Challenges for the Maintenance and Cryopreservation of Multiple Isolates of Model Microorganisms

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Challenges for the maintenance and cryopreservation of multiple isolates of model microorganisms: An example using the marine diatom *Skeletonema marinoi*

Running title: Challenges for the cryopreservation of a model alga

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Modern genomic and metabolomic tools have provided the possibility of generating and interrogating large data-sets that can provide answers to previously imponderable taxonomic, evolutionary, ecological and physiological questions. However, the curatorial tools needed to provide and maintain the relevant biological resources on which new knowledge can be built have not kept pace with this meteoric rise in scientific capacity, its associated activity, or the huge increase in published science. The availability of biological material of guaranteed identity and quality in Biological Resource Centers is fundamental for scientific research, but it crucially depends on there being adequate preservation/maintenance methods capable of ensuring phenotypic, genotypic and functional security of the biological material(s). This paper highlights the challenges to the long-term maintenance of genetic resources in general, focusing specifically on the issues associated with the maintenance of a large collection of strains of the ecologically significant diatom *Skeletonema marinoi*. This research collection, held at the Department of Marine Sciences, University of Gothenburg has been systematically tested for its capacity to survive cryopreservation. A method, involving incubation in the dark for 20-24 hours before cryopreservation, followed by cryoprotection employing 10% dimethysulphoxide (DMSO) and conventional cooling in a Passive cooler, prior to plunging into liquid nitrogen was successfully applied to approximately 80% of the strains tested. In addition, the growth characteristics of exemplar strains confirmed after storage.

Introduction

Microbial culture collections, or more broadly, "biobanks" or Biological Resource Centers (BRCs), have diverse remits, but with the exception of some of the larger service collections such as the American Type Culture Collection (ATCC) in the USA and the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Germany they commonly focus on discrete groups of organisms. These may be restricted to a major taxonomic lineage, as is the case for many bacterial or fungal collections, alternatively for algal collections it may involve the retention of a phylogenetically diverse range of microbial photoautotrophs and for some research collections restricted to a single taxon. The organisations hosting BRCs encompass a mixture of academic, public service and private, government and commercial entities that have as part of their core remit the provision of characterized cultures as 'seed' stocks:

- for use in research,
- for teaching and training purposes,
- for the development of industrial processes,
- for the control of testing for quality assurance of products,
- as referenced standards in the published scientific literature,
- as reference strains for biological assays,
- as reference material in biomedical assays,
- as type and/or authentic strains for taxonomical studies and
- as centers for conservation of biodiversity.

It is widely agreed that the provision of cultures from BRCs is of little value unless these cultures are accompanied by information on their identity, provenance and characteristics[1]. Thus, culture collections are important store-houses of information which can be accessed by direct contact with individual collections, or by contacting microorganism database organizations such as MIRCEN at http://wdcm.nig.ac.jp/. Information resources can grow with time as work on cultures from BRCs is published and increasingly individual collections provide more comprehensive data, images, bibliographic data and links to EMBL/Genbank directly from their websites e.g. the Culture Collection of Algae and Protozoa (CCAP)[2] and the Belgian Co-ordinated Collections of Micro-organisms Diatom Culture Collection[3] All of these services necessitate the long-term maintenance of "alive", genetically, phenotypically and functionally stable cultures. In the case of algae, maintenance by serial transfer has been the historic method of choice[4] and whilst there is evidence that some algae are stable in culture for many decades[5], others have been reported to lose or modify key phenotypic characters, or metabolite production on prolonged maintenance by serial transfer[6].

With the exception of the Cologne, Coimbra, Roscoff and UTEX culture collections, algal service culture collections all maintain less than 3000 strains in their open collections (Table 1), in comparison to bacterial or fungal collections, which in some cases hold tens of thousands of strains. Although almost all of these major collections now use cryopreservation as a curatorial tool, many algal taxa remain recalcitrant to conventional methodologies, and resource constraints (financial, staffing and infrastructural) limit the number of strains that can be maintained by cultivation-based methods. This is a significant restriction to the types of studies that can be undertaken on algae, for example projects which might generate 100's or even 1000's of individual isolates. Modern analytical approaches including the development of molecular sequencing and their associated bioinformatic tool-kits have revolutionized the capacity to generate and interrogate large data-sets. Furthermore, the increase in interest in the biotechnological exploitation of algae will inevitably result in the generation of thousands of mutants through selective breeding, conventional mutagenesis, as well as via genetic manipulation.

Diatoms, one of the most species-rich groups of microalgae, are of key ecological significance and hold an interesting biotechnological potential, but represent a particular challenge for long-term preservation. Due to their gradual reduction in cell size, vegetatively dividing clones cannot be kept in culture for extended time by serial transfer, as cells lose viability beyond a species-specific minimal cell size. Experimentally inducing sexual reproduction is one way to maintain species (but not genotypes) in culture, but is not possible for many species. In particular in the case of centric diatoms the sexual cycle is poorly understood and controlled sexual reproduction is currently in its infancy. Precious strains, possessing unique phenotypic characteristics, or those originating from archived sediment samples are easily lost in culture[7]. Furthermore, in the case of genetically transformed strains there is a high risk at losing insertions if strains are maintained asexually[8]. The above challenges provide a stimulus to exploring the options available to conserve large collections of strains belonging to the same taxon. Of the long-term preservation approaches employed in BRCs only cryopreservation (storage at ultra-low temperatures) has the potential to provide a practicable solution to these challenges. Additionally, this approach has the advantage that, after the initial preservation procedure has been performed, routine maintenance costs are limited to costs of refrigerant and viability can effectively be guaranteed for decades if not hundreds of years[9].

