main reason why these strains are not suitable for large-scale commercial cultivation is their weakness in outdoor culture or poor productivity. Typically, vigorous strains are selected for outdoor culture, such as strains that are less susceptible to short-term changes in culture conditions or those that can grow in various environments. *Euglena* and *Porphyridium*, which are mainly used as dietary supplements and in cosmetics (Borowitzka 2013), are currently produced on large scales, but their production capacity remains low. The commercial use of seawater algae is gradually increasing, including *Nannochloropsis*, *Tetraselmis*, *Isochrysis*, and *Chaetoceros* species as high-quality feed for juvenile fish and crustaceans in aquaculture. Cytologia only mentions the mitochondrion-dividing ring of *Nannochloropsis* once (Hashimoto 2004), so studies of these microalgae for practical use are not very popular in Cytologia.

Using biology and genetics techniques, great effort has been made to elucidate fundamental synthesis mechanisms before trying to increase target compound accumulation, focusing especially on fatty acids and astaxanthin (Han *et al.* 2013). Many scientists have applied methodologies developed for other organisms to microalgae but, unfortunately, those approaches have not been successful because of microalgae characteristics, such as their robust cell walls. In the early days, super-producing strains were usually selected, but the improvements achieved were limited. Subsequent attempts to screen for mutants were far from efficient, because random mutations usually revert to the wild-type after a few generations. Recently, innovations in molecular biology have made it possible to acquire stable overproducing strains (Dautor *et al.* 2014).

Figure 1 shows the number of papers on algae published Cytologia annually from 1929, the year it was first published, to 2019. We also investigated temporal changes in publications on major microalgae using the citation databases of “Web of Science.” Globally, there were few publications on microalgae before 1950. Numbers began to increase slowly after about 1960 and more dramatically since 1990 and 2008. The latest one might have been triggered by the Nature article “Algae bloom again” (Haag 2007). From 1960 to 1990, papers on all the microalgae examined increased moderately. After 2008, there was a remarkable increase in papers on *Chlorella*. Papers on other genera, divided into a group including *Chlamydomonas*, *Scenedesmus*, and *Nannochloropsis*, have been increasing significantly, although far fewer than on *Chlorella*, while there has been very little change in papers on the second group (*Phaeodactylum*, *Isochrysis*, and *Spirulina*).

The bibliometric analysis of all microalgae publications shows a significant increase from 1970 to 2017, especially since 2007 (Garrido-Cardenas *et al.* 2018). This is consistent with the recent rapid increase in market demand for microalgae products and the rush to construct microalgae culture facilities (Forján *et al.* 2014). *Chlorella* and *Chlamydomonas*, studied for decades, are the genera most frequently mentioned in scientific documents. Interestingly, different genera attract attention in different countries (Garrido-Cardenas *et al.* 2018). For example, in the United States there is growing interest in these two genera, while in China *Scenedesmus* is the next most prominent after *Chlorella*. The most studied microalgae in Spain are *Phaeodactylum* and *Isochrysis*. Brazil is the only country where *Spirulina* is studied the most, although it is the seventh most commonly mentioned microalgae in a keywords analysis of research publications worldwide. Although genera such as *Dunaliella*, *Euglena*, *Porphyridium*, and *Haematococcus* are not yet widespread in academic studies, the number of publications about them should increase significantly in the near future.

Microalgae first published in Cytologia

Cytologia, which was first published in 1929, has published more than 5,800 papers (Kawano 2019). In the early years of this international journal, the first description of algae appeared in 1933. That paper first mentioned *Spirogyra* in Cytologia (Shinke and Shigenaga 1933). As the title “A histochemical study of plant nuclei in rest and mitosis” indicates, stained tissue samples of various plants were compared to distinguish nuclei at the two stages. For example, the thymus nucleic acid reaction was compared in Virginia spiderwort (*Tradescantia ohiensis*), tiger lily (*Lilium tigrinum*), evening primrose (*Oenothera* sp.), broad bean (*Vicia faba*), corn (*Zea mays*), and *Marsilea quadrifolia*, which is a peculiar aquatic fern with a clover-like form. The nucleus of *Spirogyra* did not react to thymus nucleic acid, but to a protein, in striking contrast to the other plants investigated.

