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# **Culturing Diatoms**

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### 23 Abstract

In this chapter, we provide information on cultures of diatoms, starting with a brief introduction of the 24 history of diatom culture collections. We present a synopsis of diatom strains currently available in culture 25 collections and their representation in terms of habitat diversity, geographical distribution, phylogeny and 26 taxonomic diversity. We outline the major techniques for isolating and cultivating diatoms and discuss 27 the problems and possible strategies linked to diatom life cycles and habitat requirements. We provide an 28 overview of available techniques for genetic transformation of diatoms and strategies for forward genetics. 29 Finally, we summarize information and techniques available for long term preservation of diatom cultures, 30 focusing on cryopreservation. 31

#### 32 1 - Introduction

Diatoms were discovered in the 18<sup>th</sup> century, but the first cultures were not established until the middle 33 of the next century and cultivation only really developed from the early 1900s (Figure 1, Richter 1903). 34 Museums, botanical gardens, zoos and research facilities have served as repositories to preserve collections 35 of fixed specimens, notably type material, for centuries, with such facilities deemed reliable places to 36 safeguard specimens in the state in which they were first deposited, be sure that they are cared for, and be 37 able to distribute them to researchers so that scientific research can be reproduced and expanded (Smith 38 and Ryan 2012). The development of repositories for living material is more recent, Ernst Pringsheim 39 having established the first culture collection catalogue in 1928. Cultures are extremely useful for studies 40 on diatom taxonomy and life cycles, although such work can also be undertaken on natural populations 41 (Mann and Chepurnov 2004). They are, however, absolutely essential for physiological studies, in particular 42 in the domain of photosynthesis, as well as for the biochemical characterization of cells. Cultures are also 43 key for the acquisition of 'omics' data, that has played a central role for understanding the regulation of 44 cell growth and functions (Armbrust et al. 2004; Bowler et al. 2008). 45

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 isolation and culture of diatoms, detailing constraints linked to the specific life cycle of diatoms. We conclude by reviewing relatively new approaches, genetic transformation and cryopreservation.

#### <sup>52</sup> 2 - Current diversity of diatoms in culture

Diatoms are extremely diverse in terms of shapes and structures (Figure 2), as well as habitats and physiology. Only a small fraction of this diversity is currently represented in cultures.

<sup>55</sup> We identified 37 algal collections containing diatom cultures and obtained culture listings for 27 of them <sup>56</sup> (Table 1). The number of diatom cultures in these collections varies from a few to more than 2000. <sup>57</sup> Some collections are specialized for marine (e.g., RCC, NCMA) or freshwater ecosystems (e.g., TCC) while <sup>58</sup> others such as UTEX and BCCM/DCG contain marine, freshwater and terrestrial strains. In total, these <sup>59</sup> collections currently hold more than 6,400 diatom strains (Supplementary Data S1). Depending on the <sup>60</sup> collection, the amount and type of associated metadata varies considerably.

In general, strains are taxonomically identified to the species level, although in many cases only identifi-61 cation at higher taxonomic levels (e.g., class, order, family or genus) is available. The majority of strains 62 belong to the pennate Bacillariophyceae (3746), followed by the centric classes (Mediophyceae: 1625, 63 Coscinodiscophyceae: 316). 316 strains have not been assigned at the class level. Identified strains belong 64 to 216 genera and 828 species. The best represented genera in culture are Seminavis, Navicula, Nitzschia, 65 Pseudo-nitzschia, Thalassiosira, Chaeotoceros and Skeletonema, while some of the best represented species 66 are Seminavis robusta and Chaetoceros neogracilis (Figure 3). The prevalence of particular genera and 67 species in culture holdings may reflect the ease with which these species can be isolated, but may also 68 reflect the interest of a particular laboratory or a collection in specific genera or species. For example, the 69 genus Pseudo-nitzschia, some species of which can produce domoic acid (Bates et al. 2018), is an organism 70 of high research value and has been the focus of numerous projects, leading to more strains being isolated. 71 More effort has been devoted to isolation of marine diatoms (Figure 4) compared to those from freshwater, 72 while other ecosystems (e.g. soil) are particularly underrepresented. Most isolation has been performed 73 from water, plankton or ice, although microbiomes (e.g., biofilms from turtles or from macroalgae) have 74 received some attention. Most diatom cultures originate from coastal and continental habitats in Europe, 75 the US, Japan, Australia and New Zealand (Figure 5). While polar areas (both Arctic and Southern Oceans) 76

<sup>77</sup> are quite well covered, very few diatom strains have been isolated from oligotrophic oceanic regions, where <sup>78</sup> they are of course less abundant and often harbour symbionts (Foster and O'Mullan 2008), which may <sup>79</sup> hinder cultivation. Although the oldest diatom strains held in a collection were isolated prior to 1960, there <sup>80</sup> has clearly been an increase in the number of strains isolated in the last 10 years (Figure 6).

#### **3** - Isolation of diatom cultures

Sampling for diatoms (and more generally for phytoplankton) has to be adapted to the targeted ecosystem. 82 Surface plankton can be collected with a net with a mesh size (e.g. 5  $\mu$ m, 10  $\mu$ m, 20  $\mu$ m, 64  $\mu$ m) adapted 83 to the cell size of targeted taxa. In order to preserve the most fragile cells, it is critical to tow the net at 84 slow speed. Towing time will of course depend on the cell concentration in the water and will be longer 85 when sampling more oligotrophic waters. Samples can also be collected at different depths in the water 86 column with Niskin bottles attached to a cable or mounted on a Rosette sampling device. For samples from 87 benthic sediments or ice, special tools such as box corers or ice corers may be required. For ice, melting 88 at room temperature with the addition of 0.2  $\mu$ m filtered seawater may be necessary. It is important to 89 record as many parameters as possible when collecting samples, in particular substrate type, ecosystem 90 type, geographical position (longitude and latitude), depth or altitude, temperature, salinity, pH and where 91

possible nutrient concentrations (nitrate, phosphate, silicate, etc.). These parameters will be critical for
 documenting the origin of the isolated strains and may also help to define optimal growth conditions.

