

Improving quantification of bivalve larvae in mixed plankton samples using qPCR: A case study on *Mytilus edulis*

Alexander, Jenna; Malham, Shelagh; Smyth, David; Webb, Julie; Fidler, David; Bayford, Philippa; McDonald, James; Le Vay, Lewis

Aquaculture

DOI:
[10.1016/j.aquaculture.2020.736003](https://doi.org/10.1016/j.aquaculture.2020.736003)

Published: 15/02/2021

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Alexander, J., Malham, S., Smyth, D., Webb, J., Fidler, D., Bayford, P., McDonald, J., & Le Vay, L. (2021). Improving quantification of bivalve larvae in mixed plankton samples using qPCR: A case study on *Mytilus edulis*. *Aquaculture*, 532, Article 736003.
<https://doi.org/10.1016/j.aquaculture.2020.736003>

Hawliau Cyffredinol / General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 Improving quantification of bivalve larvae in mixed plankton samples using qPCR: A case
2 study on *Mytilus edulis*

3 Jenna L Alexander^a, Shelagh K Malham^a, David Smyth^a, Julie Webb^a, David Fidler^b,
4 Philippa Bayford^a, James McDonald^b, Lewis Le Vay^a

5 ^aCentre of Applied Marine Sciences, School of Ocean Sciences, Bangor University, Anglesey, LL59 5AB, UK

6 ^bSchool of Natural Sciences, Bangor University, Gwynedd, LL57 2UW, UK

7

8 * Corresponding author at: Centre of Applied Marine Sciences, School of Ocean Sciences, Bangor University,
9 Anglesey, LL59 5AB, UK
10 E-mail address: j.lalexander@bangor.ac.uk

11 Abstract

12 Problems sourcing spat from naturally occurring seed beds for relay has been the main underlying limiting
13 factor in mussel aquaculture over recent years. Attempts to address this issue require a better understanding
14 of mussel larval patterns during the initial planktonic phase prior to settlement. A crucial step in progressing
15 the detection and prediction of larval travel is the accurate identification of mussel larvae within environmental
16 samples in conjunction with hydrodynamic patterns. This requires unambiguous, high throughput methods for
17 the discrimination between larvae of morphologically-similar bivalve species. Presently methodologies require
18 direct microscopic observation with accuracy based on taxonomic skills, techniques which are impractical for
19 large-scale larval movement studies. Species-specific polymerase chain-reaction (PCR) presents a powerful
20 alternative method for species detection. In addition, the technique allows for the collection of quantitative
21 real-time PCR data which can be used for inter sample comparisons of relative larval abundance.

22 In this study Blue mussel *Mytilus edulis* D-stage larvae were used to compare and optimise DNA extraction
23 methods and to examine the quantitative potential of species-specific qPCR targeting the polyphenolic
24 adhesive protein involved in byssal thread production. Molecular data were used to create a predictive model
25 which could be employed to determine larval numbers from real-time data. Assays were then used to estimate
26 *M. edulis* abundance in vertical –tow plankton samples collected from a trial aquaculture site off the North
27 Wales coast.

28 This method offers a more effective means of temporal and spatial larval pattern analysis which will improve
29 the tracking and predictive capabilities of seed supply hydrodynamic models used for dispersal and population
30 connectivity predictions.

31 **Keywords**

32 *Mytilus edulis*, QPCR, DNA extraction, Larval settlement, Seed supply modelling

33 1. Introduction

34 The majority of the global production of marine bivalve molluscs for human consumption is provided by
35 cultured stocks (Wijsman et al., 2019). Shellfish production has increased rapidly in Asia, yet there has been
36 limited growth in Europe in recent decades, with a decrease in output from the mussel aquaculture sector
37 being the primary contributor to a gradual decrease in productivity (Hambrey & Evans, 2016). While growth
38 potential within the sector is considered strong, it is heavily constrained by the availability of wild juvenile
39 mussels known as seed or spat which derive from settlement of planktonic larvae. Spat settlement is highly
40 variable, both temporally and spatially, with first phase settled beds quickly lost through predation or storm
41 events (Kamermans & Capelle, 2018; Dankers & Zuidema, 1995; Nehls & Thiel, 1993). Variations in spat
42 abundance and location are determined by planktonic larval dispersal and settlement processes which vary
43 seasonally and locally but are poorly understood (Stirling et al., 2018; Knights et al., 2006; Dobretsov & Miron,
44 2001).

45 The use of hydrodynamic larval particle tracking models combined with in-situ time series larval identification
46 data has proved unreliable in predicting first phase settlement sites of *M. edulis* (Stirling et al., 2018). A main
47 impediment to studies of larval ecology and distribution is the lack of unambiguous methods for the
48 discrimination of bivalve species larvae with similar morphological characteristics. Established methods of
49 larval identification involve direct microscopic observation, however these are insufficient for large scale
50 studies of larval movement, and are limited by cost, time, and researcher experience (Bott et al., 2010). Whilst
51 identifying larvae to class level (Bivalvia) is simple, resolution to lower levels (family, genera, or species)
52 requires extensive taxonomic experience in marine bivalve larvae and therefore has great potential for human
53 error which is confounded by phenotypic plasticity.

54 More recent bivalve larval identification techniques can produce specific level identification in mid to late stage
55 larvae via analysis of hinge structure using scanning electron microscopy or optical compound microscopes
56 equipped with high-intensity reflected light sources (Lutz et al., 2018). However, this involves time-consuming
57 disarticulation and mounting of valves which limits use for *in-situ* field studies. Advanced techniques such as
58 Raman spectroscopy (Thompson et al., 2015) and the use of polarized light to identify colour patterns from
59 larval shells (Goodwin et al., 2018) offer an alternative but suffers from a lack of specificity and low taxonomic
60 determinations which requires sorting or isolation of individual larvae. Newer alternative methods based on
61 molecular or immunological techniques offer more accurate and precise identification. Detection using

62 directed antibodies and fluorescently labelled DNA probes have been used successfully to identify plankton
63 larvae to family, genus or in some cases species level (Perez et al., 2009; Abalde et al., 2003; Paugam et al.,
64 2003; Paugam et al., 2000; Demers et al., 1993). However, their use in field studies has been rare with both
65 methods having limitations which can slow down sample processing and can result in erroneous false positive
66 or negative identification (Heaney et al., 2011).

67 PCR-based techniques have become increasingly popular as an identification tool for aquatic species,
68 providing accurate and specific detection via the use of targeted oligonucleotide primers (Dysthe et al., 2018;
69 Sterling et al., 2018; Ludwig et al., 2014; Sanchez et al., 2014; Bott & Giblot-Ducray, 2011(a); McBeath et al.,
70 2006). Researchers have attempted to use PCR to quantify larvae on an individual basis, performing
71 extraction and analysis individually using single larvae (Sawada et al., 2008; Larsen et al., 2007). However,
72 larvae contain small amounts of tissue and correspondingly low levels of DNA (Lasota et al., 2013) with the
73 result that these assays often have a high failure rate (Christian et al., 2007) particularly with ethanol- or
74 chemically fixed samples (Goodwin et al., 2018). The potential risk of skewed results due to larval selection
75 bias is also a concern and as the whole larvae is often used in a single PCR this eliminates the possibility of
76 testing for multiple species (Larsen et al., 2005; Hosoi et al., 2004; Hare et al., 2000).

77 Environmental DNA (eDNA) studies, which analyse air, soil or water samples for the presence of intracellular
78 or extracellular target DNA, have been used to non-invasively detect a range of species (Prié et al., 2020;
79 Mychek-Londer et al., 2019; Günther et al., 2018; Klymus et al., 2017 or see Ruppert et al., 2019 for review).
80 This offers a number of benefits over traditional sampling methods, particularly when target species are less
81 abundant or at challenging survey locations and when a number of species are to be sampled from the same
82 location (Dysthe et al., 2018; Furlan et al., 2016; Rees et al., 2014(b)). These studies typically use
83 metabarcoding to identify multiple species within a sample, which requires post-amplification processing and
84 an adequate reference database (Cowart et al., 2015) or DNA-barcoding using species-specific markers can
85 be used to target short fragments of mitochondrial DNA (mtDNA) (Stirling et al., 2018; Rees et al.,
86 2014(b)). Yet the failure rate of this technique when applied to molluscs can be unsatisfactorily high, up to
87 43.6% in some cases (Cahill et al., 2018; Barco et al., 2016). Unresolved criticisms of this technique concern
88 both experimental design and analytical methodology (e.g. primer bias (Couton et al., 2019), primer mismatch
89 (Cahill et al., 2018), inadequately 'populated' barcode reference libraries (Rosenberg. 2014), bacterial
90 infection biasing mtDNA variation (Kaya & Ciplak. 2018), anthropogenic artefacts during reference library

91 development (Weigand et al., 2019). High genetic variability and the mixing of genetic lineages within a
92 species, along with hybridisation and introgression events may also confuse taxonomic boundaries and
93 hamper accurate taxonomic identifications (Trivedi et al., 2016; Sun et al., 2016). Hybridization, introgression
94 and heteroplasmy of mtDNA are known to occur in *Mytilus* mussels where species boundaries overlap (Barco
95 et al., 2016; Kijewski et al., 2006; Rawson & Hilbish. 1998), a consequence of backcrossing with one or both
96 parental taxa and of the peculiar mtDNA inheritance systems observed in these and other bivalves (Schizas.
97 2012; Breton et al., 2007; Theologidis et al., 2008). Marine mussels of the order Mytiloida exhibit an
98 inheritance mechanism known as doubly uniparental inheritance (DUI) (Zbawicka et al., 2007; Breton et al.,
99 2007; Theologidis et al., 2008). The implications for this are the possibility of concurrently amplifying M and F
100 DNA when targeting mitochondrial gene regions during PCR, which depends on sequence divergence
101 between M and F types; average sequence divergence in the 3 *Mytilus* species is ~8.3% but levels can be
102 >20% (Śmietanka et al., 2016; Schizas, 2012). Both DUI and non-DUI heteroplasmy have been observed in
103 crossings between blue mussels (Kijewski et al. 2006), and this along with other issues inherent in DNA
104 barcoding/ metabarcoding, some of which are mentioned above, can have significant implications for accurate
105 taxonomic classification and ultimate barcoding success (Larraín et al. 2019).

