Direct multiplex PCR-NALFIA to inform marine conservation: Use of an innovative diagnostic tool for the detection of Ostrea edulis larvae

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Received 8 June 2021 / Accepted 20 September 2021

Handling Editor: Carlos Saavedra

Abstract – The European oyster Ostrea edulis played a key role in the North Sea by providing several ecosystem functions and services. Today, O. edulis is classified as severely degraded or functionally extinct in Europe. Marine conservation is focusing on biogenic reef restoration, namely the restoration of O. edulis in Natura 2000 sites of the North Sea. The identification of oyster larvae related to natural spatfalls of restored reefs and monitoring of larval drift is a key aspect of marine protected area management. Morphological identification and distinction from other abundant bivalve larvae using microscopy is difficult. Existing molecular biological methods are expensive and bound to stationary laboratory equipment, or are inadequate in the visualization. In this study, we identified nucleic acid lateral flow immunoassay (NALFIA), a well-established tool in human pathogen diagnostics, as an efficient approach for point-of-care (POC) testing in marine monitoring. Based on the genetic sequence of the mitochondrial cytochrome b of O. edulis, forward and reverse primers were developed. The reverse primer was labelled with fluorescent dye FITC, forward primer with biotin. Reaction on the lateral flow stripe could be realized with a single O. edulis larva in direct PCR with multiplex primers in a portable PCR-cycler. The established NALFIA system can distinguish O. edulis larvae from Crassostrea gigas and Mytilus edulis larvae, respectively. This method offers new approaches in POC testing in marine research and monitoring. It gives quick and clear results, is inexpensive, and could be easily adapted to other species of interest.

Keywords: Nucleic acid lateral flow immunoassay / oyster restoration / larvae detection / European oyster / point-of-care detection / biogenic reef restoration

1 Introduction

As an ecosystem engineer, the native European oyster played a key role in the North Sea ecosystem by sustaining a biogenic and three-dimensional habitat for a highly diverse associated community. Ostrea edulis beds provide settlement substrate, spawning grounds, a shelter for juveniles, and nutrition (Pogoda, 2019). In general, oyster beds are biodiversity hotspots, with key ecological functions in temperate marine seas. Due to multiple anthropogenic stressors, mainly fisheries and disease, more than 85% of oyster reefs worldwide have been lost so far (Beck et al., 2011). O. edulis is classified as severely degraded or even as functionally extinct in several European regions and there is broad agreement, that protection alone is not enough (Jackson and Buceta Miller, 2009; Aronson and Alexander, 2013; Fariñas-Franco et al., 2018; Pogoda et al., 2019, 2020). Following up on successful oyster restoration approaches in the USA and Australia, marine nature conservation in Europe is now gaining momentum to increase conservation and restoration efforts against the background of the Habitats Directive, the Marine Strategy Framework Directive, and OSPAR recommendations (Pogoda, 2019; Pogoda et al., 2020). The ecological restoration of biogenic reefs, namely oyster reefs is a focus of future marine protected area (MPA) management in the North Sea, considering population reinforcement, habitat restoration and species reintroduction in the respective areas and is linked to global approaches of the UN Decade for Ecosystem Restoration (UN General Assembly, 2019; Bundesamt für Naturschutz, 2020).
The detection of *O. edulis* larvae in the field is of paramount importance to identify potential reproductive performance of degraded populations or of restored populations. Furthermore, it allows to define substrate-limited areas versus recruitment-limited areas (Westby et al., 2019; Fitzsimons et al., 2020), to inform about the ecological connectivity of MPAs and to support the practical implementation of tailor-made conservation measures. Currently, no high throughput or easy to use method exists to determine the existence and abundance of *O. edulis* larvae in the field and to distinguish the larvae from other bivalve species, such as *Crassostrea gigas* and *Mytilus edulis*.

The diagnosis and distinction of bivalve larvae via microscopy and morphological traits are often complicated and relies on well-trained experts to generate reliable results. In addition, this approach is accompanied by a low detection limit and a time-consuming examination. Rapid detection tests, such as antibody-based detection methods on a lateral flow system, are promising alternatives to determine the larvae species at the point-of-care (POC) under certain circumstances, but also often fail due to low specificity and sensitivity. In this context we define POC as an application directly in the field such as on a ship with little or no special laboratory equipment to carry out sophisticated analyses.

