Evaluación del impacto genético por vertido de fuel, sobre poblaciones de *Littorina saxatilis*

Una de las formas más drásticas de degradación del medio marino ocurre cuando se producen vertidos accidentales o deliberados de sustancias contaminantes. Dichos vertidos suelen producir la desaparición de una gran parte de los organismos de las áreas afectadas. La estrategia clásica para evaluar el daño ambiental ocasionado es la de vigilar las poblaciones afectadas hasta su aparente recuperación en cuanto a presencia y número de ejemplares. Esta estrategia, sin embargo, no considera un posible daño que pueden sufrir las especies y que no es observable a simple vista: el hecho de que se haya visto afectada su variabilidad genética. La variabilidad genética es el patrimonio evolutivo más importante que posee cualquier ser vivo, ya que de su magnitud depende la capacidad intrínseca de adaptarse a nuevos cambios ambientales, es decir, de evolucionar. De ahí que después de un proceso de contaminación y su correspondiente estudio, nos encontremos ante la posibilidad de que en una especie determinada se hayan recuperado las densidades poblacionales características de la especie, pero ésta presente unos niveles de variabilidad genética deteriorados.

Como consecuencia del hundimiento del buque *Prestige*, y de las consiguientes oleadas de mareas negras que éste produjo, se planteó la necesidad de investigar las áreas más afectadas para evaluar el daño genético ocasionado en poblaciones aparentemente recuperadas. Este daño genético se espera que sea máximo en especies de baja movilidad (que no pueden escapar al contaminante) y de baja capacidad dispersiva (con reducida capacidad para colonizar de nuevo el área afectada) que habitan en la región superior del intermareal de las costas rocosas (las áreas más afectadas por las mareas de petróleo). Para ello, se eligió una especie con estas características, el caracol *Littorina saxatilis*, que además posee típicamente altas densidades poblacionales y una amplia distribución en las costas gallegas. Se eligieron siete áreas que fueron localmente muy afectadas por los vertidos (Cedeira, Laxe, Camelle, Muxía, Aguiño, Ons y Mougás), y en cada una de ellas se estudiaron una localidad impactada y dos controles (típicamente a ambos lados de la impactada). Las inspecciones previas indicaban que la especie había quedado aparentemente extinguida en la localidad afectada y sus alrededores (50-100 m de costa). En cada localidad, además, se recogieron dos muestras separadas por unos 100 metros, y en cada muestra alrededor de 30 ejemplares. Se analizó la variación genética para caracteres

morfológicos de la concha (utilizando métodos de morfometría geométrica e información de los embriones que portan las hembras en su interior) y para marcadores moleculares de ADN (3 microsatélites y 800 AFLPs). El objetivo era investigar si las poblaciones afectadas por el derrame, un año y medio después de haber ocurrido el mismo (hasta 3-4 generaciones en esta especie), presentaban alguna reducción apreciable de su variabilidad genética para estos caracteres.

Los resultados fueron bastante tranquilizadores en cuanto a los efectos de pérdida de variabilidad genética. Para ninguno de los marcadores estudiados (marcadores de ADN o morfológicos) se detectó una reducción significativa de la variabilidad genética en las poblaciones afectadas por la contaminación de petróleo, en relación a sus respectivos controles. Esto sugiere que los organismos que presentan amplias distribuciones y densidades abundantes son muy resistentes a la pérdida de variabilidad genética como consecuencia de procesos de contaminación puntuales, aunque esto podría ser muy diferente ante procesos de contaminación continuada. Esta conclusión puede extenderse a otros organismos con las mismas características que la especie estudiada y, con mayor motivo, a otros de mayor capacidad de dispersión, como el mejillón o el percebe. A pesar de no detectar una reducción de la variabilidad genética, sí se detectaron algunos efectos genéticos, que podrían indicar procesos de adaptación de las poblaciones afectadas por el derrame de petróleo. Similares efectos de adaptación genética ya han sido descritos en otras poblaciones naturales ante la presencia de diferentes tipos de contaminantes. Será interesante comprobar en el futuro si estos procesos de adaptación se mantienen en el tiempo o si desaparecen con el paso de los años. En cualquier caso y, afortunadamente, los resultados sugieren que las especies con amplia distribución en nuestras costas son capaces de recuperar los daños genéticos como consecuencia del derrame en un periodo de tiempo relativamente corto. Obviamente, las conclusiones de nuestro estudio no se aplican a especies que ya con anterioridad estuviesen en una situación más delicada, como aquéllas en peligro de extinción, alto grado de fragmentación o elevado nivel de capturas por razones comerciales.

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Research Report

Genetic impact of the Prestige oil spill in wild populations of a poor dispersal marine snail from intertidal rocky shores

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Abstract

In November 2002, the sinking of the Prestige cargo ship produced an oil spill of 60,000 tons that affected many areas along the Galician coast (in the northwest of Spain). In a number of rocky shore sites, most organisms (particularly marine mollusks) were nearly extinct at a local scale. We tested whether the local bottleneck/extinction that occurred in affected localities caused any detectable reduction of the genetic diversity in the marine snail *Littorina saxatilis*, an ovoviviparous rocky shore model species characterized by a low dispersal ability, high population density, and wide distribution range. We compared the level of genetic variation and population differentiation between affected (polluted) and control sites located in seven geographical areas (three sites per area, one impacted and two controls, and two replicates per site) one and a half years after the spill. The analysis included molecular marker variation (microsatellite and AFLP loci) and quantitative trait genetic variation for shell variables in embryos extracted from pregnant females. Our results indicate that the affected populations did not show a significant overall reduction in genetic diversity when compared to the controls, suggesting that the species is highly resistant to losing genetic variability as a consequence of a local short-term pollution process in spite of its low dispersal ability and direct development. However, some genetic effects were detected in the polluted populations, particularly for quantitative shell traits and AFLPs, consistent with local adaptations resulting from the fuel contamination.
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Keywords: *Littorina saxatilis*; Heterozygosity; Bottleneck; Natural selection; Quantitative variation; Heritability

1. Introduction

One of the most extreme habitat degradation processes occurs when accidental or deliberate contaminants are spilled into the marine ecosystems, causing a number of biological and ecological consequences (Suchanek, 1993). For example, as a result of the Exxon Valdez oil spill, several alterations were found in different species of fishes and mollusks (Hose et al., 1996; Fukuyama et al., 2000; Carls et al., 2004). After a long enough period of time, the affected populations may recover the census numbers and coverage that existed prior to the pollution event. However, even apparently fully recovered populations could be evolutionarily affected if they suffered a genetic diversity loss that could partially affect their long-term capability of adaptation. This possible genetic diversity loss through population bottlenecks is expected to be more intense in species with low dispersal rates, as these are expected to have a more reduced capacity for recolonization.

