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A field study of the molecular response of brown macroalgae to heavy metal exposure: An (epi)genetic approach

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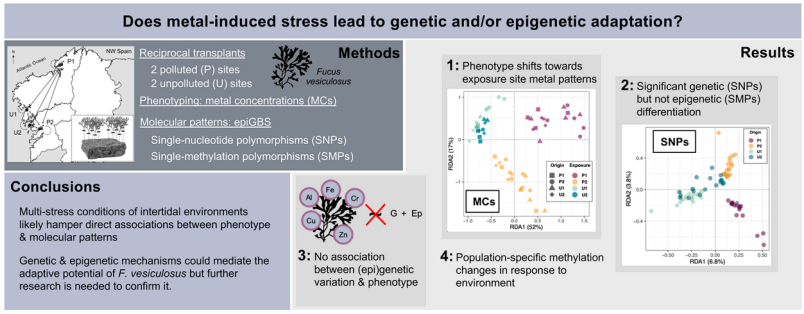
HIGHLIGHTS

- Exposure history leads to phenotypic differences in macroalgae metal uptake capacity.
- Genetic and epigenetic differentiation do not explain these phenotypic differences.
- Environmental complexity of natural habitats might explain this lack of association.
- Change of environment induces population-specific epigenetic changes in macroalgae.
- Genetic and epigenetic mechanisms may influence the adaptive potential of macroalgae.

GRAPHICAL ABSTRACT



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ABSTRACT

Our understanding of the relative contribution of genetic and epigenetic mechanisms to organismal response to stress is largely biased towards specific taxonomic groups (e.g. seed plants) and environmental stresses (e.g. drought and salinity). In previous work, we found intraspecific differences in heavy metal (HM) uptake capacity in the brown macroalgae *Fucus vesiculosus*. The molecular mechanisms underlying these differences, however, remained unknown. Here, we evaluated the concentrations of HMs, and characterized the genetic (single nucleotide polymorphisms) and epigenetic (cytosine DNA methylation) variability in reciprocal transplants of *F. vesiculosus* between two polluted and two unpolluted sites on the NW Spanish coast after 90 days. Genetic and epigenetic differentiation did not explain the phenotypic differentiation observed, possibly due to the combined effect of multiple environmental factors acting on the algae in their natural habitats. Nonetheless, we provide further evidence of intraspecific genetic differentiation in *F. vesiculosus* at short spatial scales, as well as first evidence of population-specific epigenetic changes in brown macroalgae in response to changes in environmental conditions (i.e. transplantation ex situ). We propose that both genetic and, to some extent, epigenetic

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mechanisms might impinge upon the adaptive potential of this species to environmental change, but this needs to be further addressed.

1. Introduction

Marine macroalgae are a diverse and globally distributed group of photosynthetic organisms considered to be fundamental components of coastal ecosystems [1]. Recent studies have revealed their key contribution to coastal productivity [2], ecosystem structure, functioning [3] and services [4,5]. In their natural environments, macroalgae are continuously exposed to multiple environmental stressors that affect their communities and, hence, compromise the ecosystems they support [6]. In particular, those inhabiting intertidal rocky shores are exposed to a physically demanding environment with steep abiotic gradients and drastic environmental fluctuations at small spatial scales. Desiccation, irradiance, salinity, temperature, grazing or epibiontism, are some of the factors causing physiological stress to algae from these environments [7]. In addition, declines in abundance and the redistribution of coastal macroalgae (brown seaweed in particular) on a global scale have been attributed to anthropogenic pressure and climate change (see examples reviewed in e.g. [8-11]).

Heavy metal pollution has emerged as a major environmental concern, threatening coastal ecosystems and human health [12,13]. Although some heavy metals are essential in trace amounts (e.g. Cu, Fe, Zn and V), playing important roles in metabolic processes, all metals are highly toxic at high concentrations, which eventually results in oxidative stress damage due to generation of reactive oxygen species (ROS) [14-16]. Increasing evidence suggest that metal induced stress can affect photosynthetic activity, growth, development, survival, and reproduction in macroalgae [17-20]. Some species of macroalgae have adapted to living in highly polluted environments, accumulating levels of heavy metals that greatly exceed the tolerance limits of most other living organisms in the marine environment [21-23]. Scientific evidence has shown that algae may display inherent tolerance to metals, and are considered “hypertolerant”, e.g. the Cu-tolerant red algae *Gracilariopsis longissima* [24], while others have developed metal-tolerant ecotypes (i.e. genetically differentiated populations with differential tolerance to metal excess) after long-term exposure to metal pollution, e.g. the Cu-tolerant brown algae *Scytosiphon lomentaria* [25] and *Ectocarpus siliculosus* [26].

Macroalgae can mitigate metal-induced oxidative stress through cellular metal-exclusion and accumulation mechanisms, syntheses of metal-chelating compounds, and the activation of antioxidant metabolism [16,27–29]. These processes are generally mediated by the overexpression of genes that take part in heavy metal transport and homeostasis (e.g. ABC transporters, metallothioneins and phytochelatins, antioxidant enzymes and molecules) [30-32]. Most of this knowledge is derived from laboratory-based bioassays [15,27,28] which limits our understanding of macroalgal response in complex natural conditions. Reciprocal transplant experiments in the field have shown, for example, that macroalgae transplanted from a control site to a contaminated site accumulated significantly higher amounts of metals than the resident populations [33,34]. The mechanistic basis of such phenotypic differentiation, however, has not been elucidated.

Genetic and epigenetic variation both shape phenotypic variation in living organisms [35-38]. Epigenetic variation, more specifically DNA methylation, is known to change genome function under exogenous influence without changes in DNA sequence [39]. This chemical modification is more dynamic than DNA sequence variation [40-42] and is one mechanism that allows organisms to respond efficiently to external stimuli. Heavy metals, for example, can induce changes in cytosine methylation levels throughout the genome. For instance, DNA hypermethylation was reported in response to high levels of Cd in *Posidonia oceanica* [43], Cr in *Brassica napus* [44], Pb in *Zea mays* (Agar, 2014), or Cd and Cu in the metallophyte moss *Scopelophila cataractae* [45]. In contrast, DNA hypomethylation was detected in response to high levels of Cd, Cr and Ni in the metal-sensitive *Trifolium repens* and in the metal-tolerant *Cannabis sativa* [46]. When metal stress is prolonged in time, changes in DNA methylation can also be inherited in the next generation [47,48].

In algae, the role of DNA methylation in response to heavy metal stress has not been well studied [49]. Among the few studies available, most focus on green microalgae and the effects of Cr and Cd on DNA methylation. For example, Cozza et al. [50] showed that DNA methylation changes leading to changes in nuclear chromatin conformation were associated with differences in Cr tolerance between two strains of the microalgae *Scenedesmus acutus* with different sensitivities to Cr. More recently, Ferrari et al. [51] found that differences in DNA methylation between these two strains were also associated with differential expression of genes involved in the sulfate pathway, which is related to Cr tolerance. Regarding Cd, treatment with this metal resulted in increased DNA methylation levels in *Chlamydomonas reinhardtii* and *Scenedesmus quadricauda* [52]. The only study assessing DNA methylation changes in macroalgae induced by metal stress, reported significant hypomethylation in the red algae *Gracilaria dura* in response to Cd [53]. Still, these studies were carried out in laboratory conditions, with cultivated species, and metal concentrations that exceeded levels usually found in nature. Therefore, their results may not necessarily reflect what would happen in the natural environment, where many interacting environmental and physiological factors condition the heavy-metal effects on algae.

