Controlling populations of invasive pygmy mussel (Xenostrobus securis) through citizen science and environmental DNA

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ABSTRACT

Early detection of dangerous exotic species is crucial for stopping marine invasions. The New Zealand pygmy mussel *Xenostrobus securis* is a problematic species in coasts of temperate regions in the northern hemisphere. In this study we have controlled a population of this invader that recently expanded in a north Iberian estuary with both a participatory approach involving researchers and citizens, and employing a sensitive eDNA-based tool to monitor the population expansion in the estuary. Results demonstrate successful eradication of pygmy mussels in the outer part of the estuary with citizen science and the practical utility of eDNA for controlling biological invasions.

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1. Introduction

As a generalized problem of ecosystems worldwide, biological invasions require a complex management that involves multiple actors. Researchers, environmental managers and ordinary citizens should work together for successful control of biological pollutants, which only occurs when it is supported both from the relevant agencies and the citizens (see examples in Mack et al., 2000; Hershdorfer et al., 2007, Larson et al., 2011). Enormous workforce is needed for surveying natural and artificial spaces to detect new arrivals of exotic species, as well as for eradicating already established populations. Citizen scientists and volunteers are involved in research and control of biological pests worldwide (e.g. Simberloff, 2003; Crall et al., 2010; Ingwell and Preisser, 2011; Larson et al., 2011).

However direct observational approaches are a problem when the exotic species are elusive, can be confounded with native biota (cryptic species), or are just too small for easy observation. DNA helps to distinguish exotics from the rest of biota (e.g. Geller et al., 2010; Pejovic et al., 2016). New technologies based on environmental DNA (eDNA) may be a solution for surveillance of biological pests. DNA can be extracted from water, soil and sediments, and the species present in the habitat can be recognized from phylogenetically informative DNA sequences. Aquatic invaders can be detected from water using PCR-based species-specific markers; for example molluscs (e.g. Ardura et al., 2015, 2016) and others species. Full implementation of eDNA methodology in monitoring programs is in progress (see a review in Goldberg et al., 2015), and has already been used with the collaboration of citizen scientists for surveying the endangered great crested newt in the UK (Biggs et al., 2015). In this study we explore its efficiency in a case of biological invasions.

The black pygmy mussel *Xenostrobus securis* is a recognized invader native to New Zealand that threatens coastal and estuarine biota in different regions of the world, from Asian (Kimura et al., 1999; Morton and Leung, 2015) to Mediterranean (e.g. Gofas and Zenetos, 2003) to north Atlantic coasts (e.g. Pascual et al., 2010; Adarraga and Martínez, 2012, Devloo-Delva et al., 2016). The newest recording outside its normal geographic distribution in New Zealand occurred in one marina port inside an estuary of southwest Bay of Biscay in Spain in 2014 (Pejovic et al., 2016), at low frequency. In 2015, the species had already occupied many new areas of the estuary (Devloo-Delva et al., 2016). Its early detection and rapid eradication are therefore essential. However, it grows on rocks and artificial structures together with other native mussels, which makes it relatively difficult to detect new settlement spots when the density is still low. Environmental DNA can be examined for detection of this pest since a species-specific marker has been recently developed for this purpose (Devloo-Delva et al., 2016).

Rapid eradication and surveillance of possible remnants or propagules is essential for controlling invasions. In the particular case of *X. securis* manual removal of the adult individuals and the sessile organisms that may harbor small *X. securis* juveniles seems to be the only environmental-friendly eradication method available today (Iwasaki and Yamamoto, 2014). Here, we have explored the efficiency of manual removal relying on citizen collaboration (multi-aged volunteers). We employed the eDNA marker recently developed (Devloo-Delva et al., 2016), coupled with conventional sampling, to check the real disappearance of the species in the trial sites. The case study was Ria de Aviles, the estuary recently colonized by the species. To our knowledge it is the first time that citizen environmental activities are evaluated using this novel molecular approach.