In this study the centric diatom *Skeletonema* was selected because it is an important contributor to the pelagic community and often dominates phytoplankton assemblages in temperate coastal regions. It is a widespread genus, except in the polar regions[10], and to date thirteen species have been described[11-14]. In Scandinavian waters so far only one species, *Skeletonema marinoi* Sarno et Zingone, has been reported[15,16] and it is especially abundant during spring bloom, when densities of millions of cells per litre occur. The predominant means of propagation is through vegetative division, but the formation of

auxospores and sexual reproduction has been documented[17]. The generation time is 24 hours which makes it ideal for studies of phenotypic response. Furthermore, benthic cells act as a resting stage, and can survive for at least hundred years and provide a short-term evolutionary archive in sediments[7]. *S. marinoi* is easy to collect, isolate, and maintain in culture, and the survival of monoclonal cultures after single cell isolation is almost 100%. *Skeletonema* is routinely used as a model in ecological and other studies[18-20] and at present hundreds of strains are held in the, Göteborg University Marine Algal Culture Collection (GUMACC) as an "in house" research resource. Like all such collections the maintenance of the resource is a challenge with respect to its curatorial costs and the long-term conservation of the materials cannot be guaranteed. Initially a pilot study was undertaken on three well documented strains. This was subsequently expanded to test the suitability and robustness of a standardized protocol across the holdings of the GUMACC.

Materials and Methods

Isolates studied and cultivation conditions

Skeletonema marinoi CCAP 1077/5 was obtained from the Culture Collection of Algae and Protozoa (CCAP), all other isolates studied were from the Göteborg University Marine Algal Culture Collection and their origin, isolation date, and where available Genebank barcode data are listed in Table 2. All the strains were grown in 50 ml Nunc Nunclon™Δ EasYFlasks™ with a vent closure permitting gas exchange, at irradiance ca. 40 µmol photons m^{-2} s^{-1} (measured at lid height and provided by fluorescence tubes, L36W/865 Lumilux® Cool Daylight, Osram GmbH, Augsburg, Germany), with a 12h:12h light-dark photoperiod at ca. 10^{o}C. Growth medium was prepared with filtered seawater autoclaved and enriched with nutrients according to the standard recipe for f/2 medium[21] plus sodium metasilicate.

Cryopreservation methods

Controlled rate cooler (CRC) methods (Methods A-D)

A 10% v/v solution of DMSO 99.9% (Sigma-Aldrich Ltd., UK), was prepared and filter-sterilized. A 2.5ml aliquot of the 10% (v/v) DMSO solution was added to 2.5ml of late log phase cells to give a final DMSO concentration of 5% (v/v). Aliquots (1.0ml) were dispensed into cryovials (Greiner Bio-One GmbH, Frickenhausen, Germany). These were then incubated at room temperature (ca.20°C) for 15 min prior to cryopreservation, to ensure that the cryoprotectant had entered the cells. The cryovials were then transferred to the cooling chamber of a Planer Kryo 360-3.3 (Planer, UK) programmable freezer and cooled from room temperature at -1°C $min.^{-1}$ (Method A), -5°C $min.^{-1}$ (Method B) or -10°C $min.^{-1}$ (Method C) to -40°C and then plunged into a dewar of liquid nitrogen (LN2) as previously detailed by Day and Deville[25].

Alternatively, samples were cooled employing the standard protocol used at the National Center for Marine Algae and Microbiota (NCMA) (Method D), as detailed by Day and Brand (2005)[26]. Aliquots (0.5ml) of cryoprotected culture, as outlined above, were held at room temperature (ca. 20°C) for 15 min, then the cryovials were transferred to the Planer Kryo 360-3.3 programmable freezer and cooled at -1°C min^{-1} to -9°C, then at -20°C min^{-1} to -45°C, then warmed at +20°C min^{-1} to -12°C, then cooled at -1°C min^{-1} to -45°C, then cooled at -20°C min^{-1} to -90°C and finally plunged into LN2.

The samples were then transferred to a cryostorage container and the location logged on the CCAP cryo-storage database for overnight storage prior to being thawed.

Passive freezer methods (Methods E & F)

Method E: Cryoprotectant solutions and cultures were prepared as outlined above and 1ml aliquots aseptically transferred into pre-labelled cryovials. The Passive freezer unit, Mr. Frosty™ (Nalgene NUNC International, Rochester, NY) was prepared as per instructions, with the addition of 250ml of isopropanol to the reservoir adjacent to the cryovials tube holder and was then placed in a refrigerator overnight to equilibrate at approx 4°C. The cryovials containing cryoprotectant-treated algae were transferred into the Mr. Frosty (which has been designed to have a cooling rate of ca. -1°C min^{-1}) and the unit sealed and placed in a –80°C freezer. This was then held in the freezer for 1.5 h, by which time the temperature of the contents of the vial is less than –50°C. The frozen samples were then plunged into LN2, transferred to a cryostorage container and the location logged on the CCAP or GUMACC cryo-storage database for overnight storage prior to being thawed.

Method F: An alternative approach, previously successfully applied in the BCCM (Chepurnova and Vyverman, unpublished data) was also employed. This involved placing late log phase *S. marinoi* cultures in complete darkness for 20-24 hours before cryopreservation. The Mr Frosty™ unit was prepared as above. A 40% (v/v) solution of DMSO was prepared, in the culture medium used to grow the alga, and filter sterilized. Then for each strain 6 ml of culture and 2 ml of 40% DMSO were pipetted into 15 ml Falcon tubes to give a final cryoprotectant concentration of 10% (v/v). The tubes were inverted several times to mix the cryoprotectant (CPA) uniformly. The cultures with CPA were then transferred into pre-labeled 2 ml cryogenic vials and left to equilibrate at room temperatures in darkness for at least 20 minutes. The cryogenic vials were then transferred into the pre-chilled Mr Frosty™ and placed in an -80°C freezer for 90 minutes, as outlined above. The frozen samples were then plunged into LN2, transferred to a cryostorage container and their locations logged on the CCAP or GUMACC cryo-storage database for overnight storage prior to being thawed.

Thawing and culture recovery

The frozen cryogenic vials were rapidly transferred to a 37°C water bath. The vials placed in floating vial holders and were gently agitated by hand to facilitate thawing. As soon as all ice had melted, 1 ml from each vial was immediately transferred into a 250 ml flask containing 50 ml of fresh culture medium. This was then mixed to ensure homogeneous resuspension of the culture and reduction in the concentration of the CPA to < 0.2%. The cultures were allowed to recover in complete darkness overnight and then incubated at 10°C, under a 12h:12h light-dark photoperiod (40 μmol photons m^2 s^{-1}). Samples were monitored for up to 48 days post-thaw and fresh medium added to the culture as soon as growth was observed.