Brown macroalgae (Class Phaeophyceae) are among the most efficient heavy metal accumulators in coastal systems [22,23,54]. Specifically, *Fucus* species are highly tolerant to heavy metals and resilient to abiotic stressors, dominating a variety of polluted environments in the Northern hemisphere [7,55,56]. In previous work, we observed limited heavy metal uptake capacity in *F. vesiculosus* populations exposed to long-term metal pollution in the NW coast of Spain [33]. These results were consistent with the existence of a potential adaptive response to the stress caused by chronic heavy metal exposure. The application of high-resolution genomic techniques combined with multivariate statistics can help unravel the mechanisms of adaptation of species to variable and local environmental conditions [57,58]. In this study, we used a reduced representation bisulfite sequencing method (epiGBS; [59]) to simultaneously examine the potential role of single-nucleotide polymorphisms (SNPs) and variation DNA methylation on the response of natural macroalgae populations to environmental heavy metal exposure.

Specifically, using reciprocal transplants in natural field sites, we aimed to answer the following questions: i) are *F. vesiculosus* populations with distinct metal pollution history (epi)genetically differentiated? ii) does *F. vesiculosus* experience (epi)genetic changes after long-term exposure to environments with distinct metal pollution levels? and iii) is epigenetic variation related to genetic variation in *F. vesiculosus*?

2. Material and methods

2.1. Sample collection

We collected individual thalli of the brown macroalga *Fucus vesiculosus* L. (Phaeophyceae) in four distant populations experiencing contrasting local environmental conditions in Galicia (NW Spain), near the southern limit of its geographic range (Fig. 1A). Two of the populations were located in industrialized areas within rias (i.e., coastal inlet formed at the lower end of a river in which estuarine processes only

dominate in the inner part; [60]) where they were subjected to different levels of metal pollution (P, polluted sites), at least since the 1990s [61]. One was located in the surroundings of an iron and steel plant (P1: 43º29'52.7''N 8º10'20.5''W), and the other was close to a paper pulp industry (P2: 42º24'24.1''N 8º41'06.4''W). Both factories discharge high levels of metals and metalloids (e.g. As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb and Zn) into seawater [62]. The other two populations were located in open-shore areas away from focal pollution points, representative of reference, unpolluted (U) sites (U1: 42º44'03.9''N 8º59'38.9''W; U2: 42º27'40.7''N 8º54'54.0''W), as per the low metal concentrations (e.g. Cr, Cu, Hg, Ni, Pb and Zn below regional background values) found in this species at these sites [61]. Mean concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, and Zn in *F. vesiculosus* at the beginning of the experiment in each of the study sites are shown in Table S1. The levels of Al, Co, Cr, Cu, Fe, Pb and Zn in P1 were between 3 and 29 times higher than in U1; those of Al, Co, Cr, Cu, Fe, Hg, Ni, Pb, and Zn were between 2.4 and 10 times higher than in U2. Overall, P2 showed lower metal concentrations than P1 (except for Cd, Cu, and Hg), however, the concentrations of Al, Cu, Fe, Hg, and Zn, as well as those of Cr, Cu, Hg, and Zn at this site were $\geq$ 2 times higher than in U1 and U2 respectively.

At each population, we collected 200 reproductive thalli (adults) of *F. vesiculosus* evenly distributed across the intertidal zone, in plots of about 25 × 25 m, during low tide. All thalli were washed in the surrounding seawater to remove surface particulate material, and transported to the laboratory in refrigerated conditions where they were stored (5 ± 1ºC) for a maximum of 3 days until preparation of the transplants. To establish population-specific baseline conditions (t_0), we collected three additional samples per population that were not exposed as transplants (unexposed samples). Each of these consisted of a pool of the apical most segments (~1 cm), i.e., the most recent and physiologically active tissue, of five individual thalli. These were separated and immediately flash frozen in liquid N in the field before storage at −80ºC. An additional pooled sample was collected within each population, in the same way, to establish baseline concentrations of heavy metals at each site (more details in [33]).

2.2. Reciprocal transplant experiment

To test for population-specific phenotypic (metal concentrations in macroalgae) and epigenetic (DNA methylation) changes in response to pollutant exposure, we conducted a reciprocal transplant experiment using the 200 thalli collected in early spring (end of March) as described above. Within each site, we transplanted living thalli from the same site (home transplants) and from the other three sites (foreign transplants) (Fig. 1A). Each transplant consisted of ten thalli attached to a rock and covered with a mesh net (Fig. 1B). Half of these thalli were used for metal concentration analysis (results published in [33]); the other half, were used for the (epi)genetic analyses in this study. Thus, a total of 20 transplants (n = 5 replicate transplants per origin population, Fig. 1C) were exposed at each site in the intertidal zone, amongst the native populations of *F. vesiculosus*. After 90 days (from the beginning of April to the beginning of July), considered enough time for element concentrations to equilibrate with those in the environment [63,64], the thalli were detached from the rocks, and cleaned in the surrounding seawater. We combined the apical segments (~1 cm length) of the five thalli belonging to each transplant and flash froze the pooled sample in liquid N. We expected these apical segments were completely developed within the site where they were exposed according to the growth rates defined by García-Seoane et al. [65]. We stored the samples at −80ºC until DNA extraction. More information on transplant preparation and exposure can be found in García-Seoane et al. [33].

2.3. DNA extraction

We isolated genomic DNA from a total of 92 samples: 12 samples (3 replicates per population) not subjected to the reciprocal transplant experiment, which serve as a "reference" for the initial status of each population, and 80 transplants (5 replicates x 4 origin populations x 4 sites). We followed the cetyltrimethylammonium bromide (CTAB) high quality DNA extraction protocol for recalcitrant plant tissues adapted from McLay [66] (https://www.protocols.io/view/high-quality-dna-extraction-protocol-from-recalcit-i8jchun). Briefly, we ground 20–80 mg of frozen tissue from each sample inside a 2-mL Eppendorf tube with a 1 mm stainless steel bead in a TyssueLyser II (Qiagen) for 3 min. We immersed the samples in liquid N every 30 s to prevent thawing. After grinding, we added to each sample 1 mL STE extraction buffer: 8 % sucrose, 3 % Tris-HCl pH 8 (1 M), 10 % EDTA (0.5 M) and H_2O. We vortexed and centrifuged the mixture at 5000 rpm for 10 min, discarding the supernatant. Then, we added to each tube 500 μL of pre-warmed CTAB buffer: 10 % Tris-HCl pH 8 (1 M), 30 % NaCl (5 M), 5 % EDTA (0.5 M), 2 % CTAB, 2 % PVP, 0.2 % BME and H_2O, plus 100 μL of NaCl (5 M):BSA (4 %) solution (5:1). We vortexed and incubated the mixture overnight at 65ºC. Thereafter, we added 450 μL of chloroform-isoamyl alcohol (24:1) to each sample and vigorously inverted by hand for 5 min and centrifuged each tube for other 5 min at maximum speed. We recovered the top aqueous phase (~400 μL) to a new tube and repeated the chloroform step. Again, we recovered the top aqueous phase to a 1.5-mL tube and added 500 μL of 2-propanol to aid DNA precipitation. We inverted by hand for 5 min and incubated each tube for 20 min at room temperature. After centrifugation for 10 min at maximum speed,

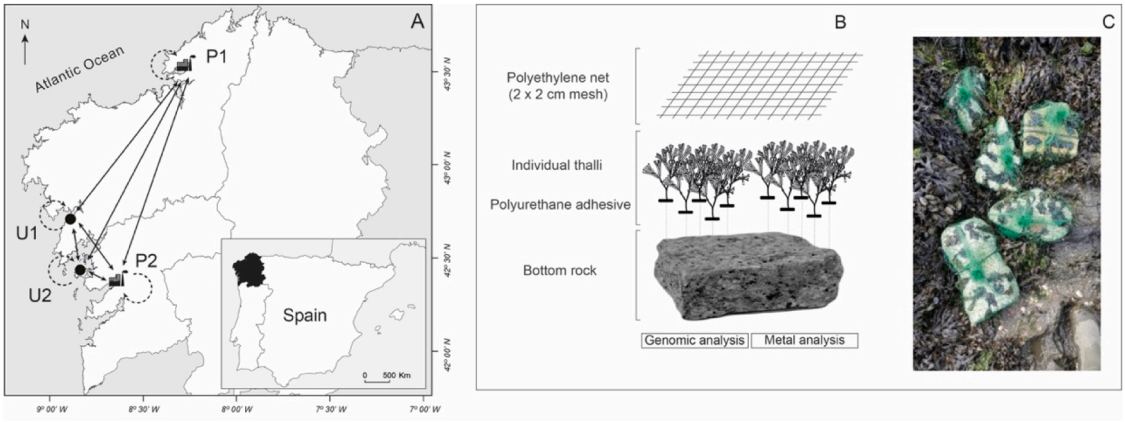

Fig. 1. Experimental design. A) Map showing the location of the four sites, two "polluted" (P1 and P2) and two "unpolluted" reference (U1 and U2) sites, where *Fucus vesiculosus* samples were collected (original populations) and reciprocally transplanted in Galicia (NW Spain). Dashed arrows represent samples transplanted back in to their home environment whereas solid arrows represent samples transplanted into foreign or "away" sites. B) Illustration of an algae transplant consisting of 10 individual thalli, 5 for (epi)genetic and 5 for metal analyses. C) Image of 5 replicate transplants exposed in the field.