In 1939, the fluidity of cytoplasmic streaming in *Spirogyra* was reported to be increased by a micro-current (Northen and MacVicar 1939). Cytoplasmic streaming is the flow inside the cytoplasm that transports various molecules to organelles. Using ATP as an energy source, fluidity is generated by the interaction between actin filaments forming the cytoskeleton and the motor protein myosin. At that time, the mechanism of this phenomenon was not well understood. Research using *Chara* and slime molds has been ongoing since 1772, when Bonaventura Corti of Italy published his microscopic observations of the circulating cell contents. *Spirogyra* was considered a model cell for studying cytoplasmic streaming. Several similar papers were published subsequently (Northen 1939, Nishina *et al.* 1940, Yamaha and Suita 1940).

Yotsuyanagi (1953) wrote the earliest paper on *Chara corallina* in Cytologia, titled “Recherches sur les phénomènes moteurs dans les fragments de protoplasme isolés [Research on the active phenomenon in isolated cytoplasm].” He investigated whether cytoplasmic movement occurred in protoplasmic fragments using *Chara* cells separated from the cytoplasmic structure by cleaving. Sasaki (1935) reported the morphology of the spermatozoid in *Chara* and *Nitella* with reference to the structure of the motile apparatus. The earliest paper on *Nitella* alone was a German paper on chloroplast abnormalities in *Nitella* culture (Kusunoki 1944). In 1953, observations of the nucleolar organization and nucleolar organizing chromosome during mitosis in *Spirogyra* were reported (Oura 1953). These are the first papers on the characteristics of specific algae in Cytologia.

Spirogyra belongs to the Conjugatophyceae, which is thought to be the group most closely related to land plants, along with Charophyceae to which *Nitella* and *Chara* belong (Wodniok *et al.* 2011, Timme *et al.* 2012, Ruhfel *et al.* 2014, Wickett *et al.* 2014, O.T.P.T.I. 2019). Many researchers consider Conjugatophyceae to be

very different organisms from other algae. In 1954, new knowledge on reproduction and reproductive cells of the freshwater alga *Chaetomorpha okamurae* Ueda was reported (Hirose 1954). *Chaetomorpha* belongs to the Phylum Chlorophyta, Class Ulvophyceae, and researchers are unanimous that it is an alga. Other studies of the Class Ulvophyceae include a study on the cytology and life-history of *Cladophora uberrima* Lambert, from Ranchi, India (Sinha and Ahmed 1973), a karyotype analysis of three *Rhizoclonium* Kütz species (Verma 1980), and the ultrastructural changes of *Chaetomorpha brachygona* growing in a metal environment (Chan and Wong 1987).

“Algae” is a general term for organisms that perform oxygen-generating photosynthesis, excluding mosses, ferns, and terrestrial seed plants. “Algae” used to be considered a monophyletic group of lower plants, but are now considered polyphyletic. “Microalgae” is a generic term for diverse taxa of single-cell organisms performing photosynthesis and includes polyphyletic groups. However, red algae (Rhodophyta) are thought to belong to the monophyletic Archaeplastida, together with the Glaucophyta plus the green algae (Chlorophyta) (Adl *et al.* 2019).

