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Culturing Diatoms

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Abstract
In this chapter, we provide information on cultures of diatoms, starting with a brief introduction of the history of diatom culture collections. We present a synopsis of diatom strains currently available in culture collections and their representation in terms of habitat diversity, geographical distribution, phylogeny and taxonomic diversity. We outline the major techniques for isolating and cultivating diatoms and discuss the problems and possible strategies linked to diatom life cycles and habitat requirements. We summarize information and techniques available for long-term preservation of diatom cultures. Finally, we also describe the main strategies to make diatom strains amenable to genetic engineering.

***Keywords*:** Diatoms, culture, life cycles, genetic engineering, cryopreservation

14.1 Introduction

Diatoms were first discovered in the 18th century, but it was not until the middle of the next century that the first cultures were established. The actual cultivation of diatoms was significantly developed at the end of the 19th century (Figure 14.1, Richter 1903) pioneered by Miquel (1892). Museums, botanical gardens, zoos and research facilities have served as repositories to preserve collections of fixed specimens, notably type material, for centuries, with such facilities deemed reliable places to safeguard specimens in the state in which they were first deposited, be sure that they are cared for, and be

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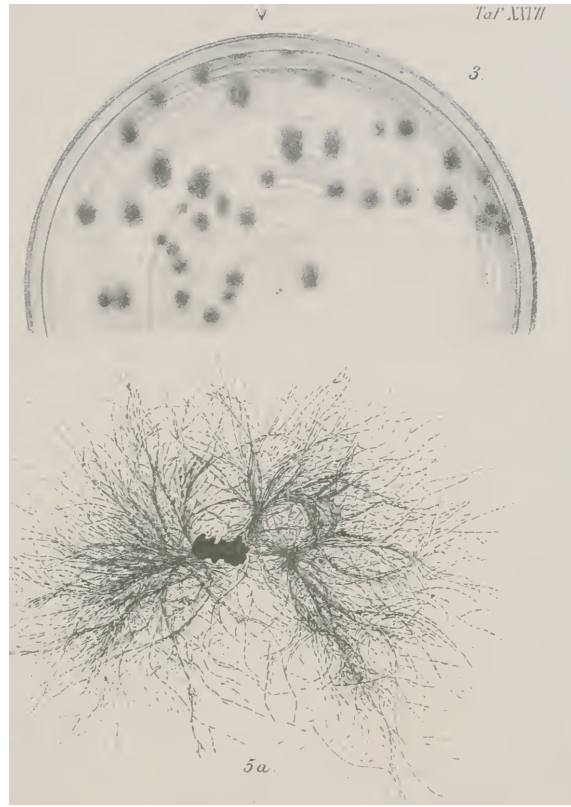

Figure 14.1 Colonies of *Nitzschia palea* growing on agar (reprinted from Richter 1903).

able to distribute them to researchers so that scientific research can be reproduced and expanded (Smith and Ryan 2012). The development of repositories for living material is more recent, Ernst Pringsheim having established the first culture collection catalogue in 1928. Cultures are extremely useful for studies on diatom taxonomy and life cycles, although such work can also be undertaken on natural populations (Mann and Chepurnov 2004). Cultures are, however, absolutely essential for physiological studies, in particular in the domain of photosynthesis, as well as for the biochemical characterization of cells. Cultures are also key for the acquisition of "omics" data, which is playing a central role for understanding the regulation of cell growth and functions (Armbrust *et al*. 2004; Bowler *et al*. 2008).

In recent decades, the development of large public microalgal culture collections has increased the quantity and diversity of cultures at the disposal of diatom researchers, in particular from environments such as the polar regions for which few strains were previously available. In this chapter, we inventory culture collections that host diatom strains and provide information on the major approaches to isolate, culture and preserve diatoms, detailing constraints linked to the specific life cycle of diatoms. We end by reviewing recent advances in the development of techniques for the genetic transformation of cultured diatoms, which are opening new avenues for the production of novel functional knowledge.

14.2 Current Diversity of Diatoms in Culture

Diatoms are extremely diverse in terms of shapes and structures (Figure 14.2), as well as habitats and physiology. Only a small fraction of this diversity is currently represented in cultures.

We identified 27 algal collections for which we were able to obtain listings of the diatom cultures they currently maintain (Table 14.1). More collections contain diatom strains, but they either escaped our search or did not answer our request for information. Some collections harboring many diatom strains have also disappeared (for example, the Scandinavian Culture Collection for Algae & Protozoa, Copenhagen, Denmark or the Korea Marine Microalgae Culture Center; Hur *et al*. 2015). Some of these collections are registered with the World Data Center for Microorganisms (WDCM, https://ccinfo.wdcm.org), while some do not have a website where researchers can order strains. The number of diatom cultures in these collections varies from a few to more than two thousand. Some collections

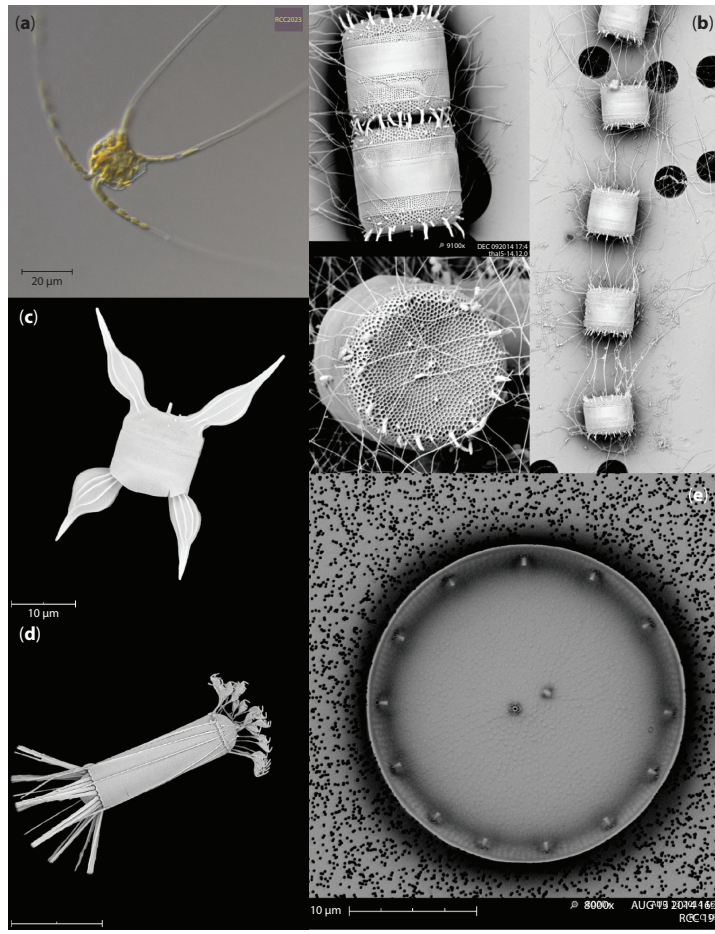

Figure 14.2 Examples of marine diatoms. (a) *Chaetoceros peruvianus* RCC2023. (b) *Thalassiosira delicatula* RCC2560. (c) *Chaetoceros bulbosus* from a natural sample off New Zealand. (d) *Corethron pennatum* from a natural sample off New Zealand. (e) *Shionodiscus bioculatus* RCC1991.

Table 14.1 List of culture collections containing diatoms. Only collections that contain diatoms and answered our survey are included. WDCM corresponds to collection ID in the World Data Center for Microorganisms (https://ccinfo.wdcm.org). Some website URLs have been shortened.

Acronym	WDCM	Name	# of diatom strains	Country	Website	Email
ACOI		Coimbra Collection of Algae	16	Portugal	http://acoi.ci.uc.pt/index.php	algoteca@ci.uc.pt
ACUF		Algal Collection University Frederico II	20	Italy	http://www.acuf.net/index.php?lang=en	gabpinto@unina.it
ALISU		Culture Collection of the Universidade de Lisboa	9	Portugal		aaferreira@fc.ul.pt
ANACC		Australian National Algae Culture Collection	336	Australia	https://www.csiro.au/ANACC	ian.jameson@csiro.au
ARC	1096	Algal Resources Collection	9	USA	https://www.algalresourcescollection.com/	desouzac@uncw.edu
BCCM/DCG	1039	BCCM/DCG Diatoms Collection	536	Belgium	https://bccm.belspo.be/about-us/bccm-dcg	bccm.dcg@ugent.be
BMAK		Aidar & Kutner Microorganisms Collection	73	Brazil	https://bit.ly/3IdfDA6	fsalcorr@usp.br
CCALA	905	Culture Collection of Autotrophic Organisms	4	Czech Republic	https://ccala.butbn.cas.cz/	Josef.Juran@ibot.cas.cz
CCAP	522	Culture Collection of Algae and Protozoa	165	UK	https://www.ccap.ac.uk/	ccap@sams.ac.uk

(*Continued*)

Table 14.1 List of culture collections containing diatoms. Only collections that contain diatoms and answered our survey are included. WDCM corresponds to collection ID in the World Data Center for Microorganisms (https://ccinfo.wdcm.org). Some website URLs have been shortened. (*Continued*)

Acronym	WDCM	Name	# of diatom strains	Country	Website	Email
CICCM	1042	Cawthron Institute Culture Collection of Micro-algae	135	New Zealand	https://cultures.cawthron.org.nz/	Krystyna.Ponikla@cawthron.org.nz
CMR RBI		Culture collection of the Center for Marine Research, Ruder Boskovic Institute	152	Croatia		ruso@cim.irb.hr
CPCC	605	Canadian Phycological Culture Centre	31	Canada	https://bit.ly/3IgecRA	hdroshon@uwaterloo.ca
DSMZ	274	Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zelllkulturen	50	Germany	https://www.dsmz.de/	Silke.Pradella@dsmz.de
FINMARI CC		FINMARI Culture Collection	30	Finland		heidi.hallfors@syke.fi
MBRU		Russian Marine Biobanks	24	Russia	https://bit.ly/42T57Ws	naizdaicher@mail.ru
MCC	591	National Institute for Environmental Studies Collection	124	Japan	https://mcc.nies.go.jp/	mcc@nies.go.jp
MEDIC		Marine Epibiotic Diatom Collection	126	South Africa	http://ipt.sanbi.org.za/iptsanbi/resource?r=diatom	roksana.majewska@nwu.ac.za