The tanker Prestige sank at 120 miles from the Galician coasts (in northwestern Spain) in November 2002. In this ecological disaster, around 60,000 tons of heavy fuel oil were spilled, affecting a great extension of the rocky shores and marine bottoms (Albaigés et al., 2006). A thick deposit of heavy fuel arrived at the coast, asphyxiating many organisms, such as snails, limpets and other mollusks, which are among the species most sensitive to physical habitat destruction (Moore et al., 1995). The large devastation produced by the contamination and the subsequent cleaning activities (sometimes even more damaging than the contamination *per se*; Moore et al., 1995) led to the apparent complete elimination of some populations, especially

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on the intertidal rocks. After the impact, many sites presented a deteriorated aspect, characterized by the absence of the species typically contributing to the stratification at the intertidal vertical shore gradient.

In a period of one or two years a recolonization of the most affected areas took place, but the question remains as to whether the affected populations were able to recover the levels of genetic variability present in unaffected areas. The starting hypothesis is that species with a low dispersal capability and direct life cycle could suffer from drastic reductions of their genetic variability as a result of eventual population bottlenecks, either from the survival of a low number of individuals or, if completely extinct, from the recolonization by a few individuals from other populations. A model species that fulfills these requirements and has an extensive distribution on the affected areas is *Littorina saxatilis*. This dioecious gastropod has low dispersal ability (rates of dispersal have been estimated to be of the order of 1–2 m per month; Erlandsson and Rolán-Alvarez, 1998), internal fertilization and direct, non-pelagic, development.

The genetic impact of contaminants on aquatic organisms are usually assessed by neutral molecular genetic variation (see review by Belfiore and Anderson, 2001). This variation is the simplest way to infer overall levels of genetic diversity, but may overlook the impact of contaminants on relevant characteristics such as morphological or life-history traits. In fact, theoretical analyses indicate that quantitative genetic variation can detect human impact on diversity that are not detected using molecular markers (Carvajal-Rodríguez et al., 2005a). Although this type of variation is difficult to assess in wild populations, *L. saxatilis* presents the advantage that females carry a brood pouch with shelled embryos. Thus, estimates of quantitative genetic variation, at least for morphological traits, can be obtained assuming that the embryos from a female constitute a full sib family (Newkirk and Doyle, 1975; Carballo et al., 2001; Conde-Padín et al., 2007).

The objective of the present work was to determine whether the contamination due to the Prestige oil spill produced any effect on the level of genetic variability of apparently recovered natural populations of *L. saxatilis* living on the Galician coast one and a half years after the spill. With this purpose, we assessed the levels of genetic variation and differentiation of these populations using both molecular (microsatellites and Amplified Fragment Length Polymorphism (AFLP)) and morphological (shell size and shell shape) markers. This is the first study assessing the impact of the Prestige oil spill on overall levels of genetic variation in marine organisms.

2. Methodology

2.1. Experimental design and sampling

In the absence of data from an affected population before and after the pollution event, the experimental design needs to compare polluted and control populations using ANOVA (Underwood, 1981). However, because such a comparison may confound natural (geographical/local) variability with the differences between polluted and control populations, the best solution is to replicate the control populations using an asymmetric ANOVA design, i.e. using at least two controls for each pollution site (Glasby, 1997; Glasby and Underwood, 1996). In this case, the differences between the polluted and control populations can account for the natural diversity present between controls. Thus, an asymmetric ANOVA design was used to infer the consequences of the oil spill on the genetic variation of *L. saxatilis* populations. The polluted sites (i.e. sites directly affected by the oil spill) were chosen based on information given by the Galician local government (http://www.ccmm-prestige.cesga.es/), as well as by direct inspection across repeated visits to some localities before sampling (see also Fig. 1 from González et al., 2006). The polluted sites were completely covered by fuel along a stretch of 50 or more meters, and *L. saxatilis* pop-

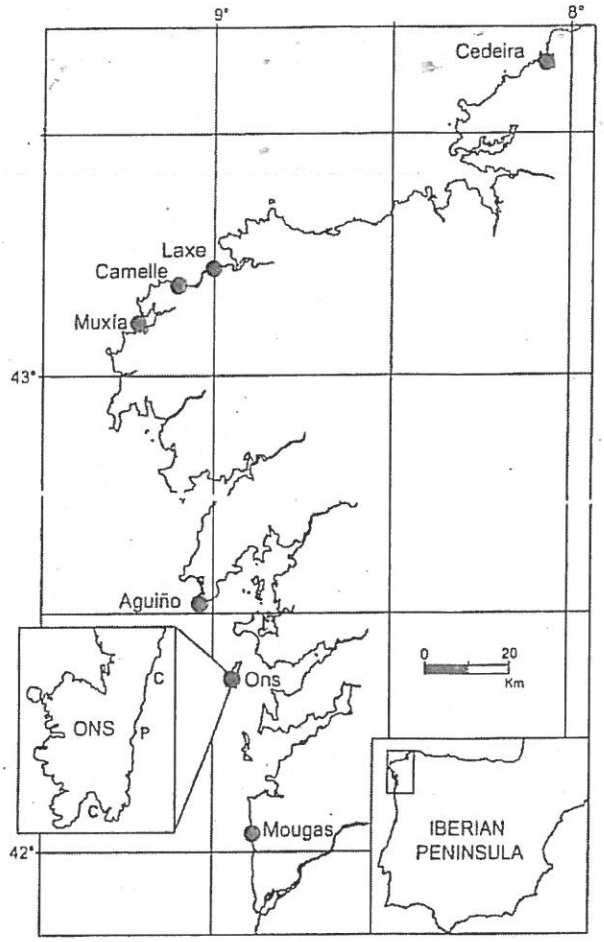


Fig. 1. Map showing the seven sampling areas in NW Spain. In each area three sites were sampled, one polluted (P) and two non-polluted (control, C) (see the example for the Isle of Ons). Typically the polluted site was situated between the controls (except for Laxe).

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ulations were apparently depleted in these areas after the cleaning process. The control sites, however, were sampled in proximal areas close to the affected sites (1.4 km on average) that were not directly affected by the oil spill (based on information from local and regional authorities as well as by the absence of rests of fuel), and showed usual population densities and algae coverage. Samples were mostly gathered in June–July 2004 (one and a half years after the oil spill, equivalent to around three generations of *L. saxatilis*) from seven areas of the western coast of Galicia (Fig. 1). Three sites were sampled in each area, separated from one another by a few kilometers (range 0.5–2 km, average 1.2 km). One site was polluted, and the other two non-polluted and used as controls.

