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2
3 A qPCR MGB probe based eDNA assay for freshwater pearl mussel (*Margaritifera margaritifera* L.) in an Irish
4 River
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26 **Introduction max 2000 words**
27

28 Freshwater pearl mussel *Margaritifera margaritifera* L., are large (~14 cm), long lived (>100 years) bivalves
29 that are native to clean, fast-flowing, soft-water rivers and streams across Western and Northern Europe (Bauer
30 1986, 1992; Moorkens 1999; Young et al. 2001). Due to a degeneration habitat quality *M. Margaritifera* have

31 declined significantly across their range and are classified as critically endangered by the IUCN. Although large
32 populations still exist, recruitment is low and the isolation of the subpopulations ensures low to no gene
33 exchange occurs (Moorkens, 2011).

34
35 Within Western Europe, this has precipitated protection and conservation actions under national and
36 international legislation including the listing of *M. margaritifera* under Annex II and V of the Habitats Directive
37 (92/43: EEC). This has ensured that many rivers with *M. Margaritifera* sub populations have been designated as
38 Special Areas of Conservation (SAC). The species is further protected under national legislation and is legally
39 protected in Ireland under Schedule 1 of the Wildlife Act (Protection of Wild Animals) (Statutory Instrument
40 No. 112, 1990) and the European Communities (Natural Habitats) Regulations (Statutory Instrument No. 94,
41 1997).

42
43 Although recent estimates have suggested a total 1282 sub populations, this is expected to be reduced to 204 sub
44 populations by 2100. The island of Ireland currently supports 139 sub populations, with an expected number of
45 subpopulations being reduced to 6 by 2100 (Mookhens et al., 2011). However, currently the species is still
46 widespread in Ireland, although abundances have declined (Geist 2005). These subpopulations may provide a
47 potential source for re-colonisation, given proposed improvements in future water and habitat quality-levels as
48 outlined under the European Water Framework.

49
50 There is a recognised linkage between healthy stocks of salmonids such as brown trout (*Salmo trutta* L.) and
51 Atlantic salmon (*S. salar* L.) and the endurance, growth and propagation and of *M. margaritifera* sub
52 populations (Bauer 1979; Ziuganov and Nezhlin 1988; Ziuganov *et al.* 1994). Studies have suggested a symbiotic
53 relationship between salmonids and *M. margaritifera* - the mussels maintain water quality required by the
54 salmonids and have been shown to reduce senility in salmon, thereby extending their life expectancy (Ziuganov
55 2005!). The salmonids gills host *M. margaritifera* glochidium, a larval stage of *M. margaritifera* that requires
56 salmonids for dispersal. The chances of a glochidium successfully finding a host in waters with healthy
57 salmonid stocks is as low as 0.0004%, with only 5% of these successfully attaching which can only survive for
58 (Young and Williams 1984;). The pearl mussels have evolved to live in ultra- oligotrophic waters, where
59 obligate salmonid host populations (e.g. brown trout) were never high and depend on the anadromous sea trout
60 and salmon. Reductions in anadromous salmonid populations therefore have a drastic and immediate impact on

the successful attachment of glochidium and by extension recruitment and population viability of *M. margaritifera* (Bauer 1979). Therefore, any recovery *M. margaritifera* populations is dependent on and concomitant with the promotion of healthy salmonid populations. As an added value, the presence of *M. margaritifera* beds aids the legal designation and maintenance of high quality salmonid nursery habitats for through complementary legal protection.

M. margaritifera are important members of the food web in soft-water nutrient poor ecosystems; transferring nutrients and energy from the water column to the sediments through filter feeding, stimulating production across trophic levels (Spoonner and Vaughn 2006). A previous study (Stoeckle et al. 2015) developed *M. margaritifera* specific primers anchored in 16S mitochondrial (mt)DNA region and successfully deployed the assay on environmental (e)DNA samples from Central Europe. However, while 16S sequences from different organisms are abundant in public repositories, it would be advantageous to focus on the barcode of life gene (mtDNA COI - <http://www.barcodeoflife.org>) as repositories of COI sequences contain representatives from many more organisms than any other gene sequence repository. Further improvements of eDNA assays include adding species specific probes with higher fidelity (e.g. minor groove binding, MGB probes) than both assays based solely on species specific primers or those also incorporating TaqMan® probes not using the MGB group (Kutyavin et al. 2000).

The aim of the current study was to develop an eDNA assay that can detect the presence and relative abundances of *M. margaritifera* without hard sampling. This approach may allow for the detection of previously unrecorded populations that would require recognition and a measure of protection. Remnant populations may exist at densities too low for observation by traditional studies; acting as a potential source of recruits for repopulation. The approach would also allow for the identification of *M. margaritifera* hotspots; zones that support relatively a larger proportion of the mussel population.

