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Extending cryoprotectant strategies from *Phaeocystis antarctica* to *Fragilariopsis kerguelensis*

Submitted by:	Tanja Sedlacek
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Examiner:	Prof. Dr. rer. nat. Stephan Frickenhaus
Co-examiner:	Dr. rer. nat. Steffi Gäbler-Schwarz

I. Abstract

The diatom *Fragilariopsis kerguelensis* and the prymnesiophyte *Phaeocystis antarctica* play an important role in the phytoplankton community in the Southern Ocean and contribute to a great extent to the global primary production. At the Alfred-Wegener-Institute in Bremerhaven Germany both species are kept by means of serial subculturing for research purposes. Because of several disadvantages concerning serial subculturing such as high costs, time, genetic drift and possible contamination, several cryoprotective strategies were tested in this study to cryopreserve these algal species. Cryopreservation is the storage of organic material in liquid Nitrogen (LN_2) at -196°C. Most common fluids such as algal medium and other intracellular fluids in algae form ice when being frozen, which harms the cells during cryopreservation. Therefore special strategies must be applied to ensure that after having thawed the samples the algae will recover and grow. These strategies include the treatment with cryoprotective agents (CPA) before freezing, the freezing and thawing method and the post-cryopreservation treatment. In this study a combination of penetrating (p) and non-penetrating (np) CPA were tested as well as the single application of npCPA when plunging the samples directly into LN_2. After thawing, both algal species showed viability for each of the CPA treatments proved by vital staining. In some cases there was a statistical significant difference in viability between samples treated with only npCPA and those treated with npCPA+pCPA. It was revealed for the colony-forming *P. antarctica* samples and for *F kerguelensis* samples that directly after thawing the differences in mean viability levels were significant between the methyl cellulose (MC) and the fish gelatine (FG) treated samples, the two npCPA. The highest percent numbers of living cells of *F. kerguelensis* were reached when using MC as npCPA. For the colony-forming *P. antarctica* samples the combination of a pCPA with MC led to the highest mean viability values. Whereas for the attached aggregates (AA) *P. antarctica* samples, those treated with a combination of methanol and FG (MeOH+FG) resulted in the highest mean viability values. Two weeks after thawing, in almost all samples viable cells were observed in the vital staining analysis. However four weeks after thawing, no algae growth was recorded. Instead, growth of bacteria especially in all FG treated samples was observed. It was presumed that the main factor, having an impact on recovery and growth, was the behaviour of algae being dependent on seasons. Results of other studies and a self-implemented preliminary study showed good growth despite bacteria when performed at another time than the Antarctic winter season.

II. Zusammenfassung

Die Kieselalge *Fragilariopsis kerguelensis* und die Prymnesiophyte *Phaeocystis antarctica* spielen beide eine wichtige Rolle in der Phytoplankton-Gemeinschaft im Antarktischen Ozean und bei der globalen Primärproduktion. Am Alfred-Wegener-Institut in Bremerhaven werden beide Algenarten durch kontinuierliches Überimpfen der Zellkulturen für Forschungszwecke gehalten. Da diese Methode Nachteile aufweist, wie ein hoher Kosten- und Zeitaufwand und auch die Gefahr birgt die Kulturen zu kontaminieren, wurden in dieser Studie kryoprotektive Strategien getestet um die Algen kryokonserviert zu lagern. Kryokonservierung ist die Aufbewahrung von Proben in flüssigem Stickstoff bei -196°C. Da die Flüssigkeiten wie das Algenmedium und intrazelluläre Algenflüssigkeiten bei diesen Temperaturen zu Eis werden und durch Eiskristallbildung die Zellen geschädigt werden, müssen spezielle Strategien ergriffen werden, damit nach dem Auftauen die Algenproben sich erholen und wachsen. Diese bestehen aus der Anwendung von Kryoprotektiva (CPA) zur Behandlung vor dem Einfrieren, dem Einfrierungs- und Auftauprozess und der weiteren Behandlung. In dieser Studie wurde eine Kombination von penetrierenden (p) und nicht-penetrierenden (np) CPA und die Anwendung von nur npCPA getestet, wenn die Probe direkt in den Flüssigstickstoff gegeben wurde. Direkt nach dem Auftauen zeigte eine Vitalitätsfärbung, dass jede Probe lebende Zellen enthielt. In einigen Fällen wurde ein statistisch signifikanter Unterschied zwischen den Proben, die mit der CPA-Kombination und die nur mit npCPA behandelt worden sind, festgestellt. Für die Proben von *F. kerguelensis* und Kolonie-formende Proben von *P. antarctica* gab es einen signifikanten Unterschied, zwischen der Verwendung von Methylcellulose (MC) und Fischgelatine (FG) als npCPA. Die höchste Prozentanzahl an lebenden *F. kerguelensis* Zellen wurde durch die Behandlung mit MC erreicht, bei den Kolonie-formenden Proben von *P. antarctica* die Kombination eines pCPA+MC. Wohingegen bei *P. antarctica* Proben mit „attached aggregate“ Zellformen die Kombination aus Methanol und FG (MeOH+FG) am erfolgreichsten war. Obwohl eine Vitalitätsfärbung zwei Wochen nach dem Auftauen zeigte, dass fast alle Proben lebende Zellen enthielten, konnte kein Wachstum nach weiteren zwei Wochen festgestellt werden. Stattdessen wuchsen Bakterien. Eine selbst durchgeführte Vorstudie und Ergebnisse anderer Studien haben gezeigt, dass trotz des Vorhandenseins von Bakterien Algen gewachsen sind, wenn die Versuche zu einer Zeit, die nicht dem antarktischen Winter entspricht, durchgeführt wurden. Das saisonabhängige Verhalten der Algen spielt womöglich eine entscheidende Rolle für die Erholungsphase und das Wachstum der Algen nach dem Auftauen.

III. Confirmation

I hereby confirm that this Bachelor's thesis is my own work and that it has been written by me in its entirety.
I also confirm that, to the best of my knowledge and belief, this thesis contains no material by any other person or previously published except where due reference is made in the text.

Name: Tanja Sedlacek

Place and date: Bremerhaven, 30/10/2013 Signature: 

IV. Table of Contents

V. List of abbreviations

AA	Attached aggregates
bp	base pairs
CFDA-SE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CPA	Cryoprotective agents
DMSO	Dimethyl sulfoxide
e-cup	2mL safe-lock tube (Eppendorf, Germany)
Eq.	equation
F/2-Ant	Antarctic sea water based F/2 medium
F/2-bidest	F/2 based on bidestilled water
FG	Fish gelatine
F. kerguelensis	*Fragilariopsis kerguelensis*
GP5-Ant	Antarctic sea water based GP5 medium
GP5-bidest	GP5 based on bidestilled water
LN_2	Liquid nitrogen
MC	Methyl cellulose
MeOH	Methanol
npCPA	Non-penetrating cryoprotective agents
P. antarctica	*Phaeocystis antarctica*
pCPA	Penetrating cryoprotective agents
RT	Room temperature
SO	Southern Ocean
TAE	Tris-acetate-EDTA
wb-bacteria	bacteria from the waterbath

1. Introduction

The diatom *Fragilariopsis kerguelensis* and the prymnesiophyte *Phaeocystis antarctica* belong to the phytoplankton community in the Southern Ocean and play both an important role in this ecosystem. Due to this, both polar micro algae are investigated extensively within the scientific community. For the testing of several experimental parameters and for distinguishing their genetic potential, algal culture collections of these species are needed. Several disadvantages can be the downfall within culture collections, high costs, time, genetic drift and possible contamination during transfer, when maintaining them by means of serial subculturing.

In this study cryoprotective strategies from Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*) applied to *P. antarctica* were adopted, modified and further tested for the cryopreservation of *F. kerguelensis* and *P. antarctica*.

Cryopreservation is the ultra-low temperature storage of organic material in liquid Nitrogen (LN_2) at -196°C. Two different methods exist before plunging the sample into LN_2: 1) the traditional 2-step controlled rate freezing in computer programmable freezers and 2) the vitrification method with plunging the sample directly into LN_2. In both cases cryoprotective strategies must be applied to prevent and minimize the impact of injuries due to ice crystal formation and dehydration being described as the "two-factor hypothesis of freezing injury" by Mazur et al. (1972). Cryoinjury is for example prevented or reduced by realising the prime objective of achieving vitrification. Hereby the cell viscosity is increased to a critical point using different options: "1. Evaporative desiccation by air-drying (e.g. still or laminar flow, 2. Chemical desiccants (silica gel), 3. Osmotic dehydration (e.g. high concentrations of sugars), 4. Alginate encapsulation/dehydration, 5. Chemical cryoprotectants (penetrating and non-penetrating)" (SAMS 2012).

"Under these high viscosity conditions water forms an amorphous, non-crystalline glassy state" (Harding et al 2004). Which results in no more ice nucleation because the movement of the water molecules is restricted due to the high intracellular viscosity and the mechanical and physical properties resembling those of the solid state are reached.

Cryoprotectants and cryoinjury might lead to an change in the DNA structure. A common method for assessing genetic stability is "Amplified Fragment Length Polymorphism" (AFLP) described by Vos et al. (1995) (Gäbler et al 2007, Müller et al. 2007). AFLP "is based on the

selective PCR amplification of restriction fragments from a total digest of genomic DNA" (Vos et al. 1995). One important advantage of this DNA fingerprinting technique is that no foreknowledge of the DNA sequence is needed. The first step is the restriction of the whole genomic DNA using a rare and a frequent enzyme cutter followed by ligation with enzyme-specific oligonucleotides. After preselective PCR amplification, enzyme-specific primers with selective nucleotides are used for the selective PCR amplification. So only a part of the restriction sites will be amplified but results can be compared regarding the presence of fragments with the same base pairs (bp)-length.

Suitable DNA is pure, integer and high in molecular weight (Müller et al. 2007, John et al. 2004). Contamination caused by bacteria or other foreign DNA should be avoided to ensure comparable and reliable results.

P. antarctica, one of the tested organisms in this study, belongs to the genus *Phaeocystis*. Six different *Phaeocystis* species are reviewed in Medlin & Zingone (2007) and Rousseau et al. (2007): *P. jahnii* and *P. cordata*, *P. globosa*, *P. pouchetii*, *P. antarctica* and *P. scrobiculata*. Genetic analyses have been done and revealed phylogenetic relationships within the various species. Based on these results, it is suggested that further *Phaeocystis* species exist (Medlin & Zingone 2007, Lange et al. 2002). *P. antarctica* is one of the colony-forming species (Verity & Medlin 2003). It dominates besides diatoms the phytoplankton community in the Southern Ocean (SO) (Arrigo et al. 1999). The morphological characterization is described in Zingone et al. (2011): colonies being generally spherical with diameters up to 2000µm consist of single cells or single cells and flagellates. Single cells and flagellates also occur outside colonies. The diploid or haploid flagellates are motile with two unequal flagella and one haptonema. A further cell stage, the attached aggregates (AA) is assumed to be either a zygote suggesting a sexual cycle within the life cycle or an overwintering stage (Gäbler-Schwarz et al. 2010).

Morphotypes of *P. antarctica* may vary in response to the physical appearance of ice, availability of nutrients and irradiance (Kennedy et al. 2012, Van Leeuwe & Stefels 2007). An alteration of morphotypes after cryopreservation was also observed (Gäbler-Schwarz et al. 2013, accepted in *Cryoletters*).

The endemic diatom species to the SO with the highest abundance is *Fragilariopsis kerguelensis*. Thus this pennate diatom species plays a significant role in the global silicon cycle and also contributes to the global primary productivity (Zielinski & Gersonde 1997).

Reproduction happens through cell division or sexual. In the process of cell division the mean size within the population is decreased because “one sibling cell uses the epitheca of the parent cell as a size guide to generate a new hypotheca, whereas the other uses the parental hypotheca, which therefore becomes the epitheca of the daughter cell” (Zurzolo & Bowler 2001). The epitheca is one half of the silica cell wall (known as frustule) overlapping the other smaller half the hypotheca. The reproduction by sexual events does not decrease the mean cell size. The size is restored by forming an auxospore. This cell stage is also reported for *F. kerguelensis* in Assmy et al. 2006.

Both species are maintained by serial subculturing at the Alfred Wegener Institute in Bremerhaven, Germany. Since they are of great interest regarding research about their genetics, complex life cycle and their ecology, cultures need to be maintained with another method than serial subculturing. The corresponding disadvantages mentioned above seem to be reduced by applying the method of cryopreservation. Additionally, access to the strains for a wider scientific community would also be simplified.

Results of cryopreservation experiments already performed with algae suggest that there is the need to empirically develop cryopreservation protocols applicable only to the examined organism/species of interest because some of them might be cryo-recalcitrant (Rhodes et al. 2006, Harding et al. 2004).

As mentioned above, selected cryoprotective strategies for *P. antarctica* have successfully been implemented, using a programmable freezer and viable cells 48h after thawing have been reported (Gäbler-Schwarz et al. 2013, accepted in *Cryoletters*). In this study these strategies will be tested for both species of interest, *P. antarctica* and *F. kerguelensis*, with plunging the sample directly into LN_2 and also new strategies will be investigated (see 2.2.1 Cryoprotective agents (CPA)).

One objective of this thesis is to find a combination of non-penetrating CPA (npCPA) and penetrating CPA (pCPA) or single application of npCPA which results in high viability rates after thawing, as well as recovery of cells post-cryo after a few weeks of growth time. Another objective is to further investigate the single application of npCPA for both species.

Preliminary results for *P. antarctica* (Gäbler-Schwarz et al. 2013, accepted in *Cryoletters*) have already shown that there was high viability of cells when plunging the sample into LN_2 treated with only a single npCPA.

For *F. kerguelensis* no tests have been made yet and the samples in contrast to *P. antarctica* have not been examined whether the applied pCPA have a toxic effect or not. The use of npCPA and pCPA might lead to higher viability rates because extra and intra cellular ice crystals are prevented. However pCPA could also have a toxic effect and cells exposed to these chemicals after cryopreservation might not be able to recover.

So it will be tested, whether there is a difference in viability of algal cells between the treatments npCPA with pCPA or single application of a npCPA.

2. Material and Methods

All pictures of algal cultures of section 3. Results were taken with the Axio Observer D1 microscope (Zeiss, Germany) with AxioCamMR3.

All work steps were performed on ice under a laminar flow clean bench (Schulz Lufttechnik, Germany) while working with living algae.

2.1. Algae cultures and their corresponding medium

One diatom species *F. kerguelensis* and eight genetically different *P. antarctica* strains were investigated (see Table 1). Cultures were maintained in Corning® flasks (Corning, NY, USA) filled with the corresponding medium. The prepared media were based on Antarctic seawater, which was taken on site in regions being poor in plankton in 2011. Surface water from 61°31.11 south and 64°30.42 west to 62°10.22 south and 65°27.65 west was filled in plastic tanks and kept in a cool and dark place for at least four to six months. The storage time should ensure that there was a stable nutrition and salt concentration and no more biological activity.

Six 150cm² rectangular canted neck cell culture flasks with vent caps filled with 400mL Antarctic sea water based F/2 medium (F/2-Ant) were used for *F. kerguelensis*. See annex a) for preparation of F/2-Ant medium and ingredients based on Guillard & Ryther (1962).

F. kerguelensis was kept in a 3°C storage room under a 16h/8h light/dark – regime. Eight 75cm² rectangular canted neck cell culture flasks with plug seal caps filled with 200mL Antarctic sea water based GP5 medium (GP5-Ant) were used for each *P. antarctica* strain. See annex b) for preparation of GP5-Ant and ingredients based on Loeblich and Smith (1968,

p. 7). *P. antarctica* was kept in a 1°C storage room under a 12h/12h light/dark – regime. Two weeks prior to the freezing procedure the *P. antarctica* cultures were transferred to the 3°C storage room to increase growth.

The cultures were inoculated (2mL algae culture) with sterile disposable pasteur pipettes (Roth, Germany) to new sterile flasks (75cm²) filled with 4°C corresponding medium to have enough cell material for DNA extraction and maintaining purposes. The subculturing was performed on ice under a laminar flow clean bench (Schulz Lufttechnik, Germany).

Table 1: Overview of investigated algal cultures for the experiment.

Culture name or identification number for *P. antarctica*	**origin**	**latitude / longitude [°]**	**morphotype**
F. kerguelensis	Antarctic ocean	not applicable	long chains of cells
#25	Ross Sea	-76.09000 / -170.13000	colonial cells and colonies
69/109-2	Lazarev Sea	-62.51300 / -5.99300	
69/875-86	Prydz Bay	-65.25850 / 82.83233	
69/905-100	Indian Ocean	-56.16017 / 76.44267	
VIMS 119-1	McMurdo	-77.839977 / 166.67557	flagellates and attached aggregates
69/71-5	Lazarev Sea	-64.00220 / -0.00400	
69/82-10	Lazarev Sea	-69.4984 / -0.03	
69/770-50	Riiser-Larsen Sea	-68.01483 / 23.7705	

2.2. Cryopreservation

2.2.1. Cryoprotective agents (CPA)

Two penetrating cryoprotective agents (pCPA) – methanol (MeOH) and dimethyl sulfoxide (DMSO) – and two non-penetrating ones (npCPA) – fish gelatine (FG) and methyl cellulose (MC) – were used.