Two hundred individuals from two replicates (10–20 m away) per site (three sites per area, and seven areas) were sampled and transferred to the laboratory and frozen at −80 °C. For the morphological analysis of adults, twenty individuals per replicate (840 individuals in total) were randomly chosen. These individuals were also used for the molecular analyses described below. For the genetic component analysis, all embryos with a shell height larger than 0.5 mm (developed embryos) were extracted from each of 20 randomly chosen females per replicate and laid in a numbered grill. Four embryos were then chosen at random for analysis (3360 embryos in total).

2.2. Molecular variability

Genomic DNA was purified from head to foot tissue of each individual using a modified version of the procedure described by Winnepenninckx et al. (1993).

We assessed variation at three polymorphic microsatellite loci (Lsax6CAA, Lx-12 and Lx-18) isolated from the *L. saxatilis* genome (Sokolov et al., 2002). PCR (Polymerase Chain Reaction) primers and amplification conditions of these three loci were as described previously (Sokolov et al., 2002), except that annealing temperatures of 61 °C were used for Lsax6CAA and of 60 °C for Lx-12 and Lx-18. The reverse primer was labeled with one of the fluorescent phosphoramidite dyes: 6-FAM (Lsax6CAA), HEX (Lx-12) or NED (Lx-18). The PCR products were separated on an ABI PRISM 310 DNA automatic sequencer. Data for fluorescent loci were scored using GENOTYPER software version 2.1 (Applied Biosystems). Basic statistics were calculated using GENETIX 4.0.3 (Belkhir, 2004) and FSTAT 2.9.3.2 (Goudet, 1995). Two diversity measures were computed: the number of alleles per locus (n_a) and the expected heterozygosity (H_e). In addition, two genetic diversity measures aimed at detecting possible population bottlenecks in polluted versus control populations were also calculated: the heterozygosity excess (H_x) (Luikart and Cornuet, 1998), and the M coefficient (Garza and Williamson, 2001). More negative values for H_x or closer to zero values for the M coefficient are expected to appear on populations affected by bottlenecks. Finally, fixation index (F_{ST}, Wright, 1951) values were estimated for all pairwise comparisons among sites.

AFLP analysis was performed using a modified version of the procedure described by Vos et al. (1995). Briefly, the genomic DNA was double digested with *Eco* RI and *Mse* I restriction enzymes. The DNA fragments were ligated with *Eco* RI and *Mse* I adaptors and pre-amplified with primers carrying one selective nucleotide (5′-GACTGCGTACCA-ATTC + A-3′ for *Eco* RI adapter, and 5′-GATGAGT-CCTGAGTAA + C-3′ for *Mse* I adapter). The resulting product was diluted 1:20 and used for the second amplification using *Eco* RI and *Mse* I primers (both carrying three selective nucleotides at the 3′ end). Amplification was conducted using three fluorescent-labeled primer pairs (Table 1). The final products were separated on an ABI PRISM 310 DNA automatic sequencer. Fragments in a range of 75 to 500 bp were scored using GeneScan. The software AFLPapp (Benham, 1997) and AFLPsurv 1.0 (Vekemans, 2002) were used to estimate the number of polymorphic loci (PL), the expected heterozygosity (H_e), and the fixation index (F_{ST}) for all pairwise comparisons among sites.

2.3. Morphological analysis

Morphological variation of adults and embryos was studied using the Geometric Morphometric methodology (Adams et al., 2004; Zelditch et al., 2004). This technique allows us to study the changes in size and shape from the displacement in the plane or in the space of a set of morphometric points or landmarks, to be combined with statistical multivariate procedures. The images of the 840 adult individuals and 3360 embryos from the 42 samples were captured using a Leica MZ12 stereoscopic microscope, with the specimens always placed in the same position (Fig. 2). The images were digitized using a Leica digital ICA video camera and QWin Lite v. 2.2 software. Twelve representative landmarks of the shell (eleven in embryos) were used in this study (Fig. 2; see also Carvajal-Rodríguez et al., 2005b), and obtained from the digitized shell image from Image Tool 3.0 (available at http://ddsdx.uthsc-sa.edu/dig/itdesc.html).

Table 1
Primer combinations used in the amplification of the AFLP fragments

Eco RI primer (5′–3′)	*Mse* I primer (5′–3′)
FAM-GAC TGC GTA CCA ATT C + ACT	GAT GAG TCC TGA GTA A + CAA
HEX-GAC TGC GTA CCA ATT C + AAG	GAT GAG TCC TGA GTA A + CAA
NED-GAC TGC GTA CCA ATT C + AGC	GAT GAG TCC TGA GTA A + CAA

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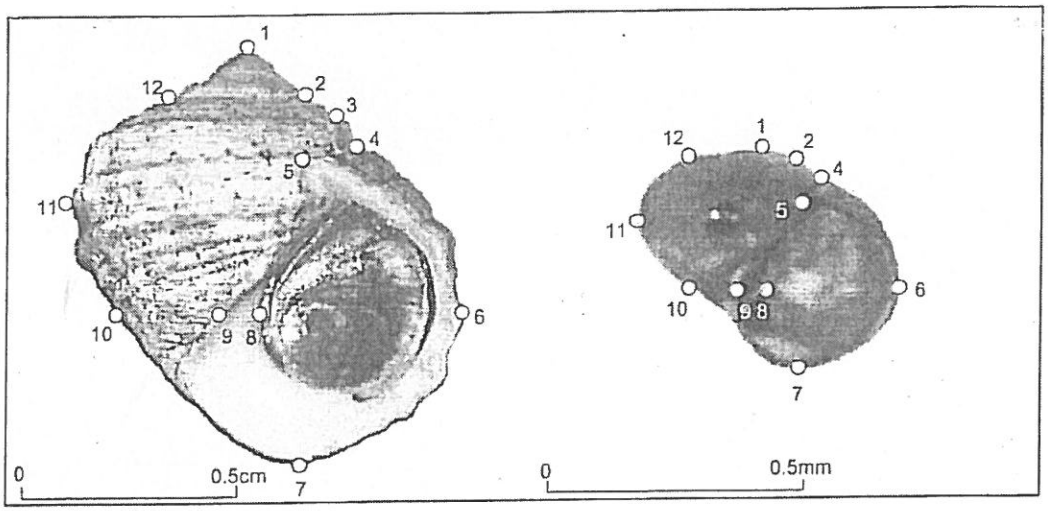


Fig. 2. Shell picture of an adult (left) and embryo (right) and corresponding landmarks (coordinates) used in the geometric morphometric analysis.