106 Of particular concern is the uncertain ability of metabarcoding to produce quantitative results, with many
107 studies maintaining that read numbers do not accurately represent the proportions of each species (Piñol et
108 al., 2019; Klymus et al., 2017; Sun et al., 2015). Real-time PCR using targeted primers presents a powerful
109 alternative method to detect species within environmental samples, simultaneously identifying and quantifying
110 target DNA and allowing relative sample comparisons (Peñarrubia et al., 2016). A number of studies have
111 examined the potential of qPCR to detect and potentially quantify eggs and larvae from marine and freshwater
112 species (Odero et al., 2018; Sanchez et al., 2014; Jensen et al., 2012; Pan et al., 2008), including *M. edulis*.
113 Dias et al, (2009) estimated mussel larval numbers from plankton samples gathered in Loch Etive using
114 nuclear primers developed by Inoue et al, (1995), giving an upper limit for predicted larvae based on a
115 standard curve established using a single stage (D larvae). In this study we aimed to further develop this
116 technique in order to provide more accurate measures of larval abundance in plankton samples, using blue
117 mussel *M. edulis* D-stage larvae obtained from single species culture to compare and optimise DNA extraction
118 methods for larval samples. Spawned larvae were also used to create a model which predicts approximate
119 mussel larval numbers from real-time data, allowing a range of reported values which more accurately mirrors
120 the expected variation resulting from biological (variations in larval size and/ or molecular copies,

121 nonhomogeneous distribution of template DNA in sample), sample preparation (liquid retention and adhesion
122 of target molecules to pipette tips, etc.) and assay (intra- and inter-plate variation, improper background
123 subtraction) variation. This method was used to analyse the bivalve content of vertical –tow plankton samples
124 collected from the site of a trial mussel longline system in North Wales

125 In order to address the industry concerns of diminishing mussel seed beds effective tracking of larval supply is
126 essential and hence the development of a high throughput tool for rapid identification and quantification of
127 *Mytilus edulis* larvae within mixed plankton samples is a priority. The eventual methodology should be high
128 throughput, cost effective and robust enough to be applied to field samples which can be highly variable in
129 terms of organic and inorganic content as well as the condition of preservation.

130 **2. Methods**

131 *2. 1. Comparison of DNA extraction methods for generating larval standard curves in qPCR*

132 Thirty adult *Mytilus edulis* were gathered from the Menai Strait, Anglesey, to generate standards for
133 qPCR and as positive control DNA. Specimens were dissected and approximately 30 mg of adductor or
134 mantle tissue were extracted using a modified DNEasy Powersoil kit (Qiagen) protocol, where the 10 minute
135 vortex step to homogenise tissues was replaced with a 2 x30 s bead beating steps at 5.5 m/s in a benchtop
136 homogeniser (Precellys 24 (Bertin Instruments)). *M. edulis* larvae were obtained from single species
137 experimental culture and fixed in 99.5% ETOH. D-stage larvae were counted under a light microscope and
138 manually transferred using a pipette to 1.5ml tubes containing 70% ETOH. Tubes containing 1, 10, or
139 100 larvae were collected in triplicate and standard enumeration and volumetric determination were used to
140 generate batches of approximately 1000 larvae.

141 Prior to extraction, 1, 10, 100, or 1000 larvae were filtered on to 0.45 µm cellulose nitrate (C-N) filter papers
142 using a vacuum filter rig before being cut in to strips to improve chemical digestion. Genomic DNA was
143 extracted using the protocols described below.

144 *2. 1. 1. Adapted DNEasy Powersoil Kit (Qiagen)*

145 Extraction was performed according to the DNEasy Powersoil kit protocol, with modifications (see A.1 in
146 Supplementary material, Appendix A). DNA was eluted in to 70ul 0.1 µM TE buffer.

147 To test the effect of homogenisation time on extraction efficacy, three methods were tested: samples were
148 homogenised using a benchtop homogeniser for two cycles of 20, 40, or 60 s.

149 2. 1. 2. *Adapted E.Z.N.A Mollusk kit (Omega Biotek)*

150 Extraction was performed according to the E.Z.N.A Mollusk DNA extraction kit with modifications (see A.2 in
151 Supplementary material, Appendix A). DNA was eluted in to 70 µl 0.1 µM TE buffer.

152 To test the effect of incubation time on extraction efficacy, samples were incubated for 90, 180, or 270
153 minutes.

154 2. 1. 3. *Cetyltrimethylammonium bromine (CTAB) buffer extraction*

155 DNA was extracted based on a method used by Balasingham, et al. (2018) using cetyl-
156 trimethylammonium bromide (CTAB) extraction buffer (see A.3 in Supplementary material, Appendix A). DNA
157 was resuspended in 70 µl 10 µM TE buffer and 0.5 µl RNase A.

158 2. 1. 4. *qPCR analysis of DNA extraction efficiency*

159 DNA yields were quantified using a Qubit 3.0 fluorometer and Quant-iT™ dsDNA high sensitivity reagents for
160 a direct comparison of extraction efficacy. qPCR was carried out in a QuantStudio™ Flex 6 Real-Time PCR
161 System (Applied Biosystems, USA) using nuclear Me15/16 *Mytilus* primers developed by Inoue, et al. (1995).
162 PCR reactions consisted of 10 µl 1x Kapa SYBR FAST Low ROX mix, 0.2 µM of each primer, 2 µl of DNA
163 template, and molecular grade H₂O in a total reaction volume of 20 µl. Cycling conditions were set to 95 °C for
164 2 minutes, followed by 40 cycles of 95 °C for 3 s, 60 °C for 20 s, and 72 °C for 20 s. Standard curves were
165 constructed using serial dilutions of target amplicons (1/10 fold dilutions from 1 x 10⁷-1 x 10¹ molecules/ µl),
166 and negative (no template) and positive controls were included in the form of PCR-g H20 and *M. edulis* gDNA
167 replacing DNA template, respectively. Samples were run in triplicate. Threshold values were set during the
168 exponential phase of the reaction, allowing cycle threshold (Ct) values to be determined for each sample.

169 2. 2. Quantitative PCR development

170 2. 2. 1. *Specificity, efficiency and precision of M. edulis qPCR assay*

171 To determine the specificity of the primer set used, the sequences were tested *in silico* against
172 the NCBI database using Primer-BLAST (<https://blast.ncbi.nlm.nih.gov>) and tested for specificity and cross-
173 reactivity through PCR with DNA from other bivalve species of commercial interest which potentially occur in
174 the area (*Cerastoderma edule*, *Pecten maximus*, *Aquiptecten opercularis*, *Ensis siliqua*, *Crassostrea gigas*,
175 *Ostrea chilensis*). To ensure non-occurrence of cross-reaction was not due to absence of amplifiable
176 DNA, end-point PCR was carried out on all bivalve DNA using 'universal' invertebrate primer pair LCO₁₄₉₀ and
177 HCO₂₁₉₈ developed by Folmor, et al. (1994) targeting a 710-bp fragment of the mitochondrial cytochrome c
178 oxidase subunit I gene (COI). Samples were composed of 1 x MyTaq Redmix (Bioline), 0.4 µM of each
179 primer, 2 µl of DNA template, and molecular grade H₂O for a total reaction volume of 25 µl. Cycling conditions
180 were set to 95 °C for 3 minutes, followed by 30 cycles of 95 °C for 20 s, 45 °C for 20 s, and 70 °C for 30 s.

181 The efficiency of the PCR reaction was assessed using triplicate ten-fold serial dilutions (1 x 10⁷-1 x 10¹
182 molecules/ µl) of target amplicons generated from DNA extracted from adult *M. edulis* tissue. Assay precision
183 was analysed by calculating the intra-assay coefficient of variation (COV) for Ct values generated
184 from triplicate serial dilutions (STDS 1-3).

185 2. 2. 2. PCR-inhibition from extracted samples

186 To test the inhibiting effects of planktonic matter which may affect overall accuracy and sensitivity of the qPCR
187 assay we spiked DNA extracted from 20ml plankton and water samples containing 100 *M. edulis* larvae with 1
188 ng of control DNA from a pure culture of the bacterial species, *Gibbsiella quercinecans* (Brady, et al. 2010).
189 The potential for inhibiting compounds resulting from the extraction process itself was examined by
190 including extracts from negative controls of distilled water. All samples were processed with the
191 E.Z.N.A Mollusk extraction kit with a 180 minute incubation step, and DNA extracts were spiked with *G.*
192 *quercinecans* DNA. Subsequently, a Taqman MGB-based assay targeting *G. quercinecans* was employed to
193 detect inhibition. Reactions consisted of 1 x SensiFast mix (Bioline), 0.4 µM of each primer, 0.1 µM of probe, 1
194 µl of DNA template, and molecular grade H₂O for a total reaction volume of 20 µl. All qPCR assays were
195 carried out in a QuantStudio™ Flex 6 Real-Time PCR System (Applied Biosystems, USA); the initial
196 denaturation was 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 10 s and 60 °C for 40 s. Standards
197 were run in triplicate in 1/10 fold dilutions from 3 x 10⁸ - 3 x 10³ molecules/ µl. Negative controls were included
198 by replacing DNA template with 1 µl molecular-grade H₂O.

199 Reactions were initially run with unspiked sample extracts to ensure no unwanted cross-amplification
200 occurred which could affect results (data not included).

201 2. 2. 3. Quantitative potential of qPCR for predicting *M. edulis* larval abundance

202 D-stage larvae were isolated to 1.5 ml tubes containing 70% ETOH. Tubes containing 1 - 1000 larvae were
203 collected in triplicate and filtered through 0.45 µm cellulose nitrate filter papers prior to genomic DNA
204 extraction using E.Z.N.A Mollusk extraction kit with 180 minute incubation step. Quantitative PCR was carried
205 out under the conditions listed in Section 2.1.4, with each sample being run in triplicate. Template DNA
206 quantity was calculated by plotting Ct values to standard curves obtained from serial dilutions of target
207 amplicons (1/10 fold dilutions from 1×10^7 - 1×10^1 molecules/ µl) and negative and positive controls were
208 included in the form of PCR-g H₂O and *M. edulis* gDNA.

209 Actual extracted larval numbers and predicted values for number of larvae per PCR reaction are given in
210 Table 1.

211 Table 1. Number of DNA-extracted *M. edulis* larvae and corresponding number input per PCR reaction.

Extracted no. <i>M. edulis</i> larvae	1000	200	100	60	40	30	20	10	8	6	5	4	2	1
Predicted no. larvae/ PCR reaction	22.22	4.44	2.22	1.33	0.89	0.67	0.44	0.22	0.18	0.13	0.11	0.09	0.04	0.02

212

213 The specificity of the reactions was examined by the generation of melt curves after amplification. At low
214 template concentration, non-specific amplification can cause fluorescence to reach detectable levels. In order
215 to avoid false positive results, samples which had no visible melt curve corresponding with the target
216 amplicon, had reported molecular copies of <100 and were deemed to have no target molecules were given a
217 value of 0. The reported numbers of gene copies were log (x+1) -transformed prior to statistical analyses, in
218 order to improve the normality of the data and the homogeneity of variance. A singular effects LMM
219 (generalised linear mixed effects model) with a Gamma error distribution was fitted for molecular copies and
220 Ct values, where copies or Ct values are explained by the log₁₀ of larval number per PCR reaction, with
221 random factor of sample accounting for single samples contributing separate measurements (done
222 using glmer function from the lme4 package for R (version 3.5.3) (Bates, et al. 2014).