Molecular biological methods, such as PCR, are highly sensitive and specific but require endpoint determination using gel electrophoresis (GE), sometimes toxic dyes and UV light equipment or expensive equipment to determine the amplification of DNA in real-time (Zieritz et al., 2012). In addition, DNA has to be isolated and purified before PCR application. Several improvements and further developments of PCR have already been described in many publications (Bakheit et al., 2008; Watts et al., 2019). That includes, for example, isothermal PCR based on either the loop-mediated isothermal amplification system (Notomi et al., 2000), helicase-dependent amplification (Vincent et al., 2004), strand displacement amplification (Walker et al., 1992), or recombinase polymerase amplification (Piepenburg et al., 2006). The detection of the PCR amplicons is again achieved by GE and thus is lab dependent (Jiang et al., 2020). The next-generation sequencing (NGS) method is developed to identify marine bivalve larvae enabling a new and efficient approach for the point of care testing and thus filling technological gaps in marine conservation ecology.

### 2 Materials and methods

#### 2.1 Primer design

In this study, sets of primer pairs were designed targeting the mitochondrial cytochrome b (mtCyB) gene of *Ostrea edulis* (GenBank accession no. JF274008.1), *Crassostrea gigas* (GenBank accession no. NC_001276.1), and *Mytilus edulis* (GenBank accession no. NC_006161.1). The sequences for the mtCyB of the three species were downloaded from GenBank (Geer et al., 2010). The Primer3 web application Version 4.1.0 (Untergasser et al., 2012) was used to pick 20 bp long forward and reverse primer pairs (Tab. 1) for each species.

All forward primers were labelled with biotin at 5′ ends and *O. edulis* reverse primer was labelled with FITC at 5′ end (Eurofins Genomics Germany GmbH, Ebersberg). The reverse primers of *C. gigas* and *M. edulis* were labelled with commercial fluorescent markers DY-632 and with DY-495 at 5′ ends, respectively (IBA GmbH, Göttingen). Within the PCR forward and reverse primer will form an amplicon with a biotin label at one end and fluorescent label at the other end, if the respective DNA is present. The analogue antibodies were coated on the lateral flow strip to immobilised the analyte.

#### 2.2 Lateral flow design

Lateral flow test strips, to be seen in Figure 1, based on a nitrocellulose membrane (Senova Gesellschaft für Biowissenschaft und Technik mbH, Weimar), customized with a conjugate of anti-biotin gold nanoparticles in the conjugate pad followed by three test zones coated with a FITC-antibody,
DY-632-antibody, and DY-495-antibody, respectively. The control zone at the end of the strip was coated with bovine serum albumin and biotin. With the presence of an analyte the anti-biotin gold nanoparticles bounded to the biotin labelled end of the analyte to realise a red colour coding at the respective zone on the lateral flow strip which was coated with the respective antibody to the fluorescent labelled end of the analyte. If no analyte was present the anti-biotin gold nanoparticles were immobilised at the biotin coated control zone to realise a red colour coding.

2.3 PCR analysis and NALFIA application

The PCR master mix with a total volume of 10 µl consisted of 5 µl PerfeCTa qPCR ToughMix UNG (Quantabio, Beverly, Mass.), 0.05 µl (each) forward primer, 0.05 µl (each) reverse primer, 2.5 µl DNA template and sufficient volume of Ampuwa (Fresenius Kabi Deutschland GmbH, Bad Homburg) to match the total volume.

The PCR was run in a Primus 25 advanced thermocycler (Peqlab Biotechnologie GmbH, Erlangen). The program started with uracil-DNA glycosylase (UNG) carry over process at 45 °C for 5 min followed by initial denaturation at 95 °C for 3 min. The performed PCR included denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, and the elongation at 72 °C for 45 s in a repeating loop of 35 cycles, followed by the final elongation at 72 °C for 7 min and a temperature decreasing ramp of 3 °C s⁻¹ down to 8 °C.

Extraction of DNA from deep-frozen tissue of adult *O. edulis*, *C. gigas*, and *M. edulis* individuals was realized by using a commercial NucleoSpin® Tissue extraction kit (Macherey-Nagel GmbH & Co. KG, Düren). In contrast extraction from *O. edulis* larvae was achieved by thermal lysis at 95 °C for 3 min embedded in the PCR as a direct PCR. After PCR amplification, the target genes were detected with a 3% agarose gel electrophoresis (GE) and comparatively nucleic acid lateral flow immunoassay (NALFIA).

Table 1. Primer pairs targeting the mitochondrial cytochrome b of *Ostrea edulis*, *Crassostrea gigas*, and *Mytilus edulis*. The reverse primers are labelled at 5' with a specific dye, respectively the forward primers are labelled at 5' with biotin for later implementation to nucleic acid lateral flow immunoassay.