we discarded the supernatant and washed the pellet in 500 μL of 70 % ethanol, incubated for 30 min at room temperature and centrifuged for 5 min at maximum speed. Finally, we discarded the ethanol, air-dried (~30 min) and resuspended the pellets in 50 μL of molecular water.

We checked the quality of extracted DNA using the NanoDrop (Nanodrop™ 8000 Spectrophotometer; Thermo Scientific), selecting only samples with high purity ($A_{260/280}$ and $A_{260/230}$ ratios of at least 1.8 and 1, respectively). For the samples that did not meet the purity standards, we performed an extra purification step using SPRI (Solid Phase Reversible Immobilization) magnetic beads (HighPrep™ PCR; MAGBIO Genomics, Inc.), as recommended by Fort et al. [67] and Catarina Medeiros (personal communication). For this, we added 0.6x SPRI beads (at room temperature) to the DNA, mixed them thoroughly using the pipette, and incubated for 5 min at room temperature. Then, we placed the samples on a magnetic rack (DynaMag™−2 Magnet, Thermo Fisher Scientific) until the solution cleared, and removed the supernatant from the tubes carefully, avoiding disturbing the beads. We washed the beads twice with 500 μL of 80 % ethanol for 30 s and then removed the ethanol from the tubes. After that, we air-dried the beads for 10 min to remove residual ethanol and added 50 μL of molecular water. We incubated the mixture for 5 min and again placed the tubes on the magnet until the solution cleared. Finally, we transferred the DNA to a new tube. Four out of the 92 samples did not meet the DNA quality standards even after the purification step and were not included in downstream analyses.

We quantified the concentrations of DNA using the Qubit 3.0 Fluorometric dsDNA BR assay kit (Q32851; Life Technologies).

2.4. epiGBS library preparation and DNA sequencing

We digested 400 ng of DNA from each sample overnight at 37 °C in a volume of 40 μL, containing 1x NEBuffer 3.1, 125 μg BSA (NEB, B9000S) and 2 μL/40 units of the restriction enzyme *Pst*I (NEB, R0140S). Then, we ligated 1200 pg (of both forward and reverse) methylated and non-phosphorylated barcoded adapters to digested DNA fragments in a reaction containing 40 μL DNA digest, 1x T4 DNA ligase buffer and 4000 units T4 DNA ligase (NEB, M0202M/L). We ran the ligation reaction for 3 h at 22 °C followed by 4 °C overnight. We allocated the samples across eight pools and cleaned the pools using the QIAquick PCR purification procedure (Qiagen 28104). We size-selected DNA fragments greater than 100 bp using 0.8x SPRI beads. We performed nick translation (1 h at 15 °C) in a reaction containing 18 μL of the purified library, 2.5 μL of 10 mM 5-methylcytosine dNTP Mix (Zymo research, D1030), 1x NEBuffer 2 and 7.5 units of DNA polymerase I (NEB, M0209S). We used the EZ DNA Methylation-Lightning™ Kit (Zymo Research) to bisulfite treat the DNA (20 μL of the nick-translated library). We performed epiGBS PCR Library amplification with the KAPA HiFi HotStart Uracil+ ReadyMix (Roche) under the following PCR conditions: initial denaturation step at 95 ºC for 3 min; 20 cycles of 98 ºC for 10 s, 65 ºC for 15 s, 72 ºC for 15 s; and 72 ºC for 5 min. We pooled libraries at equimolar concentrations and assessed their quality using a High Sensitivity DNA chip on a 2100 Bioanalyzer system (Agilent). Libraries were considered suitable for sequencing if the majority of DNA fragments were 100–600 bp. Finally, libraries were sequenced on the Illumina HiSeq X™ Ten Sequencing System (2 ×150 bp) at Novogene (HK) Company Limited in Hong Kong.

2.5. Data processing and filtering

We used the pipeline provided by [59] with a bug-fix modification (https://github.com/MWSchmid/epiGBS_Nov_2017_fixed) to process the raw sequencing files. First, this pipeline involves raw read demultiplexing and read quality trimming. Then, these reads are used to create a *de novo* reference, and later mapped to the *de novo* reference to perform strand-specific single nucleotide polymorphisms (SNPs) and single methylation polymorphisms (SMPs) calling. The unfiltered SNP and SMP datasets consisted of 492,414 SNPs and 1514,636 SMPs respectively across 88 samples. These were filtered as specified below.

First, we performed a "soft" filtering step by removing SNPs and SMPs without a minimum coverage of 3 and a maximum coverage equal to the 99th percentile of the read coverage distribution in at least one replicate sample per group out of 20 groups in total. Group was defined as each unique combination of "*origin site*" (with four levels corresponding to each of the four populations), "*exposure site*" (with four levels corresponding to each of the populations and one extra level corresponding to the unexposed samples that served as a reference status of the macroalgae at the beginning of the experiment), and "*pollution level*" (with two levels corresponding to the pollution status of the exposure site – polluted vs. unpolluted). Second, we removed the samples with very poor sequencing output, i.e., lacking more than 50 % of the SNPs and 60 % of the SMPs. Finally, after removing 15 samples with poor sequencing, we removed SNPs and SMPs without a minimum coverage of 10 and a maximum coverage equal to the 99th percentile of the read coverage distribution in at least one replicate sample per group. The filtered datasets consisted of 36,131 SNPs and 67,252 SMPs across 73 samples (11 unexposed samples and 62 transplants with a minimum of 2 and a maximum of 5 replicate samples per group). In the methylation dataset, we additionally removed 3,506 SMPs called on the same cytosine as a SNP resulting in a final working dataset of 63,746 SMPs.

2.6. Statistical analysis

All analyses were performed in R v.3.5.1 (R [68]) running under R Studio v.1.2.5019 (RStudio [69]).

2.6.1. Phenotypic analyses

To test whether populations differed significantly in their heavy metal content profiles (phenotype) before and after the transplantation experiment, we used distance-based redundancy analysis (dbRDA) [70]. This is a constrained ordination technique that summarizes the main patterns of variation in a response distance matrix that can be explained by a set of explanatory variables. For this, we first created a phenotypic Euclidean distance matrix using the function *daisy* from the R package cluster [71] on the standardized concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, and Zn across 70 samples – the concentrations of all metals were not available for 3 of the samples included in the SNP and SMP datasets. Then, we ran two different models using the function *capscale* implemented within the vegan package [72]. In the first one, we tested for phenotypic differences before transplantation using the subset of 11 unexposed samples (3 replicate samples per population except U2 which had 2 replicates) with the model: "*Phenotypic Distance Matrix ~ Origin Site*". In the second one, we tested for phenotypic differences after transplantation using the subset of 59 transplants with the model: "*Phenotypic Distance Matrix ~ Origin Site: Exposure Site*". We tested the significance of the model using a permutation test with 9999 permutations and obtained adjusted R^2 values using the function *RsquareAdj* from the vegan package. We visualized the structure of the data in a 2-D space using the constrained ordination axes (RDA axes). Finally, we performed multilevel pairwise comparisons to assess which populations differed in the heavy metal content profiles using the function *pairwise.adonis* implemented within the pairwiseAdonis package [73], with 999 permutations and adjusted *p* values using the false discovery rate (fdr) method.