Viability staining

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a non-fluorescent, membrane permeable molecule. When inside a viable cell, due to enzymatic modification, CFSE becomes highly fluorescent; in addition, it losses permeability to the cell membrane, and it can remain detectable inside the cell for several days[27]. CFSE in the form of a 5mM stock-solution in DMSO was freshly prepared and added to the samples to give a final concentration of 2μM. The CFSE labelled cells were incubated at room temperature for 15 min before examination under an Axioskop 2 epifluorescence microscope and micrographs taken under fluorescence and phase contrast at 400x magnification. Viability assessment was estimated, by calculating the percentage of CFSE positives (live cells, stained in green) out of a total of ca. 50 cells observed per sample. This was performed after 24 h incubation[26] to reduce the number of false positive cells that were damaged by the procedure, but despite not being able to recover, still retain metabolic capabilities immediately after thawing.

Regrowth assays

Comparisons were made on the maximum growth rates, during the exponential phase, before and after cryopreservation to investigate possible phenotypical changes. The growth rates were obtained spectrophotometrically using optical density (OD, λ 600 nm).

Three 250 ml culture flasks for each strain were filled with 160 ml of culture containing a concentration of 5,000 cells ml^{-1}. The flasks were kept in a controlled environment (10°C, under a 12h:12h light-dark photoperiod ca. 40 μmol photons m^2 s^{-1}) for 15 days. Each day 700 μl from each sample were withdrawn onto a 48 well plate and density estimated by optical density using Varioscan Flash plate reader (Thermo Scientific). Post-cryopreservation samples were taken identically as pre-cryopreservation samples from the surviving cultures.

Statistical analysis

Growth rate (μ_{max}), defined as instantaneous rate of increase, were based on the longest possible period of exponential growth for each strain pre- and post-cryopreservation according to: $\mu_{max} = (\ln OD_t - \ln OD_{t-1})/ \Delta t$

where t = time, and OD = optical density reading at 600nm[28]. The interval of exponential growth was determined from growth curves established from each experimental culture replicate. The statistical difference between pre- and post- cryopreservation growth was assessed with a t-test.

Results

A pilot study was undertaken at the CCAP using a CCAP strain (*S. marinoi* CCAP 1077/5) and two GUMACC, strains one isolated from a sediment sample (*S. marinoi* St54) and the second from a water sample (*S. marinoi* 0603AA) to test five commonly employed protocols. These methods utilized either a controllable programmable cooler, which allows the cooling rate to be varied mechanically, or a passive cooler with a nominal cooling rate of ca. -1^{o}C min^{-1}. Post-thaw viability levels were assessed by light microscopy and vital staining using fluorescence microscopy (Fig. 1), and the capacity to regenerate "normal" cultures. Cells with ultrastructural damage induced by cryopreservation were readily identified using phase-contrast microscopy and vital staining confirmed that they were metabolically inactive and/or had a ruptured cell membrane. On the basis of the vital staining data all methods tested were effective for the CCAP strain with high levels (64-85%) of viable/ metabolically functional cells being observed and revival of all the cultures (Table 3). Levels of post-thaw viability, on the basis of vital staining, were much lower for the GUMACC strains (0-39%) and Controlled rate cooling at 1^{o}C min^{-1} using the standard CCAP protocol resulted in the best recovery with all 3 replicated re-growing for *S. marinoi* St54 and two of the three replicates of *S. marinoi* 0603AA regenerating normal cultures (Table 3). It was noted that the vital staining method of viability assessment was not robust and that although high levels of CFSE positive cells were observed 24 after thawing, for cultures that failed to recover these levels fell on subsequent incubation under standard cultivation conditions and after prolonged incubation no morphologically normal or CFSE positively stained cells were observed.

As part of the EU funded ASSEMBLE project Trans National Access programme a cryopreservation campaign was undertaken at the CCAP. In total 10 batches of four GUMACC strains with 15 replicates per strain were cryopreserved and banked in the CCAP cryobank. The logistics of applying a campaign of cryopreservation to the GUMACC collection worked well, but subsequent recovery regrowth was much poorer than anticipated. Recovery of all 3 replicates, within 4 weeks, was only observed in two strains (*S. marinoi* St54 and *S. marinoi* GF04-3A). However, on prolonged incubation full recovery was observed in two additional strains (*S. marinoi* 0603AA and *S. marinoi*

Oslo AE). Of the remaining 36 strains, recovery of two replicates was observed for four strains and recovery of one replicate for a further seven strains after prolonged incubation, with the remaining 25 strains tested failing to show any capacity to re-grow (Fig. 2).

Controlled Rate Cooling equipment is not available at the GUMACC; therefore, further optimisation of this approach "in house" was not practicable, so improvement of the Passive cooler approach was prioritised. Using a protocol trialled at the BCCM post-thaw viability was improved for the reference genome strain *S. marinoi* St54 with all three replicates tested showing recovery and the time taken to regenerate a healthy culture reduced from 40 to <30 days (Table 4). Furthermore, the method was applicable to strains that had failed to recover when tested using the CCAP passive cooling protocol including: S. *marinoi* A22, *S. marinoi* HAKH and *S. marinoi* R05AC (Table 4). This approach has been tested for a total of 17 *S. marinoi* strains, with 14 showing recovery and only 3 strains failing to grow on thawing (Table 4). The stability/ growth performance after employing this cryopreservation method was assessed for nine *S. marinoi* strains. On assessing algal biomass daily, based on optical density (600 nm), no significant difference in maximum growth rate (μ_{max}) before and after cryopreservation was observed in any isolate tested, with the exception of *S. marinoi* HAKH, which displayed significant lower μmax after cryopreservation (P=0.01) (Fig. 3). Furthermore, these cultures were subsequently maintained by serial transfer for > 6 months, with transfers every 3-4 weeks without any observable change to the cells or their growth characteristics.

