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# Genetic heterogeneity in natural beds of the razor clam *Ensis siliqua* revealed by microsatellites

MIGUEL A. VARELA, ANDRÉS MARTÍNEZ-LAGE AND ANA M. GONZÁLEZ-TIZÓN

Departamento de Bioloxía Celular e Molecular, Universidade da Coruña, A Zapateira s/n, A Coruña E-15071, Spain

*The aim of this study was to analyse the genetic diversity and population structure in the razor clam* Ensis siliqua *along the European Atlantic coast taking into account their recent history of exploitation and the 'Prestige' oil spill. To this end we examined the genetic variability of microsatellite markers in 211 razor clams from five populations in Ireland, Portugal and Spain. Microsatellite data revealed a low genetic differentiation between the Spanish and Portuguese populations ($F_{ST} = 0–0.032$) and a moderate differentiation of these populations and the Irish samples ($F_{ST} = 0.071–0.100$). Although we observed changes in genetic diversity in accordance with the level of exploitation and the distribution of the oil spill, these changes were mild and not significant after Bonferroni correction. This could be the result of a genuine low impact, lack of statistical power and/or the capacity of this species to recolonize quickly after the impact of anthropogenic stressors. Supporting the latter argument we found a significant temporal heterogeneity of allelic frequencies in samples coming from the same sampling locality that could be attributed to the movement of adults or larvae from unaffected source populations.*

**Keywords:** conservation, *Ensis siliqua*, genetic diversity, microsatellites, 'Prestige', oil spill, razor clams

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## INTRODUCTION

The razor clam *Ensis siliqua* (Linnaeus, 1758) is an infaunal marine bivalve that burrows vertically into sandy and muddy bottoms in low intertidal and subtidal zones on the European Atlantic coast from Norway to Spain, in the Mediterranean and in the north-west of Africa. Razor clams feed filtering water and, therefore, they are sensitive to the presence of toxic substances in the water column that they assimilate and accumulate in their tissues (Pearce & Mann, 2006). *Ensis siliqua* is one of the razor clams more widely exploited in Europe where it reaches important values in the market and it has been overexploited in some regions of the continent (Gaspar *et al.*, 1999; Tuck *et al.*, 2000; Fahy & Gaffney, 2001). The sustainability of this commercial exploitation and the survival of this species in many zones of its present distribution could be now in danger because of human activities that can lead to environmental pollution and overexploitation.

Marine pollution is one of the main anthropogenic effects that can occur in a marine environment and causes habitat and ecosystem degradation. When contaminants are spilled into marine ecosystems, the ecological consequences can be extreme. In November 2002, the 'Prestige' oil tanker ran aground off 120 miles from the Galician coasts (north-west Spain). It spilled between 40,000 and 63,000 tonnes of heavy fuel oil into the Atlantic Ocean, which caused pollution mainly on the north-western coast of Spain, and damaged rocky shores, marine bottoms, and a great diversity of marine fauna and flora (Marcos *et al.*, 2004; de la Huz *et al.*, 2005; Peteiro *et al.*, 2006; Sánchez *et al.*, 2006; Díez *et al.*, 2007; Piñeira *et al.*, 2008; Varela *et al.*, 2009; Viñas *et al.*, 2009).

The 'Prestige' oil spill affected a great extension of shores and large quantities of emulsified oil and a thick deposit of fuel arrived in several tides at the marine bottoms that razor clams inhabit for the months following the accident affecting *Ensis* spp. populations. Thus, Viñas *et al.* (2009) showed that the concentration of polycyclic aromatic hydrocarbons (PAHs) increased significantly in *Ensis arcuatus* and *Ensis siliqua* after the 'Prestige' oil spill and decreased 6–7 months later close to background levels for the region. Pollution increased again in the following winter, probably as a result of storms that reintroduced more oil into the water column (Viñas *et al.*, 2009). Previous studies on the effects of this oil spill on other molluscs showed a lower growth (Peteiro *et al.*, 2006) in oil-exposed mussels, and high levels of mortality in marine snails (Piñeira *et al.*, 2008). On the other hand, high levels of mortality of *Ensis* have been reported after the wreckage of the 'Amoco Cadiz' in Brittany (Southward, 1978), 'Torrey Canyon' (Smith, 1968) and 'Braer' (Glegg & Rowland, 1996) in Great Britain. Although after a period of time populations may recover from the toxic effects of the oil spill, an acute decrease in the effective population size could result in a loss of genetic diversity that can compromise the future of the population.