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2. Material & methods

2.1. The case study

Aviles estuary is represented in a map in Fig. 1, with the open sea in the southwest (P13) and the mouth of River Avilés in the east of this map (P7). Thirteen points within the estuary were surveyed for *Xenostrobus securis* three times: in May (before reproduction peak), in October (just after reproduction peak), and December 2015 (after eradication from five points). A qualitative visual survey was done, according to the Rapid Assessment Survey (RAS) protocol (Pederson et al., 2005; Minchin, 2006). This design uses haphazard search patterns. It is only restricted by the accessibility of sites. To equalise the sampling effort each site was sampled for one hour. In these 13 points there is a mussel bed growing on rocky substrate. The community is composed by native *Mytilus* species and, since 2014 (Pejovic et al., 2016) different proportions of *X. securis*.

One eradication trial was carried out by volunteers and researchers on 28th November 2015, a date of a maximum low tide level (− 1.9 m; the maximum registered in the Aviles is − 2.3 m) to be sure all intertidal individuals were accessible. The area of eradication trial is marked in the map (point P4 in Fig. 1). The point was selected because it was the most advanced area in the outer part of the estuary, thus it represented the invasion front to be stopped. Removal started from the water level upwards at 11:30 a.m. and endured 2:30 h. The area that had been examined the day before by the researchers to determine the extension occupied by the pygmy mussel was distributed among the volunteers. All visible individuals were manually removed and counted before disposal in plastic containers.

The trial site P4 was surveyed by the researchers the day after the citizen eradication. No *X. securis* were found. Then the researchers removed manually all the visible *Xenostrobus* individuals from the sites P2, P3, P5 and P11. On 31 December 2015 a new survey of mussel individuals was carried out and water samples taken from these sites.

Four sites were chosen for more detailed analysis and performing eDNA study: two in the inner part of the estuary near the river mouth where eradication was not done (P7 and P8, Fig. 1), and two in the outer part where eradication was carried out (P2 and P4). Environmental data were monitored from the points analysed monthly over the studied period, with three independent measurements each time. The data were acquired using the Horiba multiparameter water quality checker (model 'U-52'). Abiotic factors were substrate, water temperature (TEMP), salinity (SAL), pH, dissolved oxygen (DO), oxygen reduction potential (ORP) and turbidity (TURB). These were all obtained at low tide. Sampling was carried out in these points as indicated in Devloo-Delva et al. (2016). Briefly, it is a modified version of the Bernice P. Bishop Museum (BPBM) protocol (Bishop and Hutchings, 2011), with three replicates of 100 cm^2 quadrat. *X. securis* relative abundance was estimated from the proportion of *X. securis* individuals over the total number of mussels. Water samples (three aliquots of 1 l separated 20 m to each other along the area of each sampling site) were taken four times: May, October, December in 2015 and April in 2016, and immediately frozen until DNA extraction.

2.2. Ethics statement

The presence of *X. securis* in the zone was declared to the Spanish Ministry of Agriculture, Fisheries and Foods that has the competences on invasive species, using the official channel specified for this purpose in Spanish regulations. The Regional authorities (General Directorate of Natural Resources, Service of Protected Spaces and Biodiversity, and Section of Analysis and Conservation of Biodiversity) were also informed.