Concentration, types and combination of CPA are based on and modified after Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*). See Table 2 for an overview of adopted CPA combinations as well as new CPA applications.

All CPA solutions were prepared in sterile autoclaved DURAN® laboratory glass bottles (Duran, Germany).

Tests regarding the toxicity of pCPA were done for one hour and 24h incubation in Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*) for *P. antarctica*. These tests were not repeated to ensure applicability and so far no tests have been performed for *F. kerguelensis.*

CPA preparation for *P. antarctica*

20% (v/v) stock solutions of DMSO (Sigma-Aldrich, Germany) and MeOH (Merck, Germany) were prepared by adding 20mL each to 80mL of GP5-Ant. They were stored in a 4°C fridge. 160g of dried FG powder (lot 08205KD, FIB Foods, Netherlands) were dissolved in about 300mL of GP5-Ant by adding the powder little by little under continuous stirring within one day. The solution was topped up to 400mL with GP5-Ant to get a 40% (w/v) FG solution and sterile-filtered using a 500mL Stericup® Filter Unit (Millipore, MA, USA). The FG was stored at room temperature (RT). For the second npCPA 18g of MC powder (Sigma-Aldrich, Germany) were dissolved in 500ml of GP5 based on bidestilled water (GP5-bidest) in the same way as dissolving the FG powder and topped up to 600mL with GP5-bidest to get a 3% (w/v) MC solution. To salt up the MC solution, 12g of HW Meersalz (Wiegandt, Germany) were added little by little within two days and dissolved. Then the solution was autoclaved and stored at RT.

CPA preparation for *F. kerguelensis*

The CPA solutions were prepared in the same way as those for *P. antarctica* with the modification of using F/2-Ant and F/2-bidest instead of GP5-Ant and GP5-bidest. 50mL each of 20% (v/v) stock solutions of DMSO and MeOH, 100mL of 40% (w/v) FG solution and 100mL 3% (w/v) MC solution were prepared.

2.2.2. Cryopreservation procedure

All tubes and flasks filled with algae culture were kept on ice under a laminar flow clean bench. An Eppendorf-Centrifuge 5810 R (Eppendorf, Germany) was cooled down to 4°C. 45mL of one algae culture was filled into a 50mL centrifuge tube (Sarstedt, Germany) and centrifuged at 3500rpm for 1min. 40mL of supernatant was carefully discarded, algae culture was added and the steps repeated until the Corning® flask was empty. The remaining 5mL of high cell density algae culture were used for one cryopreservation procedure.

2.2.2.1. Pre-treatments

See Table 2 for an overview of the treatments performed with which algae cultures. For each treatment six cryovials per culture were filled with a combination of pCPA and npCPA or only npCPA.

pCPA and npCPA combined treatment:

5mL of 20% (v/v) DMSO or 20% (v/v) MeOH were added to the 5mL high cell density algae culture and incubated on ice for 2 hours. 10mL of 3% (w/v) MC or 40% (w/v) FG were added using 10mL serological pipette (Sarstedt, Germany) and the solution was mixed by inverting several times. Samples were labelled as DMSO+FG or MC and MeOH+FG or MC.

npCPA treatment:

5mL of 3% (w/v) MC or 40% (w/v) FG were added to the 5mL high cell density algae culture and mixed by inverting several times. The npCPA treatment was done twice. Once the samples labelled as FG and MC were directly plunged into LN_2 after adding the npCPA. The second method was to store the sample labelled as FG-s or MC-s at the 0°C room for a few days (see Table 2 for storage time).

2.2.2.2. Freezing

For freezing in LN_2 1mL of the algae with CPA mixture was pipetted into CryoPure 2.0mL tubes with external thread (Sarstedt, Germany). The vials were put in a small Dewar vessel (Roth, Germany) half-full with LN_2 by using long forceps. The vials stayed in LN_2 for at least one hour.

2.2.2.3. Thawing

Prior usage a water bath SW1-VL (Julabo, Germany) was cleaned with 70% (v/v) ethanol and wiped again with Ethanol 99 % denatured with 1 % MEK p. A. (AppliChem, Germany). Deionised water was poured inside and heated up to 40°C.

Six vials at a time were taken out of the LN_2 using long forceps. They were put into a polystyrene sheet with holes so that the external thread with cap did not come into contact with the water and were then rewarmed in the water bath. As soon as all visible ice crystals in the vials had melted they were taken out, wiped with 70% (v/v) ethanol and put on ice.

2.2.2.4. Post-treatment

The thawed vials were wiped with 70% (v/v) ethanol a second time before putting them on ice under the laminar flow clean bench. 1mL medium was pipetted into the cryo vial then the solution was transferred into the remaining 1mL medium in the well plate. A 5mL serological pipette (Sarstedt, Germany) was used. So the samples were transferred into a 12-well plate (BD Falcon, USA); each well filled with 2mL GP5-Ant or GP5-F/2 in total.

One of the six samples per culture was used directly after thawing for microscopy. The lid of the well plate was secured with Parafilm and then the plate was wrapped in aluminium foil to ensure darkness for the samples and stored in the 1°C room. For *F. kerguelensis* the plates were stored in the 3°C room. After 72 hours the plates were unwrapped but not directly exposed to light. After two weeks the content of each well was inoculated into 25cm² rectangular canted neck cell culture flasks with plug seal screw caps filled with 40mL of the corresponding medium. 1mL each out of two wells per culture was taken for microscopy.

For *P. antarctica* two flasks for each treatment were stored in the 1°C room and three flasks in the 3°C room. For *F. kerguelensis* all five flasks were stored in the 3°C room.

Every two days the flasks were gently shaken to mix the content.

2.3. Viability analysis by light and fluorescence microscopy

For fluorescence microscopy an Axio Observer D1 microscope (Zeiss, Germany) with AxioCamMR3 and Filter Set 34 (DAPI/BFP) was used. The samples were stained with a 5mM stock solution of 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE) (Sigma-Aldrich, Germany) dissolved in DMSO. 2.8mg of CFDA-SE were dissolved in 1mL DMSO and stored in a dark box in a 4°C fridge.

CFDA-SE is highly membrane permeant but only fluorescences in viable cells by active enzymes removing the two acetate groups and turning the CFDA-SE into carboxyfluorescein succinimidyl ester (CFSE) (Parish 1999). Viable cells fluoresced green, dead ones appeared red due to the chloroplasts' own fluorescence.

P. antarctica triplicates each consisting of three times 10µL samples were analysed by counting living and dead single cells, flagellates and single floating AA at 20-fold magnification. For the colonies and clusters of AA, viability was analysed at 10-fold magnification by counting the number and by dividing them up into large, middle and small sizes and 0%, 25%, 50%, 75% and 100% of green fluorescence parts of the colonies and clusters of AA.

F. kerguelensis triplicates each consisting of three times 10µL samples were analysed by counting living and dead cells.

For each 10µL sample the percentage of living cells from the total number was calculated and the mean value with its standard deviation for each triplicate was determined using the following equations (Eq.):

$$\bar{x} = \frac{\sum_i x_i}{n} \qquad \text{Eq. (1)}$$

$\bar{x} = mean\ value$

$n = number\ of\ samples\ (in\ this\ case\ n = 3, unless\ otherwhise\ stated)$

$$s = \sqrt{\frac{\sum_i (x_i - \bar{x})^2}{n-1}} \qquad \text{Eq. (2)}$$

$s = empirical\ standard\ deviation$

$$\bar{s} = t * \frac{s}{\sqrt{n}} \qquad \text{Eq. (3)}$$

$t = student\ factor\ from\ the\ Student's\ t - ditribution$

$confidence\ level\ P = 95\%\ and\ n = number\ of\ samples = degrees\ of\ freedom$

$for\ n = 3{:}\ \ t = 2{,}353\ and\ for\ n = 2{:}\ \ t = 2{,}920$

For the number of colonies and AA the calculation was done in the same way as for the cells whereby viability levels of 0% were counted as dead.

The viability of colonies for each 10µL sample was determined by multiplying the number of colonies with the respective percentage of green fluorescence then adding these up and dividing it by the total number of colonies. With the result for each sample the mean viability percentage and its standard deviation was determined using Eq. (1) to Eq. (3). The same calculation was done with the cultures containing AA.

The software R (R Core Team 2012) was used to visualise and evaluate the results statistically.

Therefore the percent data for AA, colonies and cells of each 10µL sample were used calculated by using Eq. (1). The level of significance was set as α=5%.

Outliers were determined and eliminated by having a look at the boxplots.

The results were tested for deviations from normal distribution (Shapiro-Wilk test) and for homogeneity of variances (Bartlett test and F-test) before they were analysed using the following tests: two-way ANOVA, Tukey's HSD post-hoc test, Welch two sample t-test or Kruskal-Wallis test. In case of significant differences between variances or deviation from the normal distribution results were summarised or arcsine-sqrt transformed.

Counting-analyses were performed directly after thawing and with the post-cryo samples after two weeks.

Directly after thawing the content of one vial per treatment per culture was transferred into a 15mL centrifugation tube (Sarstedt, Germany) filled with 5mL corresponding medium. The tube was shortly centrifuged at 3500rpm. The lower part of the sample was transferred to a 2mL safe-lock tube (Eppendorf, Germany) (e-cup). After 10min sedimentation time 27µL from the bottom were transferred to a 0.2mL PCR tube (Eppendorf, Germany). 3µL of 5mM CFDA-SE in DMSO were added and mixed by stirring with the tip of the pipette. The samples were wrapped in aluminium foil and incubated on ice for 30min.

Two weeks after the day of freezing, samples for microscopy were taken again. 1mL each out of two wells per treatment per culture was pipetted into an e-cup and after 10min sedimentation time 9µL from one tube and 18µL from the other tube from the bottom were transferred to 0.2mL PCR tubes (Eppendorf, Germany). To 9µL sample 1µL of 5mM CFDA-SE in DMSO was added and to 18µL sample 2µL 5mM CFDA-SE in DMSO were added. The samples were wrapped in aluminium foil and incubated on ice for 30min.

Four weeks after the day of freezing, each 25cm² culture flask was observed using Axiovert 25 Inverted Microscope (Zeiss, Germany).

Table 2: Overview about the performed treatments*

	DMSO+ FG	DMSO+ MC	MeOH+ FG	MeOH+ MC	FG	MC	FG-s	MC-s
F. kerguelensis	4wk	4wk	4wk	4wk	-	4wk	4wk (13d)	-
#25	4wk	3wk	4wk	3wk 1d	4wk	3wk	4wk 5d (6d)	4wk (6d)
69/109-2	4wk 2d	4wk 2d	3wk 1d	3wk 1d	3wk 4d	4wk 5d	3wk 4d (6d)	5wk 1d (6d)
69/875-86	2wk 6d	4wk 1d	2wk 6d	4wk	2wk 6d	3wk	4wk 6d (6d)	5wk 1d (6d)
69/905-100	5wk	3wk 6d	5wk	4wk 6d	3wk 5d	3wk 5d	3wk 5d (7d)	5wk 5d (6d)
VIMS 119-1	4wk 1d	5wk 1d	5wk 2d	4wk	3wk 6d	5wk	5wk 5d (14d)	4wk 5d (6d)
69/71-5	5wk 1d	4wk 1d	5wk 2d	4wk 1d	4wk 5d	5wk 2d	-	5wk 6d (5d)
69/82-10	-	-	-	-	4wk 6d	4wk 6d	4wk 6d (7d)	5wk 5d (6d)
69/770-50	8wk 1d	-	8wk 1d	-	8wk 1d	-	-	-

Abbreviations used in this table: wk: weeks d: days

The weeks and days indicate the growth time from the latest inoculation until the date of freezing including the storage time prior to freezing given in brackets for the FG-s and MC-s treatments.

- sign: the treatment was not performed with this algae culture

*: The treatments in the first five columns were adopted from Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*). MC, FG-s and MC-s are newly tested treatments.

2.4. Antibiotic treatment

Algae cultures were treated with antibiotics to obtain axenic cultures.

The algae cultures were treated with a combination of three antibiotics. Penicillin G sodium salt and Streptomycin sulphate salt (Sigma-Aldrich, Germany) were each dissolved in bidestilled water to obtain a stock solution of 100mg/mL for Penicillin and a stock solution of 25mg/mL for Streptomycin.

The solutions were sterile-filtered using Nalgene ™ Syringe Filters (Thermo Scientific, Germany) and stored in 50mL centrifuge tubes (Sarstedt, Germany) at -20°C.

1mL each of Penicillin and Streptomycin were pipetted to 200mL algae culture. 100µL of Ciprofloxacin Hydrochloride 10mg/ML (USBiological, MA, USA) were added.

The treatment was performed five times including a one week break in between the treatments.

2.5. DNA extraction

One flask per strain was harvested according to the harvesting protocol (see annex c)) from Gäbler-Schwarz (2009) to get one DNA sample. Harvesting and DNA extraction was performed several times to obtain high molecular weight DNA suitable for AFLP.

Since previous DNA extraction results were not satisfying regarding high molecular weight DNA two different kits for extraction were used: NucleoSpin® Plant II (Macherei-Nagel, Germany) and E.Z.N.A.® SP Plant DNA Kit (Omega Bio-Tek, GA, USA).

Steps of the Genomic DNA from plant protocol (NucleoSpin® Plant II) were performed with the following modifications: Instead of the steps 1, 2a and 2b 400µL of buffer PL1 were added into the 15mL centrifugation tube containing the harvested algae. After adding 10µL of RNase A the tube was inverted a few times. The samples were incubated at 65°C for 10min in a thermo block (Dri-Block DB-1, Techne, UK) and inverted every 2min. The sample was transferred to a 2mL e-cup and centrifuged for 5min 11000xg at RT.

The sample was vortexed in step 4. At step 7 instead of incubating at 65°C the sample was incubated at RT for 10min. A new tube was used for the second elution. Here the incubation time at RT was 20min.

The steps of the SP Plant DNA Fresh/Frozen Sample protocol (E.Z.N.A.® SP Plant DNA Kit) were performed with the following modifications:

All centrifugation steps were performed with 13000xg.

Step 1: Buffer SP1 was preheated to 37°C. Instead of using the provided RNase, 5µL of 100mg/mL RNase A from Qiagen (Germany) was used. The sample was mixed by inverting.

Before step 8 was performed 100µL of Equilibration Buffer was pipetted onto a HiBind DNA Mini Column placed into a 2mL collection tube. It was centrifuged at 13000xg for 2min.

Step 13: 50µL of Elution Buffer were applied. The sample was incubated at RT for 10min.

Step 14: A new tube was used. 50µL of Elution Buffer were applied. The sample was incubated at RT for 20min.

2.6. Analysing DNA quality and quantity

The concentration of nucleic acid was measured with the Thermo Scientific NanoDrop™ 1000 spectrophotometer (DE, USA). For the blanc-measurement the corresponding elution buffer was applied. 2µL of the sample were used.

The quality whether high molecular weight DNA fragments were isolated was checked by running a gel electrophorese on a 0.8% (w/v) agarose gel at 80V for 40min. Depending on the number of isolated DNA samples 40mL of gel or 80mL of gel were prepared. 0.32g of agarose (AppliChem, Germany) (respectively 0.64g) were dissolved in 1x TAE (Tris-acetate-EDTA) buffer (800mL of 50x TAE provided stock solution: 193,6g Tris, 200mL 0,2M Na2-EDTA pH=8) by microwaving at 700W for one minute with a few interruptions for swirling the solution. 5µL of GelRed Nucleic Acid Stain (10,000x in water, Biotium, Ca, USA) per 100mL gel were added to the handwarm solution. After solidification the gel was loaded with 2µL of DNA sample mixed with 6x DNA loading dye (Thermo Scientific, Germany). DNA-Sizer III (peqlab, Germany) was used for reference by which the lengths of DNA strands were estimated.

For gel documentation a UV-imager V03 from Vilber Lourmat (France) was used.

2.7. Amplified fragment length polymorphism

AFLP was performed twice with DNA from one extraction and a third time from another DNA extraction to ensure reliable results. For AFLP materials and chemicals including but not limited to the ones in Table 3 were used.

The 0.2mL tubes were used for the processes in a thermocycler (Mastercycler gradient, Eppendorf, Germany). The 1.5mL tubes were used for preparing the mastermix and for the samples in step 3 when adding TE buffer.

All ingredients and the tubes to be used were kept on ice.

DNA samples were diluted with deionised water if applicable to reach a nucleic acid concentration between 150ng/µL to 300ng/µL in a total volume of 30µL DNA/deionised water solution.

AFLP was performed after the following protocol modified after Vos et al. (1995) and Zabeau & Vos (1993). The mastermix preparation in each step is described for one sample.