(*Continued*)

Table 14.1 List of culture collections containing diatoms. Only collections that contain diatoms and answered our survey are included. WDCM corresponds to collection ID in the World Data Center for Microorganisms (https://ccinfo.wdcm.org). Some website URLs have been shortened. (*Continued*)

Acronym	WDCM	Name	# of diatom strains	Country	Website	Email
MNHN-ALCP		Collection du Museum d'Histoire Naturelle, Paris	3	France	https://bit.ly/3o7jec0	sahima.hamlaoui@mnhn.fr
MUC		Mersin University Collection	16	Turkey		elifeker@gmail.com
NBRC	825	Culture Collection Division, National Institute of Technology and Evaluation	14	Japan	https://www.nite.go.jp/nbrc/catalogue/	
NCMA	2	Provasoli-Guillard National Center for Marine Algae and Microbiota	585	USA	https://ncma.bigelow.org/	ncma@bigelow.org
NORCCA	498	Norwegian Culture Collection of Algae	290	Norway	https://norcca.scrol.net/	bente.edvardsen@bio.uio.no
RCC	829	Roscoff Culture Collection	939	France	https://roscoff-culture-collection.org/	rcc@sb-roscoff.fr
SZCZ		Szczecin Diatom Culture Collection	2151	Poland	https://bit.ly/432Rl3X	andrzej.witkowski@usz.edu.pl
TCC	1030	Thonon Culture Collection	193	France	https://www6.inrae.fr/carrtel-collection_eng/	frederic.rimet@inrae.fr
UTEX	606	Culture Collection of Algae at the University of Texas at Austin	390	USA	https://utex.org/	dnobles@austin.utexas.edu

are specialized for marine (e.g., RCC, NCMA) or freshwater (e.g., TCC) ecosystems, while others such as UTEX and BCCM/DCG contain marine, freshwater and terrestrial strains. In total, these collections currently hold more than 6,400 diatom strains (Supplementary Data S1). Depending on the collection, the amount and type of associated metadata varies considerably.

In general, strains are taxonomically identified at the species level, although in many cases only identification at higher taxonomic levels (e.g., class, order, family or genus) is available. The majority of strains belong to the pennate Bacillariophyceae (3,746), followed by the centric classes (Mediophyceae: 1,625, Coscinodiscophyceae: 316). More than 300 strains have not been assigned at the class level. Identified strains belong to 216 genera and 828 species. This represents less than 5% of the 18,537 diatoms species listed in Algaebase (Guiry 2012), underlining the need to develop isolation efforts. The best represented genera in culture are *Seminavis*, *Navicula*, *Nitzschia*, *Pseudo-nitzschia*, *Thalassiosira*, *Chaeotoceros* and *Skeletonema*, while some of the best represented species are the coastal benthic *Seminavis robusta* and the polar pelagic *Chaetoceros neogracilis* (Figure 14.3). The prevalence of particular genera and species in culture holdings may reflect the ease with which these species can be isolated but may also reflect the interest of a particular laboratory or collection in specific genera or species. For example, the genus *Pseudo-nitzschia*, some species of which can produce domoic acid (Bates *et al.* 2018), has been the focus of numerous research projects, leading to numerous strains being isolated.

More effort has been devoted to isolation of marine diatoms (Figure 14.4) compared with those from freshwater, while terrestrial ecosystems (e.g., soil) are underrepresented. Most isolation has been performed from water column or benthic samples, although microbiomes from animals or plants (e.g., biofilms from turtles or from macroalgae) and ice have received some attention. Most diatom cultures originate from coastal and continental habitats in Europe, the US, Japan, Australia and New Zealand (Figure 14.5). While polar areas (both Arctic and Southern Oceans) are quite well covered, very few diatom strains have been isolated from oligotrophic oceanic regions, where they are of course less abundant and often harbor symbionts (Foster and O'Mullan 2008), which may hinder cultivation. Although the oldest diatom strains held in a collection were isolated prior to 1960, there has clearly been an increase in the number of strains isolated in the last 10 years (Figure 14.6). A peak was reached around 2016, but this could be simply due to a larger number of cruises from which diatoms were isolated. For example, the Green Edge expedition in the Arctic in 2016 led to the isolation of several hundred diatom strains (Gérikas Ribeiro *et al.* 2020).

14.3 Isolation of Diatom Cultures

14.3.1 Sampling

Sampling for diatoms (and more generally for phytoplankton) has to be adapted to the targeted ecosystem. Surface plankton can be collected with a net with a mesh size adapted to the cell size of targeted taxa (e.g., 5 μm, 10 μm, 20 μm, 64 μm). In order to preserve the most fragile cells, it is critical to tow the net at slow speed. Towing time will of course depend on the cell concentration in the water and will be longer when sampling more oligotrophic waters. Samples can also be collected at different depths in the water column with Niskin

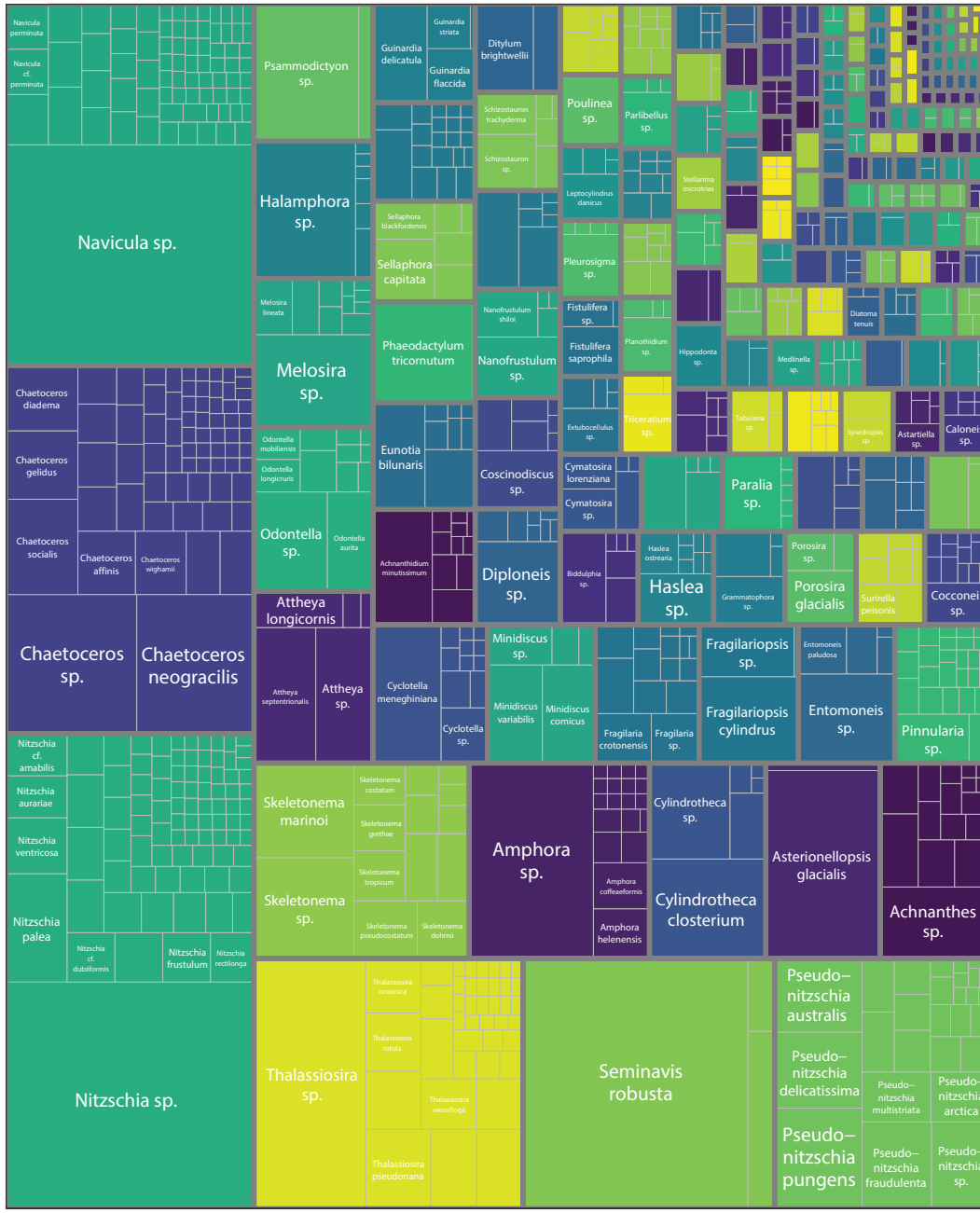

Figure 14.3 Diatom genera and species available in culture collections. Rectangle surfaces are proportional to the number of strains.

bottles attached to a cable or mounted on a Rosette sampling device. For samples from benthic sediments or ice, special tools such as box corers or ice corers may be required. For ice, melting at room temperature with the addition of 0.2 μm filtered seawater may be necessary. It is important to record as many parameters as possible when collecting samples, in particular substrate type, ecosystem type, geographical position (longitude and latitude),

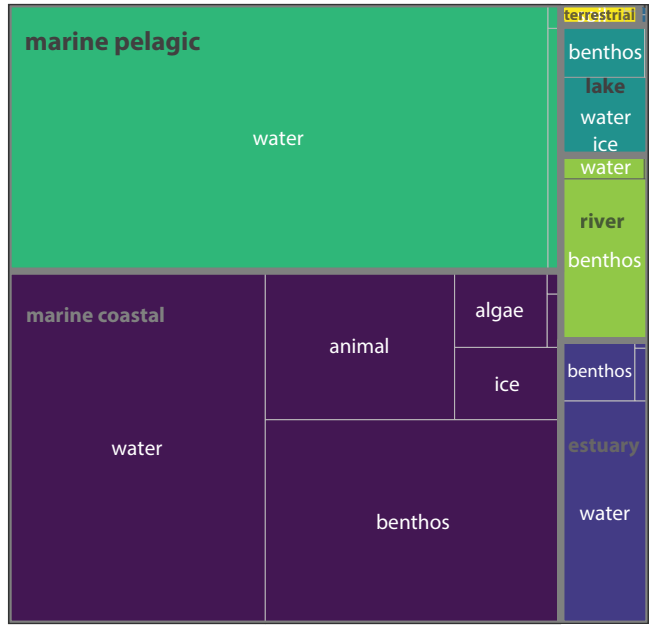

Figure 14.4 Origin of diatom cultures according to ecosystems and substrates. Rectangle surfaces are proportional to the number of strains.