The estimate of shell size was the centroid size (CS), which is the square root of the sum of squared distances of a set of landmarks from their centroid, this being the center of gravity of a configuration of points (Bookstein, 1991). The shape (geometric information that remains after eliminating the effects of translation, rotation and scale) can be decomposed into uniform and non-uniform components (Bookstein, 1991; Zelditch et al., 2004). The uniform component describes the global variation of the shell (affecting all landmarks simultaneously, for example elongation/compression deformations) and, in turn, is decomposed into two components (U1 and U2), that represent changes in the vertical and the horizontal axis. However, only results averaged for both components (hereafter U) are presented for simplicity. The non-uniform components describe local shape deformations of a reference configuration at different spatial scales (representing local changes in the landmarks). Non-uniform shape measurements were computed by Relative Warp Analysis (Bookstein, 1991; Rohlf, 1993; Zelditch et al., 2004). Two main relative warps (accounting for 54% of the non-uniform variation) were used but, again, results were averaged for these two local components for simplicity (hereafter L).

2.4. Estimation of genetic components from embryos

Estimates of genetic variation were obtained for shell size (CS) and for each of the geometric morphometrics shell size components (U and L) in each of the 42 samples. Assuming that the chosen embryos of each female are full sibs (see Conde-Padín et al., 2007), twice the variance among families ($V_{b,fam}$), obtained from an analysis of variance, provides an estimate of the additive genetic variance (V_A) with possible bias due to dominance and environmental factors common to the members of the families (Falconer and Mackay, 1996). Note that, in the context of the present study, this possible bias is irrelevant, provided it is of similar magnitude in polluted and control populations. The heritability, or ratio between V_A and the phenotypic variance, V_P, was estimated as $h^2 = 2V_{b,fam}/(V_{b,fam} + V_{w,fam})$, where $V_{w,fam}$ is the component of within-family variance. The inter-population genetic differentiation, analogous to F_{ST} for molecular markers, was estimated as $Q_{ST} = V_{b,pop}/(V_{b,\,pop} + 2V_{w,pop})$, where $V_{b,pop}$ and $V_{w,pop}$ are the estimates of between-population and within-population genetic components of variance, respectively, the latter being equal to $2V_{b,fam}$. All the quantitative genetic estimates were obtained using the program MODICOS (Carvajal-Rodríguez and Gandarela, 2004).

2.5. Data analysis

The possible impact of fuel contamination on the within-population genetic variation of *L. saxatilis* populations was analysed by an asymmetric ANOVA (Underwood, 1991; Glasby, 1997). As stated above, this approach is the most powerful to analyze post-impact data. The analysis involves three stages: a hypothetical symmetric ANOVA, an analytic phase with three further ANOVAS, and a final asymmetric ANOVA (see Glasby, 1997, for details). Here, only the results of the third stage will be shown.

In order to study whether fuel contamination affected the level of genetic differentiation between-populations, we carried out two analyses. In the first one, genetic differentiation (F_{ST} or Q_{ST}) was estimated between the two control sites of each area and between one of the controls (chosen at random) and the polluted site. Because the compared estimates were not independent (the same control site was included in both estimates), a paired *t*-test was used for testing significance (Sokal and Rohlf, 1996). The objective was to test whether the polluted site had increased the genetic differentiation between sites within each area. In the second analysis, genetic differentiation estimates (F_{ST}

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and Q_{ST}) were obtained for all pairs of sites. A non-parametric test (modified from the Tsutakawa and Hewett, 1977, test for comparing groups across regression lines), was used for assessing the overall impact of fuel on Q_{ST} relative to F_{ST}. Each value of (Q_{ST}, F_{ST}) was plotted on a graph distinguishing pairs between control sites (C–C) and between control and polluted sites (C–P) (pairs involving two polluted sites were excluded for clarity, as they did not affect the trends or the significance of the tests). The neutral additive expectation ($Q_{ST} = F_{ST}$) was represented as a line, so that pair values above (below) this line imply that Q_{ST} is larger (smaller) than F_{ST}, which is generally interpreted as a consequence of divergent (convergent) selection affecting the quantitative trait (see review by Toro and Caballero, 2005). If the fuel pollution did not affect differentially the magnitudes of Q_{ST} and F_{ST}, points (C–C) and (C–P) should be equally likely above and below the line, and this was tested by a chi-square test. Statistical analyses were done with the SPSS/PC v. 14.0.

3. Results

3.1. Genetic and phenotypic population diversity

The averages of the different genetic diversity estimators for microsatellites and AFLPs for polluted and control populations are shown in Table 2. The F_{IS} index was significantly different from zero for microsatellites (mean $F_{IS} = 0.214$) and, thus, AFLPs were analysed either assuming this value or $F_{IS} = 0$ (Hardy–Weinberg equilibrium). However, both analyses gave similar results and only the latter are shown. Although there was a slight tendency for polluted sites to show smaller levels of variability (n_a, H_e and PL) than control sites, the average estimates of diversity were very close to each other for both types of populations. Values of H_x and M were, on average, lower for polluted than for control sites, but the differences were small and non-significant.

Mean values for morphological variables (Table 3), gave a similar overall picture. There was a tendency for the estimates of additive genetic variances and heritabilities to be somewhat smaller for polluted than for control sites, particularly for the uniform component of shell shape variation (U), but the differences were generally small.

All the above comparisons between averages of statistics, however, do not disentangle site and area effects, interactions with the main treatment or within-site variation. Thus, a formal analysis of the effect of pollution was carried out with the asymmetric ANOVA (Table 4). With respect to molecular marker variation, there were significant differences between areas for most diversity estimators (n_a, PL, and H_e for micros but not for AFLPs), in accordance with the relatively large geographic separation between some of the areas studied (see Fig. 1), but no significant effects were detected for the bottleneck estimators (H_x and M). The most relevant result is that the factor Treatment (polluted vs. control) was not significant for any of the molecular diversity estimates, suggesting that the polluted populations did not suffer from a reduction in genetic variation relative to the control sites. The only significant effect was the interaction term (A × T) for the number of polymorphic loci (PL) in AFLPs. An inspection of the average values of PL and H_e for AFLPs in each area (Fig. 3) reveals that polluted and control sites differed in molecular diversity, but the sign of the difference varied from one area to another. Thus, in some areas (e.g. Muxía or Mougás) the control sites showed larger genetic diversity than the polluted sites, but in others (e.g. Ons) the observation was the opposite.

The analysis carried out on the phenotypic measures in adults only detected significant effects between control sites within areas (Table 4), suggesting that the most important pattern of adult shell variation is between locations separated by a few hundred meters.

