Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review

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Abstract

Protocols for the cryopreservation of fish gametes have been developed for many different fish species, in special, freshwater salmonids and cyprinids. Methods for sperm freezing have progressed during the last decades due to the increasing number of potential applications: aquaculture (genetic improvement programs, broodstock management, helping with species having reproductive problems), biotechnology studies using model fish species (preservation of transgenic or mutant lines), cryobanking of genetic resources from endangered species, etc.

This mini-review tries to give an overview of the present situation of this area of research, identifying the main challenges and perspectives, redirecting the reader to more in-depth reviews and papers.

Keywords

Sperm; oocyte; aquaculture; cryobanking; endangered species; biotechnology

Highlights

- Freezing protocols have been developed for many fish species
- Gamete cryopreservation has applications in aquaculture, biotechnology or cryobanking
- The lack of standardization limits the industrial use of fish gamete cryopreservation
- PGCs, spermatogonia or somatic cells are alternatives for fish genome preservation
- The improvement of techniques for sperm quality evaluation is required
1. Progress

1.1. Applications of fish gamete cryopreservation

Cryopreservation of fish gametes has evolved during the last decades due to the increasing number of potential applications. The most evident is its use for aquaculture purposes, allowing the improvement of broodstock management at hatcheries (for example, modifying the offspring production season), preserving the genetically selected strains resulting from genetic improvement programs, or helping with species having reproductive problems as lack of synchronization in the gamete production of male and females (as in the case of the European eel, *Anguilla anguilla*; Asturiano et al., 2004) or with those having a low sperm production (as in the case of F1 Senegalese sole, *Solea senegalensis*; Cabrita et al., 2006).

Another potential application is the preservation of genetic material from individuals of natural populations of fish species in the initial phases of the domestication process and genetic modifications. This can assist in maintaining the original wild genotypes for the recovery of genes in the future (becoming a phenotypic backup), contrarily with happened for example in the case of the domestication process of bovine cattle (Vandeputte, 2011). Other possible conservation-related uses include the storage of genetic resources of the increasing number of fish in the lists of endangered species, allowing cryobanking for biodiversity (Van Der Walt et al., 1993; Martínez-Páramo et al., 2009, 2016), or in the case of fish species recently attracting the interest of cryobiologists and aquaculturists, mainly in South America and Asia (Viveiros and Godinho, 2009).

Moreover, the increasing use of aquatic models such as zebrafish in studies of biotechnology, toxicology or pharmacology, requires the use of transgenic lines, knockout and mutant strains that need adequate storage (Kollár et al., 2015; Tiersch et
1.2. Cryopreservation of fish sperm

Fish genome cryobanking has been attempted using different cell types (see section 2.3.; Labbé et al., 2013). However, spermatozoa have been the objective of most of the studies, making sperm cryopreservation the most established and commercialized technique. The choice of this type of cell is because it is easy to collect in most of the fish species, has a simple cellular structure and a small size and high chilling resistance, making these cells easy to preserve in many fish species. Moreover, reconstruction of individuals can be done by normal fertilization (or androgenesis), but it allows the preservation of only male germplasm.

Some previous publications have reviewed fish sperm cryopreservation subject (Suquet et al., 2000; Cabrita et al., 2009a; Kopeika and Kopeika, 2008; Tiersch and Green, 2011; Figueroa et al., 2014). The Table 1 summarizes studies on cryopreservation of sperm from fish species published during the last 15 years, including the cryoprotectants used (and their concentrations) and the best results obtained in each case in terms of post-thaw motility, cell viability and fertilization rates.

2. Challenges

2.1. When biodiversity means problems

With 25,000 to 30,000 species, fish are the largest group of vertebrates, displaying an extreme biodiversity (Near et al., 2013). This biodiversity is evident in the significant differences found in gamete (spermatozoa) morphology and biology (Mattei, 1991). During the cryopreservation steps of cooling, freezing and thawing, some biophysical and chemical processes such as osmotic changes, dehydration and
rehydration, cell volume changes, ice crystals formation, cryoprotectants toxicity, etc., occur and cells (gametes or others) are more or less sensitive to these changes being species-specific (Cabrita et al., 2014). Thus, cryopreservation protocols must be adapted to find species-specific compromises, and the increasing numbers of studies describing methods to cryopreserve sperm in many species, evidences this diversity (Cabrita et al., 2009a).