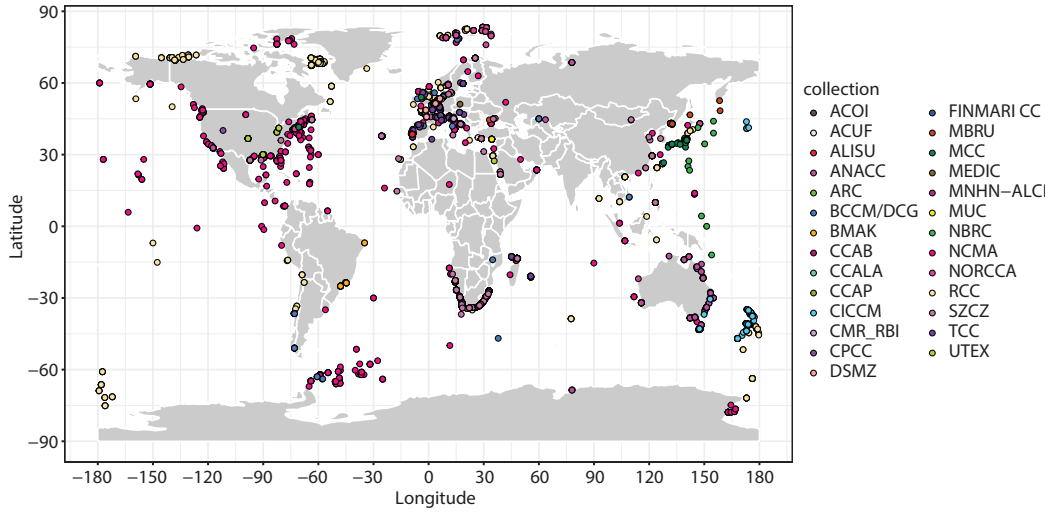

Figure 14.5 Isolation sites for diatom cultures from the different collections.

depth or altitude, temperature, salinity, pH and, where possible, nutrient concentrations (e.g., nitrates, phosphates, silicates). These parameters will be critical for documenting the origin of the isolated strains, in particular to assess the status of the strain relative to Access and Benefit Sharing regulations (see below), and may also help to define optimal growth conditions.

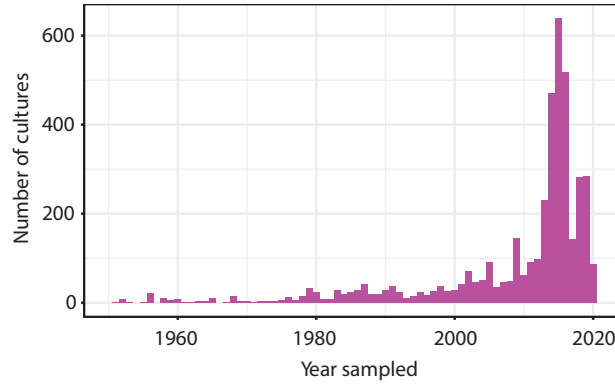

Figure 14.6 Year of sampling for diatoms listed in Supplementary Data S1. The peak around 2016 could be linked to the large number of sampling expeditions during this period.

After collection, samples are often passed through a 100 to 200 μm mesh to remove large particles and unwanted zooplanktonic organisms. If necessary, samples can be concentrated either by gravity filtration onto 0.8 or 3 μm filters and then resuspended in filtered seawater, or by tangential flow filtration (TFF), which is a reverse filtration process that allows gradual removal of water from the sample through a membrane filter and thus concentration of the sample (typically from 2 L down to 25 ml). Although TFF uses a peristaltic pump, it is nevertheless amenable to the preservation of delicate cells (Vaulot 2017).

The samples can either be processed immediately or enriched by adding a small volume of culture medium (typically 1 to 10% of the volume of the sample) or of filtered natural seawater collected at the same location (to mimic as much as possible *in situ* conditions). Samples are then incubated at a temperature and photoperiod matching those prevailing in the environment from which the sample was taken. Enriched samples (also called "pre-cultures") should be placed in a culture cabinet and regularly monitored by techniques such as optical microscopy or flow cytometry. Pre-culturing can be used to select specific characteristics, for example, by inoculating a plankton sample in nitrogen-depleted medium to pre-select strains containing diazotrophs.

14.3.2 Isolation Strategies

Several isolation strategies can be employed on the same sample to maximize the chances of success and the diversity of cultures obtained (for a detailed description of algal isolation techniques, refer to the book by Andersen 2005).

Serial dilution: Serial dilution, consisting of repeatedly diluting a sample or an enrichment by transferring a small volume of each successive dilution to fresh medium, is one of the most common approaches (Andersen 2005). The principle of serial dilution is to dilute the sample to extinction, such that the last dilution that contains any cells is likely to contain just one cell, from which a pure (mono-specific) culture is initiated. Serial dilution is most easily carried out in multi-well plates (typically 24, 48 or 96 wells), but tubes or flasks can also be used. Dilutions are maintained in optimal growth conditions (temperature, light) and culture growth should be observed within a few days, but it is advisable to wait for 1 to 2 weeks before transferring in order to verify the purity of the isolates. Serial dilution is

cheap to perform and does not require specific skills or equipment, but is a non-targeted method, meaning there is no guarantee of selecting taxa of interest. It can, however, lead to successful isolation of rare and/or fragile taxa from mixed samples that may be difficult to isolate by other methods. Another significant drawback of this method is that there is no practical way of knowing whether the culture was initiated from a single cell (and hence is clonal) or from two or more cells of the same species. If clonal cultures are required, it is therefore recommended to re-isolate cultures resulting from successful serial dilution using one of the other methods listed below.

Single cell isolation: Single cell isolation is usually undertaken with a drawn-out Pasteur pipette, which allows cells to be manually selected one at a time from the natural sample or the enrichment under a microscope (ideally an inverted microscope which has more space for manipulating cells). The collected cell is placed in a drop of clean culture medium to be washed. This step will be repeated as many times as necessary until the cell is free from other protists (Figure 14.7a). Aspiration is controlled by adding either a suction bulb or a tube with a mouthpiece to the end of the pipette. Single cells isolated in this manner are placed in sterile culture medium in a tube or a multi-well plate and maintained in optimal growth conditions in terms of temperature and light. These different steps can be viewed at www.youtube.com/watch?v=0hNWVtRGeXI. Micropipette isolation is very targeted and should result in clonal cultures of species of interest. However, it requires considerable technical expertise and is most effective for reasonably large cells (typically above 10 μm).

Solid medium: More commonly employed for bacterial culture, the use of solid or semi-solid agar media can also be useful for the isolation of diatom cultures. For both streaking and inclusion techniques, 0.5-2% agarose is added to the culture medium (supplemented with silica for diatoms). For streaking, a drop of a few microlitres (3-25, depending on the richness of the natural sample) is streaked onto the agar plate using the quadrant method (Figure 14.7b). Using the ring of the loop, tight striations are very delicately drawn on the first dial, so as not to damage the agar. Then the loop is flamed and left to cool. The box is turned by 90° towards the second dial and a trace is drawn in the same way as the first. This is repeated for the third dial, this time inoculating it with streaks that are not tightly

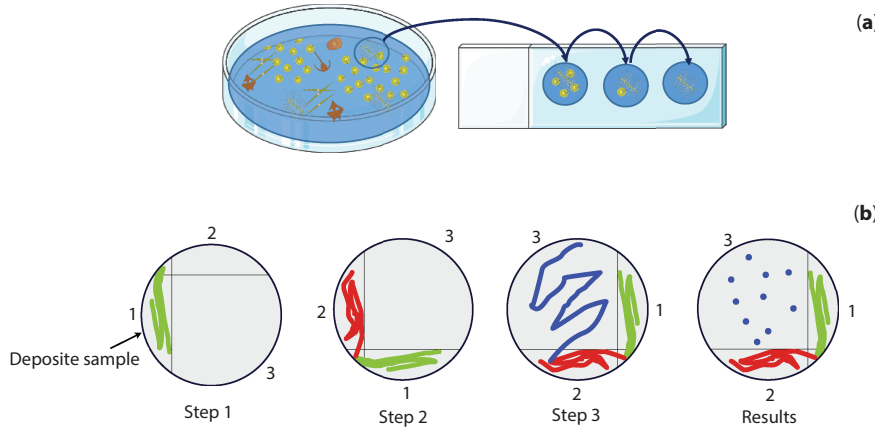

Figure 14.7 Culture isolation strategies. (a) Single cell isolation. (b) Quadrant method on agar plates.

spaced, that do not overlap the previous streaks. This simple technique allows separation of taxa that will appear after a few weeks in the form of spots (corresponding to colonies originating from a single cell) on the agar. To guarantee a clonal culture, it is recommended to transfer the colony to liquid medium and repeat the agar streaking operation at least once. While this streaking method is more specific to isolation from natural samples, the alternative inclusion technique is dedicated to strain purification and is detailed in the last section of this chapter. Once a pure culture has been obtained by either of these methods, it can be cultured on agar plates or transferred to liquid medium. This method is quite easy and economical, but it may take several weeks to establish pure cultures.

Flow cytometry sorting: An alternative method for cell isolation is the use of a sorting flow cytometer. Flow cytometry can distinguish populations according to pigment autofluorescence (chlorophyll, phycoerythrin) and light scatter, which is a proxy of size. A flow cytometer with sorting capacity makes it possible to select cells according to their characteristics and to deposit one or several cells in tubes or multi-well plates containing a suitable culture medium. Many diatoms form chains or colonies which hinder flow cytometric analysis and sorting, a constraint that can potentially be offset by vigorous shaking or vortexing of the sample prior to sorting. Due to the presence of the frustule, most diatoms are relatively robust compared to other microalgae and thus survival rates upon passage through a flow cytometer tend to be relatively high. For fragile species, Marie *et al.* (2017) described a method involving the use of bovine albumin serum (BSA) and antibiotics to mitigate the effect of the stress to which the cells are subjected during flow cytometry (laser, shear stress, bacterial dominance, etc), thereby increasing the success rate of culture isolation. Flow cytometry sorting allows the very rapid preparation of a large number of isolates, but it requires a sorting flow cytometer, which is expansive and in general is difficult to operate. Its success rate can be low, and it is impossible to differentiate diatoms from other phytoplankton based on their flow cytometry characteristics, so, in the case of diatoms, this method is non-targeted.