2.6.2. Genetic analyses

We tested for genetic differentiation among populations using two different methods, dbRDA and the estimation of fixation index values (Fst) [74]. For dbRDA, we created a genetic distance matrix calculating the average distance of all per-SNP differences between two samples using the filtered SNP dataset (36,131 SNPs across 73 samples). For each SNP, the distance was set to 0 if both alleles were identical, 1 if both alleles were different, and 0.5 if one allele was different. Then, we ran

the model "*Genetic Distance Matrix ~ Origin Site*" to test for overall genetic differentiation among populations, and tested the significance of the model and obtained the adjusted R^2 value as explained above. We visualized the structure of the data using the RDA axes. Finally, we ran multilevel pairwise comparisons to assess which population pairs differed significantly as described above.

To estimate the overall (across all populations) and pairwise Fst values, we used the functions *wc* and *genet.dist* respectively, from the package hierfstat [75] on the working SNP matrix (14,033 SNPs across 11 unexposed samples). To look for evidence of significant population differentiation we tested whether pairwise Fst values were significantly different from zero, by calculating the confidence intervals of the pairwise Fst values with the function *boot.ppfst*, from hierfstat, and 999 permutations.

Finally, we used Google Earth v10.52.0.0 (https://earth.google.com) to estimate the linear geographic distances between each pair of populations and visualize it against the pairwise Fst values.

2.6.3. Epigenetic analyses

First, we estimated the methylation level of each SMP within each individual sample as the number of reads mapping to one position showing evidence of methylation divided by the total number of reads mapping to that position, using the filtered DNA methylation matrix (67,252 SMPs across 73 samples). Second, we calculated mean and standard deviation of DNA methylation for each sequence context (CG, CHG, CHH) and across all contexts together for each group.

Then, we used dbRDA to test whether population of origin, exposure site, or their interaction explained a significant proportion of genome-wide epigenetic variation in the samples. For this, we created a pairwise epigenetic distance matrix by calculating the average difference in DNA methylation level across all available cytosines between each pair of samples using the filtered SMP matrix (63,746 SMPs across 73 samples). To test for epigenetic differentiation among populations before the reciprocal transplant experiment, we ran the model "*Epigenetic Distance Matrix ~ Origin Site*" on the subset of 11 unexposed samples for all contexts together, and separately for each sequence context. To test for epigenetic differentiation after the reciprocal transplant experiment, we ran the model "*Epigenetic Distance Matrix ~ Origin Site: Exposure Site*" on the subset of 62 transplants, for all contexts together and separately for each sequence context. We tested the significance of the models, obtained the adjusted R^2 values and visualized the structure of the data as explained above. For the models that were significant, we ran an additional partial constrained dbRDA that allowed us to assess whether our predictors were still significant after controlling for the epigenetic variation explained by the genetic variation. For this, we first summarized the genetic data using principal component analysis (PCA), and selected the first 10 principal components, which explained 24.3 % of the total genetic variation, and then ran the models "*Epigenetic Distance Matrix ~ Origin Site: Exposure Site + Condition (PCs from genetic data)*" for all contexts together and separately for each sequence context.

Additionally, we used multiple matrix regression with randomization (MMRR) analysis as in Wang [76] and adapted from Herrera et al. [77] to simultaneously assess the effect of (i) genetic, phenotypic, and environmental distances (predictors) on the epigenetic distance matrix (response) – for all contexts together and separately for each sequence context – and (ii) genetic, epigenetic (all contexts together), and environmental distances (predictors) on the phenotypic distance matrix (response). The pairwise environmental distance matrix was obtained using the *daisy* function with the Gower metric (0 for polluted sites and 1 for unpolluted sites), based on whether the samples shared origin, exposure site, both, or none. Hence, samples from P sites transplanted to P sites would have a value of (00), samples from U sites transplanted to U sites would have a value of (11), samples from P sites transplanted to U sites would have a value of (01), and samples from U sites transplanted to P sites would have a value of (10). Both origin and exposure site contributed equally to the distance value. Before the analyses, we scaled and centered (mean = 0, SD = 1) each dissimilarity matrix to obtain comparable standardized linear regression coefficients between predictor matrices. Then, we used the *MMRR* function in R, developed by Wang [76] and available from the Dryad Data Repository (https://doi.org/10.5061/dryad.kt71r), with 9999 permutations. This analysis was performed on a subset of 70 samples that were common to all four datasets.

Finally, we identified single differentially methylated cytosine positions (DMPs) in response to transplantation using the R package DSS [78] and a matrix of 8343 cytosines that were present in at least two samples per group (1877 positions in CG – 22 %; 2690 positions in CHG – 32 %; and 3776 positions in CHH – 45 %). For this, we first used the function *DMLfit.multifactor* to model the methylation frequency at each cytosine position within each group using a beta-binomial distribution with arcsine link function and the formula "$\sim 0 + group$" (no intercept). Then, we used the function *DMLtest.multifactor*, that performs Wald tests to detect differential methylation between groups at each position and reports adjusted *p* values using the fdr method. Only cytosines with fdr ≤ 0.05 and a methylation change between groups of at least 10 % were considered DMPs. We used the following contrasts within each population: (i) to test the effect of transplantation sensu stricto (i.e., transplantation in situ), we compared methylation levels between transplants in their home sites and unexposed samples for each population; (ii) to test the effect of transplantation into foreign sites (ex situ), we compared methylation levels between "home" versus "away" transplants for each population.

3. Results

3.1. Phenotypic differentiation

The dbRDA showed that populations differed significantly in their heavy metal concentration profiles both before and after the transplantation experiment (before: F value = 154.7, *p*-value = 0.003; after: F value = 8983, *p*-value = 0.0001; Table S2). Before transplantation, samples clearly grouped by population of origin (Fig. 2A), which explained 97.9 % of the phenotypic variation (Table S2). The first constrained ordination axis separated the samples by pollution level, with U1 and U2 samples located towards the left-hand side of the plot, and P1 and P2 samples located towards the right-hand side of the plot (Fig. 2A). The permutation test carried out to perform multiple pairwise comparisons, however, showed no significant differences between any of the population pairs (Table S3), which may be due to the low number of replicate samples within each population of origin (between 2–3 per population).

After transplantation, samples grouped by transplant site regardless of their population of origin (Fig. 2B). The full model explained 67.4 % of the phenotypic variation and both main effects were significant (origin and transplant site), but not their interaction (Origin: F value = 4.612, p value = 0.0002; Exposure: F value = 36.71, p value = 0.0001; Origin: Exposure: F value = 1.197, p value = 0.252; Table S2). The first constrained ordination axis also separated, though less clearly, the samples by the pollution level of the site where they were transplanted (Fig. 2B). The permutation test carried out to perform multiple pairwise comparisons for samples grouped by origin site (the first main effect) showed again no significant differences between any of the population pairs. Yet, when grouped by transplant site, the test showed significant differences between all pairwise comparisons (Table S3).