Discussions

Science built on the use of live materials, whether fundamental or applied, has an absolute requirement for the biological materials employed to retain reproducible characteristics. This need for reproducibility has profound implications for those who use, hold and/or distribute microbial cultures including microalgae. A traditional example of this is in taxonomy where the botanical taxonomic community, including those who work with algae, have a requirement for type materials (i.e. the originally described organism to which the scientific name has been attached) that do not change, indeed this is a prerequisite in the International Code of Botanical Nomenclature[29]. Most commonly type specimens are held as preserved specimens, in the case of diatoms as permanent slides, or alternatively holotypes such as illustrations published in the hard-copy scientific literature; however, since the year 2000 fungi and algae, "if preserved in a metabolically inactive state (e.g., by lyophilization/ freeze-drying, or deep-freezing), are acceptable as types"[30]. It has subsequently been suggested in the era of genomics and metabolomics that alternative strategies, such as cryopreservation at ultra-low temperatures that allow the possibility of restoration of metabolism could be used to conserve type materials and that this would allow more comprehensive scientific exploitation of the type materials[31]. In addition to taxonomy, ecological, physiological and

biotechnological orientated users of biological resources all have a requirement for "biological standards" and optimally these should be held /maintained in such a way as to minimize any genotypic, phenotypic or functional change in the organism conserved[32].

In total over 2.4 million microbial strains are held by BRCs registered at the World Data Centre for Microorganisms (WDCM). The vast majority of these holdings are bacterial and fungal and although there are exceptions, the use of long-term conservation methods, such as lyophilization, or cryopreservation, is the norm. For algae, whilst freeze-drying may be applicable for some cyanobacteria[33] this method results in unacceptably low levels, or no, viability when used for eukaryotic algae[26,34]. However, cryopreservation has increasingly become a standard approach in larger algal culture collections (Table 1) and methodologies have been developed for a wide range of algal taxa[26,35].

A further stimulus to the need for cryopreservation methodologies for algae is the logistic and resource implications of routine maintenance. The number of strains held in the major algal collections is constrained by the cost and staffing needs and in recent years these collections have stabilized in size, or in some cases such as the CCAP, reduced slightly as more strains have been lost through culture failure and Q/A exercises than have been accessed. Furthermore, significant expansion of ecologically orientated collections like the GUMACC and their long-term maintenance, without guaranteed long-term core funding, are extremely challenging. New large projects necessitating accession of multiple isolates, or the development of genetically transformed algae, which generate 1000's of transformed clones[36,37] are equally problematic and the instability of some transformed algae, including the model diatom *Phaeodactylum tricornutum*[37] mean that conventional *ex situ* maintenance by serial transfer is inappropriate and not sustainable.

Cryopreservation provides a methodology that is capable of holding preserved material in effective "suspended animation" so that their genetic or physiological potential remains unchanged. There is a growing literature (evidence-base) on this in algae where the capacity to retain genetic integrity, morphology, metabolic functionality and growth characteristics have been reported in organisms that have been revived from cryopreservation[8,38-40]. For diatoms this is particularly relevant as on repeated serial transfer of inoculum, irreversible shrinkage can occur in some species[41] and this has been a major constraint on their use in sequential scientific publications, as well as the capacity of collections to maintain them in culture and to provide identical strains to different research groups. Thus, particularly in studies, or applications, where stability is critical to performance cryopreservation provides the most practicable solution to ensuring a "fit for purpose " inoculum is employed.

As outlined above there is an expanding published literature on the cryopreservation of algae. In the case of diatoms much of the emphasis has been on methodological development, particularly of taxa with biotechnological or aquaculture relevance[42-44]. These papers explored the influence of key parameters including culture age, cryoprotectant choice, cooling rate etc. with the objective of developing methodologies applicable to a range of algal taxa including diatoms. Other workers have

focused specifically on diatoms exploring both the cryobiological implications of subjecting diatoms to ultra-low temperatures and how this knowledge may be employed to improve post-thaw viability and cell recovery[45-4849]. To date there have been no publications exploring the logistics of attempting to conserve a large diatom collection by cryopreservation; however, Morris[49] previously reported the successful application of a conventional two-step cryopreservation method to >250 members of the Chlorococcocales. The method employed was similar to the optimal approach used in this study insofar as it utilized a relatively cheap, constant temperature alcohol bath (Fryka model KB 3000) maintained at a sub-zero temperature, with subsequent immersion into liquid nitrogen. In this study despite the possibility of more strictly controlling the cooling profile the methodology that had been optimized in a pilot study for three *S. marinoi* strains proved unsuccessful when applied to a much larger number of strains, with only ca.35% of the strains tested surviving. This was unexpected as one might have anticipated on the basis of prior experience[25,26,35] that slow cooling using a controlled rate cooler would result in more success than the alternative use of less controlled passive coolers. The standard CCAP Passive cooler cryopreservation protocol[26] was found to be effective for some strains e.g. *S. marinoi* GB3, with recovery being observed in all culture replicates, but unsuitable for other strains with no recovery observed after up to 48 days post-thaw incubation. However, employing higher levels of cryoprotectant, 10% in the BCCM method (Method F), versus 5% in the CCAP method (Method E) resulted in survival and regrowth in >80% of strains tested. The use of higher levels of DMSO has been observed in the BCCM to improve survival in a range of marine diatoms with concentrations in the range 10-15% being optimal. This has also been observed for *S. marinoi* CCMP2092, which has been cryopreserved using the standard NCMA/CCMP cryopreservation protocol (Method D) using 15% DMSO as cryoprotectant[50]. The BCCM protocol also has the advantage of practicality and involves use of standard lab equipment, rather than an expensive specialists cooling unit. For most practitioners, particularly those in smaller laboratories where there is not access to specialist cryobiological cooling devices, the use of low-tech approaches are the only realistic option. Here a conventional two-step, colligative cryopreservation approach was used successfully with good recovery in most strains and acceptable culture performance in terms of growth characteristics on thawing. An alternative strategy that was not explored in this study is the use of vitrification, whereby cells are subjected to treatments that result in high levels of intracellular dehydration and then ultra-rapid cooling, which leaves samples in a vitrified glassy state. This has been successfully used to cryopreserve the marine diatoms *Nitzschia closterium* and *Chaetoceros muelleri* using pre-treatment with a high osmotic potential solution (PVS2), followed by encapsulation in calcium alginate beads and rapid cooling in liquid nitrogen[51]. Such methodologies have potential to be more widely employed; however, from a practicality perspective they have a greater number of handling steps, with their potential for error and contamination. Furthermore, conventional vitrification solutions, such as PVS2, are known to be toxic to a range of algae, so

encapsulation dehydration methodologies using sucrose as a vitrification solution and/or air flow dehydration have been found to be more applicable to a range of microalgae[52-54].