14.3.3 Culture Media for Isolation

Each of these approaches has its advantages and disadvantages, but it should be emphasized that the isolation medium is a critical element that can select for certain taxa. For marine diatoms, for example, f/2 (Guillard 1975) enriched with silica is often used. Media can be used at different dilutions and can be prepared with seawater originating from different environments. Standard media can also be modified by adding for example soil extract and there is undoubtedly considerable scope for the formulation of new media to broaden the list of diatoms that can be successfully cultured. To limit the growth of bacteria, and thus maximize the chances of successful isolation, the culture medium can be supplemented with a mix of antibiotics (e.g. Penicillin - Streptomycin - Neomycin solution, Sigma-Aldrich P4083). However, there may be cases when microalgal growth depends on bacteria (Riquelm *et al*. 1988) and antibiotics may be detrimental.

14.3.4 Characterization of Isolates

Once single diatom cells have been separated by these different approaches, isolations should be regularly monitored either by light microscopy with or without fluorescence

or by flow cytometry. Cultures that appear pure should rapidly be transferred to fresh medium. After a period of a few months (beyond which cultures are generally considered to be acclimated), cultures can be taxonomically characterized, which for diatoms typically involves observation by scanning electron microscopy (SEM) and/or sequencing of a short genetic barcode. This information is useful to interpret field metabarcoding surveys and help to provide to external researchers cultures that are reliably identified. Due to the presence of the silica frustule, preparation of diatom cultures for SEM is generally relatively straightforward (filtration onto a membrane filter, drying, sputter coating), but for species that produce organic material that covers the frustule, a cleaning step (typically in boiling nitric acid) may be necessary in order to be able to clearly visualize the frustule (Seaborn and Wolny 2000). For diatoms, barcoding typically involves sequencing of the V4 region of the 18S rRNA gene, which is a short region located roughly between 500 and 1000 base pairs. In some cases the 18S rRNA gene is not variable enough to discriminate between closely related species or subspecies, and it may therefore be necessary to sequence other genes, such as the 28S rRNA gene and/or the ITS region of the rRNA operon, or plastidial (e.g. *rbcL*) or mitochondrial (e.g. *cox1*) markers (Evans *et al*. 2007; Rimet *et al*. 2019). Characterization of cultures allows dereplication of duplicate strains, with several potential strategies that can be used to keep strains of interest. For example, if the same species has been isolated several times from several stations, only one or two strains from each sampling station may be kept.

14.3.5 Information Management

It is very important to keep all of the information about the isolated strains in a database. Each strain should have a unique and permanent identifier and it is important to add as much metadata as possible related to the sampling site (see above), sampling permits, culture isolation (isolation method, isolator, medium used for isolation, etc.), as well as information pertaining to the taxonomy (ideally including links to reference databases such as AlgaeBase, www.algaebase.org), genotype (links to barcode and genome sequences) and phenotype (images, size, pigment composition, etc.) of the strains. This information is particularly relevant in the context of possible restrictions on the use of strains (and potentially also digital sequence information issuing from strains) related to the Nagoya Protocol of the Convention for Biological Diversity, which sets out guidelines for regulation of access to genetic resources and the fair and equitable sharing of benefits arising from their utilization (so-called ”ABS” legislation). Culture collections are ideally placed to facilitate implementation of, and adherence to, such regulations that depend on longterm traceability of information related to strains. For culture collections, it is therefore recommended to avoid using Excel files that are only local to a computer and do not allow easy sharing of information. The best option is a SQL relational database (e.g. MySQL) hosted on a cloud server. Such a database allows all personnel from the culture collection to simultaneously access and update data. It also allows the data to be displayed on a website that external users can access to obtain information about the strains and eventually order them. Although such a database can be developed and maintained in-house using tools such as R which allow both the management of the database and the development of websites using R shiny (https://shiny.posit.co), another solution is to subcontract to a web development company that will ensure the maintenance of the website, in particular if the website is used to order strains.

14.4 Culture of Diatoms

Diatom strains are generally considered to be relatively easy to grow (at least in the short term) using standard microalgal culturing techniques. A wide range of media have been developed for culturing marine microalgae (for an exhaustive list, refer to the book by Andersen 2005). For marine diatoms, one of the first media to be employed was the simple Erd-Schreiber's (ES) medium containing soil extract (Föyn 1934). In the last 50 years, this has been replaced by more sophisticated media such as f/2 (Guillard 1975), K (Keller *et al.* 1987) and L1 (Guillard and Hargraves 1993). f/2 can be made from its constituent components, but can also be purchased commercially. Low nutrient media such as MET-44 (or f/2 diluted 10 times) can be useful for diatoms that originate from oligotrophic ecosystems. The main specificity for culturing diatoms is that media should contain silica (usually in the form of sodium silicate), which is required by diatoms for production of their frustules. Natural seawater may contain a sufficient quantity of silica to maintain diatom growth without adding a supplement, particularly for species with thin frustules and/or if the water is collected outside periods of diatom blooms which deplete natural silica levels. However, silicate is generally added (at concentrations up to 100 μM) as a precaution when culturing diatoms, and silica supplements are absolutely required when using artificial seawater-based medium recipes. For cultures in liquid media, plasticware (polystyrene flasks, tubes or multi-well plates) or glass recipients (Erlenmeyer flasks, tubes, etc.) can be used (Figure 14.8). Some diatom species may die out in certain brands of plastic flasks, whereas

Figure 14.8 Cultures of diatoms at the Roscoff Culture Collection using either small culture flasks, tubes or larger glass containers with a bubbling system.

they may grow fine in other brands or glass tubes. The reason for this is unclear. The advantage of using pre-sterilized plasticware is to reduce preparation time and the risk of contamination, but it generates a lot of waste and thus has a strong environmental impact. For cultures on solid medium, the recipe is supplemented with 0.5-2% agar and following autoclaving the medium is distributed (before setting) either in Petri dishes or as slants in glass tubes. All cultivation procedures should be conducted under a laminar flow hood to limit bacterial contamination.

With respect to temperature, once diatom cultures are established, it is not necessary to exactly reproduce the isolation temperature. Typical routine maintenance temperatures are 4 °C for polar species, 13 °C for temperate species and 20 °C for tropical species. Diatoms contain chlorophylls *a* and *c*, which absorb light in the blue and red parts of the light spectrum, and the main accessory pigment is fucoxanthin, which absorbs light in the 450-540 nm wavelength range. It is generally therefore best to use daylight-quality fluorescent tubes or LED lights which reproduce the natural spectrum, combining several wavelengths (blue, green, red). Although it is possible to grow some diatoms in continuous light, the best results are usually obtained by applying a photoperiod such as 12L:12D. Light intensity is typically set at 80-100 $\mu E.m^{-2}.s^{-1}$ for strains established from surface samples and around 30 $\mu E.m^{-2}.s^{-1}$ for strains established from deeper samples. For long-term maintenance of microalgal strains, it is recommended to use dedicated incubators with temperature and light control. Alternatively, walk-in temperature-controlled rooms equipped with shelves and light ramps are very convenient for large collections.

For growth in liquid media, transfer to fresh medium should typically be conducted every 2 to 4 weeks, depending on the species and light conditions. Lowering the temperature and/or light level may result in longer periods between transfers and reduce the labor involved. Growth is generally slower on solid medium, and transfer frequency is typically between 2 and 3 months. In most cases, growth of the newly transferred culture can be verified simply by observing the color, but it may be necessary to use optical microscopy or flow cytometry.

It is important to record the growth preferences of each culture (photoperiod, medium, transfer period, etc.) and any relevant information (e.g., change of morphology) in the culture database (see above). In order to limit sources of error and contamination linked to human activity during transfers, it is highly recommended to use a unique code for each strain (see database above) and to identify each culture container with a computerized label (rather than a manual indication which can be misinterpreted or erased) displaying at least the culture code, taxonomy, medium used and date of last transfer. Again, working with a cloud-based database makes this relatively easy to implement.

Diatoms have commercial applications in fields such as cosmetics, pharmaceuticals or aquaculture (Sharma *et al.* 2021) since they produce high-value-added molecules (e.g., pigments, lipids, polyunsaturated fatty acids). For such applications, it is often necessary to increase the volume of cultures in order to collect large amounts of biomass. This requires optimizing some parameters (medium, light) compared to standard culture conditions. One approach is to gradually increase culture volume starting with a tube (a few milliliters) up to Erlenmeyer flasks (typically several hundreds of milliliters) and then working up to liter scale in dedicated infrastructures such as photobioreactors (Figure 14.8). Such gradual increase should limit stress. Various parameters can be modified depending on the result to be achieved. For example, increase in photoperiod (up to 24 h continuous light) and light

intensity will induce greater photosynthetic activity and change in pigment content, such as antheraxanthin and zeaxanthin, known for their photoprotective and antioxidant activities (Goss *et al.* 2020). Factors such as pH, temperature, salinity and nutrient concentration are essential for optimal biomass production. When diatoms are used to feed larger organisms such as shellfish or zooplankton it may be necessary to alter medium composition (Liu *et al.* 2016; Nghiem Xuan *et al.* 2020). Air bubbling may prevent diatoms from settling or adhering to the walls of containers. Over the last decades, numerous large-capacity microalgae culture systems have been developed for biorefining and waste treatment (e.g., Matsumoto *et al.* 2017). While conditions in closed systems are easier to control, this is not the case in open systems (ponds and raceways of up to several hectares) for which external conditions such as seasonality, irradiation, temperature and variable rainfall present additional challenges (Wang and Seibert 2017).