Finally, the results of the asymmetric ANOVA on the genetic estimates of variation for morphological traits is shown in the last part of Table 4. Apart from the variation

Table 2
Averaged values for different estimators of genetic variation from microsatellite loci and AFLPs in polluted and control sites of *L. saxatilis*

	Polluted	Control
Microsatellites		
n_a	11.1 ± 0.653	11.3 ± 0.470
H_e	0.80 ± 0.022	0.81 ± 0.018
H_x	−1.47 ± 0.381	−1.15 ± 0.361
M	0.62 ± 0.019	0.68 ± 0.047
F_{IS}	0.19 ± 0.036	0.22 ± 0.031
AFLPs		
PL	761.2 ± 15.18	764.3 ± 7.00
H_e	0.31 ± 0.006	0.31 ± 0.002

n_a: number of alleles. H_e: expected heterozygosity. H_x: excess of heterozygosis. M: Garza and Williamson (2001) M coefficient. F_{IS}: deviation from Hardy–Weinberg proportions. PL: number of polymorphic loci. The AFLP analysis assumes $F_{IS} = 0$, but results assuming $F_{IS} = 0.214$ were very similar (not shown). Averages based on 7 (Polluted) and 14 (Control) data.

Table 3
Mean values in polluted and control sites for the morphological variables of adults (CS, U and L) and their corresponding additive variances (V_a) and heritabilities (h^2) estimated from embryos

	Polluted	Control
Adults		
CS	1.240 ± 0.076	1.070 ± 0.049
U	0.001 ± 0.001	−0.001 ± 0.001
L	0.005 ± 0.003	−0.003 ± 0.004
Embryos		
$V_{a(CS)}$	0.019 ± 0.002	0.019 ± 0.002
$V_{a(U)}$	0.243 ± 0.044	0.336 ± 0.031
$V_{a(L)}$	0.825 ± 0.100	0.889 ± 0.099
$h^2_{(CS)}$	0.77 ± 0.044	0.81 ± 0.057
$h^2_{(U)}$	0.24 ± 0.053	0.34 ± 0.047
$h^2_{(L)}$	0.31 ± 0.040	0.31 ± 0.043

Averages based on 7 (Polluted) and 14 (Control) data.

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Table 4
F values and their significances for the asymmetrical analyses of variance for the different variables studied

Source of variation	df	Microsatellites				AFLP	
		n_a	H_e	H_x	M	PL	H_J
Molecular markers							
Area (A)	6	12.20[a]	4.10[b]	3.81	0.82	5.68[a]	1.75
Treatment ($T = P$ vs. C)	1	0.13	0.21	2.03	1.00	0.02	0.00
$A \times T$	6	1.78	1.13	0.25	0.46	2.83[a]	1.75
Control sites (within A)	7	0.86	2.12	0.81	1.21	0.90	3.00[a]
Residual	21						
	df	CS		U		L	
Shell variables in adults							
Area (A)	6	2.11		1.11		3.56	
Treatment ($T = P$ vs. C)	1	3.37		1.80		1.28	
$A \times T$	6	1.93		0.58		1.82	
Control sites (within A)	7	21.13[c]		16.07[c]		11.22[c]	
Residual	765						
	df	$V_{a(CS)}$	$V_{a(U)}$	$V_{a(L)}$	$h^2_{(CS)}$	$h^2_{(U)}$	$h^2_{(L)}$
Genetic variables from shells variables in embryos							
Area (A)	6	–	2.44	1.61	1.67	5.29[a]	1.86
Treatment ($T = P$ vs. C)	1	0.01	1.99[d]	0.14	0.38	0.64[d]	0.00
$A \times T$	6	–	3.24[d]	1.70	1.55	3.32[d]	2.28
Control sites (within A)	7	0.00	0.38	0.72	0.77	0.52	0.51
Residual	21						

n_a: numbers of alleles. H_e: observed heterozygosity. H_x: excess of heterozygosis. M: Garza and Williamson (2001) M coefficient. PL: number of polymorphic loci. CS: centroid size; U: mean uniform component. L: mean local non-uniform component. V_a: additive genetic variance. h^2: heritability.
[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.001$.
[d] $P < 0.1$.

.between areas for the heritability of the uniform component of shell shape variation (U), no other factors were significant. However, the factor comparing polluted and control populations and the A × T interaction were marginally significant again for the U component. The mean values of heritability and additive variance for this component in polluted and control sites for each area are shown in Fig. 4. In all areas, except in Mougás, genetic variation was lower in polluted than in control sites. As expected, if Mougás was excluded from the analysis, the factor Treatment became highly significant.

3.2. Between-population genetic differentiation

The possible impact of pollution on genetic differentiation was analysed comparing the levels of F_{ST} and Q_{ST} between the control sites and between control and polluted sites. In a first analysis, the mean differentiation between the two control sites of each area was compared with that between one control (randomly chosen) and the polluted site (seven independent replicates). Similar results were obtained irrespective of the control used in this latter estimate (not shown). Mean values of genetic differentiation are shown in Table 5 for the two molecular markers (F_{ST}) and the main shell traits (Q_{ST}). There were significant differences between treatments exclusively for shell shape components (U and L), the genetic differentiation between polluted and control sites being larger than that between two controls. Interestingly, the strongest effect was detected, again, for the uniform component of shell variation (U), a result consistent with the previous ones for within-population variability. It should be noted, in addition, that the average distance between the two control sites of each area was typically larger (about 60%) than that between the polluted site and any of the controls, because in all cases (except Laxe) the polluted site was situated geographically within the two control sites. Thus, a larger differentiation because of isolation by distance would operate in the opposite way to that observed.

In a second analysis, estimates of F_{ST} vs. Q_{ST} between all pairs of sites from all areas were compared, involving two controls (C–C) or one control and one polluted site (C–P). A representation of the pairs (F_{ST}, Q_{ST}) is given in Fig. 5, where white and black circles refer to pairs C–C and C–P, respectively, and the plotted line represents the neutral additive expectation ($F_{ST} = Q_{ST}$) for reference. Values above this line imply that Q_{ST} is larger than F_{ST}, which is generally interpreted as a consequence of divergent selection affecting the quantitative trait. Thus, the null hypothesis is that both comparisons (C–C vs. C–P) should show the same proportion of points above and below the line. A test for this hypothesis is shown in Table 6. Yet again a significant trend was only found for the uniform component of shell shape variation, indicating that C–C