223 2. 3. Plankton trials

224 Plankton samples were collected from a trial aquaculture site operated by Bangor University and Deepdock
 225 Ltd off the North Wales coast (53°18'60.7"N 3°59'39.2"W), using 100 µm mesh plankton net with ballast
 226 weight deployed vertically from approximately 1-2 m above the sea bed and fixed following the protocol
 227 recommended by Black and Dodson (2003). Five subsamples were used to record the number of individual
 228 larvae belonging to the class Bivalvia and bivalve density (larvae m⁻³) was calculated by multiplying the
 229 average number of larvae by the total volume of the entire plankton sample, divided by the volume (m³) of
 230 seawater sampled. From each sample, 20ml was passed through a 0.45 µm C-N filter; one half of the filter
 231 was stored at -80 °C and the second half was cut in to strips and used for DNA extraction using the modified
 232 E.Z.N.A mollusc extraction method with 180 minute incubation. Samples were analysed by the qPCR assay
 233 for *M. edulis* detailed in Section 2. 1. 4. and template DNA quantity was calculated by plotting Ct values to
 234 standard curves obtained from serial dilutions of target amplicons (1/10 fold dilutions from 1 x 10⁷-1 x 10¹
 235 molecules/ µl). The model generated in Section 2. 2. 3. was used to generate mean and upper and lower
 236 prediction values for number of larvae per sample, which were converted to larvae/ m³ for direct comparison
 237 with observed larval numbers. Efficiency and precision values were also generated for the reaction.

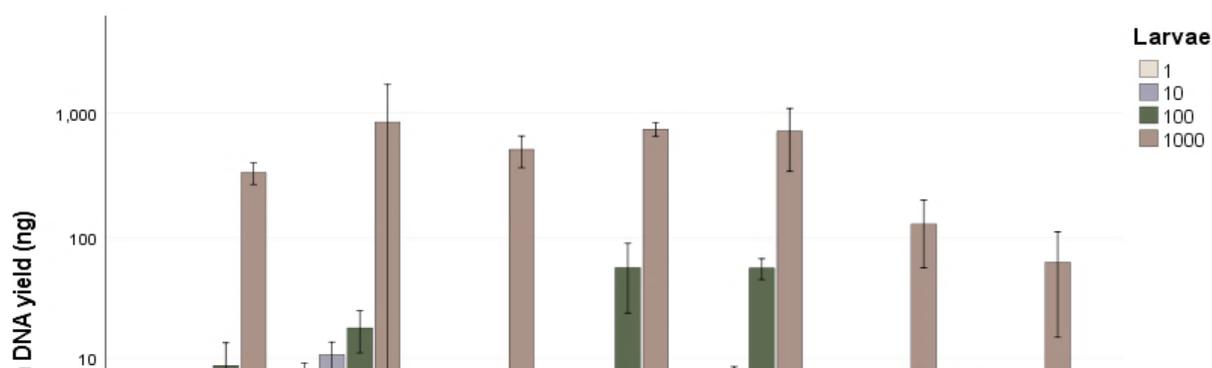
238 3. Results

239 3. 1. Comparison of DNA extraction methods for generating larval standard curves in qPCR

240 A comparison of extraction from 1, 10, 100, or 1000 larvae showed that the extraction kit and method used
 241 has a significant effect on DNA yield ($F(4, 51) = 2.715, p = .04$) (Figure 1) and therefore subsequent qPCR
 242 detection sensitivity. Extraction using the Balasingham CTAB method resulted in the lowest DNA yields
 243 overall and was the least promising method for further experimentation. Both the E.Z.N.A and Powersoil kits
 244 yielded sufficient DNA overall but incubation or homogenisation time had a clear effect on yield: too much
 245 (270m or 2 x 60s cycles) or too little ((90m or 2 x 20s cycles) of each resulted in reduced DNA recovery.
 246 Ultimately the E.Z.N.A kit extracted DNA of ample quantity after 90 and 180m incubation periods and was
 247 less variable in efficiency than the Powersoil kit even under optimal conditions (2 x 40s homogenisation
 248 cycles).

249

250



251

252

253

254

255

256

257

Fig 1. Mean log DNA yield (ng) for 1, 10, 100, and 1000 *M. edulis* larvae using modified DNEasy Powersoil kit, E.Z.N.A Mollusk kit and Balasingham CTAB extraction protocols. Error bars represent standard deviations over three replicates. X-axis values refer to duration (in seconds) of tissue homogenisation cycle (Powersoil – 20, 40, 60) or tissue incubation time (in minutes) (EZNA – 90, 180, 270).

258

from serial dilutions of template DNA. From these standard curves, we aimed to determine the linearity (on a

259

log scale) of the qPCR on samples extracted using each method and possible detection limits for the assay

260

(Figure 2). DNA extracted from 1 – 1000 larvae using the E.Z.N.A extraction method with 180m incubation

261

step fitted to the standard curve with narrowest confidence interval and greatest differentiation between larval

262

values. This interaction between extraction method and larvae number was significantly different between

263

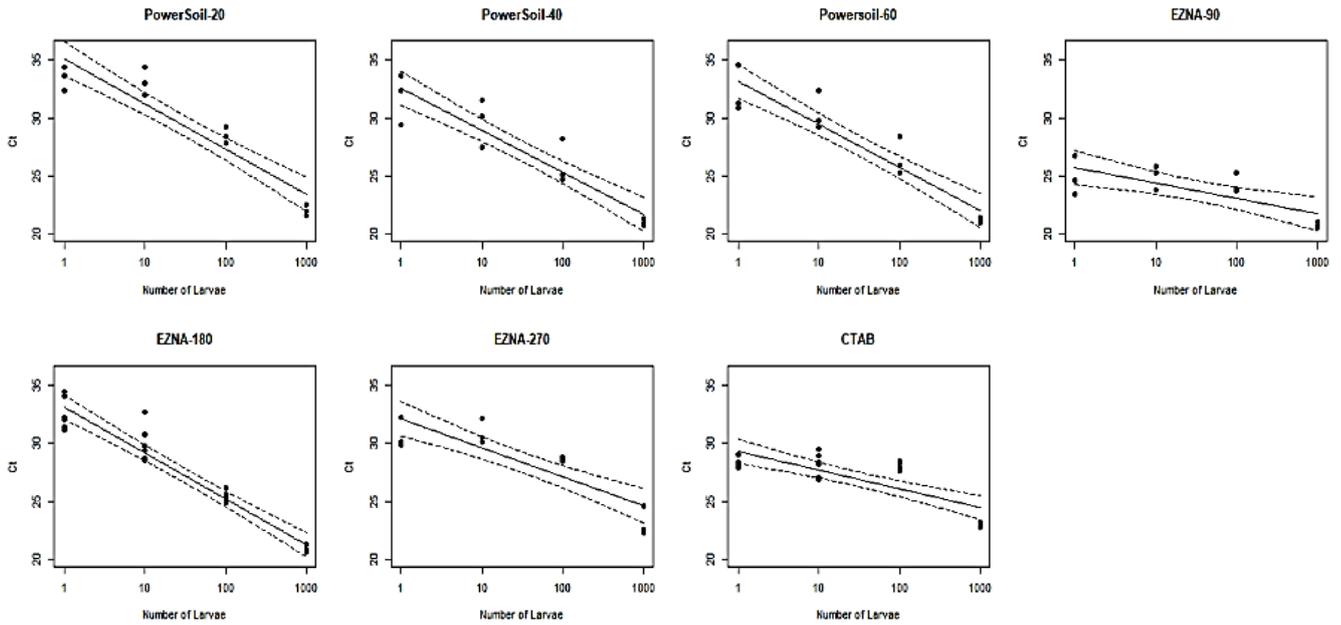
methods ($F(12, 80) = 5.93, p < 0.001$). Based on these results and DNA extraction yield, we determined this

264

method had the greatest potential for optimal qPCR assay sensitivity and accuracy, and was therefore used

265

in any further experimentation.



266

267

268

Fig 2. Ct values obtained for 1, 10, 100, 1000 *M. edulis* larvae extracted using modified DNEasy Powersoil kit, E.Z.N.A Mollusk kit and Balasingham CTAB extraction protocols and mapped to standard curves created from 1/10 fold serial dilutions of target amplicon X-axis values refer to duration (in seconds) of tissue homogenisation cycle (Powersoil – 20, 40, 60) or tissue incubation time (in minutes) (EZNA – 90, 180, 270).

269

3. 2. 1. Specificity, efficiency and precision of *M. edulis* qPCR assay

270

271

272

273

274

275

276

277

278

279

280

281

Primer sequences tested *in silico* against the NCBI database aligned only to members of the *Mytilus edulis* complex. When tested for cross-reactivity with a number of bivalve species found at our sample site, cross-reaction occurred with *Crassostrea gigas* and to a lesser extent *Cerastoderma edule*. All other species showed negligible amplification. Cycle threshold (Ct) values obtained from *C. edule* gDNA were sufficiently high (35.12 ± 0.64) and reported copies low (average 109.2 molecules) that the number of larvae in a sample would have to be substantial to have any significant effect on amplification. Higher detectable fluorescent signals were detected from *C. gigas* gDNA, where the reported molecular copies were 3804.1 ± 3600.5 , the equivalent of approximately 51 larvae; in comparison, *M. edulis* gDNA yielded the equivalent amount of template as 430 larvae, indicating much higher affinity binding of primers to the intended *Mytilus* template. Whilst the potential level of amplification due to cross-reactivity with *C. gigas* larvae is comparatively low and unlikely to significantly skew results, it could result in false positives or exaggerations of mussel larval abundance in areas where naturally-spawning populations of both species occur.

282

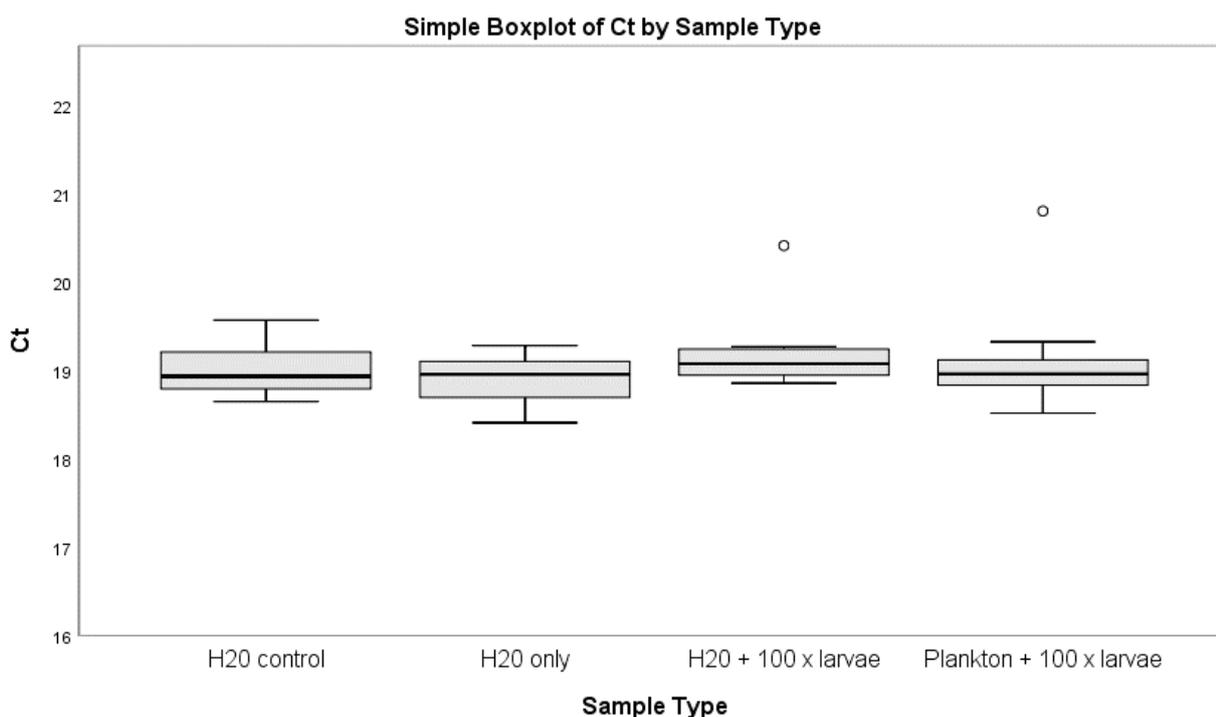
283

For series there was a high correlation between cycle number and dilution factor, R^2 0.99 and slope values of -3.49, close to the theoretical value of - 3.32, indicating an efficiency of 93.32 %. Based on Ct values and

284 coefficient of variation (CV), the mean \pm S.D. intra-assay reproducibility of triplicate intra-assay serial dilution
285 tests (STD 1-3) was 0.82 ± 0.59 %, values considered acceptable (<5%) for validating assay precision.