Permit for sampling this species inside the port were allowed by Aviles port authority. Outside the port the permits were allowed by the competent regional authority (Consejeria de Agroganadería y Re-
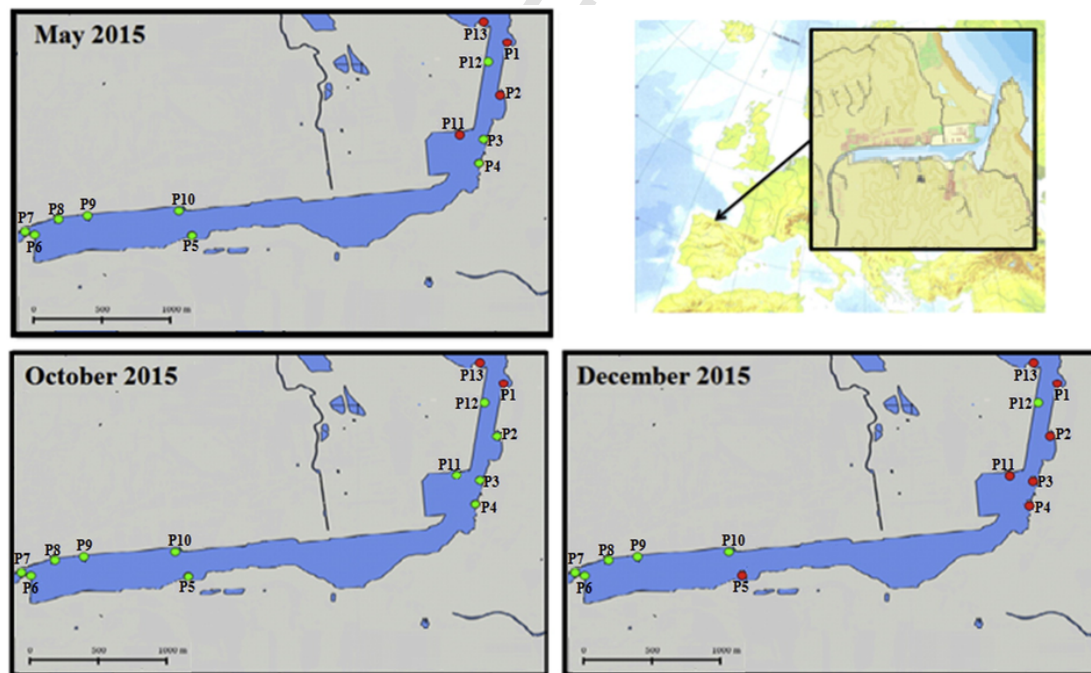

Fig. 1. Maps showing the evolution of pygmy mussel population in Aviles estuary (north Iberia). Presence or absence was monitored by visual identification of individuals.

cursos Autóctonos del Gobierno del Principado de Asturias, date of permit: 13th January to 31st December 2015). The land accessed is not privately owned nor protected. Protected species and the rest of native species were not sampled, disturbed or damaged in any case. This study adheres to the European Code of Conduct for Research Integrity.

Since *X. securis* is an invasive species, disposal of the individuals removed from the coast was arranged with the regional service in charge of destroying biological waste (Consortium of Management of Solid Residues of Asturias, COGERSA; http://www.cogersa.es/metaspace/portal/14498/18718). The biological waste and the materials and overalls employed for manual removal were carried to the next Clean Point and disposed there together with the plastic containers.

The adult volunteers signed an informed consent for participating in the activity following the instructions given by the researchers, and committing to not to conserve or transport out of the sampling area any living *X. securis*. The children that participated were always accompanied by their parent/s, who signed the due participation permit. Safety measures were explained before starting the eradication trial. First-aid kits and cell phones with connectivity for emergency calls were available for each participant.

2.3. Recruitment of citizen scientists

In 2015 direct contact was established separately with different groups: children aged 4–16 enrolled in a surf school and accompanied by their parents; adults enrolled in lifelong education programs of the University of Oviedo (*Evolution Club*). To each group we explained the problem of marine biological invasions and the particular risks derived from the recently detected presence of *X. securis* in Aviles estuary. Children were encouraged to further share the problem with their parents. Contact data (name, telephone and email) of adult participants, or parents of children participants, interested in the control of *X. securis* population of Aviles estuary were taken. The potential volunteers were convoked to a 2-hour training session on species' recognition and differentiation from native mussels, using graphic material and real mussel shells. Then, the appointment for mussel eradication was set.

2.4. Species-specific primers and their sensitivity in water samples

The primers designed by Devloo-Delva et al. (2016) were chosen as species-specific marker. They amplify a 310-nucleotide fragment within the COI (cytochrome oxidase subunit I) gene of *Xenostrobus securis*. The sequences are:

XSminiCOI-F: 5′-TCTATGGAYATRATYT TTCCTCG-3′.
XSminiCOI-R: 5′-GCAGTYACAGYYATAGACCA-3′.