<table>
<thead>
<tr>
<th>Primer:</th>
<th>Sequence:</th>
<th>5'-end label:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. edulis</em> forward</td>
<td>TAA AGT ATC CCT GTC CGC CC</td>
<td>Biotin</td>
</tr>
<tr>
<td><em>O. edulis</em> reverse</td>
<td>AAC CCT CCG AAT AGC TCA CC</td>
<td>FITC</td>
</tr>
<tr>
<td><em>C. gigas</em> forward</td>
<td>TCA TAA AGC AGG TGG GTG GT</td>
<td>Biotin</td>
</tr>
<tr>
<td><em>C. gigas</em> reverse</td>
<td>TCC TGA GCA CCA ATA ACC GT</td>
<td>DY-632</td>
</tr>
<tr>
<td><em>M. edulis</em> forward</td>
<td>GCC TAT TCA CGT TCA GCC TG</td>
<td>Biotin</td>
</tr>
<tr>
<td><em>M. edulis</em> reverse</td>
<td>GGA CGA GCA CCA ATT CAT GT</td>
<td>DY-495</td>
</tr>
</tbody>
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![Fig. 1. Schematic illustration of the lateral flow test stripes with the exact location of the different zones. (Modified after Senova Gesellschaft für Biowissenschaft und Technik mbH).](image-url)
FITC-antibody on the first test line. *C. gigas* and *M. edulis* was visualized on the second and third test line by an immunochromatographic reaction of DY-632 labelled amplicon with DY-632 antibodies, and DY-495 labelled amplicon with DY-495 antibodies.

In order to gradually approach a multiplex system, PCR reaction series started with a combination of single DNA templates with single primer pairs followed by a PCR reaction involving duplex primer pairs and triplex primer pairs, respectively. In the next step, the PCR reaction series was conducted with two DNA templates in combination with triplex primer pairs and three DNA templates in combination with triplex primer pairs, respectively.

In order to confirm the performance of the system for the detection of one single larva, one individual *O. edulis* larva was transferred to a PCR tube with 10 µl PCR master mix. The veliger larvae were removed mechanically from an oyster shortly before natural spawning and then fixed in ethanol. The DNA extraction of the larva was carried out by thermal lysis within the PCR as a direct PCR with an extension of the initial denaturation at 95 °C to 5 min.

### 2.4 Exclusivity test

The focus was on the development of methods for the detection of *O. edulis*. To check whether the selected primers hybridise exclusively to *O. edulis* in the PCR, cross-reactions with *C. gigas* and *M. edulis* DNA were also performed. In addition, the effects of a multiplex approach on the results were performed by adding the primers for *C. gigas* and *M. edulis*. In a further test, DNA from the sea anemone *Sagartiogeton laceratus* and the oaten pipes hydroid *Tubularia indivisa* was also tested for reaction with the *O. edulis* primer.

### 3 Results

#### 3.1 Single DNA template – monoplex PCR

The nucleic acid lateral flow immunoassay (NALFIA) testing for single DNA templates with the respective single primer pairs in Figure 2 showed the expected test lines for the positive controls (+) of *Ostreia edulis*, *Crassostrea gigas*, and *Mytilus edulis*. Each test showed a control line, confirming the complete flow of the sample through the sample pad. The respective non-template controls (−) did not show visible test lines. The PCR master mix was realized with a total concentration of 100 pmol µl⁻¹ for each primer.

The gel electrophoresis (GE) for the quality control of the PCR product showed the expected fragment sizes of the amplicons. PCR with DNA templates of *O. edulis* on lane 6 and lane 7 showed a 191 bp long amplicon, *C. gigas* DNA templates on lane 12 and lane 13 shows a 174 bp long amplicon, and *M. edulis* DNA templates on lane 18 and lane 19 a 234 bp long amplicon, respectively. The respective non-template controls on lanes 3, 4, 9, 10, 15, and 16 showed no visible amplicons.

#### 3.2 Single DNA template – duplex PCR

For the combination of *O. edulis* primers (OE) and *C. gigas* primers (CG) with *C. gigas* DNA template in part A of Figure 3, the NALFIA showed beside the expected strong visible test line for *C. gigas* also a light coloration at the test line for *O. edulis*. This light coloration also was evident in the negative control. However, the GE of the PCR product showed no amplicons in the negative control and the expected single fragment of 174 bp for *C. gigas* DNA template without any other amplicons on lane 5 and lane 6. The result of the NALFIA with *O. edulis* DNA template in combination with OE and CG primers showed the expected test line on the NALFIA as well on the GE on lane 3 and lane 4 with an amplicon of 191 bp.