286 3. 2. 2. PCR-inhibition from extracted samples

287 Prior analysis using *Gibbsiella quercinecans* primer sets on un-spiked extracts showed negligible amplification
288 from samples, therefore results were not affected by sample contamination. No significant inhibition of PCR
289 was observed in spiked samples compared to controls ($F(3,50) = 1.207$, $p = .317$) regardless of sample type
290 (Figure 3), indicating the E.Z.N.A extraction kit efficacy in removing potential inhibitors, or the ability of the
291 qPCR reagent mix to successfully overcome inhibitors, or a combination of both. Assay inhibition from
292 samples extracted using this method is likely to be low even from mixed plankton samples and from relatively
293 high numbers of larvae (≤ 100). As a consequence DNA from environmental samples will not need to be
294 diluted which could reduce assay sensitivity.



295

Fig 3. Ct values \pm SE obtained for DNA samples extracted from distilled water without larvae (H2O only) and containing 100 *M. edulis* larvae (H2O + 100 larvae), and plankton samples containing 100 larvae (Plankton + 100 larvae) spiked with *G. quercinecans* gDNA as compared with spiked control (H2O control). Outliers (>3 x interquartile (IQ) range) are marked with a circle (O) on the boxplot.

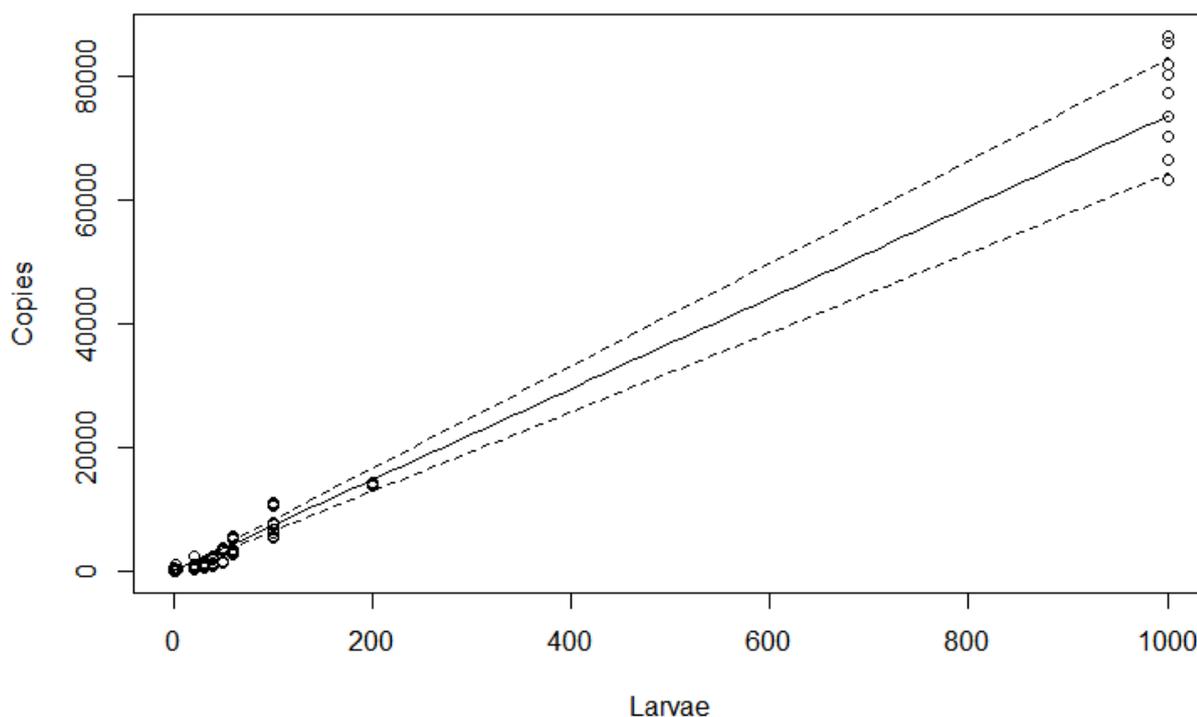
296

297 3. 2. 3. Quantitative potential of qPCR for predicting *M. edulis* larval abundance

298 Spawned *M. edulis* larvae were used to examine the quantitative potential of qPCR, where data generated
299 from differing concentrations of larvae were used to generate a model which predicts larval numbers from
300 real-time data (Figure 4). Number of larvae with random factor of sample can be used as a predictor of
301 molecular copies (LMM: $t = 15.46$, $p < 0.001$), with the model line of best fit which predicts 7.34×10^1 copies/
302 larvae $SE \pm 4.75 \times 10^0$.

303 Reaction efficiency was less than optimal ((76-76.95 %) during this reaction however high R^2 values of 0.99
304 and CV values demonstrated high intra- and inter-assay precision, where the mean \pm S.D. between three runs
305 (Plate 1 - 3) with standard dilutions was $1.78 \pm 0.6 \%$.

306 A distinctive melt curve representing the amplicon of interest was used to diagnose the presence of target
307 DNA when occurring in all 3 replicate values for a given samples, reducing the potential for contamination
308 effecting false positives. Reliable limit of detection was 0.04 larvae, correspondent to 2 larvae per extract,
309 where all samples satisfied criteria for detection. Samples with an input of 0.02 larvae satisfied these criteria in
310 50 % of samples, therefore extracts with a single larvae can be expected to be identified in half of samples. To
311 confirm positive results obtained from single larvae, DNA was sequenced and returned 100 % match with the
312 reference sequence from GenBank and the sequences generated from adult *M. edulis* gDNA. In low-template
313 samples (0.02 - 0.09 larvae) additional peaks were detected and attributed to primer-primer interactions.



314

Fig 4. Line of best fit and 95% prediction intervals for number of extracted larvae as a predictor of molecular copies of *M. edulis* DNA generated by qPCR assay.

315

316 3. 3. *Plankton trials*

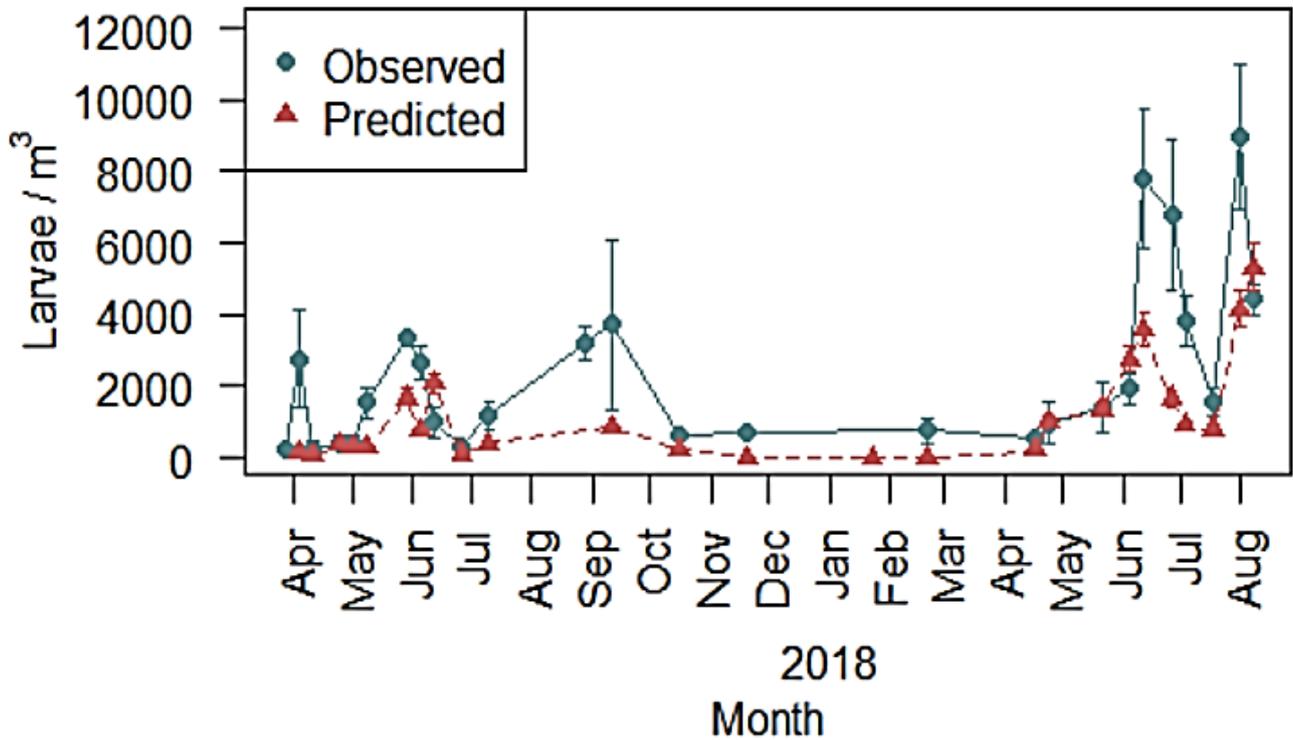
317 The results obtained from plankton sample analysis by visual identification and real-time PCR are in Figure 5.

318 The standard curve efficiency for the assay was 101.9 %.

319 Bivalve larvae were visually detected throughout the sampling period. Visual counts and qPCR estimates of
320 abundance followed a similar pattern (Figure 5) with typically lower magnitude peaks for *M. edulis*.

321 Significantly more larvae were detected in samples gathered in 2018, both via assessment of visual counts
322 and qPCR abundance estimates. Peak abundance values for 2018 were 8,956 SD \pm 1,997 and 3,653 – 4,713
323 nm⁻³ (no. larvae/m⁻³) for bivalve (via visual counts) and *M. edulis* larvae (via qPCR abundance estimates),
324 respectively, compared to 3,712 SD \pm 2,353 nm⁻³ and 1,808 – 2,364 nm⁻³ in 2017.