After collection, samples are often passed through a 100 to 200  $\mu$ m mesh to remove large particles and unwanted zooplanktonic organisms. If necessary, samples can be concentrated either by gravity filtration onto 0.8 or 3  $\mu$ m filters and then resuspension 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 2L down to 25ml). TFF uses a peristaltic pump, but 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) and incubated at a temperature and photoperiod matching those prevailing in the environment from which the sample was taken. Enriched samples (also called "precultures") should be placed in a culture cabinet and regularly monitored by techniques such as optical microscopy or flow cytometry.

<sup>105</sup> Several isolation strategies can be employed on the same sample to maximize the chances of success and <sup>106</sup> the diversity of cultures obtained (Andersen 2005).

Serial dilution. Serial dilution, consisting of repeatedly diluting a sample or an enrichment by transferring 107 a small volume of each successive dilution to fresh medium, is one of the most common approaches. The 108 principle of serial dilution is to dilute the sample to extinction, such that the last dilution that contains any 109 cells is likely to contain just one cell, from which a pure (mono-specific) culture is initiated. Serial dilution 110 is most easily carried out in multi-well plates (typically 24, 48 or 96 wells), but tubes or flasks can also 111 be used. Dilutions are maintained in optimal growth conditions (temperature, light) and culture growth 112 should be observed within a few days, but it is advisable to wait for 1 to 2 weeks before transferring in 113 order to verify the purity of the isolates. 114

Single cell isolation. Single cell isolation is usually undertaken with a tapered Pasteur pipette, which 115 allows cells to be manually selected one at a time from the natural sample or the enrichment under a 116 microscope (ideally an inverted microscope which has more space for manipulating cells). The collected 117 cell is placed in a drop of clean culture medium to be washed. This step will be repeated as many 118 times as necessary until the cell is free from other protists (Figure 7). Aspiration is controlled by adding 119 either a suction bulb or a tube with a mouthpiece to the end of the pipette. Single cells isolated in this 120 manner are placed in sterile culture medium in a tube or a multi-well plate and maintained in optimal 121 growth conditions in terms of temperature and light. These different steps can be viewed on video at 122 www.youtube.com/watch?v=0hNWVtRGeXI. 123

Solid medium. More commonly employed for bacterial culture, the use of solid or semi-solid agar media 124 can also be useful for the isolation of diatom cultures. For both streaking and inclusion techniques, 0.5-2% 125 agarose is added to the culture medium (supplemented with silica for diatoms). For streaking, a drop of 126 a few microlitres (3-25, depending on the richness of the natural sample) is streaked onto the agar plate 127 using the dial method. This simple technique allows separation of taxa that will appear after a few weeks 128 in the form of spots (corresponding to colonies originating from a single cell) on the agar. To guarantee 129 a clonal culture, it is recommended to transfer the colony to liquid medium and repeat the agar streaking 130 operation at least once. While the streaking method is more specific to isolation from natural samples, 131 the inclusion technique is dedicated to strain purification and is detailed in the "Diatom transformation 132 and genetic engineering" section of this chapter. Once a pure culture has been obtained by either of these 133 methods, it can be cultured on agar plates or transferred to liquid medium. 134

Flow cytometry sorting. An alternative method for cell isolation is the use of a sorting flow cytometer. 135 Flow cytometry can distinguish populations according to pigment autofluorescence (chlorophyll, phycoery-136 thrin) and light scatter, which is a proxy of size. A flow cytometer with sorting capacity makes it possible 137 to select cells according to their characteristics and to deposit one or several cells in tubes or multi-well 138 plates containing a suitable culture medium. Many diatoms form chains or colonies which hinder flow 139 cytometric analysis and sorting, a constraint that can potentially be offset by vigorous shaking or vortexing 140 of the sample prior to sorting. Due to the presence of the frustule, most diatoms are relatively robust 141 compared to other microalgae and thus survival rates upon passage through a flow cytometer tend to be 142 relatively high. For fragile species, Marie et al. (2017) described a method involving the use of bovine 143 albumin serum (BSA) and antibiotics to mitigate the effect of the stress to which the cells are subjected 144 during flow cytometry (laser, shear stress, bacterial dominance, etc.) and thereby increase success rate of 145 culture isolation. 146

Each of these approaches has its advantages and disadvantages. Serial dilution is cheap to perform and 147 does not require specific skills or equipment, but is a non-targeted method, meaning there is no guarantee 148 of selecting taxa of interest. It can, however, lead to successful isolation of rare and/or fragile taxa from 149 mixed samples that may be difficult to isolate by other methods. A significant drawback of this method 150 is that there is no practical way of knowing whether a mono-specific culture was initiated from a single 151 cell (and hence is clonal) or from two or more cells of the same species. If clonal cultures are required, 152 it is therefore recommended to re-isolate cultures resulting from successful serial dilution using one of the 153 other methods presented here. Micropipette isolation is very targeted and should result in clonal cultures of 154 species of interest. However, it requires considerable technical expertise and is most effective for reasonably 155 large cells (typically above 10  $\mu$ m). Flow cytometry sorting allows very rapid preparation of a large number 156

of isolates, but requires a very expensive sorting flow cytometer, which 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.

In all of these approaches, the isolation medium is a critical element that can select for certain taxa. For 160 marine diatoms, for example, f/2 (Guillard 1975) enriched with silica is often used. Media can be used at 161 different dilutions and can be prepared with seawater originating from different environments. Standard 162 media can also be modified by adding for example soil extract and there is undoubtedly considerable scope 163 for the formulation of new media to broaden the list of diatoms that can be successfully cultured. To limit 164 the growth of bacteria, and thus maximize the chances of successful isolation, the culture medium can be 165 supplemented with a mix of antibiotics (e.g. Penicillin - Streptomycin - Neomycin solution, Sigma-Aldrich 166 P4083). However, there may be cases when microalgal growth depends on bacteria (Riquelm et al. 1988) 167 and antibiotics may be detrimental. 168