The combination of OE and *M. edulis* primers (ME) with *O. edulis* and *M. edulis* DNA templates in part B of Figure 3 showed the expected NALFIA results for *O. edulis* and *M. edulis* DNA templates at the respective test lines. As well the correlating GE of the PCR products confirmed the expected fragment sizes for *O. edulis* with 191 bp on lane 3 and lane 4, respectively 234 bp for *M. edulis* on lane 5 and lane 6. The non-template control on lane 1 and lane 2 showed no visible fragments.

The combination of ME and CG primer pair with *O. edulis* and *M. edulis* DNA templates in part C of Figure 3 showed the expected NALFIA results for *O. edulis* and *C. gigas* DNA templates at the respective test lines. As well the correlating GE of the PCR products confirmed the expected fragment sizes for *M. edulis* with 234 bp on lane 3 and lane 4, respectively 174 bp for *C. gigas* on lane 5 and lane 6. The non-template control on lane 1 and lane 2 showed no visible fragments. The total concentration of each primer in the master mix was 100 pmol µl⁻¹.

#### 3.3 Single DNA template – triplex PCR

Total primer concentration in the PCR master mix was reduced to 10 pmol µl⁻¹ to remove the false-positive result with light coloration at the *O. edulis* test line with no such DNA template involved. The NALFIA in Figure 4 with DNA templates of *O. edulis*, *C. gigas*, and *M. edulis*, respectively, showed all expected test lines. No test lines were visible in the non-template control, and all tests showed a visible control line. The corresponding GE showed no visible amplicons on lane 3 and lane 4. The positive controls of *O. edulis* on lane 6 and lane 7 showed a 191 bp long fragment. For *C. gigas* on lane 9 and lane 10 a 174 bp long fragment was visible, respectively a 234 bp long fragment on lane 12 and lane 13 for *M. edulis*.

#### 3.4 Double and triple DNA templates – triplex PCR

Figure 5 showed the NALFIA with a combination of the three primer pairs OE, CG, and ME, with double, respectively, three DNA templates. OE and CG primers were applied in a total concentration of 10 pmol µl⁻¹, and ME primer at a total concentration of 50 pmol µl⁻¹ within the master mix.

All five NALFIA had been running through the lateral flow stripe correctly, as the visible control lines were showing. The non-template control was showing no visible bands at the test zones. The cartridge with the *O. edulis* and *C. gigas* DNA templates was showing two visible red lines at the test zone for *O. edulis* DNA and the test zone for *C. gigas* DNA. The cartridge with NALFIA using the *O. edulis* and *M. edulis* DNA.
The corresponding GE for quality control of the PCR products showed no amplicons for the non-template control on lane 3 and lane 4. Lane 6 and lane 7 with the PCR product of *O. edulis* DNA and the test zone for *M. edulis* DNA. The cartridge with NALFIA running *C. gigas* and *M. edulis* DNA template was showing two visible red lines at the test zone for *C. gigas* DNA and the test zone for *M. edulis* DNA. The cartridge with the NALFIA running with all three DNA templates showing three red lines at all test zone.

The PCR product of *C. gigas* and *M. edulis* DNA template showed two fragments on both lines with 174 bp and 234 bp for *C. gigas* and *M. edulis*, respectively. On lane 17, one can see the PCR product with all three DNA templates and three fragments with 174 bp, 191 bp, and 234 bp for *C. gigas*, *O. edulis*, and *M. edulis*, respectively.