14.5 Life Cycles

Although many diatoms can be easily isolated from environmental samples (see above) and grow well on commercially available algal culture growth media, long-term maintenance of strains is often challenging because of their peculiar life cycle, resulting in a gradual cell size diminution and final loss of cultures. Unlike most other microalgae, the majority of diatoms have a diplontic life cycle (Figure 14.9), with a long vegetative phase with diploid mitotically dividing cells, alternating with a comparatively short sexual phase with short-lived haploid gametes (Chepurnov *et al.* 2004). During mitotic cell division, two slightly unequal daughter cells are formed due to the peculiar architecture of the siliceous cell wall (frustule) and the biosynthesis of new valves inside the confines of the parental cell wall. As a result,

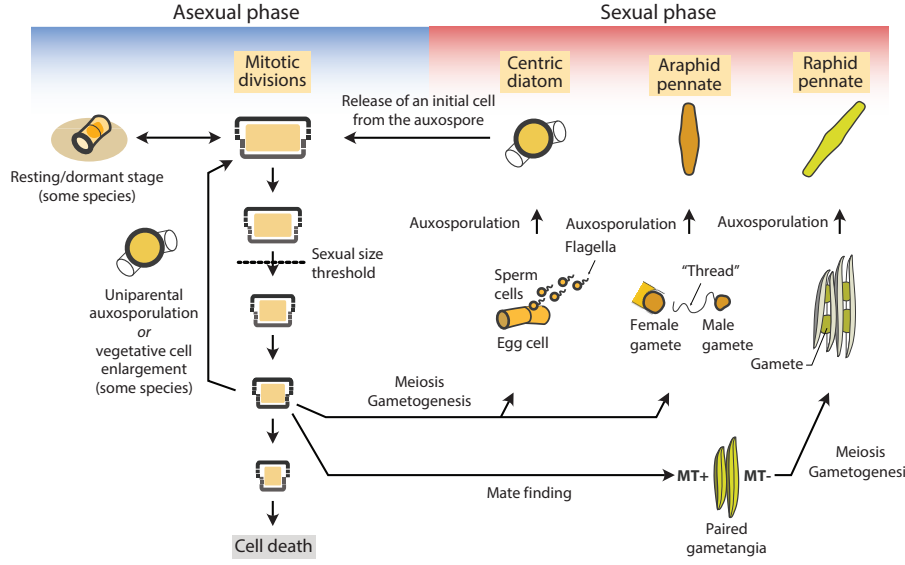

Figure 14.9 The life cycle of diatoms: a graphical representation of vegetative cell size decrease and the main strategies to restore cell size.

the mean cell size of a clonal population gradually decreases during repeated rounds of mitotic divisions, known as the MacDonald-Pfitzer rule (Macdonald 1869; Pfitzer 1869). Ultimately, cells become critically small, resulting in malformations and eventually cell death (Mann 2011). While this appears to be the general rule, a minority of diatom species do not decrease in cell size when dividing, while others are able to restore their cell size asexually (Kaczmarska *et al.* 2022, 2013; Mann 2011; Rose and Cox 2013). In most species studied, however, sexual reproduction and the subsequent expansion of a zygote into the auxospore is the most prevalent mechanism to restore large sized cells. Spontaneous and experimentally induced abrupt cell size reduction demonstrates that sexual reproduction in diatoms is dependent on cell size and can take place once cells pass a species-specific size threshold (SST, Chepurnov *et al.* 2004). Although studied in detail for only a few species, this endogenous cell size sensing mechanism, also referred to as the diatom sex clock, results in an alternation between brief sexual reproduction events and long intervals of vegetative growth, which can span several years (Lewis 1984; Mann 2011). While the processes of cell size decrease and auxosporulation are conserved among most diatoms, diverse mating strategies exist, largely coinciding with the main morphological groups of centric, araphid pennate and raphid pennate diatoms (Figure 14.9). Most centric diatoms are oogamous, producing large egg cells which are fertilized by small, flagellate spermatozoa. In general, they are homothallic (self-fertile), and clonal cells below the SST can, depending on their size, differentiate into egg or sperm cells. However, exceptions to this general pattern exist (e.g., Davidovich *et al.* 2017). In contrast, species from the evolutionary younger group of pennate diatoms, are often heterothallic, although homothallic species and mixed strategies are also known (Davidovich *et al.* 2010, 2009; Mann and Poulíčková 2019; Quijano-Scheggia *et al.* 2009; Vanstechelman *et al.* 2013), and sexual reproduction therefore requires a partner of the compatible mating type. The phylogenetically older group of araphid pennate diatoms is characterized by non-flagellate motile male gametes and larger immobile female gametes (anisogamy). In contrast, most raphid pennate species typically produce morphologically indistinguishable gametes (isogamy). A key difference with araphid diatoms is that a transfer of function from gametes to the gametangia has evolved, where diploid sexualized cells from opposite mating types interact to form a mating pair in which gametogenesis is subsequently initiated. Pheromone signaling plays a crucial role during diatom sexual reproduction (Frenkel *et al.* 2014; Sato *et al.* 2011). The system appears to be most evolved in raphid pennate diatoms and includes both sex-inducing pheromones and attraction pheromones influencing the behavior of gametangial cells, as well as pheromones, so far uncharacterized, involved in gamete attraction (Gillard *et al.* 2013; Klapper *et al.* 2021; Moeys *et al.* 2016).

Although cell size is the precondition for sexual reproduction to occur, appropriate environmental conditions should also be present to permit auxosporulation or to trigger it. This is best known for centric diatoms, where spermatogenesis and/or oogenesis can be induced by a shift in environmental conditions such as salinity, temperature, irradiance and its spectral composition, or photoperiod (Amato 2010; Baatz 1941; Godhe *et al.* 2014; Moore *et al.* 2017). In pennate diatoms, it appears that growing conditions should be favorable, although the topic has been little explored in a systematic manner. For example, sexual reproduction of the pennate *Pseudo-nitzschia multistriata* was most successful when cultures were in the exponential growth phase (Scalco *et al.* 2014). In addition, auxosporulation appears to be

light dependent, both in terms of irradiance as well as spectral composition (Bilcke *et al.* 2022; Davidovich 1998; Gillard *et al.* 2013; Mouget *et al.* 2009).

Understanding the processes involved in cell size reduction and restoration is essential for the long-term maintenance of diatom species in culture collections. To our knowledge, very little is known about possible controls of vegetative cell enlargement and asexual auxosporulation and how widespread it is among diatoms. Such information would be very interesting as it would allow preservation of the original genotype, in contrast to meiotic recombination during sexual auxosporulation which results in new genotypes. In addition, repeated selfing of homothallic strains may be applied to increase the level of homozygosity, which may be of interest for both fundamental and applied research. In any case, the use of sexual reproduction in the context of maintaining species or strains in a culture collection implies knowledge of the mating strategy SST and of conditions favoring sexual reproduction. In the case of heterothallic species, the availability of multiple strains of compatible genotypes is an obvious additional requirement. Increasingly, the importance of diatom-bacteria interactions is being revealed, including both beneficial as well as inhibitory effects on diatom sexual reproduction (Cirri *et al.* 2019). However, as this information and resources are available for only few species, many diatom strains may be maintained for variable periods in culture collections, but are eventually bound to be lost.

Ideally, strains of large-sized diatoms (well above SST) would be cryopreserved (see below). However, if cryopreservation of large-sized cells is not possible, inducing sexual reproduction in such species is a solution to maintain those species in a collection. It should be noted, however, that this may also promote selection of genotypes adapted to laboratory conditions.

One alternative, but as yet little explored, avenue for long-term storage of diatom species in culture collections is exploiting the fact that some species are able to form resting stages (Figure 14.9), including spores or resting cells. While the former are morphologically distinct from vegetative cells, resting cells have valves that are indistinguishable compared to their vegetative counterparts, but they are characterized by a condensed protoplast (Kaczmarska *et al.* 2013). In diatoms, resting spore formation is fundamentally different from auxosporulation. Auxosporulation involves swelling of a zygote and the subsequent formation of large initial cells, which then divide into cells with a species-specific morphology. In contrast to many other groups of microalgae where resting spores are formed from zygotes, diatom resting spores develop from mitotically dividing cells, usually as a result of environmental stressors such as, e.g., nitrogen deprivation. In some species it has been demonstrated that these spores remain viable for centuries to millennia (Härnström *et al.* 2011; Kaczmarska *et al.* 2013; Sanyal *et al.* 2022), opening the prospect of exploring anoxic, dark conditions to maintain such species in culture.

14.6 Cryopreservation

Most algal strains are maintained in a perpetual active culture state and must therefore be periodically transferred to fresh culture medium. Despite being the most commonly employed method of maintenance, perpetual active culture results in a host of potentially significant problems, perhaps the most important being that of genetic drift. As old methods are improved and new methods developed, enigmatic scientific results issuing from

studies using cultures can be reevaluated, but this presupposes that the biological material does not change in phenology or genome content over time. Researchers have known for decades that transferred cell lines have their own selective pressures, and that spontaneous mutation rates are higher for microbes in culture than for the same microbe in the natural environment (Lakeman *et al.* 2009; Phillips *et al.* 1994). Cryopreservation is an alternative to perpetual culture that ensures genotypic (and consequently physiological) stability over time, but not all microalgal strains can be/have been successfully cryopreserved.

Cryopreservation is the storage of viable cells at ultra-low temperatures of liquid nitrogen (-196 °C) and/or its vapor phase (-156 °C). The underlying principle of cryopreservation is that at ultra-low temperatures, vital functions, such as enzymatic activity and cell division, are slowed to the point of cessation, but not death (Benson 2008). A fundamental discovery in implementing successful cryopreservation was made by Polge *et al.* (1949) working with fowl sperm. They observed that at low temperatures, the sperm could be "protected" by using glycerol. Since then, cryopreservation has been used as a means to store (or "cryobank") biological materials including seeds, viruses, bacteria, and mammalian and plant tissue culture cells as a hedge against loss of valuable research resources. Unicellular algae have been cryopreserved since the early 1960s, although new methods were required due to poor success rates and alteration of algal ultrastructure (Morris 1976; Plattner *et al.* 1972). Morris (1978) successfully cryopreserved 252 of 284 strains of Chlorococcales (green algae) using a two-step cooling protocol. Cryopreservation methodology for algae has continued to evolve (Day *et al.* 2010).

The primary challenge in cryopreservation is to prevent formation of ice crystals that can lead to damage to intracellular material. Achieving this goal has been approached through methodological aspects, namely rate and/or type of freezing process and use of cryoprotectants.