Methods

eDNA qPCR assay development

89 All DNA tissue originated from a naturally diseased individual *M. margaritifera*. Found on the bank of the
 90 River Munster Blackwater (Fran Igoe personal comments). The tissue sample was received and total DNA was
 91 extracted from these tissue samples using the Qiagen Dneasy kit (Qiagen, Valencia, CA). Extracted DNA was
 92 used as template for assay validation and standard curves for qPCR. Species-specific primers for *M.*
 93 *margaritifera* (forward primer: 5'- TTG TTG ATT CGT GCT GAG TTA GG-3', and reverse primer: 5'- GCA
 94 TGA GCC GTA ACA ATA ACA TTG-3') and 5'-6-FAM labelled TaqMan® minor groove binding probe (5'-
 95 CCT GGT TCT TTG CTG GGT-3') targeting region within the mtDNA cytochrome oxidase I (COI) region
 96 were designed using PRIMER EXPRESS 3.0 (Applied Biosystems-Roche, Branchburg, NJ). The total amplicon
 97 size, including primers, was 83 bp. Probe and primer sequences were matched against the National Centre for
 98 Biotechnology Information (NCBI - <http://www.ncbi.nlm.nih.gov/>) nucleotide database with BLASTn (Basic
 99 Local Alignment Search Tool) to confirm the species specificity for *M. margaritifera* in *in-silico* assays. The
 100 specificity and amplification capability of the assay was confirmed by conventional PCR amplification and
 101 DNA visualisation on a 1.5% agarose gel stained with SYBR® Safe - DNA Gel Stain (Life Technologies). In
 102 addition, to the qPCR eDNA assay for *M. margaritifera*, we included a previously developed eDNA qPCR
 103 assay (Gustavson et al. 2015) for brown trout (*S. trutta*) as a positive control for presence of amplifiable eDNA
 104 in water samples.

105

106 *eDNA filtering and extraction of field samples*

107 Water samples were collected from **x** locations where live *M. margaritifera* had been observed were in Munster
 108 Blackwater River in sterile 3 L PET bottles and kept frozen until analysed. Water samples were thawed in
 109 ambient temperature and 1 L per sample was filtered through individual 0.45 µm Whatman nitrate filters. The
 110 amount of water filtered was recorded for each water sample to the closest cL. Filters were subsequently
 111 dehydrated with 100% EtOH before storage at -20°C. Each filter was cut into halves (half for analysis and half
 112 for archival storage) and shredded to increase surface area for eDNA extraction using Qiagen QIAshredder
 113 (Qiagen, Valencia, CA). Total eDNA was extracted using a Qiagen DNeasy kit (Qiagen, Valencia, CA).
 114 Extracted eDNA was stored at -20°C until further processing.

115

116 *eDNA assay deployment*

117 Concentrations of eDNA were determined by qPCR using an Applied Biosystems ViiA™ 7 (Life Technologies,
 118 Inc., Applied Biosystems, Foster City, CA) quantitative thermocycler. Amplification reactions for each species

119 included: 15 µl of TaqMan® Environmental Master Mix 2.0 (Life Technologies., Applied Biosystems, Foster
 120 City, CA), 3 µl of each primers (final concentration of 0.2 µM), probe (final concentration of 0.2 µM), ddH₂O,
 121 and DNA template (3 µl), forming the 30µL reaction volume. The qPCR run method used warm-up conditions
 122 of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles between 95°C for 15 s and 60°C for 1 min. The
 123 standard curve for *M. margaritifera* was generated with quantified (NanoDrop®-1000, Thermo Scientific,
 124 Wilmington, DE) DNA extractions from a tissue sample (DNA concentrations of 122.0 pg/L) using seven 10:1
 125 serial dilutions as template for qPCR. The standard curve for *M. margaritifera* ($y = -3.4058x + 38.238$, $R^2 =$
 126 0.9997 , efficiency = 96.62%) was generated using 3 µl DNA template in a total reaction volume of 30 µl,
 127 respectively. The lowest concentrations of DNA (dynamic range) equalling 0.122 pg/L in the standard curves
 128 were detected at C_q (quantification cycle) 34.8 (average over three technical replicates, SD = 0.073). Results
 129 from the standard curve (Fig. 1.) indicate a dynamic range and lowest eDNA detection level at C_q 38.238
 130 (equivalent to a *M. margaritifera* DNA concentration of 0.333 pg/L). All field samples were quantified in
 131 duplicates (two technical replicates), to ensure consistency, with two laboratory negative controls and *M.*
 132 *margaritifera*. Three water samples within the Munster Blackwater River system were used as template for *S.*
 133 *trutta* qPCR to validate the presence of amplifiable eDNA. The average C_q across technical replicates (n=2)
 134 were used for quantification.