Marker sensitivity was determined in vitro from serial dilutions (1:1, 1:5, 1:10, 1:15, 1:25, 1:50, 1:100, 1:150, 1:300, 1:400, 1:500, 1:1000) of 352 ng/ml DNA obtained from *X. securis* muscle and dissolved in bidistilled water.

DNA was extracted from water samples as explained in Devloo-Delva et al. (2016).

2.5. PCR methodology

PCR amplification was conducted in a Veriti Blue Thermal Cycler, following Devloo-Delva et al. (2016) in a final volume of 20 µl. The PCR mix was 1 × Taq buffer, 1.5 mM MgCl2, 0.25 mM dNTPs, 1 µM of each primer, 200 ng/µl Bovine Serum Albumin (BSA), 0.0325 U/µl Taq polymerase (Promega) and 8 µl of target DNA. PCR conditions were: initial step at 95 °C for 5′; then 45 cycles of 30 s at 95 °C, annealing at 62 °C for 30 s, extension at 72 °C for 30 s; final extension at 72 °C for 7 min; final step of 20 °C for 1 min. PCR products were visualized on a 2% agarose gel. DNA concentration as a proxy of amplicon quantity was estimated on gel by comparison with Low DNA Mass Ladder Invitrogen (Thermo Fisher Scientific).

2.6. Statistical analysis

The clustering of the sampling sites from their environmental characteristics was examined. Using PRIMER 6.1.16 (Clarke and Gorley, 2006) a principal component analysis (PCA) was performed to analyse the characteristics of each site. Next, the differences of important variables were plotted over the sites with a non-metric multidimensional scaling. Afterwards, it was possible to construct a regression model to explain the abundance of *X. securis* with a General Regression Model (GRM). These analyses were done with PAST v3.0 (Hammer et al., 2001).

Correlation between pygmy mussel density in a site and *Xenostrobus*-specific PCR amplicon quantity estimated in gel obtained from that site was checked using Pearson's r tests. The significance threshold was $p < 0.05$.

3. Results

3.1. Advance of Xenostrobus securis invasion in Aviles estuary

The field surveys carried out in different months revealed the rapid advance of the pygmy mussel population front (Fig. 1). In May the species was found in 9 of the 13 points considered. In October two more points, P11 and P2, contained visible *X. securis* individuals. Moreover, the relative abundance of *X. securis* within the mussel bed increased in all the points analysed in depth (Table 1, columns at

Table 1
Xenostrobus securis abundance (P, proportion of *X. securis* over the total number of individuals in the mussel bed; in italics, proxy values estimated from less intensive sampling) observed in four sampling points within Aviles estuary, and proxy of amplicon quantity (in nanograms) of the specific DNA marker from water samples (eDNA), as estimated from Mass Ladder in agarose gel. Salinity, average salinity over the studied period (max and min in parenthesis).

Point	Salinity ppm (min–max)	Sampling time							
		May 2015		October 2015		December 2015		April 2016	
		P (%)	eDNA	P	eDNA	P	eDNA	P	eDNA
2	27.85 (26.0–29.8)	–	–	16.50	10	–	–	–	–
4	23.22 (21.9–24.5)	35.29	20	39.71	20	–	–	11.97	30
7	12.42 (4.6–18.6)	67.69	40	98.54	40	99.2	30	99.82	50
8	19.33 (11.1–24.2)	91.80	40	95.15	40	97	20	98.98	40

left), especially in P7 (innermost point of the estuary) where this species represented almost the totality of the mussels.

From the abiotic parameters measured (Supplementary Table 1), the sites with abundant *X. securis* were grouped together in a quadrant (Fig. 2). No significant relationship between salinity and the relative abundance of *X. securis* was found, although it is evident that the latter was higher in the inner part of the estuary where salinity is lower. Notwithstanding it, the species expansion occurred towards the outer part of the estuary where salinity is clearly higher.