Table 3: Part of the materials and chemicals used for AFLP

materials and chemicals	producer
0.2mL PCR tube	Eppendorf, Germany
1.5mL safe-lock tube	
dNTP (1mM)	
Taq polymerase (5U/µL)	
restriction buffer: NEBuffer 2 B7002S	New England Biolabs, MA, USA
BSA 1:10 (diluted from Purified BSA 100x 10mg/ml)	
MseI enzyme (10,000U/mL)	
EcoRI enzyme (20,000U/mL)	
T4 DNA Ligase Reaction Buffer	
T4 DNA Ligase (400,000U/mL)	
MseI primer and EcoRI primer	MWG-Biotech AG, Germany
10xPCR buffer	Applied Biosystems, Roche, Germany
Rox (Gene Scan 500 Rox Size Standard)	Applied Biosystems, UK
Hi-Di Formamide	

1. Restriction digest:

20µL of mastermix (1) (see Table 4) was added to 30µL of the DNA/deionised water solution. The sample was incubated in the thermocycler using the programme in

Table 5.

2. Ligation of adapter:

17µL of the digestion mix were added to 8µL of mastermix (2) (see Table 6) in a new tube. After applying the programme see Table 7, the ligation mix was diluted with 100µL of 0.1xTE buffer and stored at -20°C until further use.

3. Preselective Amplification:

10µL of the ligation mix were added to 20µL of mastermix (3) (see Table 8) in a new tube. After applying the programme see Table 9, a 1% (w/v) Agarose gel was run at 80V for 45min to check the results. peqGOLD 100bp DNA ladder (peqlab, Germany) was used for reference. 5µL of the preselective amplification mix were loaded onto the gel. The remaining 25µL were pipetted into 1.5mL tubes containing 225µL of 0.1xTE buffer and stored at -20°C until further use.

4. Selective amplification:

5µL of the preselective amplification mix were added to 8µL of mastermix (4) (see Table 10) in a new tube. The EcoRI+A* primer was marked with 6 FAM fluorescent dye.

After applying the programme see Table 11, a 1.5% (w/v) Agarose gel was run at 80V for 45min to check the results. peqGOLD 100bp DNA ladder (peqlab, Germany) was used for reference 5µL of the preselective amplification mix were loaded onto the gel. The remaining 15µL of the sample were stored in an aluminium foil wrapped box at -20°C

5. Preparation for the fragment analysis and evaluation of the results:

15.3µL of Mastermix (5) consisting of 0.3µL Rox and 15µL Hi-Di Formamide were added to 1µL preselective amplification mix in a MicroAmp^{R} optical 96-well plate (Applied Biosystems by life technologies, China). A 3130x Genetic Analyser (Applied Biosystems Hitachi, Germany) was used and the results were analysed using GeneMapper^{R} (Applied Biosystems, Germany). From 50 base pairs to 500 base pairs the results were transferred to 1 when there was a measured signal and to 0 when there was no measured signal.

For each base pair the fragment existed in the sample when the result of the triplicate was bigger or equal to 2.

Table 4: Components of mastermix (1) for AFLP - restriction digest

chemical	volume in µL
restriction buffer	5
BSA 1:10	5
MseI enzyme	1
EcoRI enzyme	1
deionised water	8

Table 5: Thermocycler programme for AFLP – restriction digest

lid temperature on	110°C			
loop start				
	step 1:	37°C	5min	repeat
	step 2:	37°C	5min	96 times
loop end				
keep sample at	65°C		10min	
lid temperature off				
keep sample at	4°C			

Table 6: Components of mastermix (2) for AFLP – ligation of adapter

chemical	volume in µL
deionised water	2.5
ATP (10mM)	1.7
T4 DNA Ligase Reaction Buffer	0.8
MseI adapter (50µM)	1.0
EcoRI adapter (5µM)	1.0
T4 DNA Ligase	1.0

Table 7: Thermocycler programme for AFLP – ligation of adapter

lid temperature on	110°C			
loop start				
	step 1:	24°C	5min	repeat
	step 2:	24°C	5min	42 times
loop end				
keep sample at	65°C		10min	
lid temperature off				
keep sample at	4°C			

Table 8: Components of mastermix (3) for AFLP – preselective amplification

chemical	volume in µL
deionised water	12.5
10xPCR buffer	3.0
MseI+0 primer (10pmol/µL)	0.5
EcoRI+0 primer (10pmol/µL)	0.5
dNTP's (1.00mM)	3.0
Taq polymerase	0.5

Table 9: Thermocycler programme for AFLP – preselective amplification

lid temperature on	110°C			
loop start				
	step 1:	94°C	30sec	repeat
	step 2:	56°C	30sec	35 times
	step 3:	72°C	1min	
loop end				
keep sample at	4°C			

Table 10: Components of mastermix (4) for AFLP – selective amplification

chemical	volume in µL
deionised water	9.9
10xPCR buffer	2.0
dNTP's (1.00mM)	2.0
MseI+C primer (10pmol/µL)	0.1
EcoRI+A* primer (10pmol/µL)	0.6
Taq polymerase	0.4

Table 11: Thermocycler programme for AFLP – selective amplification

lid temperature on	110°C			
keep sample at	94°C		30sec	
first loop start				
	step 1:	94°C	30sec	repeat 12 times
	step 2:	65°C	30sec	
	step 3:	72°C	1min	
first loop end				
second loop start				
	step 1:	94°C	30sec	repeat 23 times
	step 2:	56°C	30sec	
	step 3:	72°C	1min	
second loop end				
keep sample at	72°C		10min	
keep sample at	4°C			

3. Results

3.1. Cryopreservation

In general all samples examined with vital staining directly after thawing, had accordingly to their species viable cells, colonies or AA (see Table 12 and annex d)).

Two weeks after the day of thawing in most of the samples selected for vital staining green fluorescent cells were observed. Green pigmentation of *P. antarctica* samples was visible to the naked eye. Despite that there were still viable cells, four to six weeks old post-cryo samples had no algae growth and no more green pigmented *P. antarctica* algae were visible.

3.1.1. *Fragilariopsis kerguelensis*

Directly after thawing the samples used for microscopy and treated with CFDA-SE showed green fluorescent cells for all cryoprotective strategies applied to *F. kerguelensis*. The analyses of the cell counting demonstrated that the treatments with MC resulted in higher viability levels than those with FG (see Table 12). A two-way ANOVA showed that there was a significant effect whether MC or FG was applied ($p=1.07e-08 < \alpha$) and no significant effects of the application of DMSO, MeOH and only npCPA ($p=0.0617 > \alpha$) nor an interactive effect ($p=0.5162 > \alpha$). The mean viability values for samples treated with FG were significantly lower than the ones treated with MC (Welch two sample t-test, one sided: $p= 3.802e-08 < \alpha$). In both cases where only the npCPA was used the highest percentage of living cells was observed compared to the results of the same npCPA+pCPA (see Table 12). Results within one group (all MC treated samples or FG respectively) were analysed and no significant difference was found between the mean percent number of living cells treated with DMSO, MeOH or only npCPA (one-way ANOVA: $p_{FG}=0.198$, $p_{MC}=0.217 > \alpha$)

After two weeks a significant difference whether MC or FG was applied with an interactive effect was found (two-way ANOVA: $p_{MC,\ FG} = 0.00537$ and $p_{interactive}=0.01089 < \alpha$). The interactive effect can be seen on basis of comparing the boxplots in Figure 1. This time the mean viability values for samples treated with FG were significantly greater than the ones treated with MC (Welch two sample t-test: one sided $p= 0.0093 < \alpha$). Furthermore two weeks old samples used for vital staining treated with only MC and pCPA+MC had a decrease in viability whereas an increase in viability in the samples with only FG and pCPA+FG was recorded (see Table 12).

Summarising the two-weeks-results of samples treated with DMSO+FG and MeOH+FG they had significantly lower viability mean values than those treated with only FG, whereas summarised results of treatments with DMSO+MC and MeOH+MC had a significantly higher viability than with only MC (Welch two sample t-test, one sided: p_{FG}= 0.03375 < α, p_{MC}= 0.01922< α).

In week four after thawing selected samples for vital staining treated with MeOH+FG and FG had viable cells (see Figure 2). Whereas in samples with MC as npCPA, cells neither had green fluorescence nor the red fluorescence from the chloroplasts (see Figure 3). Under the light microscope they didn't show any green pigmentation anymore and the inside of the cell looked empty (see Figure 3). Figure 4 shows healthy looking cells of a sample not used for cryopreservation. It was noted that the fluorescence colouring in Figure 4 on the right side was evenly spread inside the cells. This was not the case for all cells in post-cryo samples (see Figure 2).

Table 12: Post-cryo viability of *F. kerguelensis*: percent mean value (living cells from total number of cells) with its standard deviation (after Eq. (1) to (3))

	DMSO+FG	MeOH+FG	FG-s	DMSO+MC	MeOH+MC	MC
after thawing	25±15%	30±7%	39±11%	71±1%	75±9%	77±3%
after two weeks	57±5%	57±14%	79±16%	59±28%	45±13%	34±5%

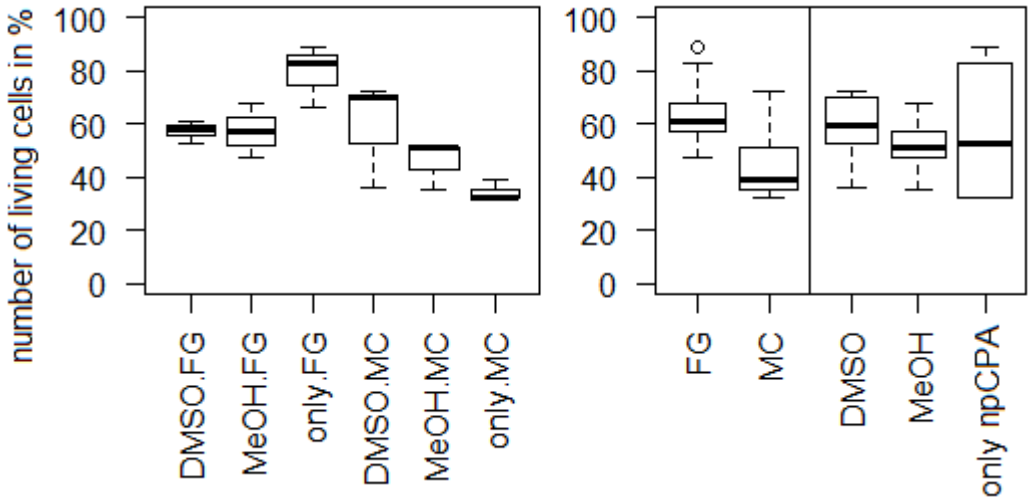


Figure 1: Boxplot of viability results two weeks after thawing for *F. kerguelensis*
Left: results per treatment. Right: results summarised into FG and MC group.

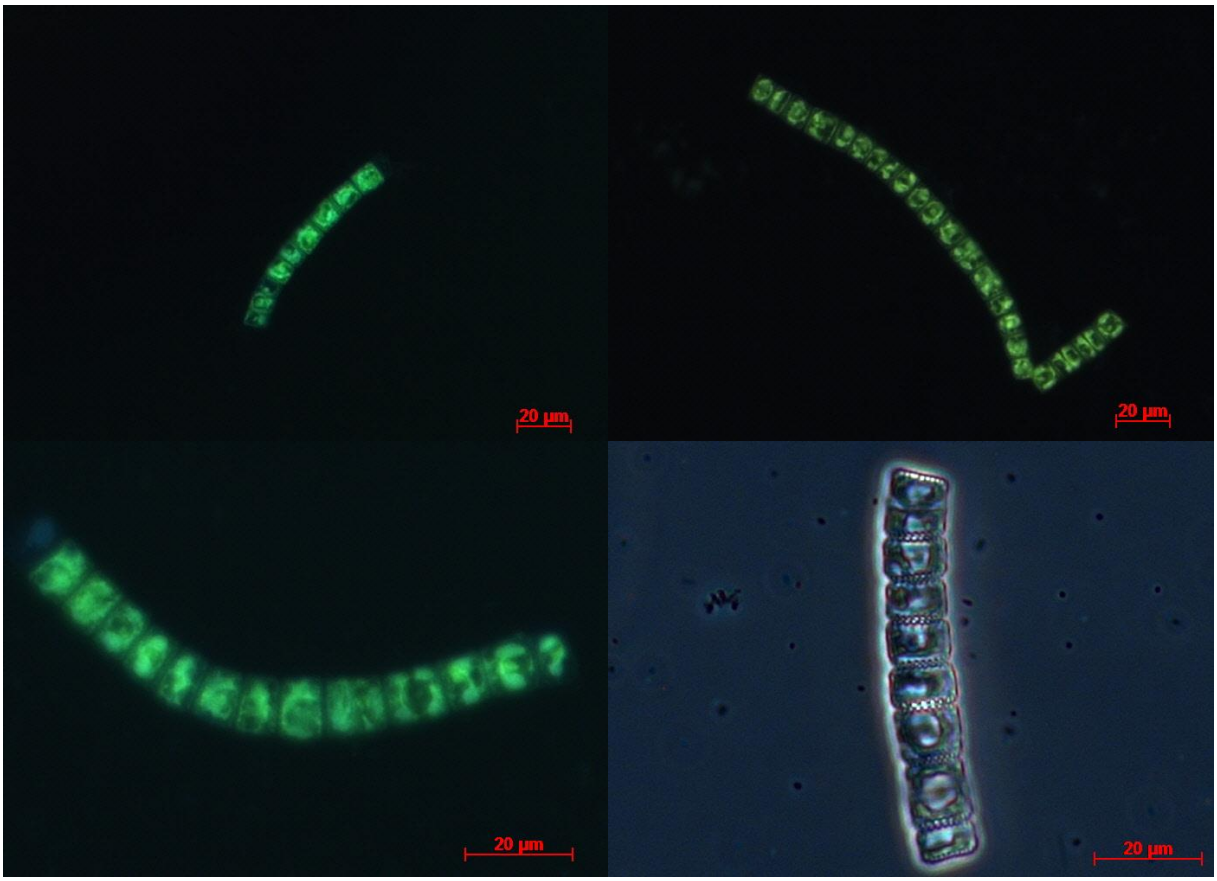


Figure 2: **Four weeks old post-cryo *F. kerguelensis* samples.**
Top left: sample treated with MeOH+FG. Top right: sample treated with FG-s. Bottom left and right: samples treated with FG-s

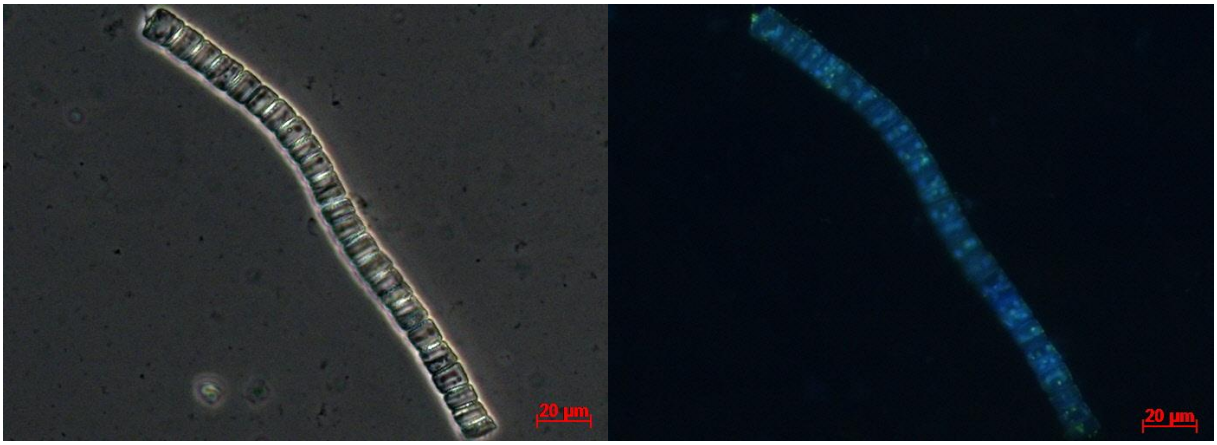


Figure 3: **Four weeks old post-cryo *F. kerguelensis* sample treated with MC.**
Left: light microscopy. Right: fluorescence microscopy

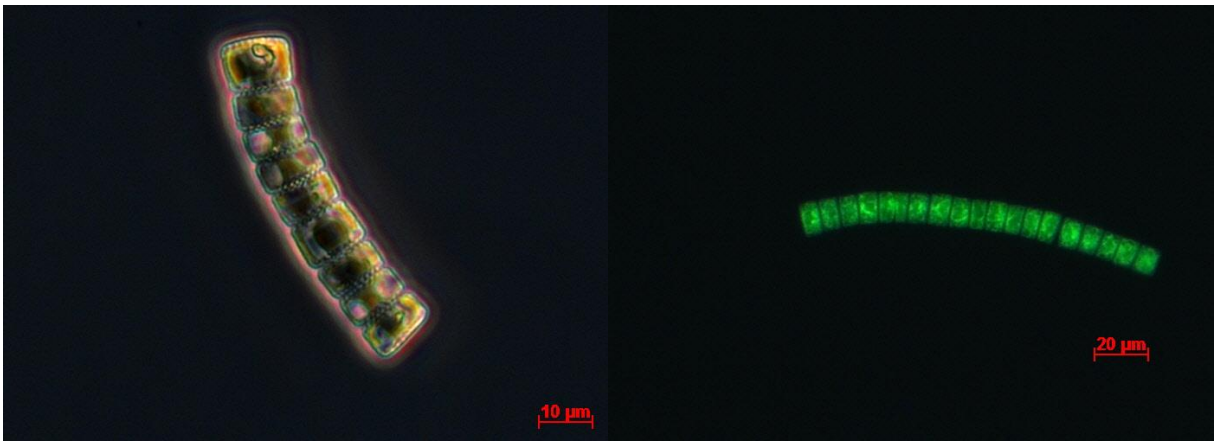


Figure 4: ***F. kerguelensis*.**
Left: light microscopy. Right: sample stained with CFDA-SE, fluorescence microscopy.

