This is the accepted manuscript of the following article: Folgueira, I., de Felipe, A.P., Sueiro, R.A., **Lamas,** J. & **Leiro,** J. (2018). Protocol for cryopreservation of the turbot parasite *Philasterides dicentrarchi* (Ciliophora, Scuticociliatia). Cryobiology, 80, 77-83. doi: 10.1016/j.cryobiol.2017.11.010. © <Ano> Elsevier B.V. This manuscript version is made available under the CC-BY-NC-ND 4.0 license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

# Protocol for cryopreservation of the turbot parasite *Philasterides dicentrarchi (Ciliophora, Scuticociliatia)*

Folgueira, I.[1], de Felipe, A.P.[1] Sueiro, R.A.[1,2], Lamas, J.[2], Leiro, J.[1,*]

[1]*Departamento de Microbiología y Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*

[2]*Departamento de Biología Funcional, Instituto de Acuicultura, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*

SHORT TITLE: Cryopreservation of *Philasterides dicentrarchi*

[*]**Correspondence**

José M. Leiro, Laboratorio de Parasitología, Instituto de Investigación y Análisis Alimentarios, c/ Constantino Candeira s/n, 15782, Santiago de Compostela (A Coruña), Spain; Tel: 34981563100; Fax: 34881816070; E-mail: josemanuel.leiro@usc.es

## Abstract

*Philasterides dicentrarchi* is a free-living marine ciliate that can become an endoparasite that causes a severe disease called scuticociliatosis in cultured fish. Long-term maintenance of this scuticociliate in the laboratory is currently only possible by subculture, with periodic passage in fish to maintain the virulence of the isolates. In this study, we developed and optimized a cryopreservation protocol similar to that used for the long-term storage of scuticociliates of the genus *Miamiensis*. The cryogenic medium comprised ATCC medium 1651 and a combination of 11% dimethylsulfoxide and 5% glycerol. We have verified that the most important factor ensuring the efficiency of the cryopreservation procedure is the growth phase of the culture, and that ciliates should be cryopreserved at the stationary phase (around the sixth day of culture). The cryopreservation protocol described here can be used for all strains of *P. dicentrarchi* as well as commercial strains of *Miamiensis* and enables the virulence of the strains to be maintained. Finally, this cryopreservation protocol has been shown to be more effective than others routinely applied to scuticociliates, yielding a higher survival rate with a lower initial concentration of ciliates. The results obtained indicate that the cropreservation protocol enables the long-term storage of scuticociliate parasites while maintaining the virulence of the isolates. The protocol is therefore suitable for use in vaccine production and related studies.

**Keywords:** *Philasterides dicentrarchi*; *Miamiensis avidus*; freezing; cryoprotectants; cryopreservation; optimization.

49 ## 1. Introduction

50 Scuticociliates (Ciliophora: Scuticociliatia) are ubiquitous members of
51 phagotrophic marine free-living ciliates that colonize the marine environment and are
52 abundant in coastal waters [30]. Some species can also act as opportunistic histophagous
53 parasites, causing systemic infections in marine cultured fish worldwide [26]. Such
54 infections usually lead to rapid death of the host [15, 36]. Scuticociliatosis, which is
55 caused by *Philaterides dicentarchi* and affects cultured flatfish such as the turbot
56 *Scophthalmus maximus*, the olive flounder *Paralichthys olivaceus* and the fine flounder
57 *Paralichthys adspersus*, is particularly serious and causes high mortalities in infected fish
58 [9, 18, 21].

59 Techniques, used to maintain protozoan species and strains in the laboratory for
60 long periods of time without frequent subculture, include drying, freeze-drying, and in
61 particular, cryopreservation [20, 24].

62 Cryopreservation at ultra-low temperatures has been applied to several species of
63 parasites, thus eliminating the need to maintain cultures by repeated passage *in vitro*.
64 Cryopreservation is the method of choice for long-term maintenance as it overcomes the
65 disadvantages associated with *in vitro* propagation, e.g. the length of the procedures used,
66 difficulty in initial isolation, loss of strains, bacterial and fungal contamination during
67 handling, changes in the original biological and metabolic characteristics, and the re-
68 establishment of infections *in vivo* [10, 20, 24]. Serial subculture, particularly of axenic
69 strains, can also result in the loss of biological characteristics such as virulence [22].

70 Although successful cryopreservation of some non-encysting ciliates is quite easy
71 to achieve, the procedure is difficult for other ciliates, including the scuticociliates, and
72 modifications must be made to the normal thawing procedure [28]. Strains of the marine

73 scuticociliates belonging to the genera *Methanophrys, Miamiensis, Parauronema,*
74 *Paranophrys, Pseudocohnilembus* and *Uronema* can be maintained in an axenic state [34]
75 and cryopreserved in formulations containing glycerol, methanol and dimethyl
76 sulphoxide ($Me_2SO$) as cryoprotective agents [2, 33]. *P. dicentrachi* can also be
77 maintained in axenic cultures in Leibovitz L15 medium supplemented with foetal bovine
78 serum, lipids, nucleosides and glucose [17]. The ciliates obtained by this method retain
79 their capacity to infect turbot after at least twenty passages *in vitro*. However, the
80 virulence of the isolates is modified by long-term *in vitro* culture [1, 35], and isolates
81 must be passaged in turbot to recover their virulence [31]. Several studies have
82 demonstrated the existence of an intraspecific variation in *P. dicentrarchi* that displays
83 clear antigenic differences between strains and which can be altered during serial
84 subculture, thus affecting the protection afforded by vaccination [6,7,32].
85 Cryopreservation would overcome the disadvantages associated with serial culture (also
86 observed in other protozoan parasites), such as the labour-intensive techniques involved,
87 bacterial / fungal contamination of cultures, genetic drift, and loss of the infectivity and
88 immunogenicity of the strains [5, 24, 36].

89 Cryopreservation of the scuticociliate parasite *P. dicentrarchi* has not yet
90 previously been reported. The objective of this study was to develop and optimize an
91 effective cryopreservation protocol for the long-term storage of the turbot parasite
92 *Philasterides dicentrarchi*.

93

94

95

96

97 ## 2. Materials and Methods

98

99 *2.1. Scuticociliate isolation and culture*

100 Isolates of the scuticociliate *P. dicentrarchi* were obtained from the ascitic fluid
101 of naturally infected turbot during an outbreak of scuticociliatosis affecting different
102 turbot farms in Galicia (NW Spain) and Portugal [6]. The farms were located in the Ria
103 de Muros-Noia (isolate S1), the Ria de Arousa (isolates I1, C1, D2, D3; isolates D2 and
104 D3 were obtained from the same fish farm), the Ria de Vigo (isolate B1) and the Algarve
105 (southern Portugal; isolate Po). Two isolates of *P. dicentrarchi*, denominated Pe5 and
106 Pe7, were also obtained from the ascitic fluid of naturally infected specimens of the fine
107 flounder *Paralichthys adspersus* from a fish farm in Peru (Ancash, Huarmey Province)
108 [9], as previously described [18]. The *P. dicentrarchi* isolates were maintained in the
109 laboratory in Leibovitz L15 medium supplemented with 10% foetal bovine serum, lipids
110 (lecithin and Tween 80), nucleosides and glucose (standard complete L15 medium),
111 under the culture conditions described by Iglesias et al. [17].

