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# Impact of sewage pollution on two species of sea urchins in the Mediterranean Sea (Cortiou, France): Radial asymmetry as a bioindicator of stress

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ABSTRACT

*Background:* The surroundings of the Cortiou sewage are among the most polluted environments of the French Mediterranean Sea (Marseilles, France). So far, no studies have precisely quantified the impact of pollution on the development of organisms in this area.
*Methods:* We used a fluctuating asymmetry (FA) measure of developmental instability (DI) to assess environmental stress in two species of radially symmetric sea urchins (*Arbacia lixula* and *Paracentrotus lividus*). For six sampling sites (Cortiou, Riou, Maire, East Maire, Mejean, and Niolon), levels of FA were calculated from continuous and discrete skeletal measures of ambulacral length, number of pore pairs and primary tubercles.
*Results:* For both species, the most polluted sampling site, Cortiou, displayed the highest level of FA, while the Maire and East Maire sampling sites displayed the lowest levels. *A. lixula* revealed systematic differences in FA among sampling sites for all characters and *P. lividus* showed differences in FA for the number of primary tubercles.
*Conclusions:* Statistical analyses of FA show a concordance between the spatial patterns of FA among sampling sites and the spatial distribution of sewage discharge pollutants in the Cortiou area. High developmental stress in these sampling sites is associated with exposure to high concentrations of heavy metals and many harmful organic substances contained in wastewater. FA estimated from structures with complex symmetry appears to be a fast and reliable tool to detect subtle differences in FA. Its use in biomonitoring programs for inferring anthropogenic and natural environmental stress is suggested.
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## 1. Introduction

The vicinity of the Cortiou sewage (Marseilles, France) is one of the most polluted environments of the French Mediterranean Sea. A daily 250 000 $m^3$ of wastewater is released by the Cortiou sewage and negative impact of the domestic and industrial waste on the species richness of macrobenthic communities in the polluted zones has been documented since 1965 (for a review see Bellan et al., 1999; Demoulin, 2009; Perez et al., 2003, 2005; Sauzade et al., 2007). To the northwest of Cortiou, another sewage located at Niolon releases a daily 40 to 110 $m^3$ of domestic waste (Perez et al., 2005). There, domestic effluents have been reported to strongly influence the level of contamination in sponge communities (Perez et al., 2005; Sauzade et al., 2007). In 1979, a sewage treatment was installed at Niolon to reduce the flow of pollutants and heavy metals that were released into the sea by precipitating the suspended material using physico-chemical decantation, then this plant underwent renovation in 1998 and new installations are underway to update the facilities to the latest norms and security regulations in 2015 (Marseille Provence Metropole, 2013). In

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**Table 1**
Abbreviations and Definition of the terms used in this study.

| Abbreviations | |
|---|---|
| MEDICIS | Mediterranean Contaminants Integrated Survey |
| Ifremer | Institut Français de Recherche pour l'Exploitation de la Mer<br>English: French Research Institute for Exploitation of the Sea |
| WFD | Water framework directive |
| PAH | polycyclic aromatic hydrocarbon |
| PCB | polychlorinated biphenyl |
| DDT | dichlorodiphenyltrichloroethane |
| DI | developmental instability |
| FA | fluctuating asymmetry |
| ANOVA | analysis of variance |
| ME | measurement error |
| LME | linear mixed effects model |
| REML | REstricted maximum likelihood |
| OPR | Ocular Plate Rule |

1987, a treatment plant was installed at Cortiou to reduce the discharge of chemicals and filter out the suspended material following three steps: first, a primary treatment uses grids to filter out coarse elements from the waste water; second, a physico-chemical treatment separates by decantation the suspended material such as oils and fats from sandy particles and heavy compounds; and third, a series of inclined plates called lamella clarifiers further remove the smaller solid particles from the liquid ones (Le Méditerranéen, 1998). Perez et al. (2005) showed that the concentrations of chemicals have been drastically reduced compared to their levels prior to the installation of the plant (e.g. 40% reduction for copper and 75% for lead). In 2003, the project MEDICIS (Mediterranean Contaminants Integrated Survey) was launched by Ifremer (Institut Français de Recherche pour l'Exploitation de la Mer, English: French Research Institute for Exploitation of the Sea) to gather data for a full survey of the contamination status in the West part of the Mediterranean Sea (Demoulin, 2009; Sauzade et al., 2007, see Table 1 for abbreviations). During the same year the European Water Framework Directive (WFD) established a new list of contaminants that are considered to have a dangerous impact on the environment, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dichlorodiphenyltrichloroethanes (DDTs) (Demoulin, 2009; Sauzade et al., 2007). Despite being banned since 1987 PCBs were still detected around the Cortiou sewage with the highest concentration in Cortiou and the lowest in Maire and Riou (Perez et al., 2003). The flow of pollutants tends to be carried towards the West by currents and generate a complex spatial gradient of pollution in the area (Perez et al., 2003, 2005). The resulting heterogeneous distribution of pollutants in the vicinity of Cortiou imposes spatially variable stresses on benthic communities.