Freezing Methods: Although a range of freezing protocols has been developed for the cryopreservation of algae (Ramon *et al.* 2002), they fall into two main categories: (1) rapid cooling, and (2) "two-step" cooling. The rapid cooling technique consists of plunging the algal material, suspended in culture medium with an appropriate cryoprotective agent, rapidly into liquid nitrogen at −196 °C. At such rapid cooling rates, however, the internal solution of the material becomes supercooled, increasing the possibility of damaging intracellular structure due to ice formation (Karlsson and Toner 1996; Meryman 1966). Most freezing protocols avoid this by utilizing a two-step cooling process, with controlled or semi-controlled cooling from room temperature (generally at a rate of ca. −1 °C min^{-1}) to a holding temperature of around −30 °C before the material is plunged into liquid nitrogen to complete the freezing process. The first step can be performed using simple devices such as Mr. Frosty© or more sophisticated controlled-rate freezers. Damage is thought to be prevented using this method because the reduced cooling rate allows sufficient time for osmotic equilibrium to be maintained by shrinkage of the cell. Another method, common in higher plants, to minimize intracellular damage upon freezing is the process of vitrification, which is ultra-rapid cooling in the presence of high concentrations of cryoprotective agents, (first described by Rall and Fahy 1985). Although not widely used to date, vitrification does appear to have some potential use in the cryopreservation of algal material. A more detailed description of vitrification methods can be found in Steponkus *et al.* (1992) and Karlsson and Toner (1996). A more recently employed method with algae is encapsulation-dehydration (Hirata *et al.* 1996). This technique involves the dehydration

of encapsulated algal cells by means of sterile air drying followed by immersion and storage in liquid N_2. The method has the advantage that no toxic cryoprotectants are required: hence, upon thawing there can be direct cultivation without the need for repeated washing of the sample.

Cryoprotectants: Cryoprotective agents (CPAs) are added to offer protection from cell damage during freezing and thawing processes. CPAs are low molecular weight compounds that passively traverse the plasma membrane to equilibrate the solute concentration between the cell interior and the extracellular matrix (Day and Brand 2005). Different CPAs may act in different ways such as lowering the temperature at which intracellular water freezes (Franks 1985), minimizing osmotically driven decreases in cell volume during freezing (Canavate and Lubian 1995) or altering membrane properties such as solute permeability (Santarius 1996). As an important mode of action of CPAs is the stabilization of membranes, and considering that membrane damage can be a primary initiator of free radical reactions, it is not surprising to find that several CPAs may also act as free radical scavengers (Benson 1990; Canavate and Lubian 1995). Hubálek (2003) reviewed the use of cryoprotectants in microorganisms. For microalgae, DMSO is the most commonly used CPA.

Cryopreservation of marine algae without CPAs has been reported in a very few instances, and the results for some species are not consistent between studies. Freezing-induced membrane damage results primarily from severe dehydration. Phospholipids are the major component of plant membranes, and their degree of fatty acid unsaturation and length naturally influence both flexibility and permeability of membranes (Ramon *et al.* 2002). In addition, strains that are desiccation-resistant may be predisposed to successful cryopreservation. It has been suggested that extracellular freezing and desiccation ultimately stress cells similarly, namely that both lead to an increase in the osmotic stress on the cell through the deprivation of free water. A number of diatom strains held by NCMA have been cryopreserved with and without the addition of CPA (Figure 14.10). While some were not successfully cryopreserved without CPA (i.e., CCMP581, CCMP1500, CCMP151, CCMP552), many were successfully cryopreserved in both treatments. A primary observation, however, was a delayed growback time (time to reach pre-cryopreservation cell abundance) in the samples cryopreserved without CPA.

Thawing: Thawing is critical for the reactivation of cultures. Several parameters are critical, notably osmotic and toxic shocks and light stress. In order to limit osmotic shocks and the intracellular and resulting extracellular damages, frozen cultures are preferably thawed rapidly by immersing them for about three minutes in a water bath at 27°C (e.g., at RCC) or 37°C (e.g., at DCG). The CPA can have a toxic effect on the cells, and to prevent this, it is important to rapidly dilute the thawed aliquot in a sufficient volume of fresh culture medium. It is possible to include a centrifugation step to pellet the cells and remove the CPA before re-culturing, but this step induces additional stress and decreases survival chances. In order to slowly revive the cultures, they are placed in optimal culture conditions (medium, temperature), while light is introduced progressively after a 24- to 48-hour phase of darkness or semi-darkness, thus limiting the light stress. It takes a few weeks for the cultures to recover a growth rate identical to that before cryopreservation. During these few weeks, monitoring is performed by light microscopy and/or flow cytometry.

Overall Success of Cryopreservation for Diatoms: Cryopreservation success for microalgae varies widely between different taxonomic groups. For example, dinoflagellates

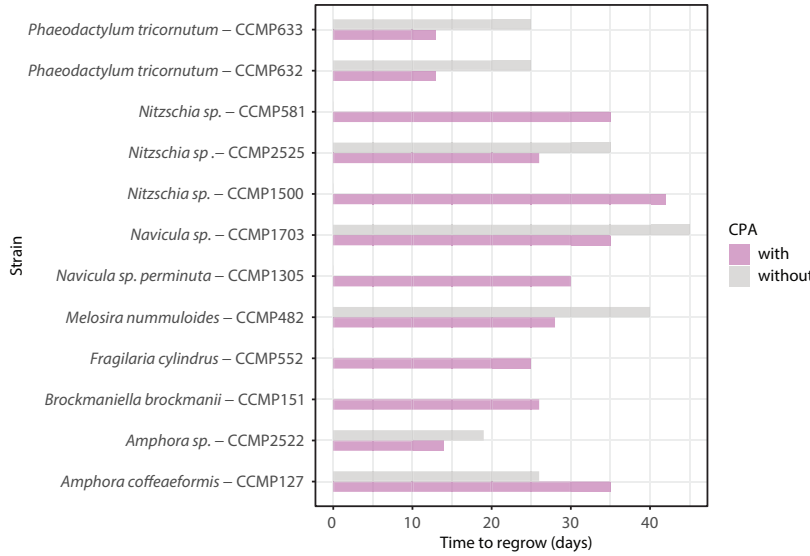

Figure 14.10 Grow back times for diatom strains cryopreserved with and without cryoprotective agents (CPA) added.

are very difficult to cryopreserve (Hagedorn *et al*. 2015). Diatoms are relatively easy to cryopreserve, although success rates vary significantly among diatom lineages (Stock *et al*. 2018). In the collections we surveyed (Table 14.2), pennate diatoms appear easier to cryopreserve than centric diatoms, especially the Coscinodiscophyceae. However, there are large variations between genera (Table S1). For example, while all strains of *Seminavis* tested can be cryopreserved, only 50% of *Thalassiosira* strains are successful. This could be an effect of cell size, as Paredes *et al.* (2021) reported that in terms of estimated cell volume, approximately two-thirds of the small (< 1000 μm^3) and intermediate (1000–2500 μm^3) categories of diatoms from the RCC collection for which cryopreservation was attempted were successfully cryopreserved, whereas few of the larger (> 2500 μm^3) category survived cryopreservation. Paredes *et al.* (2021) also reported that in terms of geographic origin, approximately only two-thirds of temperate and polar diatom strains for which cryopreservation was attempted were successfully cryopreserved, whereas almost all tropical strains (albeit from a lower total number) grew after thawing.

Table 14.2 Cryopreservation status by class for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). Plus sign corresponds to success and Minus sign to no success. Cultures not tested were not included. Refer to Table S1 for data at the genus level.

Class	+	-	**% success**
Bacillariophyceae	921	304	75
Bacillariophyta others	25	3	89
Coscinodiscophyceae	11	45	20
Mediophyceae	436	263	62

Long-term Conservation: Although some studies have shown that long-term storage (15 years) maintains the same properties as short-term storage (less than one year) (Nakanishi *et al*. 2012), other studies have raised some potentially significant issues. Müller *et al.* (2007) found that genomic alterations occurred after cryopreservation of some microalgae cultures, as repeatable differences in AFLP patterns, although no visible phenotypic changes were observed. They highlighted that although all biological functions cease at cryogenic storage temperatures, prolonged storage can induce some chemical damage, for example, due to the formation of free radicals and ionizing radiation, which can damage nucleic acids and ultimately influence genetic stability. With the drastic reduction in sequencing costs, Foo *et al.* (2023) advocate that whole genome analysis would provide comprehensive information on whether cryopreservation alters the genetic and functional characteristics of a strain of interest.

14.7 Diatom Strains Amenable to Genetic Engineering

The study of the molecular basis of diatom life began in the 1990s with the development of DNA-mediated transformation in a few selected species. This important achievement to analyze and modulate the function of diatom genes was first achieved by biolistic transformation in *Cyclotella cryptica* and *Navicula saprophyla* via the pioneering work of Dunahay *et al.* (1995). Subsequently, this method was adapted to other species such as *Phaeodactylum tricornutum* (Apt *et al*. 1996; Falciatore *et al*. 1999), *Thalassiosira weissflogii* (Falciatore *et al*. 1999), *Cylindrotheca fusiformis* (Poulsen and Kröger 2005) and several others (Table 14.3). Biolistic transformation is based on the helium-accelerated particle bombardment of diatoms with exogenous DNA coated on gold or tungsten particles, followed by selection of the transformed cells with antibiotics.

There are also some reports describing diatom transformation by electroporation, where electric currents are used to increase cell membrane permeability and DNA delivery (Table 14.3).

More recently, bacterial-mediated conjugation transformation was developed for the well studied diatom model species *P. tricornutum* and *T. pseudonana* (Karas *et al*. 2015). With this approach, DNA is delivered as an episome or artificial chromosome using genetically engineered *Escherichia coli* bacteria that promote DNA transfer via direct contact with diatoms via cell-to-cell pili. Following transformation, the transgenic material is replicated and maintained in the diatom cells due to the presence of a centromeric element, CEN6-ARSH4-HIS3, included in the episome. Bacterial conjugation is becoming the method of choice for diatom synthetic biology because the efficiency of transformation is very high (100-1000 transformants per 10^8 cells with bacterial conjugation vs 1-800 tranformants per 10^8 cells with biolistics, Moosburner *et al*. 2022). It also allows reproducible expression levels of transgenes in different transgenic lines because the episome is not integrated into the diatom genome. Conversely, with the biolistic and electroporation transformation, plastid-containing DNA is integrated into multiple copies and in multiple sites in the genome, meaning independent transgenic lines show very different expression levels (George *et al*. 2020). Moreover, random integration into the genome can also result in undesirable changes in genomic loci at integration sites or surrounding regions. To overcome these problems and to obtain conclusive functional information, it is always

Table 14.3 Summary of published transformable diatom species and of the methods used for genetic transformation.