112 Strains Ma and Ma/2 of *Miamiensis avidus* deposited by A.T. Soldo and E. B.
113 Small under the name *Miamiensis avidus* Thompson and Moewus (ATCC® 50179™ and
114 ATCC® 50180™, respectively) were acquired from the American Type Culture
115 Collection (ATCC, USA). Strains Ma and Ma/2 of *M. avidus* were cultivated axenically
116 in ATCC® medium 1651 MA (LGC Standards, Spain) at 25 ºC and were subcultured
117 every 3-5 days.

118

119

120

121 *2.2. Freezing protocol for cryopreservation*

122 The ciliates were collected from cultures at different stages of growth and
123 centrifuged at 700 xg for 5 min. Aliquots of 0.5 x $10^6$ ciliates were resuspended in 0.5
124 mL of 1651 MA medium diluted 1:1 in double distilled water (MM), in Leibovittz L15
125 medium, or in artificial sea water (in g/L solution: 23.926 g NaCl, 4.008 g $Na_2SO_4$, 0.677
126 g KCl, 0.196 g $NaHCO_3$, 0.098g KBr, 0.026 g $H_3BO_3$, 10 g $Cl_2Mg$, 2.22 g $CaCl_2$) (ASW),
127 plus 5, 7.5 or 10% glycerol or 11% $Me_2SO$, in 1.8 mL sterile plastic screw-top cryotubes
128 (Nunc, Thermo Scientific, China). Cell preparation took between 5-10 min and never
129 exceeded 15 min. Finally, the cryotubes were placed in a cell freezing container
130 (CoolCell, Biocision), which decreased the temperature by approximately 1ºC /min,
131 before being transferred to a freezer (-80 ºC) for 2h and then stored in liquid nitrogen. In
132 some experiments, the same medium with cryoprotectant additives (CPA) was used, but
133 with the glycerol replaced by 5% methanol.

134

135 *2.3. Thawing steps*

136 For thawing, the cryovials were removed from the liquid nitrogen and
137 immediately placed in a water bath at 37 ° C for 2 min, without shaking, ensuring that the
138 frozen samples were completely submerged. The cryoprotective solution was then
139 removed by centrifugation at 750 g for 5 min at room temperature. The cell pellet was
140 resuspended in 0.5 mL of MM, under aseptic conditions, and the mixture was transferred
141 to a sterile 15 mL Falcon ™ conical bottom tube with an additional 2.0 mL of MM
142 medium. Two cryovials were used in each experiment, and the contents (total 5.0 mL)
143 were aseptically transferred to a 25 $cm^2$ vented flask (Corning™ Cell Culture Treated
144 Flasks). The flasks were incubated horizontally in a cooled incubator at 21 °C, and cell
145 growth was monitored daily by examination under an inverted microscope.

146 *2.4. Viability/survival assessment and* in vitro *growth*

147 Cultures were examined on an inverted microscope (Eclipse CFL60, Nikon, 148 Japan) at 200x magnification to check viability and ciliate morphology. The 149 viability/survival of *P. dicentrarchi* trophozoites was assessed by motility. The number 150 of motile ciliates present in different parts of the flask were counted. Results were 151 expressed as the number of motile ciliates (NMC) per flak, calculated using the following 152 formula:

153 **Survival level folloving cryopreservation = NMC/flask = (NMC1 x S / Sf x Nf) x V**,

154 where,

155 NMC1: Number of motile ciliates present in the sample after thawing

156 S: flask surface (25 x $10^2$ $mm^2$)

157 Sf: Surface area of a visual field in the 20X objective lens (0.95 $mm^2$)

158 Nf: Number of fields displayed (n = 5)

159 V: Total sample volume (5 mL)

160 Three flasks were examined at each time point and the mean values (and standard 161 deviations) for recovery quantified at 24, 48 and 72 h after thawing were calculated, and 162 expressed as a percentage (%) of the total number of ciliates for each experiment.

163 To determine the *in vitro* growth of the different strains of *P. dicentrarchi*, 75 $cm^2$ 164 flasks were inoculated with $5x10^4$ ciliates / mL in a final volume of 30 mL of MM 165 medium. Aliquots (15 µL) of medium were removed daily from each flask and ciliates 166 inactivated by addition of 0.25% glutaraldehyde (Sigma-Aldrich) before being quantified 167 using a haemocytometer [17].

168

169

170

171 *2.5. Experimental design*

172 We investigated the effects of two controllable components of cryopreservation
173 on the viability of *P. dicentrarchi*: the concentration of the cryoprotectant glycerol (factor
174 A) and the day of culture (factor B). Experiments were designed using STATGRAPHICS
175 Centurion XVI version 16.1.15 Windows (Statgraphics.net, Madrid) by application of a
176 3^2 factorial design, as previously described [23]. StatAdvisor created a factorial design
177 with 10 experimental runs to study the effects of each factor on the viability of samples
178 24 h after thawing (Table 2). Experiments were executed in a single block with the order
179 randomized to limit the effect of hidden variables.

180 For experimental infections, two groups of fifteen fish were infected
181 intraperitoneally with 0.1 ml of $10^6$ ciliates/ml in phosphate-buffered saline (PBS)
182 containing 10 mM $Na_2HPO_4$, 2mM $KH_2PO_4$, 2.7 mM KCl, and 137 mM NaCl, as
183 previously described [31]. The fish were held according to the criteria of protection,
184 control, care and welfare of animals and the legislative requirements relating to the use
185 of animals for experimentation (EU Directive 86/609 / EEC), the Declaration of Helsinki,
186 and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated
187 by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The
188 Institutional Animal Care and Use Committee of the University of Santiago de
189 Compostela approved all experimental protocols.

190 The fish were observed daily for signs of infection and mortality. Infection was
191 confirmed *post mortem* by the presence of ciliates in organs and tissues.

192

193 *2.6. Statistical analysis*

194 The results, expressed as mean values ± standard deviation (n = 5) were analysed by
195 unpaired *t*-test, and the Kolmogorov and Smirnov method was used to check for normality

196 of distribution. The Kaplan–Meier test were used to evaluate differences in survival rates.
197 Differences between groups were considered significant at $P \leq 0.05$.
198

199 ## 3. Results

200 *3.1. Influence of growth phase and glycerol concentration on cryopreservation of* P.
201 dicentrarchi *isolate I1*

202 The ciliates were maintained for 6 days before cryopreservation in one of three
203 different culture media (see Material and Methods): 1) MM medium, 2) Complete L15
204 medium, and 3) artificial sea water (ASW) (Fig. 1). Growth to stationary phase in MM
205 medium with 5% glycerol yielded the highest survival level (Fig. 1B). In the MM and in
206 complete L15 media, the culture entered in the stationary phase on day 3, whereas in
207 ASW growth remained unchanged throughout the study period (Fig. 2A). The highest
208 growth rate was obtained with the MM medium (Fig. 2A), in which most of the ciliates
209 appeared as microstomes, and a minority as tomites on day 6 of culture (Fig. 2B). By
210 contrast, by day 6 most ciliates cultured in ASW were tomites (Fig. 2B).
211

212 *3.2. Optimization of the cryopreservation protocol*

213 To optimize the cryopreservation method, two factors were varied (Table 1): the
214 concentration of glycerol ($X_1$) and harvest day from culture ($X_2$) assessed by ciliate
215 recovery at 24 hours post thawing (Table 1A). We applied multiple regression analysis to
216 determine the effect of these factors on the ciliate viability (Table 1B).