Developmental instability (DI) is defined as the inability of an organism to buffer developmental errors and results from random processes that disturb the development of traits along their regular developmental trajectory within a specific environment (e.g. Debat and David, 2001; Klingenberg, 2003; Palmer and Strobeck, 2003; Waddington, 1957). DI is usually measured by fluctuating asymmetry (FA). FA refers to small random differences between left and right sides of bilaterally symmetric organisms (e.g. Graham et al., 1993b; Leamy, 1984; Ludwig, 1932; Palmer and Strobeck, 1986; Palmer and Strobeck, 2003; Van Valen, 1962) or among repeated structures (e.g. Freeman et al., 1993; Graham et al., 2010; Raz et al., 2012; Savriama et al., 2010, 2012; Savriama and Klingenberg, 2011). These minor inconstancies arise from random disturbances in the developmental processes that produce the traits (e.g. Clarke, 1993; Graham et al., 1993a; Leamy and Klingenberg, 2005; Palmer and Strobeck, 1992). Genomic stresses (e.g. inbreeding and outbreeding depression) as well as environmental pressures (e.g. extreme temperatures, competition for resources, food deprivation, toxic conditions) can affect the expression of symmetry at the phenotypic level (e.g. Alibert and Auffray, 2003; Alibert et al., 1994; Klingenberg and Nijhout, 1999). FA has been found to increase with stress and was also used as a measure of individual quality (e.g. Debat et al., 2011; Leary and Allendorf, 1989; Parsons, 1990, 1992; Valentine et al., 1973).

Here we apply a method designed for the statistical analysis of FA to two species of radially symmetric ('regular') sea urchins, *Arbacia lixula* and *Paracentrotus lividus*, both well known and traditionally used as bioindicators of pollution, particularly considering bioaccumulation (e.g. Beiras et al., 2003; Chinchon et al., 2000; Delmas and Regis, 1984; Geffard et al., 2001; His et al., 1999; Lillie, 1921; Pagano et al., 1996; Pena-Mendez et al., 1999; Pinheiro et al., 2015; Warnau et al., 1998). Our aim is to assess the impact of Cortiou and Niolon sewage discharges on the DI of these organisms. We quantify the degree of DI as inferred from FA analysis to test for a potential relationship between levels of DI and levels of pollution.

## 2. Materials and methods

### 2.1. Sampling sites and design

Six sampling sites were investigated in the Marseilles area (Fig. 1). They exhibited different levels of pollution with respect to PAHs, heavy metals, as well as pesticides concentrations measured in the sediment (Sauzade et al., 2007) depending on their distances from the two main sewages at Cortiou and Niolon (Fig. 2). In the East part, effluents from Cortiou, the most polluted sampling site of all, led to moderate levels of pollution in Maire and East Maire that marked the maximum reach of the pollution originating from Cortiou, while a sheltered cove in Riou was considered as a clean sampling site (Augier, 2013; Demoulin, 2009; Jany and Zebracki, 2012; Perez et al., 2003, 2005; Sauzade et al., 2007). In the North West part, Niolon and Mejean exhibited similar levels of pollution but not as high as at Cortiou (Sauzade et al., 2007). In September 2004, 548 adult specimens of two species of sea urchins (*P. lividus* and *A. lixula*) were collected by scuba diving in these six sampling sites at depths ranging from 10 to 15 m (34 to 50 specimens per sampling site and per species; Fig. 1).

### 2.2. Data collection

Prior to any measurement, all soft tissues were removed by dissolution in hydrochloric acid baths and only skeletal structures of the corona were considered. In 'regular' sea urchins, the geometric arrangement of skeletal plates into ten pairs of juxtaposed columns displays a five-fold radial symmetry (Fig. 3). Five pairs of columns perforated by several pairs of pores are named ambulacra. The other five pairs that alternate with the ambulacra are the interambulacra (Mooi and David, 1997; Ruppert et al., 1994). All plates bear spines that are attached to tubercles (Smith, 1984). A single large primary tubercle is attached on each interambulacral plate, and several small tubercles and granules grow along the outer margins of interambulacral plates and on the suture between ambulacral columns (David and Néraudeau, 1989; Gosselin and Jangoux, 1998). The corona of sea urchins comprises five growth zones that originate from each ocular plate (Fig. 3, David et al., 1995; Mooi and David, 2008; Mooi et al., 1994, 2005; Morris and Byrne, 2005) and numbered according to a specific morphogenetic sequence (Lovén, 1874). These growth zones are homologous in a way comparable to the left and right sides of bilaterally symmetric organisms (David et al., 1995) and therefore are suitable for studies of FA (Lawrence et al., 1998; Saucède et al., 2006; Stige et al., 2006).