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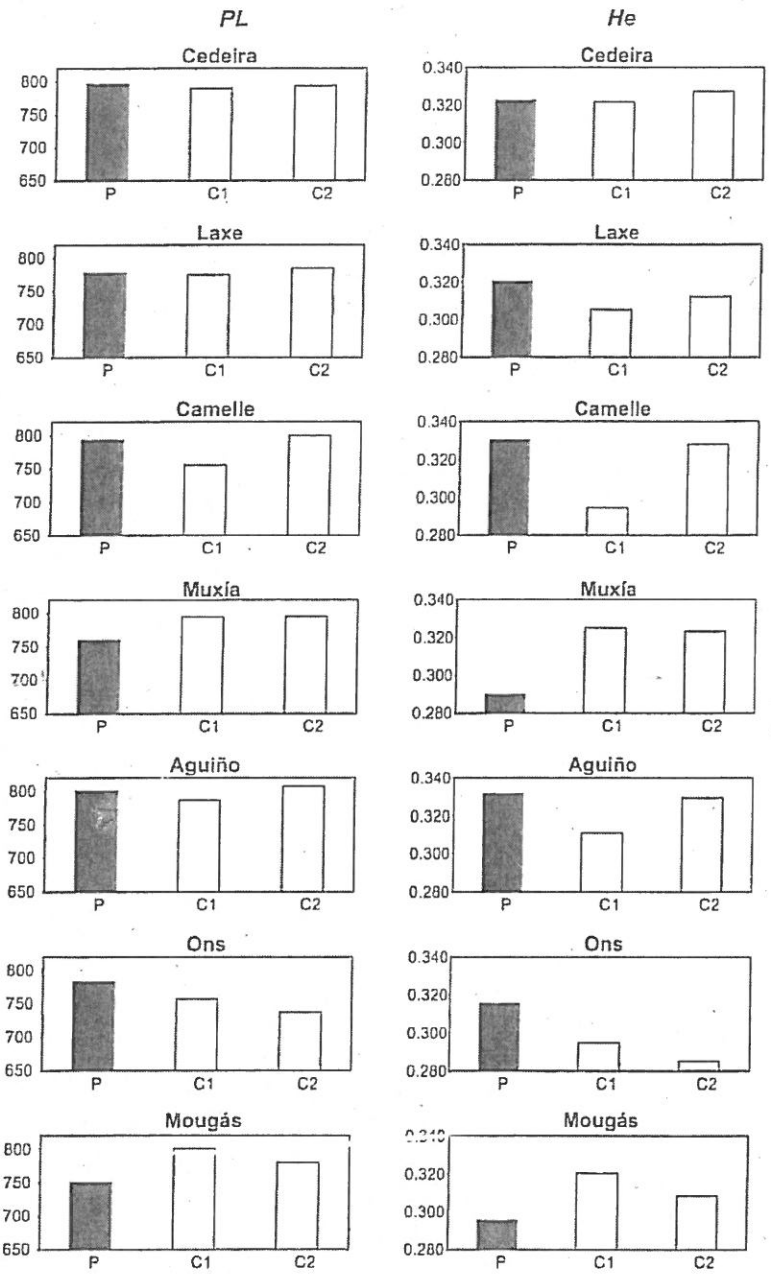


Fig. 3. Graphic comparison between polluted (*P*, black bars) and control (*C*, open bars) sites in each area studied for the variables number of polymorphic loci (PL) and expected heterozygosis (H_e) of AFLPs (Hardy–Weinberg equilibrium is assumed).

pairs tend to be below the neutral expectation whereas C–P pairs tend to be above it. The interpretation is that the inclusion of the polluted site produces an increase in quantitative genetic differentiation for the mean U shell variable with respect to the molecular marker differentiation.

4. Discussion

About 20,000 tons of heavy fuel washed up on some parts of the Galician coast in November 2002, and up to 40,000 tons arrived later. The characteristics of this catastrophe, with many localities heavily and locally affected but relatively dispersed along the Galician shores (Albaigés et al., 2006), provides a good opportunity to check the genetic impact caused by fuel pollution on natural populations of common and low dispersal species. *L. saxatilis* was chosen as a model organism in this study because it lives on the upper part of the shore (the habitat more affected by the spill), it shows one of the lowest dispersal abilities among grazer mollusks, and it has a high density and wide-

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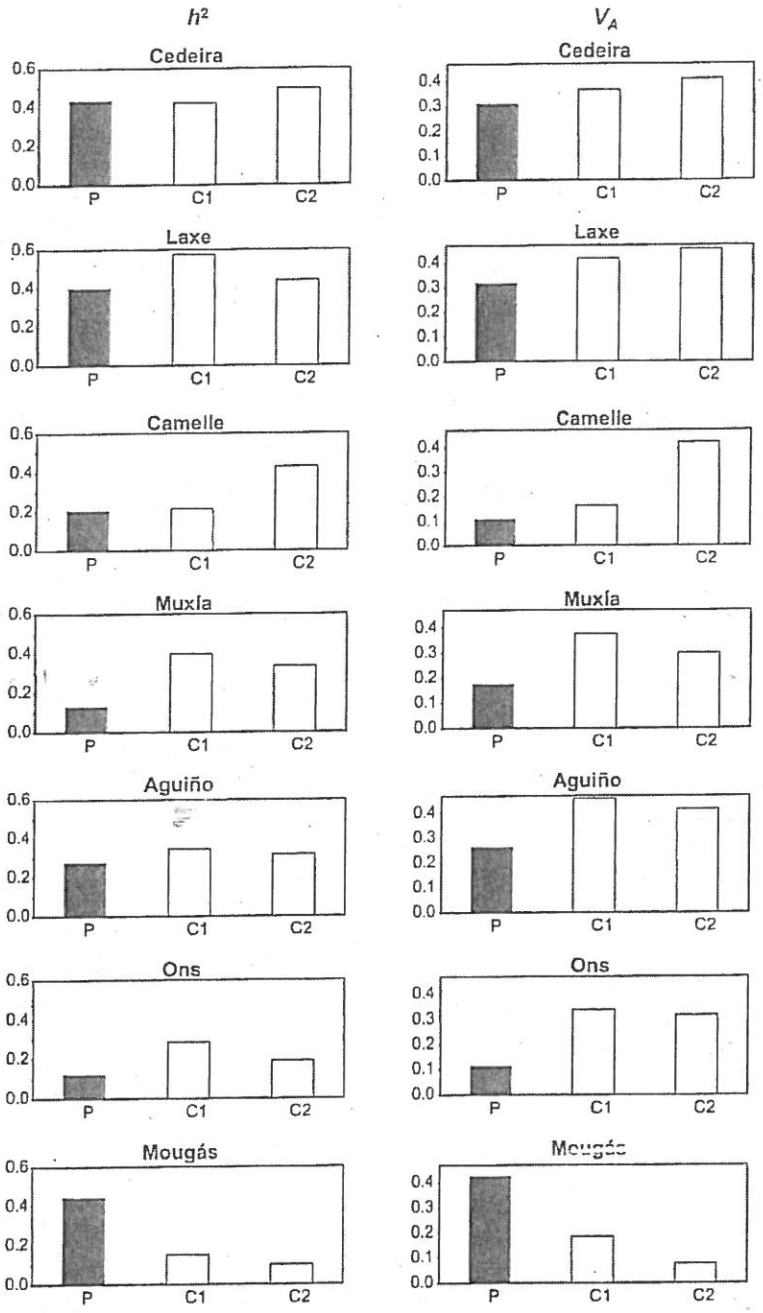


