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A qPCR MGB probe based eDNA assay for freshwater pearl mussel (Margaritifera margaritifera L.) in an Irish River

1Area 52 Research Group, School of Biology & Environmental Science/Earth Institute, University College Dublin, Belfield, Dublin 4, Ireland.

2Queen's University Marine Laboratory (QML), 12–13 The Strand, Portaferry, Co. Down, Northern Ireland, BT221PF UK.

3School of Biology & Environmental Science, University College Dublin, Belfield, Dublin 4, Ireland.

4FRAN'S ADDRESS

Corresponding Author:
Email: nettan11@gmail.com, jeanette.carlsson@ucd.ie,
Telephone: +353 (01) 716 2395

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Introduction max 2000 words

Freshwater pearl mussel Margaritifera margaritifera L., are large (~14 cm), long lived (>100 years) bivalves that are native to clean, fast-flowing, soft-water rivers and streams across Western and Northern Europe (Bauer 1986, 1992; Moorkens 1999; Young et al. 2001). Due to a degeneration habitat quality M. Margaritifera have
declined significantly across their range and are classified as critically endangered by the IUCN. Although large populations still exist, recruitment is low and the isolation of the subpopulations ensures low to no gene exchange occurs (Moorkens, 2011).

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

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

There is a recognised linkage between healthy stocks of salmonids such as brown trout (*Salmo trutta* L.) and Atlantic salmon (*S. salar* L.) and the endurance, growth and propagation of *M. margaritifera* sub populations (Bauer 1979; Ziuganov and Nezlin 1988; Ziuganov et al. 1994). Studies have suggested a symbiotic relationship between salmonids and *M. margaritifera* - the mussels maintain water quality required by the salmonids and have been shown to reduce senility in salmon, thereby extending their life expectancy (Ziuganov 2005!). The salmonids gills host *M. margaritifera* glochidium, a larval stage of *M. margaritifera* that requires salmonids for dispersal. The chances of a glochidium successfully finding a host in waters with healthy salmonid stocks is as low as 0.0004%, with only 5% of these successfully attaching which can only survive for salmon 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 \textit{M. margaritifera} (Bauer 1979). Therefore, any recovery \textit{M. margaritifera} populations is dependent on and concomitant with the promotion of healthy salmonid populations. As an added value, the presence of \textit{M. margaritifera} beds aids the legal designation and maintenance of high quality salmonid nursery habitats for through complementary legal protection.

\textit{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 (Spooner and Vaughn 2006). A previous study (Stoeckle et al. 2015) developed \textit{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 \textit{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 \textit{M. margaritifera} hotspots; zones that support relatively a larger proportion of the mussel population.

\textbf{Methods}

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

eDNA filtering and extraction of field samples

Water samples were collected from 8 locations where live *M. margaritifera* had been observed were in Munster Blackwater River in sterile 3 L PET bottles and kept frozen until analysed. Water samples were thawed in ambient temperature and 1 L per sample was filtered through individual 0.45 µm Whatman nitrate filters. The amount of water filtered was recorded for each water sample to the closest cL. Filters were subsequently dehydrated with 100% EtOH before storage at -20°C. Each filter was cut into halves (half for analysis and half for archival storage) and shredded to increase surface area for eDNA extraction using Qiagen QIAshredder (Qiagen, Valencia, CA). Total eDNA was extracted using a Qiagen DNeasy kit (Qiagen, Valencia, CA). Extracted eDNA was stored at -20°C until further processing.

