frontiers in Marine Science

PERSPECTIVE
published: 24 July 2019
doi: 10.3389/fmars.2019.00454

The Use of Cryopreserved Biological Material for Water Quality Assessment

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The stated aim of this perspective article is to present new developments and discuss future directions on the applications of cryopreserved organisms to marine water quality assessment. To facilitate this, the authors provide a background of essential knowledge of cryopreservation when applied to ecotoxicology, as well as, practical examples available in literature. An integrated approach with combined monitoring of chemical status plus measurements of biological effects has been recommended extensively by international institutions for the assessment of marine pollution. Among the available techniques, bioassays have been considered as sufficiently robust to be incorporated in marine pollution monitoring programs. However, the routine application of bioassays has also allowed the identification of one of the factors that limits a more extensive use of such biological methods: the availability of biological material throughout the year, regardless of natural spawning periods. A solution to this limitation is the application of cryopreservation techniques. Cryopreservation may, for instance, provide access to stable quality biological material when test species are out of the reproductive season, without the need for maintaining and conditioning organisms in the laboratory. It also guarantees access to a large variety of species that might not be available at the same time of the year and, on top of that, cryopreservation provides opportunities to laboratories that might not have the facilities to keep all these organisms in culture.

Keywords: cryopreservation, water quality assessment, cryobiology, bioassay, model organisms

OPEN ACCESS

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Specialty section:
This article was submitted to Marine Pollution, a section of the journal Frontiers in Marine Science

Received: *12 December 2018*
Accepted: *08 July 2019*
Published: *24 July 2019*

Citation:
Paredes E and Bellas J (2019) The Use of Cryopreserved Biological Material for Water Quality Assessment. Front. Mar. Sci. 6:454. doi: 10.3389/fmars.2019.00454

INTRODUCTION

Water quality assessment is crucial for achieving good chemical and biological status throughout coastal waters and current approaches include the monitoring of responses at different levels of biological organization to indicate effects on the ecosystem. Integrative approaches, intended for the protection of the marine environment, are based mainly on the use of biological tools at different trophic levels in combination with chemical measures, in order to establish environmental damage thresholds (Lyons et al., 2010). In fact, the European Union Marine Strategy Framework Directive (2008/56/EC), which has the objective of achieving and maintaining the Good Environmental Status (GES) in European seas by 2020, emphasizes the need to evaluate and keep within acceptable limits the biological effects of pollutants.

Chemical analyses can identify many contaminants present in the environment, whilst biological methods permit to obtain ecologically relevant information. Among the biological tools that have been considered sufficiently robust for marine pollution assessment, ecotoxicological bioassays present several advantages such as: the detection of new pollutants for which analytical techniques have not yet been developed, provide information about the bioavailability of the pollutants (i.e., the fraction of pollutant that can be incorporated by the organism); they allow to integrate the toxic effects of the different substances present in the environment, and present a good cost/effect ratio (e.g., Stebbing et al., 1980; Calow, 1993).

As useful as they can be, the application of biological techniques using bioassays in routine monitoring has allowed to identify one of the factors that limit a more extensive use of this tools: obtaining biological material of stable high quality throughout the year, regardless of the natural spawning periods (His et al., 1999a).

A great number of response variables can be measured at different levels of biological organization and at different trophic levels in order to determine the GES of the marine environment (e.g., Lyons et al., 2010; Davies and Vethaak, 2012). A wide range of organisms have been considered for marine pollution monitoring, including microorganisms like marine bacteria (Gellert, 2000; Parvez et al., 2006), microalgae (Debelius et al., 2009; Aylagas et al., 2014; Araujo and Moreno-Garrido, 2015), marine invertebrates (Snell and Persoone, 1989; His et al., 1999a; Bellas et al., 2005; Bellas, 2008; Laranjeiro et al., 2015; Perez Fernández et al., 2015) or fish (Hutchinson et al., 1994; EPA, 2002), in all these examples the endpoints are either hatching, growth or normal development along time.