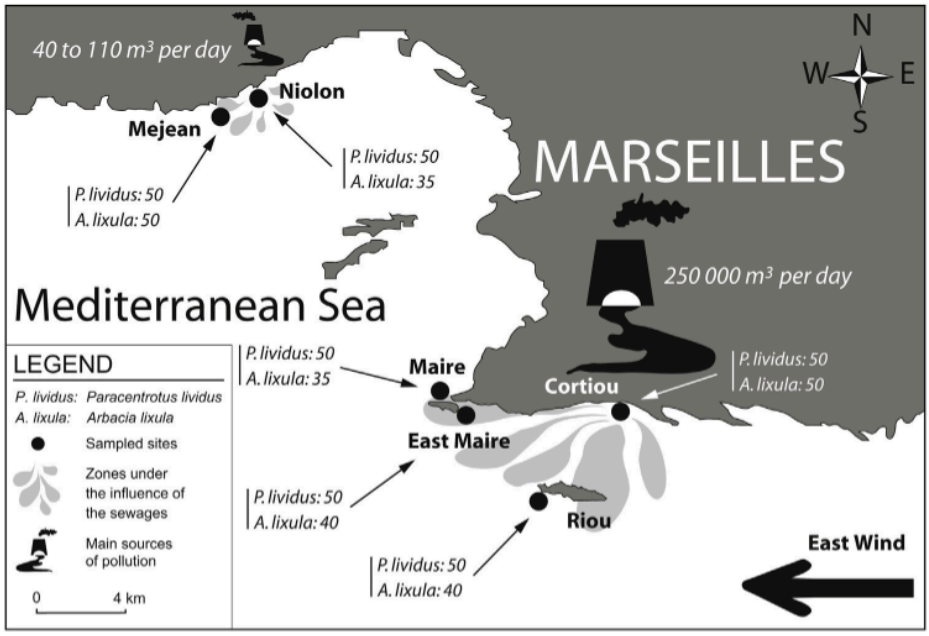


**Fig. 1.** Map showing the geographic locations of the sampling sites and the number of specimens sampled for each species.

For both species, the lengths of the five homologous ambulacra were measured from the tip of each ocular plate to the rim of the peristome. Primary tubercles in *P. lividus* and pore pairs in *A. lixula* were counted on homologous ambulacra (pore pairs in *P. lividus* and primary tubercles in *A. lixula* were not considered because the skeletal morphology of the two species makes these traits prone to measurement error). All measurements were taken under a Leica x1 Wild MZ 9.5 Stereomicroscope objective at ×40 to increase the precision of their readings. All data were taken twice to assess measurement error.

### 2.3. Statistical analysis of complex symmetric structures

For studies of bilaterally symmetric structures, a two-way mixed model analysis of variance (ANOVA) is traditionally used to quantify symmetric variation and asymmetry (Leamy, 1984; Palmer and Strobeck, 1986, 2003). In this design, the 'individual' main effect represents variation among individuals, the 'side' main effect stands for directional asymmetry (DA: on average one side is consistently more developed than the other; e.g. American lobster), the interaction term 'individual-by-side' accounts for FA, and measurement error (ME) can be quantified if replicates have been made. However, this design has been criticised since it does not take into account heterogeneity in asymmetry or ME (Stige et al., 2006; van Dongen et al., 1999). An alternative method has been suggested that uses linear mixed effects models (LMEs) and REstricted Maximum Likelihood (REML) (Pinheiro and Bates, 2000) to calculate robust estimates of asymmetry and take into account heterogeneity in FA, DA and ME (Stige et al., 2005, 2006; van Dongen, 2006; van Dongen et al., 1999). We followed this latter approach to estimate FA, DA, and ME and we generalised van Dongen et al.'s framework (1999) for the analysis of asymmetry in radially symmetric organisms. In our extended approach of the aforementioned framework, the 'side' variable was replaced by 'repeated structure'. In our case, this 'repeated structure' is five-fold and corresponds to the ambulacrum that confers symmetry to the whole organism. The 'repeated structure' variable was categorical with levels 1, 2, . . ., 5 and the modelled fixed effect of 'repeated structure' represents (five-fold) DA (see equation below). Note that the 'repeated structure' numbering is unambiguously defined here, because the

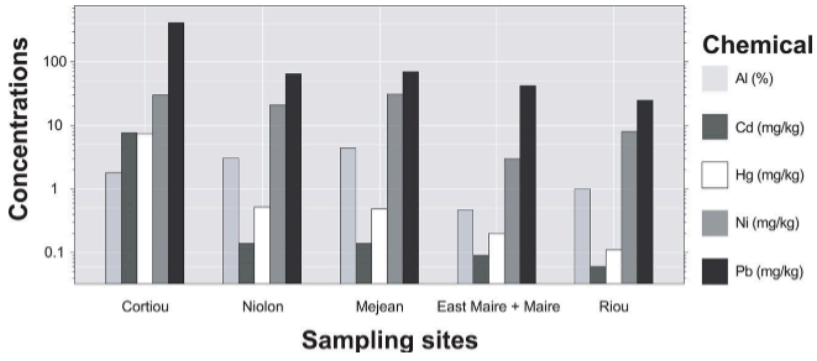


**Fig. 2.** Concentrations of the following important metal wastes Al (light grey column), Cd (black grey), Hg (white), Ni (medium grey), and Pb (black) found at the sampling sites presented in Fig. 1. Succession of the sampling sites from left to right represents increasing distance from the source of pollution. Al concentrations are expressed in percentages of elements that occur in clay sediment with high percentage indicating higher property of the sediment to adsorb contaminants. Metal concentrations in mg per 1 kg of sediment (dry weight). Data have been log-transformed to even out the weight of very high versus low values and allow all values to fit in a single graph (data compiled from Sauzade et al., 2007).