3.1.2. *Phaeocystis antarctica*

The results were separated into all AA-forming samples and colony-forming samples.

Comparing the results summarised to FG treated samples and MC treated samples directly after thawing, the mean number of living colonies and their viability were higher than those of the AA and their viability except for the different kind of cells (flagellates and colonial cells) (see Table 13) . Here the mean values (FG and MC groups) for the percentage of living cells for AA-forming samples were higher. In some cases these results were significantly lower or higher or only a significant difference could be determined (see Table 13). Furthermore a clear difference between the results of the FG group compared to MC group for colony forming samples could be seen which was examined in more detail below; whereas this was not observed for the AA-forming samples (see Table 13). Values in Table 13 showed that in general after two weeks viability decreased for all samples. It was observed that the results were higher for AA-forming samples when treated with FG as npCPA but lower when treated with MC. This applied in the opposite way to the results of colony-forming samples. Having a closer look at the respective treatment with higher results there was a clear decrease of living cells for FG treated AA-forming samples but not noted for the results of living AA and their viability; whereas the number of living cells in the MC treated colony-forming samples slightly increased.

Table 13: Mean values of viability analyse (calculated with R (R Core Team 2012)) for *P. antarctica* samples directly after thawing and significance test results.

		AA-forming samples	colony forming samples	test method and significance test results[1)]
FG treated samples	**Living cells**	47 ±17%	32 ±17%	Welch two sample t-test (alternative greater): **p=0.0002549**
	Living AA / colonies	52 ±30%	69 ±20%	Welch two sample t-test (alternative less): **p=0.01514**
	viability of AA / colonies	26 ±18%	30 ±14%	Welch two sample t-test (alternative less): p=0.0772
MC treated samples	**Living cells**	44 ±17%	41 ±19%	Welch two sample t-test: p=0.4447
	Living AA / colonies	52 ±27%	75 ±24%	Kruskal-Wallis test: **p=1.517e-05**
	viability of AA / colonies	24 ±14%	40 ±20%	Welch two sample t-test (alternative less): **p=0.0001039**

[1)] p-values are shown in bold for significant differences

Table 14: Mean values of viability analyse (calculated with R (R Core Team 2012)) for *P. antarctica* samples after two weeks.

		AA-forming samples	colony forming samples
FG treated samples	**Living cells**	28 ±25%	17 ±14%
	Living AA / colonies	47 ±32%	36 ±33%
	viability of AA / colonies	25 ±19%	13 ±15%
MC treated samples	**Living cells**	12 ±14%	43 ±29%
	Living AA / colonies	13 ±23%	57 ±38%
	viability of AA / colonies	6 ±13%	28 ±25%

1) Colony-forming samples (#25, 69/875-86, 69/109-2, 69/905-100)

When looking at Figure 5, Figure 6 and Figure 7 (top part) it is seen that directly after thawing the mean values (see Table 15) of the treatments DMSO+MC and MeOH+MC were the highest. Furthermore the lowest values reached in these treatments were also higher compared to the other treatments. Using only MC as npCPA as treatment it covered the widest range of living cells or colonies and viability of colonies directly after thawing (see at Figure 5, Figure 6 and Figure 7, top part).

Table 15: Highest mean values of colony-forming *P. antarctica* samples directly after thawing.

	DMSO+MC	**MeOH+MC**
number of living cells	46 ±15%	39 ±14%
number of living colonies	81 ±14%	82 ±13%
viability rates of colonies	48 ±17%	40 ±10%

a) Living cells

Regarding the percentage of living cells summarised from all colony-forming samples directly after thawing a two-way ANOVA showed that there is a significant effect between the application of either FG or MC, no interactive effect with and no significant effect between the treatments DMSO, MeOH, stored npCPA or only npCPA ($p_{MC,\ FG}$=0.00828 < α, $p_{interactive}$=0.94545 and p=0.17428 > α). Lower mean viability values were obtained when using FG as npCPA than when using MC as npCPA (see Figure 5). This was found to be significant (Welch two sample t-test, one sided: p=0.004257 < α).

Within the group of samples treated with FG and MC respectively there was a significant difference in homogeneity of variances between the treatments DMSO, MeOH, stored npCPA or only npCPA (Bartlett test: p_{FG}=0.003937 and p_{MC}=0.01838 < α). The Kruskal-Wallis test showed that there was no significant difference between the treatments DMSO, MeOH, stored npCPA and only npCPA within the groups of FG (p=0.7335 > α). The same holds true for the group of samples treated with MC (p_{MC}=0.3933 > α).

After two weeks mean values for the percentage of living cells decreased for the FG treated samples, the results were distributed between 10% and 20% and before between 20% and 40%. In the MC group the mean values lay in the same range as directly after thawing. But the distribution of the results of MC treated samples became greater. (See Figure 5)

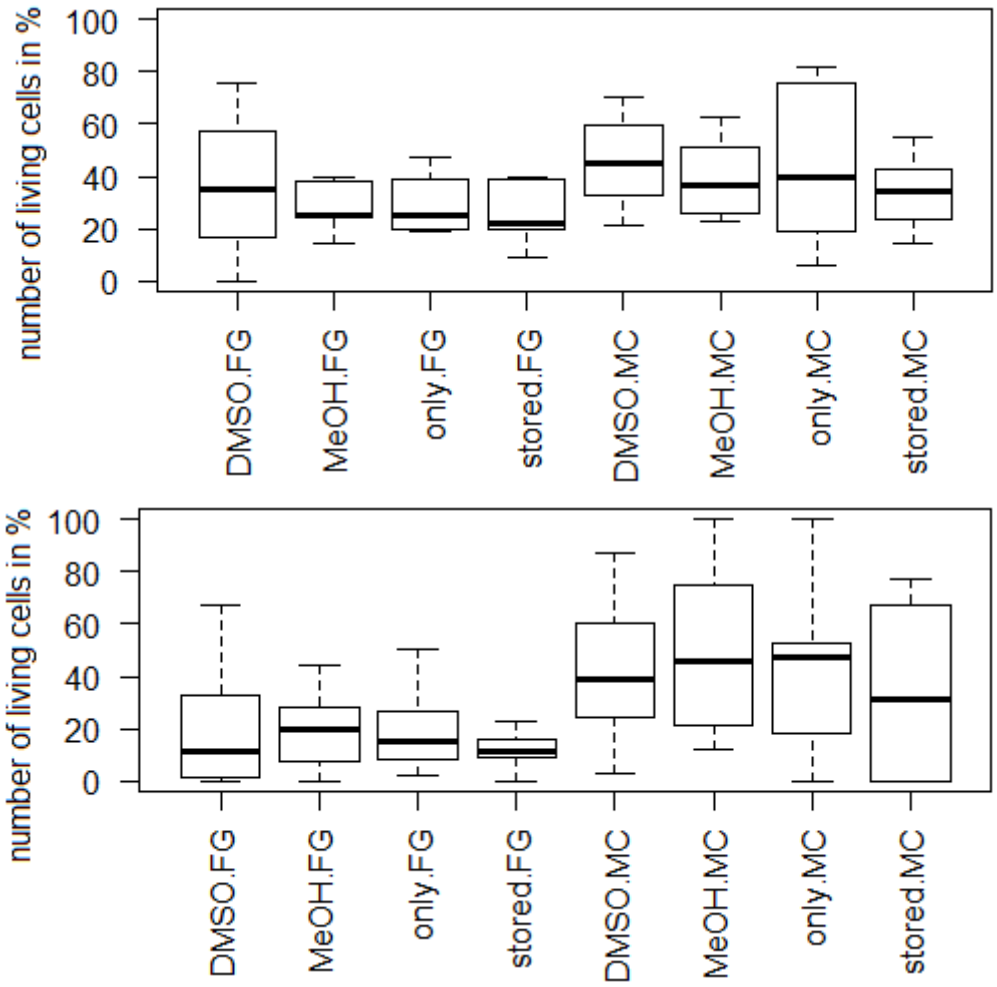


Figure 5: Percentage of living cells of colony-forming *P. antarctica* samples.

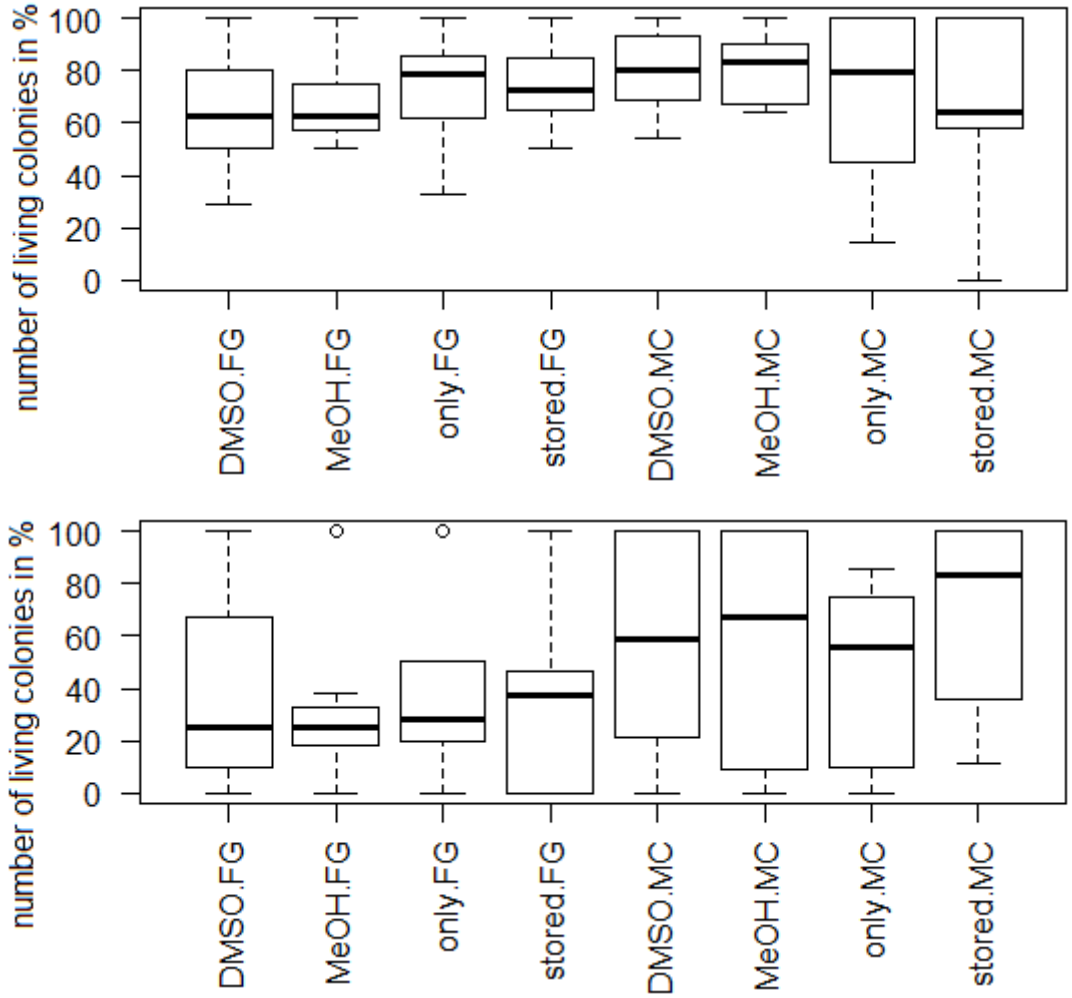
Top: directly after thawing (Outlier #25-MeOH+FG-72% was eliminated). Bottom: after two weeks.

b) Living colonies and their viability

After an arcsine-sqrt transformation and eliminating two outliers (#25-stored FG-100% and 69/875-86-MeOH+FG-100%) the results after thawing were still not normally distributed when regarding samples in groups of MC or FG treated samples (Shapiro-Wilk test: p_{MC}=0.002396 and p_{FG}=0.03802 < α). Applying the Kruskal-Wallis test there was a significant difference between the results of MC group and FG group (p=0.02251 < α). A one-way ANOVA showed that in groups of MeOH, DMSO, only npCPA or stored npCPA there was no significant effect between the arcsine-sqrt transformed results of these treatments (p=0.889 > α).

After two weeks a decrease of the mean percentage of colonies for the FG treated samples was recorded and most of the treatments covered a wider range of the percentage of living colonies (see Figure 6) which means that there was a sample in the counting-analyse with all

colonies being alive and the second sample out of the triplicate with all colonies being dead per treatment.



<u>Figure 6:</u> Arcsine-sqrt transformed percentage of living colonies of colony-forming *P. antarctica* samples.

Top: directly after thawing. Bottom: after two weeks.

Percent viability of colonies directly after thawing:

Outlier #25-singly FG-8% was eliminated. A two-way ANOVA outlined that there was a significant effect whether FG or MC was applied, but no interactive effect with or a significant effect between the application of DMSO, MeOH, only npCPA or stored npCPA was found ($p_{FG,MC}$=0.0236 < α, $p_{interactive}$=0.1112 and p=0.2145 > α). The Welch two-sample t-test showed that mean viability levels in the FG group were significantly lower than those in the MC group (one-sided: p=0.00846 < α).

Within both groups there was a significant deviation from homogeneity of variances between the results treated with DMSO, MeOH, stored npCPA or only npCPA (Bartlett-test: p_{FG}= 5.61e-05 and p_{MC}=0.005546 < α). Regarding results in the FG group there was a significant difference between the treatments and in the MC group there was none (Kruskal-Wallis test: p_{FG}=5.61e-05 < α, p_{MC}=0.1842 > α).

After two weeks a decrease in mean viability values was recorded for both groups except for the stored MC treatment. Furthermore the covered range of percent viability stayed around the same, but results were shifted towards lower percent viability levels (see Figure 7).

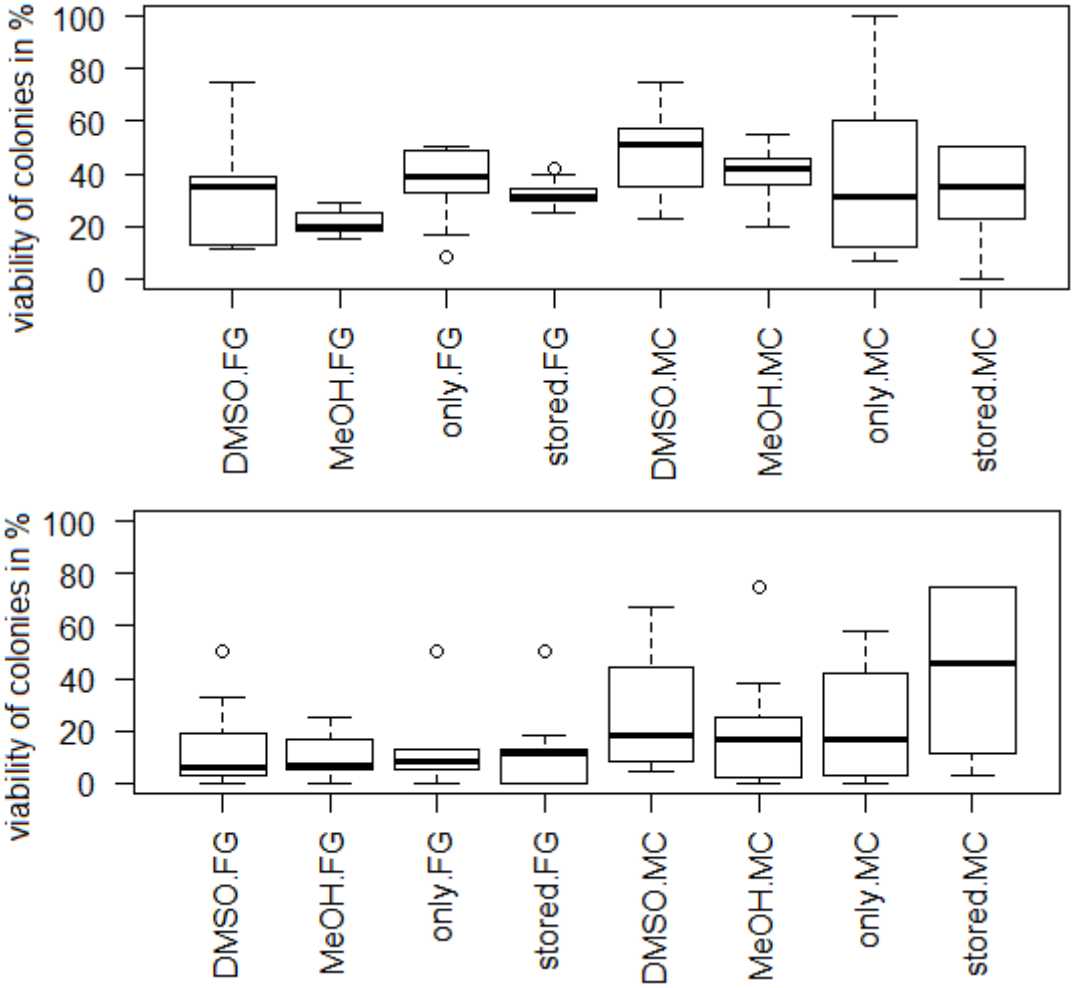


Figure 7: Percent viability of colonies of colony-forming P. antarctica samples.