### 3.5 Single larva detection

Figure 6 showed the results of a direct multiplex PCR product from a single larva on a NALFIA. At all cartridges, the red line at the control zone was visible, indicating that the sample moved through the entire lateral flow strip. The negative control at the cartridge on the far-left side showed no visible red lines at the test zones. All other cartridges,
Fig. 3. PCR-nucleic acid lateral flow immunoassay (NALFIA) with a combination of two primer pairs and one DNA template. The label on the left side of every pair of cartridges is indicating the test line for the existence of *Ostrea edulis* (FITC OE), *Crassostrea gigas* (632 CG), and *Mytilus edulis* (495 ME). The most upper line on the cartridges is indicating the control line of each NALFIA. Primer combinations above the cartridges are indicated with OE, CG, and ME for the primers of *O. edulis*, *C. gigas*, and *M. edulis*, respectively. Part A of the figure shows OE and CG primers in combination with *O. edulis* DNA (left cartridge), *C. gigas* DNA (middle cartridge) and a negative control with no DNA template. Part B of the figure shows OE and ME primers in combination with *O. edulis* DNA (left cartridge), *M. edulis* DNA (middle cartridge) and a negative control with no DNA template. Part C of the figure shows ME and CG primers in combination with *M. edulis* DNA (left cartridge), *C. gigas* DNA (middle cartridge) and a negative control with no DNA template. The image of the 3% agarose gel electrophoresis shows the quality control of the PCR products. The scheme of each gel slots: 1: non-template replicate 1, 2: non-template replicate 2, 3: left cartridge replicate 1, 4: left cartridge replicate 2, 5: middle cartridge replicate 1, 6: middle cartridge replicate 2, M: 50 bp marker, (--) empty.
respectively, replicates with a single larva showed a visible red line at the FITC test zone indexing for _O. edulis_. The control of the GE beneath the cartridges showed the 100 bp marker on lane 1 and the negative control on lane 3 and lane 4 with no visible bands. On lane 6 to lane 9, the four replicates showed a single fragment of 191 bp.

### 3.6 Exclusivity test

The exclusivity test in Figure 7 showed no visible test lines but the control line in the cartridge with the negative control on the left side. The second cartridge with _Sagartiogeton laceratus_ and _Tubularia indivisa_ DNA showed no test line but the control line. The third cartridge with _O. edulis_, _S. laceratus_ and _T. indivisa_ DNA showed the control line and test line for _O. edulis_. The cartridge on the right side showed the positive control for _O. edulis_, _C. gigas_ and _M. edulis_ DNA and the control line. The GE image showed the expected fragments for _O. edulis_, _C. gigas_ and _M. edulis_ on lane 9, 10 and 12, 13, respectively.

### 4 Discussion

This study aimed to expand the molecular biological detection method of nucleic acid lateral flow immunoassay (NLFIA) into a marine nature conservation context. NLFIA is already very well-established in human pathogen diagnostic plus providing a promising potential for further application fields (Posthuma-Trumpie et al., 2009; Buscher, 2011; Wang et al., 2013; Brunauer et al., 2021). We identified it as an effective tool for the identification and distinction of larvae of marine key species. In a multi-stage process, the primer and PCR program were optimized for a multiplex approach so that the amplicon leads to a clear result on the NLFIA. This enables new approaches in point-of-care (POC) testing in marine research and monitoring. A species of specific interest is the native European oyster _Ostrea edulis_, classified as functionally extinct in the German Bight, with small populations in adjacent water bodies, such as in the Netherlands, France, around UK and Denmark and a high potential for ecological restoration (Pogoda et al., 2019).

#### 4.1 NLFIA and primer design for closely related species

Lateral Flow Immunoassays (LFD) impress with their simple use and the quick presentation of precise results, regardless of whether they are protein- or nucleotide-based (Anfossi et al., 2018). The main problem and most significant challenge, especially with multiplex approaches, are the potential occurrences of false-positive or false-negative results (Posthuma-Trumpie et al., 2008). With NLFIA, there is a risk of a later false-positive result in an unsuitable primer design, which leads to unspecific binding in PCR due to primer dimerization. When separated in the gel electrophoresis (GE), these amplicons can be recognized by their unexpected base pair size (see Appendix A for examples). Still, the immunological reactions of such non-specific amplicons on the lateral flow strips can lead to false-positive results that remain undetected. In some cases, we noticed single small red dots especially at the first test line coated with FITC antibodies. We are assuming that this effect is due to not precisely applied antibodies within the manufacturing process of the lateral flow test stripes. The FITC dye showed a very high affinity and colouring power, we already noticed that in the early development state and therefore already reduced the concentration of the primers. Furthermore, a narrow point is not to be seen as an indication of a positive result. A pale but continuous line formation can be seen even with the smallest amounts of the target gene. The Basic Local Alignment Search Tool (BLAST) (Ye et al., 2012) provides the first indication of a suitable primer design with a low probability of later false-positive results. As part of this work, primer design for _Crassostrea gigas_ and _Mytilus edulis_ was also integrated into the multiplex approach to address a direct ecological question with a regional reference to the North Sea. Larvae of these two bivalve species are highly abundant during the spawning season in spring and identification and distinction of _O. edulis_ among these morphologically similar bivalve larvae is difficult (Garland and Zimmer, 2002). For the primer design of _C. gigas_ and _M. edulis_, however, it must be mentioned that there was no further exclusivity test with other closely related species. In _C. gigas_, the BLAST indicates that bonds with _Crassostrea angulata_, _Crassostrea sikamea_, and _Crassostrea hongkongensis_ can also occur (Kim et al., 1999). However, considering the local use of this test, this can be neglected considering the natural habitats and current ecological distribution of these species (Boudry et al., 1998; Lam and Morton, 2004; Sekino, 2009). For the primer design of the mitochondrial cytochrome b (mtCytB) of _M. edulis_, BLAST provides an even higher probability of binding to the very closely related species _Mytilus trossulus_, _Mytilus galloprovincialis_, and _Mytilus chilenis_. The difficulties in differentiating individual Mytilus species, especially those between _M. edulis_ and _M. trossulus_, are well known, also on the molecular level (Larrain et al., 2019). It is also challenging to delimit these species locally from one another since some of them are even sympatric. Concerning the aforementioned regional reference of sampling _O. edulis_ larvae in the German Bight, this problem was also considered less relevant within the question, since it is sufficient for the circumstances to distinguish whether it is _O. edulis_ or a Mytilus species. If one wants to transfer this NLFIA system to a question for which it is necessary to make a very precise statement about a _Mytilus_ species, then a more considerable effort must be made in the primer design.