**Table 2**
Analysis of directional Asymmetry (DA) using the dataset of all ambulacra for *Paracentrotus lividus* and *Arbacia lixula* for ambulacral length, number of tubercles, and number of pore pairs. DA is estimated from mixed-effects REML models and assessed by a three-way ANOVA. The fixed term 'repeated structure' represents DA, that is, consistent differences among the five sides in trait size. The interaction terms test whether DA varies with body size and among sampling sites. numDF: numerator degrees of freedom, denDF: denominator degrees of freedom. The sampling sites are presented in Fig. 1.

| | | *P. lividus* | | *A. lixula* | |
|---|---|---|---|---|---|
| | | Ambulacral length | Number of primary tubercles | Ambulacral length | Number of pore pairs |
| **Source of variation** | **Statistic** | | | | |
| Repeated Structure (DA) | numDF | 4 | 4 | 4 | 4 |
| | denDF | 1166 | 1159 | 935 | 808 |
| | *F*-value | 61.6 | 74.1 | 117.4 | 95.3 |
| | *P*-value | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Body size | numDF | 1 | 1 | 1 | 1 |
| | denDF | 292 | 291 | 247 | 240 |
| | *F*-value | 34022.2 | 20863.4 | 24061.1 | 30180.2 |
| | *P*-value | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Sampling site | numDF | 5 | 5 | 5 | 5 |
| | denDF | 292 | 291 | 247 | 240 |
| | *F*-value | 2.7 | 0.2 | 1.6 | 1.9 |
| | *P*-value | 0.0206 | 0.9351 | 0.1697 | 0.0952 |
| Repeated structure-by-body size | numDF | 4 | 4 | 4 | 4 |
| | denDF | 1166 | 1159 | 935 | 808 |
| | *F*-value | 1.5 | 2.7 | 1.2 | 1.8 |
| | *P*-value | 0.206 | 0.0281 | 0.3166 | 0.1234 |
| Repeated structure-by-sampling site | numDF | 20 | 20 | 20 | 20 |
| | denDF | 1166 | 1159 | 935 | 808 |
| | *F*-value | 3.4 | 1.3 | 0.8 | 1.1 |
| | *P*-value | <0.0001 | 0.1396 | 0.6672 | 0.2977 |

corona of 'regular' sea urchins includes features that are not radially symmetric (i.e. genital Plate 2 in Fig. 3). The random individual effect represents variation among specimens in trait size, the random within-individual 'repeated structure' effect represents FA, and variation among replicate measurements represents ME. We combined van Dongen et al. (1999) and Stige et al. (2006)'s approaches to test for heterogeneity in DA by including interaction terms between the fixed effect of 'repeated structure' on the one hand, and body size (measured as the average of all measurements for the considered trait) or location on the other (Table 2). All fixed effects were tested by *F*-tests (Pinheiro and Bates, 2000). Individual FA estimates corrected for DA, size dependence and heterogeneous ME (see Appendix in Supplementary data) were provided by the random 'repeated structure' estimates from the best model. The standard deviation of these estimates (calculated for each individual) is equivalent to the index FA10a of Palmer and Strobeck (2003) (Stige et al., 2005; van Dongen et al., 1999). To test for the significance of FA compared to ME, two LMEs were compared in each case using likelihood ratio tests. One model included all parameters (full model) and the other did not include the effect under interest, i.e., FA (reduced model). This procedure tested how much variance is explained by the effect under study in the full model (Pinheiro and Bates, 2000). In summary, the statistical testing of FA used the models below (Greek symbols indicate fixed effects and Latin letters stand for random effects):