Top: directly after thawing. Bottom: after two weeks.

2) AA-forming samples (69/71-5, 69/82-10, 69/770-50, VIMS 119-1)

Highest viability mean values (see Table 16) were reached for the treatment of MeOH+FG

The treatments DMSO+FG and FG or MC only covered in all cases directly after thawing a wide range of viability levels. The mean viability values were the lowest for the treatments singly FG or MC and stored FG or MC. (See Figure 8, Figure 9 and Figure 11, top part)

After two weeks a large decrease in the percentage of living cells, AA and their viability was recorded for the treatments with MC as npCPA (see Figure 8, Figure 9, Figure 11).

Table 16: Highest mean values of AA-forming *P. antarctica* samples directly after thawing.

	MeOH+FG
number of living cells	53 ±8%
number of living AA	70 ±18%
viability rates of colonies	42 ±20%

a) Living cells

Directly after thawing: a two-way ANOVA showed that there were no significant effects between applying either FG or MC, between the various applications of DMSO, MeOH, singly npCPA and stored npCPA and no interactive effect ($p_{MC,FG}$=0.877, p=0.556 and $p_{interactive}$=0.866 > α) (Outlier VIMS 119-1-MeOH+FG-77% was eliminated).

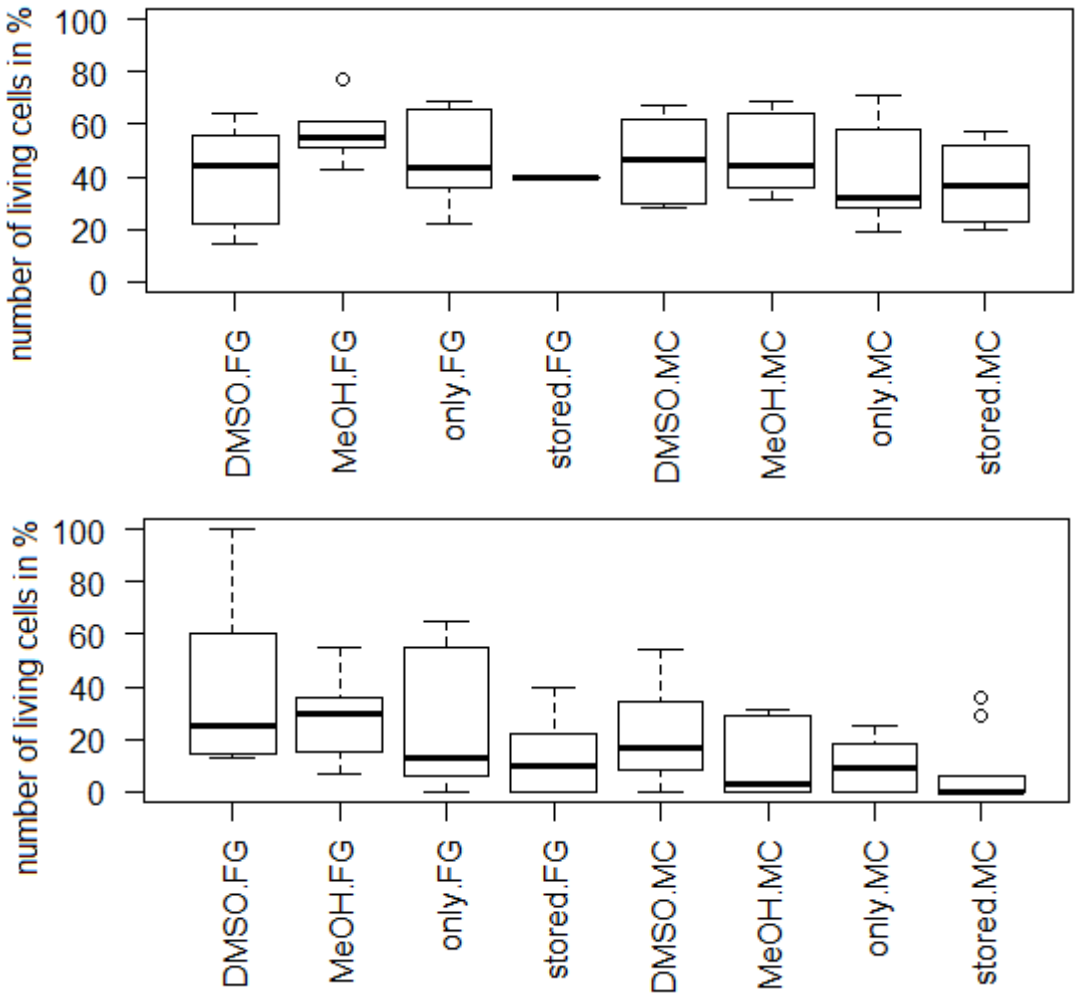


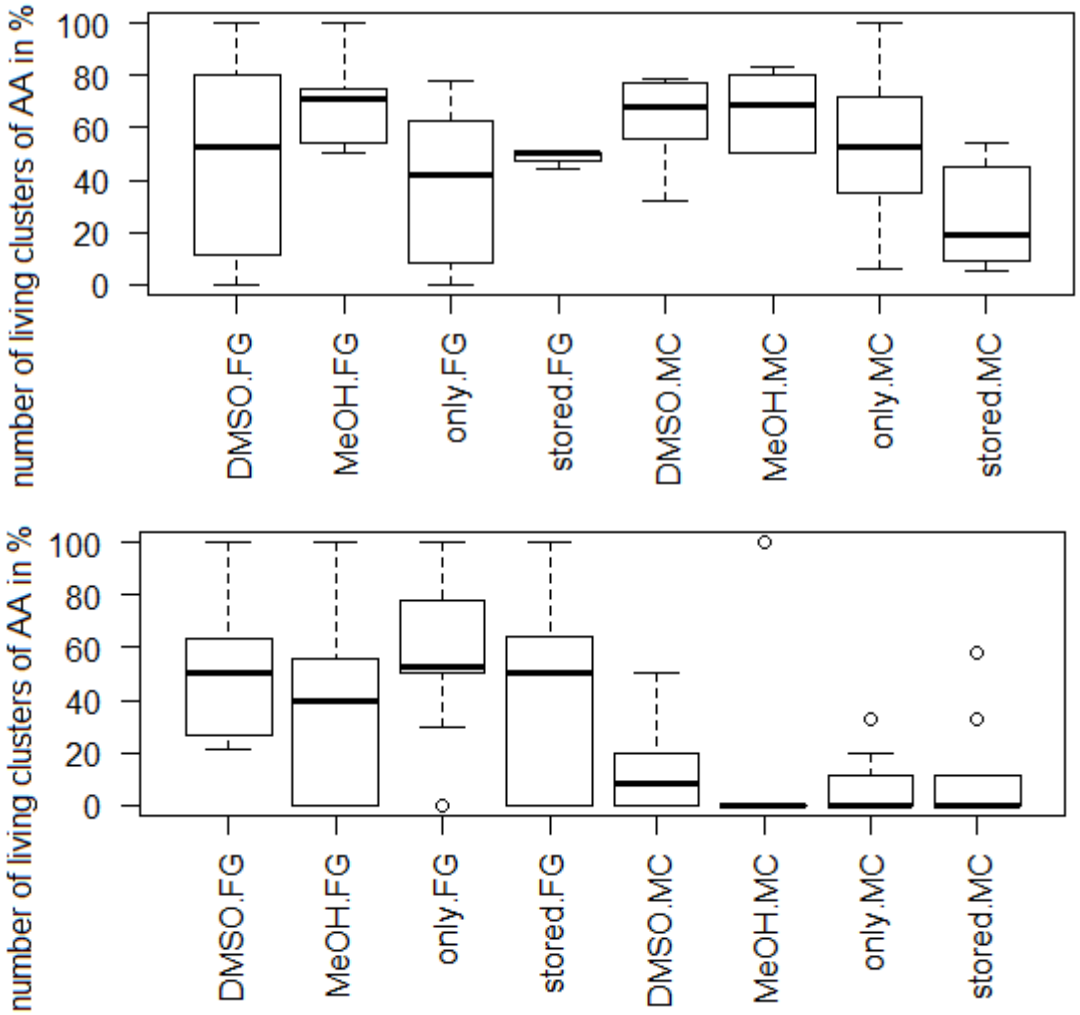
Figure 8: Percentage of living cells of AA-forming *P. antarctica* samples.

Top: directly after thawing. Bottom: after two weeks.

b) Living clusters of AA and their viability

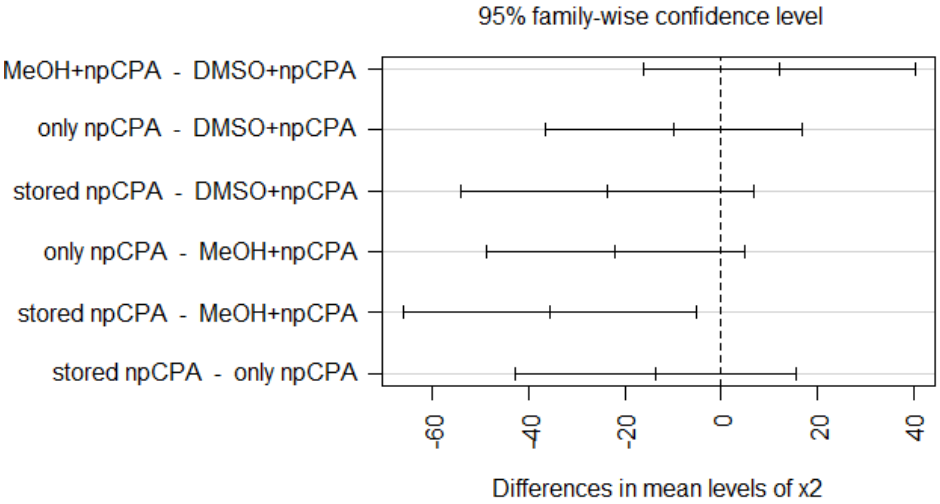
Directly after thawing for the number of living AA there was a significant effect in choosing MeOH, DMSO, singly or stored npCPA with no interactive effect and no significant effect of FG or MC (two-way ANOVA $p=0.0228 < \alpha$, $p_{MC,FG}=0.7272$ and $p_{interactive}=0.3792 > \alpha$).

Tukey's HSD post-hoc test showed that the application of stored npCPA results in significantly lower mean levels than MeOH+npCPA ($p=0.0161748 < \alpha$, see Figure 10).



<u>Figure 9:</u> Percentage of living AA of AA-forming *P. antarctica* samples.

Top: directly after thawing. Bottom: after two weeks.



<u>Figure 10:</u> Plot of Tukey's HSD post-hoc test results for AA-forming *P. antarctica* after thawing: number of living AA without taking into account whether FG or MC was applied. x2=DMSO, only, MeOH or stored

Viability of AA directly after thawing:

Outlier 69/71-5-DMSO+MC-11% was eliminated. A two way ANOVA showed that there is no significant effect between the application of FG or MC ($p=0.8729 > \alpha$), but the choice of DMSO, MeOH, only npCPA or stored npCPA had a significant effect ($p=0.0127 < \alpha$) with an interactive effect ($p=0.0440 < \alpha$), which can be seen on the basis of comparing Figure 11 and Figure 12.

Tukey's HSD post-hoc test (see Figure 13) showed that regarding the interactive effect the mean viability of stored MC treated samples is significantly lower than the ones treated with MeOH+FG ($p=0.0091081 < \alpha$) and without taking into account whether MC or FG was applied stored npCPA viability results were significantly lower than MeOH+npCPA viability results ($p=0.0074786 < \alpha$)

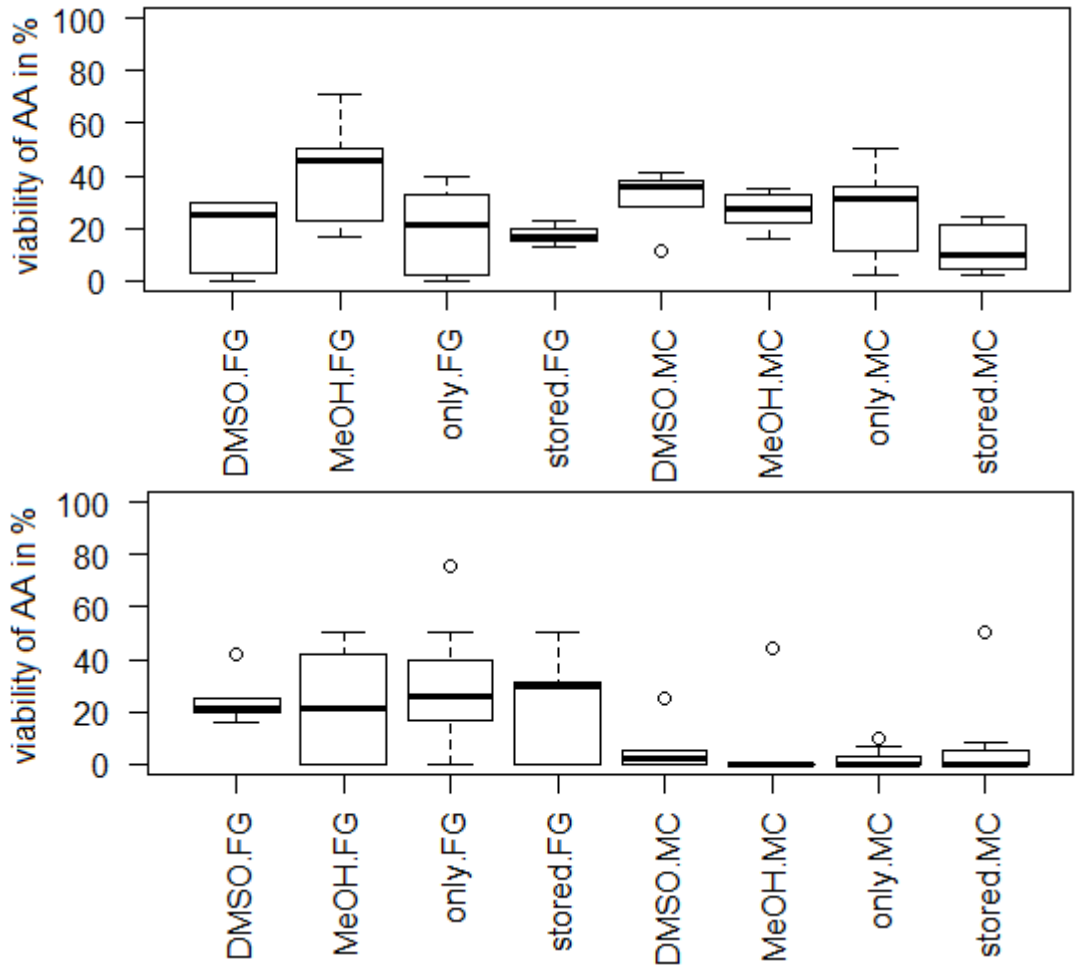


Figure 11: Percent viability of AA-forming *P. antarctica* samples.

Top: directly after thawing. Bottom: after two weeks.

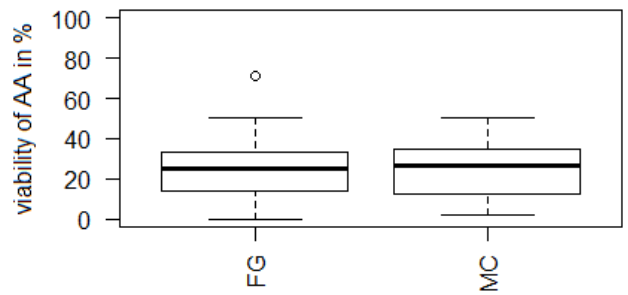


Figure 12: Percent viability of AA-forming *P. antarctica* samples directly after thawing separated into FG treated group and MC treated group.