Species	Method	Marker gene	Antibiotic	References
Cyclotella cryptica	Biolistic	nptll	G418	Dunahay *et al.* 1995
Navicula saprophila	Biolistic	nptll	G418	Dunahay *et al.* 1995
Phaeodactylum tricomutum	Biolistic	sh-ble	Phleomycin/Zeocin	Apt *et al.* 1996
	Electroporation	natl	Nourseothricin	Falciatore *et al.* 1999
	Bacterial conjugation	nptll	Neomycin	Zaslavskaia *et al.* 2001
		sat	Streptothricin	Buck *et al.* 2018
		bsr	Blasticidin-S	Niu *et al.* 2012 Karas *et al.* 2015
Thalassiosira weissflogii	Biolistic	sh-ble	Phleomycin/Zeocin	Falciatore *et al.* 1999
Cylindrotheca fusiformis	Biolistic	sh-ble	Zeocin	Fischer *et al.* 1999 Poulsen and Kröger 2005
Thalassiosira pseudonana	Biolistic	natl	Nourseothricin	Poulsen and Kröger 2005
	Electroporation	sh-ble	Phleomycin/Zeocin	Bugge and Robertson 2019
	Bacterial conjugation			Karas *et al.* 2015
Amphora coffeaeformis	Biolistic	natl	Phleomycin/Zeocin	Buhmann *et al.* 2014
		sh-ble	Nourseothricin	
Fistulifera solaris	Biolistic	nptll	G418	Muto *et al.* 2015
Pseudo-nitzschia multistrata	Biolistic	sh-ble	Phleomycin/Zeocin	Sabatino *et al.* 2015
Pseudo-nitzschia arenysensis	Biolistic	sh-ble	Phleomycin/Zeocin	Sabatino *et al.* 2015
Fragilariopsis cylindrus	Biolistic	sh-ble	Zeocin	Faktorov *et al.* 2020
Skeletonema marinoi	Electroporation	sh-ble	Zeocin	Johansson *et al.* 2019
Chaetoceros muelleri	Biolistic	natl	Nourseothricin	Miyagawa-Yamaguchi *et al.* 2011
	Electroporation	sh-ble	Zeocin	Ifuku *et al.* 2015
		bsr	Blasticidin-S	Yin and Hu 2021

Abbreviations: sh-ble: *Streptoalloteichus hindustanus* zeocin/bleomycin resistance gene; nat1: nourseothricin acetyl transferase; nptII: neomycin phosphotransferase II; sat: streptothricin acetyl transferase; bsr: blasticidin-S deaminase.

necessary to characterize independent transgenic lines. Episomal genetic elements may, however, be lost in the absence of antibiotic selection. Due to the high cost of the antibiotics commonly used for selection, this represents a disadvantage if a large quantity of transgenic lines is required for functional characterization of diatom gene products or biotechnological exploitation of transgenic cell extracts. On the other hand, loss of the episome by removal of antibiotics may also be an advantage in achieving transient expression of the transgene, which is a desirable effect in gene functional studies (e.g., to compare phenotypes related to the presence or absence of a specific gene) or to remove transgenic proteins that may modify the DNA, and thus generate an unintended genomic change (Moosburner *et al*. 2022).

Several factors are critical to the success of genetic transformation, which may limit the application of these tools to certain diatom species (Table 14.3). The ability to maintain the selected species under laboratory conditions is obviously the first important requirement, but many diatom species currently remain refractory to laboratory culture. Moreover, as different transformation methods also cause cell damage and mortality, transformation of diatoms is usually performed with a high quantity of cells (typically around 10^8) as starting material. For diatom species that do not reach a high cell density under laboratory conditions, it is therefore necessary to use large culture volumes and a cell concentration step prior to transformation, which may represent a limitation to routine use of the technology. In addition, genetic transformation relies on the use of an appropriate selection marker to isolate transgenic lines in a population of untransformed cells. Diatoms, like many algae, are resistant to many commercially available antibiotics. However, some antibiotics have been identified that kill diatoms under particular conditions of cell concentration and salinity (to be defined for each species) and these can be used to isolate transgenic lines expressing specific antibiotic resistance genes (Table 14.3). For successful transformation, it is also preferable to use axenic cultures, as bacterial contamination can alter the growth of diatoms on the selection medium and thus lead to the growth of false transgenic lines. However, many diatoms do not grow properly in the absence of bacteria, which can complicate genetic transformation efforts.

Transformation also relies on the identification of appropriate regulatory regions (promoter and terminator) for controlled expression of endogenous (i.e., from the same species to be transformed) or exogenous (from other diatom species or other organisms) genes in the diatoms. For most transformed diatoms, transgene expression has been successfully achieved by using endogenous regulatory regions. For only a few species, transformation has been achieved using regulatory regions from other diatoms or viral promoters (see Falciatore *et al*. 2020). The identification of endogenous regulatory regions does not represent a major challenge if genomic and transcriptomic information are available, but such information is currently only available for a limited number of diatom species (Mock *et al*. 2022).

Co-transformation of two vectors is also documented in several diatom species, whereby a selectable marker gene on a plasmid can be used to co-deliver in the same diatom cell another non-selectable transgene, such as a gene expressing a protein of interest fused to a protein tag or a reporter gene. However, the possibility to use multiple selectable markers largely facilitates genetic manipulations requiring the expression of different transgenes. This can be useful to simultaneously modify multiple genes or for the characterization of the function of a gene (e.g., gene mutagenesis in a first transformation round, followed by

complementation of a wild-type or a mutated version of the gene of interest in a second transformation, see Giovagnetti *et al.* 2022). Multiple selectable antibiotics have so far only been identified for a few species (Table 14.3). For *P. tricornutum*, chemical-based selectable markers have also been recently developed (Serif *et al.* 2018), based on the APT (Adenine Phosphoribosyltransferase, enzyme of the adenine salvage pathway) and UMPS (Uridine-5'-monophosphate synthase, enzyme of the *de novo* pyrimidine biosynthesis pathways) genes, whose inactivation results in the resistance to the toxic compounds 2-fluoroadenine and 5-fluoroorotic acid, respectively.

Independently of the transformation method employed, transgenic lines are visible after several weeks of growth on the selectable media (between 2–5 weeks depending on the species and the method). Growth of the transformed diatom cells on an agar plate greatly facilitates the isolation and characterization of clonal transgenic lines derived from the same transformation event. For some species, such as *P. multistriata* and *Pseudo-nitzschia arenysensis*, that are difficult to grow on plates, the selection of transgenic lines following transformation is undertaken in liquid media in the presence of the antibiotic (Sabatino *et al.* 2015). However, this protocol is more time-consuming as it requires the subsequent isolation of individual transgenic cells from a pool of transformed cells in order to perform molecular characterization. In some centric species, such as *T. pseudonana* or *Cyclotella cryptica*, which are also difficult to grow on agar plates, the isolation of transgenic lines can be successfully achieved by growing cells inside a low % (0.25%) agar matrix (Turnsek and Dupont 2017) or on the agar surface, but by using high purity agar.

Over the last twenty years, the main application of genetic transformation has been the study of diatom gene function. Powered by the availability of -omic information and tools to modulate gene expression, these studies have been instrumental in characterizing specific cellular and metabolic features of diatoms and in elucidating the mechanisms contributing to their ecological success in the environment. The readers could refer to Falciatore and Mock (2022) for a recent overview on the "Molecular Life of Diatoms" and to Moosburner *et al.* (2022) and the Chapter "Genetic Regulation of Diatom Photosynthesis: Understanding and Exploiting Genetic Diversity" in this book for additional details on the tools currently available for genetic engineering and genome editing in various diatom species.

Seminal information to drive genetic engineering in diatoms has also increased the potential of these algae for biotechnological exploitation. Diatoms naturally generate relevant foodstuff substances (e.g., omega-3 fatty acids like EPA) and produce lipids, especially triacylglycerides (TAGs), for carbon storage (Bozarth *et al.* 2009). Thus, efforts have been made to establish diatoms as source material in various industrial applications (e.g., production of health foods, biomolecules, feed for aquaculture, jet fuels) and to overcome limitations of biomass production. It has already been shown that the production of high-added-value molecules such as lipids can be increased by modifying specific diatom genes (Hao *et al.* 2018; Muto *et al.* 2015). Diatoms can also be exploited in targeted drug delivery using genetically engineered diatom biosilica (Delalat *et al.* 2015) or as a platform for the production of heterologous products such as plant-derived monoterpenoids (Fabris *et al.* 2020) or monoclonal antibodies (Hempel *et al.* 2017). The application of genetic engineering to a wide range of diatom species will further strengthen the field of diatom biotechnology in the future.

14.8 Conclusion

Culture collections are critical for diatom research. Less than 5% of described species are currently maintained in culture, which is a small proportion given that the real extent of diatom biodiversity is estimated to be in the region of 100,000 species. This highlights the necessity to continue and amplify isolation efforts, especially for species from environments that have been relatively undersampled to date, such as plant and animal microbiomes or sediments. It is also important that researchers that isolate diatoms (and other algae) routinely deposit them in public culture collections, and for public culture collections to provide clear information on their strain deposition policies. In most instances, this will require communication with public collections prior to sample collection, and perhaps even a written sample management plan if dedicated funding is sought.