3.2. Genetic structure

The dbRDA showed that populations were significantly genetically differentiated (F value = 3.275, p-value = 0.0001; Table S2), with population of origin explaining 9 % of the genetic variation (Fig. 3). The permutation test carried out to perform multiple pairwise comparisons showed significant differences between all pairs (Table S3). The overall

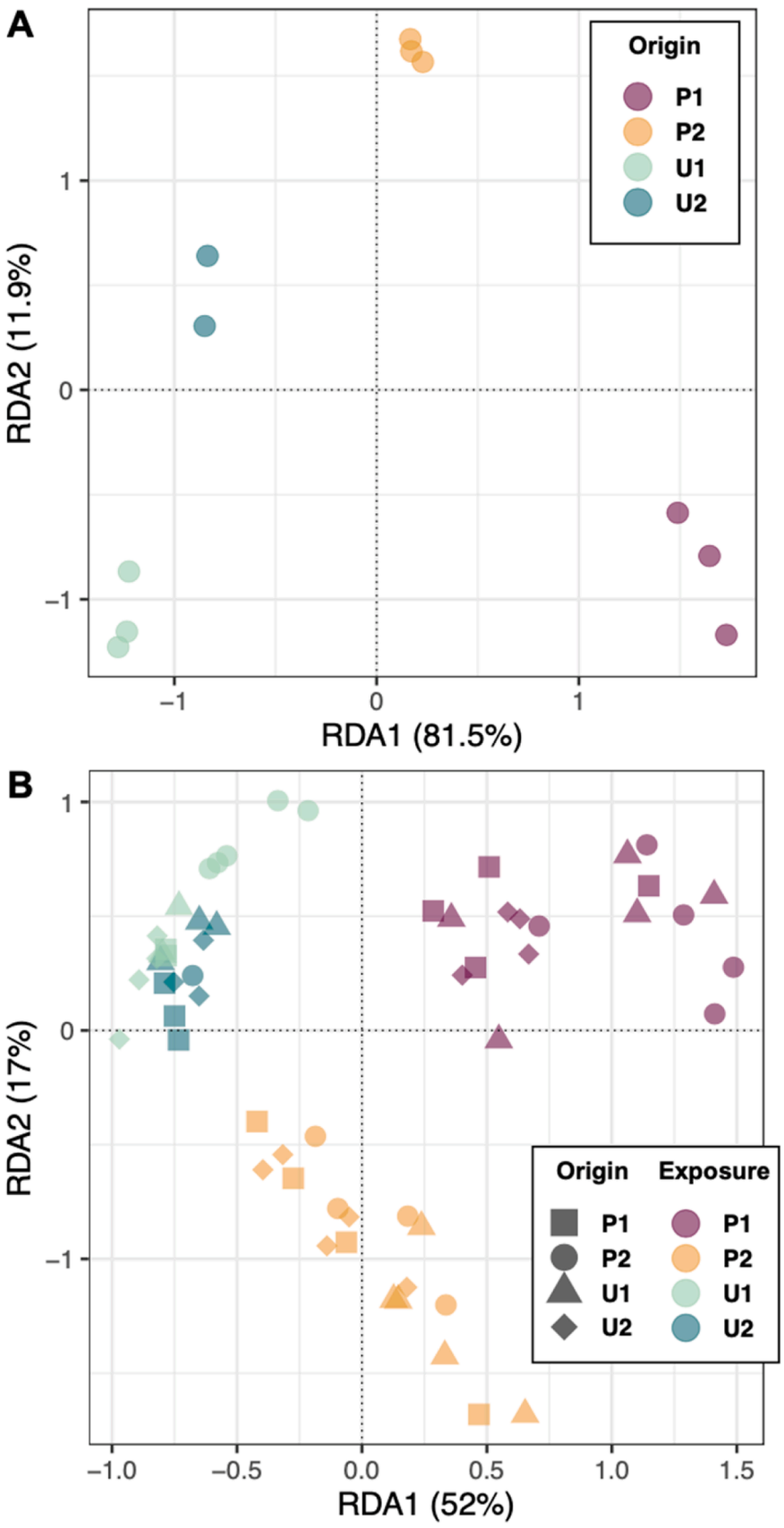

Fig. 2. Phenotypic differentiation plots. Ordination plots of the distance-based redundancy analysis (dbRDA) carried out to test for phenotypic differences – based on the heavy metal content profiles of the samples – among *Fucus vesiculosus* populations before (A) and after the transplantation experiment (B). RDA1: first constrained ordination axis; RDA2: second constrained ordination axis. Samples are colored by population of origin in (A). In (B), symbol shapes represent the population of origin and colors represent the site where samples were exposed during the experiment. U1, U2: unpolluted sites 1 and 2; P1, P2: polluted sites 1 and 2.

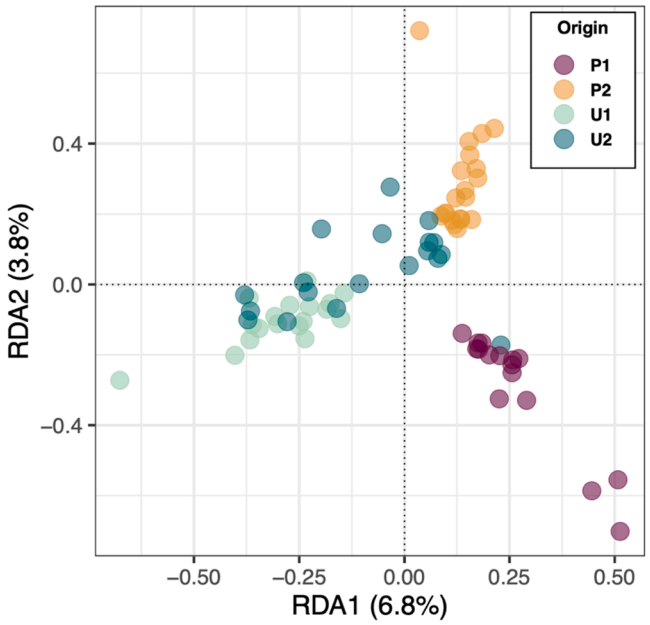

Fig. 3. Genetic structure plot. Ordination plot of the distance-based redundancy analysis (dbRDA) carried out to test for genetic differences among four populations of *Fucus vesiculosus*. RDA1: first constrained ordination axis; RDA2: second constrained ordination axis. Samples are colored by population of origin. U1, U2: unpolluted sites 1 and 2; P1, P2: polluted sites 1 and 2.

Fst value was 0.108, which is indicative of moderate genetic differentiation among populations. Pairwise Fst values ranged between 0.067 (between U2 and P2) and 0.138 (between U1 and P2), and the permutation test corroborated that all population pairs differed significantly (Table 1).

The pairwise Fst values did not increase linearly with the increase in the geographic distance between populations (Fig. S1). Instead, the two more geographically distant populations pairs (P1-P2 and P1-U2) showed the lowest pairwise genetic differentiation values.

3.3. DNA methylation levels and changes in response to transplantation

Mean DNA methylation levels per group ranged between 10.0–15.7 % in all contexts together, and 4.4–10.5 %, 22.8–27.5 %, and 4.3–10.5 % in the CG, CHG and CHH contexts respectively (Fig. 4; Table S4).

Populations were not significantly epigenetically differentiated before the reciprocal transplant experiment (Table 2, Fig. S2). At the end of the experiment, the model including origin, exposure site and their interaction was significant for all contexts together and the CHG and CHH contexts (Table 2, Fig. S3). The variance explained by these models was, however, rather low (adjusted R^2 values ranged between 1.8 % and 2.0 %). The interaction term was significant in the models ran with all contexts together and CHG and CHH contexts, whereas the main effect of population of origin was only significant in the CHG context (Table 2). Yet, when accounting for the variance explained by the genetic component, epigenetic differentiation was no longer significant (Table 2).

The overall regression model in the MMRR analysis significantly explained 14.1 % of the variation in phenotypic distances (F p-value = 0.001; Table 3), and between 33.5 % (CHG context) and 38.1 % (CHH context) of the variation in the epigenetic distances across samples (F p-values = 0.001; Table 3). Only environmental distances explained a significant proportion of the variation in phenotypic distances (regression coefficient = 0.361; t p-value = 0.001), whereas the variation in epigenetic distances was only significantly explained by genetic distances (regression coefficients ranged between −0.442 and −0.517; t p-values = 0.001).