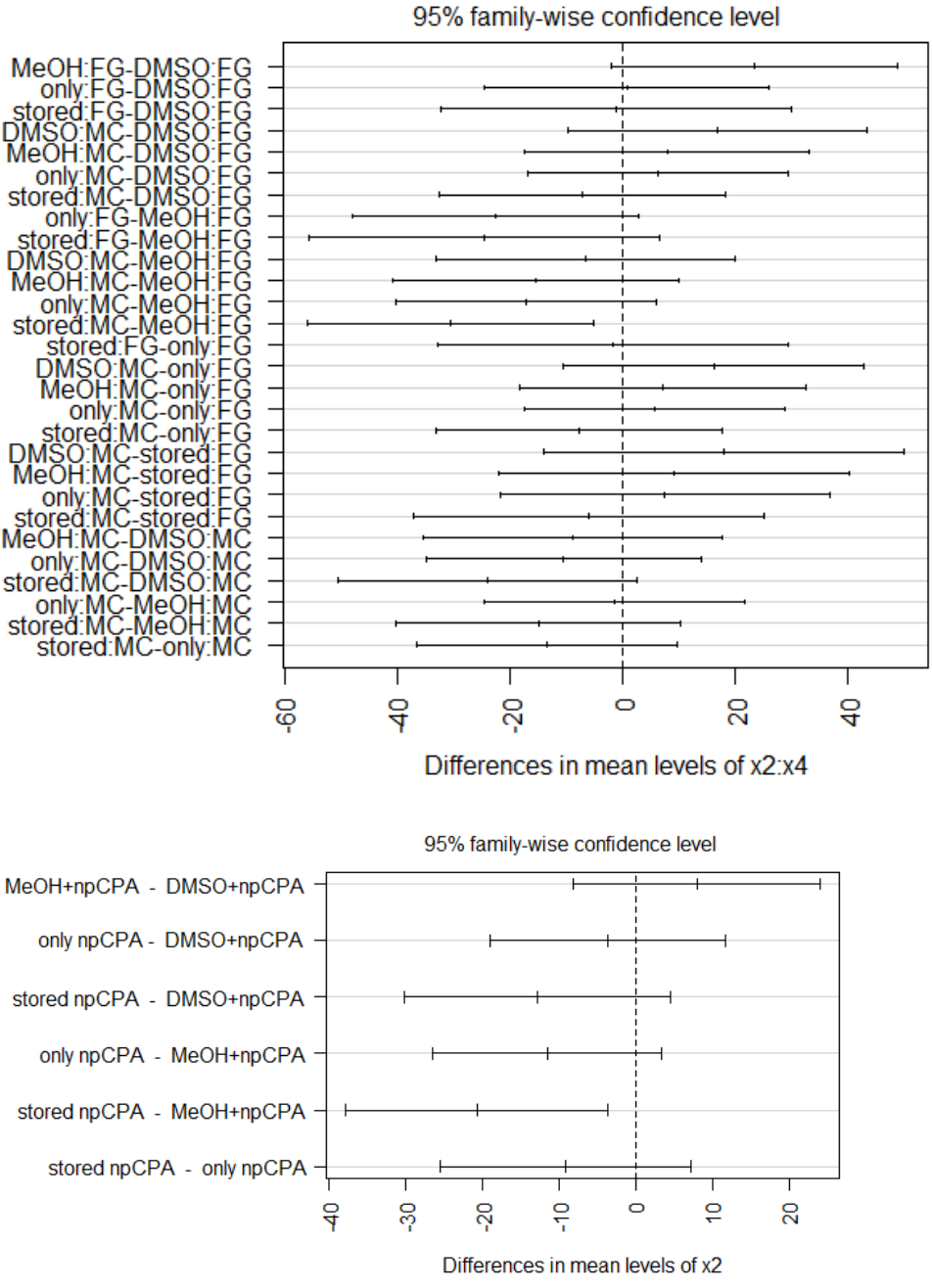


Figure 13: Plot of Tukey's HSD post-hoc test results for AA-forming *P. antarctica* samples. Viability of AA directly after thawing. Top: with interactive effect. Bottom: all results without taking into account whether FG or MC was applied. x2=DMSO or singly or MeOH or stored, x4=FG or MC.

3.1.3. Bacterial Growth and contamination

All samples treated with any kind of CPA treatment showed high bacterial growth (dot-shaped bacteria) (see Figure 14 and Figure 15 bottom right) four weeks after thawing. These bacteria were observed to accumulate in round shapes decomposing the algae (see Figure 14). The same bacteria, but in much less concentration was found in samples not used for cryopreservation. In comparison to this observation, treatments with MC as npCPA showed less growth of these bacteria. Here another type of bacteria (see Figure 15) was observed which was also detected in the waterbath (see Figure 15 top right), hereafter called wb-bacteria. Wb-bacteria were also found in samples treated with FG (see Figure 15, bottom right and left).

Samples stored in the 1°C room had less bacterial growth compared to the ones in the 3°C room but after another one to two weeks samples treated with FG also went turbid like those in the 3°C room which showed high turbidity due to bacterial growth already four weeks after thawing. Samples with MC as npCPA did not become turbid until the final observation five to six weeks after the day of thawing whereby still less bacteria compared to FG treated samples were observed.

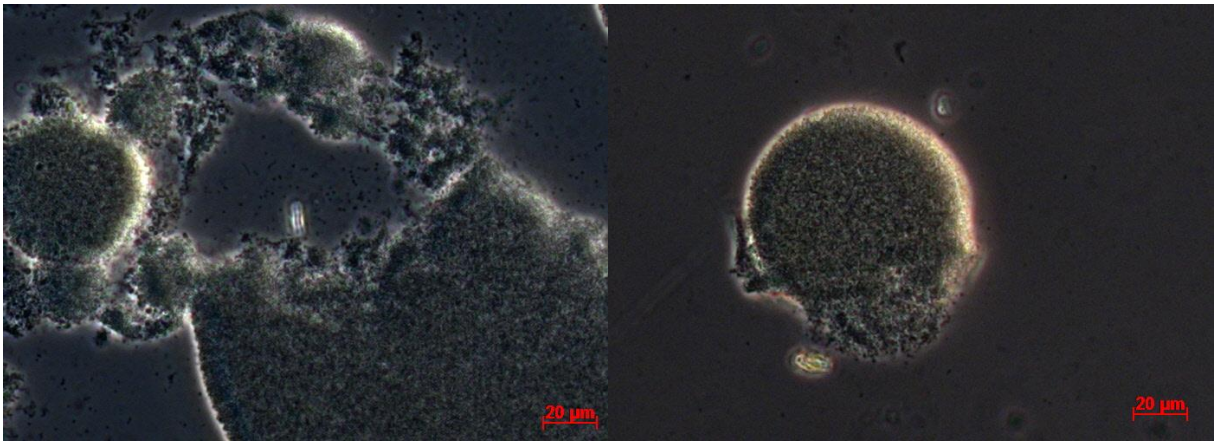


Figure 14: Left and right: dot-shaped bacteria and decomposed dead cell material in 4 weeks old post-cryo sample VIMS119-1 treated with FG stored in the 1°C room.

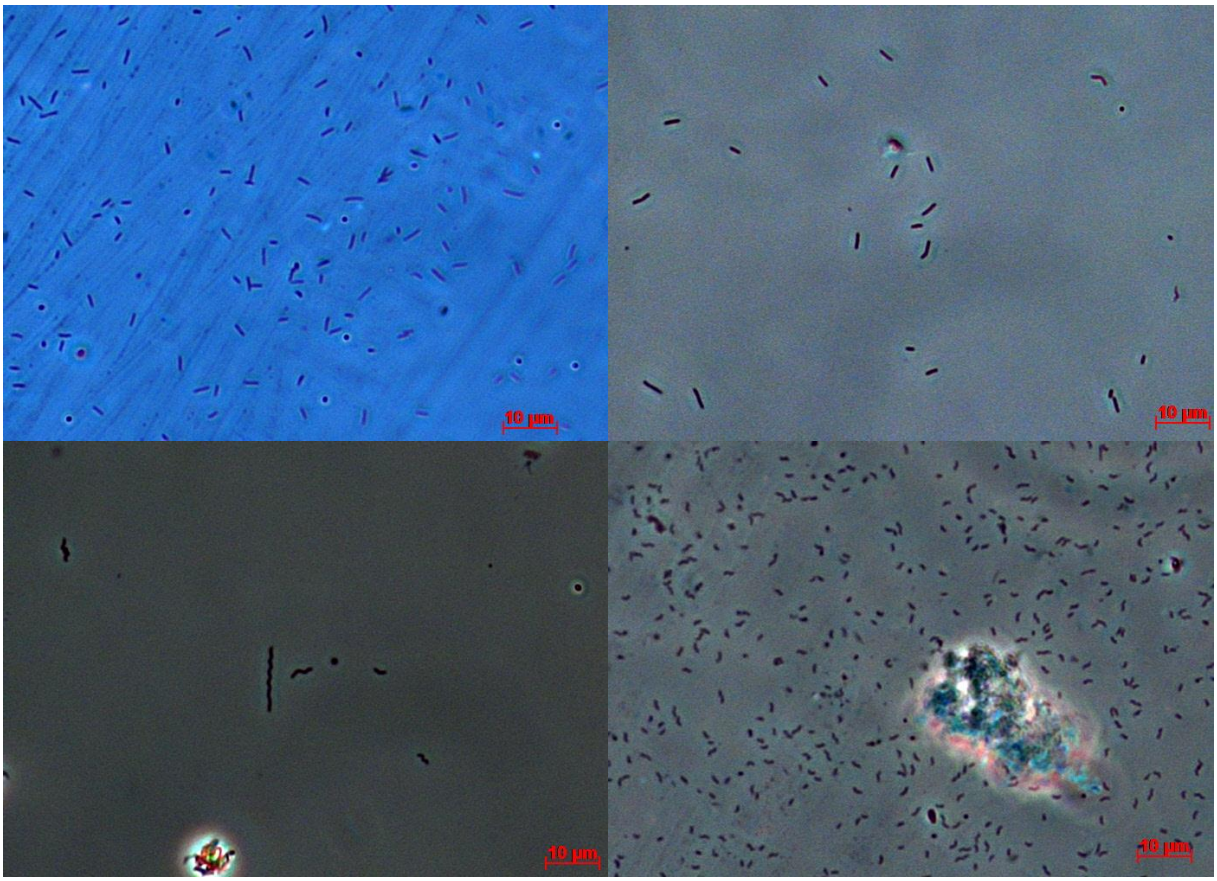


Figure 15: Pictures of bacteria

Top left: wb-bacteria in four weeks old post-cryo sample *F. kerguelensis* treated with MeOH+MC stored in the 3°C room. Top right: bacteria found in water mixed with GP5-Ant after 1 week stored in the 3°C room. Bottom left: wavelike wb-bacteria in four weeks old post-cryo sample 69/770-50 treated with DMSO+FG stored in the 1°C room. Bottom right: wb-bacteria and dot-shaped bacteria in 4 weeks old post-cryo sample VIMS119-1 treated with FG-s in the 3°C room.

3.2. Antibiotic treatment

Even after the 5th treatment bacteria were found in all *P. antarctica* samples and in *F. kerguelensis.*

In *P. antarctica* cultures #25 less colonies and in 69/82-10 less AA were observed. In both cases there were more flagellates than any other cell formations.

In *F. kerguelensis* samples, algal cells did not look alive anymore after the treatments. Most of the cells were broken or penetrated by rod-shaped bacteria.

So it was not possible to obtain axenic cultures after five treatments of the antibiotics Penicillin, Streptomycin and Ciprofloxacin Hydrochloride within the used protocol.

3.3. DNA extraction and amplified length polymorphism

In both DNA extraction methods the DNA of colony-forming *P. antarctica* samples showed various fragments with different bp-lengths in the first elution compared to AA-forming samples (see Figure 16 and Figure 17) except for the AA samples in Figure 16 top.

In the second elution there were usually high molecular weight DNA strands however most of the time the concentration was too low so that these samples could unfortunately not be used for AFLP.

Since there was no growth for post-cryo cultures, no post-cryo AFLP could be done. That is why no comparison between axenic cultures and cultures with bacteria was possible.

For the pre-cryo non-axenic samples the AFLP results were as follows: in one pre-selective amplification the primers EcoRI+A and MseI+C were used followed by selective amplification with the primers EcoRI+AT* MseI+CT. For both amplifications gel electrophoresis showed that there were only very small fragments (below 100bp) (see Figure 18).

Using a combination of EcoR+0 and MseI+0 for preselective amplification and EcoRI+A* and MseI+C for selective amplification there were fragments with different lengths. The intense dark bands seen in Figure 18 show that most of the fragments had a length between 200bp and 400bp after selective amplification.

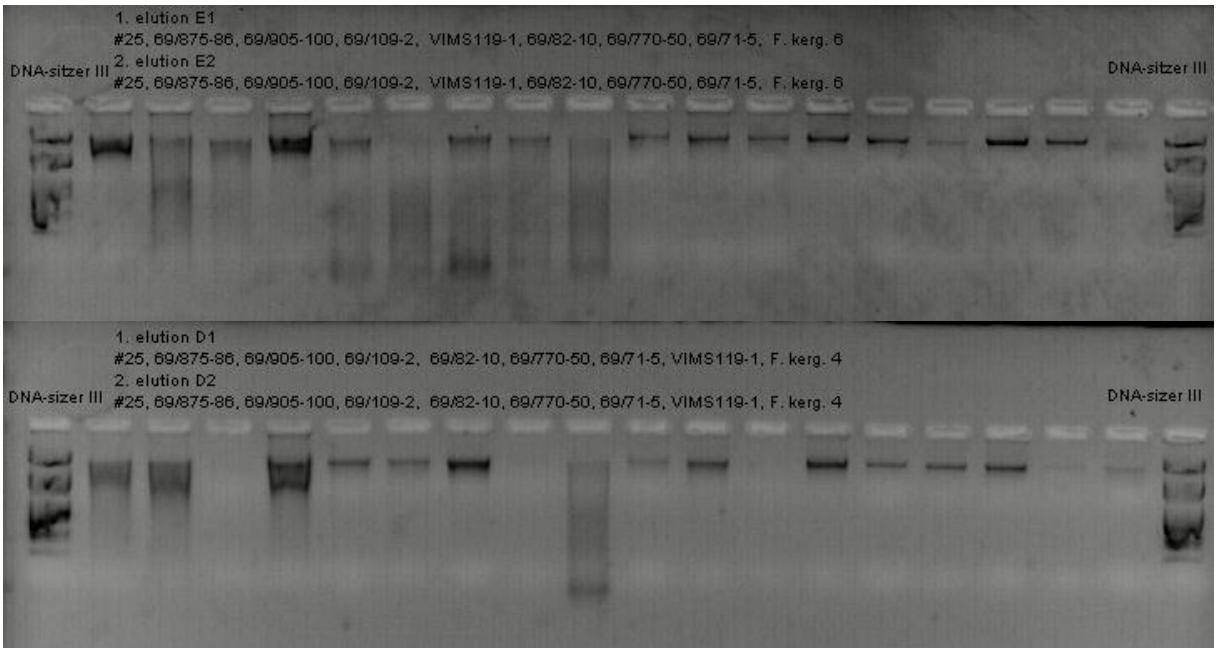


Figure 16: Gel electrophoresis results of DNA samples extracted by using NucleoSpin® Plant II (Macherei-Nagel, Germany)