3.2. Result of the pilot trial of Xenostrobus securis eradication

In total 20 volunteers participated in the eradication trial carried out on November 28, 2015: 17 adults (41% females, 59% males) and 3 children under 18 (33.3% females, 66.7% males). A total number of 774 *X. securis* were removed. Individual inspection made by the researchers revealed that no one was mistaken for *Mytilus galloprovincialis*. The eradication area was surveyed the next day by the researchers, in order to confirm the species was really eradicated. No one *X. securis* was found in the area.

Following this successful experience with citizen scientists, researchers removed all the visible *Xenostrobus* individuals from the sites P2, P3, P5 and P11. On December 31, 2015, the estuary was surveyed again. No one *X. securis* was found in these four sites, neither in site P4 which was not recolonized in one month (Fig. 1, bottom). The effect of eradication is shown in Fig. 3: after cleaning, the relative abundance of this species decreased in all the sites.

3.3. Results of eDNA and Xenostrobus securis relative abundance

Assayed in serial DNA dilutions, clear PCR amplification bands were observed in the agarose gel for dilution down to 1:500. For the latter dilution of 1:500 a weak but visible band was observed (Fig. 4). The marker sensitivity limit, visualizing the amplicon in agarose gel, was therefore between 0.352 and 0.704 ng/ml.

The four field sites examined for *X. securis* relative abundance and eDNA provided data consistent with rapid surveys. In May 2015 the species was dominant in P7 and P8, especially in the latter point, and represented near 35% mussels in P4 (Table 1). In October 2015 the species had increased in relative abundance in both P7 and P8, being > 95% of mussels, as well as in P4 up to 40%. Moreover it ap-

peared in P2 where it represented > 16% mussels in the mussel bed. After cleaning the sites P2 and P4 the detailed site inspection confirmed the previous rapid survey, since no *X. securis* were found in December 2015 (Table 1), while in P7 and P8 it was the only species present. The results obtained in April 2016 revealed that the species had reached again the site P4, although in low density. The site P2 was still clean (Table 1).

From water samples, positive PCR amplification was obtained for all sites where the species was visually detected, and nor for the sites where it was not found (Table 1). This was consistent in all the cases including the samples obtained after *X. securis* eradication. In December 2015 the eDNA extracted from water samples taken from P2 and P4, the cleaned sites, did not provide positive PCR amplification with the *Xenostrobus*-specific marker (Table 1). In contrast clear amplicons were obtained from water sampled in the sites not cleaned, P7 and P8 (Fig. 5II, above). In April 2016 a weak band was obtained in site P4 with the species-specific marker (Fig. 5III below). The absence of *Xenostrobus*-specific amplicon band from a water sample was not due to PCR failure (due to presence of inhibitors or any other reason) because the universal primers provided clear positive bands of the expected amplicon size in all cases (Fig. 5I and II). The evolution of the *X. securis* population in the four sites is clearly seen from eDNA in Fig. 5III, with positive bands of *X. securis* in the infested P7 and P8 sites in all samplings; in P2 only in October 2015; in P4 in October 2015 and in April 2016 but not in December 2015.

The correlation between the *X. securis* abundance and the proxy of amplicon quantity was positive and statistically significant (r = 0.661, 10 d.f., P = 0.019). However, in P7 and P8 the proxy of the amplicon quantity was lower in December than in October water samples (30 and 20 in P7 and P8 respectively in December versus 40 in October), while the relative abundance of *X. securis* was higher in December than in October.