Once single diatom cells have been separated by these different approaches, isolations should be regularly 169 monitored either by light microscopy with or without fluorescence or by flow cytometry. Cultures that 170 appear pure should rapidly be transferred to fresh medium. After a period of a few months (beyond which 171 cultures are generally considered to be stable), cultures can be characterized, which for diatoms typically 172 involves observation by scanning electron microscopy (SEM) and/or sequencing of a short genetic barcode. 173 Due to the presence of the silica frustule, preparation of diatom cultures for SEM is in general relatively 174 straightforward (filtration onto a membrane filter, drying, sputter coating), but for species that produce 175 organic material that covers the frustule, a cleaning step (typically in boiling nitric acid) may be necessary 176 in order to be able to clearly visualize the frustule. For diatoms, barcoding typically involves sequencing of 177 the V4 region of the 18S rRNA gene, which is a short region located roughly between 500 and 1000 base 178 pairs. In some cases the 18S rRNA gene is not variable enough to discriminate between closely related 179 species or subspecies, and it may therefore be necessary to sequence other genes, such as the 28S rRNA 180 gene and/or the ITS region of rRNA operon, or plastidial (e.g. rbcL) or mitochondrial (e.g. cox1) markers 181 (Evans et al. 2007; Rimet et al. 2019). Characterization of cultures allows dereplication of duplicate strains, 182 with several potential strategies can be used to keep strains of interest. For example, if the same species 183 has been isolated several times from several stations, one or two strains from each sampling station can 184 be kept. When isolated strains are entered into a culture collection database, it is important to add as 185 much metadata as possible, in particular related to the sampling site, but also related to the isolation 186 (enrichment, isolation method, isolator, medium used for isolation, etc.). 187

#### **4** - Culture of diatoms

Once diatom strains are established, they are generally considered to be relatively easy to grow (at least 189 on the short term) using standard microalgal culturing techniques. A wide range of media have been 190 developed for culturing marine microalgae (for an exhaustive list refer to the book by Andersen 2005). For 191 marine diatoms, commonly used media used are f/2 (Guillard 1975), L1 (Guillard and Hargraves 1993) 192 and K (Keller et al. 1987). The main specificity for culturing diatoms is that media should contain silica 193 (usually in the form of sodium silicate), which is required by diatoms for production of their frustules. 194 Natural seawater may contain a sufficient quantity of silica to maintain diatom growth without adding 195 a supplement, particularly for species with thin frustules and/or if the water is collected outside periods 196 of diatom blooms (when silica levels are depleted). However, when culturing diatoms silicate is generally 197 added (at concentrations up to 100  $\mu$ M) as a precaution, and silica supplements are absolutely required 198 when using artificial seawater based medium recipes. For cultures in liquid media, plastic ware (polystyrene 199 flasks, tubes or multi well plates) or glass recipients (Erlenmeyer flasks, tubes, etc.) can be used (Figure 8). 200 The advantage of using pre-sterilized plastic ware is to reduce the risk of contamination, but it generates 201 a lot of waste and thus has a strong environmental impact. For cultures on solid medium, the recipe is 202 supplemented with 1 to 3% agar and following autoclaving the medium is distributed (before setting) either 203 in Petri dishes or as slants in glass tubes. All cultivation procedures should be conducted under a laminar 204 flow hood to limit bacterial contamination. 205

With respect to temperature, once diatom cultures are established, it is not necessary to exactly reproduce 206 the isolation temperature. Typical routine maintenance temperatures are 4 °C for polar species, 13 °C 207 for temperate species and 20  $^{\circ}$ C for tropical species. Diatoms contain chlorophylls a and c which absorb 208 light in the blue and red parts of the light spectrum and the main accessory pigment is fucoxanthin which 209 absorbs light in the 450-540 nm wavelength range. It is generally therefore best to use daylight quality 210 fluorescent tubes or LED lights which reproduce the natural spectrum, combining several wavelengths (blue, 211 green, red). Although it is possible to grow some diatoms in continuous light, the best results are usually 212 obtained by applying a photoperiod such as 12L:12D. Light intensity is typically set at 80-100  $\mu$ E.m<sup>-2</sup>.s<sup>-1</sup> 213 for strains established from surface samples and around 30  $\mu$ E.m<sup>-2</sup>.s<sup>-1</sup> for strains established from deeper 214 samples. For long-term maintenance of microalgal strains, it is recommended to use dedicated incubators 215 with temperature and light control. Alternatively, walk-in temperature-controlled rooms equipped with 216 shelves and light ramps are very convenient for large collections. 217

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 labour 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 colour, but it may be necessary to use optical microscopy or flow cytometry.

#### <sup>224</sup> 5 - Life cycles

Although many diatoms can be easily isolated from environmental samples (see above Andersen 2005) 225 and grow well on commercially available algal culture growth media, long-term maintenance of strains 226 is often challenging because of their peculiar life cycle, resulting in a gradual cell size diminution and 227 final loss of cultures. Unlike most other microalgae, diatoms have a diplontic life cycle (Figure 9), with 228 a long vegetative phase with diploid mitotically dividing cells, alternated by a comparatively short sexual 229 phase with short-lived haploid gametes (Chepurnov et al. 2004). During mitotic cell division, two slightly 230 unequal daughter cells are formed due to the peculiar architecture of the siliceous cell wall (frustule) 231 and the biosynthesis of new valves inside the confines of the parental cell wall. As a result, the mean 232 cell size of a clonal population gradually decreases during repeated rounds of mitotic divisions, known 233 as the MacDonald-Pfitzer rule (Macdonald 1869; Pfitzer 1869). Ultimately, cells become critically small, 234 resulting in malformations and eventually cell death (Mann 2011). While this appears to be the general 235 rule, a minority of diatom species do not decrease in cell size when dividing, while others are able to restore 236 their cell size asexually (Kaczmarska et al. 2022, 2013; Mann 2011; Rose and Cox 2013). In most species 237 studied, however, sexual reproduction and the subsequent expansion of a zygote into the auxospore is the 238 most prevalent mechanism to restore large sized cells. Spontaneous and experimentally induced abrupt cell 239 size reduction demonstrates that sexual reproduction in diatoms is dependent on cell size and can take 240 place once cells pass a species-specific size threshold (SST, Chepurnov et al. 2004). Although studied in 241 detail for only a few species, this endogenous cell size sensing mechanism, also referred to as the diatom 242 sex clock, results in an alternation between brief sexual reproduction events and long intervals of vegetative 243 growth, which can span several years (Lewis 1984; Mann 2011). While the processes of cell size decrease 244 and auxosporulation are conserved among most diatoms, diverse mating strategies exist, largely coinciding 245 with the main morphological groups of centric, araphid pennate and raphid pennate diatoms (Figure 9). 246 Most centric diatoms are oogamous, producing large egg cells which are fertilized by small, flagellate 247 spermatozoa. In general, they are homothallic (self-fertile), and clonal cells below the SST can, depending 248 on their size, differentiate into egg or sperm cells. However, exceptions to this general pattern exist (e.g. 249 Davidovich et al. 2017). In contrast, species from the evolutionary younger group of pennate diatoms, 250