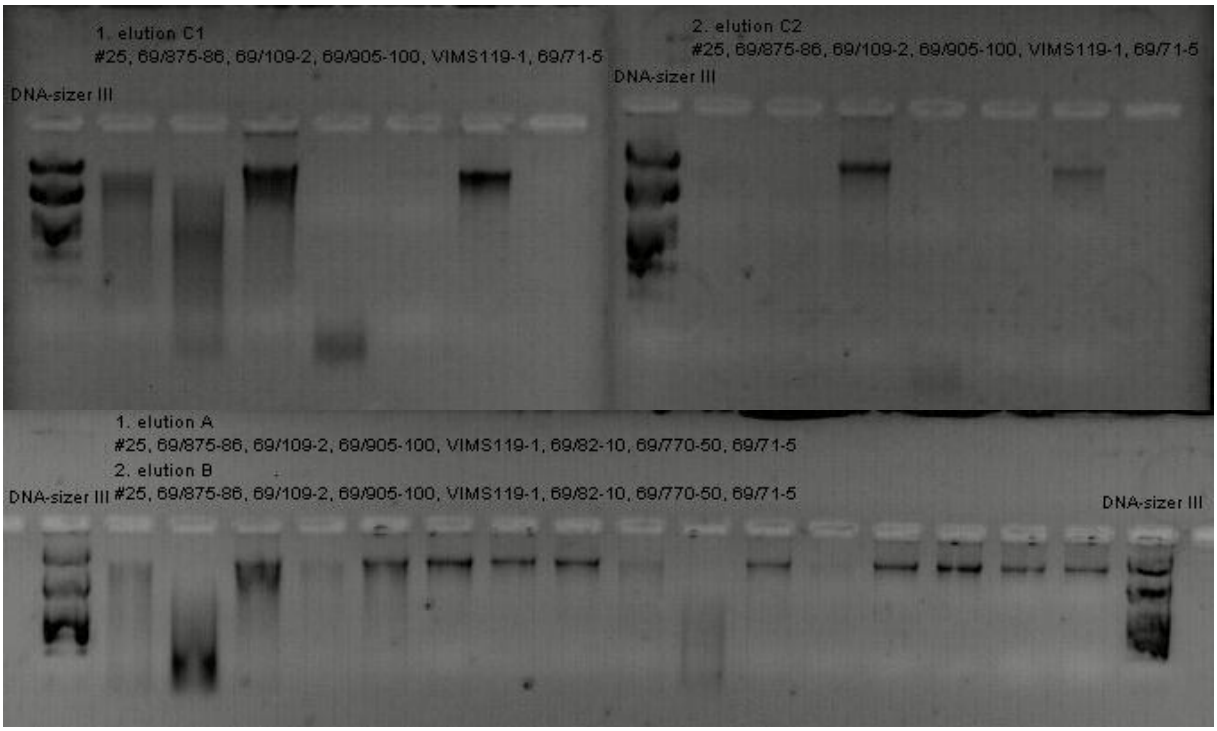


Figure 17: Gel electrophoresis results of DNA samples extracted by using E.Z.N.A.® SP Plant DNA Kit (Omega Bio-Tek, GA, USA)

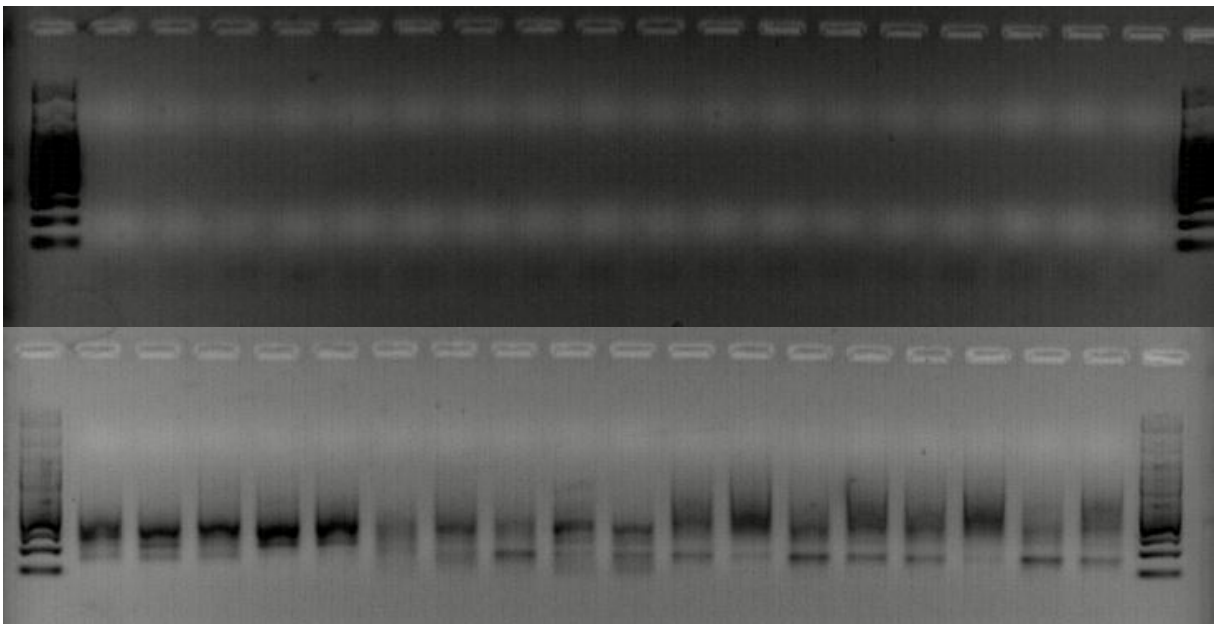


Figure 18: Picture of the gel electrophoresis result with samples after selective amplification. Top: primers EcoRI+AT* and MseI+CT (preselective amplification with primers EcoRI+A and MseI+C) Bottom: primers EcoRI+A* and MseI+C (preselective amplification with primers EcoR+0 and MseI+0)

4. Discussion

A lot of different algae species have already been successfully cryopreserved with different methods and varying viability results after thawing (Abreu et al. 2012, Nakanishi et al. 2012, Rhodes et al. 2006, Taylor & Fletcher 1999). The primary objective of "Finding a combination of npCPA and pCPA or single application of npCPA which results in high viability rates after thawing" was achieved (see Table 12, Table 15 and Table 16). Unfortunately the recovery of algal cells post-cryo after a few weeks of growth time failed. One possibility for the failed recovery could be the high bacterial growth and additional contamination of the samples through wb-bacteria. High attention was paid to prevent contamination because earlier studies showed the same problem (Friesen 2013). Therefore the vials were put into a floating rack and cleaned with ethanol right after the waterbath and again before opening under the clean bench. However the freezing and thawing process can lead to fine cracks of the cryovials or lightly loosen caps enabling contamination which was also stated by the manufacturer of the used cryo vials (Sarstedt 2011, page 204).

Another possibility besides the bacterial growth could be the time of the performance of the testing. It is observed for *P. antarctica* that while maintaining them by serial subculturing at the same temperature and light regime in the Alfred-Wegener-Institute for several years, they still show a seasonal growth pattern. That means these isolates show slower and poor growth during the Antarctic winter despite being stored in a room with steady light and temperature conditions. This study was performed during this time meaning that their behaviour might have had an impact on recovery as well. So the additional stress through cryopreservation in combination with less "active" algae but vivid bacteria could have led to no growth. Results of Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*) show that there is algal growth, when performing the experiments during Antarctic summer/autumn time despite bacterial contaminations and also preliminary results gained prior to this study during Antarctic autumn with isolates #25 and 69/875-86 showed this as well (practical pre-study course of the author in March/April 2013). For MC treated samples there was good growth observed two weeks after thawing. So the seasonal growth is suggested to be a main factor influencing the chance of recovery after cryopreservation.

Besides the wrong timing also the stress of the complete cryopreservation handling procedure itself could be an issue. So there was no cell recovery because of the high stress

level due to freezing and the algae simply could not grow under these circumstances. Rhodes et al. (2006) stated that control samples treated the same way as the cryopreserved samples with the exception of not freezing them failed to grow as well.
This could have also happened to *F. kerguelensis* samples treated with MC discussed later.
Furthermore centrifugation to increase cell density prior to plunging them into LN_2 on the same day could have induced additional stress (Day & Brand 2005) to the algae. But it seemed that there was no impact on viability levels through this factor in this study because no significant differences for *P. antarctica* samples were found if the stored samples or those directly plunged into LN_2 using the same npCPA were compared. In general the viability results of these treatments covered a wide range of viability values after thawing and after two weeks. The method showed unpredictable viability results for freezing *P. antarctica* samples because on one hand there could have been samples with high viability or on the other hand samples with low viability leading probably to no recovery. Additionally significantly lower values were reached for stored npCPA treatments compared to npCPA+pCPA for number of living AA and their viability (see Figure 10 and Figure 13) and thus confirming the suggested hypothesis that there could be a difference in viability of algal cells between the treatments npCPA with pCPA or single application of a npCPA.

Viability of algae after cryopreservation is dependent on various factors. They can be separated into treatment of the algae prior and after freezing and the freezing method in itself. Prior freezing algae should be tested whether the applied CPA's have a toxic effect or not, especially with those that penetrate the cells.
P. antarctica samples were tested on different concentrations of DMSO and MeOH (Gäbler-Schwarz et al. 2013, accepted in *Cryoletters*) and no toxic effect was found. But through the stress which the algae was exposed to, DMSO and MeOH might have had an impact in this study. *F. kerguelensis* samples were unfortunately not tested prior cryopreservation. While freezing a combination of npCPA+pCPA can prevent the intra and extra cellular ice nucleation but the single application of FG has already showed good results (Gäbler-Schwarz et al. 2013, accepted in *Cryoletters*). Hence it was suggested that there is a difference in viability of algae between the treatments npCPA with pCPA or single application of a npCPA. This hypothesis was verified but not in all cases.

F. kerguelensis showed higher viability after thawing when applying only MC or FG compared to the results of pCPA with the respective npCPA (see Table 12). These differences were not significant. Furthermore it cannot be stated whether DMSO or MeOH had a toxic effect or not because viability results after two weeks showed that there was an increase in the number of living cells for the samples treated with pCPA+FG, however these results were significantly lower than those of samples treated with only FG (see Table 12). But no recovery was observed for any FG treated samples after four weeks growth time. It seemed that recovery in FG samples was inhibited by growth of bacteria decomposing the algae.

In the case of MC treated samples there was high viability directly after freezing but a decrease after two weeks. So it is suggested that MC did not provide suitable protection of extracellular ice nucleation leading to fatal injuries and therefore a drop in viability after two weeks. Though fewer bacteria were observed in MC treatments also no algae growth was observed. These observations also applied to AA-forming *P. antarctica* samples, indicating that MC might inhibit and thereby slow down the growth of bacteria.

Vital staining after thawing and two weeks showed that *F. kerguelensis* cells looked empty shown in Figure 3. In both cases (FG or MC as npCPA) cells were observed in which the fluorescence colouring was not as evenly spread as in healthy cells (see Figure 2 compared to Figure 4). This seems to indicate that cell organelles ruptured while freezing or thawing so that no growth or recovery was possible. The red fluorescence from the chloroplasts also was not observed, so these organelles were fatal injured and in this case Mitbavkar & Anil (2006) state as well for another diatom species that there is no growth after thawing.

Ensuring that cells are not killed while the freezing process itself several methods of pCPA+npCPA or singly npCPA were tested in this study. But not only the choice of CPA plays a major role, other factors including cooling rates, CPA composition and concentration and a combination of these factors (Pegg 2007, Taylor & Fletcher 1999, Cañavate & Lubian 1997 and 1995) can lead to different results.

Regarding the impact of cooling rates through plunging the sample directly into LN_2 excessive dehydration was prevented. But this causes formation of intracellular ice in contrast to thawing where the faster the change in temperature the more recrystallization is minimized. (Day & Brand 2005)

The first aspect was considered using CPA. But regarding the thawing it seems that this was one of the crucial steps for *P. antarctica* and *F. kerguelensis* where most of the damaging through ice crystals could have happened. The samples needed between three to five minutes to be completely thawed. During this time some samples turned milky or were already milky when getting them out of the LN_2. This means that the substances crystallized (Harding et al. 2004). Rewarming is a critical step for *P. antarctica* and *F. kerguelensis* in two ways. The first one is the just described formation of ice crystals being threatening for every species cryopreserved (Mazur et al. 1972). The second one is that both species are from Antarctica. Exposure to high temperatures leads to death of cells. So every step was performed on ice and therefore handling of the cultures was made difficult.

It can be confirmed that depending on the cooling and rewarming rate different or similar viability results are achieved (Cañavate & Lubian 1997). In this study results of *P. antarctica* strain 69/905-100 used for the MeOH+MC treatment was compared to the ones from Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*) 48h after freezing (see Table 17).

Similar results shown in Table 17 were achieved for the same CPA treatment and thawing method using different freezing methods: plunging the sample directly into LN_2 or applying a controlled rate cooling programme. Whereas for the treatment of MeOH+FG totally different results shown in Table 17 were obtained for *P. antarctica* strain 69/770-50.

Table 17: Selected mean viability values for *P. antarctica* samples after thawing.

		this study	**Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*)**
69/905-100 treated with MeOH+MC	**number of living colonies**	90 ±15%,	91 ±9%,
	number of living cells	48 ±12%	56 ±10%
69/770-50 treated with MeOH+FG	**number of living AA**	64 ±17%	5 ±9%
	number of living cells	52 ±12%	13 ±8%

Regarding the CPA composition DMSO, MeOH and FG stock solutions were prepared in the respective medium GP5-Ant and F/2-Ant and MC in the bidest. versions later salted up so that osmotic stress induced through different salinity levels was prevented.

The right treatment after freezing is also important. Since the freezing procedure means a lot of stress for the algae the samples were diluted with 2mL of fresh medium and covered in aluminium foil for 72h to improve recovery. But there was a viability drop after two weeks for some samples so it might have been better to dilute them using a greater volume of medium already after thawing, also suggested in Day & Brand (2005).

The experiment was performed with four colony-forming and four AA-forming *P. antarctica* strains. Clear differences in viability results were observed (see Table 13). Despite the fact that AA have a sticky tough membrane (Gäbler-Schwarz et al. 2010) and observing the formation of AA in colony-forming samples after freezing stated in Gäbler-Schwarz et al. (2013, accepted in *Cryoletters*) as well as in this study for #25 and 69/875-86, viability rates were in general lower compared to colonies after thawing (see Table 13). This also stands in contrast to the suggestion of Gäbler-Schwarz et al. 2010 that AA are an overwintering stage.
After thawing cells showed higher as well as lower results compared to colonies, AA and their viability (see annex d)) but after two weeks there was a great decrease in the living number of cells except for MC treated cells in colony-forming samples (see Table 13 and Table 14). This supports the suggestion "that they were more sensitive to the physical and chemical stress induced by freezing and thawing" (Gäbler-Schwarz et al. 2013, accepted in *Cryoletters*).
Different behaviour was observed depending on their viability. It was shown that after thawing there were significant differences for colony-forming samples between applying FG and MC as npCPA but they had no significant effect on viability of AA-forming samples.
It is suggested that this is due to their morphotype and it was planned to see whether there were also significant differences in AFLP results. However it was not possible to obtain axenic cultures so that clean algal DNA could be extracted. The results of AFLP using DNA extracted from non-axenic cultures could lead to falsified assumptions because it was not known whether the fragments occurred through bacterial DNA or not.

5. Conclusion

It is suggested that experiments should be performed again for both species with the treatments leading to highest viability results after thawing at the beginning of Antarctica summer time. In this way it can be tested whether the seasons also have an impact on the recovery of *F. kerguelensis*.

Furthermore establishing axenic cultures especially in the case of *P. antarctica* would allow genetic analyses by AFLP of the different morphotypes used for cryopreservation so that possible relations between morphotypes, AFLP results and viability could be examined. Axenic cultures could also improve recovery since no bacteria would overgrow the algae.

6. Acknowledgements

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8. List of figures

9. List of tables

Annex

a) F/2-Ant modified after Guillard & Ryther (1962)

Stock solutions:

1) Sodium nitrate ($NaNO_3$): dissolve 75g in 1000mL aqua dest.
2) Disodium hydrogen phosphate (NA_2HPO_4 x $2H_2O$): prepare a solution of 6,25g/L
3) Sodium metasilicate (NA_2SIO3 x $9H_2O$): dissolve 15g in 1000mL auqa dest.
4) Metal mix solution:

   Solution a: dissolve the following ingredients in 100mL aqua dest.:

   - $ZnSO_4$ x H_2O: 150mg of zinc sulphate
   - $CuSO_4$ x $5H_2O$: 100mg of copper sulphate
   - $CoSO_4$ x $7H_2O$: 120mg of cobalt sulphate
   - $MnSO_4$ x H_2O: 2000mg of manganese sulphate

   Solution b:

   - $FeCl_3$ x $6H_2O$: dissolve 5000mg of iron chloride in 100mL aqua dest.

   Solution c:

   - Na_2MoO_4 x $2H_2O$: dissolve 65mg of sodium molybdenum in 100mL aqua dest.

   Solution d:

   - Na_2 EDTA x $2H_2O$: dissolve 5000mg of Titriplex III in 100mL aqua dest.

   Mix 100mL of solution d with 10mL each of solution a, b, and c. Top up to 800mL with aqua dest. and adjust pH-level with NaOH to 7.5. Top up to 1000mL with aqua dest.
5) Vitamins solution (store at -20°C):

   Dissolve 1mL each in 100mL aqua dest. of the following stock solutions: vitamin B12 (1mg dissolved in10mL aqua dest.) and biotin (1mg dissolve in 10mL aqua dest.). Mix with 20mg Thiamin x HCl.

   Sterile filter through a 0.2µm acetate filter.

Add the following volumes to Antarctic seawater and top up to 1000mL:

- 1mL each of solution 1, 2, 4 and 5
- 2mL of solution 3

The F/2-Ant is filtered through a 0,2µm Vacu Cap Filter(Pall, USA) using a peristaltic pump (Heidolph, Germany).

b) GP5-Ant modified after Loeblich and Smith (1968, p. 7)

Prepare the stock solutions listed in Table 18.

Add the following concentrations of the stock solutions to 1000mL Antarctic sea water:

- 400µL ofKNO_3
- 200µL of K_2HPO_4
- 200µL of vitamins solution
- 1mL of PII-metals
- 3mL of soil extract

The Gp5-Ant is filtered through a 0,2µm Vacu Cap Filter(Pall, USA) using a peristaltic pump (Heidolph, Germany).