Overexploitation of marine resources can also generate an important environmental impact on the ecosystems. The traditional fishing methods of harvesting these bivalves involve the small-scale collection of animals on foot by hand or using rakes or small dredges. In some European regions this

**Corresponding author:**
A.M. González-Tizón
Email: hakuna@udc.es

traditional fishing has been replaced by hydraulic dredging. This is a more destructive method that causes disturbance to the substrate that leaves the remained animals more vulnerable to predators (Gaspar *et al.*, 1999, 2003; Tuck *et al.*, 2000; Alves *et al.*, 2003; Falcao *et al.*, 2003; Fahy & Carroll, 2007). Although it has been shown that most of fauna dislodged by the dredge retained the ability to rebury successfully (Hauton *et al.*, 2003), the fact that modern hydraulic dredges are extremely efficient and have the potential to remove the majority of a clam population (Hauton *et al.*, 2007) could easily lead to overexploitation in non-monitored fisheries.

Monitoring the effects of marine pollution or different fishing techniques on the genetic diversity of populations of *Ensis siliqua* is essential to ensure a sustainable exploitation of this fishery. The anthropogenic effects on genetic diversity can be assessed by genetic markers such as microsatellite markers. Microsatellites are routinely used to investigate the genetic structure and provide values of heterozygosity and allelic richness of natural populations (Balloux & Lugon-Moulin, 2002; England *et al.*, 2003). Microsatellite markers have already been developed in *Ensis siliqua* (Varela *et al.*, 2007a) and other species of razor clams such as *Ensis arcuatus* (Varela *et al.*, 2009), *Solen marginatus* (Francisco-Candeira *et al.*, 2007) and *Sinonovacula constricta* (Niu *et al.*, 2008) but, to date, microsatellites have not been used to study populations of *Ensis siliqua*.

The aim of this work was to analyse the genetic differentiation of *Ensis siliqua* on the European Atlantic coast and study the possible impact of human activities on the genetic variability of razor clam populations. To this end we examined the genetic variability of microsatellite markers in samples captured before and after the 'Prestige' oil spill and in regions where razor clams are exploited under different fishing techniques.

## MATERIALS AND METHODS

### Sampling and DNA extraction

Specimens of *Ensis siliqua* were obtained from subtidal areas on the European Atlantic coast. All samples were adult razor clams ranging in size from 10 to 15 cm. The geographical situation of each sampling locality is indicated in Figure 1. In total we sampled 211 razor clams from five natural beds (Table 1). Strangford Lough (east coast of Ireland) is a large shallow inlet with high biodiversity. It is an area where small-scale collection of bivalves is made on foot by hand or using rakes. The Spanish populations were the only ones affected by the 'Prestige' oil spill. In the Portuguese populations hydraulic dredging is allowed for razor clam harvesting.

DNA extraction was carried out as described by Winnepenninckx *et al.* (1993) from 25 mg of adductor muscles and mantle tissues. Samples stored in ethanol were rehydrated in PBS (0.137 M NaCl, 2.68 mM KCl, 10.1 mM Na2 HPO4, 1.76 mM KH2PO4) and distilled water before DNA extraction.

### Microsatellite characterization and data analysis

Five microsatellite loci were isolated from the sequences of intersimple sequence repeat (ISSR) markers (Fisher *et al.*, 1996; Varela *et al.*, 2007b) as described in Varela *et al.* (2007a). Microsatellite fragments were amplified under the following conditions: 94°C (2 minutes), followed by 30 cycles at 92°C (1 minute), annealing temperature at 60°C (1 minute), and 72°C (30 seconds) with a final extension step at 72°C (10 minutes). Polymerase chain reactions were carried out in a total volume of 25 µl containing 0.2 µM each of forward and reverse primers, 0.2 mM dNTPs, 2–2.4 mM $MgCl_2$, 0.75 U Roche Taq DNA polymerase, and 20 ng DNA template (Table 2). An Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies) was used to carry out an initial screening and five loci were scorable and polymorphic. Reverse primers were 5′-labelled with fluorescent dyes (WellRED oligos, Proligo) and then amplification products were electrophoresed in a CEQ 8000 Genetic Analysis System apparatus (Beckman Coulter).