The cryopreservation and cryobanking of test organisms to be used for marine quality assessment, could ensure the accessibility to organisms or their reproductive material all year round as an alternative to either conditioning adults or continuous culture efforts for availability of biological material, which is a very time consuming and expensive process. Biobanking these test organisms in a stable manner (below −135°C) is possible, either using liquid nitrogen or ultrafreezers. At this low temperature, no chemical reactions take place and cellular metabolism is on hold. These stored cells are stopped in time and their viability would only be affected by background radiation, which at normal level will take 2000 years to become a hazard to stored cells (Glenister et al., 1984). There are not many marine cells biobanks apart from culture collections (usually microalgae and/or bacteria), but this is beginning to change (mainly at local level) as cryopreservation becomes a more popular tool and many Marine Biological Research Stations acquire biobanking equipment.

The aim of this perspective paper is to present new developments and discuss future directions on the applications of cryopreserved organisms to marine water quality assessment. To facilitate this, the authors provide a background of essential knowledge of cryopreservation when applied to ecotoxicology, as well as, practical examples available in literature.

CRYOPRESERVATION AND MARINE WATER QUALITY ASSESSMENT

The application of cryopreservation techniques to marine water quality assessment requires the development and standardization of specific cryopreservation protocols for different types of organisms. The main question that needed to be answered was if cryopreserved organisms would be sensitive enough to detect gradual increases of toxic compounds in the water. If so, they could be used to obtain dose-response curves. It was also necessary to compare and establish the differences, or lack thereof, in sensitivity when using fresh and cryopreserved biological material. Regarding the first point, as listed below it has been proved that cryopreserved organisms can be used to detect gradual increases in the concentrations of chemical compounds present in the water, both with single chemicals and with complex natural samples. Cryopreserved organisms can therefore be used to produce dose-response curves and to obtain the No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC), or 10 and 50% Effective Concentrations (EC10 or EC50), as well as their fresh counterparts. In this paper we present a comprehensive list indicating examples of bioassays that specifically reported the use of cryopreserved organisms as an alternative to standard bioassays with fresh organisms (methodological information is indicated in **Table 1**), each case will be discussed in terms of their comparability with the standard method (toxicological information is indicated in **Table 2**).

Cryopreserved Microalgae

Microalgae are an important part of the food chain in the ocean. A disruption of the basis of the food chain would have deep long lasting effects in the ecosystems and therefore they are of high ecotoxicological relevance (Arensberg et al., 1995; Geis et al., 2000). It has been shown that microalgae are more sensitive than other test organisms to some compounds like metals (Wong and Beaver, 1980; Satoh et al., 2005; Araújo et al., 2010) detergents (Lewis, 1990) or herbicides (Pavlic et al., 2006).

Use of cryopreserved freshwater algae *Selenastrum capricornutum* in ecotoxicity testing has been evaluated by Benhra et al. (1997). Experiments compared the performance of this method, named Cryoalgotox, versus the classic microplate test using fresh algae. *S. capricornutum* was cryopreserved by slow cooling (**Table 1**) using 10% (v/w) polyvinylpyrrolidone (PVP) as a cryoprotecting agent (CPA) giving comparable toxicity results. After 72 h incubation, Cryoalgotox produced lower 50% effective concentrations (EC50s) for Cd^{2+}, Cu^{2+}, Cr^{6+}, and atrazine (i.e., higher sensitivity) than the classical microplate tests, which was explained by the periodic renewal of the test medium in the semistatic procedure. This test assay using cryopreserved microalgae produced highly repeatable results (low coefficients of variation).

Hundreds of cryopreservation protocols have been published for both freshwater and marine microalgae that could potentially be used to develop more bioassays with cryopreserved material. Despite most of the microalgae currently held in culture collections are kept cryopreserved and, therefore, most of the microalgae toxicity test are probably carried out with algae

TABLE 1 | Cryopreserved marine organisms that had been used as an alternative to fresh standard methods for evaluating marine water quality.