Table 18: Stock solutions for GP5-Ant.

solution	ingredients	concentration in g/L in deionised water
KNO_3	potassium nitrate	100.00
K_2HPO_4	di-potassium hydrogen orthophosphate	34.80
vitamins solution	cyanocobalamin (B12)	0.001
	thiamine (B1)	1.000
	biotin (B7)	0.002
PII-metals*	$Na_2EDTA \times 2H_2O$ ethylenediaminotetra-acetic acid disodium salt (Boehringer 808261)	6.000
	$FeCl_3 \times 6H_2O$ ferric chloride (Sigma F2877)	0.290
	H_3BO_3 orthoboric acid (Sigma B6768)	6.850
	$MnCl_2 \times 4H_2O$ manganese chloride (Merck 1.05927.0100)	0.860
	$ZnCl_2$ zinc chloride (Sigma-Aldrich 208086 – 5g)	0.060
	$CoCl_2 \times 6H_2O$ cobaltous chloride (Sigma C2911)	0.026
Soil-Extract	as per particulars given below**	

*Pre-treatment of prepared PII-metals solution: stirring for 20min to 30min at 40°C

**Preparation steps for Soil-Extract:

- 2cm-layer garden soil without chemical contamination in a 1L flask covered with 1L distilled water, after leaving it for a few hours autoclave it for 30min
- Let it cool down and allow to settle then pour off the supernatant and centrifugate
- Pour in 200mL DURAN® flask (DURAN Group, Mainz, Germany) and sterilize again
- Let it cool down and freeze in labelled 50mL Falcon™ tubes (BD Biosciences Franklin Lakes, US-NJ)

c) Harvesting Protocol

1. Cool down the centrifuge (Eppendorf-Centrifuge 5810 R) to 4°C
2. Fill up a small Dewar vessel (Roth, Germany) with liquid nitrogen
3. Label 8 cultures: A,B,C,D,E,F,G,H
4. Label for each culture 3x50mL centrifuge tubes: 3xA, 3xB, etc.
5. Loosen cells with a cell scraper and fill up the 50mL tubes with culture

Steps in one line are performed at the same time

A,B,C,D [1]	**E,F,G,H** [1]
centrifuge A,B,C,D 50mL tubes: 4min, 2500rpm*	
Discard the supernatant Divide the rest of the culture to the 50mL tubes Fill up the falcons with Antarctic seawater (H_20-Ant) to balance weights	centrifuge E,F,G,H 50mL tubes: 4min, 2500rpm*
centrifuge A,B,C,D 50mL tubes: 4min, 2500rpm*	Discard the supernatant Divide the rest of the culture to the 50mL tubes Fill up the falcons with Antarctic seawater to balance weights
First washing step: Discard the supernatant and fill up to 40mL with H_20-Ant, resuspend the pellet	centrifuge E,F,G,H 50mL tubes: 4min, 2500rpm*
centrifuge A,B,C,D 50mL tubes: 4min, 2500rpm*	First washing step: Discard the supernatant and fill up to 40mL with H_20-Ant, resuspend the pellet
Second washing step: Discard the supernatant and fill up to 40mL with H_20-Ant, resuspend the pellet	centrifuge E,F,G,H 50mL tubes: 4min, 2500rpm*
centrifuge A,B,C,D 50mL tubes: 4min, 2500rpm*	Second washing step: Discard the supernatant and fill up to 40mL with H_20-Ant, resuspend the pellet
Discard the supernatant Resuspend the pellets in max. 5ml H_20-Ant These 5ml of the three 50ml falcons for each isolate are filled into one labelled 15ml tube.	centrifuge E,F,G,H 50mL tubes: 4min, 2500rpm* Discard the supernatant Resuspend the pellets in max. 5ml H_20-Ant These 5ml of the three 50ml falcons for each isolate are filled into one labelled 15ml tube.
Centrifuge all eight 15mL tubes: 6min, 4000rpm	
Discard the supernatant and freeze the tubes with the pellet immediately in liquid N_2 → after 10min store the frozen samples at -20°C	

* Up to 3100rpm if no pellet is established

[1] the four cultures centrifuged at the same time in the 50mL tubes should either be all colony-forming cultures or cultures with attached aggregates, since mostly colony-forming cultures need higher rpm-numbers

d) Mean viability data set

The following tables show the mean viability values with their standard deviation of each culture for each treatment calculated using the equations Eq. (1) to Eq. (3).

Table 19: mean viability values of cryopreserved *P. antarctica* samples used for vital staining

	69/82-10	FG	FG-stored	MC	MC-stored	69/770-50	DMSO+FG	MeOH+FG	FG
after thawing	**living cells**	no data	no data	40±22%	31±13%	**living cells**	24±13%	52±12%	60±19%
	living AA			56±10%	11±10%	**living AA**	14±21%	64±17%	65±16%
	viability of AA			31±8%	6±7%	**viability of AA**	11±23%	57±16%	34±8%
after two weeks	**living cells**	7±9%	23±23%	11±7%	24±21%	**living cells**	59±3%	44±13%	61±7%
	living AA	60±21%	71±35%	18±23%	34±32%	**living AA**	25±5%	51±12%	55±34%
	viability of AA	24±11%	37±15%	6±7%	21±34%	**viability of AA**	20±6%	39±8%	47±35%

Table 20: mean viability values of cryopreserved *P. antarctica* samples used for vital staining

		DMSO+FG	DMSO+MC	MeOH+FG	MeOH+MC	FG	FG-stored	MC	MC-stored
#25 after thawing	**living cells**	39±29%	33±2%	41±37%	29±7%	28±12%	15±7%	19±19%	26±17%
	living colonies	71±46%	71±6%	67±20%	77±16%	56±52%	74±34%	31±20%	64±13%
	viability of colonies	40±44%	50±17%	22±8%	36±23%	25±30%	32±12%	9±2%	36±6%
#25 after two weeks	**living cells**	9±10%	48±7%	16±11%	**67±64%	27±28%	14±4%	40±26%	31±4%
	living colonies	34±40%	**68±3%	**64±70%	*100±0%	18±23%	48±17%	**53±5%	*100±0%
	viability of colonies	9±12%	**21±6%	**16±17%	*25±0%	4±6%	15±5%	**16±1%	*75±0%
69/109-2 after thawing	**living cells**	8±10%	52±8%	24±1%	53±13%	43±6%	39±1%	78±5%	40±24%
	living colonies	**58±24%	80±32%	62±17%	78±26%	80±26%	78±24%	100±0%	*100±0%
	viability of colonies	**27±43%	75±72%	18±4%	46±10%	40±12%	34±8%	81±22%	*50±0%
69/109-2 after two weeks	**living cells**	23±51%	28±7%	8±20%	17±6%	27±10%	9±13%	32±38%	**33±97%
	living colonies	**0±0%	**35±43%	**10±29%	13±21%	50±68%	0±0%	*75±0%	*100±0%
	viability of colonies	**0±0%	**13±16%	**5±15%	4±8%	21±35%	0±0%	*31±0%	*50±0%
69/875-86 after thawing	**living cells**	63±17%	32±15%	26±16%	25±3%	20±1%	22±2%	20±18%	26±6%
	living colonies	58±37%	80±17%	62±9%	82±18%	82±7%	71±6%	68±25%	47±35%
	viability of colonies	32±17%	36±20%	22±7%	34±4%	45±7%	31±1%	21±11%	17±13%
69/875-86 after two weeks	**living cells**	33±2%	14±15%	32±16%	**78±7%	9±9%	7±4%	*7±0%	7±16%
	living colonies	48±61%	16±21%	27±15%	**83±49%	26±4%	40±7%	6±7%	28±25%
	viability of colonies	15±22%	6±3%	11±14%	**27±30%	8±1%	11±1%	1±2%	8±8%

*one 10µL sample out of the triplicate existed, the other two samples didn't contain any algae

**two 10µL samples out of the triplicate existed, the other one sample didn't contain any algae

Table 21: mean viability values of cryopreserved *P. antarctica* samples used for vital staining

		DMSO+FG	DMSO+MC	MeOH+FG	MeOH+MC	FG	FG-stored	MC	MC-stored
69/905-100 after thawing	**living cells**	**39±42%	65±6%	**39±3%	48±12%	no data	no data	60±21%	43±6%
	living colonies	*50±0%	91±16%	*100±0%	90±15%			91±14%	67±78%
	viability of colonies	*13±0%	53±23%	*25±0%	44±7%			42±13%	33±39%
69/905-100 after two weeks	**living cells**	10±23%	80±16%	19±19%	48±31%	8±7%	17±8%	78±39%	63±23%
	living colonies	**75±73%	100±0%	-	67±78%	*100±0%	**50±146%	76±21%	89±26%
	viability of colonies	**31±55%	53±16%	-	33±52%	*50±0%	**25±73%	52±13%	64±26%
VIMS 119-1 after thawing	**living cells**	58±7%	41±30%	**66±33%	62±12%	34±15%	**40±0%	66±12%	44±25%
	living AA	85±18%	73±11%	76±31%	81±3%	13±21%	48±4%	82±21%	39±26%
	viability of AA	27±4%	36±2%	27±17%	31±7%	5±10%	17±7%	39±13%	18±12%
VIMS 119-1 after two weeks	**living cells**	43±67%	8±11%	29±6%	22±18%	9±14%	5±11%	0±0%	0±0%
	living AA	*100±0%	7±16%	24±34%	**50±146%	56±69%	**0±0%	0±0%	0±0%
	viability of AA	*42±0%	2±4%	9±15%	**22±64%	22±35%	**0±0%	0±0%	0±0%
69/71-5 after thawing	**living cells**	no data	51±14%	no data	35±4%	no data	not performed	24±7%	no data
	living AA		53±27%		52±6%			17±21%	
	viability of AA		27±21%		22±9%			5±7%	
69/71-5 after two weeks	**living cells**	17±10%	35±26%	10±6%	0±0%	no data		20±5%	0±0%
	living AA	59±11%	22±32%	33±78%	0±0%			7±8%	0±0%
	viability of AA	22±3%	10±18%	17±39%	0±0%			2±2%	0±0%

*one 10µL sample out of the triplicate existed, the other two samples didn't contain any algae

**two 10µL samples out of the triplicate existed, the other one sample didn't contain any algae

e) Counting data set

The following tables show the percent values calculated from the counting analyse.

Table 22: viability values in % of cryopreserved *P. antarctica* 10µL samples used for vital staining

	DMSO+FG		MeOH+FG		FG-s		FG		DMSO+MC		MeOH+MC		MC-s		MC	
#25	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks
living cells	63	17	26	10	9	14	25	9	32	54	29	100	14	33	16	18
	20	5	72	25	17	10	38	23	35	44	24	33	26	29	6	53
	35	4	24	14	20	17	20	50	33	46	34	-	38	-	34	48
living colonies	80	67	60	27	71	47	33	20	67	67	81	100	60	100	14	50
	100	25	57	100	100	61	100	33	75	70	86	-	57	-	36	56
	33	10	83	-	50	36	33	0	71	-	64	-	75	-	43	-
viability of colonies	35	19	20	7	30	14	8	5	58	17	36	25	35	75	7	16
	75	6	18	25	42	18	50	8	56	25	53	-	32	-	9	17
	11	3	29	-	25	11	17	0	36	-	20	-	41	-	11	-
69/109-2																
living cells	0	67	23	0	40	0	44	33	47	33	63	18	55	67	82	50
	9	3	24	0	39	19	39	30	50	24	53	21	20	0	78	0
	14	0	24	25	40	9	47	19	58	25	43	12	44	-	75	47
living colonies	67	0	60	20	85	0	79	0	86	21	100	31	100	100	100	75
	50	0	50	0	57	0	62	50	100	50	67	9	100	-	100	-
	-	-	75	-	90	0	100	100	54	-	67	0	-	-	100	-
viability of colonies	42	0	20	10	34	0	38	0	57	8	55	11	50	50	100	31
	13	0	15	0	29	0	33	13	75	19	42	2	50	-	75	-
	-	-	19	-	40	0	50	50	26	-	42	0	-	-	69	-

-no algae

Table 23: viability values in % of cryopreserved *P. antarctica* 10µL samples used for vital staining

	DMSO+FG		**MeOH+FG**		**FG-s**		**FG**		**DMSO+MC**		**MeOH+MC**		**MC-s**		**MC**	
69/875-86	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks
living cells	76	33	38	20	24	4	21	10	32	24	27	80	27	0	22	7
	61	32	14	44	20	9	20	16	43	16	23	75	30	0	7	-
	51	35	26	31	22	9	19	2	21	3	24	-	21	21	32	-
living colonies	82	100	67	38	65	37	85	23	91	31	89	67	59	24	79	10
	29	25	55	17	74	46	76	27	82	16	89	100	17	11	77	0
	63	20	65	25	73	38	86	29	67	0	67	-	64	47	47	8
viability of colonies	39	33	27	23	31	11	46	8	52	8	35	17	22	8	30	3
	18	6	17	4	32	12	39	9	34	4	37	38	5	3	21	0
	39	5	21	6	31	11	49	7	23	5	31	-	23	14	13	2
69/905-100																
living cells	25	0	38	5	no data	23		14	61	87	50	22	42	68	76	90
	54	0	40	19		12		7	70	67	57	58	40	44	58	46
	-	-	29	33		15		4	64	87	39	64	48	77	45	100
living colonies	50	100	100	-		100		100	100	100	100	0	100	67	92	83
	-	50	-	-		0		-	78	100	92	100	0	100	80	58
	-	-	-	-		-		-	95	100	78	100	100	100	100	86
viability of colonies	13	50	25	-		50		50	70	67	42	0	50	42	52	58
	-	13	-	-		0		-	36	44	50	25	0	75	40	42
	-	-	-	-		-		-	51	50	42	75	50	75	33	57

-no algae

Table 24: viability values in % of cryopreserved *P. antarctica* 10µL samples used for vital staining

	DMSO+FG		MeOH+FG		FG-s		FG		DMSO+MC		MeOH+MC		MC-s		MC	
VIMS 119-1	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks
living cells	64	14	77	30	40	14	22	6	67	8	69	31	52	0	56	0
	55	14	55	24	40	0	36	0	28	17	52	29	57	0	71	0
	56	100	-	32	-	0	44	20	30	0	64	6	23	0	71	0
living AA	75	100	54	0	50	0	30	100	79	20	80	100	45	0	74	0
	80	-	75	23	44	0	8	0	64	0	83	0	18	0	100	0
	100	-	100	50	50	-	0	67	77	0	80	-	54	0	72	0
viability of AA	25	42	17	0	17	0	14	50	38	5	35	44	21	0	36	0
	30	-	23	6	13	0	2	0	35	0	33	0	7	0	50	0
	25	-	42	21	23	-	0	17	36	0	25	-	24	0	31	0
69/71-5																
living cells	no data	25	no data	7	not performed		no data	no data	42	34	31	0	no data	0	26	18
		13		15					51	16	36	0		0	28	18
		13		10					62	54	37	0		0	19	25
living AA	no data	67	no data	0			no data	no data	72	8	57	0	no data	0	35	10
		60		100					56	9	50	0		0	6	11
		50		0					32	50	50	0		0	10	0
viability of AA	no data	21	no data	0			no data	no data	41	2	29	0	no data	0	11	3
		25		50					28	2	16	0		0	2	3
		21		0					11	25	22	0		0	2	0

-no algae

Table 25: viability values in % of cryopreserved 69/82-10 10µL samples used for vital staining

	FG-s		FG		MC-s		MC	
	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks
living cells	no data	6	no data	7	35	29	58	7
		22		13	20	6	32	9
		40		0	38	36	29	17
living AA	no data	64	no data	50	20	58	64	33
		100		78	5	33	49	20
		50		53	9	11	53	0
viability of AA	no data	30	no data	15	12	50	36	10
		50		31	4	8	24	7
		31		25	2	5	34	0

Table 26: viability values in % of cryopreserved 69/770-50 10µL samples used for vital staining

	DMSO+FG		MeOH+FG		FG	
	thawing	2 weeks	thawing	2 weeks	thawing	2 weeks
living cells	34	60	51	55	66	62
	14	60	43	40	43	65
	22	56	61	36	69	55
living AA	11	28	75	56	54	53
	0	25	50	40	78	81
	30	21	67	56	63	30
viability of AA	3	19	71	42	40	40
	0	25	50	33	33	76
	30	16	50	44	28	26

Table 27: viability values in % of cryopreserved *F. kerguelensis* 10µL samples used for vital staining

	DMSO+FG	MeOH+FG	FG-s	DMSO+MC	MeOH+MC	MC
living cells after thawing	35	28	45	70	78	74
	28	35	30	71	68	79
	13	26	43	71	80	78
living cells after 2 weeks	61	47	83	72	51	39
	53	57	66	36	35	32
	58	68	89	70	51	32