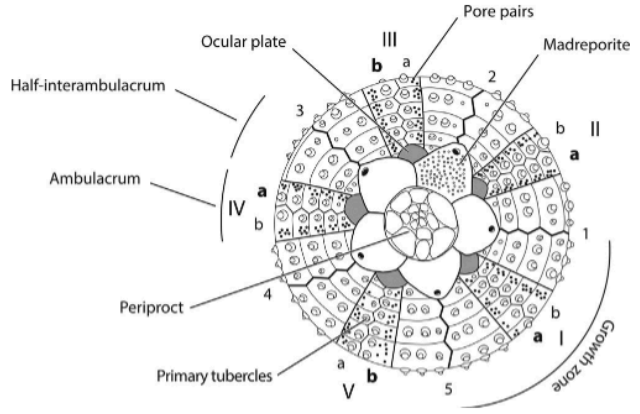


**Fig. 3.** Aboral view of the corona of a 'regular' sea urchin. The corona comprises five growth zones. Each growth zone originates from the ocular plates and is composed of one ambulacrum (numbered counterclockwise from I to V, with two column pairs, following the same rotation, individual columns labelled with a and b bold letters indicate homology of ambulacral columns underlain by the Ocular Plate Rule (OPR, David et al., 1995; Mooi et al., 2005) and two half-interambulacra (with the numbers 1 to 5). In our study, measurements were all taken on ambulacra. During development, the new ambulacral plates form right next to their corresponding ocular plate and are laid down according to the OPR: plates are alternately added to one and then the other of the two ambulacral columns. As growth proceeds and new plates are added to the corona, this repeated pattern draws a typical zigzag suture and the oldest plates progressively shift away from the aboral region. We followed Lovén's rule (1874) for numbering ambulacra and interambulacra: a differentiated genital plate, the madreporite, is used as a reference and faces the thus-defined second interambulacrum.

$$\text{Full model}: Y_{ijk} = \mu + \beta_j + b1_i + +b2_{ij} + E_{ijk}$$

$$\text{Reduced model}: Y_{ijk} = \mu + \beta_j + b1_i + +b2_{ij} + E_{ijk}$$

where $Y_{ijk}$ is the measurement for individual $i$ ($i = 1, \ldots, n$), for the 'repeated structure' $j$ ($j = 1, \ldots, 5$), and replicate $k$ ($k = 1,2$). Parameter $\mu$ is the grand mean, $\beta_j$ is five-fold directional asymmetry (DA), $b1_i$ is the among-individual variation $\sim N(0, \sigma 1)$, $b2_{ij}$ is fluctuating asymmetry (FA) $\sim N(0, \sigma 2)$, and $E_{ijk}$ is measurement error (ME) $\sim N(0, \sigma 3)$.

We used an analysis of variance (ANOVA) to investigate whether FA varies among sampling sites and the F-statistic as a measure of the difference in FA means among sampling sites. However, we did not consider *P*-values calculated from the F-distribution since the residuals for the ANOVA model were not normally distributed (see Appendix in Supplementary data). Instead, we used permutation tests to perform a non-parametric version of the *F*-test that does not make the normality assumption of the *F*-test. To test for differences in FA among sampling sites, we first used a permutation test (10 000 randomisations) where individuals were

**Table 3**
Directional asymmetry (DA), fluctuating asymmetry (FA), and measurement error (ME) using the dataset of all ambulacra for *Paracentrotus lividus* and *Arbacia lixula* for ambulacral length, number of tubercles, and number of pore pairs. Skewness and kurtosis are given separately for the five ambulacra. DA, FA, and ME are estimated from mixed-effects REML models. DA is an estimated mean deviation, FA and ME are estimated standard deviations. [a] Pearson's correlation coefficient between FA and log-transformed trait size ($^{*}$ $P<0.05$, $^{**}$ $P<0.01$, $^{***}$ $P<0.001$).

| Species | Trait | Ambulacrum | Corr FA log(Size)[a] | DA | FA | ME | Skewness | Kurtosis |
|---|---|---|---|---|---|---|---|---|
| *P. lividus* | Ambulacral length | Ia | 0.122 | 0.004$^{***}$ | 0.012 | 0.013 | 0.01 | 0.08 |
| | | IIa | | | | | 0.03 | 0.22 |
| | | IIIb | | | | | −0.2 | −0.32 |
| | | IVa | | | | | 0.07 | 0.26 |
| | | Vb | | | | | 0.2 | −0.05 |
| | Number of primary tubercles | Ia | 0.052 | 0.009$^{***}$ | 0.020$^{**}$ | 0.066 | −0.16 | 0.07 |
| | | IIa | | | | | −0.15 | 0.31 |
| | | IIIb | | | | | 0.06 | 0.27 |
| | | IVa | | | | | 0.07 | 0.11 |
| | | Vb | | | | | −0.15 | 0.38 |
| *A. lixula* | Ambulacral length | Ia | 0.099 | 0.003$^{***}$ | 0.012$^{**}$ | 0.018 | 0.43$^{**}$ | 1.84$^{***}$ |
| | | IIa | | | | | −0.1 | 0.55 |
| | | IIIb | | | | | 0.15 | 0.28 |
| | | IVa | | | | | −0.19 | 0.1 |
| | | Vb | | | | | −0.11 | 0.16 |
| | Number of pore pairs | Ia | 0.074 | 0.002$^{***}$ | 0.015$^{***}$ | 0.018 | −0.18 | 0.39 |
| | | IIa | | | | | −0.13 | 0.65$^{*}$ |
| | | IIIb | | | | | −0.25 | 0.80$^{*}$ |
| | | IVa | | | | | −0.13 | 0.01 |
| | | Vb | | | | | −0.94$^{***}$ | 3.49$^{**}$ |