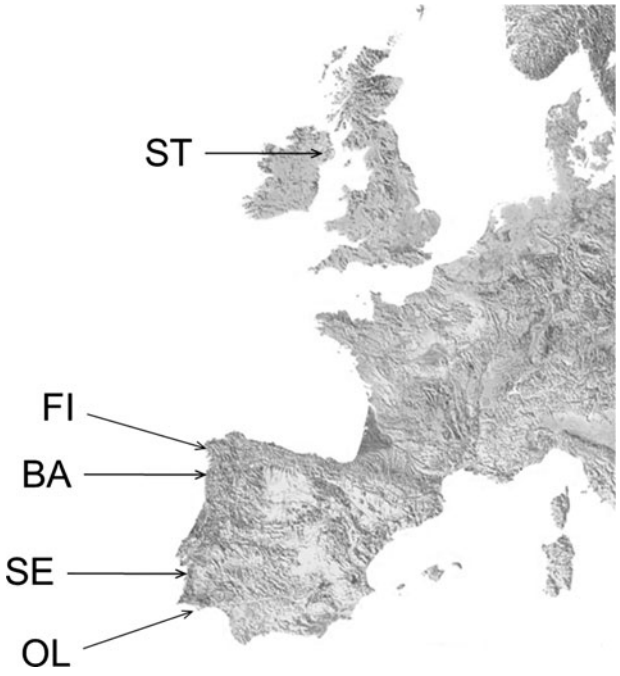


**Fig. 1.** Sampling locations of *Ensis siliqua* along the European Atlantic coast, Barra (BA), Finisterre (FI), Olhao (OL), Setubal (SE) and Strangford Lough (ST).

We used Genepop version 3.3 (Raymond & Rousset, 1995) to calculate observed and expected heterozygosities and Fisher's exact tests of allelic differentiation. The allelic variation of these markers was compared among sampling localities using *FSTAT* Version 2.9.3 (Goudet, 2001). Estimates of the allelic richness (AR) were corrected by rarefaction for differences in sample size. This method uses the frequency distribution of alleles at a locus to estimate the number of alleles that would occur in smaller samples (Leberg, 2002). Subsequently, we carried out a factorial correspondence analysis (FCA) and calculated estimators of $F_{ST}$ (Weir & Cockerham, 1984) using *Genetix* (Belkhir *et al.*, 2003). Lastly, changes in genetic diversity were tested by Friedman and Wilcoxon tests using the program SPSS version 15 (SPSS Inc, Chicago, Ill).

## RESULTS

We began by studying the changes in genetic diversity for different sampling locations (Table 3). We detected

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**Table 1.** Sampling locations of the razor clam *Ensis siliqua* populations included in the study. Names of populations make reference to the date of capture.

| Locality | Sample | Date | N | Country | Latitude | Longitude |
|---|---|---|---|---|---|---|
| Finisterre | Finisterre 00 | 17-03-2000 | 34 | Spain | 42°55′N | 9°14′W |
| | Finisterre 03 | 03-09-2003 | 27 | | | |
| | Finisterre 05 | 01-02-2005 | 21 | | | |
| Barra | Barra 02 | 20-05-2002 | 27 | Spain | 42°15′N | 8°46′W |
| | Barra 04 | 04-10-2004 | 26 | | | |
| Olhao | Olhao 04 | 29-04-2004 | 24 | Portugal | 37°01′N | 7°50′W |
| Setubal | Setubal 04 | 28-04-2004 | 16 | Portugal | 38°01′N | 8°53′W |
| Strangford Lough | Strangford 04 | 25-05-2004 | 36 | Ireland | 54°29′N | 5°36′W |