#### 4.2 NLFIA and primer design for _O. edulis_

This study chose mtDNA because (1) it is relatively simple and small compared to nDNA due to its lack for noncoding sequences (introns), pseudogenes, repetitive DNA, and transposable elements; (2) it is relatively easy to extract; (3) it does not undergo genetic rearrangements such as recombination; and (4) sequence ambiguities resulting from
Fig. 4. PCR-nucleic acid lateral flow immunoassay (NALFIA) with a combination of three primer pairs and one DNA template of Ostrea edulis, Crassostrea gigas, and Mytilus edulis. The label on the left side of every pair of cartridges is indicating the test line for the existence of O. edulis (FITC OE), C. gigas (632 CG), and M. edulis (495 ME). The most upper line on the cartridges is indicating the control line of each NALFIA. The image of the 3% agarose gel electrophoresis is showing the quality control of the PCR products. The scheme of the gel slots: 1: 100 bp marker, 2: empty, 3: OE / CG / ME x non-template replicate 1, 4: OE / CG / ME x non-template replicate 2, 5: empty, 6: OE / CG / ME x O. edulis replicate 1, 7: OE / CG / ME x O. edulis replicate 2, 8: empty, 9: OE / CG / ME x C. gigas replicate 1, 10: OE / CG / ME x C. gigas replicate 2, 11: empty, 12: OE / CG / ME x M. edulis replicate 1, 13: OE / CG / ME x M. edulis replicate 2, 14: empty, 15: 100 bp marker.
Fig. 5. PCR-nucleic acid lateral flow immunoassay (NALFIA) with a combination of three primer pairs and two, respectively three, DNA template of *Ostrea edulis*, *Crassostrea gigas*, and *Mytilus edulis*. The label on the left side of every pair of cartridges is indicating the test line for the existence of *O. edulis* (FITC OE), *C. gigas* (632 CG), and *M. edulis* (495 ME). The most upper line on the cartridges is indicating the control line of each NALFIA. The combinations for DNA templates are *O. edulis* / *C. gigas*, *O. edulis* / *M. edulis*, *C. gigas* / *M. edulis*, with triple primer combination of OE / CG / ME primer pairs. OE and CG primer total concentration is 10 pmol µl⁻¹, and the total ME primer concentration is 50 pmol µl⁻¹. The cartridge on the far-left side shows the non-template control of the multiplex primer combination. Other cartridges are indicating the respective combination of the DNA template above. The image of the 3% agarose gel electrophoresis is showing the quality control of the NALFIA. The scheme of the gel slots: 1: 100 bp marker, 2: empty, 3: OE / CG / ME x non-template replicate 1, 4: OE / CG / ME x non-template replicate 2, 5: empty, 6: OE / CG / ME x *O. edulis* / *C. gigas* replicate 1, 7: OE / CG / ME x *O. edulis* / *C. gigas* replicate 2, 8: empty, 9: OE / CG / ME x *O. edulis* / *M. edulis* replicate 1, 10: OE / CG / ME x *O. edulis* / *M. edulis* replicate 2, 11: empty, 12: OE / CG / ME x *C. gigas* / *M. edulis* replicate 1, 13: OE / CG / ME x *C. gigas* / *M. edulis* replicate 2, 14: empty, 15: 100 bp marker, 16: empty, 17: OE / CG / ME x *O. edulis* / *C. gigas* / *M. edulis*. 
heterozygous genotypes are avoided. Especially the mitochondrial cytochrome b (mtCytB) is particularly suitable as a species-specific target because mtCytB is maternal inherited and interspecifically conserved, so even closely related and ubiquitous species can be distinguished (Cronin et al., 1991; Danic-Tchaleu et al., 2011; Mortz et al., 2021).