randomly allocated to populations and individual FA was calculated as the standard deviation of the asymmetry values ($b2_{ij}$ from the full model) across the five sides. The statistical significance was assessed by comparing the observed test statistic with the null-distribution obtained by the permutation procedure. To assess the robustness of the findings, we did two other tests in which FA was calculated as the absolute value of the asymmetry. One tested for the main effect of sampling site on FA (as the first test did) and the other tested for the interaction between DA and sampling site on FA (1000 randomisations, also with random allocation of individuals to populations). A nonsignificant interaction effect was taken as support for our interpretation that variability among sampling sites in FA truly reflected variability in DI rather than DA. We used a bootstrap procedure corrected for multiple pairwise comparisons (Bonferroni correction) to test for differences in FA among sampling sites. Additionally, we used Cohen's *d* to assess the effect size of the geographic location on FA (Cohen, 1969; Hedges and Olkin, 1985; Olejnik and Algina, 2000). Further information about the study design and statistical testing can be found in Appendix in Supplementary data. All statistical analyses were carried out using the functions *lm* and *anova* from the stats package (R Development Core Team, 2014) and *lme* from the *nlme* package (Pinheiro et al., 2015) in the statistical software R (version 3.0.3, 2014) with R scripts written specifically for the permutation tests and the bootstrap procedures.

## 3. Results

### 3.1. Directional asymmetry

Mean DA is highly significant in all three traits and for both species (Tables 2 and 3). However, there is no significant difference in DA among sampling sites except in ambulacral length for *P. lividus* (for further statistical tests about the heterogeneity in DA see Table 2). The non-parametric tests indicate that interaction between DA and sampling site on FA is not significant (Table 4). This suggests that variability among sampling sites in FA is mainly due to variability in DI rather than DA. On average, DA is marked by the fact that columns IIa and IIIb are the shortest, with less primary tubercles across all sampling sites for *P. lividus* and higher number of pore pairs in *A. lixula*.

### 3.2. Fluctuating asymmetry

Table 3 summarises the results for the analysis of FA in all traits and for both species. As the residuals of the ANOVA model are not normally distributed, we only present results from non-parametric approaches (the details of the statistical tests used to test for normality of data are shown in Appendix in Supplementary data). Table 4 summarises the results for the permutation tests that test for the statistical significance of differences in FA among sampling sites for each trait and both species. Table 5 summarises the results for the bootstrap procedure that tests for the statistical significance of all pairwise comparisons in FA among sampling sites for each trait and both species (for a detailed account of the results from the bootstrap procedure, see supplementary Table 1). Results of effect size analyses are reported in Table 6.

Overall, the bootstrap and effect size approaches give the same results. For *P. lividus*, FA in ambulacral length does not vary among sampling sites. On the contrary, FA in number of primary tubercles depends on sampling site and the pairwise comparisons reveal a significantly higher level of FA in Cortiou than in Maire (1.8 times greater), and East Maire (1.5 times greater).

For *A. lixula*, both FA in ambulacral length and number of pore pairs depend on sampling site. Pairwise comparisons for ambulacral length show a significantly higher level of FA in Cortiou than in Maire (1.34 times higher), and East Maire (1.28 times higher). For the number of pore pairs, pairwise comparisons show higher level of FA in Cortiou than in Maire (1.29 times higher), East Maire (1.24 times higher), and Mejean (1.21 times higher). Fig. 4 presents the mean levels of FA for each trait and both species according to their distance from the main sources of pollution in Cortiou. For both species and all characters considered, the highest levels of FA are encountered at Cortiou and Riou.

## 4. Discussion

Our analyses highlight contrasted levels of FA measures in ambulacral length, number of pore pairs and primary tubercles in specimens of *P. lividus* and *A. lixula* sampled from six sampling sites variously exposed to pollutants. These pollutants include heavy metals that have been reported in ecotoxicological studies to affect the normal development of sea urchins. For instance, high

**Table 4**
Statistical significance of Fluctuating Asymmetry (FA) using the dataset of all ambulacra for Paracentrotus lividus and Arbacia lixula ambulacral length, number of tubercles, and number of pore pairs. The sampling sites are presented in Fig.1. Permutation tests were used to test for differences in mean FA among sampling sites. [a] Permutation test (10 000 randomisations): differences in mean FA among sampling sites with FA calculated as Standard Deviation of asymmetry (SD). [b]Permutation tests (1000 randomisations): differences in mean FA among sampling sites with FA calculated as ABSolute value of asymmetry (ABS). One tested for the main effect of sampling site on FA (as the first test did) and the other tested for the interaction between 'repeated structure' (DA) and sampling site on FA. ($^{*}$ $P<0.05$, $^{**}$ $P<0.01$, $^{***}$ $P<0.001$).

| Species | Trait | FA calculated as SD[a] | | FA calculated as ABS[b] | | | |
|---|---|---|---|---|---|---|---|
| | | $F(df_{Sampling\ site}, df_{error})$ | *P*-value | $F(df_{Sampling\ site}, df_{error})$ | *P*-value | $F(df_{Repeated\ structure\text{-}by\text{-}Sampling\ site}, df_{error})$ | *P*-value |
| *P. lividus* | Ambulacral length | $F(5, 290)=0.67$ | 0.6446 | $F(5, 290)=0.72$ | 0.614 | $F(4, 1162)=6.64$ | 0.381 |
| | Number of primary tubercles | $F(5, 290)=3.50$ | 0.0049$^{**}$ | $F(5, 290)=2.45$ | 0.046$^{*}$ | $F(20, 1160)=1.20$ | 0.235 |
| *A. lixula* | Ambulacral length | $F(5, 246)=3.66$ | 0.0035$^{**}$ | $F(5, 246)=3.78$ | 0.001$^{**}$ | $F(20, 935)=0.94$ | 0.541 |
| | Number of pore pairs | $F(5, 237)=4.57$ | 0.0004$^{***}$ | $F(5, 237)=4.30$ | <0.0001$^{***}$ | $F(20, 800)=1.26$ | 0.189 |