Focusing on microalgae in Cytologia, Ueda (1958) reported optical and electron microscopy examinations of the structure of pure *Euglena gracilis* cultures. This was the second of five papers published by Ueda in a series titled “Structure of plant cells with special reference to lower plants” from 1956 to 1961. The others were studies of *Spirogyra setiformis* mitosis (Ueda 1956), the structure of *Trachelomonas* a genus of flagellates closely related to *Euglena* (Ueda 1960a), nuclear division in *Oedogonium* (Ueda 1960b), and a comparison of the fine structure of 21 algae chloroplasts (Ueda 1961). Subsequent studies of *Euglena* included papers on mitosis in *Euglena acus*, *E. spirogyra*, and *E. viridis* (Leedale 1959), depolymerization patterns of the nuclear constituents in the interphasic and mitotic stages of *Euglena gracilis* var. *bacillaris* treated with DNase, RNase, trypsin, and α -chymotrypsin *in vivo* (O'Donnell 1965), and nuclear changes following treatment with actinomycin D (O'Donnell-Alvelda 1967). Then, there were no papers on *Euglena* in Cytologia for 40 years until Sumida *et al.* (2007). In the 1960s, Ueda (1966) reported the fine structure of *Chlorogonium elongatum* (Chlorophyceae), especially the origin and development of vacuoles.

The most frequently reported microalga in Cytologia is the primitive red alga *Cyanidioschyzon merolae*, which is studied in 27 papers. *C. merolae* is a unicellular red alga with a diameter of about 1.5 μ m isolated from a hot spring in Italy; its cell division is easily synchronized by controlling light conditions. Therefore, research on the cell division mechanism, especially on the mitochondrial and chloroplast division machinery, has been conducted for many years. The first whole ge-

nome sequence of a eukaryotic alga was completed by Kuroiwa's group (Matsuzaki *et al.* 2004). The second most prominent species in the microalgae papers of Cytologia is *Chlamydomonas reinhardtii*, which is a model organism in molecular studies, especially flagellar movement, chloroplast dynamics, developmental biology, and genetics. At least 12 papers on *C. reinhardtii* have been reported in Cytologia. Since 2000, it has been possible to transform *C. reinhardtii* using selectable markers after its genome was completed, establishing it as a model organism. The culmination of this is an inducible, repressible gene expression system developed by Miyagishima and Fujiwara (2020).

Cytologia today and microalgae

Spermatophyta is prominent in Cytologia, as the subject of 70 to 83% of the papers in the last two years. The subject species are very diverse, and include more detailed studies of well-known model plants such as *Arabidopsis thaliana* (Hayashi *et al.* 2018b, Shibuta and Matsunaga 2019, Shimahara *et al.* 2019, Toyoda and Matsunaga 2019) and onion, *Allium cepa* (Barbhuiya *et al.* 2018, Verma and Srivastava 2018, Basu *et al.* 2019, Roy and Roy 2019, Tütüncü *et al.* 2019). Species with regional characteristics have also been reported, as pointed out in a “Cytologia Focus” two years ago (Kawano 2018).

Some examples of local plant research include the cytology of male meiosis in 17 species in 11 genera of the family Poaceae from Haryana and the Shiwalik hills (Dhaliwal *et al.* 2018b), a cytomorphological study of important medicinal plants of *Cenchrus* L. from north India (Dhaliwal *et al.* 2018a), a comparative karyotype analysis of five strains of *Colocasia esculenta* in Thailand (Senavongse *et al.* 2018), chromosome reports for 26 species of Asteraceae from cold deserts of the western Himalayas (Gupta *et al.* 2018), male meiosis in 29 species of Lamiaceae from the Sirmaur district, Himachal Pradesh, India (Singh *et al.* 2018), and new chromosome reports on Commelinaceae from Indian hot desert in Rajasthan (Kaur and Gupta 2018).

Two Indian research groups reported three papers on the induction of mutants of coriander *Coriandrum sativum* L. by gamma irradiation (Pramanik *et al.* 2018a, 2018b, Kumar and Pandey 2019). Coriander is an annual herb native to the eastern Mediterranean; it was one of the most commonly used herbs in ancient Greece and Rome, and is now widely used in cooking around the world. It was described in Pliny's “Naturalis Historia.”