In conclusion, there is an increasing demand for the maintenance of both a wider diversity of taxa and many more cultures of individual microalgal species. The traditional approach of employing serial transfer and maintenance under conditions that extend the culturing interval[4] is no longer capable of meeting this challenge, without significant investment in infrastructure and manpower. Only cryopreservation has the potential as a long-term conservation approach suitable for maintaining viability and functionality. Choice of methodology is crucial as methods need to be robust, reproducible and applicable to as large a number of strains as possible. The designing of individual methodologies for each isolate is neither practicable, nor efficient, so compromises need to be accepted with regards what is an appropriate viability level or how long is required to regenerate a healthy normal culture. The choice of method and cooling equipment is dictated by resource availability, but in general standardized simple methodologies that do not require specialist equipment will be more readily adoptable by the user community.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Day_et_al_FIG_legends

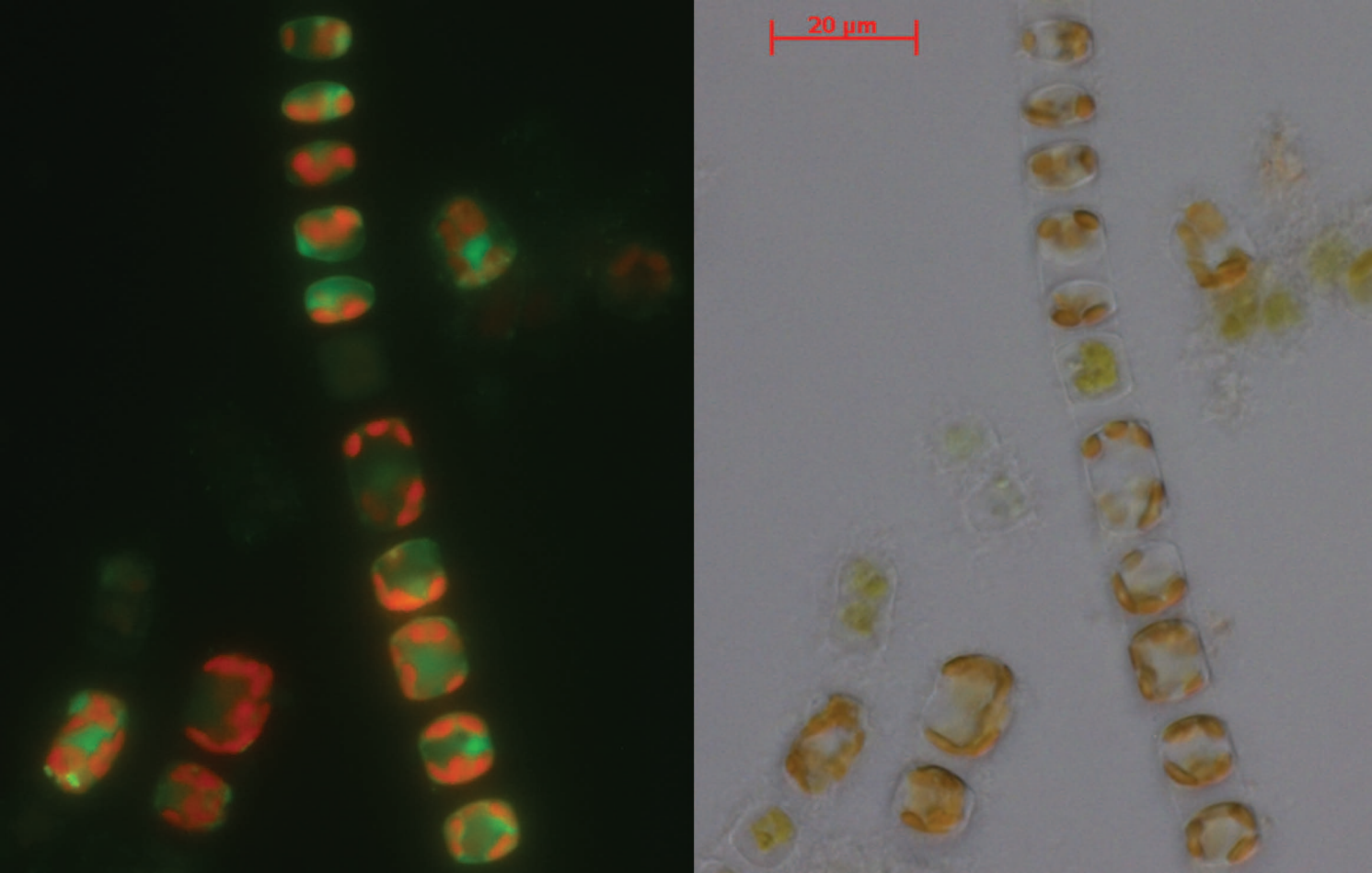
FIG. 1. Viability assessment by microscopy