The total number of DMPs ranged between 38 (0.5 % of all tested positions in home transplants from P1) and 268 (3.2 % in foreign transplants from U2 in U1) when considering all contexts together (Table S5). Even though the number of DMPs within each sequence context differed quite a bit in some contrasts, the proportion of DMPs out of the total number of cytosines analyzed per context was rather similar within each contrast (Table S5). Transplantation in situ resulted in more point cytosine methylation changes in samples from unpolluted sites (with 2.4 % and 2.5 % of DMPs in U1 and U2 respectively) than in samples from polluted sites (with 0.5 % and 1.2 % of DMPs in P1 and P2 respectively). The highest proportion of DMPs was found in foreign transplants in P1 and U1 (% of DMPs $\geq$ 3 %). Transplantation into their home sites resulted in a much higher proportion of hypermethylated (increase in methylation level) than hypomethylated (decrease in methylation level) DMPs in U1 and P1 (in transplants from U2 and P2, the proportion of hyper- and hypomethylated DMPs was more balanced) (Fig. 4). Interestingly, transplantation into foreign sites resulted in hypomethylation of most DMPs in transplants from U1 and U2, whereas in those from P1 the vast majority of DMPs were hypermethylated (Fig. 4).

Table 1
Pairwise Fst values between four populations of *Fucus vesiculosus* assessed in this study. All population pairs were significantly genetically differentiated. U1, U2: unpolluted sites 1 and 2; P1, P2: polluted sites 1 and 2. The overall Fst value was 0.108.

	U1	P2	U2
P2	0.138		
U2	0.104	0.067	
P1	0.131	0.075	0.071

4. Discussion

In a former study, we showed that populations of *F. vesiculosus* growing naturally in relatively unpolluted areas that were transplanted into polluted sites reached significantly higher metal concentrations in their thalli than populations native to those polluted environments and transplanted within their site of origin (i.e., U1 and U2 transplants exposed in P1 and P2 showed higher levels of most metals than home transplants of P1 and P2 respectively - Table S1). Hence, we hypothesized that macroalgae that had been chronically exposed to high concentrations of metals could have responded to this pressure by limiting their metal uptake capacity. Here, we performed next generation sequencing on samples from the same reciprocal transplant experiment to elucidate some of the molecular mechanisms - DNA sequence and DNA methylation polymorphisms - associated with such phenotypic differentiation. Our results showed that the populations studied had distinctive heavy metal content profiles and that, when transplanted into other sites, these profiles shifted towards those of the local residents of the sites. This result is consistent with the fact that environmental distances experienced by the transplants explained a significant proportion of the variation in the phenotypic distances. Yet, a great deal of such variation remained unexplained (85.9 %), and neither genetic nor epigenetic differentiation accounted for a significant fraction of it. We hypothesize that the lack of association between molecular patterns and phenotypic patterns could be due to three main reasons: limitations of the sequencing approach, the high complexity of the environment, and a potentially limited role of DNA methylation in brown macroalgae adaptation to environmental stress.

Reduced representation bisulfite sequencing tools, have boosted important advances in ecological and evolutionary epigenetics research in the recent years. For example, using epiGBS, Ibañez et al. [79] suggested that DNA methylation can be used to trace genealogical relationships in clonal plant species with very limited genetic variation.

Table 2
Results of the distance-based redundancy analysis (dbRDA) carried out to test the effect of population of origin (Origin), transplant site (Exposure), or their interaction (Origin: exposure) on genome-wide variation in DNA methylation before (EpiDist-Initial) and after the transplantation experiment (EpiDist-Final) for all context together (all) and independently for each sequence context (CG, CHG, CHH). The results of the partial constrained dbRDA to account for the variance explained by the genetic structure of the samples (EpiDist_Final ~ Origin: Exposure + Condition(PCs_genetics)), as well as the results of the ANOVA performed to test for the significance of each predictor in the model with the interaction term (ANOVA by terms) are also shown. Ctxt: sequence context; Df: degrees of freedom; SumOfSqs: Sum of squares; F: value of the F statistic; Pr(>F): p value; ns: not significant; * : $p < 0.05$.

Model	Ctxt		df	SumOfSqs	F	Pr (>F)
EpiDist_Initial ~ Origin	all	model	3	16.20	1.052	0.262 ^{ns}
		residual	7	35.95		
	CG	model	3	17.82	1.058	0.270 ^{ns}
		residual	7	39.31		
	CHG	model	3	13.43	1.102	0.115 ^{ns}
		residual	7	28.44		
	CHH	model	3	17.05	1.002	0.487 ^{ns}
		residual	7	39.72		
EpiDist_Final ~ Origin: Exposure	all	model	15	0.115	1.071	0.032*
		residual	46	0.330		
	CG	model	15	20.84	1.050	0.096 ^{ns}
		residual	46	60.86		
	CHG	model	15	15.32	1.082	0.016*
		residual	46	43.43		
	CHH	model	15	20.33	1.069	0.044*
		residual	46	58.30		
EpiDist_Final ~ Origin: Exposure + Condition(PCs_genetics)	all	model	15	19.96	1.064	0.153 ^{ns}
		residual	36	45.04		
	CG	model	15	22.26	1.049	0.230 ^{ns}
		residual	36	50.92		
	CHG	model	15	15.81	1.054	0.184 ^{ns}
		residual	36	36.01		
	CHH	model	15	21.71	1.072	0.137 ^{ns}
		residual	36	48.59		

ANOVA by terms	Ctxt	Predictor	df	Variance	F	Pr(>F)
EpiDist_Final ~ Origin: Exposure	all	Origin	3	3.661	1.038	0.187 ^{ns}
		Exposure	3	3.648	1.034	0.212 ^{ns}
		orig: exp	9	11.57	1.094	0.026*
		Residual	46	54.08		
	CHG	Origin	3	3.166	1.118	0.025*
		Exposure	3	2.935	1.036	0.213 ^{ns}
		orig: exp	9	9.219	1.085	0.031*
		Residual	46	43.43		
	CHH	Origin	3	3.887	1.022	0.279 ^{ns}
		Exposure	3	3.938	1.036	0.220 ^{ns}
		orig: exp	9	12.51	1.096	0.030*
		Residual	46	58.30		

Using the same tool, Mounger et al. [80] found that epigenetic variation may be important for the persistence of genetically depauperate plant species in challenging environments. These authors also provided evidence of a significant association of epigenetic variation with phenotypic variation in natural plant populations of the foundation species *Spartina alterniflora* [57]. More closely related to this study, Boquete et al. [45] found a limited, but population-specific epigenetic response to heavy metal exposure in bryophytes. The utility of epiGBS to reliably characterize epigenetic changes in response to stress in plants, has recently been corroborated by Troyee et al. [81] in a comparative study of the results of epiGBS and whole genome bisulfite sequencing (WGBS) on the effects of herbivory on DNA methylation in the Lombardy poplar tree.

Even though the results presented above are promising, interpreting the magnitude of the evidence presented should be taken with care. Several of the previous studies were done on clonal plant species which simplifies the genomic landscape and its potential interactions with the epigenome [45,79,81]. Additionally, the effects of heavy metal exposure, low light, and herbivory on DNA methylation in these studies were tested in common garden experiments which greatly simplifies the environment, and the potential interacting effect of other environmental conditions. The three studies carried out in field-collected plants showed significant, but limited results. For example, Mounger et al. [80] showed that genome-wide epigenetic variation was significantly structured among six field populations of the red mangle *Rhizophora mangle*; the proportion of epigenetic variation explained by population, however, was low (1.96 % across all contexts together and 2.97 % in the CG context). In Mounger et al. [57], most of the epigenetic variation in field plants of *S. alterniflora* was explained by genetic variation. Finally, van Moorsel et al. [82], showed evidence of significant DNA methylation divergence in response to selection history in several grass species, however, whether such divergence was independent of the underlying genetics was unclear. Hence, as pointed out by McNew et al. [83], complex ecological epigenetics studies demand higher sequencing power to detect subtle or more complex effects. The cost of sufficiently increasing sequencing power, however, may be prohibitive.