325 Similar abundance patterns were observed during 2017 and 2018, with peaks in June and during the
326 Summer-Autumn period. An initial increase in larvae identified visually in April 2017 (2,762 SD \pm 1,353 nm⁻³)
327 did not correspond to an increase in *M. edulis* larvae detected by qPCR, suggesting the majority of larvae in
328 the water column during this time could be assigned to other non-Mytilus bivalve taxa. Abundance of *M. edulis*
329 during Summer-Autumn 2017 was lower than expected (considering observed trend correlation and the high
330 abundance of bivalve larvae during this period); no molecular data was available from August to mid-
331 September so potentially peak abundance was missed. The mean density of visually detected bivalve larvae
332 per m³ of seawater was lowest in samples taken from November to March (<766 nm⁻³). No *M. edulis* larvae
333 were detected by qPCR during this period. QPCR specificity was confirmed with melt curve analysis and the
334 sequencing of a random selection of positive samples which returned 100% positive matches with the
335 reference sequence from GenBank and sequences generated from adult *M. edulis* gDNA.



336

Fig 5. Observed (visually counted) larvae belonging to Class Bivalvia and predicted (estimated by qPCR abundance) *M. edulis* larvae in vertical-tow environmental plankton samples taken over a 2 year period. Error bars for observed larvae represent standard deviations over five replicate subsamples. Error bars for predicted larvae represent larval numbers calculated from 95% prediction intervals generated using a singular effects LMM (generalised linear mixed effects model) with a Gamma error distribution fitted for molecular copies.

337

338 **4. Discussion**

339 Species level identification of bivalve larvae within planktonic samples can be particularly challenging as a
 340 high degree of taxonomic proficiency is required, even when skilled an average visual classification can
 341 exceed 90 minutes (Vadopalas et al., 2006). A lack of expertise within this discipline often leads to significant
 342 bottlenecks in large-scale plankton species assessments. This can impact on research capabilities within such
 343 studies as budgets are substantially impacted due to the length of time required to process samples (Paugam
 344 et al., 2000). Subsequently, researchers have explored a number of alternative methodologies to address the
 345 taxonomic issues which have arisen in large-scale environmental plankton surveys (Lorenzo et al., 2005). A
 346 number of these are readily available and operationally superior to the recognised standard of visually keyed
 347 microscope identifications (Le Goff-Vitry et al., 2007). Presently, plankton studies which incorporate standard
 348 PCR analysis and post-PCR processing as a species verification tool are approximately seven times faster
 349 than conventional microscopic protocols (Boeger et al., 2007).

350 Here we present a real-time PCR assay capable of identifying and quantifying *M. edulis* larvae within unsorted
351 mixed field samples. The methodology does not require the time-consuming preparation of reagents,
352 antibodies, or hybridomas and negates the need to manually quantify larvae (Johnson et al., 2015; Heaney et
353 al., 2011; Perez et al., 2009). The procedure can be applied directly thereby substantially reducing the time
354 allocated to sample handling. After DNA extraction standard processing time was <2 hr. Inhibition was
355 negligible, even in high density (>100) larvae samples, demonstrating the efficacy of our chosen DNA
356 extraction method in removing inhibitors and/ or the ability of utilised SYBR reagents to overcome inhibition of
357 primer-template binding and Taq Polymerase activity (Keele et al., 2014). Furthermore, extracts could be used
358 undiluted, increasing the probability of detection in samples with low target species (Xia et al., 2018).

359 The use of molecular tools in identifying invertebrate larvae has previously proved successful in the detection
360 of *Corbicula* clams (Ludwig et al., 2014), the Golden mussel (*Limnoperna fortunei*) and Quagga mussel
361 (*Dreissena bugensis*) (Peñarrubia, et al. 2016; Boeger et al., 2007; Pie et al., 2006). The absolute or semi-
362 quantification of larval values within mixed environmental samples using real-time PCR is still in its infancy.
363 However, results have been promising using the technique, with strong correlations between real and
364 predicted numbers for the larvae of crab, barnacle, sea lice, abalone and oyster samples (Sanchez et al.,
365 2014; Endo et al., 2010; Pan et al., 2008; Mcbeath et al., 2006; Vadopalas et al., 2006). Dias et al., (2009)
366 developed an assay which specifically targeted nuclear DNA to quantify *M. edulis* larvae, but emphasised that
367 values were an approximation and unlikely to be entirely accurate. By providing a 'most probable value' for
368 larval numbers and including the range of variation and uncertainty expected from field sampling, from the
369 DNA extraction and from the amplification method we aimed to improve the statistical degree of confidence
370 regarding larval abundance.

371 However, if the use of targeted DNA is to be employed with confidence in the determination of species-
372 specific larval density loads then an increase in precision is required. A high specificity and sensitivity can be
373 achieved through qPCR and the use of species-specific primers in conjunction with real-time technology.
374 Muniesa et al., (2014) demonstrated that under optimal conditions the technique can detect single-fold
375 changes within a gene copy number. Pan et al., (2008) demonstrated its efficiency as a working tool while
376 examining artificially modified plankton samples by detecting single *Liocarcinus* sp. and copepodid larvae
377 within mixed community samples. In the present study the assay demonstrated a 100% limit of detection
378 (LoD) of 0.04 larvae/ reaction on laboratory samples, the equivalent of 2 whole larvae per sample. The assay

379 proved sensitive enough to detect solitary *M. edulis* larvae within pooled unsorted field samples. However, the
380 research findings suggest that when larval numbers are anticipated to be low for example outside known
381 spawning seasons *in-situ* sample replication should be increased if false negatives are to be minimised
382 (Ficetola et al., 2015). Rees et al., (2014(b)) suggests three replicates as sufficient for improving the likelihood
383 of detection while also decreasing the number of biological variables such as distribution and patchiness
384 (Taylor et al., 2019) and the findings of this study would concur.

385 Field samples are considerably more complex in terms of organic content and at lower larval numbers the risk
386 of false negative results due to the 'masking' effect of primer-dimer or amplification of DNA from non-target
387 species increases (Xia et al., 2018). Therefore, samples which had no visible melt curve corresponding with
388 the target amplicon and reported molecular copies of <100 were deemed to have no target molecules and
389 were given a value of 0 copies/ larvae. This allowed the detection of a single larvae within the DNA extracts
390 and thereby confirmed the improved sensitivity of the method. However, non-specific amplification observed
391 within low-density larval samples may have artificially inflated abundance values. Further investigative studies
392 to quantify this inflation are required and could be carried out using spiking experiments on artificial
393 predetermined plankton samples.

394 An important consideration when undertaking assessments using DNA analysis is that the total amount of
395 mitochondrial (Mt) and nuclear DNA can vary during ontogeny (Peñarrubia et al., 2016). As larvae progress
396 through the life cycle these variations continue and as larval size increases there will be an effect on
397 amplification and quantification success (Wood et al., 2003). This study used primers targeting a nuclear
398 genome region to increase the likelihood of accurate larval quantification. This is because a known number of
399 copies (two) are found per cell; in contrast numbers of Mt DNA molecules can vary among tissue types during
400 the cell cycle and in response to stress (Cole, 2016), compounding quantification errors.

401 Nevertheless larval age, or more specifically size may have an effect on amplification and quantification
402 success (Wood et al., 2003). Whilst there is some evidence to suggest that late stage larvae are too closely
403 associated with the sea bed to be successfully sampled (Knights, Crowe, & Burnell, 2006) and therefore have
404 little to no effect on 'skewing' qPCR results in environmental samples, a study of the effect of larval size on
405 quantification would be pertinent. The results of such a study could be used to further refine and improve the
406 predictive capabilities of the model.

407 The primers selected for this study offer an added advantage of being able to detect alleles belonging to all
408 members within the *Mytilus edulis* complex; *M. edulis*, *M. galloprovincialis* and *M. trossulus* (Inoue et al.,
409 1995). The nuclear Me15/16 DNA marker follows a Mendelian inheritance pattern and is the most commonly
410 used for routine identification of *Mytilus* mussels due its robustness and reliability (Larraín et al., 2019). Whilst
411 single locus genotyping using this marker has limited potential for analysing patterns of hybridisation or
412 genome introgression (Wilson et al., 2018; Beaumont et al., 2008) and can underestimate levels of
413 hybridisation in populations (Larraín et al., 2019; Kijewski et al., 2011), it can be used to identify size-specific
414 gene fragments unique to each of the *Mytilus* species (Wilson et al., 2018; Kijewski et al., 2006). The
415 detection of more than one of these alleles in a population is evidence of hybridisation and may prompt a
416 more comprehensive study of population structure utilising multilocus approaches (Larraín et al., 2019), single
417 nucleotide polymorphism (SNP) genotyping (Wenne et al., 2020; Wilson et al., 2018; Zbawicka et al., 2012) or
418 polymorphic microsatellite analysis (Lallias et al., 2009).

419 Allele-detection using the Me15/16 marker may be successful in characterising single larvae, however
420 analysis of mixed environmental samples containing more than one *Mytilus* species would be considerably
421 more complex (Wood et al., 2003). Some level of clarification is possible and was outlined by Dias et al.,
422 (2008) when probes based on the Me15/16 marker were applied to screen for *M. edulis*, *M. galloprovincialis*
423 and *M. trossulus* alleles. Another simple cost-effective method for verification of sequence variation was also
424 achieved by Pryor and Wittwer, (2006) through the screening of melt curves.

425 While real-time PCR can be useful in specificity analysis of taxon when compared to other identification
426 methods, the level of accuracy will be reliant on the existence of an adequate taxonomically referenced
427 database, which is unrealistic (Weigand et al., 2019). During this present research an alignment of primers
428 against a reference database indicated high specificity for *Mytilus* spp, yet cross-amplification was observed
429 with *C. gigas* and to a lesser extent *C. edule*. In the event that numbers of *C. edule* larvae in a sample were
430 sufficiently abundant to cause detectable amplification, the inflation of larval predictions would be so low as to
431 be insignificant. In contrast, the potential level of amplification due to cross-reactivity with *C. gigas* larvae was
432 comparatively low and therefore unlikely to significantly skew results but may result in false positives or
433 exaggerations of mussel larval abundance. In areas where naturally spawning *C. gigas* and *M. edulis* overlap
434 this is a factor worth considering as a more specific assay or the design of a probe-based assay may be
435 needed. *Crassostrea gigas* has considerably expanded its range following introductions into Europe in the

436 1960s, forming naturalised populations. Only sparse patchy aggregations of *C. gigas* occur in the Menai Strait
437 (Robins et al., 2020) with population structure indicating only intermittent settlement, suggesting limited
438 release of larvae. Furthermore, hydrodynamic modelling indicates limited dispersal potential of larvae from the
439 source populations in the Menai Strait to the location plankton samples were taken in the present study
440 (Robins et al., 2020). Nonetheless, further investigation will be needed to confirm whether cross-reaction is
441 occurring and the effect of this on *M. edulis* abundance estimates.