2.2. Lack of standardization: a problem to compare results and to arrive to the industry

The main objective has always been maintaining a high sperm fertilizing ability after thawing. However, the difficulties in obtaining reproducible results using sperm cryopreserved using the published methods have limited the use of cryopreserved sperm in production.

Recent scientific discussion have evidenced the need of standardization in different aspects of this area of research, as definition of basic concepts (extenders, cryoprotectant concentrations, dilution ratios), work protocols (sperm concentration determination, sperm cryopreservation methods, equilibration time, handling of straws, polystyrene box or controlled-rate freezer, type of vials, thawing systems, calculation of fertilization and hatching rates, osmolality measurements, sperm quality evaluation, etc) or even reporting of results (Rosenthal et al., 2010; Horváth et al., 2012a).

Although some recent efforts have been made in this regard (Benson et al., 2013; Gallego et al., 2012, 2013; Kása et al., 2014, 2015; Vilchez et al., 2014), new efforts must be made for a complete description and standardization of protocols for sperm cryopreservation, including a very wide area of topics: determination or estimation of sperm motility, substances used for activation of sperm, details of dilution of sperm with extender and cryoprotectants (new ones as the antifreeze proteins, AFPs, or better
combinations of classic ones), use of straws (sealed or unsealed), cooling of samples (dry ice vs. liquid nitrogen, styrofoam box vs. programmable freezer), methods of calculating fertilization and hatching results.

Regardless of the very high number of publications on this topic, few of the published methods have been adapted to aquaculture practice. There can be several reasons for this failure of application; however, one of them is beyond doubt the lack of standardization not only in methodologies but also in reporting them correctly. The difficulties in interpretation and replication of methods lead to a disappointment and ultimately rejection by the aquaculture industry. We also need to understand that in most fish species sperm is not a limiting factor during induced spawning and, moreover, individual selection is not as advanced in fish as it is in terrestrial livestock.

2.3. Alternative cells

Fish genome cryobanking has been attempted using different cell types: spermatozoa, oocytes, spermatogonia and primordial germ cells (PGCs), as well as somatic cells, blastomeres and embryos (Labbé et al., 2013).

The cryopreservation of fish oocytes has severe limitations because of their large cell volume, the presence of a chorion, the low permeability to cryoprotectants, and a high chilling sensitivity. Different studies have been carried out in zebrafish, as well as other marine and freshwater species, including cryoprotectant toxicity, chilling sensitivity, membrane permeability and cryopreservation (cooling rates, vitrification) of oocytes at different stages of development or ovarian fragments (Zhang et al., 2007; Godoy et al., 2013; Streit Jr. et al., 2014; Marques et al., 2015; reviewed by Martínez-Páramo et al., 2016). However, development of protocols for in vitro maturation of ovarian follicles after cryopreservation is required for the use of cryopreserved oocytes (Seki et al., 2008,
Thus, oocyte cryopreservation is still in its experimental phase and far from aquaculture applications. The preservation of spermatogonia and primordial germ cell guarantees the full individual genome. These cells have been cryopreserved successfully in several fish species (Yoshizaki et al., 2011; Robles et al., in press). However, its use requires the development of specific biotechnological tools, such as transplantation.

Fish embryo cryopreservation could be perfect for the establishment and management of genetic selection programs in fish farms. However, they have low membrane permeability, low surface-to-volume ratio, large size, high yolk content and high chilling sensitivity (Hagedorn and Kleinhans, 2000), which is the primary reason for the very limited number of preliminary positive results (Chen and Tian, 2005; Martínez-Páramo et al., 2008; Robles et al., 2005).

The cryopreservation of somatic (diploid) or embryonic cells (including PGCs) is an alternative to the cryobanking of gametes. They can be a good source of diploid genome to reconstruct fish (reviewed by Labbé et al., 2013). Moreover, they can easily be collected (e.g.: fins clips that regenerate easily). However, the use of these cells means to develop a series of complex and specific techniques as cell culture, nuclear transfer or the transplantation of the thawed cells into recipient fish (of the same or related species) for individual restoration (Siripattarapravat et al., 2011; Chenais et al., 2014), that must be explored in different fish species (reviewed by Martínez-Páramo et al., in press).

2.4. Vitrification

This technique tries to prevent the negative effects of crystallization happening in the conventional cryopreservation methods mixing cryoprotectants at very high concentration and using very high freezing rates, getting the solidification of external...
and internal media into an amorphous/glassy state without formation of harmful ice
crystals (Fahy et al., 1984).