are often heterothallic, although homothallic species and mixed strategies are also known (Davidovich et 251 al. 2010, 2009; Mann and Poulíčková 2019; Quijano-Scheggia et al. 2009; Vanstechelman et al. 2013), 252 and sexual reproduction therefore requires a partner of the compatible mating type. The phylogenetically 253 older group of araphid pennate diatoms is characterized by non-flagellate motile male gametes and larger 254 immobile female gametes (anisogamy). In contrast, most raphid pennate species produce morphologically 255 indistinguishable gametes (isogamy). A key difference with araphid diatoms is that a transfer of function 256 from gametes to the gametangia has evolved, where diploid sexualized cells from opposite mating types 257 interact to form a mating pair in which gametogenesis is subsequently initiated. Pheromone signalling plays 258 a crucial role during diatom sexual reproduction (Frenkel et al. 2014; Sato et al. 2011). The system appears 259 to be most evolved in raphid pennate diatoms and includes both sex inducing pheromones and attraction 260 pheromones influencing the behaviour of gametangial cells, as well as pheromones, so far uncharacterised, 261 involved in gamete attraction (Gillard et al. 2013; Klapper et al. 2021; Moeys et al. 2016). 262

Although cell size is the precondition for sexual reproduction to occur, appropriate environmental condi-263 tions should be also present to permit auxosporulation or to trigger it. This is best known for centric 264 diatoms, where spermatogenesis and/or oogenesis can be induced by a shift in environmental conditions 265 such as salinity, temperature, irradiance and its spectral composition, or photoperiod (Amato 2010; Baatz 266 1941; Godhe et al. 2014; Moore et al. 2017). In pennate diatoms, it appears that growing conditions 267 should be favourable. For example, sexual reproduction of the pennate Pseudo-nitzschia multistriata was 268 most successful when cultures were in the exponential growth phase (Scalco et al. 2014). In addition, 269 auxosporulation appears to be light dependent, both in terms of irradiance as well as spectral composition 270 (Bilcke et al. 2022; Davidovich 1998; Gillard et al. 2013; Mouget et al. 2009). 271

Understanding the process of sexual reproduction and the conditions under which it takes place is essential 272 for the long-term maintenance of diatom species in culture collections. This implies knowing the mating 273 strategy, SST, and conditions favouring sexual reproduction. In the case of heterothallic species, the avail-274 ability of multiple strains of compatible genotypes is an obvious additional requirement. Increasingly, the 275 importance of diatom-bacteria interactions is being revealed, including both beneficial as well as inhibitory 276 effects on diatom sexual reproduction (Cirri et al. 2019). However, as this information and resources 277 are available for only few species, many diatom strains may be maintained for variable periods in culture 278 collections, but are eventually bound to be lost. 279

Ideally, strains of large-sized (well above SST) can be cryopreserved, especially in the case of strains that are used as model species or for whole genome sequencing. Increasingly, studies are demonstrating significant genotype-related differences in physiological traits of diatom strains of the same species as well as strong differences in gene expression patterns (Anderson and Rynearson 2020; Pinseel et al. 2022), implying that the loss of genome-sequenced strains can have important implications for studies using other strains. If cryopreservation of large-sized cells is not possible (often small-sized cells are more easily cryopreserved, Chepurnova et al. unpublished) inducing sexual reproduction in such species is a solution to maintain those species in a collection. It should be noted, however, that apart from losing the original genotype(s), 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 9), including spores or resting cells. While the former are morphologically distinct from vegetative cells, resting stages have valves that are indistinguishable compared to their vegetative counterparts, but they are characterized by a condensed protoplast (Kaczmarska et al. 2013). 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.

#### <sup>296</sup> 6 - Diatom transformation and genetic engineering

The study of the molecular basis of diatom life began in the 1990s with the development of DNA-mediated 207 transformation in a few selected species. This important achievement to analyse and modulate the function 298 of diatom genes was first achieved by biolistic transformation in Cyclotella cryptica and Navicula saprophyla 299 via the pioneering work of Dunahay et al. (1995). Subsequently, this method was adapted to other species 300 such as Phaeodactylum tricornutum (Apt et al. 1996; Falciatore et al. 1999), Thalassiosira weissflogii 301 (Falciatore et al. 1999), Cylindrotheca fusiformis (Poulsen and Kröger 2005) and several others (Table 2). 302 Biolistic transformation is based on the helium-accelerated particle bombardment of diatoms with exogenous 303 DNA coated on gold or tungsten particles, followed by selection of the transformed cells with antibiotics. 304 There are also some reports describing diatom transformation by electroporation, where electric currents 305 are used to increase cell membrane permeability and DNA delivery (Table 2). Although theoretically faster 306 and less expensive than biolistic transformation, this method is less popular, largely due to the limited 307 reproducibility of the protocol in different laboratories. 308