Species	Viability	Cryopreservation and conservation	Advantages	Comparison with standard
S. capricornutum (Benhra et al., 1997)	Toxicity tests with cryopreserved algae lead to lower EC50s than standard methods. High repeatability and reliability.	Cryoprotectant used is 10 %PVP (w/v), Addition (1:1) of the cryoprotectant to the algae and allow to equilibrate for 30 min at 22°C under light. Cells were cooled at 1.5°C min^{-1} until −30°C and faster at 10.5°C/min until −80°C. Storage in a −80°C freezer up to 90 days. Thawing by immersion in a water bath 37°C until ice melting. No washing of the PVP needed prior inoculation for culture.	Rapid method, no preculture needed. Cost-effectiveness by elimination of algal stock cultures.	Ratio between EC50s obtained by classic/cryoalgotox rages from 1.3 to 1.4
C. gigas and *T. philippinarum* (McFadzen, 1992)	They concluded that cryopreserved D-veliger larvae were sensitive to environmentally realistic levels of contaminants and can be used for water quality assessment.	Patent number PCT/GB90/01267 Filled on 13/08/1990. Cooling from 20°C to −20°C at 16°C min^{-1} and then at 45°C min^{-1} to −45°C then stored in liquid nitrogen preferably. Cryprotecting agents used were 15% Me_2SO (v/v) + 1 M Trehalose and 0.5 $mgml^{-1}$ crystallized cholesterol. Thawing in water bath at 22–28°C	Immediate access to biological material all year-round.	N/A
P. lividus (Paredes and Bellas, 2015)	Comparative bioassays with fresh/cryopreserved sea urchin embryos. Cryopreserved embryos usually yielded more sensitive results. Can be used for water quality assessment.	Cryopreservation protocol using Me_2SO 1.5 M + 0.04 M Trehalose. One milliliter of CPA solution was added in 15 equimolar steps 1 min apart. The cooling ramp started with a hold at 4°C for 2 min, cooled at a rate of 1°C min^{-1} to −12°C, followed by cooling at 1°C min^{-1} to −80°C and vials were transferred to liquid nitrogen for storage. Thawing was performed by immersion into a 17°C water bath until the ice was melted. CPAs were then removed in 12 equimolar steps.	Immediate access to biological material all year-round.	Correlation between EC50s obtained with classic/cryopreserved sea urchin embryos is $y = 0.68x+0.53$, $n = 4$
S. aurata (Fabbrocini et al., 2013)	Toxicity tests with cryopreserved sperm. Analysis of motility parameters visually and sperm velocity with CASA. Cryopreserved sperm can be sufficiently sensitive to be used for bioassays.	Cryopreservation protocol detailed in Fabbrocini et al. (2000). The Cryoprotecting agent used was 5% Me_2SO. Cooled in straws at 10–15°C min^{-1} to −150 and stored in liquid nitrogen. Thawing at 15°C min^{-1}.	Rapid and easy method.	Computer assisted analysis of the samples lead to significantly lower NOEC/LOEC values than visual examination of motility. CASA parameters produce a LOEC Coherent with other fish sperm samples.

Me_2SO stands for Dimethyl sulfoxide.

that had been cryopreserved at some point, there are no other published comparisons for cultured vs. cryopreserved marine microalgae as far as the authors know.

Cryopreserved Molluscs

Molluscs have been extensively used for several ecotoxicological tests, among which stands out the embryo-larval bioassay (International Council for the Exploration of the Sea (ICES), 1991; His et al., 1999b). The high sensitivity of early-life stages allows the detection of low pollution levels by the identification of effects in the embryonic development (delays or morphological abnormalities) after a short period of exposure/incubation in the presence of a toxicant or a water sample of unknown quality. Oysters, such as *Crassostrea gigas* (His et al., 1999b; Leverett and Thain, 2013) and mussels, such as *Mytilus edulis* (Nolan and Duke, 1983) or *Mytilus galloprovincialis* (His et al., 1997; Beiras and Bellas, 2008), are the star test species for this procedure for being well known and studies species but also for their worldwide distribution.

Cryopreserved bivalve larvae (*C. gigas* and *Tapes philippinarum* larvae) have been exposed to different water samples and shown to be sensitive to environmentally realistic levels of contaminants for field monitoring of water quality (McFadzen, 1992). This was the first attempt to use cryopreserved cells of any type for ecotoxicology studies proving that those cells retain the sensitivity to chemicals and could be used for bioassays.