First applications of vitrification to the cryopreservation of sperm of different species
have been published (channel catfish, Ictalurus punctatus; Cuevas-Uribe et al., 2011a;
green swordtail, Xiphophorus hellerii; Cuevas-Uribe et al., 2011b; rainbow trout,
Oncorhynchus mykiss; Figueroa et al., 2013; Atlantic salmon, Salmo salar; Figueroa et
al., 2015; Tambaqui, Colossoma macropomum; Varela Jr. et al., 2015; Eurasian perch,
Perca fluviatilis and European eel, Anguilla anguilla; Kása et al., in press). However,
concentrated cryoprotective solutions are toxic for cells and very high freezing rates can
be difficult to achieve with large samples, limiting the applicability of the vitrification
to low sperm volumes (2-4 µl; using cryoloops or cryotops). Thus, it does not seem to
be able to replace conventional freezing used in most fish species. A real practical
application is just feasible in small model species as zebrafish due to the low volumes of
sperm that they produce.

Vitrification presents a viable alternative to freezing for the cryopreservation of various
teleost tissue types. Vitrification has been tested on somatic cells such as caudal fin
explants (Cardona-Costa et al., 2006) as well as testicular cells (Bono-Mestre et al.,
2009), embryo blastomeres (Cardona-Costa et al., 2007), oocytes (Guan et al., 2010)
and ovarian tissue (Streit et al., 2015). In case of testicular tissue, vitrification was
found to be more effective than conventional freezing in terms of cell survival (Bono-
Mestre et al., 2009). The efficiency of testicular tissue vitrification and its potential for
transplantation and production of germ-line chimeras has been recognized in avian and
mammalian species (Liu et al., 2013; Gouk et al., 2011), thus opening the possibilities
of its application in teleost species, as well.
2.5. Evaluation of gamete quality

Fast and accurate techniques for the evaluation of the quality of fish gametes are needed both for the selection of sperm samples and for the establishment of sperm cryopreservation programs by companies. Many different techniques have been developed for gamete quality evaluation (Cosson et al., 2008; Figueroa et al., 2016; Pérez et al., 2009; Sørensen et al., 2013; Valdebenito et al., 2015) including sperm volume, color and density, spermatozoa motility and morphometry parameters (CASA and ASMA software; reviewed by Mylonas et al., in press), and seminal plasma composition (Pérez et al., 2003; Lahnsteiner, 2009).

However, new techniques require new approaches and parameters to evaluate the freezing-thawing processes, and to provide in-depth information on the effects of these processes undergoing during freezing-thawing that reduce sperm quality (Bobe and Labbé, 2010; Cabrita et al., 2009b; Martínez-Páramo et al., in press; Mylonas et al., in press). For example, the cryopreservation process can induce different types of damage to the spermatozoa, such as DNA fragmentation (Bungum et al., 2011; Cabrita et al., 2005b, 2014; Chohan et al., 2006; Pérez-Cerezales et al., 2010; Riesco et al., 2011), and changes in the protein profile of spermatozoa (Zilli and Vilella, 2012) as well as increases on the production of reactive oxygen species (ROS) inducing alterations at DNA level (Aitken and Baker, 2006; Martínez-Páramo et al., 2012; Thomson et al., 2009). Other techniques provide information about specific damage to certain genes and mRNA (Cartón-García et al., 2013; Guerra et al., 2013) and potential epigenetic damages (Labbé et al., submitted). Specifically, in cryopreserved PGCs, different methylation patterns were found in several genes (e.g *vasa*) (Riesco and Robles, 2013).

The improvement of these techniques is allowing the development of better cryopreservation methods, although an evident lack of standardization can compromise
the comparison of results between different laboratories and further applications (see section 2.2.).

3. Perspectives

3.1. Cryopreservation and industry

In mammals (both cattle and humans) cryopreservation means an important commercial business, while this is yet to happen in aquatic species. Trying to solve the lack of commercial-scale know-how for scaling-up to industry and practical aquaculture, national or supranational specialized centers should be created to improve the standardization (definitions, methodologies, reporting), and offer quality assessment and cryobanking services (linked to genetic programs, endangered species management, or preservation of special samples). Unfortunately, this activity is currently carried out primarily by research centers that concentrate on conservation-related issues (O’Reilly and Doyle, 2007; Streit et al., 2013) and not by commercial companies that use cryopreserved sperm in their genetic improvement programs.