The four populations of *F. vesiculosus* studied here inhabit one of the most spatially and temporally heterogeneous habitats, the intertidal rocky shore [84,85]. Over time, and due to the tide action, these algae are exposed to daily cycles of desiccation, temperature extremes, UV exposure, waves, and winds [86]. The relative impact of these stressors also varies seasonally. Across space, the presence of rock crevices and overhangs create a great variety of microenvironments where individual thalli are differentially exposed to waves, wind, light, and other conditions [86]. Additionally, the two polluted populations (P1 and P2) were located in the inner part of the rias where they are more protected from wave and wind action, and more exposed to tide action and fresh water flowing from the rivers, than the reference populations (U1 and U2) located in open shore habitats [87]. Finally, our sampling strategy, aimed to gather as much variability - phenotypic, genetic, epigenetic - as

Table 3
Results of the multiple matrix regression with randomization (MMRR) analysis carried out to test the joint effect of pairwise genetic (GenDist), epigenetic (EpiDist) and environmental (EnvironDist) distances on the phenotypic (PhenoDist) distance matrix, and the joint effect of pairwise genetic, phenotypic and environmental distances on the epigenetic distance matrices (for all contexts together - EpiDist-all - and separately for CG - EpiDist-CG -, CHG - EpiDist-CHG -, and CHH - EpiDist-CHH). Coeff.: model regression coefficients for each predictor; tStat: value of the t statistic; tPvalue: p value of the t statistic; Fstat: value of the F statistic; FpVal: p value of the F statistic; R^2: total variance explained by the model; ns: not significant; * : $p < 0.05$; ** : $p<0.01$.

Response	Predictor	Coeff.	tStat	tPvalue	Fstat	FpVal	R^2
PhenoDist	Intercept	0.018	0.772	0.770 ^{ns}			
	GenDist	0.030	0.981	0.744 ^{ns}			
	EpiDist	−0.015	−0.389	0.914 ^{ns}			
	EnvironDist	0.361	16.44	0.001**			
	Overall model				93.1	0.001**	0.141
EpiDist-all	Intercept	0.203	14.51	0.002**			
	GenDist	−0.495	−31.62	0.001**			
	PhenoDist	−0.006	−0.389	0.931 ^{ns}			
	EnvironDist	0.036	2.421	0.289 ^{ns}			
	Overall model				334.2	0.001**	0.370
EpiDist-CG	Intercept	0.206	14.54	0.002**			
	GenDist	−0.512	−32.23	0.001**			
	PhenoDist	0.002	0.153	0.971 ^{ns}			
	EnvironDist	0.036	2.375	0.319 ^{ns}			
	Overall model				346.6	0.001**	0.379
EpiDist-CHG	Intercept	0.200	14.86	0.003**			
	GenDist	−0.442	−29.30	0.001**			
	PhenoDist	−0.010	−0.652	0.861 ^{ns}			
	EnvironDist	0.033	2.256	0.345 ^{ns}			
	Overall model				287.2	0.001**	0.335
EpiDist-CHH	Intercept	0.199	13.99	0.005**			
	GenDist	−0.517	−32.38	0.001**			
	PhenoDist	−0.008	−0.501	0.914 ^{ns}			
	EnvironDist	0.039	2.550	0.288 ^{ns}			
	Overall model				350.5	0.001**	0.381

possible within each population. Taken together, the high variability in the degree of exposure to multiple environmental stressors (whose effects may be cumulative, synergistic or antagonistic), together with the technical limitations mentioned above, may explain our inability to identify a signature of genome-wide (epi)genetic patterns associated with the phenotypic differentiation in this study.

The DNA methylation levels found in this study for *F. vesiculosus*, and in other studies for brown algae [88-91], are very low compared to, for example, flowering plants [92,93]. It has been recently suggested that low DNA methylation levels in brown algae could be due to the loss genes encoding DNA methylation enzymes during the emergence of this group [94]. Hence, we cannot rule out that the lack of significant epigenetic differentiation could be also due to, at least in part, a limited role of DNA methylation in epigenetic regulation in brown macroalgae or limited heritability in this group. A more in-depth analysis of the *F. vesiculosus* methylome, and the study of other epigenetic mechanisms (e.g., histone modifications or small, non-coding RNAs) may help elucidate the actual role of epigenetic mechanisms on brown algae adaptation to environmental stress.

In spite of the lack of association with phenotype, we found evidence of genetic differentiation among the studied populations. Genetic differentiation has been previously reported in this species [95-98] even at very short spatial scales – i.e., between stands within populations [98]. This genetic differentiation has sometimes conformed to an isolation-by-distance (IBD) model whereby genetic differentiation levels increased with geographic distance (e.g., [96,98]). The reproductive mode of the species could explain such results. *Fucus vesiculosus* is dioecious, that is, male and female gametes are produced in different thalli and released into the environment for fertilization. Both gametes are short lived which makes it unlikely that they travel long distances before fertilization [99-101]. After fertilization, the zygote settles relatively close to the parent plants. Additionally, clonality has been reported several times within this species [96,102,103], which may also contribute to population differentiation at relatively short spatial scales (but see [104] for evidence of long distance dispersal of floating *F. vesiculosus* propagules).

Genetic differentiation in this study, however, does not fit an IBD model. This is not surprising given the extreme complexity of the hydrodynamics of the Galician coast, which may prevent dispersal even within relatively short distances. The movement of water in the rias is strongly influenced by the direction of the predominant winds, entrance of seawater from the continental shelf, inputs of freshwater from the rivers, orientation of the rias, local topology of the coast, the tides, etc. [105,106]. Hence, gene flow could be strongly restricted, especially for populations inhabiting the innermost parts of the rias. Natural selection could thus partly explain the genetic patterns observed in this study. This includes but is not limited to, pollutant exposure - which varies in intensity within and between our study sites. Evidence of genetic differentiation was previously found in species of the same genus in response to salinity [107], and genotypic differences in tolerance to (a) biotic stress have been reported for *F. vesiculosus* (e.g., [108-110]). With our data, we cannot decipher which selective pressure(s), if any, might be responsible for such patterns.

Home transplantation induced significant point DNA methylation changes. This is not surprising, since thallus manipulation can cause some mechanical and osmotic stress [111]. Transplant shock - a minor or major setback in plant growth in response to transplantation - is frequently considered in agriculture and forestry [112-114]. The proportion of DMPs due to transplantation of thalli from both polluted sites into their home sites, however, was generally lower than the proportion due to transplantation into foreign sites (Fig. 4). Thus, changing environmental conditions seem to have a greater effect on DNA methylation than manipulation in these populations.

Transplantation to a different habitat led to population-specific changes in DNA methylation in *F. vesiculosus*, which is consistent with the findings of Boquete et al. [45]. These authors reported more hyperthan hypomethylated DMPs in the less metal tolerant populations of the copper moss *Scopelophila cataractae* in response to Cu and/or Cd exposure, whereas the more tolerant populations showed, in general, more hypo- than hypermethylated DMPs. Here, algae from the reference populations (U1 and U2) showed more hypomethylated DMPs whereas those from P1 showed more hypermethylated DMPs (Fig. 5). This