differences in genetic diversity between the populations of Spain, Ireland and Portugal. The Irish population, which is not commercially exploited, had the highest levels of genetic diversity both in terms of allelic richness (AR = 7.499) and heterozygosity ($H_O = 0.783$). The lowest levels of genetic diversity were observed in the two populations from Portugal, non-affected by the 'Prestige' oil spill but where the use of hydraulic dredges is allowed, which can lead to overexploitation (Gaspar *et al.*, 1994, 1999; Tuck *et al.*, 2000; Fahy & Gaffney, 2001; Chícharo *et al.*, 2002). In particular, samples from Setubal 04 showed the lowest allelic richness (AR = 6.046) and Olhao 04 the lowest heterozygosity ($H_O = 0.594$). On the other hand, the Spanish populations were of intermediate genetic diversity value. These differences in genetic diversity were not significant when heterozygosity was compared between populations by Wilcoxon tests whereas differences in allelic richness were significant ($P < 0.05$) between Setubal 04-Finisterre 00 and Barra 04-Strangford 04. Nevertheless, after adjusting to multiple testing using the Bonferroni correction, differences in allele richness did not remain significant ($P > 0.0018$), neither when comparing all samples by a Friedman test ($P > 0.05$).

Afterwards we studied the temporal variation of genetic diversity in two Spanish populations affected by the 'Prestige' oil spill, Barra and Finisterre. The results showed a tendency of loss of heterozygosity and alleles in both populations (Table 3). Again differences in genetic diversity were not significant when heterozygosity was compared between samples of the same population in Finisterre and Barra. The loss of allelic richness was significant between the samples of Finisterre 00 and Finisterre 05 (Wilcoxon test, $P < 0.05$) but not after Bonferroni correction. Additionally, a significant heterogeneity of the allelic frequencies was detected in samples coming from the same locality using Fisher's exact tests with the program Genepop 3.3 (Raymond & Rousset, 1995) in Finisterre ($P < 0.0001$) and Barra ($P < 0.0001$).

We then studied the genetic differentiation of all samples and examined the population structure performing a FCA (Figure 2). The two-dimensional plot shows two major groups of individuals. One included the Irish population, whereas the other comprised the Portuguese and Spanish samples. Figure 2 also suggests a slight genetic differentiation between the Spanish and Portuguese sampling localities, the latter having a tendency towards higher values of factor 2. Furthermore, Figure 2 shows the genetic differentiation of samples coming from the same locality, with Finisterre 03 having in general lower values of factor 2 than Finisterre 05 and specially Finisterre 00. The estimators of $F_{ST}$ (Wright, 1978; Weir & Cockerham, 1984) between samples indicated a low genetic differentiation between the Spanish and Portuguese populations ($F_{ST} = 0–0.032$) and a moderate differentiation of these populations and the Irish samples ($F_{ST} = 0.071–0.100$) (Table 4), which supports that the Irish population is the most genetically different.

## DISCUSSION

In this work we used microsatellite markers to provide estimates of genetic differentiation and diversity in several razor clam populations of the European Atlantic coast. We did not observe statistically significant changes in the genetic diversity of populations affected by the 'Prestige' oil spill and in beds of *Ensis siliqua* that are commercially

**Table 2.** Characterization of five microsatellite markers in the razor clam *Ensis siliqua*.

| Locus | EMBL no. | Repeat motif (5′–3′) | Primer sequence (5′–3′) | [$MgCl_2$] | $t_a$ |
|---|---|---|---|---|---|
| Es263 | AM182601 | $(TAG)_{29}$ $(GTT)_7$ | F: AATTACTTCTGGAACCTTATTTACGCA<br>R: CTATTTACCCGAACATATACTGCCG | 2.4 mM | 60 |
| Es177 | AM182587 | $(GA)_{10}$ | F: ATTACCTCCAATACTAGGAGAGCCG<br>R: CCGTAACCGTGTTCTTCTCCG | 2 mM | 60 |
| Es136 | AM182580 | $(CT)_3$ GTATGT $(CT)_5$ | F: TGACCAACACTACCACCCCATC<br>R: AGAAGGGTGTGAATGAGAGATAGGG | 2.4 mM | 60 |
| Es129 | AM182574 | $(ATT)_{10}$ | F: TAATGCATACCCGTCTCTGATAAGC<br>R: AATTAGCCTAAATTGTGCAGAAACG | 2.4 mM | 60 |
| Es128 | AM182578 | $(GA)_{40}$ | F: GAAAGAGAGAAGGGAGATAATTGGG<br>R: GTTTTTGTGTATGTGTGTGCGTCTT | 2.4 mM | 60 |

$t_a$, annealing temperature.