Today, the import and export of agricultural products is a major industry. Increasingly, cutting-edge technology will be applied to accelerate spice breeding. Cytologia has reported studies on stable capsaicinoid biosynthesis during fruit development in *Capsicum baccatum* (Sugiyama 2019) and karyotype analysis

from aerial roots of *Piper nigrum* based on Giemsa and fluorochrome banding (Jha 2019). In Japan, male sterile plants were confirmed in two major domestic cultivars of *Brassica rapa*: “Kida-aokabu” and “Tennhoji-kabu” (Ozeki *et al.* 2019).

Studies in Cytologia have also examined fish chromosomes. Alongklod Tanomtong’s group from Khon Kaen University, Thailand, is playing an active role. They have investigated the karyotype and chromosome characteristics of nucleolar organizing regions (NORs) in Thai fishes, including the snakeheads *Channa marulius* and *C. maruloides* (Perciformes: Channidae) (Sarasan *et al.* 2018), hihgfin barb, *Cyclocheilichthys armatus* (Cypriniformes, Cyprinidae) from the Chao Phraya River (Chaiyasan *et al.* 2018), and longfin carp, *Labiobarbus leptocheilus* (Cypriniformes, Cyprinidae) from the Mekong Basin (Saenjundaeng *et al.* 2018). They have also published papers on the tropical oyster *Crassostrea iredalei* (Ostreoida, Ostreidae) from Nakhon Si Thammarat province (Chooasangjaew *et al.* 2018) and black lancer, *Bagrichthys majusculus* (Siluriformes, Bagridae) from Nakhon Phanom and Sing Buri provinces (Supiwong *et al.* 2018).

The Turkish researchers Ayata and Gaffaroğlu (2019) published a chromosome study of an endemic fish, *Luciobarbus kottelati* (Teleostei, Cyprinidae). As model organisms, there are also technical notes on medaka,

Oryzias latipes by Watanabe-Asaka *et al.* (2018) and zebrafish, *Danio rerio* (Iwasaki and Wada 2019), which were illustrated on front covers of the journal. Other papers studied *Drosophila melanogaster* (Takase and Suzuki 2018, Natori and Kojima 2019) and *Xenopus laevis* (Nakamura and Yamamoto 2019); it is obvious that model organisms are well studied not only in plants but also in animals.

Regarding microalgae, Cytologia does not directly reflect the recent global trend and the number of papers has not increased dramatically. However, Matsunaga (2018) published a focus paper titled “Planimal cells: Artificial photosynthetic animal cells inspired by endosymbiosis and photosynthetic animals” that sets out microalgae as the most fascinating autotrophic organisms for creating planimal cells. Uchida *et al.* (2018) wrote a review titled “Algal genes encoding enzymes for photosynthesis and hydrocarbon biosynthesis as candidates for genetic engineering.” This review focused on *Botryococcus braunii* and is the first mention of this species in Cytologia. In addition, Ota *et al.* (2019) wrote an original review-style paper titled “The *Parachlorella* genome and transcriptome endorse active RWP-RK, meiosis and flagellar genes in trebouxiphycean algae,” considering the expression of MID-related RWP-RK genes and meiosis-specific and flagellar proteins in trebouxiphycean algae. The only other microalgae paper deals with *C. merolae*

Fig. 2. Twenty years of algae biotechnology: from biofuels to functionality. A diagram showing the changes in US energy policy and trends in functional biotechnology since the Bush administration began in 2001. Largely expanded and redrawn after Yue *et al.* (2014).

(Hayashi *et al.* 2018a). There are also reports on the marine green macroalga *Chaetomorpha spiralis* (Miyamura *et al.* 2018), the giant-celled green alga *Valonia utricularis* (Mine *et al.* 2018), the filamentous charophyte alga *Klebsormidium nitens* (Takano *et al.* 2018), and female and male gametes of the marine green alga *Ulva arasaki* (Miyamura *et al.* 2019).