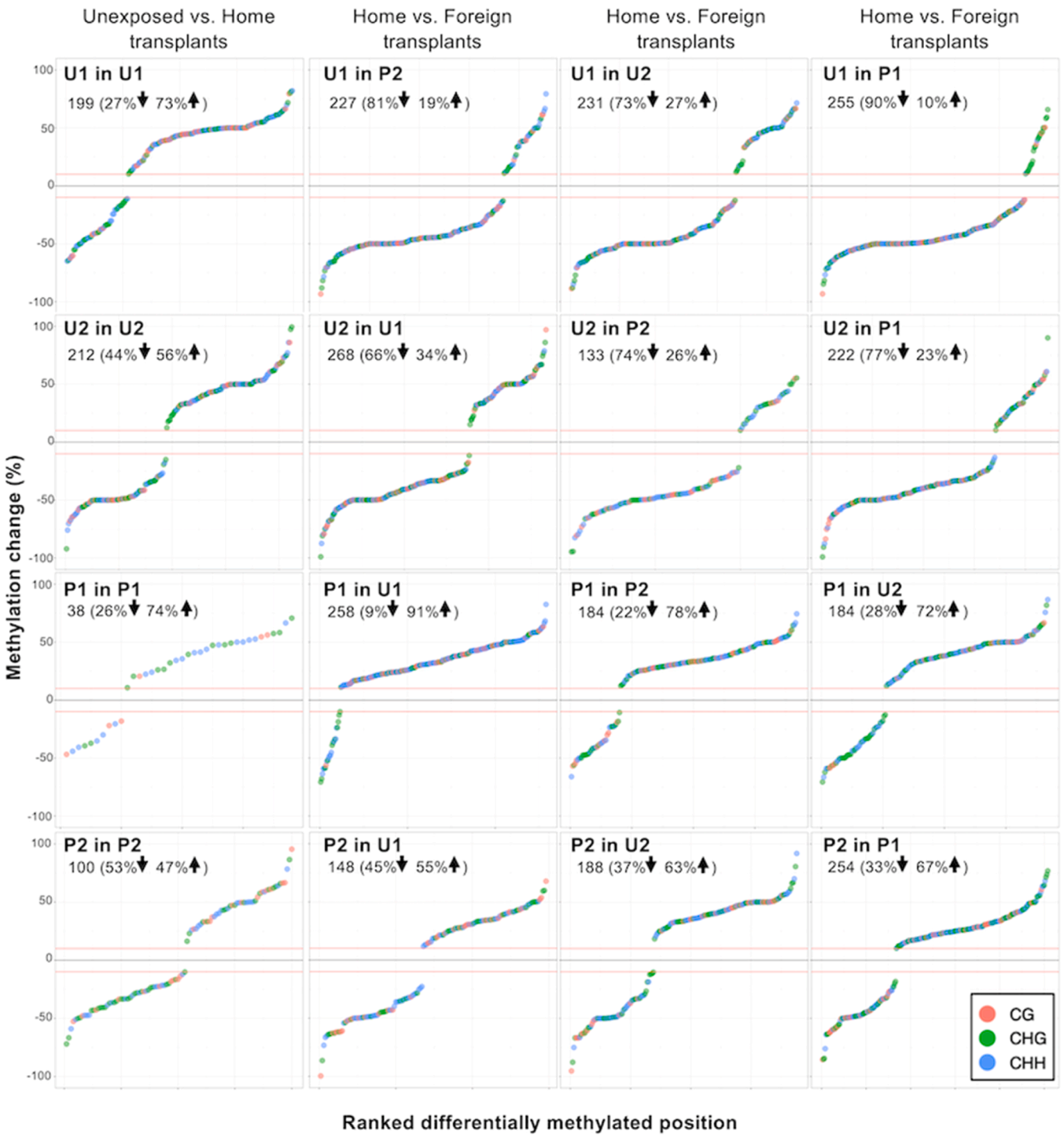

Fig. 4. Differential methylation analysis results. Methylation change of differentially methylated cytosine positions (DMPs; fdr < 0.05) between unexposed samples and transplants into their home environment (Unexposed vs. Home transplants' column) and between home transplants and foreign transplants (Home vs. Foreign transplants' columns) from each population of *Fucus vesiculosus* assessed in this study. Each graph contains information about the contrast represented, the total number of DMPs detected for that contrast, and the proportion of hyper (↑) and hypomethylated (↓) DMPs out of the total. The colors in the dots represent DMPs belonging to the CG (red), CHG (green), and CHH (blue) context. Red lines delimit the area in which absolute methylation differences are lower than 10 %. U1, U2: unpolluted sites 1 and 2; P1, P2: polluted sites 1 and 2.

contrasting result could be due to the combined effect of several abiotic stresses in the transplant sites, and not only to the effect of pollution. Additionally, considering that the transplantation time was rather long and – spanning the period of maximum growth for this species [115], we cannot rule out that some of the observed DNA methylation changes could be due to phenological changes, as previously reported in other plant groups (e.g., [116]). Yet, provided the relatively small geographic range in which our populations were sampled, we would expect that phenology-driven DNA methylation changes would not contribute too much to the population-specific DMPs reported here.

Finally, literature on the effect of metal exposure on DNA methylation has shown mixed results. For example, there is evidence of overall cytosine hypermethylation in response to Cd in *Posidonia oceanica* [43], radish [117], and rice [118], to Cr in *Brassica napus* [44], and Pb in *Zea*

mays [119]. Cadmium, Cr and Ni, however, induced cytosine hypomethylation in *Trifolium repens* and *Cannabis sativa* [46]. Hence, while DNA methylation changes in response to metal exposure have been shown to be metal- and species-specific, our results and those from Boquete et al. [45] support the contention that these changes are also population-specific.

5. Conclusions

In this study, we investigated the molecular basis of the intraspecific differentiation for heavy metal uptake capacity in the brown macroalgae *F. vesiculosus*. Our results showed that neither genetic nor epigenetic differentiation was associated with this phenotypic differentiation. We hypothesize that the complexity of the natural environment in which this study was carried out, where the combined effect of multiple stress factors with added, synergistic and/or antagonistic effects, could have generated more complex molecular patterns that could not be unraveled with the sequencing technique applied. Nonetheless, we provide additional evidence of intraspecific genetic differentiation in *F. vesiculosus* at rather limited spatial scales, as well as the first evidence of population-specific epigenetic changes in brown macroalgae in response to changes in environmental conditions (i.e. transplantation ex situ). These findings point towards a potentially important capacity of this species to adjust to changing environmental conditions. Yet, the extent of this capacity needs to be further investigated through, for example, targeted laboratory experiments, or using more powerful tools like whole genome bisulfite sequencing. These findings are important in the current context of global change, in which key foundation species like brown macroalgae, and hence, the ecosystems they sustain in coastal areas, are threatened by: pollution, anthropogenic development, tourism, invasive species, sea level rise, changes in water temperature and acidification, increased frequency of severe storms, etc.

Environmental implication

Heavy metals are highly toxic and impinge an important selective pressure upon all living organisms. Understanding how organisms respond to heavy metal exposure is thus pivotal to predict its potential impacts in natural ecosystems and optimize environmental protection efforts. Here, we provide the first evidence of population-specific epigenetic changes in brown macroalgae in response to changes in environmental conditions, including pollutant exposure levels. Although this needs further exploration, our results suggest that epigenetic mechanisms might contribute to the adaptive potential of this and other foundation species to pollution and other environmental stressors.

Author statement

RGS, **JRA**, **JAF** and **MTB** conceived and designed the experiments; **RGS** performed the field work; **RGS** and **MTB** performed the lab work; **MWS** and **MTB** performed the epiGBS data pre-processing; **MTB** performed the statistical analyses; **RGS** and **MTB** wrote the initial draft of the manuscript; **CLR**, **JRA**, **JAF** and **MWS** critically reviewed and edited the manuscript; **JRA**, **JAF** and **MTB** secured the funding for this study.

CRediT authorship contribution statement

M. Teresa Boquete: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marc W. Schmid:** Writing – review & editing, Methodology, Investigation, Data curation. **J. Ángel Fernández:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Jesús R. Aboal:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Christina L. Richards:** Writing – review & editing, Supervision, Methodology, Investigation. **Rita García-Seoane:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Conceptualization.

Data availability statement

The pipeline scripts used for this study are available at:https://github.com/thomasvangurp/epiGBS, with a bug-fix modification (https://github.com/MWSchmid/epiGBS_Nov_2017_fixed). The raw sequence datafiles for epiGBS (Illumina paired end reads) have been submitted to theSequence Read Archive (SRA) of NCBI under project number PRJNA1122084 (http://www.ncbi.nlm.nih.gov/bioproject/1122084).The barcodes (Fv_barcodes.tsv)required to process the raw epiGBS sequencing data available on SRA, thereference contigs (de_novo_reference.fa.gz), the SNPs (snps.vcf.gz), andthe methylation data (methylation.bed.gz) are available on zenodo (https://doi.org/10.5281/zenodo.11550298).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: there is nothing to declare If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136304.

Data availability

All the data will be publicly available upon publication. The links are available in the Data availability statement section.

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