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**Table 3.** Summary of genetic variation parameters in different samples of *Ensis siliqua*: allelic richness (AR), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$).

| Locus and parameter | Olhao 04 | Setubal 04 | Barra 02 | Barra 04 | Finisterre 00 | Finisterre 03 | Finisterre 05 | Strangford 04 |
|---|---|---|---|---|---|---|---|---|
| Mean | | | | | | | | |
| AR | 6.389 | 6.046 | 6.375 | 6.087 | 7.120 | 6.592 | 6.318 | 7.499 |
| $H_O$ | 0.594 | 0.752 | 0.696 | 0.675 | 0.712 | 0.663 | 0.661 | 0.783 |
| $H_E$ | 0.738 | 0.702 | 0.740 | 0.699 | 0.746 | 0.733 | 0.705 | 0.789 |
| Es128 | | | | | | | | |
| AR | 2.563 | 2.692 | 3.045 | 3.026 | 3.020 | 3.850 | 2.994 | 4.218 |
| $H_O$ | 0.813 | 0.923 | 0.963 | 0.909 | 0.879 | 0.833 | 0.857 | 0.857 |
| $H_E$ | 0.529 | 0.536 | 0.565 | 0.539 | 0.553 | 0.675 | 0.635 | 0.661 |
| Es129 | | | | | | | | |
| AR | 7.135 | 7.721 | 8.410 | 8.995 | 8.789 | 8.291 | 6.928 | 9.959 |
| $H_O$ | 0.500* | 0.846 | 0.750 | 0.870 | 0.758* | 0.870 | 0.800 | 0.852 |
| $H_E$ | 0.846 | 0.834 | 0.848 | 0.889 | 0.882 | 0.861 | 0.784 | 0.894 |
| Es177 | | | | | | | | |
| AR | 6.066 | 6.151 | 6.648 | 4.867 | 7.251 | 5.512 | 6.674 | 5.763 |
| $H_O$ | 0.867 | 0.929 | 0.765 | 0.737 | 0.939 | 0.750 | 0.846 | 0.808 |
| $H_E$ | 0.766 | 0.781 | 0.739 | 0.612 | 0.811 | 0.744 | 0.728 | 0.677 |
| Es194 | | | | | | | | |
| AR | 4.526 | 3.666 | 3.000 | 4.201 | 4.093 | 3.412 | 3.447 | 5.919 |
| $H_O$ | 0.474* | 0.615 | 0.500 | 0.333 | 0.455 | 0.526 | 0.333 | 0.706 |
| $H_E$ | 0.630 | 0.482 | 0.635 | 0.573 | 0.543 | 0.460 | 0.464 | 0.786 |
| Es263 | | | | | | | | |
| AR | 11.653 | 10.000 | 10.774 | 9.348 | 12.446 | 11.897 | 11.546 | 11.636 |
| $H_O$ | 0.316* | 0.444* | 0.500* | 0.529* | 0.531* | 0.333* | 0.467* | 0.720* |
| $H_E$ | 0.920 | 0.877 | 0.911 | 0.881 | 0.939 | 0.924 | 0.913 | 0.922 |

*, significant deficit of heterozygous after Bonferroni correction ($P < 0.00625$).

exploited. Although this could be the result of a genuine low impact or lack of statistical power, the significant temporal heterogeneity of allelic frequencies in samples coming from the same sampling locality suggests that the movement of adults or larvae from unaffected source populations could enable this species to recolonize quickly after the impact of anthropogenic stressors.