135

136 Results and Discussion

137

138 The present study successfully developed an eDNA assay with very high sensitivity for *M. margaritifera*. All
 139 analysed samples yielded detectable eDNA (with C_q within the dynamic range) for both *M. margaritifera* and *S.*
 140 *trutta* (presence of *S. trutta* eDNA was validated in three sample locations), indicating that amplifiable target
 141 eDNA was present in all water samples. Resulting C_q values from the qPCR assays were transformed to
 142 pgDNA/L (based on the standard curve, Fig 1.). The concentrations of eDNA ranged from 0.462 pg/L in Rowls
 143 Aldworth West Bridge (right bank sample) to the highest of 109.884 pg/L in the Leader's Bridge Allow (left
 144 bank sample). Environmental DNA concentrations were relatively stable across transects within location (right
 145 bank, middle and left bank samples). Average eDNA concentrations (across transects) ranged from the lowest at
 146 in Rowls Aldworth West Bridge (1.056 pg/L) to the highest at Cullen Bridge (79.412 pg/L). Two graphs were
 147 plotted to visualise eDNA concentrations (Figs. 2 and 3). These results indicate variable eDNA concentrations
 148 among localities. The developed eDNA assay can be used to assess concentrations of eDNA which should be

Commented [JC1]: What do we do here. We do not need to have tons of locations. Just one field sample and one negative field sample...

149 related to the biomass of *M. margaritifera* and could be used for monitoring the status of *M. margaritifera* in
150 individual locations and river systems. However, utilising the quantifying capabilities of eDNA assays requires
151 careful planning, standardised and coordinated sampling efforts (exact GPS positions, dates, time of day, water
152 levels, weather conditions, details about where in the water body samples were acquired from, etc.) to ensure
153 that samples are of the highest quality. Nevertheless, the eDNA assay developed here can be used for rapid
154 detection of *M. margaritifera* presence throughout Ireland and the natural range of *M. margaritifera*. Wide scale
155 deployment of the assay can help detecting cryptic populations in watersheds where *M. margaritifera* has not
156 previously been reported or where *M. margaritifera* are considered to have gone extinct.

157
158 This might aid and inform conservation efforts through the translocation of existing, although previously
159 unreported, unviable subpopulations of *M. Margaritifera* in suboptimal habitats to either recently refurbished or
160 pre-existing optimal habitats (clean water and salmonids). This will ensure gene transfer between sub
161 populations and maintain genetic diversity in existing sub populations. The transferral of genetic material is
162 pertinent given the additional stresses of climate change. Unfortunately, given the low levels of funding and
163 political priority for conservation, this approach requires a hierarchical valuation of *M. margaritifera* habitats
164 and the focusing of efforts on SAC designated viable habitats.

165

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170 transport of samples.

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173 **Conflict of Interest:** The authors declare that they have no conflict of interest.

174

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Fig. 1. Standard curve used to quantify *Margaritifera margaritifera* eDNA concentrations. This curve is based on known concentrations and dilution series (10X) from a starting concentration of 0.122pg/L, Cq – quantification cycle.

Fig. 2. Average concentrations (error bars indicate 95% C.I within locations) of *Margaritifera margaritifera* eDNA among water samples from the Munster Blackwater river system.

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221 *Fig. 3. Average concentrations (error bars indicate observed max and min eDNA concentrations within*
222 *locations) of Margaritifera margaritifera eDNA among water samples from the Munster Blackwater river system.*
223

224 *Appendix. Margaritifera margaritifera eDNA concentrations within and among sites in the Munster Blackwater*
225 *river system. (loc – location number, site L – left bank sample, site M - mid river sample, site R – right bank*
226 *sample, Cq - quantification cycle, T1 – technical replicate 1, T2 – technical replicate 2).*

Location name	Loc	site	CqT1	CqT2	Average Cq	Average eDNA Conc across technical replicates pg/LI	Average eDNA Conc across location pg/L
Leader's Bridge Allow	1	L	29.662	30.049	29.8555	96.410	62.796
		M	30.796	30.786	30.791	51.220	
		R	31.216	31.042	31.129	40.757	
Kanturk Castle Brogeen	2	L	35.053	34.082	34.5675	7.973	8.405
		M	36.844	35.107	35.9755	3.078	
		R	33.478	33.957	33.7175	14.165	
Grenane Park Allow	4	L	37.733	37.176	37.4545	1.132	2.660
		M	37.856	N/A	37.856	0.863	
		R	34.924	35.06	34.992	5.984	
John's Bridge Allow	7	L	32.672	33.373	33.0225	22.660	36.946
		M	32.806	32.355	32.5805	30.552	
		R	31.426	31.858	31.642	57.624	
Metal Bridge Allow	8	L	35.783	35.661	35.722	3.653	4.941
		M	34.412	35.289	34.8505	6.585	
		R	35.77	35.002	35.386	4.585	
Freemount Bridge Allow	9	R	31.772	31.471	31.6215	58.428	46.389
		M	32.498	32.361	32.4295	33.836	
		L	31.841	32.052	31.9465	46.903	
Rowls Aldworth West Bridge	12	L	N/A	N/A			1.056
		M	37.233	N/A	37.233	1.315	
		R	38.779	37.166	37.9725	0.798	
Hayes Crossroad Bridge	14	R	33.235	33.555	33.395	17.615	24.708
		M	34.458	34.378	34.418	8.821	
		L	32.133	31.711	31.922	47.686	
Cullen Bridge	16	R	30.779	30.881	30.83	99.775	79.412
		M	31.304	31.367	31.3355	70.892	
		L	31.385	31.428	31.4065	67.570	

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