More recently, bacterial-mediated conjugation transformation was developed for the most 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 314 for diatom synthetic biology because the efficiency of transformation is very high (100-1000 transformants 315 per  $10^8$  cells with bacterial conjugation vs 1-800 tranformants per  $10^8$  cells with biolistics, Moosburner 316 et al. 2022). It also allows reproducible expression levels of transgenes in different transgenic lines because 317 the episome is not integrated into the diatom genome. Conversely, with the biolistic and electroporation 318 transformation, plastid-containing DNA is integrated into multiple copies and in multiple sites in the 319 genome, meaning independent transgenic lines show very different expression levels (George et al. 2020). 320 Moreover, random integration into the genome can also result in undesirable changes in genomic loci at 321 integration sites or surrounding regions. To overcome these problems and to obtain conclusive functional 322 information, it is always necessary to characterize independent transgenic lines. Episomal genetic elements 323 may, however, be lost in the absence of antibiotic selection. Due to the high cost of the antibiotics 324 commonly used for selection, this represents a disadvantage if a large quantity of transgenic lines is required 325 for functional characterisation of diatom gene products or biotechnological exploitation of transgenic cell 326 extracts. On the other hand, loss of the episome by removal of antibiotics may also be an advantage in 327 achieving transient expression of the transgene, which is a desirable effect in gene functional studies (e.g., 328 to compare phenotypes related to the presence or absence of a specific gene) or to remove transgenic 329 proteins that may modify the DNA, and thus generate an unintended genomic change (Moosburner et al. 330 2022). 331

Several factors are critical to the success of genetic transformation, which may limit the application of these 332 tools to certain diatom species (Table 2): The ability to maintain the selected species under laboratory 333 conditions is obviously the first important requirement, but many diatom species currently remain refractory 334 to laboratory culture. Moreover, as different transformation methods also cause cell damage and mortality, 335 transformation of diatoms is usually performed with a high quantity of cells (typically around  $10^8$ ) as 336 starting material. For diatom species that do not reach a high cell density under laboratory conditions, it 337 is therefore necessary to use large culture volumes and a cell concentration step prior to transformation, 338 which may represent a limitation to routine use of the technology. In addition, genetic transformation relies 339 on the use of an appropriate selection marker to isolate transgenic lines in a population of untransformed 340 cells. Diatoms, like many algae, are resistant to many commercially available antibiotics. However, some 341 antibiotics have been identified that kill diatoms under particular conditions of cell concentration and 342 salinity (to be defined for each species) and these can be used to isolate transgenic lines expressing specific 343 antibiotic resistance genes (Table 2). For successful transformation, it is also preferable to use axenic 344 cultures, as bacterial contamination can alter the growth of diatoms on the selection medium and thus 345 lead to the growth of false transgenic lines. However, many diatoms do not grow properly in the absence 346

<sup>347</sup> of bacteria, which can complicate genetic transformation efforts.

Transformation also relies on the identification of appropriate regulatory regions (promoter and terminator) 348 for controlled expression of endogenous (i.e. from the same species to be transformed) or exogenous (from 349 other diatom species or other organisms) genes in the diatoms. For most transformed diatoms, transgene 350 expression has been successfully achieved by using endogenous regulatory regions. For only few species, 351 transformation has been achieved using regulatory regions from other diatoms or viral promoters (see 352 Falciatore et al. 2020). The identification of endogenous regulatory regions does not represent a major 353 challenge if genomic and transcriptomic information are available, but such information is currently only 354 available for a limited number of diatom species (Mock et al. 2022). 355

Co-transformation of two vectors is also documented in several diatom species, whereby a selectable marker 356 gene on a plasmid can be used to co-deliver in the same diatom cell another non-selectable transgene, such 357 as a gene expressing a protein of interest fused to a protein tag or a reporter gene. However, the possibility to 358 use multiple selectable markers largely facilitates genetic manipulations requiring the expression of different 359 transgenes. This can be useful to simultaneously modify multiple genes or for the characterisation of the 360 function of a gene (e.g., gene mutagenesis in a first transformation round, followed by complementation of 361 a wild-type or a mutated version of the gene of interest in a second transformation, see Giovagnetti et al. 362 2022). Multiple selectable antibiotics have so far only been identified for a few species (Table 2). For P. 363 tricornutum, chemical based selectable markers have also been recently developed (Serif et al. 2018), based 364 on the APT (Adenine Phosphoribosyltransferase, enzyme of the adenine salvage pathway) and UMPS 365 (Uridine-5'-monophosphate synthase, enzyme of the de novo pyrimidine biosynthesis pathways) genes, 366 whose inactivation results in the resistance to the toxic compounds 2-fluoroadenine and 5-fluoroorotic acid, 367 respectively. 368

Independently of the transformation method employed, transgenic lines are visible after several weeks of 369 growth on the selectable media (between 2-5 weeks depending on the species and the method). Growth of 370 the transformed diatom cells on an agar plate greatly facilitates the isolation and characterisation of clonal 371 transgenic lines derived from the same transformation event. For some species such as P. multistriata 372 and Pseudo-nitzschia arenysensis that are difficult to grow on plates, the selection of transgenic lines 373 following transformation is undertaken in liquid media in the presence of the antibiotic (Sabatino et al. 374 2015). However, this protocol is more time-consuming as it requires the subsequent isolation of individual 375 transgenic cells from a pool of transformed cells in order to perform molecular characterization. In some 376 centric species such as T. pseudonana or Cyclotella cryptica, which are also difficult to grow on agar plates, 377 the isolation of transgenic lines can be successfully achieved by growing cells inside a low % (0.25%) agar 378