217 Statistical analysis of the data yielded a quadratic model described by the
218 following equation:

219 $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{1,2} X_1 X_2 + \beta_{1,1}(X_1)^2 + \beta_{2,2}(X_2)^2$

220 where $\beta_0$, $\beta_1$, $\beta_2$, $\beta_{1,2}$, $\beta_{1,1}$, $\beta_{2,2,}$ are the regression coefficients and $X_1$ and, $X_2$ the

221 independent variables.

222 Based on estimation of statistical significance of model coefficients, the

223 concentration of glycerol and the culture phase significantly affected ciliate viability ($\alpha$

224 $= 0.05$) (Table 1B). The value of the $R^2$ statistic indicates that the model fit explained

225 73.69 % of the variability in the viability after thawing. The Durbin-Watson test, used to

226 detect any autocorrelation, produced a value of 2.5 ($P$ = 0.76), indicating correlation

227 between the independent variables included in the regression model (Table 1B).

228

229 *3.3. Application of the cryopreservation method to other strains of* Philasterides *and*

230 Miamiensis

231 Optimization of the cryopreservation protocol was performed with *P. dicentrarchi*

232 isolate I1, and further experiments were carried out to determine whether this method

233 could also be applied to other strains of *P. dicentrarchi* and to strains of related species

234 such as *Miamiensis*. We initially determined the growth rates of all strains in MM medium

235 for 6 days. The growth curves obtained with strains B1, C1, D2, D3, I1, Pe5, Pe7 and Po

236 of *P. dicentrarchi* and with Ma strains of *Miamiensis* sp. and Ma / 2 of *M. avidus* are

237 shown in Fig. 3. Most strains enter stationary phase after three days. As the optimization

238 model indicated that ciliates in stationary phase were the most suitable for

239 cryopreservation, we collected ciliates on day 6. Viable ciliates of all strains were

240 obtained post thawing (Table 2). The strain that recovered most quickly was *M. avidus*

241 strain Ma / 2, in which mobile ciliates were observed within a few hours of thawing,

242 followed by strains C1, D3 and I1, in which motile ciliates were observed on day 1, and

243 by strains B1 and D2, in which motile ciliates appeared on day 2 (Table 2). Samples of

244 *Miamiensis* sp. strain Ma and *P. dicentrarchi* strain Pe5 contained motile ciliates on day

245 3 after thawing, and samples of strains Pe5 and Po contained motile ciliates on day 4 after
246 thawing (Table 2).
247
248 *3.3. Maintenance of virulence in cryopreserved ciliates*
249 We found that ciliates (isolate I1) maintained in culture and those cultured after
250 being cryopreserved exhibited the same level of virulence in turbot (Fig. 4).
251
252 *3.4. Comparison between the cryopreservation protocol developed in the present study*
253 *and the protocol routinely used for cryopreservation of* Miamiensis
254 We compared the cryopreservation protocol described here and used by the
255 American Type Culture Collection (ATCC) for the Ma strain of *Miamiensis sp.* (ATCC®
256 50179™; https://www.lgcstandards-atcc.org/products/all/50179.aspx?geo_country=es)
257 and Ma/2 strain of *M. avidus* (ATCC® 50180™; https://www.lgcstandards-
258 atcc.org/Products/All/50180.aspx). Although the protocols are similar, we used glycerol
259 as the cryoprotectant instead of methanol. The use of glycerol appeared to improve
260 recovery after cryopreservation, increasing the survival of both strains of *Miamiensis*
261 tested (Table 3). Recovery was inversely correlated with ciliate concentration (Table 3).
262
263 **4. Discussion**
264 ***4.1.*** *Effect of culture medium*
265 Numerous factors affect the efficacy of cryopreservation of microorganisms,
266 including species type, strain, cell size and shape, growth stage and rate, incubation
267 temperature, growth medium composition, pH, osmolarity, cellular water content, lipid
268 content and cell composition, freezing medium composition, cooling and thawing rate,

269 storage temperature and duration, and recovery medium [16]. The most important factor
270 affecting the efficacy of cryopreservation is the type of medium used to suspend the
271 microorganisms during the freezing process, although it usually assumed that the medium
272 should be the same as that used to propagate the cells [24]. We observed that MM medium
273 was good for growth and cryopreservation of *P. dicentrarchi*, probably due to the high
274 content of CPA such as peptone [34].
275
276 ***4.2.*** *Effect of growth phase*
277 It is traditionally considered that cryopreservation of any cell type should be
278 performed at the late lag or early log (exponential) phase to yield optimal survival with
279 traditional slow prefreezing protocols [8]. Although in the present study surviving ciliates
280 were obtained after cryopreservation of log-phase and stationary phase cultures, cell
281 viability was maximal for ciliates present at an advanced phase of stationary growth after
282 culture on MM medium. On the other hand, when the ciliates used for cryopreservation
283 were grown in ASW, maximum viability was obtained at the beginning of the culture;
284 however, under these conditions, the ciliates were already at the stationary phase of
285 growth. The biological cycle of *P. dicentrarchi* includes microstomes and tomites [10].
286 Our study findings show that both forms that occur in the biological cycle of *P.*
287 *dicentrarchi* are resistant to cryopreservation. Similar results were also obtained in
288 relation to the cryopreservation of the tomont and teront forms of the ciliate parasite
289 *Icththyophthirius multifiliis* [12].
290 Prior optimization of any cryopreservation protocol is therefore necessary [4, 24,
291 29]. Optimization experiments based on factorial designs have been used in
292 cryopreservation studies to identify the most important factors determining cell viability
293 after cryopreservation [3]. We analysed the influence of factors such as glycerol

294 concentration and the culture phase to optimize the cryopreservation protocol for *P.*
295 *dicentrarchi*. The multiple regression analysis revealed that the factor with most
296 significant influence on ciliate viability during freezing is the growth stage of the ciliate
297 at the time of cryopreservation. The optimal stage is the stationary phase, as also observed
298 for other organisms [19].

299 We also observed differences in the viability of the different strains, confirming
300 the need for the protocol to be optimized for each strain, as done for other strains of
301 parasite species [27].

302 A decrease in virulence may also occur after *in vitro* subculture of strains for a
303 long time in the laboratory [17]. In this study, the cryopreservation protocol also
304 preserved the virulence of *P. dicentrarchi.* Other cryopreservation protocols using 20%
305 $Me_2SO$ as the CPA also maintain the infectivity of cryopreserved samples of parasites
306 [11].

307

308 ***4.3.*** *Effect of CPA*

309 $Me_2SO$ is the most widely used CPA with most protozoan species [37]. The
310 concentration of $Me_2SO$ used depends on the strain and can vary from 2.5% to 12.5%
311 and, in some scuticociliate species, from 9-11% [28]. Glycerol (1,2,3-propanetriol) is also
312 a widely used CPA in microbiology and it was first used by [13] to cryopreserve parasitic
313 protozoans, at concentrations ranging from 2-55% (mean 10%) [25]. For
314 cryopreservation of some microorganisms, methanol is as effective as $Me_2SO$ or glycerol
315 as a CPA [16]. The concentrations of methanol used in cryomix formulations typically
316 range from 2-10% (mean 5%) [14]. The use of different concentrations of $Me_2SO$ and
317 glycerol, has also been widely studied in relation to the cryopreservation of parasites [24].
318 We found that a combination of 11% $Me_2SO$ and 5% glycerol, with no equilibration in

319 the cryomix, was the most effective for the cryopreservation of *P. dicentrarchi* strain I1,
320 and was more effective for cryopreservation of strains Ma and Ma / 2 of *Miamiensis* than
321 the combination used by the ATCC (https://www.atcc.org/products/all/50180.aspx).