Commercial application of fish sperm cryopreservation is hindered by several factors. Sperm cryopreservation came to the aid of dairy cattle farming exactly when the industry needed it in the 1950-ies. Due to a continuously increasing selection pressure and the development of artificial insemination (AI) techniques, the individual value of dairy bulls increased significantly (Chandler and Godke, 2011). Cryopreservation solved a very stressing problem: how to use the sperm of a few valuable bulls to simultaneously fertilize the oocytes of several thousand cows on various continents. However, all this was a result of a very long domestication process. Aquaculture on the other hand is still in its infancy: higher yields can still be achieved using technologies that do not require genetic improvement (e.g. formulation of feeds, stocking rates,
vaccination, etc.). Thus, sperm cryopreservation will be achieved commercially in some species where the protocols are better developed, the aquaculture industry has demanded such tools and there are economic interests. For other species, sperm cryopreservation will be used occasionally or in niche areas for solving acute problems. Nevertheless, aquaculture is also a very rapidly developing industry. During the last years the development of genetic improvement programs has happened and there is a growing offer of cryobanking services for several salmonids and other species (reviewed by Martínez-Páramo et al., in press). Several companies that supply the cryopreservation industry have a line of products specifically for fish, such as extenders and activating solutions and there is at least one company based in Norway that offers commercial cryopreservation services to the aquaculture industry, exclusively. All this means that cryopreservation in aquatic species is slowly gaining momentum along with the development of aquaculture, especially in the salmon industry where marker assisted selection has already been introduced and QTL (Quantitative Trait Loci) mapping is becoming increasingly important (Yue, 2014; Tsai et al., 2015).

3.2. Development of alternative techniques

The techniques dedicated to preserving oocytes, embryos or larvae could be a key area of research, although much effort has been made with little success. An alternative or complementary way is to improve emerging biotechnological techniques, as the use of PGCs, spermatogonia or alternative diploid cell sources for genome preservation and transplantation (Labbé et al., 2013; Chenais et al., 2014). However, these techniques will need improvement in several technical aspects (reviewed by Martínez-Páramo et al., in press) such as cell isolation, identification, labelling, transplantation, nuclear transference or genome inactivation of the recipient. Moreover, better and simpler
methods are required for DNA integrity evaluation, types of cryodamage (induced by freezing, thawing or cryoprotectants) on the chromatin and cellular structures and their epigenetics consequences, or regarding the production and effects of reactive oxygen species, to guarantee the sperm viability after every step of these processes and the embryo survival (Chenais et al., 2014). And basic research is required on aspects such as cell reprogramming, to regenerate fish producing gametes from somatic cells after transplantation, or germ cell pluripotency (Martínez-Páramo et al., in press; Robles et al., in press).

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Zilli, L., Vilella, S., 2012. Effect of cryopreservation on bio-chemical parameters, DNA Integrity, protein profile and phosphorylation state of proteins of seawater fish
Table 1. Studies on sperm cryopreservation of fish species (published from year 2000 on) indicating the cryoprotectant(s) used in each case and giving (when mentioned) the best results on post-thaw motility (% motile cells), viability (% live cells) and fertilization rate (%).