eDNA assay deployment

Concentrations of eDNA were determined by qPCR using an Applied Biosystems ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City, CA) quantitative thermocycler. Amplification reactions for each species
included: 15 µl of TaqMan® Environmental Master Mix 2.0 (Life Technologies., Applied Biosystems, Foster City, CA), 3 µl of each primers (final concentration of 0.2 µM), probe (final concentration of 0.2 µM), ddH2O, and DNA template (3 µl), forming the 30µL reaction volume. The qPCR run method used warm-up conditions 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 standard curve for *M. margaritifera* was generated with quantified (NanoDrop®-1000, Thermo Scientific, Wilmington, DE) DNA extractions from a tissue sample (DNA concentrations of 122.0 pg/L) using seven 10:1 serial dilutions as template for qPCR. The standard curve for *M. margaritifera* (*y = -3.4058x + 38.238, R² = 0.9997, efficiency ~ 96.62%) was generated using 3 µl DNA template in a total reaction volume of 30 µl, respectively. The lowest concentrations of DNA (dynamic range) equalling 0.122 pg/L in the standard curves were detected at Cq (quantification cycle) 34.8 (average over three technical replicates, SD = 0.073). Results from the standard curve (Fig. 1.) indicate a dynamic range and lowest eDNA detection level at Cq 38.238 (equivalent to a *M. margaritifera* DNA concentration of 0.333 pg/L). All field samples were quantified in duplicates (two technical replicates), to ensure consistency, with two laboratory negative controls and *M. margaritifera*. Three water samples within the Munster Blackwater River system were used as template for *S. trutta* qPCR to validate the presence of amplifiable eDNA. The average Cq across technical replicates (n=2) were used for quantification.

**Results and Discussion**

The present study successfully developed an eDNA assay with very high sensitivity for *M. margaritifera*. All analysed samples yielded detectable eDNA (with Cq within the dynamic range) for both *M. margaritifera* and *S. trutta* (presence of *S. trutta* eDNA was validated in three sample locations), indicating that amplifiable target eDNA was present in all water samples. Resulting Cq values from the qPCR assays were transformed to pgDNA/L (based on the standard curve, Fig 1.). The concentrations of eDNA ranged from 0.462 pg/L in Rowls Aldworth West Bridge (right bank sample) to the highest of 109.884 pg/L in the Leader’s Bridge Allow (left bank sample). Environmental DNA concentrations were relatively stable across transects within location (right bank, middle and left bank samples). Average eDNA concentrations (across transects) ranged from the lowest at Rowls Aldworth West Bridge (1.056 pg/L) to the highest at Cullen Bridge (79.412 pg/L). Two graphs were plotted to visualise eDNA concentrations (Figs. 2 and 3). These results indicate variable eDNA concentrations among localities. The developed eDNA assay can be used to assess concentrations of eDNA which should be...
related to the biomass of *M. margaritifera* and could be used for monitoring the status of *M. margaritifera* in individual locations and river systems. However, utilising the quantifying capabilities of eDNA assays requires careful planning, standardised and coordinated sampling efforts (exact GPS positions, dates, time of day, water levels, weather conditions, details about where in the water body samples were acquired from, etc.) to ensure that samples are of the highest quality. Nevertheless, the eDNA assay developed here can be used for rapid detection of *M. margaritifera* presence throughout Ireland and the natural range of *M. margaritifera*. Wide scale deployment of the assay can help detecting cryptic populations in watersheds where *M. margaritifera* has not previously been reported or where *M. margaritifera* are considered to have gone extinct.

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

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Conflict of Interest: The authors declare that they have no conflict of interest.
References MAX 30


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.

\[ y = -3.4058x + 38.238 \]
\[ R^2 = 0.9997 \]

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

Commented [jc2]: Spara John’s Bridge, Rowls Vilken mer?
Fig. 3. Average concentrations (error bars indicate observed max and min eDNA concentrations within locations) of Margaritifera margaritifera eDNA among water samples from the Munster Blackwater river system.
**Appendix. Margaritifera margaritifera eDNA concentrations within and among sites in the Munster Blackwater river system.** (loc – location number, site L – left bank sample, site M - mid river sample, site R – right bank sample, Cq - quantification cycle, T1 – technical replicate 1, T2 – technical replicate 2).

<table>
<thead>
<tr>
<th>Location name</th>
<th>Loc</th>
<th>site</th>
<th>CqT1</th>
<th>CqT2</th>
<th>Average Cq</th>
<th>Average eDNA Conc across technical replicates pg/L</th>
<th>Average eDNA Conc across location pg/L</th>
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<tr>
<td>Leader's Bridge</td>
<td>1</td>
<td>L</td>
<td>29.662</td>
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<td>M</td>
<td>32.806</td>
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