322 In conclusion, the cryopreservation protocol developed was highly effective
323 regarding the viability of strains of *P. dicentrarchi* and other strains of scuticociliates,
324 confirming that the growth phase, the culture medium and the concentration of the CPA
325 glycerol used are key factors in the procedure. Use of this cryopreservation protocol
326 yielded ciliates with intact virulence, allowing long-term maintenance of virulent strains
327 without the need for passage in fish to recover the virulence. Cryopreserved ciliates could
328 therefore be used to produce vaccines and also in diagnostics and immunoassays.

329

330 ## Acknowledgements

331 This study was financially supported by grant AGL2014-57125-R from the Ministerio de
332 Economía y Competitividad (Spain), by grant GPC2014/069 from the Xunta de Galicia
333 (Spain) and by PARAFISHCONTROL project, which received funding from the
334 *European Union's Horizon 2020 research and innovation programme* under grant
335 agreement No. 634429. This publication only reflects the views of the authors, and the
336 European Commission cannot be held responsible for any use which may be made of the
337 information contained therein.

338

339 ## References

340 [1] P. Álvarez-Pellitero, O. Palenzuela, F. Padrós, A. Sitjà-Bobadilla, A. Riaza, R. Silva,
341 J. Arán, Histophagous scuticociliatids (Ciliophora) parasitizing turbot

342 *Scophthalmus maximus*: morphology, *in vitro* culture and virulence. Folia
343 Parasitol. (Praha) 51(2004) 177–187.
344 [2] S.A. Anderson, D.A. Hulston, S.M. McVeagh, V.L. Webb, P.J. Smith, *In vitro* culture
345 and cryopreservation of *Uronema marinum* isolated from farmed New Zeland
346 grouper (*Polyprion oxygeneios*). J. Microbiol. Methods 79 (2009) 62-66.
347 [3] I. Babiak, E. Brzuska, J. Perkowski, Fractional factorial design of screening
348 experiments on cryopreservation of fish sperm. Aquacul. Res. 31 (2000) 273-282.
349 [4] J.G. Baust, D.G. Gao, J.M. Baust, Cryopreservation, An emerging paradigm change.
350 Cryogenesis 5 (2009) 90-96.
351 [5] K.S. Booth , E.R. James , I. Popiel, Cryopreservation of an attenuated vaccine strain
352 of the protozoan parasite *Toxoplasma gondii*. Cryobiology 33 (1996) 330-337.
353 [6] B. Budiño, J. Lamas, M.P. Pata, J.A. Arranz, M.L. Sanmartín, J. Leiro, Intraspecific
354 variability in several isolates of *Philasterides dicentrarchi* (syn. *Miamiensis*
355 *avidus*), a scuticociliate parasite of farmed turbot. Vet. Parasitol. 175 (2011) 260-
356 272.
357 [7] B. Budiño, J. Leiro, S. Cabaleiro, J. Lamas, Characterization of *Philasterides*
358 *dicentrarchi* isolates that are pathogenic to turbot: serology and cross-protective
359 immunity. Aquaculture 364-365 (2012) 130-136.
360 [8] J.G. Day. Cryopreservation: fundamentals, mechanisms of damage on
361 freezing/thawing and application in culture collections. Nova Hedwigia 79 (2004)
362 191-205.
363 [9] A.P. De Felipe, J. Lamas, R.A. Sueiro, I. Folgueira, J.M. Leiro, New data on flatfish
364 scuticociliatosis reveal that *Miamiensis avidus* and *Philasterides dicentrarchi* are
365 different species. Parasitology (2017) doi: 10.1017/S0031182017000749.

366 [10] P. De Paoli, Biobanking in microbiology: From sample collection to epidemiology,
367 diagnosis and research. FEMS Microbiol. Rev. 29 (2005) 897-910.
368 [11] J. de Rezende, C.P. Rangel, D. McIntosh, J.A.G. Silveira, N.C. Cunha, C.A.N.
369 Ramos, A.H. Fonseca, *In vitro* cultivation and cryopreservation of *Babesia*
370 *bigemina* sporokinetes in hemocytes of *Rhipicephalus microplus.* Vet. Parasitol.
371 212 (2015) 400-403.
372 [12] K.D.E. Everett, J.R. Knight, H.W. Dickerson, Comparing tolerance of
373 *Icththyophthirius multifiliis* and *Tetrahymena thermophila* for new
374 cryopreservation methods. J. Protozool. 88 (2002) 41-46.
375 [13] J. D. Fulton, A. V. Smith, Preservation of *Entamoeba histolytica* at -79 ºC in the
376 presence of glycerol. Ann. Trop. Med. Parasit. 47 (1953) 240-246.
377 [14] S. Gäbler-Schwarz, C. Rad-Menéndez, U.E.M. Achilles-Day, C.N. Campbell, J.G.
378 Day, Cryopreservation of *Phaeocystis Antarctica*. CryoLetters 34 (2013) 561-
379 570.
380 [15] R. Harikrishnan, C. Balasundaram, M.S. Heo, "Scuticociliatosis and its recent
381 prophylactic measures in aquaculture with special reference to South Korea
382 Taxonomy, diversity and diagnosis of scuticociliatosis": Part I Control strategies
383 of scuticociliatosis: Part II. Fish Shellfish Immunol. 29 (2010) 15-31.
384 [16] Z. Hubélek, Protectants used in the cryopreservation of microorganisms.
385 Cryobiology 46 (2003) 205-29.
386 [17] R. Iglesias, A. Paramá, M.F. Álvarez, J. Leiro, C. Aja, M.L. Sanmartín, *In vitro*
387 growth requeriments for the fish pathogen *Philasterides dicentrarchi* (Ciliophora,
388 Scuticociliatida). Vet. Parasitol. 111 (2003) 19-30.
389 [18] R. Iglesias, A. Paramá, M.F. Álvarez, J. Leiro, J. Fernández, M.L. Sanmartín,
390 *Philasterides dicentrarchi* (Ciliophora, Scuticociliatida) as the causative agent of

391 scuticociliatosis in farmed turbot *Scophthalmus maximus* in Galicia (NW Spain).
392 Dis. Aquat. Organ. 46 (2001) 47-55.
393 [19] M. Ishikawa, M. Suzuki, T. Nakamura, T. Kishimoto, A.J. Robertson, L.V. Gusta,
394 Effect of growth phase on survival of bromegrass suspension cells following
395 cryopreservation and abiotic stresses. Ann Bot. 97 (2006) 453-459.
396 [20] E.R. James, Parasite cryopreservation by vitrification. Cryobiology, 49 (2004) 201-
397 210.
398 [21] S.M. Kim, J.B. Cho, S.K. Kim, Y.K. Nam, K.H. Kim, Occurrence of scuticociliatosis
399 in olive flounder *Paralichthys olivaceus* by *Philasterides dicentrarchi*
400 (Ciliophora: scuticociliatida). Dis. Aquat. Organ. 62 (2004) 233-238.
401 [22] S.R. Kwon, C.S. Kim, K.H. Kim, Differences between short- and long-term cultures
402 of *Uronema marinum* (Ciliophora: Scuticociliatida) in chemiluminescence
403 inhibitory activity, antioxidative enzyme and protease activity. Aquaculture 221
404 (2003) 107–114.
405 [23] J. Lamas, A.I. Paramá, R. Castro, S. Cabaleiro, M.V. Ruiz de Ocenda, J.L. Barja, J.
406 Leiro, Optimization of an inactivated vaccine against a scuticociliate parasite of
407 turbot: Effect of antigen, formalin and adjuvant concentration on antibody
408 response and protection against the pathogen. Aquaculture 278 (2008) 22-26.
409 [24] Y. Miyake, P. Karanis, S. Uga, Cryopreservation of protozoan parasites.
410 Cryobiology 48 (2004) 1-7.
411 [25] A. Miyata, Cryo-preservation of the parasitic protozoa. Jap. J. Trop. Med. Hyg. 3
412 (1975) 161-200.
413 [26] E.M.M. Moustafa, M. Naota, T. Morita, N. Tange, A. Shimada, Pathological study
414 on the scuticociliatosis affecting farmed Japanese Flounder (*Paralichthys*
415 *olivaceus*) in Japan. J. Vet. Med. Sci. 72 (2010) 1359-1362.