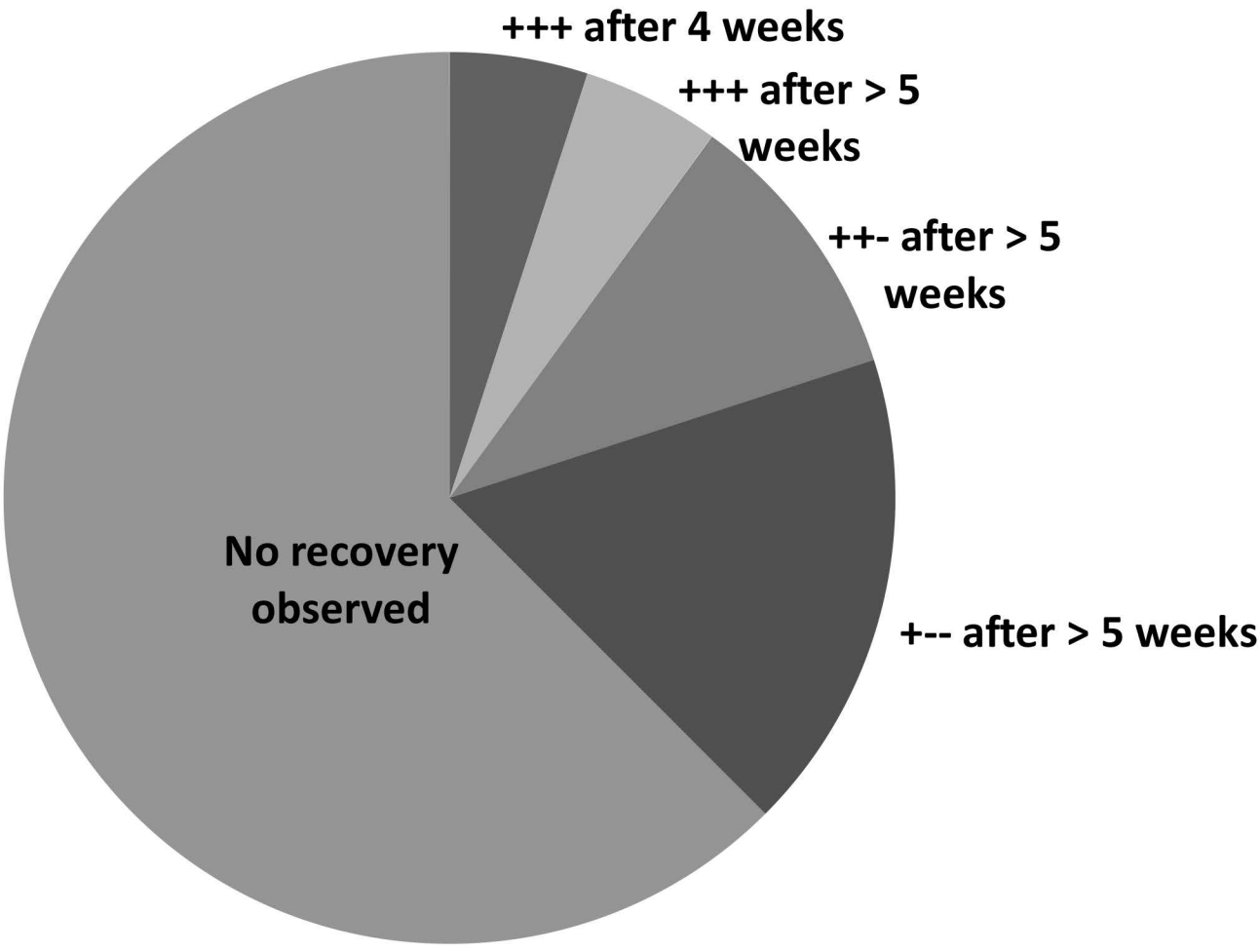
Epifluorescence and phase-contrast micrographs of *Skeletonema marinoi*. Live cells stained green with CFSE correlate with morphologically normal cells under phase contrast. Dead and damaged cells remain unstained, or where cells were intact appeared red due to auto-fluorescence of chlorophyll.

FIG. 2. Application of the standard CCAP two-step controlled rate cryopreservation protocol (Method A) to 40 GUMACC *Skeletonema marinoi* strains

Regrowth (+), or failure to grow (-), in replicate thawed samples

FIG. 3. Growth curves of *Skeletonema marinoi* strains before (black) and after (white) cryopreservation. Algal biomass was estimated daily based on optical density (600 nm). There was no significant difference in maximum growth rate (μ_{max}) before and after cryopreservation except for strain HAKH, which displayed significant lower μ_{max} after cryopreservation (P=0.01). **A**. St54 **B**. GF0410J **C**. GB3 **D**. A22 **E**. 32.3 **F**. A14 **G**. 0602I **H**. 080922P **I**. HAKH


20 µm


+++ after 4 weeks
+++ after > 5 weeks
++- after > 5 weeks
+-- after > 5 weeks
No recovery observed