Larvae were cryopreserved at 24 h for *C. gigas* and 48 h for *T. philippinarum* at the late trochophore/early D-veliger stages (**Table 1**) and stored in liquid nitrogen at (−196°C), while using 15% dimethyl sulfoxide (v/v) with 1.0 M Trehalose and

TABLE 2 | Available toxicological information for different contaminants using cryopreserved cells.

Organism	Compound	Standard method (μgL^{-1})	Cryopreserved (μgL^{-1})
S. capricornutum	Cd^{2+}	72 h EC50 ± *SD* = 43.5 ± 3.4	72 h EC50 ± *SD* = 31.8 ± 0.9
S. aurata		N/A	CASA Motility parameters LOEC = 10
S. capricornutum	Cu^{2+}	72 h EC50 ± *SD* = 28.5 ± 2.8	72 h EC50 ± *SD* = 21.7 ± 0.8
P. lividus		48 h EC50 95% c.i. = 34.1 (31.9–63.4)	96 h EC50 95% c.i. = 53.7 (51.9–55.5)
S. capricornutum	Cr^{6+}	48 h EC50 ± *SD* = 139.1 ± 31.1	96 h EC50 ± *SD* = 74.3 ± 5
S. capricornutum	Antrazine	48 h EC50 ± *SD* = 164.3 ± 37	96 h EC50 ± *SD* = 92.9 ± 2
P. lividus	Pb^{2+}	48 h EC50 95% c.i. = 425 (236.8–590.1)	96 h EC50 95% c.i. = 81 (79.1–83.0)
P. lividus	BP-3	48 h EC50 95% c.i. = 4048.6 (1950.6–6218.7)	96 h EC50 95% c.i. = 1541 (1257.5–1824.5)
P. lividus	4-MBC	48 h EC50 95% c.i. = 389.2 (254.8–523.6)	96 h EC50 95% c.i. = 300.6 (141.2–460.0)

Heavy metals like Cadmium (Cd^{2+}), Copper (Cu^{2+}), Chrome (Cr^{6+}), or Lead (Pb^{2+}), pesticides like Antrazine or emerging pollutants like UV-filters like 4-methylbenzylidene-camphor (4-MBC) and benzophenone-3 (BP-3). EC50 data provided with either the standard deviation or 95% confidence intervals (C.I.) for: microalgae (S. capricornutum),fish sperm (S. aurata), and sea urchin embryos (P. lividus). References available in ***Table 1****.*

0.5 mg/ml cholesterol as CPAs. Survival was reported as highly variable upon thawing. Despite no comparison between fresh and cryopreserved cells was carried out at the time, cryopreserved cells responded to toxicity and allowed for the calculation of toxicological parameters.

The description of cryopreservation protocols for marine invertebrates is also flourishing and protocols for molluscs like the mussels *M. galloprovincialis* (Paredes et al., 2013) and *Perna canaliculus* (Paredes et al., 2012) have been developed. Results with bivalves are promising, since the cryopreservation methods for these organisms have been proven to be reliable, repeatable and sensitive, being on an advanced stage of development. A way forward would be to test the comparison between the procedures with cryopreserved organisms and standard tests, which have not yet been performed.

Cryopreserved Echinoids

Sea urchins are other of the classic models (Bellas et al., 2005; Durán and Beiras, 2010) for water quality testing. Paredes and Bellas (2015) established for the first time a bioassay using cryopreserved sea-urchin embryos (*Paracentrotus lividus*) (Paredes et al., 2011) and provided a comparison with the already standardized sea urchin embryo larval bioassay for standard chemicals like copper and lead (**Figure 1**).