416 [27] A. Mzabi, S. Escotte-Binet, R. Le Naour, N. Ortis, S. Audonnet, M.L. Dardé, D.
417 Aubert, I. Villena. Optimization of the cryopreservation of biological resources,
418 Toxoplasma gondii tachyzoites, using flow cytometry. Cryobiology 71 (2015)
419 459-463.
420 [28] T.A. Nerad, P-M. Daggett, Cryopreservation of non-encysting ciliates, in: J.J. Lee,
421 A.T. Soldo (eds), Protocols in Protozoology, Allen Press Inc, Lawrence, Kansas,
422 1992, pp. A-92.1-A-92.4.
423 [29] E. Nsabimana, S. Kisidayová, D. Macheboeuf, C.J. Newbold, J.P. Jouany, Two-step
424 freezing procedure for cryopreservation of rumen ciliates, an effective tool for
425 creation of a frozen rumen Protozoa Bank. Appl. Environ. Microbiol. 69 (2003)
426 3826-3832.
427 [30] X. Pan, X. Fan, S.A. Al-Farraj, S. Gao, Y. Chen, Taxonomy and morphology of
428 four "ophrys-related" scuticociliates (Protista, Ciliophora, Scuticociliatia), with
429 the description of a new genus, *Paramesanophrys* gen. nov. Eur. J. Taxon. 191
430 (2016) 1-18.
431 [31] A. Paramá, R. Iglesias, M.F. Álvarez, J. Leiro, C. Aja, M.L. Sanmartín, *Philasterides*
432 *dicentrarchi* (Ciliophora, Scuticociliatida): Experimental infection and possible
433 routes of entry in farmed turbot (*Scophthalmus maximus*). Aquaculture 217 (2003)
434 73-80.
435 [32] C. Piazzon, J. Lamas, R. Castro, B. Budiño, S. Cabaleiro, M.L. Sanmartín, J. Leiro,
436 Antigenic and cross-protection studies on two turbot scuticociliate isolates. Fish
437 Shellfish Immunol. 25 (2008) 417-424.
438 [33] E.E. Simione, E.M. Brown, (eds) ATCC preservation methods: freezing and freeze-
439 drying. American Type Culture Collection, Rockville, MD, (1991) 42 pp.1-18.

440 [34] A.T. Soldo, E.J. Merlin, The cultivation of symbiote-free ciliates in axenic medium.
441 J. Protozool. 19 (1972) 519-524.
442 [35] J.Y. Song, S. Kitamura, M.J. Oh, H.S. Kang, J.H. Lee, S.J. Tanaka, S.J. Jung,
443 Pathogenicity of *Miamiensis avidus* (syn. Philasterides dicentrarchi),
444 *Pseudocohnilembus persalinus*, *Pseudocohnilembus hargisi* and *Uronema*
445 *marinum* (Ciliophora, Scuticociliatida). Dis. Aquat. Organ. 83 (2009) 133-143.
446 [36] M.F. Stidworthy, M.M. Garner, D.S, Bradway, B.D. Westfall, B. Joseph, S. Repetto,
447 E. Guglielmi, H. Schmidt-Posthaus, S.M. Thornton, Systemic Scuticociliatosis
448 (*Philasterides dicentrarchi*) in sharks. Vet. Pathol. 51 (2014) 628-632.
449 [37] P.J. Walker, M.J. Ashwood-Smith, Dimethyl sulphoxide, an alternative to glycerol,
450 for the low-temperature preservation of trypanosomes. Ann. Trop. Med. Parasit.
451 55 (1961) 93-96.
452
453
454
455
456
457
458
459
460
461
462
463

464 ## Figures and Tables

465

466 **Figure 1.** Effects of culture medium, growth stage and the cryoprotective agent glycerol
467 on the efficacy of cryopreservation of the strain I1 of *Philasterides dicentrarchi*.
468 Trophozoites were cultured for 6 days in ATCC® medium 1651 MA prepared in artificial
469 sea water (ASW) diluted 1:1 in distilled water (MM), in complete L15 medium (L15),
470 and in artificial ASW. Cryovials (0.5 ml) containing $10^5$ trophozoites / mL from a culture
471 in different growth phase (2, 4 and 6 days) were cryopreserved in a liquid $N_2$ container,
472 and maintained in a cryomix consisting of MM supplemented with 11 % dimethyl
473 sulfoxide ($ME_2SO$) and different concentrations of glycerol: 2.5 (A), 5.0 (B), 7.5 (C) or
474 10.0 % (D) as cryoprotectants. Post thawing survival was expressed as the number of
475 motile trophozoites (NMC) /flask, determined after 24, 48 and 72 hours. The data
476 presented correspond to the means ± standard deviations for the NMC/flask and five
477 cryovials.

478

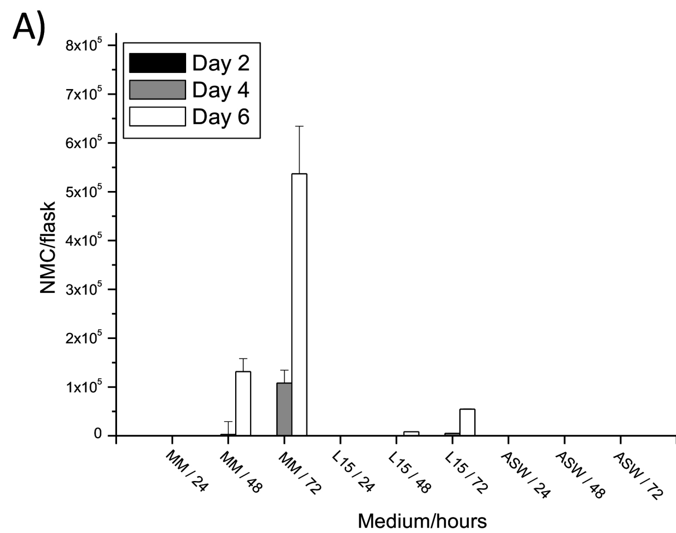
479 **Figure 2.** (A) Growth curve, and (B) percentage of tomites of isolate I1 of *Philasterides*
480 *dicentrarchi* in ATCC® Medium 1651 prepared in artificial sea water (ASW) diluted 1:1
481 in distilled water (MM), in standard complete L15 medium (L15), and in ASW, incubated
482 for 6 days at 21ºC. Each point represents the mean value ± standard deviations for five
483 assays. The photomicrographs on the right show the microstome (m) and tomite (t) forms
484 present in the MM medium, complete L15 medium and in ASW on day 6 of culture.