## Genetic differentiation among populations

The study of the population structure of razor clam populations along the European Atlantic coast showed that there is a low genetic differentiation between the Spanish and Portuguese populations. Besides, Strangford Lough is the most genetically different population, which suggests that there is a low gene flow between the Irish population, which

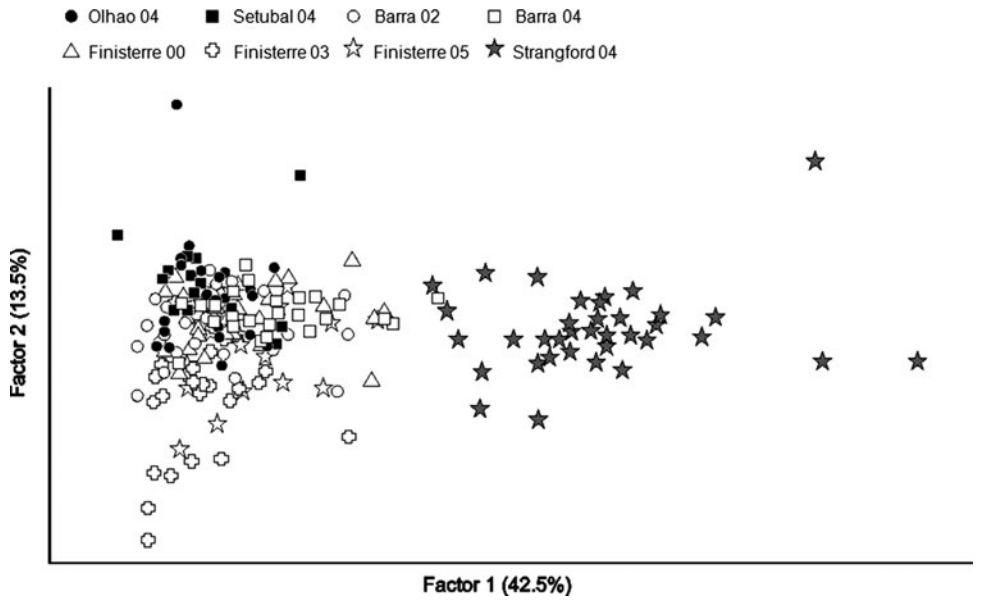


**Fig. 2.** Factorial correspondence analysis of eight samples from five populations of *Ensis siliqua* based on microsatellite allele frequencies of 211 individuals.

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**Table 4.** Pairwise $F_{ST}$ estimators between sampling localities of *Ensis siliqua*.

| | Olhao 04 | Setubal 04 | Barra 02 | Barra 04 | Finisterre 00 | Finisterre 03 | Finisterre 05 |
|---|---|---|---|---|---|---|---|
| **Olhao 04** | | | | | | | |
| **Setubal 04** | −0.009 | | | | | | |
| **Barra 02** | −0.003 | 0.015 | | | | | |
| **Barra 04** | 0.009 | *0.022 | 0.015 | | | | |
| **Finisterre 00** | −0.001 | 0.003 | 0.008 | 0.008 | | | |
| **Finisterre 03** | 0.020 | *0.022 | *0.031 | *0.032 | 0.020 | | |
| **Finisterre 05** | 0.006 | *0.021 | 0.015 | 0.006 | 0.001 | 0.009 | |
| **Strangford 04** | *0.071 | *0.092 | *0.078 | *0.091 | *0.074 | *0.100 | 0.088 |

*, estimators of $F_{ST}$ different from zero with degree of signification of 5% calculated by 1000 permutations.

is also the most distant geographically, and the rest of sampling localities. Furthermore, Strangford Lough is a fjardic inlet of the Irish Sea connected to the Irish Sea by a long and narrow strait that could restrict larvae flow from populations outside the lagoon (Kennedy & Roberts, 2006). These results of genetic differentiation are in accordance with other studies that show lack of clear genetic structure in razor clams and other species with planktonic larvae along the west coast of the Iberian Peninsula (Fernández-Tajes *et al.*, 2007; Silva *et al.*, 2009).