4. Discussion

This study provides different results useful for application in the management of biological invasions. One is the utility of the work of volunteers for extirpation of invasive species. Confirming *de visu* observations, negative results obtained from eDNA indicate that, if any, the abundance of *X. securis* is almost negligible. Since the DNA marker employed in this study can detect eDNA in concentrations as
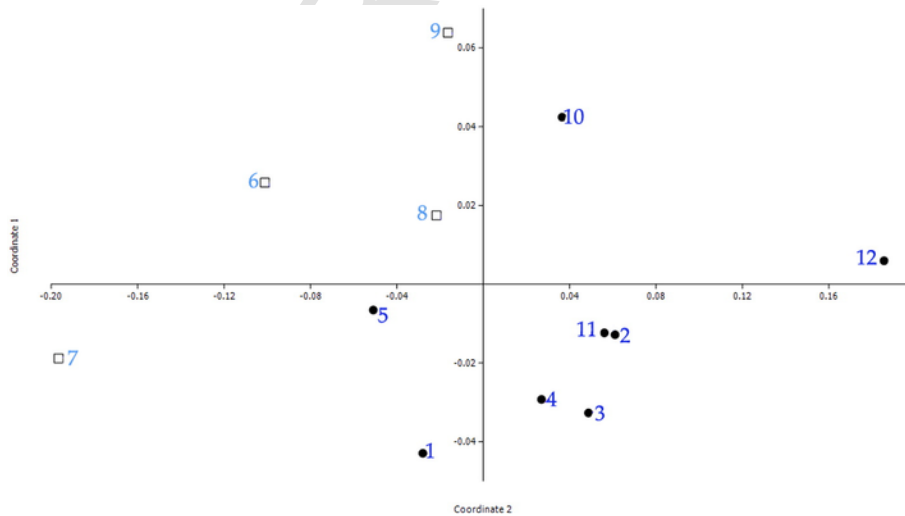

Fig. 2. Graph showing 2D Non-Metric Multidimensional Scaling of the sampling points. Squares and circles represent sites with > 50% and < 50% *X. securis*, respectively.
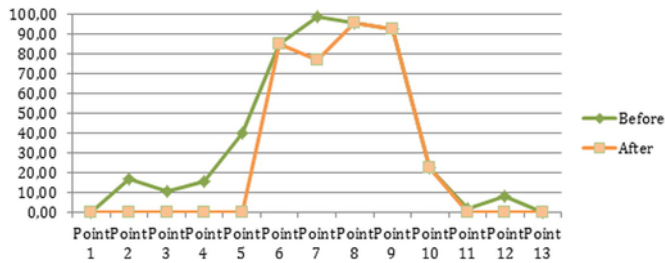
Fig. 3. Proportion of *X. securis* before (October 2015) and after (December 2015) the pilot eradication trial in the 13 sampling sites considered within Avilés estuary.
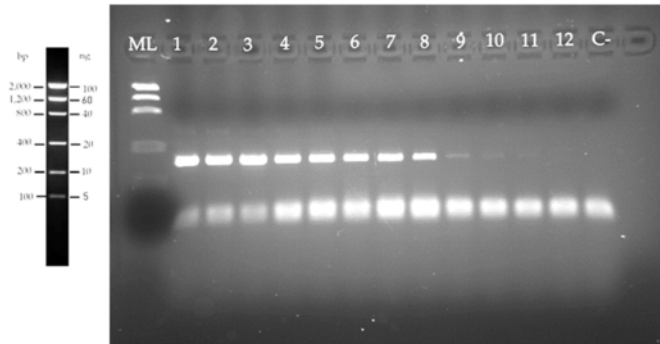

Fig. 4. Marker sensitivity: Agarose gel showing the marker detection limit from serial dilutions (from 1 to 12, 1:1, 1:5, 1:10, 1:15, 1:25, 1:50, 1:100, 1:150, 1:300, 1:400, 1:500, 1:1000; C −, negative control) of 352 ng/ml DNA obtained from muscle of *X. securis.*

low as 0.35 ng/ml it seems that the species did no longer occur there. This is good news for biodiversity conservation. If manual removal is sufficient and effective for eradicating introduced populations, as suggested from the results of this pilot study, the species can be controlled. Moreover, organized volunteers may be in charge of -or at least contributing to- that control. Public outreach of biotic invaders should be enhanced to draw the attention of general public to this generalized problem (e.g. Kaiser, 1999; Mack et al., 2000; Lodge et al., 2006), in order to promote informed, stable and committed participation of volunteers in control programs.