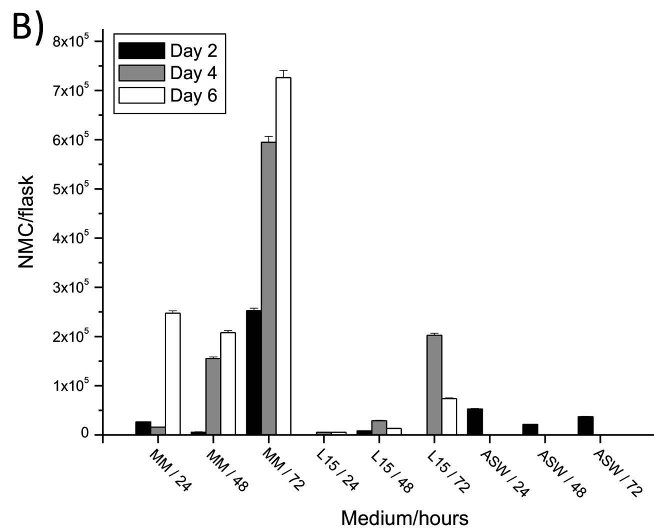
485

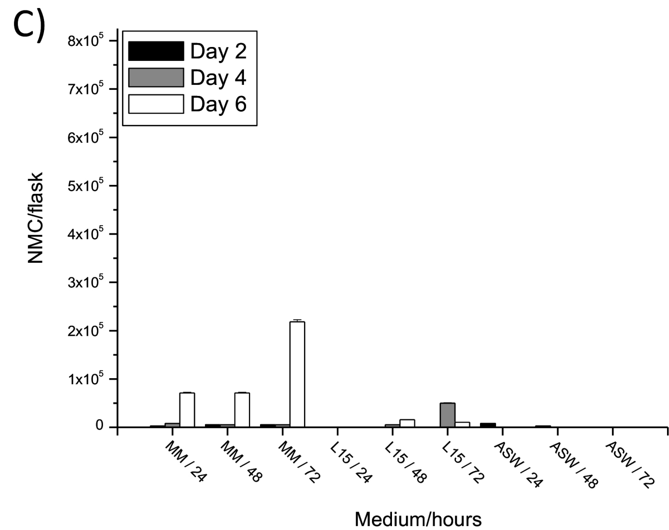
486 **Figure 3.** Growth curves of several strains of *Philasterides dicentrarchi* (B1, C1, D2,
487 D3, I1, Pe5, Pe7, Po), *Miamiensis avidus* (strain Ma/2) and *Miamiensis* sp. (strain Ma) in

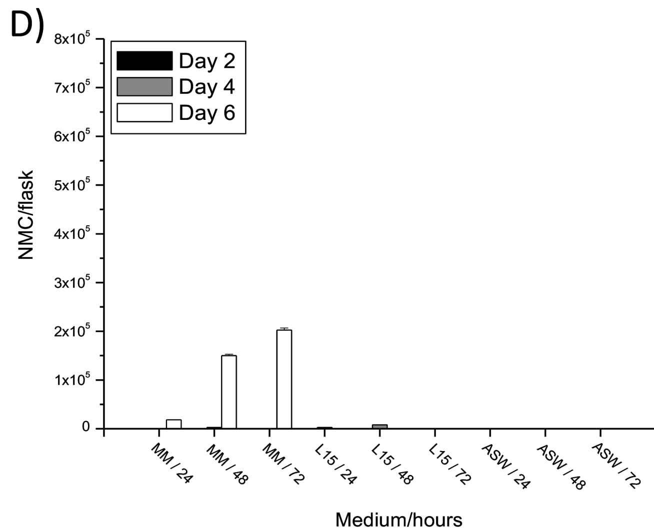
488 ATCC® Medium 1651 prepared in artificial sea water (ASW) diluted 1:1 in distilled water
489 (MM) at 21 °C. The ciliates obtained at day 6 of culture were used for cryopreservation.
490 Each symbol represents the mean value ± standard deviation of five cultures.
491
492 **Figure 4.** Cumulative mortality curve obtained after the experimental infection of turbot
493 inoculated intraperitoneally with $5x10^5$ ciliates from fresh and from cryopreserved and
494 thawed stocks. Values indicate the mean of the percentage cumulative mortality observed
495 during 10 days after challenge ± standard deviations (SD; n=3).
496
497 **Table 1**. Optimization of cryopreservation protocol. (A) Independent variables, their
498 correspondence with coded values in the design and the levels of viability achieved at 24
499 h after thawing according to the experimental design. (B) Coefficients of determination,
500 $R^2$ (fitted by degrees of freedom), Durbin-Watson statistic of multiple regression analysis
501 and level of significance for variance analysis (*P*). (**P* < 0.05).
502
503 **Table 2.** Post thawing survival of several strains of *Philasterides dicentrarchi* (B1, C1,
504 D2, D3, I1, Pe5, Pe7, P0), *Miamiensis avidus* (strain Ma/2) and *Miamiensis* sp. (strain
505 Ma) cryopreserved in ATCC® medium 1651 MA prepared in artificial sea water (ASW)
506 diluted 1:1 in distilled water (MM medium) containing 11% dimethyl sulfoxide ($Me_2SO$)
507 and 5% glycerol. The symbols (+) indicate the presence of motile ciliates on the day of
508 thawing.
509
510 **Table 3**. Survival of trophozoites from strains Ma and Ma/2 of *Miamiensis avidus* from
511 the American Type Culture Collection (ATCC), cryopreserved in two cryogenic mixtures

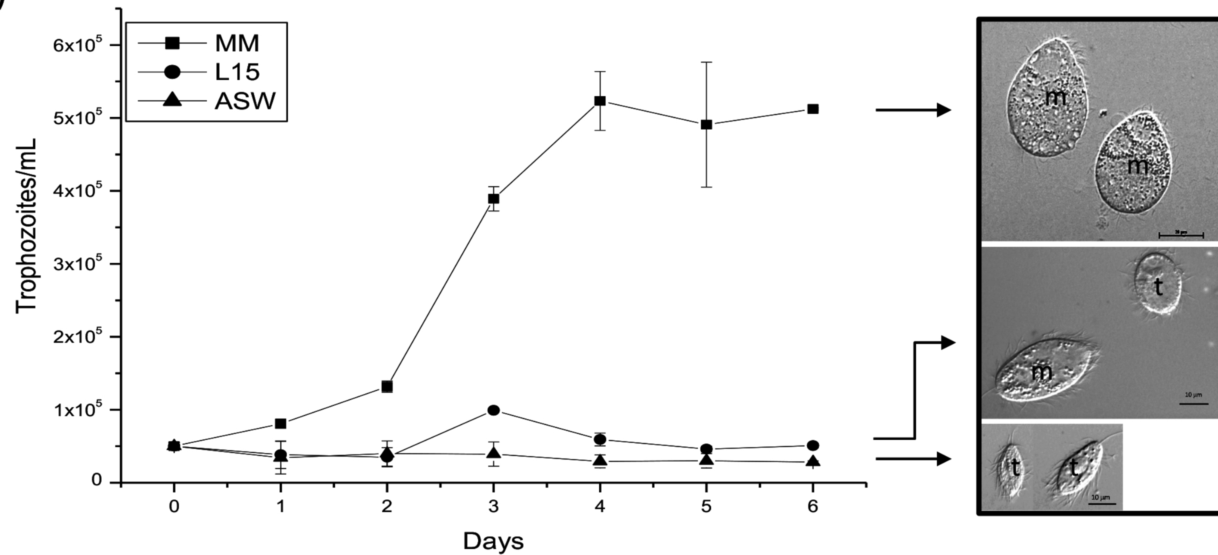
512 containing 11% dimethyl sulfoxide ($Me_2SO$) and 5% glycerol and 5% methanol as
513 cryoprotective agents. Different concentrations of ciliates were cryopreserved, and the
514 survival, expressed in number of motile trophozoites (NMC) /flask, were obtained 72 h
515 after thawing and expressed as mean values ± standard deviations for 5 cryovials.
516 Asterisks indicate the statistical significance (*P* <0.05). † indicates the presence of motile
517 ciliates 98 h after thawing, ††indicates the presence of motile ciliates 146 h after thawing.