Knowing measures of genetic differentiation and genetic diversity of razor clam populations can be important in the management of this resource to designate protected areas and conserve the integrity of the native gene pool when stocking depleted populations with non-native specimens (Allendorf *et al.*, 1997). For example, to have success in the re-stocking of a population in the north-west of Spain ideally breeders should be individuals from this region rather than from populations genetically distinct such as Strangford Lough where the gene pool could be adapted to different environmental conditions. We believe that this molecular approach will provide a fruitful route by which management decisions could be taken analysing these and other molecular markers in razor clam populations.

## Genetic diversity and anthropogenic stressors

Razor clams are under two main anthropogenic stressors that could affect genetic diversity, namely overexploitation and environmental pollution. The scarcity of samples before a punctual event in time such as an oil spill can limit the power of any analysis trying to find evidence of changes in genetic diversity. Here the availability of samples obtained from two Spanish populations before the 'Prestige' oil spill allowed us to investigate if the spill affected the genetic variability of razor clam populations at least at these two particular places and time frames. Changes in genetic diversity were in accordance with the level of commercial exploitation and the distribution of the oil spill. However, these changes were mild and not significant after Bonferroni correction. These results are in agreement with a previous analysis of *Ensis arcuatus* from another area affected by the oil spill (Varela *et al.*, 2009). The abundance of these razor clams and the movement of adults or larvae from non-affected areas by the oil spill could have avoided the effect of a bottleneck on genetic diversity.

Pollution produced by the 'Prestige' oil spill was very extensive but heterogeneous, alternating intensely affected zones with other less affected or non-affected at all because of local currents or the placement of floating oil barriers. Razor clams can move massively to colonize a new area as indicated by Fahy & Gaffney (2001) and produce a great number of offspring, which in some cases could allow adults or larvae coming from other areas to occupy a substrate in a short period of time. Thus, even if the oil spill caused a high mortality at some points of the coast, population sizes recovered during the months that beds were closed to exploitation after the oil spill and it did not affect production in the long term (Figure 3).

The mortality that the oil spill could have induced in some beds of razor clams could be added to the unpredictability of conditions that are necessary for spawning, fertilization and larval recruitment and provoke temporal stochastic variations in the genetic composition of populations of marine invertebrates as described in clams (David *et al.*, 1997; Vadopalas & Bentzen, 2004), oysters (Li & Hedgecock, 1998) and sea urchins (Moberg & Burton, 2000). These temporal changes have been attributed to the movement of adults or recruitment from different source populations or the high variance in reproductive success of mollusc populations (Hedgecock, 1994; Hedrick, 2005) that could show effective population sizes several orders of magnitude lower than actual population sizes. Therefore, this temporal heterogeneity is a phenomenon that is not always related to specific anthropogenic influences.

In summary, we found genetic differentiation between different razor clam populations on the European Atlantic Coast and temporal changes in the genetic composition of some of these populations but we did not observe an acute loss of genetic diversity related to the different levels of commercial exploitation or pollution by the 'Prestige' oil spill. Our

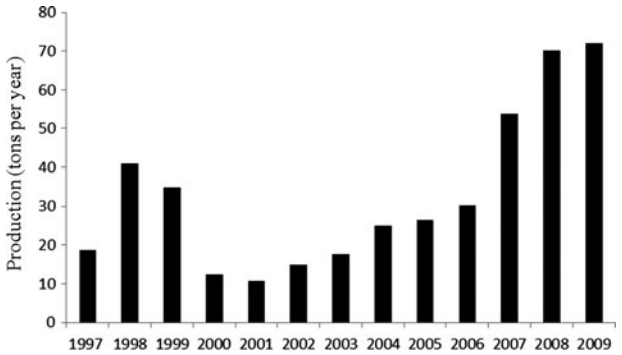


**Fig. 3.** Production of razor clams *Ensis siliqua* (tons per year) in Galicia (north-west Spain) before and after the 'Prestige' oil spill in November 2002.

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results could be explained by the movement of adults or larvae coming from unaffected areas that re-stock depleted populations.

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**Correspondence should be addressed to:**
A.M. González-Tizón
Departamento de Bioloxía Celular e Molecular
Universidade da Coruña
A Zapateira s/n, A Coruña E-15071, Spain
email: hakuna@udc.es

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