For the primer sequence used here, which is to bind to the mtCytB of *O. edulis*, Blast gives no indication of potential target sequences in other organisms. Likewise, no amplicons were formed when the primer sequence for the mtCytB of *O. edulis* was run in PCR with DNA samples from *Crassostrea gigas*, *Mytilus edulis*, *Sagartiogeton laceratus* and *Tubularia indivisa*.

Fig. 6. PCR-nucleic acid lateral flow immunoassay (NALFIA) with a product of direct multiplex PCR of a single *Ostrea edulis* larva. The label on the left side of every pair of cartridges is indicating the test line for the existence of *O. edulis* (FITC OE), *Crassostrea gigas* (632 CG), and *Mytilus edulis* (495 ME). The most upper line on the cartridges is indicating the control line of each NALFIA. The cartridge on the left side is showing the non-template control, and the following four cartridges are showing NALFIA replicates with a single larva of *O. edulis* each. The image of the 3% agarose gel electrophoresis is showing the quality control of the NALFIA. The scheme of the different gel slots: 1: 100 bp marker, 2: empty, 3: non-template replicate 1, 4: non-template replicate 2, 5: empty, 6: *O. edulis* larva replicate 1, 7: *O. edulis* larva replicate 2, 8: *O. edulis* larva replicate 3, 9: *O. edulis* larva replicate 4.
Fig. 7. PCR-nucleic acid lateral flow immunoassay (NALFIA) with multiplex primers for *Ostrea edulis*, *Crassostrea gigas*, and *Mytilus edulis*. The label on the left side of every pair of cartridges is indicating the test line for the existence of *O. edulis* (FITC OE), *C. gigas* (632 CG), and *M. edulis* (495 ME). The most upper line on the cartridges is indicating the control line of each NALFIA. The NALFIA was tested with DNA templates in the combination of *Sagartiogeton laceratus / Tubularia indivisa*, *S. laceratus / T. indivisa / O. edulis*, and *O. edulis / C. gigas / M. edulis*. The image of the 3% agarose gel electrophoresis is showing the quality control of the NALFIA. The scheme of the gel slots: 1: 100 bp marker, 2: empty, 3: OE / CG / ME x non-template replicate 1, 4: OE / CG / ME x non-template replicate 2, 5: empty, 6: OE / CG / ME x *S. laceratus / T. indivisa* replicate 1, 7: OE / CG / ME x *S. laceratus / T. indivisa* replicate 2, 8: empty, 9: OE / CG / ME x *S. laceratus / T. indivisa / O. edulis* replicate 1, 10: OE / CG / ME x *S. laceratus / T. indivisa / O. edulis* replicate 2, 11: empty, 12: OE / CG / ME x *O. edulis / C. gigas / M. edulis* replicate 1, 13: OE / CG / ME x *O. edulis / C. gigas / M. edulis* 2, 14: empty, 15: 100 bp marker.
as to be seen in Figure 7. The latter two species were chosen because they are considered *O. edulis* reef-associated species (Merk pers. comm.). Regarding the results of the NALFIA tests on *O. edulis* DNA shown here and compare the corresponding GE, one can see that a test system for *O. edulis* has been established that delivers exact positive results in both the single and the multiplex approach. However, due to the competitive PCR reaction, the multiplex approach is difficult to develop. In this case, primer limitations were necessary to balance the reaction equilibrium. Besides, to improve the applicability of the PCR at POC an uracil-n-DNA glycosylase (UNG) treatment step for the prevention of carryover of PCR amplicon was implemented (Longo et al., 1990). For further optimization and “in-house” validation of the method, further exclusivity tests with more larvae species as well as a field validation in seawater should be taken into account. Due to the user-friendly handling of the developed method at the POC and the fact that the DNA-Polymerase could not be lyophilized, it is urgently necessary to test the addition of glycerol, for example, to enhance the shelf life of the reaction (Tsai et al., 2007).