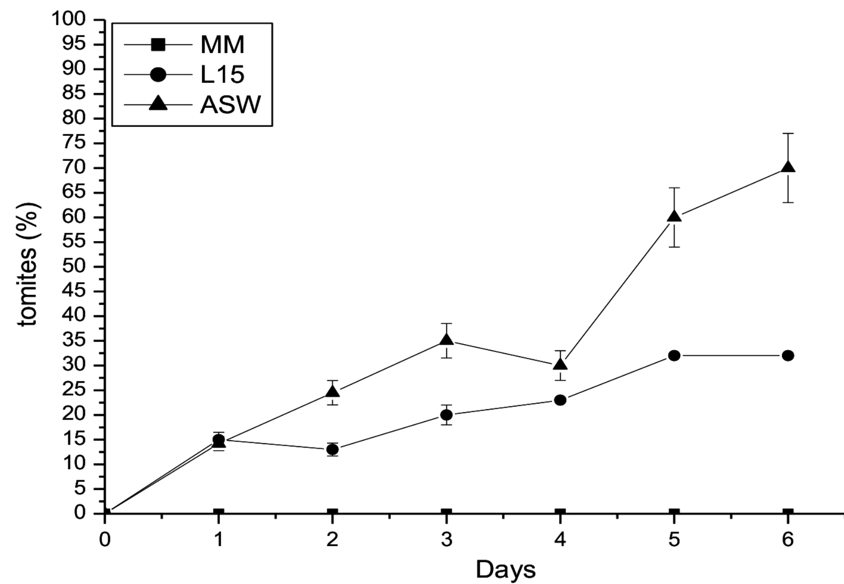

A)
Day 2
Day 4
Day 6
NMC/flask
0
1x10^5
2x10^5
3x10^5
4x10^5
5x10^5
6x10^5
7x10^5
8x10^5
MM / 24
MM / 48
MM / 72
L15 / 24
L15 / 48
L15 / 72
ASW / 24
ASW / 48
ASW / 72
Medium/hours

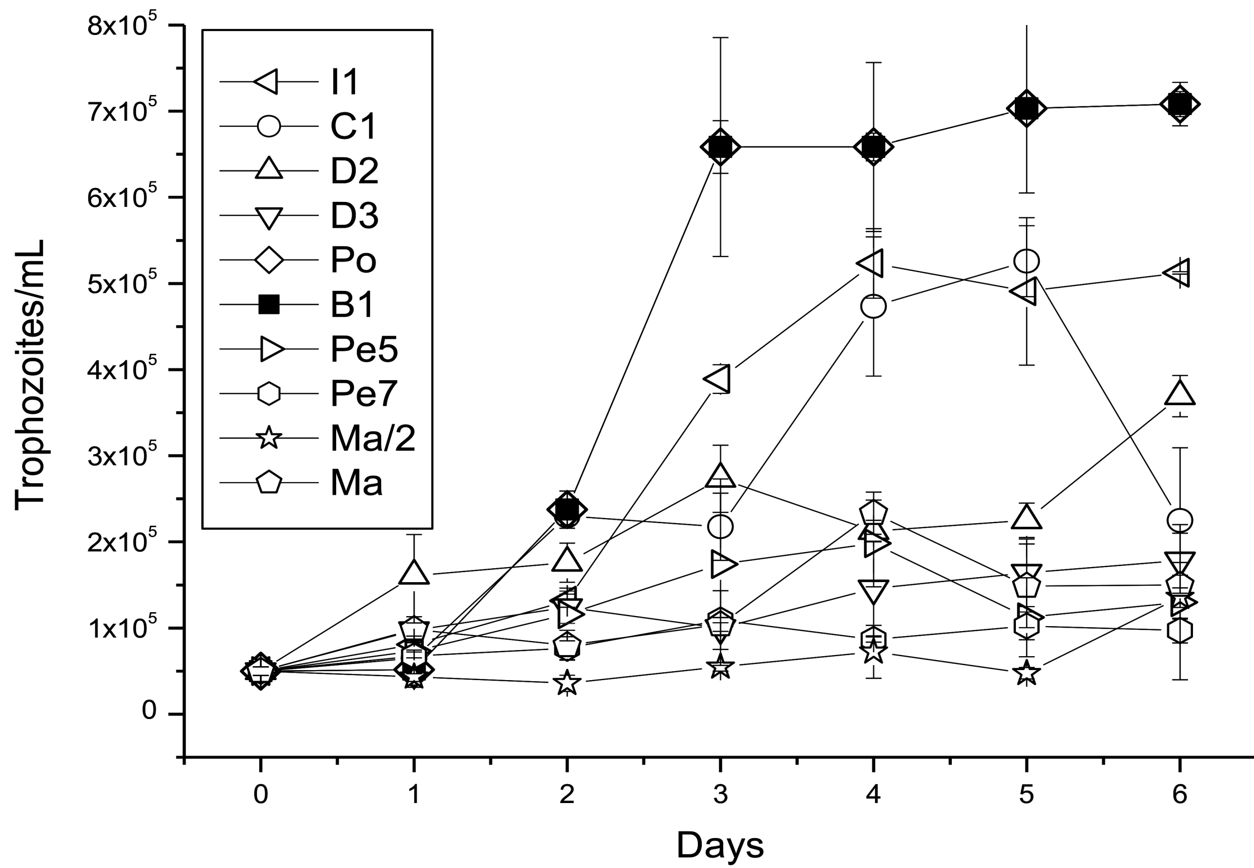

B)
Day 2
Day 4
Day 6
NMC/flask
0
1x10^5
2x10^5
3x10^5
4x10^5
5x10^5
6x10^5
7x10^5
8x10^5
MM / 24
MM / 48
MM / 72
L15 / 24
L15 / 48
L15 / 72
ASW / 24
ASW / 48
ASW / 72
Medium/hours


C)
Day 2
Day 4
Day 6
NMC/flask
0
1x10^5
2x10^5
3x10^5
4x10^5
5x10^5
6x10^5
7x10^5
8x10^5
MM / 24
MM / 48
MM / 72
L15 / 24
L15 / 48
L15 / 72
ASW / 24
ASW / 48
ASW / 72
Medium/hours