<table>
<thead>
<tr>
<th>Species</th>
<th>Motility (% or arbitrary scale*)</th>
<th>Post-thaw Viability (%)</th>
<th>Fertilization (%)</th>
<th>Cryoprotectants**</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acipenser baerii</em> (Siberian sturgeon)</td>
<td>51-40</td>
<td>19.3-39</td>
<td>6.0-29.6</td>
<td>10% Methanol</td>
<td>Glogowski et al., 2002</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Judycka et al., 2015</td>
</tr>
<tr>
<td><em>Acipenser brevirostrum</em> (Shortnose sturgeon)</td>
<td>13-15</td>
<td>5-5</td>
<td></td>
<td>10% Methanol</td>
<td>Horváth et al., 2005</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Horváth et al., 2008</td>
</tr>
<tr>
<td><em>Acipenser ruthenus</em> (Sterlet)</td>
<td>33.5</td>
<td>-</td>
<td>9.5-32.7</td>
<td>7.5% Methanol</td>
<td>Lahnsteiner et al., 2004</td>
</tr>
<tr>
<td><em>Acipenser sturio</em> (European sturgeon)</td>
<td>60-70</td>
<td>-</td>
<td></td>
<td>10% Methanol</td>
<td>Urbányi et al., 2004</td>
</tr>
<tr>
<td><em>Anguilla anguilla</em> (European eel)</td>
<td>36.6</td>
<td>-</td>
<td></td>
<td>10% DMSO</td>
<td>Asturiano et al., 2004</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td>Müller et al., 2004</td>
</tr>
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<td></td>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td>Szabó et al., 2005</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>63.2</td>
<td></td>
<td></td>
<td>Marco-Jiménez et al., 2006</td>
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<tr>
<td></td>
<td>51.9</td>
<td>58.26</td>
<td></td>
<td></td>
<td>Asturiano et al., 2007</td>
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<tr>
<td></td>
<td>~18-22</td>
<td>~30-35</td>
<td></td>
<td></td>
<td>Garzón et al., 2008</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>-</td>
<td></td>
<td></td>
<td>Peñaranda et al., 2009</td>
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<tr>
<td></td>
<td>-</td>
<td>33</td>
<td></td>
<td></td>
<td>Asturiano et al., in press</td>
</tr>
<tr>
<td><em>Anguilla japonica</em> (Japanese eel)</td>
<td>37-46.6</td>
<td>-</td>
<td></td>
<td>10% DMSO</td>
<td>Tanaka et al., 2002</td>
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<tr>
<td><em>Barbus grypus</em> (Shabout)</td>
<td>41.3</td>
<td>-</td>
<td>36.1</td>
<td>10% DMSO</td>
<td>Doğu, 2012</td>
</tr>
<tr>
<td><em>Brycon insignis</em> (Tiete tetra)</td>
<td>76-88</td>
<td>51-69</td>
<td></td>
<td>10% Methyl glycol</td>
<td>Viveiros et al., 2011</td>
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<tr>
<td><em>Brycon nattereri</em> (Pirapitinga)</td>
<td>45-72</td>
<td>-</td>
<td></td>
<td>10% Methyl glycol</td>
<td>Oliveira et al., 2007</td>
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<td><em>Brycon opalinus</em></td>
<td>18-88</td>
<td>10-80</td>
<td></td>
<td>10% Methyl glycol</td>
<td>Viveiros et al., 2012</td>
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|---------------------|---------------------------------|-----------------------------------|---------------------------------|----------------------------|------------------------|------------------------------------------|-----------------------------------|-----------------------------|-------------------------|----------------------------------------|------------------------------------|---------------------------------
<p>| 26-66               | -                               | 13.7-44                           | 27.5                            | 69                        | 35                     | -                                        | 36.8                             | 13-64                       | 45                      | 30                                  | 50-75                             | 51                               |
| 28-63               | -                               | 70.7                              | -                               | -                         | &lt;20                    | 40                                       | -                                | ~55                         | -                       | -                                    | -                                 | 63                               |
| 42                  | -                               | 52.1                              | -                               | -                         | ~60                    | 15                                       | 2.5                              | ~38-58                      | -                       | -                                    | -                                 | 11                               |
|                     | 82.2-86.7                       | -                                 | -                               | 56                        | 13                     | 15                                       | 22.5                             | 9-69                        | 87-88                   | 52.4                                | -                                 | 10                               |