As important as it is for scientists to know what level of support they may expect from public culture collections, it is even more critical that culture collections remain funded to be able to maintain existing cultures and expand the range of strains available for research and industry (Becker *et al*. 2019). There are numerous examples of culture collections containing unique diatom strains that have disappeared (e.g., the Scandinavian Culture Collection of Algae and Protozoa at the University of Copenhagen) or are demonstrably threatened (e.g., individual collections in the US that are consolidated into what is now the NCMA) due to lack of funding and annual budgets "in the red." In some countries (e.g., France), collections are supported for the long term by public institutions such as universities (e.g., Sorbonne University), in particular by the creation of permanent (centrally funded) positions for technical personnel, as well as via the improvement of infrastructures and acquisition of equipment on structural funds. They still, however, remain dependent on obtaining competitive grants to operate on a daily basis. Prevention of the loss of culture collections due to lack of funding will require development of funding models that diversify beyond the limited scope of distribution of cultures. Some examples that have proven effective are providing aligned products, such as harvested cells from larger (20 L) cultures and/or chemical extracts (e.g., DNA, RNA, protein) from strains. Leveraging long-term storage infrastructure can open up opportunities to hold "private collections" for companies, which can yield annually recurring revenues. Similarly, as economic sectors utilizing algae continue to expand, culture collections should consider the possibility of non-exclusive licensing of strains to ensure that they are not disadvantaged in the process of commercialization. While it is unlikely that there will be a single consistent financial model across all culture collections, it is important as a community that we strive for consistency where possible to ensure the broader stability of these important global resources.

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Supplementary Material

Supplementary Data S1. List of diatom strains inventoried with associated metadata (taxonomy, origin, cryopreservation status): https://doi.org/10.5281/zenodo.8163805. Taxonomic assignation of genera (i.e. class, order, family) is according to AlgaeBase (https://www. algaebase.org).

Table S1 Cryopreservation status by class and genus for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC).

Class	Genus	+	-	% success
Bacillariophyceae	*Achnanthes*	43	1	98
Bacillariophyceae	*Achnanthidium*	26	4	87
Bacillariophyceae	*Amphiprora*	2	0	100
Bacillariophyceae	*Amphora*	33	12	73
Bacillariophyceae	*Anomoeoneis*	0	7	0
Bacillariophyceae	*Astartiella*	1	0	100
Bacillariophyceae	*Asterionella*	1	0	100
Bacillariophyceae	*Asterionellopsis*	32	20	62
Bacillariophyceae	*Bacillaria*	1	0	100
Bacillariophyceae	Bacillariophyceae	43	4	91
Bacillariophyceae	*Biremis*	2	0	100
Bacillariophyceae	*Caloneis*	1	4	20
Bacillariophyceae	*Campylodiscus*	0	1	0
Bacillariophyceae	*Cocconeis*	1	1	50
Bacillariophyceae	*Craspedostauros*	2	0	100
Bacillariophyceae	*Craticula*	9	11	45
Bacillariophyceae	*Cyclophora*	0	1	0

(*Continued*)

Table S1 Cryopreservation status by class and genus for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). (*Continued*)

Class	Genus	+	-	% success
Bacillariophyceae	*Cylindrotheca*	49	5	91
Bacillariophyceae	*Cymbella*	2	4	33
Bacillariophyceae	*Decussata*	0	4	0
Bacillariophyceae	*Delphineis*	1	0	100
Bacillariophyceae	*Diadesmis*	1	2	33
Bacillariophyceae	*Diatoma*	2	0	100
Bacillariophyceae	*Encyonema*	0	2	0
Bacillariophyceae	*Entomoneis*	11	10	52
Bacillariophyceae	*Eolimna*	2	0	100
Bacillariophyceae	*Eucocconeis*	1	2	**33**
Bacillariophyceae	*Eunotia*	0	9	0
Bacillariophyceae	*Fallacia*	2	1	67
Bacillariophyceae	*Fistulifera*	1	0	100
Bacillariophyceae	*Fragilaria*	10	3	**77**
Bacillariophyceae	Fragilariaceae	21	0	100
Bacillariophyceae	Fragilariales	1	0	100
Bacillariophyceae	*Fragilariforma*	1	1	50
Bacillariophyceae	*Fragilariopsis*	24	2	92
Bacillariophyceae	*Gedaniella*	2	0	100
Bacillariophyceae	*Gomphonema*	13	10	57
Bacillariophyceae	*Gomphonemopsis*	0	1	0
Bacillariophyceae	*Grammatophora*	9	0	100
Bacillariophyceae	*Grammonema*	0	1	0
Bacillariophyceae	*Gyrosigma*	0	2	0
Bacillariophyceae	*Halamphora*	2	0	100
Bacillariophyceae	*Hantzschia*	2	9	18
Bacillariophyceae	*Haslea*	1	0	100

(*Continued*)

Table S1 Cryopreservation status by class and genus for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). (*Continued*)

Class	Genus	+	-	% success
Bacillariophyceae	*Humidophila*	8	0	100
Bacillariophyceae	*Hyalosira*	1	0	100
Bacillariophyceae	*Licmophora*	0	2	0
Bacillariophyceae	*Lucanicum*	0	1	0
Bacillariophyceae	*Luticola*	0	1	0
Bacillariophyceae	*Mayamaea*	3	0	100
Bacillariophyceae	*Nanofrustulum*	1	0	100
Bacillariophyceae	*Navicula*	70	35	67
Bacillariophyceae	Naviculales	19	0	100
Bacillariophyceae	*Navicymbula*	3	0	100
Bacillariophyceae	*Neidium*	3	8	27
Bacillariophyceae	*Neofragilaria*	0	1	0
Bacillariophyceae	*Nitzschia*	119	37	76
Bacillariophyceae	*Opephora*	1	0	100
Bacillariophyceae	*Orizaformis*	1	0	100
Bacillariophyceae	*Pauliella*	1	0	100
Bacillariophyceae	*Pinnularia*	6	16	27
Bacillariophyceae	*Placoneis*	2	1	67
Bacillariophyceae	*Plagiogramma*	1	1	50
Bacillariophyceae	*Planothidium*	1	1	50
Bacillariophyceae	*Pleurosigma*	0	1	0
Bacillariophyceae	*Psammoneis*	0	1	0
Bacillariophyceae	*Psammothidium*	0	1	0
Bacillariophyceae	*Pseudo-nitzschia*	5	8	38
Bacillariophyceae	*Pseudostaurosira*	1	0	100
Bacillariophyceae	*Rhopalodia*	0	1	0
Bacillariophyceae	*Sellaphora*	3	2	60

(*Continued*)

Table S1 Cryopreservation status by class and genus for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). (*Continued*)

Class	Genus	+	-	% success
Bacillariophyceae	*Seminavis*	269	0	100
Bacillariophyceae	*Stauroneis*	8	9	47
Bacillariophyceae	*Staurosira*	10	0	100
Bacillariophyceae	*Staurosirella*	3	1	75
Bacillariophyceae	*Stenopterobia*	0	5	0
Bacillariophyceae	*Striatella*	0	1	0
Bacillariophyceae	*Surirella*	4	21	16
Bacillariophyceae	*Synedra*	10	3	**77**
Bacillariophyceae	*Synedropsis*	9	0	100
Bacillariophyceae	*Tabellaria*	0	1	0
Bacillariophyceae	*Tabularia*	0	3	0
Bacillariophyceae	*Talaroneis*	1	0	100
Bacillariophyceae	*Thalassionema*	2	3	40
Bacillariophyceae	*Tryblionella*	1	3	25
Bacillariophyceae	*Ulnaria*	0	2	0
Bacillariophyta	*Astrosyne*	0	1	0
Bacillariophyta	Bacillariophyta	8	1	89
Bacillariophyta	*Phaeodactylum*	17	1	94
Coscinodiscophyceae	*Amphipenteras*	0	1	0
Coscinodiscophyceae	*Aulacoseira*	0	4	0
Coscinodiscophyceae	*Corethron*	0	2	0
Coscinodiscophyceae	*Coscinodiscus*	0	6	0
Coscinodiscophyceae	*Guinardia*	0	6	0
Coscinodiscophyceae	*Hyalodiscus*	2	0	100
Coscinodiscophyceae	*Melosira*	7	1	88
Coscinodiscophyceae	*Orthoseira*	0	3	0
Coscinodiscophyceae	*Paralia*	0	3	0

(*Continued*)

Table S1 Cryopreservation status by class and genus for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). (*Continued*)

Class	Genus	+	-	% success
Coscinodiscophyceae	*Podosira*	0	1	0
Coscinodiscophyceae	*Rhizosolenia*	0	1	0
Coscinodiscophyceae	*Stellarima*	0	11	0
Coscinodiscophyceae	*Stephanopyxis*	0	4	0
Coscinodiscophyceae	*Triceratium*	2	2	50
Mediophyceae	*Amphipentas*	0	1	0
Mediophyceae	*Arcocellulus*	1	0	100
Mediophyceae	*Attheya*	68	2	97
Mediophyceae	*Bacterosira*	0	1	0
Mediophyceae	*Bellerochea*	0	1	0
Mediophyceae	*Biddulphia*	0	4	0
Mediophyceae	*Brockmanniella*	1	0	100
Mediophyceae	*Cerataulus*	0	1	0
Mediophyceae	*Chaetoceros*	85	55	61
Mediophyceae	*Conticribra*	3	0	100
Mediophyceae	*Cyclostephanos*	0	1	0
Mediophyceae	*Cyclotella*	11	18	38
Mediophyceae	*Cymatosira*	1	1	50
Mediophyceae	Cymatosiraceae	23	0	100
Mediophyceae	*Detonula*	1	2	33
Mediophyceae	*Ditylum*	0	13	0
Mediophyceae	*Eucampia*	0	2	0
Mediophyceae	*Eunotogramma*	0	1	0
Mediophyceae	*Extubocellulus*	4	0	100
Mediophyceae	*Helicotheca*	0	4	0
Mediophyceae	*Leptocylindrus*	0	1	0
Mediophyceae	*Leyanella*	1	0	100

(*Continued*)

Table S1 Cryopreservation status by class and genus for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). (*Continued*)

Class	**Genus**	+	-	**% success**
Mediophyceae	*Lithodesmium*	0	3	0
Mediophyceae	*Mastodiscus*	0	1	0
Mediophyceae	*Minidiscus*	52	2	96
Mediophyceae	*Minutocellus*	15	1	94
Mediophyceae	*Odontella*	4	18	18
Mediophyceae	*Papiliocellulus*	2	0	100
Mediophyceae	*Pleurosira*	0	2	0
Mediophyceae	*Porosira*	3	15	17
Mediophyceae	*Proboscia*	0	1	0
Mediophyceae	*Skeletonema*	83	31	73
Mediophyceae	*Thalassiosira*	78	81	49

Plus sign corresponds to success and Minus no success. Cultures not tested were not included.