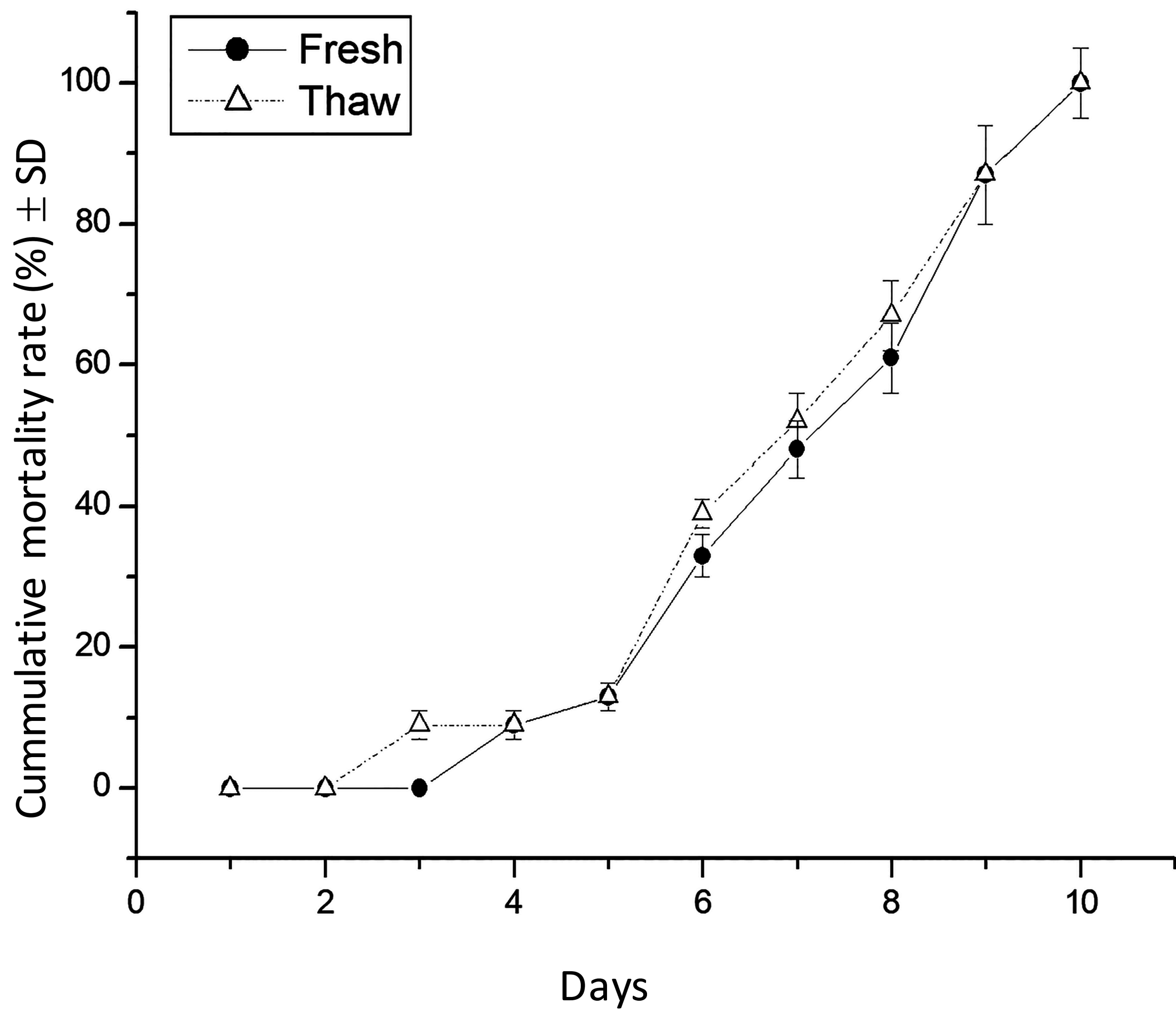

D)
Day 2
Day 4
Day 6
NMC/flask
0
1x10^5
2x10^5
3x10^5
4x10^5
5x10^5
6x10^5
7x10^5
8x10^5
MM / 24
MM / 48
MM / 72
L15 / 24
L15 / 48
L15 / 72
ASW / 24
ASW / 48
ASW / 72
Medium/hours


A)
MM
L15
ASW
Trophozoites/mL
6x10⁵
5x10⁵
4x10⁵
3x10⁵
2x10⁵
1x10⁵
0
0 1 2 3 4 5 6
Days
m
m
t
m
t
t


B)
MM
L15
ASW
tomites (%)
100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
0 1 2 3 4 5 6
Days



Trophozoites/mL
Days
0
1x10^5
2x10^5
3x10^5
4x10^5
5x10^5
6x10^5
7x10^5
8x10^5
0
1
2
3
4
5
6
I1
C1
D2
D3
Po
B1
Pe5
Pe7
Ma/2
Ma


Fresh
Thaw
Cummulative mortality rate (%) ± SD
0
20
40
60
80
100
0
2
4
6
8
10
Days

| $X_1$ | $X_2$ | Glycerol (%) | Culture phase (day) | Viability (%) |
|---|---|---|---|---|
| -1.0 | -1.0 | 5 | 2 | 2.63 |
| 0.0 | 0.0 | 7.5 | 4 | 0.79 |
| 0.0 | -1.0 | 7.5 | 2 | 1.11 |
| 1.0 | -1.0 | 10 | 2 | 0.00 |
| 0.0 | 0.0 | 7.5 | 4 | 0.89 |
| 1.0 | 0.0 | 10 | 4 | 0.00 |
| 0.0 | 1.0 | 7.5 | 6 | 7.11 |
| 1.0 | 1.0 | 10 | 6 | 1.84 |
| -1.0 | 0.0 | 5 | 4 | 1.58 |
| -1.0 | 1.0 | 5 | 6 | 24.74 |

| Independent variable | Coefficient | *P* value |
|---|---|---|
| Constant | -0.05 | |
| $X_1$ | -4.52 | 0.0459* |
| $X_2$ | 4.99 | 0.0341* |
| $(X_1)^2$ | 1.78 | 0.5210 |
| $X_1.X_2$ | -5.07 | 0.0588 |
| $(X_2)^2$ | 5.10 | 0.1143 |

**$R^2$ (fitted by degrees of freedom) (%) = 73.6947**
**Durbin-Watson Statistic = 2.4798 (*P* = 0.7649)**

| Strains | Post thawing survival (days) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| **B1** | | | + | | |
| **C1** | | + | | | |
| **D2** | | | + | | |
| **D3** | | + | | | |
| **I1** | | + | | | |
| **Ma** | | | | + | |
| **Ma/2** | + | | | | |
| **Pe5** | | | | | + |
| **Pe7** | | | | + | |
| **Po** | | | | | + |

| **No. ciliates/cryovial** | ***Miamiensis avidus* strain Ma/2** | |
|---|---|---|
| | Glycerol (5%) + $Me_2SO$ (11%) | Methanol (5%) + $Me_2SO$ (11%) |
| | NMC/flask | |
| **$10^6$** | 746,215 ± 72,185* | 529,230 ±29,320 |
| **$20x10^6$** | 731,400 ± 31,230 | 710,545 ± 60,450 |
| **$40x10^6$** | 18,655 ± 2,650* | 2,105 ± 1,985 |
| | *Miamiensis* sp. strain Ma | |
| | Glycerol (5%) + $Me_2SO$ (11%) | Methanol (5%) + $Me_2SO$ (11%) |
| | NMC/flask | |
| **$10^6$** | 1,580 ± 905 | 0† |
| **$20x10^6$** | 575 ± 510 | 0† |
| **$40x10^6$** | 0† | 0†† |