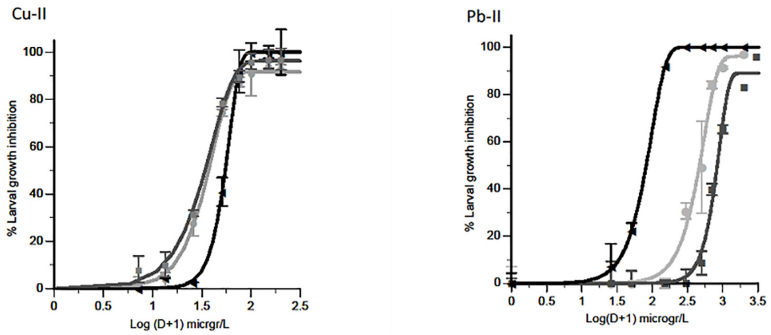
Sea urchin embryos (early blastula) were cryopreserved using 1.5 M dimethyl sulfoxide plus 0.04 M trehalose and cooled at 1°C min^{-1} (protocol in **Table 1**). Samples were then stored in liquid nitrogen. These experiments showed that there was no significant loss in sensitivity when using early blastulas instead of fresh fertilized oocytes. Paredes and Bellas (2015) did find differences in sensitivity when using cryopreserved vs. fresh cells, in some cases the differences were minimal, in other cases the cryopreserved test was clearly more sensitive (**Table 2**). This increased sensitivity may be because cryopreserved organisms are going through a recovery process after thawing, and might be more sensitive to additional stress, such as toxicant exposure.

Ribeiro et al. (2018) developed a cryopreservation protocol for *Echinometra lucunter* sperm and they are studying the cryopreservation of embryos for water quality assessment. There is a cryopreservation protocol described for *P. lividus* sperm (Fabbrocini et al., 2014) that yields good motility. Cryopreservation protocols already exist or are under development for different developmental stages for 10–14 different sea urchin species (embryos and sperm), and since sea urchins are a highly demanded model, more applications will probably be further developed soon using cryopreserved cells, including toxicology (Paredes, 2015a).

Cryopreserved Fish Sperm

The case of fish cryopreservation (but also crustaceans) is more complicated, since these organisms are very sensitive to low temperatures and have proven exceptionally difficult to cryopreserve, being fish sperm the only exception. There has been exhaustive research on marine fish sperm cryopreservation and protocols have been described for most farmed species (*Sparus aurata* by Fabbrocini et al., 2012, 2016; *Dicentrarchus labrax* by Fauvel et al., 1998; or *Mugil cephalus* by Balamurugan and Munuswamy, 2017), any of which could be used as a biomonitoring test organism.

The study by Fabbrocini et al. (2013) evaluated the feasibility of using cryopreserved *S. aurata* spermatozoa to be used in toxicity tests (**Table 1**). Sperm motility parameters were evaluated



	Embryo-larval bioassays EC50 (μg L^{-1})		
	Standard	Fresh embryo	Cryopreserved embryo
Cu^{2+}	34.1 (31.9 - 36.4)	32.2 (29.1 - 35.3)	53.7 (51.9 - 55.5)
Pb^{2+}	425 (263.8 - 590.1)	752 (688.7 - 816.2)	81 (79.1 - 83.0)

FIGURE 1 | Copper and lead toxicity tests. Larval growth inhibition at each concentration ± *SD* (*n* = 35). Light gray, dark gray, and black represent the standard bioassay, the fresh embryo bioassay and cryopreserved embryo bioassay, respectively. Table attached shows EC50s (μg L^{-1}) and 95% confidence intervals (in brackets). Figures modified from Paredes and Bellas (2015).

after thawing by a computer-assisted analysis. The sensitivity of the sperm (motility percentages and velocities) to a reference toxicant (cadmium) was comparable to what has been recorded for the fresh sperm of other aquatic species (**Table 2**). The test was found to be sensitive, rapid, easy to perform and showed good reliability.

DISCUSSION

Bioassays have been widely reported to provide a lot of information and be very useful for water quality assessment but in many cases there is either a need for maintaining breeding animals in the lab for out of season use (if possible) or some tests have a very marked seasonality (matching the spawning season of the test species). Using cryopreserved biological material is a good option to overcome this constraint, but it is crucial to be able to compare the results of the procedure with cryopreserved material to the standard tests.