<sup>379</sup> 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 380 gene function. Powered by the availability of -omic information, these studies have been instrumental 381 in characterizing specific cellular and metabolic features of diatoms and in elucidating the mechanisms 382 contributing to their ecological success in the environment (see Falciatore and Mock 2022, for a recent 383 overview on the "Molecular Life of Diatoms"). Transgenic lines have been extensively used to characterize 384 the cellular localization of diatom proteins, by fusing proteins with various reporter genes, or to monitor their 385 expression or achieve protein purification with specific tags. The development of new cloning technologies 386 for the assembly of multiple DNA fragments based on Gateway vectors (Siaut et al. 2007) and, more recently, 387 Golden Gate technology (Faktorová et al. 2020; Hopes et al. 2016) have provided important complementary 388 resources for the characterisation of diatom gene products in transgenic lines. Genetic transformation has 389 also been critical to modulate the expression of diatom genes, an essential step to understand their function 390 by performing comparative phenotypic analyses with untransformed wild-type strains. Gene over-expression 391 is currently achieved in diatoms by using a variety of strong or inducible promoters (reviewed in Falciatore 392 et al. 2020), while gene silencing can be achieved by generating transgenic lines expressing antisense 393 or inverted-repeated fragments against the gene of interest (De Riso et al. 2009). The development 394 of different gene editing tools to delete or modify diatom gene products represents an additional major 395 achievement of recent years. Nucleotide modifications were first realized in P. tricornutum with a vector 396 expressing site-specific nucleases, such as the Meganucleases (MNs) or the transcriptional Activator-Like 397 Effectors Nucleases (TALENs) (Daboussi et al. 2014). Currently, the Clustered Regularly Interspaced Short 398 Palindromic Repeats (CRISPR/Cas9) technology has been adapted as the method of choice for its ease 399 and molecular efficiency, documented in different species such as P. tricornutum (Nymark et al. 2016) and 400 T. pseudonana (Hopes et al. 2016). Stable gene modifications have also been obtained by delivering the 401 CRISPR/Cas9 system on an episome (Sharma et al. 2018) or with a RNA ribonucleoprotein complex (Serif 402 et al. 2018). These methods have the advantage that the nuclease modifying the genomes can be only 403 transiently expressed in diatom cells, reducing the risk of off-target mutagenesis and random modifications. 404 Readers can refer to Moosburner et al. (2022) for additional details on the genetic engineering and genome 405 editing tools currently available for emerging diatom model species. 406

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.

#### 419 **7** - Cryopreservation

Most algal strains are maintained in a perpetual active culture state and must therefore be periodically 420 transferred to fresh culture medium. Despite being the most commonly employed method of maintenance, 421 perpetual active culture results in a host of potentially significant problems, perhaps the most important 422 being that of genetic drift. As old methods are improved and new methods developed, enigmatic scientific 423 results issuing from studies using cultures can be reevaluated, but this presupposes that the biological 424 material does not change in phenology or genome content over time. Researchers have known for decades 425 that transferred cell lines have their own selective pressures, and that spontaneous mutation rates are 426 higher for microbes in culture than for the same microbe in the natural environment (Lakeman et al. 2009; 427 Phillips et al. 1994). Cryopreservation is an alternative to perpetual culture that ensures genotypic (and 428 consequently physiological) stability over time, but not all microalgal strains can be/have been successfully 429 cryopreserved. 430

Cryopreservation is the storage of viable cells at ultra-low temperatures of liquid nitrogen (LN2, -196  $^{\circ}$ C) 431 and/or its vapour phase (-156 °C). The underlying principle of cryopreservation is that at ultra-low tem-432 peratures, vital functions, such as enzymatic activity and cell division, are slowed to the point of cessation, 433 but not death (Benson 2008). A fundamental discovery in implementing successful cryopreservation was 434 made by Polge et al. (1949) working with fowl sperm. They observed that at low temperatures, the sperm 435 could be "protected" by using glycerol. Since then, cryopreservation has been used as a means to store 436 (or "cryobank") biological materials including seeds, viruses, bacteria, and mammalian and plant tissue 437 culture cells as a hedge against loss of valuable research resources. Unicellular algae have been cryopre-438 served since the early 1960s, although new methods were required due to poor success rates and alteration 439 of algal ultrastructure (Morris 1976; Plattner et al. 1972). Morris and colleagues published an extensive 440 experiment in 1978 successfully cryopreserving 252 of 284 strains of Chlorococcales (green algae) using a 441 two-step cooling protocol (Morris 1978). Cryopreservation methodology for algae has and must continue 442

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 have been developed for the cryopreservation of 447 algae (Ramon et al. 2002), they fall into two main categories: (1) rapid cooling, and (2) 'two-step' cooling. 448 The rapid cooling technique consists of plunging the algal material, suspended in culture medium with 449 an appropriate cryoprotective agent, rapidly into liquid nitrogen at -196 °C. At such rapid cooling rates 450 however, the internal solution of the material becomes supercooled, increasing the possibility of damaging 451 intracellular structure due to ice formation (Karlsson and Toner 1996; Meryman 1966). Most freezing 452 protocols avoid this by utilizing a two-step cooling process, with controlled or semi-controlled cooling from 453 room temperature (generally at a rate of ca. -1 °C min<sup>-1</sup>) to a holding temperature of around -30454  $^{\circ}\text{C}$  before the material is plunged into liquid nitrogen to complete the freezing process. The first step 455 can be performed using simple devices such as Mr Frosty<sup>©</sup> or more sophisticated controlled-rate freezers. 456 Damage is thought to be prevented using this method because the reduced cooling rate allows sufficient 457 time for osmotic equilibrium to be maintained by shrinkage of the cell. Another method, common in higher 458 plants, to minimize intracellular damage upon freezing is the process of vitrification, which is ultra-rapid 459 cooling in the presence of high concentrations of cryoprotective agents, (first described by Rall and Fahy 460 1985). Although not widely used to date, vitrification does appear to have some potential use in the 461 cryopreservation of algal material. A more detailed description of vitrification methods can be found in 462 Steponkus et al. (1992) and Karlsson and Toner (1996). A more recently employed method with algae is 463 encapsulation-dehydration (Hirata et al. 1996). This technique involves the dehydration of encapsulated 464 algal cells by means of sterile air drying followed by immersion and storage in liquid  $N_2$ . The method has the 465 advantage that no toxic cryoprotectants are required, hence upon thawing there can be direct cultivation 466 without the need for repeated washing of the sample. 467

**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 equilibriate 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 con-

sidering that membrane damage can be a primary initiator of free radical reactions, it is not surprising to 475 find that several CPAs may also act as free radical scavengers (Benson 1990; Canavate and Lubian 1995). 476 Hubálek (2003) reviewed the use of cryoprotectants in microorganisms. For microalgae, DMSO was far 477 and away the most commonly used CPA. While cryopreservation will conceptually arrest genetic drift (the 478 veracity of this statement is limited by the relatively short time that researchers have been successfully 479 cryopreserving microalgae), a valid question remains whether there is any genetic selection by the cryop-480 reservation event itself. Müller et al. (2005) showed that for Chlorella vulgaris AFLP patterns were identical 481 pre- and post-cryopreservation, suggesting no genetic selection during the event. 482