Fig. 4. Graphic comparison between polluted (*P*, black bars) and control (*C*, open bars) sites for the average heritability (h^2) and additive genetic variance (V_A) of the uniform shell shape component of variation, obtained with the morphological analysis of embryos.

spread distribution on Galician shores, facilitating the experimental design. In spite of the low dispersal ability of the species and its direct development, we did not find clear evidence that the populations affected by the fuel displayed a significant overall reduction in genetic variability after local extinction and recolonization (Tables 2–4), suggesting that the polluted sites either suffered a very light bottleneck or had fully recovered from a strong one. Some minor genetic effects were detected in the polluted populations (discussed below), but they do not support any overall decrease of genetic variability. A similar result has been found in the common guillemot sea bird after the Erica oil spill (Riffaut et al., 2005), and in recolonized populations of *Nucella lapillus* that had been extinct because of the presence of the TBT pollutant in English coasts. Recolonized populations showed levels of genetic variation for micro-

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Table 5
Mean F_{ST} and Q_{ST} values between sites belonging to the same area: between the two controls (C–C) and between one control and the polluted population (C–P)

	C–C	C–P	t
$F_{ST(microsatellite)}$	0.097 ± 0.006	0.099 ± 0.009	0.20
$F_{ST(AFLP)}$	0.048 ± 0.002	0.045 ± 0.002	0.99
$Q_{ST(CS)}$	0.123 ± 0.014	0.111 ± 0.018	0.51
$Q_{ST(U)}$	0.058 ± 0.007	0.143 ± 0.017	5.39[b]
$Q_{ST(L)}$	0.164 ± 0.014	0.220 ± 0.027	2.09[a]

A paired t-test is presented to verify whether the two comparisons (with seven replicates) differ significantly in mean value for different traits. A significant case supports that polluted populations are being genetically differentiated for the trait studied.
Definitions of variables as in Table 4.
[a] $P < 0.05$.
[b] $P < 0.001$.

satellites and quantitative traits similar to populations apparently unaffected by TBT (Colson and Hughes, 2004; Colson et al., 2006).

In the present study, it seems evident that, even if some genetic variability reduction could be originally caused by the pollution event, a fast and nearly complete recovery of the population density avoided its formal detection just around three generations later. Several factors could be contributing to the lack of a genetic impact of fuel pollution. Firstly, the severity of a bottleneck depends on the reduction in population size and its duration. Thus, the effect of a drastic reduction in population size on the levels of genetic variation may be very small if the duration of the bottleneck is very short. For example, a reduction in heterozygosity of about 1%, as observed for the microsatellite loci (Table 2), would occur if the effective population size is 120 individuals for a period of three generations. Secondly, most populations affected by the oil spill were apparently extinct, at least with respect to the adult population, but the possibility of juveniles hidden in crevices or behind rocks cannot be discarded, as this is natural behavior for snails (Faller-Fritsch and Emson, 1985). Finally, migration from unaffected close localities or unaffected rocks from the polluted area could have contributed to the recolonization. In fact, after events of local fuel pollution, an increase in the abundance of some seaweeds and microalgae has been observed, which may favor the recolonization by grazers from other localities (Suchanek, 1993).

The absence of a clear variability reduction does not necessarily mean that fuel pollution did not affect the exposed populations genetically. In fact, even if hundreds of snails had survived it is probable that survival would not have been random, with some particular characteristics (genotypes) being beneficial in the new habitat conditions produced by the arrival of the fuel. After an ecosystem is crashed by pollution and subsequent cleaning, an ecological transition occurs on the rocky shores (Moore et al., 1995). First, some seaweed and microalgae can resist and survive, becoming extremely abundant in a new habitat without grazers. Later on, most grazers arrive and become easily stable in the new habitat due to low competition and predation. Finally, the typical species distribution, including predators, is recovered. Thus, it is possible to speculate that when a grazer organism, such as *L. saxatilis*, recolonizes an affected site some new characteristics may be beneficial, for example particular shell shapes caused by a faster growth.

The complex pattern observed for AFLP variation in the comparison between polluted and control populations (Fig. 3) is difficult to explain, as the polluted sites presented more variation than the control ones in some areas. However, this may be hypothesized to be the result of unknown selection processes occurring on different parts of the genome. The AFLP technique provides a fast and efficient overall picture of the genome variability (in this case, about 800 genes randomly chosen along the genome). It has been already shown that the overall level of heterozygosity could be selected under particular conditions (Belfiore and Anderson, 2001) and, in fact, increases in heterozygosity have been observed under stressful conditions (Moraga et al., 2002). In addition, the fact that levels of AFLP variation (which could incorporate both neutral and selective variation) were affected in polluted populations but that no such effects were detected for microsatellites (generally assumed to be strictly neutral), argues in favor of a selective hypothesis, even if the detailed mechanisms are unknown.

We found other results which further support that natural selection may have played a role in the polluted populations. First, an overall reduction in genetic variability for the uniform shell shape component of variation was detected in polluted populations (Fig. 4; except for the Mougás area). If a bottleneck effect were the cause of such a reduction, this would affect all molecular and quantitative traits, and this did not happen. However, a reduction in variation for a particular trait can be easily explained if it is assumed to be the subject of strong selection, as this is well-known to deplete genetic variation (Falconer and Mackay, 1996). An attempt was made to find out the difference in shell shape between snails from polluted and control sites. Shells from polluted sites were, both for adults and embryos, slightly more spired than those from controls (not shown). It can be hypothesized that spired shells could be negatively selected in control sites (Johannesson and Johannesson, 1996), strongly affected by crab predation, whereas this would not occur in polluted sites where crabs would be expected to be initially absent.