442 A probe-based assay approach was attempted during this research however significant amplification of *C.*
443 *gigas* gDNA was still evident indicating a previously unrecognised homologous region in the species. This was
444 surprising, as the gene region selected was responsible for the generation of polyphenolic proteins involved in
445 biological adhesion, specifically byssal thread production specific to *Mytilus sp.* (Inoue et al., 1995). Mussel
446 adhesive proteins differ from cement proteins from other species (e.g. oysters, barnacles) due to the presence
447 of 'repetitive amino acid motifs characterized by a high polyphenolic content, high levels of the modified amino
448 acid 3, 4-DOPA, and hydroxylations to specific amino acids' (Rees et al., 2019; Silverman & Roberto, 2007).
449 However, adhesion in pediveliger oyster larvae prior to metamorphosis is distinctly different from adult
450 attachment. Secretion of byssal-like filaments by pediveliger larvae was observed before permanent adhesion
451 during final settlement in *C. gigas* (Foulon et al., 2018). Foulon et al., (2019) have suggested that similar
452 byssal secretion strategies could be used by pediveliger oyster larvae and adult mussels. An example being;
453 Tyrosinase and peroxidase-like proteins in *C. gigas* presented similarities of 47.95% (E -value: 6.5×10^{-75}) and
454 44.8% similarity (E -value: 2.6×10^{-57}) to byssal protein sequences from *Mytilus coruscus* (Foulon et al.,
455 2019). This unanticipated finding warrants a considerable amount of further research as the restoration of
456 marine bivalve ecosystems is a priority within many marine environmental management programmes (Smyth
457 et al., 2017). The settlement and attachment process is a critical phase in bivalve live cycles and a more in-
458 depth understanding of the chemical mechanisms involved would greatly benefit the success of many costly
459 restoration projects.

460 **5. Conclusions**

461 Molecular technologies such as real-time PCR offer the potential for sensitive species-specific identifications
462 in conjunction with reduced analysis time. The work presented here demonstrates the possibilities molecular
463 methods offer when compared to conventional techniques employed for larval quantification.

464 Our method allowed us to accurately identify relative peaks in larval abundance from time series or spatial
465 plankton surveys; predicted *M. edulis* larval numbers mirrored those observed using conventional methods
466 and seasonal cycles of larval abundance were consistent with known spawning patterns of *M. edulis* in the
467 Irish Sea and other temperate waters (Philippart et al., 2012). This method is high throughput and rapid; the
468 research time saved could lessen the bottleneck in time and costs incurred in lengthy taxonomic
469 identifications. The implementation and development of the techniques discussed will undoubtedly improve
470 the efficiency of field studies which focus on the temporal and spatial patterns of spawning and larval
471 transport. For example, the procedure has the potential to inform or validate particle tracking models which
472 have now become an integral tool in molluscan fishery assessments (Robins et al., 2013; Robins, et al.,
473 2017). In practical applications, results can be returned within 24 hours of receiving a sample. Used alongside
474 larval dispersal model outputs, the methodology presented here facilitate real time monitoring to inform
475 aquaculture operations of the optimal times and locations for spat collector deployment.

476 **Declaration of Competing Interest**

477 The authors declare that they have no conflicts of interest.

478 **Acknowledgments**

479 This study was supported by the Shellfish Centre RD&I operation (Grant Agreement No. c81364), part-funded
480 by the EU's West Wales and the Valleys European Regional Development Fund (ERDF) Operational
481 Programme through the Welsh Government; and the Irish Sea Portal Pilot, ISPP (Grant Agreement No.
482 80940) Project, part-funded by the European Regional Development Fund (ERDF) through the Ireland Wales
483 NTERREG Programme.

484 **Appendix A.** Supplementary data

485 A full description of experimental extraction protocols for *M. edulis* larvae

486 **References**

487 Abalde, S.L., Fuentes, J., González-Fernández, Á. (2003). Identification of *Mytilus galloprovincialis* larvae
488 from the Galician rías by mouse monoclonal antibodies. *Aquaculture*, 219, 545–559. 10.1016/s0044-
489 8486(02)00658-0.

490 Balasingham, K.D., Walter, R.P., Mandrak, N.E., Heath, D. (2018). Environmental DNA detection of rare and
491 invasive fish species in two Great Lakes tributaries. *Molecular Ecology*, 27 (1), 112–127. 10.1111/mec.14395.

492 Barco, A., Raupach, M.J., Laakmann, S., Neumann, H., Kneibelsberger, T. (2016). Identification of North Sea
493 molluscs with DNA barcoding. *Molecular Ecology Resources*, 16, 288–297. 10.1111/1755-0998.12440.

494 Bates, D., Mächler, M., Bolker, B., Walker, S. (2014). Package lme4: Linear Mixed-Effects Models Using
495 Eigen and S4. R package version, 67.

496 Beaumont, A.R., Hawkins, M.P., Doig, F.L., Davies, I.M., Snow, M. (2008). Three species of *Mytilus* and their
497 hybrids identified in a Scottish Loch: natives, relicts and invaders? *Journal of Experimental Marine Biology
498 and Ecology*, 367 (2), 100 – 110. 10.1016/j.jembe.2008.08.021

499 Black, A.R., S. I. Dodson. (2003). Ethanol: a better preservation technique for Daphnia. *Limnology and
500 Oceanography: Methods*, 1 (1), 45–50. 10.4319/lom.2003.1.45.

501 Boeger, W.A., Pie, M.R., Falleiros, R.M., Ostrensky, A., Darrigran, G., Mansur, M.C.D., Belz, C.E. (2007).
502 Testing a molecular protocol to monitor the presence of golden mussel larvae (*Limnoperna fortunei*) in
503 plankton samples. *Journal of Plankton Research*, 29 (11), 1015-1019. 10.1093/plankt/fbm075.

504 Bott, N.J., Giblot-Ducray, D. (2011). Molecular tools for detection of marine pests: *Musculista senhousia*,
505 *Corbula gibba* and *Perna canaliculus* quantitative PCR assays. SARDI Research Report No. 587, Biosecurity
506 SA. South Australian Research and Development Institute (Aquatic Sciences), Adelaide.

507 Bott, N.J., Ophel-Keller, K.M., Sierp, M.T., Herdina, Rowling, K.P., McKay, A.C., Loo, M.G., Tanner, J.E.,
508 Deveney, M.R. (2010). Toward routine, DNA-based detection methods for marine pests. *Biotechnology
509 Advances*, 28 (6), 706–714. 10.1016/j.biotechadv.2010.05.018.

510 Brady, C., Denman, S., Kirk, S., Venter, S., Rodríguez-Palenzuela, P., Coutinho, T. (2010). Description of
511 *Gibbsiella quercinecans* gen. nov., sp. nov., associated with Acute Oak Decline. *Systematic and Applied
512 Microbiology*, 33 (8), 444–450. 10.1016/j.syapm.2010.08.006.

513 Breton, S., Beaupré, H.D., Stewart, D.T., Hoeh, W.R., Blier, P.U. (2007). The unusual system of doubly
514 uniparental inheritance of mtDNA: isn't one enough? *Trends in Genetics*, 23 (9), 465–474.
515 10.1016/j.tig.2007.05.011.

516 Cahill, A.E., Pearman, J.K., Borja, A., Carugati, L., Carvalho, S., Danovaro, R., Dashfield, S., David, R., Féral,
517 J.-P., Olenin, S., Šiaulys, A., Somerfield, P.J., Trayanova, A., Uyarra, M.C., Chenuil, A. (2018). A comparative
518 analysis of metabarcoding and morphology-based identification of benthic communities across different
519 regional seas. *Ecology and Evolution*, 8 (17), 8908-8920. 10.1002/ece3.4283

520 Christian, A.D., Monroe, E.M., Asher, A.M., Loutsche, J.M., Berg, D.J. (2007). Methods of DNA extraction and
521 PCR amplification for individual freshwater mussel (Bivalvia: Unionidae) glochidia, with the first report of
522 multiple paternity in these organisms. *Molecular Ecology Notes*, 7, 570–573. 10.1111/j.1471-
523 8286.2007.01743.x.

524 Cole, L.W. (2016). The Evolution of Per-cell Organelle Number. *Frontiers in cell and developmental biology*, 4,
525 85. 10.3389/fcell.2016.00085.

526 Couton, M., Comtet, T., Le Cam, S., Corre, E., Viard, F. (2019). Metabarcoding on planktonic larval stages: an
527 efficient approach for detecting and investigating life cycle dynamics of benthic aliens. *Management of*
528 *Biological Invasions*, 10 (4), 657-689. 10.3391/mbi.2019.10.4.06

529 Cowart, D.A., Pinheiro, M., Mouchel, O., Maguer, M., Grall, J., Miné, J., Arnaud-Haond, S. (2015).
530 Metabarcoding Is Powerful yet Still Blind: A Comparative Analysis of Morphological and Molecular Surveys of
531 Seagrass Communities. *PLOS ONE*, 10, e0117562. <https://doi.org/10.1371/journal.pone.0117562>.

532 Dankers, N., Zuidema, D.R. (1995). The role of the mussel (*Mytilus edulis* L.) and mussel culture in the Dutch
533 Wadden Sea. *Estuaries*, 18 (1), 71–80.

534 Demers, A., Lagadeuc, Y., Dodson, J.J., Lemieux, R. (1993). Immunofluorescence identification of early life
535 history stages of scallops (Pectinidae). *Marine Ecology Progress Series*, 97 (1), 83–89.

536 Dias, P.J., Batista, F.M., Shanks, A.M., Beaumont, A.R., Davies, I.M., Snow, M. (2009). Gametogenic
537 asynchrony of mussels *Mytilus* in a mixed-species area: Implications for management. *Aquaculture*, 295 (3),
538 175–182. 10.1016/j.aquaculture.2009.07.007.

539 Dias, P. J., Sollelis L., Cook, E.J., Piertney, S.B., Davies, I.M., Snow, M. (2008). Development of a real-time
540 PCR assay for detection of *Mytilus* species specific alleles: Application to a sampling survey in
541 Scotland. *Journal of Experimental Marine Biology and Ecology*, 367 (2), 253–258.
542 10.1016/j.jembe.2008.10.011.

543 Dobretsov, S. V., Miron, G. (2001). Larval and post-larval vertical distribution of the mussel *Mytilus edulis* in
544 the White Sea. *Marine Ecology - Progress Series*, 218, 179–187. 10.3354/meps218179.

545 Dysthe, J.C., Rodgers, T., Franklin, T.W., Carim, K.J., Young, M.K., McKelvey, K.S., Mock, K.E., Schwartz,
546 M.K. (2018). Repurposing environmental DNA samples-detecting the western pearlshell (*Margaritifera falcata*)
547 as a proof of concept. *Ecology and evolution*, 8, 2659–2670. 10.1002/ece3.3898.

548 Endo, N., Sato, K., Matsumura, K., Yoshimura, E., Odaka, Y., Nogata, Y. (2010). Species-specific detection
549 and quantification of common barnacle larvae from the Japanese coast using quantitative real-time PCR.
550 *Biofouling*, 26, 901–911. 10.1080/08927014.2010.531389.

551 Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C.M.,
552 Boyer, F., Pompanon, F., Rayé, G., Taberlet, P. (2015). Replication levels, false presences and the estimation
553 of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, 15 (3), 543–556.
554 10.1111/1755-0998.12338.

555 Folmer, M. H., Black, O., Hoeh, W., Lutz, R. Vrijenhoek, R. (1994). DNA primers for amplification of
556 mitochondrial cytochrome c oxidase subunit 1 from diverse metazoan invertebrates. *Molecular Marine Biology*
557 *and Biotechnology*, 3 (5), 294–299.