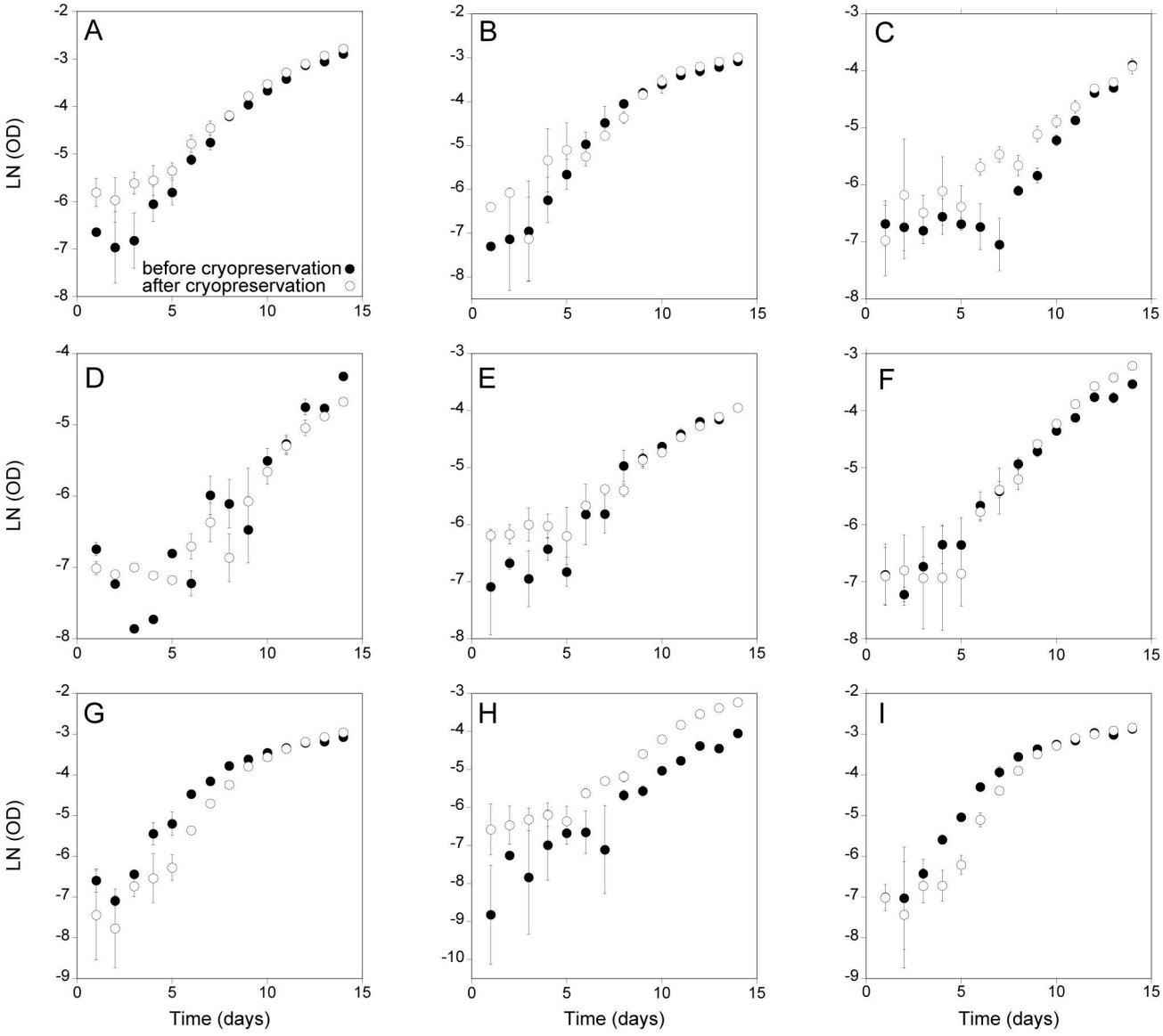

A
B
C
D
E
F
G
H
I
LN (OD)
Time (days)
before cryopreservation
after cryopreservation

TABLE 1. MAJOR ALGAL*/PROTISTAN** SERVICE CULTURE COLLECTIONS/ BIOLOGICAL RESOURCE CENTRES

Acronym	Collection name	Location	No strains held	Hold some strains in a cryo-preserved state
ACOI	Coimbra Collection of Algae	Portugal	3000	Yes
Algobank	Collection de Cultures de Microalgues - Université de Caen Basse-Normandie	France	470	No
ANACC	Australian National Algae Culture Collection	Australia	1000	No
ATCC	American Type Culture Collection	USA	>800	Yes
BCCM	Belgian Coordinated Collection of Micro-organisms	Belgium	550	Yes
CC	Chlamydomonas Resource Center	USA	2990	Yes
CCAC	**C**ulture **C**ollection of **A**lgae at the University of **C**ologne	Germany	3500	No
CAUP	Culture Collection of Algae of Charles Univ Prague	Cz Republic	250	No
CCALA	Culture Collection of Autotrophic Organisms	Cz Republic	750	No
CCAP	Culture Collection of Algae and Protozoa	UK	2800	Yes
CPCC	Canadian Phycological Culture Centre	Canada	400	Yes
DSMZ	Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH	Germany	>250	Yes
GUMACC	Göteborg University Marine Algal Culture Collection	Sweden	>400	Yes
KU-MACC	Kobe University Macroalgal Culture Collection	Japan	330	Yes
NCMA	National Center for Marine Algae and Microbiota***	USA	2600	Yes
NIES	Microbial Culture Collection at the National Institute for Environmental Studies	Japan	2800	Yes
NIVA	Norwegian Institute for Water Research	Norway	950	No
PCC	Pasteur Culture collection of Cyanobacteria	France	>700	Yes
RCC	Roscoff Culture Collection	France	3300	Yes
SAG	Sammlung von Algenkulturen Göttingen	Germany	2400	Yes
SCCAP	Scandinavian Culture Collection of Algae and Protozoa at the University of Copenhagen	Denmark	>1000	No
UTEX	UTEX Culture Collection of Algae	USA	3000	Yes

*Including the prokaryotic cyanophyta
**Numbers listed include holding of achlorophyllous heterotrophic protists

***Formerly the CCMP (Culture Collection of Marine Phytoplankton)

TABLE 2. THE *SKELETONEMA MARINOI* STRAINS USED IN THIS STUDY

Strain designation	Geographical origin	Isolation date	Isolation habitat	Estimated no. transfers since isolation	Available molecular data	Key references
CCAP 1077/5	Long Island Sound, Milford Harbour, Connecticut, USA	1956	plankton	600-700		
St54	Skagerrak, V. Koster, Sweden	2009	sediment	120	ITS rRNA Genbank: HQ111355.1 Microsatellites S.mar 1-8	Godhe et al. 2013[22]
0603AA	Skagerrak Gullmarsfjord, Sweden	2008	plankton	140	Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
GF04-3A	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
St44	Skagerrak, V. Koster, Sweden	2009	sediment	120	ITS rRNA Genbank HQ111356.1 Microsatellites S. mar 1-8	Godhe et al. 2013[22]
GC5	Skagerrak Gullmarsfjord, Sweden	2008	sediment	140	Microsatellites S. mar 1-8	Godhe & Härnström 2010[23]
090514C	Skagerrak Gullmarsfjord, Sweden	2009	plankton	120	Microsatellites S. mar 1-8	Godhe & Härnström 2010[23]
St51	Skagerrak, V. Koster, Sweden	2009	sediment	120	Microsatellites S. mar 1-8	Godhe et al. 2013[22]
Oslo AE	Skagerrak, Oslofjord, Norway	2010	sediment	100	Microsatellites S. mar 1-8	
Oslo J	Skagerrak, Oslofjord, Norway	2010	sediment	100	Microsatellites S. mar 1-8	
GF04-11G	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-11I	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
V32	Kattegat, Vinga, Sweden	2009	sediment	120	Microsatellites S.mar 1-8	Godhe et al. 2013[22]
32.27	Kattegat, Mariagerfjord, Denmark	2008	19 year old sediment	140	Microsatellites S.mar 1-8	Härnström et al. 2011[7]
GF05-6J	Skagerrak Gullmarsfjord, Sweden	2005	plankton	190		
GF04-12T	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		