According to Cairns and Pratt (1989) extrapolations from bioassays on one species to another species are not straightforward and results are only comparable in some cases. From the studies reviewed here, the same principle can be applied to the comparison between bioassays with cryopreserved cells and standard bioassays. Cryopreservation is a very useful add-on to an already developed testing methodology that could help increasing the use of bioassays. On the other hand, until an exhaustive comparison and compilation of data takes place and a robust correspondence between bioassays with cultured vs. cryopreserved organisms can be obtained with a reliable level of certainty, these results should be taken with precaution.

The advantages of using cryopreserved biological material for bioassays are many: from providing a reliable source of cells and organisms that can be stored for out of season need, to provide flexibility to the analyser. Making possible the simultaneous testing with a battery of organisms that do not reproduce at the same time of the year, without having to hold the animals in the lab for out of season production, which is costly and labor intensive. Last but not least, it also aligns with the 3R's of animal welfare principle of reduction, by allowing the storage of unused material for other experiments therefore reducing the number of animals used per trial. As more marine organisms have been successfully cryopreserved, including different cells or development stages, there is great potential for this to continue to develop (Suquet et al., 2000; Paredes, 2015b).

Many of the microalgae currently held in culture collections are kept cryopreserved, there are also available protocols for different molluscs (Wang et al., 2011; Paredes et al., 2013) and being mussels the most widely used organism for biomonitoring, this is another potential candidate for the development of a cryopreserved toxicity test in the near future. Regarding sea urchins, right now there are cryopreservation protocols developed or under development for different cells for 10–14 different sea urchin species, and being sea urchins a highly demanded model soon more applications will be developed, including toxicology (Paredes, 2015b).

Crustaceans are, as of today, not on the table as they have no reliable cryopreservation protocol. Fish are very sensitive to low temperatures and had proven exceptionally difficult to cryopreserve, being the only exception fish sperm. There has been exhaustive research on marine fish sperm cryopreservation and protocols have been described for the most farmed species (*S. aurata* by Fabbrocini et al., 2012; *D. labrax* by Fauvel et al., 1998; or *M. cephalus* by Balamurugan and Munuswamy, 2017), any of these could be used as a biomonitoring test organism and cryopreservation could enhance the possibilities of development

of this test. There is potential for the further use of this biotechnology applied to marine water quality assessment.

The parameters used as endpoints in the classic bioassays were characterized by good reliability and sensitivity but, when using cryopreserved cells those parameters might need a little adjusting in order to obtain the best results, For instance, cryopreserved cells develop slower in the first hours post-thaw, therefore experimental protocols need to be adjusted in terms of exposure duration; cryopreserved microalgae can show sensitivity to high light intensities immediately post-thaw so that light intensity needs to be lowered during the first hours of exposure. Cryopreserved samples can be easily stored and transferred, making it possible to perform bioassays in different sites or at different times and can even be part of long-term monitoring programs. Finally, the application of certain bioassays with cryopreserved material in environmental monitoring and risk assessment schemes, may allow the detection of lower concentrations of toxic substances that classical bioassays, which would offer a higher level of protection to marine ecosystems.

CONCLUSION

This is a perspective on the state of the art and critical analysis of the application of cryopreservation as a tool to improve toxicity testing. As of today, cryopreservation holds great potential as a tool to improve toxicity testing by solving, for instance, the seasonal shortage of biological material. On the other side, there is a need for extensive comparative testing in order to select those cryopreserved cells/protocols that can be more useful, either by developing new protocols for key cell types or making sure the cryopreservation outcome of the existing protocols is specifically designed to be used in a bioassay. There is also a need to obtain good and reliable correlations between methods with both fresh and cryopreserved biological material for a wide variety of chemical compounds. An extensive battery of comparisons using both methods will establish a frame of comparison that would enable researchers to use one or the other according with their practical needs and keep increasing the historical databases. Currently, the cryopreservation of *P. lividus* embryos and *S. aurata* sperm are in an advanced stage of development and present promising perspectives for their use in water quality assessment. As cryopreservation of aquatic marine resources continues to develop, the application of those preserved cells to toxicity testing will continue to expand.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the ASSEMBLE plus, grant from the European Union's Horizon 2020 Research and Innovation Programme (No. 730984).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.