Cryopreservation of marine algae without CPAs has been reported in a very few instances, and the results 483 for some species are not consistent between studies. Freezing-induced membrane damage results primarily 484 from severe dehydration. Phospholipids are the major component of plant membranes, and their degree 485 of fatty acid unsaturation and length naturally influence both flexibility and permeability of membranes 486 (Ramon et al. 2002). In addition, strains that are desiccation resistant may be predisposed to successful 487 cryopreservation. It has been suggested that extracellular freezing and desiccation ultimately stress cells 488 similarly, namely that both lead to an increase in the osmotic stress on the cell through the deprivation 489 of free water. A number of diatom strains held by NCMA have been cryopreserved with and without the 490 addition of CPA (Figure 10). While some were not successfully cryopreserved without CPA (i.e., CCMP581, 491 CCMP1500, CCMP151, CCMP552), many were successfully cryopreserved in both treatments. A primary 492 observation, however, was a delayed growback time (time to reach pre-cryopreservation cell abundance) in 493 the samples cryopreserved without CPA. 494

**Thawing.** Thawing is critical for the reactivation of cultures. Several parameters are critical, notably 495 osmotic and toxic shocks and light stress In order to limit osmotic shocks and the intracellular and resulting 496 extracellular damages, frozen cultures are preferably thawed rapidly by immersing them for about three 497 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 498 on the cells and to prevent this, it is important to rapidly dilute the thawed aliquot in a sufficient volume 499 of fresh culture medium. It is possible to include a centrifugation step to pellet the cells and remove 500 the CPA before re-culturing, but this step induces additional stress and decreases survival chances. In 501 order to slowly revive the cultures, they are placed in optimal culture conditions (medium, temperature), 502 while light is introduced progressively after a 24 to 48 hours phase of darkness or semi-darkness, thus 503 limiting the light stress. It takes a few weeks for the cultures to recover a growth rate identical to that 504 before cryopreservation. During these few weeks, monitoring is performed by light microscopy and/or flow 505 cytometry. 506

Overall success of cryopreservation for diatoms. Cryopreservation success for microalgae varies widely 507 between different taxonomic groups. For example, dinoflagellates are very difficult to cryopreserve (Hage-508 dorn et al. 2015). Diatoms are relatively easy to cryopreserve, although success rates vary significantly 509 among diatom lineages (Stock et al. 2018). In the collections we surveyed (Table 3), pennate diatoms 510 appear easier to cryopreserve than centric diatoms, especially the Coscinodiscophyceae. However, there 511 are large variations between genera (Table S1). For example, while all strains of Seminavis tested can 512 be cryopreserved, only 50% of Thalassiosira strains are successful. This could be an effect of cell size, 513 as Paredes et al. (2021) reported that in terms of estimated cell volume, approximately two thirds of the 514 small (< 1000  $\mu$ m<sup>3</sup>) and intermediate (1000–2500  $\mu$ m<sup>3</sup>) categories of diatoms from the RCC collection for 515 which cryopreservation was attempted were successfully cryopreserved, whereas few of the larger (> 2500516  $\mu$ m<sup>3</sup>) category survived cryopreservation. Paredes et al. (2021) also reported that in terms of geographic 517 origin, approximately only two thirds of temperate and polar diatom strains for which cryopreservation was 518 attempted were successfully cryopreserved, whereas almost all tropical strains (albeit from a lower total 519 number) grew after thawing. Cell size therefore appears to be an important factor in cryopreservation suc-520 cess, with cold-adapted (polar) strains apparently not showing an increased resistance to cryopreservation. 521

#### 522 8 - Conclusion

Culture collections are critical for diatom research. However, of the ca. 8,400 described diatom species 523 (Guiry 2012), only around 10% are currently maintained in culture, which is a small proportion given that 524 the real extent of diatom biodiversity is estimated to be in the region of 100,000 species. This highlights the 525 necessity to continue and amplify isolation efforts, especially for species from environments that have been 526 relatively undersampled to date, such as plant and animal microbiomes or sediments. It is also important 527 that researchers that isolate diatoms (and other algae) deposit them in public collections and it is of course 528 even more critical that culture collections remain funded to be able to maintain existing cultures and expand 529 the range of strains available for research and industry (Becker et al. 2019). 530

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- $_{\tt 536}$   $\,$  Table 1) that contributed listings of diatom strains in their holdings.
- 537 **Competing interests** The authors declare no competing financial interests.

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### Table 1: List of culture collections containing diatoms.