The analysis of population differentiation (Q_{ST}) also supports the hypothesis that natural selection could have driven changes in the uniform shell component of variation, producing a genetic differentiation between polluted and control populations (Table 5 and Fig. 5). An alternative explanation in relation to this pattern would be that Q_{ST} estimates are inflated by environmental differences between polluted and control sites. For example, high spired shells have been occasionally considered as a side effect of low growth rates in littorinids (Reid, 1996). An environmental explanation such as this could help explain

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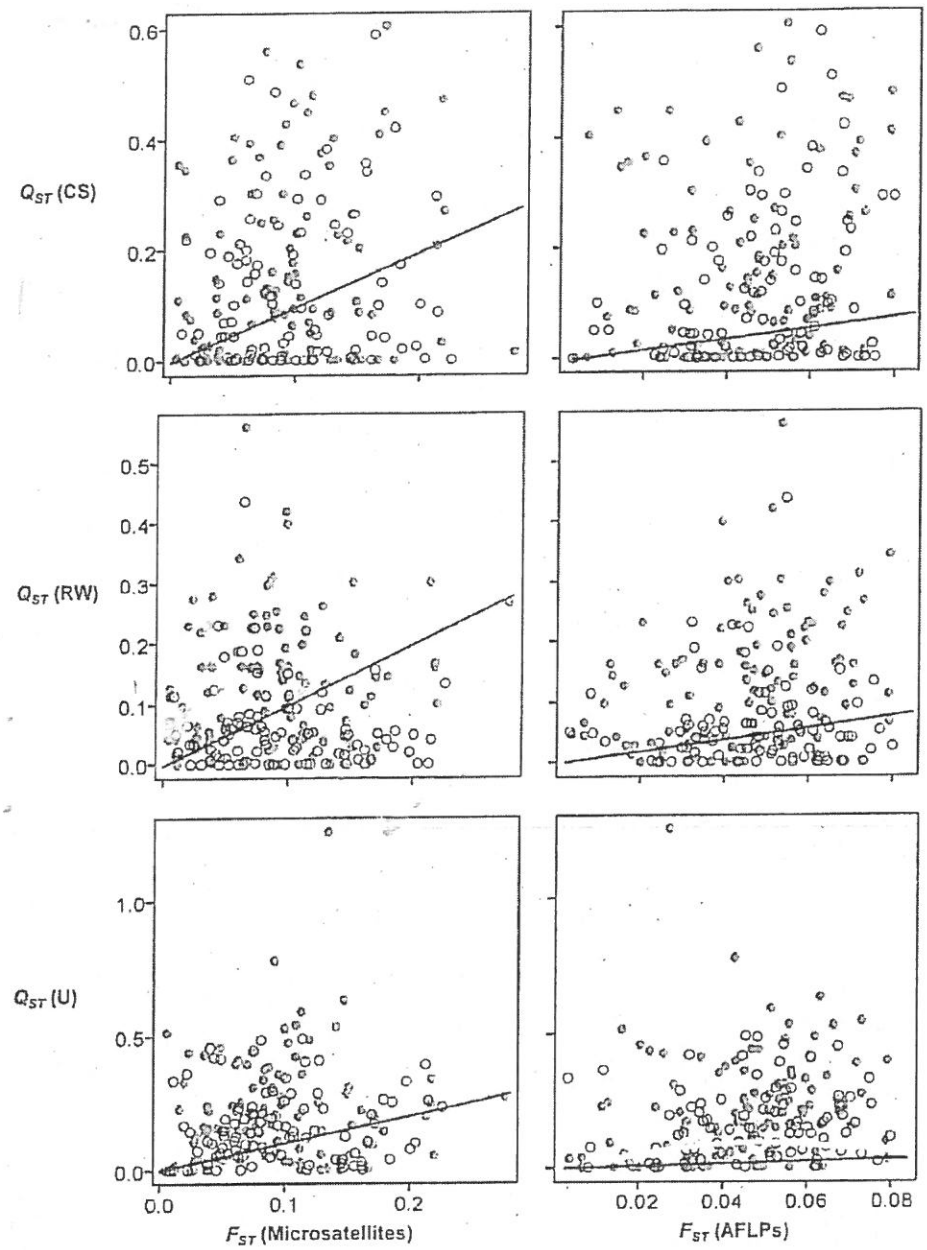


Fig. 5. Relation between F_{ST} values from microsatellites and AFLPs and Q_{ST} values obtained from morphological variables (centroid size CS, uniform component U, and local non-uniform component *L*), between pairs of sites. The black circles represent population differentiation values between one polluted and one control site (C–P), whereas white circles represent population differentiation values between control sites (C–C). The line represents the neutral additive prediction ($Q_{ST} = F_{ST}$).

the differences in shell morphology in adults. However, it should be noted that the increases in differentiation with polluted populations were also observed in embryos that had not yet been released to the external medium and, therefore, would less likely be affected by external environmental conditions (see Conde-Padín et al., 2007). In addition, a possible bias in Q_{ST} cannot explain the genetic variability reduction observed in shell shape or the complex trends on AFLP variation discussed above. Thus, a plausible interpretation is that polluted populations were more severely affected by natural selection, producing genetic changes in mean shell shape, and AFLP and shape variation.

The fact that a species with a low dispersal capability and direct development, such as *L. saxatilis*, did not suffer any overall decrease in the levels of genetic variability due to fuel pollution suggests that the conclusions of this work could be extended to other species with larger dispersal abilities and wide distribution. This would be the case of those with plancktonic larvae, such as mussels or barna-

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Table 6
Tsutakawa and Hewett (1977) test for comparing trends in F_{ST} and Q_{ST}

	Position	C–C	C–P	Chi-square
Q_{ST}(CS)–F_{ST} (Micros)	+	44	55	1.31
	–	47	42	
Q_{ST} (U)–F_{ST}(Micros)	+	26	60	21.65[a]
	–	65	36	
Q_{ST} (L)–it F_{ST} (Micros)	+	63	66	0.08
	–	28	32	
Q_{ST} (CS)–F_{ST} (AFLPs)	+	54	66	1.30
	–	37	32	
Q_{ST} (U)–F_{ST} (AFLPs)	+	41	77	22.59[a]
	–	50	21	
Q_{ST} (L)–F_{ST} (AFLPs)	+	70	81	0.96
	–	21	17	

Definitions of variables as in Table 4.
The values are the number of paired (F_{ST}, Q_{ST}) estimates above (+) or below (–) the line representing the neutral prediction ($F_{ST} = Q_{ST}$) in Fig. 6. C–C values refer to differentiation between two control sites from all areas, whereas C–P refer to differentiation between control and polluted sites from all areas. Chi-square values for one degree of freedom were used to test different proportions of points above and below the neutral expectation.
[a] $P < 0.001$.

cles, for which recolonization events would be expected to be faster. It appears thus reasonable to argue that any non-continuous perturbation, such as the case of the Prestige accident, will rarely produce a drastic reduction in genetic variability in species with high population densities and a wide range distribution. Organisms with low density populations or narrow geographical distributions, however, could be more heavily affected by pollution events. For example, the populations of *Myosotella myosotis*, a species which lives in exposed shores and is rather scarce, were drastically affected by the Prestige fuel spill. Most populations of this species have disappeared since the catastrophe (see Rolán and Guerra, 2006).

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