558 Foulon, V., Artigaud, S., Buscaglia, M., Bernay, B., Fabioux, C., Petton, B., Elies, P., Boukerma, K., Hellio, C.,
559 Guérard, F., Boudry, P. (2018). Proteinaceous secretion of bioadhesive produced during crawling and
560 settlement of *Crassostrea gigas* larvae. *Scientific Reports*, 8 (1), 5298.

561 Foulon, V., Boudry, P., Artigaud, S., Guérard, F., Hellio, C. (2019). In Silico Analysis of Pacific Oyster
562 (*Crassostrea gigas*) Transcriptome over Developmental Stages Reveals Candidate Genes for Larval
563 Settlement. *International journal of molecular sciences*, 20 (1), 197. 10.3390/ijms20010197.

564 Furlan, E.M., Gleeson, D., Hardy, C.M., Duncan, R.P. (2016). A framework for estimating the sensitivity of
565 eDNA surveys. *Molecular Ecology Resources*, 16, 641–654. 10.1111/1755-0998.12483.

566 Goodwin, J.D., Lutz, R.A., Johnson, S., Kennedy, V.S., Gallager, S. (2018). Optical Imaging and Molecular
567 Sequencing of a Preserved Collection of Bivalve Larvae. *Journal of Shellfish Research*, 37, 449–466.

568 Günther, B., Knebelsberger, T., Neumann, H., Laakmann, S., Martínez Arbizu, P. (2018). Metabarcoding of
569 marine environmental DNA based on mitochondrial and nuclear genes. *Scientific Reports*, 8 (1), 14822.

570 Hambrey, J., Evans, S. (2016). Aquaculture in England, Wales and Northern Ireland: An Analysis of the
571 Economic Contribution and Value of the Major Sub-Sectors and the Most Important Farmed Species. Seafish
572 Report SR694.

573 Hare, M.P., Palumbi, S.R., Butman, C.A. (2000). Single-step species identification of bivalve larvae using
574 multiplex polymerase chain reaction. *Marine Biology*, 137 (5), 953–961.

575 Heaney, S.A., Maloy, A.P., Slater, J. W. (2011). Evaluation of fixatives and autofluorescence reduction
576 treatments for marine bivalve larvae. *Journal of the Marine Biological Association of the United Kingdom*, 91
577 (7), 1567–1576. 10.1017/S0025315411000208.

578 Hosoi, M., Hosoi-tanabe, S., Sawada, H., Ueno, M., Toyohara, H., Hayashi, I. (2004). Sequence and
579 polymerase chain reaction-restriction fragment length polymorphism analysis of the large subunit rRNA gene
580 of bivalve: Simple and widely applicable technique for multiple species identification of bivalve larva. *Fisheries
581 Science*, 70, 629–637. 10.1111/j.1444-2906.2004.00850.x.

582 Inoue, K., Waite, J. H., Matsuoka, M., Odo, S., Harayama, S. (1994). Interspecific Variations in Adhesive
583 Protein Sequences of *Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus*. *The Biological Bulletin*, 189, 370–
584 375. 10.2307/1542155.

585 Jensen, P.C., Purcell, M.K., Morado, J.F., Eckert, G.L. (2012). Development of a Real-Time PCR Assay for
586 Detection of Planktonic Red King Crab (*Paralithodes camtschaticus*) Larvae. *Journal of Shellfish Research*,
587 31 (4), 917–924. 10.2983/035.031.0402.

588 Johnson, A.M., Rochelle, P.A., Leon, R.D., Kelly, K.L. (2015). Monoclonal Antibodies for Improved Detection
589 of Quagga Mussel Larvae. Final Report ST-2014-9640-01, Research and Development Office, U.S.
590 Department of the Interior, Bureau of Reclamation.

591 Kamermans, P., Capelle, J. (2018). Provisioning of mussel seed and its efficient use in culture. In: Smaal,
592 A.C., Ferreira, J.G., Grant, J., Petersen, J.K., Strand, Ø (eds). *Goods and services of marine bivalves*,
593 Springer International Publishing, 27–49.

594 Kaya, S., Çıplak, B. (2018). Possibility of numt co-amplification from gigantic genome of Orthoptera: testing
595 efficiency of standard PCR protocol in producing orthologous COI sequences. *Heliyon*, (4), p. e00929.
596 10.1016/j.heliyon.2018.e00929.

597 Keele, J., Carmon, J., Hosler, D. (2014). Optimization of Early Detection of Invasive Mussels with Polymerase
598 Chain Reaction. U.S. Department of the Interior, Bureau of Reclamation, Research and Development Office.

599 Kijewski, T.K., Zbawicka, M., Väinölä, R., Wenne, R. (2006). Introgression and mitochondrial DNA
600 heteroplasmy in the Baltic populations of mussels *Mytilus trossulus* and *M. edulis*. *Marine Biology*. 149, 1371–
601 1385. 10.1007/s00227-006-0316-2

602 Kijewski, T., Śmietanka, B., Zbawicka, M., Gosling, E., Hummel, H., Wenne, R. (2011). Distribution of *Mytilus*
603 taxa in European coastal areas as inferred from molecular markers. *Journal of Sea Research*. 65 (2), 224 –
604 234. 10.1016/j.seares.2010.10.004

605 Klymus, K.E., Marshall, N.T., Stepien, C.A. (2017). Environmental DNA (eDNA) metabarcoding assays to
606 detect invasive invertebrate species in the Great Lakes. *PLOS ONE*, 12, 1-24.
607 10.1371/journal.pone.0177643.

608 Knights, A.M., Crowe, T.P., Burnell, G. (2006). Mechanisms of larval transport: vertical distribution of bivalve
609 larvae varies with tidal conditions. *Marine Ecology Progress Series*, 326, 167–174. 10.3354/meps326167.

610 Lallias, D., Stockdale, R., Boudry, P., Lapègue, S., Beaumont, A.R. (2009). Characterization of Ten
611 Microsatellite Loci in the Blue Mussel *Mytilus edulis*. *Journal of Shellfish Research*, 28, 547-551.
612 10.2983/035.028.0317

613 Larraín, M.A., González, P., Pérez, C., Araneda, C (2019). Comparison between single and multi-locus
614 approaches for specimen identification in *Mytilus* mussels. *Scientific Reports*, 9, 19714. 10.1038/s41598-019-
615 55855-8

616 Larsen, J.B., Frischer, M.E., Ockelmann, K.W., Rasmussen, L.J., Hansen, B.W. (2007). Temporal occurrence
617 of planktotrophic bivalve larvae identified morphologically and by single step nested multiplex PCR. *Journal of*
618 *Plankton Research*, 29 (5), 423–436. 10.1093/plankt/fbm027.

619 Larsen, J.B., Frischer, M.E., Rasmussen, L.J., Hansen, B.W. (2005). Single-step nested multiplex PCR to
620 differentiate between various bivalve larvae. *Marine Biology*, 146 (6), 1119–1129.

621 Lasota, R., Piłczyńska, J., Williams, S.T., Wołowicz, M. (2013). Fast and easy method for total DNA
622 extraction and gene amplification from larvae, spat and adult mussels *Mytilus trossulus* from the Baltic
623 Sea. *Oceanological and Hydrobiological Studies*, 42 (4), 486–489.

624 M. Le Goff-Vitry, A. Chipman, T. Comtet. In situ hybridization on whole larvae: A novel method for monitoring
625 bivalve larvae. *Marine Ecology Progress Series*, 343 (2007), pp. 161–172. 10.3354/meps06891.

626 Lorenzo, S., González-Fernández, A., de Miguel Villegas, E., Fuentes, J. (2005). Two monoclonal antibodies
627 for the recognition of *Mytilus* spp. larvae: Studies on cultured larvae and tests on plankton
628 samples. *Aquaculture*, 250, 736–747. 1016/j.aquaculture.2005.05.039.

629 Ludwig, S., Tschá, M., Patella, R., Oliveira, A., Boeger, W. (2014). Looking for a needle in a haystack:
630 molecular detection of larvae of invasive *Corbicula* clams. *Management of biological Invasions*, 5 (2), 143–
631 149. 10.3391/mbi.2014.5.2.07.

632 Lutz, R.A., Goodwin, J.D., Baldwin, B.S., Burnell, G., Castagna, M., Chapman, S., Chestnut, A., Dabinett, P.,
633 Davis, C., Eversole, A. G., Fuller, S.C., Gallager, S.M., Goldberg, R., Goodsell, J., Grassle, J., Gustafson, R.
634 G., Hidu, H., Hu, Y.-P., Jablonski, D., Johnson, S., Kennedy, V.S., Pennec, M.L., Mann, R., Newell, C.,
635 Pooley, A.S., Tan, A.S., Vrijenhoek, R.C., Partridge, A. (2018). Scanning Electron Microscopic Aids for
636 Identification of Larval and Post-Larval Bivalves. *Journal of Shellfish Research*, 37 (2), 247–448.
637 10.2983/035.037.0202.

638 McBeath, A.J.A., Penston, M.J., Snow, M., Cook, P.F., Bricknell, I.R., Cunningham, C.O. (2006).
639 Development and application of real-time PCR for specific detection of *Lepeophtheirus salmonis* and *Caligus*
640 *elongatus* larvae in Scottish plankton samples. *Diseases of Aquatic Organisms*, 73, 141–150.
641 10.3354/dao073141.

642 Muniesa, A., Ferreira, C., Fuertes, H., Halaihel, N., de Blas, I. (2014). Estimation of the Relative Sensitivity of
643 qPCR Analysis Using Pooled Samples. *PLOS ONE*, 9 (4), 1–5. 10.1371/journal.pone.0093491.

644 Mychek-Londer, J.G., Balasingham, K.D., Heath, D.D. (2019). Using environmental DNA metabarcoding to
645 map invasive and native invertebrates in two Great Lakes tributaries. *Environmental DNA*, 1-16.
646 10.1002/edn3.56.

647 Nehls, G., Thiel, M. (1993). Large-scale distribution patterns of the mussel *Mytilus edulis* in the Wadden Sea
648 of Schleswig-Holstein: do storms structure the ecosystem? *Netherlands Journal of Sea Research*, 31 (20),
649 181–187.

650 Odero, J., Gomes, B., Fillinger, U., Weetman, D. (2018). Detection and quantification of *Anopheles gambiae*
651 *sensu lato* mosquito larvae in experimental aquatic habitats using environmental DNA (eDNA). Wellcome
652 Open Research, 3 (26). 10.12688/wellcomeopenres.14193.1.

653 Pan, M., McBeath, A.J.A., Hay, S.J., Pierce, G.J., Cunningham, C.O. (2008). Real-time PCR assay for
654 detection and relative quantification of *Liocarcinus depurator* larvae from plankton samples. Marine Biology,
655 153 (5), 859–870.

656 Passamonti, M., Ghiselli, F. (2009). Doubly uniparental inheritance: two mitochondrial genomes, one precious
657 model for organelle DNA inheritance and evolution. DNA and cell biology. 28 (2), 79–89.
658 10.1089/dna.2008.0807

659 Paugam, A., Le Pennec, M., Marhic, A., André-Fontaine, G. (2003). Immunological in situ determination of
660 *Pecten maximus* larvae and their temporal distribution. Journal of the Marine Biological Association of the
661 United Kingdom, 83 (5), 1083–1093. 10.1017/S0025315403008300h.