Acronym	Name	# of strains	Country	Web site
ACOI	Coimbra Collection of Algae	16	Portugal	http://acoi.ci.uc.pt/index.php
ACUF	Algal Collection University Frederico II	20	Italy	http://www.acuf.net/index.php?lang=en
ALGOBANK	Algobank-Caen		France	https://borea.mnhn.fr/en/algobank-caen
ALISU	Culture Collection of the Universidade de Lisboa	9	Portugal	
ANACC	Australian National Algae Culture Collection	336	Australia	https://www.csiro.au/ANACC
ARC	Algal Resources Collection	9	USA	https://www.algalresourcescollection.com/
BCCM/DCG	BCCM/DCG Diatoms Collection	536	Belgium	https://bccm.belspo.be/about-us/bccm-dcg
BEA	Banco Espaol de Algas		Spain	https://marinebiotechnology.org/en/
BMAK	Aidar & Kutner Microorganisms Collection	73	Brazil	https://www.io.usp.br/index.php/infraestrutura-io-2/banco-de-microorganismos.html
CCAC	Central Collection of Algal Cultures		Germany	https://www.uni-due.de/biology/ccac/
CCALA	Culture Collection of Autotrophic Organisms	4	Czech Republic	https://ccala.butbn.cas.cz/
CCAP	Culture Collection of Algae and Protozoa	165	UK	https://www.ccap.ac.uk/
CCBA	Culture Collection of Baltic Algae		Poland	https://ccba.ug.edu.pl/pages/en/home.php
CCCM	Canadian Center for the Culture of Microorganisms		Canada	https://cccm.botany.ubc.ca/
CICCM	Cawthron Institute Culture Collection of Micro-algae	135	New Zealand	https://cultures.cawthron.org.nz/
CMR_RBI	Culture collection of the Center for Marine Research, Ruder Boskovic Institute	152	Croatia	
CPCC	Canadian Phycological Culture Centre	31	Canada	https://uwaterloo.ca/canadian-phycological-culture-centre/
DSMZ	Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zelllkulturen	50	Germany	https://www.dsmz.de/
FACHB	Freshwater Algae Culture Collection at the Institute of Hydrobiology		China	http://algae.ihb.ac.cn/English/
FINMARI CC	FINMARI Culture Collection/SYKE Marine Research Centre and Tvorminne Zoological Station	30	Finland	
IRTA	Institute of Agriculture and Food Research and Technology of Catalonia Collection		Spain	
MBA	MBA Culture Collection		UK	https://www.mba.ac.uk/facilities/culture-collection
MBRU	Russian Marine Biobanks	24	Russia	http://marbank.dvo.ru/index.php/en/marine-genetic-resourses
MCC	Microbial Culture Collection - National Institute for Environmental Studies Collection	124	Japan	https://mcc.nies.go.jp/
MEDIC	Marine Epibiotic Diatom Collection	126	South Africa	http://ipt.sanbi.org.za/iptsanbi/resource?r=diatom
MNHN-ALCP	Collection du Musum d'Histoire Naturelle, Paris	3	France	https://www.mnhn.fr/en/collections/collection-groups/biological-resources-living-and-cryopreserved-cells/
				microalgae-and-cyanobacteria
MUC	Mersin University Collection	16	Turkey	
NBRC	Culture Collection Division, Biological Resource Center, National Institute of Technology and Evaluation	14	Japan	https://www.nite.go.jp/nbrc/catalogue/catalogueAdvSearch
NCMA	Provasoli-Guillard National Center for Marine Algae and Microbiota	585	USA	https://ncma.bigelow.org/
NORCCA	Norwegian Culture Collection of Algae	290	Norway	https://norcca.scrol.net/
RCC	Roscoff Culture Collection	939	France	https://roscoff-culture-collection.org/
SAG	Sammlung von Algenkulturen der Universitt Gttingen		Germany	https://www.uni-goettingen.de/en/culture+collection+of+algae+%28sag%29/184982.html
SMS	Singapore Marine Strains		Singapore	
SZCZ	Szczecin Diatom Culture Collection	2151	Poland	http://geocentrum.usz.edu.pl/en/szczecin-diatom-culture-collection-szcz/
SZN	Stazione Zoologica di Napoli		Italy	
TCC	Thonon Culture Collection	193	France	https://www6.inrae.fr/carrtel-collection_eng/
UTEX	Culture Collection of Algae at the University of Texas at Austin	390	USA	https://utex.org/

**Table 2:** Summary of published transformable diatom species and of the methods used for genetic transformation. 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.

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 tricornutum	Biolistic	sh-ble	Phleomycin/Zeocin	Apt et al. 1996
	Electroporation	nat1	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 Krger 2005
Thalassiosira pseudonana	Biolistic	nat1	Nourseothricin	Poulsen and Krger 2005
	Electroporation	sh-ble	Phleomycin/Zeocin	Bugge and Robertson 2019
	Bacterial conjugation			Karas et al. 2015
Amphora coffeaeformis	Biolistic	nat1	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	nat1	Nourseothricin	Miyagawa-Yamaguchi et al. 2011
	Electroporation	sh-ble	Zeocin	lfuku et al. 2015
		bsr	Blasticidin-S	Yin and Hu 2021

**Table 3:** Cryopreservation status by class for cultures from several collections (BCCM, RCC, NCMA, UTEX, DSMZ, MBRU, MCC). Plus sign corresponds to success and Minus no success. Cultures that were not tested were not included.

+	-	% success
905	294	75
25	3	89
11	45	20
436	263	62
	+ 905 25 11 436	+ - 905 294 25 3 11 45 436 263



Figure 1: Colonies of *Nitzschia palea* growing on agar (reprinted from Richter 1903).



**Figure 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.



Figure 3: Diatom genera and species available cultures. Rectangle surfaces are proportional to number of strains.



**Figure 4:** Origin of diatom cultures according to ecosystems and substrates. Rectangle surfaces are proportional to number of strains.



Figure 5: Isolation sites for diatom cultures for the different collections.



Figure 6: Year of sampling for diatom in collections.



Figure 7: Single cell isolation strategy.



Figure 8: Cultures of diatoms at the Roscoff Culture Collection using either culture flasks or tubes.



Figure 9: The life cycle of diatoms: a graphical representation of vegetative cell size decrease and the main strategies to restore cell size.



Figure 10: Growback times for diatom strains cryopreserved with and without cryoprotective agents (CPA) added.

## 835 Supplementary Material

Supplementary Data S1. List of diatom strains inventoried with associated metadata (tax onomy, origin, cryopreservation status): https://doi.org/10.5281/zenodo.7641545. 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). Plus sign corresponds to success and Minus no success. Cultures not tested were not included.

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	16	10	62
Bacillariophyceae	Bacillaria	1	0	100
Bacillariophyceae	Bacillariophyceae_XXX	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
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_X	21	0	100
Bacillariophyceae	Fragilariales_XX	1	0	100

Class	Genus	+	-	% success
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
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_XX	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

 Table S1: (continued)

Class	Genus	+	-	% success
Bacillariophyceae	Pseudostaurosira	1	0	100
Bacillariophyceae	Rhopalodia	0	1	0
Bacillariophyceae	Sellaphora	3	2	60
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_X	Astrosyne	0	1	0
Bacillariophyta_X	Bacillariophyta_XXXX	8	1	89
Bacillariophyta_X	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
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

 Table S1: (continued)

Class	Genus	+	-	% success
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_X	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
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

 Table S1: (continued)