**Table 5**
All pairwise comparisons of mean Fluctuating Asymmetry (FA) among sampling sites using the dataset of all ambulacra for *Paracentrotus lividus* and *Arbacia lixula* for ambulacral length, number of tubercles, and number of pore pairs. The sampling sites are presented in Fig. 1. Individual FA values were calculated as the standard deviation of the asymmetry values across the five sides. Bootstrap procedures were used to test for differences in mean FA for all pairwise comparisons per sampling site, per trait and per species.

| | Cortiou | Niolon | Mejean | East Maire | Maire | Riou |
|---|---|---|---|---|---|---|
| Cortiou | – | | | | | |
| Niolon | NS | – | | | | |
| Mejean | $^{*}$ap | NS | – | | | |
| East Maire | $^{*}$al/$^{*}$pt/$^{*}$ap | NS | NS | – | | |
| Maire | $^{*}$al/$^{*}$pt/$^{*}$ap | NS | NS | NS | – | |
| Riou | NS | NS | NS | NS | NS | – |

al: Pairwise comparison for mean ambulacral length in *A. lixula*. pt: Pairwise comparison for mean number of primary tubercles in *P. lividus*. ap: Pairwise comparison for mean number of pore pairs in *A. lixula*. Significance ($^{*}$ $P<0.05$) and nonsignificance (NS) are reported below the diagonal.

concentrations of Pb and Cd cause larval malformations (e.g. crossed body rods in plutei, Novelli et al., 2003) and high concentrations of Hg cause both abnormally developed plutei and developmental arrest with embryos that are unable to complete larval differentiation (e.g. embryos blocked at blastula and gastrula stages) (e.g. Cesar et al., 2004; His et al., 1999; Pagano et al., 1996; Warnau et al., 1996). Clear decreases in Pb, Cd, and Hg concentrations are observed the further we go away from the main sources of pollution, whereas this trend is less clear for Ni and Al (Fig. 2). Studies have shown that the effect of heavy metals such as Hg, Cd and Pb on sperm cell and embryo toxicity is significantly more important than those generated by Ni or Chromium (e.g. Beiras et al., 2003; Novelli et al., 2003; Volpi Ghirardini et al., 2005) with the impact of heavy metals on the development of sea urchin embryos ranked as follows Hg > Cu > Pb > Cd according to Fernández and Beiras (2001). Higher levels of heavy metals at Cortiou might concur with higher levels of FA measured at this site. There is a good spatial concordance between the patterns of FA (Fig. 4) and the concentrations of the pollutants considered in this study. As expected, the highest degree of FA was found at Cortiou that is the most polluted site of all (e.g. Cd and Hg concentrations are 98% higher in Cortiou than in Riou) and therefore the most likely to induce a stressful environment.

**Table 6**
Magnitude of differences in mean FA among sampling sites using the dataset of all ambulacra for *Paracentrotus lividus* and *Arbacia lixula* for ambulacral length, number of tubercles, and number of pore pairs. Individual FA values were calculated as the standard deviation of the asymmetry values across the five sides. Effect size analyses using Cohen's *d* was used to test for the magnitude of differences in mean FA among sampling sites. The sampling sites are presented in Fig. 1.

| Species | Trait | Treatment[a] | Cohen's *d* | CIs |
|---|---|---|---|---|
| *P. lividus* | Ambulacral length | Cortiou | 0.211 | [−0.182;0.604] |
| | | Niolon | 0.009 | [−0.383;0.401] |
| | | Mejean | 0.01 | [−0.382;0.402] |
| | | East Maire | −0.103 | [−0.495;0.290] |
| | | Riou | 0.037 | [−0.355;0.429] |
| | Number of primary tubercles | Cortiou | 0.462$^{*}$ | [0.065;0.859] |
| | | Niolon | −0.039 | [−0.431;0.353] |
| | | Mejean | −0.002 | [−0.393;0.390] |
| | | East Maire | −0.02 | [−0.412;0.372] |
| | | Riou | 0.484$^{*}$ | [0.086;0.881] |
| *A. lixula* | Ambulacral length | Cortiou | 0.811$^{*}$ | [0.362;1.260] |
| | | Niolon | 0.542$^{*}$ | [0.065;1.019] |
| | | Mejean | 0.314 | [−0.121;0.748] |
| | | East Maire | 0.195 | [−0.260;0.649] |
| | | Riou | 0.585$^{*}$ | [0.121;1.048] |
| | Number of pore pairs | Cortiou | 0.689$^{*}$ | [0.244;1.133] |
| | | Niolon | 0.196 | [−0.273;0.666] |
| | | Mejean | 0.046 | [−0.386;0.478] |
| | | East Maire | −0.052 | [−0.505;0.402] |
| | | Riou | 0.325 | [−0.131;0.782] |