HakAA	Skagerrak, Hakefjord, Sweden	2009	sediment	120	Microsatellites S.mar 1-8	Godhe et al. 2013[22]
GF05-3U	Skagerrak Gullmarsfjord, Sweden	2005	plankton	190		
GF04-10J	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
Lys6S	Skagerrak, V. Lysekil, Sweden	2009	sediment	120	Microsatellites S.mar 1-8	Godhe et al. 2013[22]
GF04-4D	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
V25	Kattegat, Vinga, Sweden	2009	sediment	120	Microsatellites S.mar 1-8	Godhe et al. 2013[22]
32.8	Kattegat, Mariagerfjord, Denmark	2008	19 year old sediment	140	Microsatellites S.mar 1-8	Härnström et al. 2011[7]
090602A	Skagerrak Gullmarsfjord, Sweden	2009	plankton	120	ITS rDNA Genbank: HQ111358.1 Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
090922P	Skagerrak Gullmarsfjord, Sweden	2009	plankton	120	Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
GF04-5C	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-5D	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
V8	Kattegat, Vinga, Sweden	2009	sediment	120	SSU rDNA Genbank: HM236348.1 Microsatellites S.mar 1-8	Godhe et al. 2013[22]
GF04-5A	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
35.15	Kattegat, Mariagerfjord, Denmark	2008	sediment	140	Microsatellites S.mar 1-8	Härnström et al. 2011[7]
St59	Skagerrak, V. Koster, Sweden	2009	sediment	120	Microsatellites S.mar 1-8	Godhe et al. 2013[22]
GF05-6L	Skagerrak Gullmarsfjord, Sweden	2005	plankton	190		
GF04-4E	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-4G	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210	Microsatellite Sequence (S.mar1, S.mar2, S.mar5, S.mar6) Genbank: EU855765.1	Almany et al. 2009[24]

					EU855768.1 EU855774.1 EU855779.1	
GF04-3M	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-3O	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-3J	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210	Microsatellite Sequence (S.mar1,2) Genbank: EU855762.1 EU855767.1	Almany et al. 2009[24]
GF04-4A	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-3P	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-2D	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-1H	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
GF04-2I	Skagerrak Gullmarsfjord, Sweden	2004	plankton	210		
A14	Kattegat, Anholt, Sweden	2009	sediment		Microsatellites S.mar 1-8	Godhe et al. 2013[22]
32.3	Kattegat, Mariagerfjord, Denmark	2008	19 year old sediment		Microsatellites S.mar 1-8	Härnström et al. 2011[7]
0521M	Skagerrak Gullmarsfjord, Sweden	2008	plankton		Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
0602l	Skagerrak Gullmarsfjord, Sweden	2008	plankton		Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
080922P	Skagerrak Gullmarsfjord, Sweden	2008	plankton		Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
A22	Kattegat, Anholt, Sweden	2009	sediment		Microsatellites S.mar 1-8	Godhe et al. 2013[22]
GB3	Skagerrak Gullmarsfjord, Sweden	2008	sediment		Microsatellites S.mar 1-8	Godhe & Härnström 2010[23]
HAK H	Skagerrak, Hakefjord, Sweden	2009	sediment		Microsatellites S.mar 1-8	Godhe et al. 2013[22]
RO5AC	Öresund, Råå, Sweden	2010	sediment		Microsatellites S.mar 1-8	Godhe et al. 2013[22]
RO5Y	Öresund, Råå, Sweden	2010	sediment		Microsatellites S.mar 1-8	Godhe et al. 2013[22]

TABLE 3. PILOT STUDY TESTING EFFICACY OF STANDARD CRYOPRESERVATION PROTOCOLS

Strain designation	CCAP CRC protocols[1]			NCMA /CCMP CRC diatom protocol[1]	CCAP passive-cooler protocol[1]
Method	A	B	C	D	E
Cooling rate[2]	1	5	10	1	1
CCAP 1077/5	+++ (77)	+++ (80)	+++ (85)	+++ (56)	+++ (64)
St54	+++ (39)	+-- (6)	--- (32)	--- (26)	+-- (21)
0603AA	++- (5)	+-- (25)	+-- (14)	+-- (17)	--- (0)

[1]Regrowth (+), or failure to grow (-)/ cells died and lysed, in replicate thawed samples. Number in parenthesis is the mean level of post thaw viability on basis of vital staining and cell appearance 24h after thawing.
[2]Cooling rate (^{o}C min^{-1}) to intermediate holding temperature.

TABLE 4. EFFICACY OF THE CCAP AND BCCM PASSIVE-COOLER CRYOPRESERVATION PROTOCOLS APPLIED TO EXEMPLAR *SKELETONEMA MARINOI* STRAINS

Strain designation	CCAP Passive cooler cryopreservation protocol		BCCM Passive cooler cryopreservation protocol	
	Method E		Method F	
	Recovery of replicate samples[1]	Time req. (days) to grow culture post-thaw[2]	Recovery of replicate samples[1]	Time req. (days) to grow culture post-thaw[2]
St54	+--	40	+++	27-33
GF04-10J	NT		+++	18-25
GB3	+++	42-47	+++	23
A22	---	-	+++	25
32.3	NT		+--	28
A14	NT		+++	31-37
0602I	+++	42-47	+++	25-32
080922P	NT		+++	32-38
HAKH	---	-	+++	20-32
GF04-3A	---	-	NT	
OsloJ	NT		+--	25
GF04-12T	NT		---	-
A14	NT		+++	31-37
32.3	NT		+--	28
0521M	NT		---	-
090922P	NT		++-	32-38
R05AC	---	-	+--	32-43
R05Y	NT		---	-

[1]Regrowth (+), or failure to grow (-), in triplicate thawed samples.
NT - Not Tested