### 4.3 Advantages of PCR-NALFIA over common molecular techniques

The PCR-NALFIA method shown here for the identification of *O. edulis* larvae offers an excellent alternative to the molecular biological detection methods described so far (Andre et al., 1999; Garland and Zimmer, 2002; Le Goff-Vitry et al., 2007; Lasota et al., 2013; Sanchez et al., 2014). The proof is faster and no sophisticated equipment is necessary. The method shown here does not require prior DNA isolation and purification to identify a single larva. This method can be used for field trials, especially on small research vessels, which only have limited laboratory equipment and capacity. For initial preliminary tests, the results can already be evaluated on board and are therefore available during day trips. The time required for clear evidence is around 60 to 90 min. In our laboratory tests, the PCR required for the NALFIA could also be carried out by using a mini-PCR machine (minipcr, Cambridge, USA). This is a small thermostycler controlled by a mobile application or laptop capable of running 8 samples at the same time. Previously, the device was already successfully used by our working group aboard on research vessels on different occasions and the practicability and accuracy in comparing to a commonly used laboratory thermocycler have been proved by Kwon et al. (2016).

The method was developed to address a critical demand for a fast, mobile, and reliable and quantitative species identification in the context of ecological restoration of a critically endangered species. It was adapted successfully to identify *O. edulis* larvae and to distinguish them from the DNA sample of two other planktivorous bivalves. Although, NALFIA can’t deliver qualitative results it is a suitable method showing rapid results when supporting unclear morphological results or for rapid random sampling. This is an important outcome with benefits for ecological restoration and marine conservation: It facilitates the confirmation of restoration progress and informs site selection by considering larval occurrence for the connectivity of ecological restoration sites. Furthermore, it supports field monitoring and the verification of larval drift models.

Further application fields in marine ecology may also benefit from this method. By developing and adjusting to organisms of interest, the NALFIA detection can be applied for specific monitoring tasks with high ecological relevance, such as tracing and identifying neobiota and invasive species which are expected to arrive in new biogeographic regions and environments as hydrodynamic conditions suggest a further distribution or active migration in climate change scenarios.

**Acknowledgements.** This work was carried out within the framework of the project PROCEED – Reinroduction of the European oyster in the German North Sea: Establishing a sustainable production of seed oysters for a long-term reintroduction program (FKZ 3517685013). The project is funded by the Federal Agency for Nature Conservation of Germany with funds from the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety as part of the Federal Program for Biological Diversity. We thank Verena Merk and colleagues of the associated project RESTORE (FKZ 3516892001) for their scientific advice and support. Furthermore, we thank the reviewers for their input to improve this manuscript.

### References


Cite this article as: Benkens A, Buchholz C, Pogoda B, Harms CG. 2021. Direct multiplex PCR-NALFIA to inform marine conservation: Use of an innovative diagnostic tool for the detection of Ostrea edulis larvae. *Aquat. Living Resour.* 34: 23
Appendix A

Examples of false-positive PCR-nucleic acid lateral flow immunoassay (NALFIA) occurring within the development process of the master mix and PCR protocol.

Fig. A1. Example of false-positive PCR-nucleic acid lateral flow immunoassay (NALFIA) with a combination of three primer pairs and one DNA template of Ostrea edulis, Crassostrea gigas, and Mytilus edulis. The NALFIA for the negative control shows a light red band for C. gigas although non-DNA template was present and the 3% agarose gel electrophoresis shows no band at the expected position for C. gigas at 174 bp. The NALFIA for O. edulis shows false-positive result for C. gigas and the NALFIA for C. gigas shows a false-positive result for O. edulis, respectively. Likewise, these two false-positive results don’t show an expected band in the gel electrophoresis on lane 6 and lane 7 at 174 bp for C. gigas and on lane 10 at 191 bp for O. edulis, respectively. The NALFIA for M. edulis shows false-positive results for O. edulis and C. gigas, but the gel electrophoresis only shows expected bands for M. edulis on lane 12 and lane 13.
Fig. A2. Example of false-positive PCR-nucleic acid lateral flow immunoassay (NALFIA) with a combination of three primer pairs and two DNA templates in combinations of Ostrea edulis, Crassostrea gigas, and Mytilus edulis. The NALFIA for the negative control, O. edulis / C. gigas and O. edulis / M. edulis DNA templates, respectively, shows the expected results and are supported by the gel electrophoresis with the expected bands of the negative control on lane 3, lane 4, O. edulis / C. gigas DNA on lane 6, lane 7 and O. edulis / M. edulis DNA on lane 9, lane 10. Nevertheless, the NALFIA for C. gigas / M. edulis shows a false-positive result for O. edulis, but with no visible band for O. edulis on the gel electrophoresis expected at 191 bp.