|                     | 10% Methyl glycol               | 10% DMSO                          | 10% Methanol                    | 10% DMSO                  | 8% Methanol            | 10% DMSO                                  | 10% DMSO                         | 10% DMSO                    | 10% Methanol             | 10% 1,2 propanediol          | 5-10% DMSO                        | 10% Methanol                  |
|                     | Maria et al., 2006              | Horváth and Urbáni, 2000          | López et al., 2015              | Linhart et al., 2000     | Yang et al., 2007      | Kollár et al., 2015                        | Cabrita et al., 2011            | DeGraaf and Berlinsky, 2004 | Nynca et al., 2015a        | Dzuba and Kopeika, 2002      | Gwo, 2010                          | Daly et al., 2008            |</p>
<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Median Survival (Days)</th>
<th>Lowest Survival (%)</th>
<th>Highest Survival (%)</th>
<th>Carcinogenic Compounds</th>
<th>References</th>
</tr>
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<tr>
<td>Melanogrammus aeglefinus (Haddock)</td>
<td>11-53</td>
<td>0.33-53</td>
<td>10% DMSO</td>
<td>DeGraaf and Berlinsky, 2004</td>
<td></td>
</tr>
<tr>
<td>Misgurnus anguillicaudatus (Loach)</td>
<td>72</td>
<td>28-32</td>
<td>15% Methanol</td>
<td>Rideout et al., 2004</td>
<td></td>
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<tr>
<td>Morone saxatilis (Striped bass)</td>
<td>45</td>
<td>54</td>
<td>7.5% DMSO+75mM glycine</td>
<td>Woods III et al., 2009</td>
<td></td>
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<tr>
<td>Mugil soiuy (Marine haarder)</td>
<td>90</td>
<td>-</td>
<td>16% DMSO</td>
<td>Dzuba and Kopeika, 2002</td>
<td></td>
</tr>
<tr>
<td>Oncorhynchus mykiss (Rainbow trout)</td>
<td>-</td>
<td>94.4</td>
<td>10% DMA</td>
<td>Babiak et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Pagrus major (Red seabream)</td>
<td>64.8</td>
<td>&gt;90</td>
<td>15% DMSO</td>
<td>Liu et al., 2007</td>
<td></td>
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<tr>
<td>Paralichthys orbignyanus (Flounder)</td>
<td>2.5*</td>
<td>78</td>
<td>10% DMSO</td>
<td>Lanes et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Perca fluviatilis (Eurasian perch)</td>
<td>43-90</td>
<td>-</td>
<td>10% Methanol</td>
<td>Bernáth et al., 2015</td>
<td></td>
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<tr>
<td>Polyodon spathula (Paddlefish)</td>
<td>85</td>
<td>80</td>
<td>10% Methanol</td>
<td>Horváth et al., 2006</td>
<td></td>
</tr>
<tr>
<td>Prochilodus lineatus (Curimbata)</td>
<td>75</td>
<td>48</td>
<td>10% Methyl glycol</td>
<td>Viveiros et al., 2010</td>
<td></td>
</tr>
<tr>
<td>Salmo marmoratus (Marble trout)</td>
<td>11-15</td>
<td>15-60</td>
<td>10% Methanol</td>
<td>Horváth et al., 2015a</td>
<td></td>
</tr>
<tr>
<td>Salmo salar (Atlantic salmon)</td>
<td>8.2</td>
<td>58.7</td>
<td>10% Methanol</td>
<td>Dziewulska et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Salmo trutta m. fario (Brown trout)</td>
<td>53-56</td>
<td>29-42</td>
<td>10% Methanol</td>
<td>Horváth et al., 2015a</td>
<td></td>
</tr>
<tr>
<td>Salvelinus fontinalis (Brook trout)</td>
<td>56.8</td>
<td>36.5</td>
<td>7.5% Methanol</td>
<td>Nynca et al., 2015b</td>
<td></td>
</tr>
<tr>
<td>Scaphyrinchus albus</td>
<td>57-70</td>
<td>79-85</td>
<td>10% Methanol</td>
<td>Horváth et al., 2005</td>
<td></td>
</tr>
<tr>
<td>Fish Species</td>
<td>Treatment</td>
<td>Efficacy</td>
<td>Solvent</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
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<td></td>
</tr>
<tr>
<td><em>Scaphthalmus maximus</em> (Turbot)</td>
<td>75/68</td>
<td>10% DMSO</td>
<td>Suquet et al., 2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sparus aurata</em> (Gilthead seabream)</td>
<td>~65/58.3-70/22.7-86.9/30-55</td>
<td>5% DMSO</td>
<td>Fabbrocini et al., 2000/Cabrita et al., 2005/Cabrita et al., 2011/Gallego et al., 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tinca tinca</em> (Tench)</td>
<td>85</td>
<td>10% Methanol</td>
<td>Lujić et al., in press</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tor tambroides</em> (Malaysian Mahseer)</td>
<td>54.9-69.4</td>
<td>10% DMSO</td>
<td>Chew et al., 2010</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tor douronensis</em> (Malaysian Mahseer)</td>
<td>74</td>
<td>10% DMSO</td>
<td>Chew et al., 2010</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thymallus thymallus</em> (Grayling)</td>
<td>~30/68</td>
<td>10% Methanol</td>
<td>Horváth et al., 2012b/10% Methanol/10% Methanol/10% Methanol/Nynca et al., 2015a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Dimethyl-acetamide (DMA); Dimethyl-formamide (DMF); Dimethyl-sulfoxide (DMSO); Propylene glycol (PG)