The results also suggest that coupling eDNA analysis and visual surveys is a good strategy for monitoring *X. securis*. Moreover, since the results obtained in this study did not reveal any discrepancy between the two methods, eDNA could replace visual observation for *in situ* surveys. Several advantages of eDNA approach are its sensitivity (e.g. Goldberg et al., 2015; Ardura et al., 2015, 2016), and the fact that it does not depend on weather conditions and personal effort as

visual surveys do. Only the time for sampling water is required (plus further laboratory work, but many samples can be analysed at the same time in the laboratory), while at least one hour is needed for exhaustive inspection of all mussels present in each quadrant. With three quadrants per site it means three-hour work for visual survey of each site. The advantage of eDNA, and the possibility of water samples to be obtained by volunteers and citizen scientists, as in the case of the great crested newt surveys (Biggs et al., 2015), make this approach attractive for controlling the evolution of *X. securis* population in Asturias.

Another result that may be important is the capacity of fast expansion of the pygmy mussel that is colonizing areas exposed to open sea for the first time in this region. Since now, in the Atlantic Iberian façade (including the Bay of Biscay) it was restricted to inner estuary areas of low salinity (Garci et al., 2007; Pascual et al., 2010; Adarraga and Martínez, 2012; Gestoso et al., 2012; Devloo-Delva et al., 2016). The same happens in Mediterranean lagoons (e.g. Zenetos et al., 2005; Barbieri et al., 2011) and Asian waters (e.g. Iwasaki and Yamamoto, 2014). However the population established in Aviles estuary seems to exhibit the capacity of expanding beyond the estuary reaching areas of high salinity. It is not strange because the species exhibits a wide range of salinity tolerance (e.g. Wilson, 1968, Kimura et al., 1995; Iwasaki and Yamamoto, 2014). This is a call of attention that emphasizes the need of early detection of this species before it is settled and the new population starts expanding. Management recommendations for this population would include periodical manual eradication. The appearance of new adults in P4 in April sample, five months after eradication, suggests that the periodicity of surveys should be at least every four months, and seasonal eradications should also be considered. The help of volunteers and organized citizens can be crucial in this and other cases.

As a final remark, the eDNA marker here employed is robust, sensitive and, since it can be checked on agarose gels, cheap enough to be applied in routine monitoring of this species. There is no need of disturbing local biota because it can be amplified from water samples. This is an advantage for accomplishing the European Code of Conduct for Research Integrity, regarding the guideline 2.3.3 in the sense that it is an alternative way to the use of animals in research. Although eDNA is still to be implemented in practical biological monitoring (Goldberg et al., 2015), the results obtained in this pilot study suggest that the technique is already mature for application in field surveys, at least for presence/absence of target species.

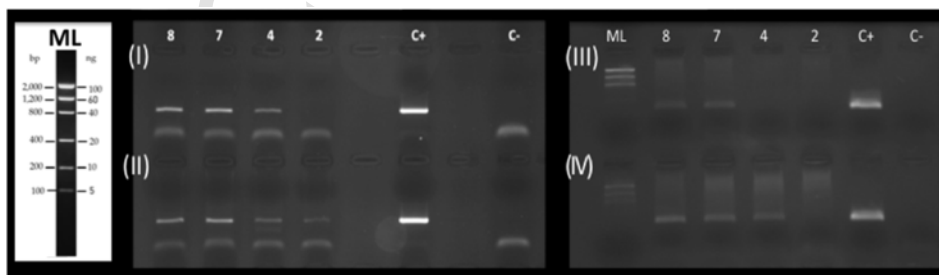## Uncited reference

Geller et al., 2013

Fig. 5. Agarose gel showing the evolution of pygmy mussel employing the PCR products of eDNA in the four selected points: two unmanaged points (7, 8) and two sites where *Xenostrobus securis* was eradicated (points 2, 4). Water samples were taken in May (I), October (II), December 2015 (III) and April 2016 (IV). Mass ladder at left, labelled as ML.

Acknowledgements

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