662 Paugam, A., Le Pennec, M., André-Fontaine, G. (2000). Immunological recognition of marine bivalve larvae
663 from plankton samples. Journal of Shellfish Research, 19, 325–331.

664 Peñarrubia, L., Alcaraz, C., Vaate, A.B.D., Sanz, N., Pla, C., Vidal, O., Viñas, J. (2016). Validated
665 methodology for quantifying infestation levels of dreissenid mussels in environmental DNA (eDNA) samples.
666 Scientific Reports, 6 (1), p. 39067.

667 Pérez, D., Lorenzo-Abalde, S., González-Fernández, Á., Fuentes, J. (2009). Immunodetection of *Mytilus*
668 *galloprovincialis* larvae using monoclonal antibodies to monitor larval abundance on the Galician coast:
669 Optimization of the method and comparison with identification by morphological traits. Aquaculture, 294 (1),
670 86–92. 10.1016/j.aquaculture.2009.05.020.

671 Philippart, C.J.M., Amaral, A., Asmus, R., van Bleijswijk, J., Bremner, J., Buchholz, F., Cabanellas-Reboredo,
672 M., Catarino, D., Cattrijsse, A., Charles, F., Comtet, T., Cunha, A., Deudero, S., Duchêne, J.-C., Fraschetti,
673 S., Gentil, F., Gittenberger, A., Guizien, K., Gonçalves, J.M., Guarnieri, G., Hendriks, I., Hussel, B., Vieira,
674 R.P., Reijnen, B.T., Sampaio, I., Serrao, E., Pinto, I.S., Thiebaut, E., Viard, F., Zuur, A.F. (2012). Spatial
675 synchronies in the seasonal occurrence of larvae of oysters (*Crassostrea gigas*) and mussels (*Mytilus*
676 *edulis/galloprovincialis*) in European coastal waters. Estuarine, Coastal and Shelf Science, 108, 52–63.

677 Pie, M.R., Boeger, W.A., Patella, L., Falleiros, R.M. (2006). A fast and accurate molecular method for the
678 detection of larvae of the golden mussel *Limnoperna fortunei* (Mollusca: Mytilidae) in plankton
679 samples. *Journal of Molluscan Studies*, 72 (2), 218–219. 10.1093/mollus/eyi070.

680 Piñol, J., Senar, M.A., Symondson, W.O.C. (2019). The choice of universal primers and the characteristics of
681 the species mixture determine when DNA metabarcoding can be quantitative. *Molecular Ecology*, 28 (2), 407–
682 419.

683 Prié, V., Valentini, A., Lopes-Lima, M., Froufe, E., Rocle, M., Poulet, N., Taberlet, P., Dejean, T. (2020).
684 Environmental DNA metabarcoding for freshwater bivalves biodiversity assessment: methods and results for
685 the Western Palearctic (European sub-region). *Hydrobiologia*.

686 Pryor, P., Wittwer, C. (2006). Real-Time Polymerase Chain Reaction and Melting Curve Analysis. *Methods in*
687 *Molecular Biology*, 336, 19–32. 10.1385/1-59745-074-X:19.

688 Rawson, P.D., Hilbish, T.J. (1998). Asymmetric introgression of mitochondrial DNA among European
689 populations of blue mussels (*Mytilus* spp.). *Evolution*, 52, 100-108. 10.1111/j.1558-5646.1998.tb05142.x

690 Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R., Gough, K.C. (2014). The detection of aquatic
691 animal species using environmental DNA – a review of eDNA as a survey tool in ecology. *Journal of Applied*
692 *Ecology*, 51 (5), 1450–1459. 10.1111/1365-2664.12306.

693 Rees, D.J., Hanifi, A., Obille, A., Alexander, R., Sone, E.D. (2019). Fingerprinting of Proteins that Mediate
694 Quagga Mussel Adhesion using a De Novo Assembled Foot Transcriptome. *Scientific Reports*, 9 (1), p. 6305.

695 Robins, P., Davies, A., Demmer, J., Wilmes, S., Smyth, D., Hayden-Hughes, M., Malham, S.
696 (2020). Hydrographic technical report for the Menai Strait. For the Welsh government (Draft).

697 Robins, P., Tita, A., King, J., Jenkins, S. (2017). Predicting the dispersal of wild Pacific oysters *Crassostrea*
698 *gigas* (Thunberg, 1793) from an existing frontier population — a numerical study. *Aquatic Invasions*, 12, 117–
699 131. 10.3391/ai.2017.12.2.01.

700 Robins, P.E., Neill, S.P., Giménez, L., Jenkins, S.R., Malham, S.K. (2013). Physical and biological controls on
701 larval dispersal and connectivity in a highly energetic shelf sea. *Limnology and Oceanography*, 58 (2), 505–
702 524. 10.4319/lo.2013.58.2.0505.

703 Rosenberg, G. (2014). A New Critical Estimate of Named Species-Level Diversity of the Recent Mollusca.
704 American Malacological Bulletin, 32 (2), 308-322. 10.4003/006.032.0204

705 Ruppert, K.M., Kline, R.J., Rahman, M.S. (2019). Past, present, and future perspectives of environmental
706 DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global
707 eDNA. Global Ecology and Conservation, 1, p. e00547. 10.1016/j.gecco.2019.e00547.

708 Sanchez, A., Quinteiro, J., Rey-Mendez, M., Perez-Martin, R.I., Sotelo, C.G. (2014). Identification and
709 quantification of two species of oyster larvae using real-time PCR. Aquatic Living Resources, 27 (3-4), 135–
710 145. 10.1051/alr/2014012.

711 Sawada, H., Saito, H., Hosoi, M., Toyohara, H. (2008). Evaluation of PCR methods for fixed bivalve larvae.
712 Journal of the Marine Biological Association of the United Kingdom, 88 (7), 1441–1449.
713 10.1017/S0025315408002154.

714 Schizas, N.V. (2012). Misconceptions regarding nuclear mitochondrial pseudogenes (Numts) may obscure
715 detection of mitochondrial evolutionary novelties. Aquatic Biology. 17, 91-96. 10.3354/ab00478

716 Silverman, H.G., Roberto, F.F. (2007). Understanding Marine Mussel Adhesion. Marine Biotechnology, 9 (6),
717 661–681.

718 Śmietanka, B., Wenne, R., Burzyński, A. (2016). Complete male mitochondrial genomes of European *Mytilus*
719 *edulis* mussels. Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis, 27 (3), 1634-1635.
720 10.3109/19401736.2014.958704.

721 Smyth, D., Mahon, A.M., Roberts, D., Kregting, L. (2018). Settlement of *Ostrea edulis* is determined by the
722 availability of hard substrata rather than by its nature: Implications for stock recovery and restoration of the
723 European oyster. Aquatic Conservation: Marine Freshwater Ecosystems, 28 (3), 662–671. 10.1002/aqc.2876.

724 Stirling, D.A., Boulcott, P., Bidault, M., Gharbi, K., Scott, B.E., Wright, P.J. (2018). Identifying the larva of the
725 fan mussel, *Atrina fragilis* (Pennant, 1777) (Pinnidae). Journal of Molluscan Studies, 84 (3), 247–258.

726 Sun, C., Zhao, Y., Li, H., Dong, Y., Maclsaac, H.J., Zhan, A. (2015). Unreliable quantitation of species
727 abundance based on high-throughput sequencing data of zooplankton communities. Aquatic Biology, 24, 9–
728 15.

729 Taylor, S.C., Nadeau, K., Abbasi, M., Lachance, C., Nguyen, M., Fenrich, J. (2019). The Ultimate qPCR
730 Experiment: Producing Publication Quality, Reproducible Data the First Time. *Trends in Biotechnology*, 37 (7),
731 761–774. 10.1016/j.tibtech.2018.12.002.

732 Theologidis, I., Fodelianakis, S., Gaspar, M. B., Zouros, E. (2008). Doubly uniparental inheritance (DUI) of
733 mitochondrial DNA in *Donax trunculus* (Bivalvia: Donacidae) and the problem of its sporadic detection in
734 Bivalvia. *Evolution; international journal of organic evolution*, 62 (4), 959–970. 10.1111/j.1558-
735 5646.2008.00329.x

736 Thompson, C.M., North, E.W., Kennedy, V.S., White, S.N. (2015). Classifying bivalve larvae using shell
737 pigments identified by Raman spectroscopy. *Analytical and Bioanalytical Chemistry*, 407, 3591–3604.
738 10.1007/s00216-015-8575-8.

739 Trivedi, S., Aloufi, A.A., Ansari, A.A., Ghosh, S.K. (2016). Role of DNA barcoding in marine biodiversity
740 assessment and conservation: An update. *Saudi Journal of Biological Sciences*, 23 (2), 161–171.
741 10.1016/j.sjbs.2015.01.001.

742 Vadopalas, B., Bouma, J.V., Jackels, C.R., Friedman, C.S. (2006). Application of real-time PCR for
743 simultaneous identification and quantification of larval abalone. *Journal of Experimental Marine Biology and*
744 *Ecology*, 334 (2), 219–228. <https://doi.org/10.1016/j.jembe.2006.02.005>.

745 Weigand, H., et al. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-
746 analysis and recommendations for future work. *Science of the Total Environment*, 678, 499–524.
747 10.1016/j.scitotenv.2019.04.247.

748 Wenne, R., Zbawicka, M., Bach, L., Strelkov, P., Gantsevich, M., Kukliński, P., Kijewski, T., McDonald, J. H.,
749 Sundaasen, K. K., Árnýasi, M., Lien, S., Kaasik, A., Herkül, K., Kotta, J. (2020). Trans-Atlantic Distribution
750 and Introgression as Inferred from Single Nucleotide Polymorphism: Mussels *Mytilus* and Environmental
751 Factors. *Genes*, 11 (5), 530. 10.3390/genes11050530

752 Wijsman, J., Troost, K., Fang, J., Roncarati, A. (2019). Global production of marine bivalves. Trends and
753 challenges. In *Goods and Services of Marine Bivalves*, Springer, 7–26.

754 Wilson, J., Matejusova, I., McIntosh, R.E., Carboni, S., Bekaert, M. (2018). New diagnostic SNP molecular
755 markers for the *Mytilus* species complex. *PLOS ONE*, 13 (7), 1–13. 10.1371/journal.pone.0200654.

756 Wood, A.R., Beaumont, A.R., Skibinski, D.O.F., Turner, G. (2003). Analysis of a nuclear-DNA marker for
757 species identification of adults and larvae in the *Mytilus edulis* complex. *Journal of Molluscan Studies*, 69 (1),
758 61–66. 10.1093/mollus/69.1.61.

759 Xia, Z., Zhan, A., Gao, Y., Zhang, L., Haffner, G.D., MacIsaac, H.J. (2018). Early detection of a highly invasive
760 bivalve based on environmental DNA (eDNA). *Biological Invasions*, 20 (2), 437–447.

761 Zbawicka, M., Drywa, A., Śmietanka, B., Wenne, R. (2012). Identification and validation of novel SNP markers
762 in European populations of marine *Mytilus* mussels. *Marine Biology*, 159 (6), 1347-1362. 10.1007/s00227-
763 012-1915-8.

764