Algae biotechnology and Cytologia

Microalgae are attracting attention as biofuels. The forecast international market for biofuels ranges from 756 billion to 20 trillion yen in 2035, which will create new core industries. Its raw materials have shifted from sugar cane and corn (first generation) to non-edible biomass such as cellulosic waste (second generation) and microalgae biomass (third generation). The transition was largely affected by international situations, such as food competition problems caused by the soaring oil prices during the 2003 Iraq War, the global financial crisis triggered by the collapse of Lehman Brothers in 2008, policy changes due to changes in government in major countries, and the Paris Agreement, a multilateral international agreement on climate change control, adopted at COP21 in 2015 (Fig. 2).

At the dawn of 21st century, supported by the high crude oil price at the time, the proposal that microalgae could be a source of biofuels attracted large energy companies and huge investment in the algal biotechnology industry. A series of highly relevant papers that established the potential of these technologies was published (Fig. 1). However, the actual biodiesel production from microalgae remains distant, at theoretical values at best, and bioenergy production from microalgae has not yet been realized. Nonetheless, the significant investment made has led to a leap in technology and production capacity, which is currently driving the expansion of commercial microalgae applications.

In recent years, technology has improved. Today, there are industrial facilities with photobioreactors and raceway reactors covering hundreds of hectares. Most of the microalgae biomass produced worldwide is still produced using the outdoor raceway method (Benemann 2013). The production costs of microalgae biomass can be reduced to 5 €kg⁻¹ and further reduced to 1 €kg⁻¹ when combined with wastewater treatment using CO₂ capture from flue gas (Acién *et al.* 2012). As a result, new microalgae applications focused on wastewater treatment are now scaled up for industrial processes, and the cheap biomass produced is suitable for use in low-value markets, such as biofertilizers. Hopefully, microalgae biotechnology will continue to grow steadily, improving both technology and products and expanding to new applications.

New strategies should increase the share of microalgae in the biodiesel and biojet fuel markets. In addition to their potential as a fuel source, microalgae can

be used to produce high-value components for various fields. Microalgae contain useful antioxidants, natural dyes, polyunsaturated fatty acids, and other raw materials and have already been used in cosmetics, dietary supplements, feeds, *etc.* New functional substances have also been proposed. Over the last decade, the focus has shifted from biofuels to functionality in algae biotechnology (Fig. 2). If a biorefinery that integrates the production of biofuel and functional substances derived from microalgae can be created, it will reduce biofuel production and enable early commercialization, which will make a significant change in social systems and industrial structures.

The market for raw material algae biotechnology in Japan, so-called “new oil,” has grown rapidly to 145 billion yen, while the traditional market for marine algae such as nori (laver sheet), kombu (kelp), and wakame (seaweed) (350–360 billion yen) has gradually lost its share. Docosahexaenoic (DHA, C22:6) and eicosapentaenoic (EPA, C20:5) acids are attracting the attention of the omega-3 fatty acid market (37 billion yen); in addition to fish oil, DHA and EPA derived from microorganisms and microalgae are starting to appear. The micro- and macro-algae markets, mainly in the form of supplements, are valued at 163.4–173.4 billion yen, while the total market in Japan was estimated to be 15,624 billion yen recently (Koyande *et al.* 2019, Kulshreshtha *et al.* 2020). The international market for functional foods and supplements is booming, but total sales of *Spirulina* and astaxanthin were only 389.5 billion yen, far below the level of biofuel, which is expected to reach 0.60–19 trillion yen (Ogunkunle and Ahmed 2019).

The major factors limiting the expansion of microalgae biotechnology are high production costs and the small scale of current production systems. As a result, fewer than 20,000 tonnes of biomass are produced worldwide at a cost of more than 5 €kg⁻¹ (Borowitzka 2013). Due to the high costs, microalgae biomass is limited to the market for high-value products, such as human food and some aquaculture feed (Vigani *et al.* 2015). To solve these problems, more robust and efficient production systems are needed to increase production capacity exponentially. Large-scale production will significantly reduce production costs. Basic research on the breeding of microalgae strains and the value of biomass will contribute to these advances. More reports on microalgae biotechnologies in Cytologia are indispensable, such as those of Ota *et al.* (2014) and Takeshita *et al.* (2015).

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