[a] Maire as the control site since it has the lowest level of FA.
$^{*}$ Indicates significant effect size based on its confidence interval CI (Hedges and Olkin, 1985)

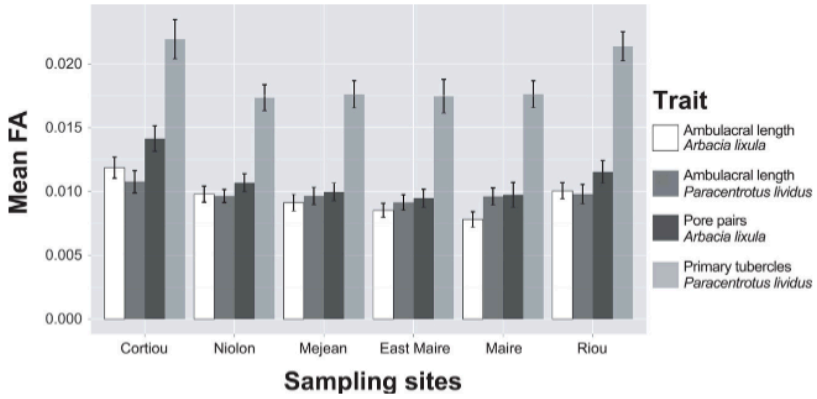


**Fig. 4.** Mean and standard error of fluctuating asymmetry (FA) levels for the ambulacral length (*Arbacia lixula*—white column; *Paracentrotus lividus*—dark grey), pore pairs (*A. lixula*—black), and primary tubercles (*P. lividus*—light grey) found at the sampling sites presented in Fig. 1. Succession of the sampling sites from left to right represents increasing distance from the source of pollution.

The only observation in contradiction to this spatial pattern was found in the Riou sampling site, which showed a surprisingly high degree of FA despite being the least polluted site. Even if the dynamics of winds and marine currents in the Cortiou area might sometimes allow the flow of pollutants from the Cortiou sewage to reach the vicinity of Riou (Perez et al., 2003, 2005), all our specimens were collected in a protected and well-known inlet, the Moyades that is at the heart of the Calanques National Park (Augier, 2013; Calanques National Park, 2013; GIP des Calanques, 2012) and is assumed to be unaffected by the sewage (Jany and Zebracki, 2012). A possible explanation for this FA anomaly could be linked to commercial fishing activities. In Cortiou, the harvesting of sea urchins is forbidden and it was a frequent practice two decades ago for some fishermen to transfer sea urchins from Cortiou to cleaner, sheltered, and easily accessible sites. The aim was to produce 'comestible' populations at convenient sampling sites and to allow the harvesting under a wider range of meteorological conditions during the commercial season. Discussions with local fishermen confirmed that such practices were still observed two years ago. Well protected from the Cortiou sewage influence and renowned for its ideal conditions for diving, the Moyades in Riou appeared as a likely candidate to relocate populations of sea urchins and this would explain its counter expected high degree of FA. Future acquisition of genetic data will help testing this hypothesis.

The method introduced in this paper appears to be efficient in recovering FA signals from only a few simple traits and suggests a link between FA and pollution. Other studies investigating associations between environmental stress and FA in bilaterally symmetric organisms have shown conflicting results (e.g. Bjorksten et al., 2000; Hoffmann and Woods, 2003; Leamy and Klingenberg, 2005; Leung and Forbes, 1996; Leung et al., 2003; van Dongen, 2006). The significant findings in our study may at least partly reflect the higher statistical power of the method to detect variation in DI compared to traditional FA measures. Indeed, the statistical power to detect differences in FA has been shown to increase in organisms that possess orders of symmetry higher than the bilaterally symmetric ones, because of their higher number of homologous structures measurable (e.g. Ambo-Rappe et al., 2008; Helsen and van Dongen, 2009; Nuche et al., 2014; Savriama et al., 2010, 2012; Savriama and Klingenberg, 2011; Sinclair and Hoffmann, 2003; Tan-Kristanto et al., 2003; van Dongen and Møller, 2007). Future studies on other stressors and other organisms with complex symmetry (e.g. flowers, colonial corals, snails, desmids and diatoms) will assess the generalisability of this approach as a standard protocol for biomonitoring anthropogenic or natural environmental stress.

## 5. Conclusions

Depending on the species under study, different patterns of FA are obtained: traits measured in *A. lixula* showed higher FA with more significant differences among sampling sites than the traits measured in *P. lividus*. Studies have already shown that these two species can have different degrees of pollutant bioaccumulation while they develop in polluted sediment, with *A. lixula* showing slightly higher sensitivity to heavy metals than *P. lividus* (e.g. Carballeira et al., 2011, 2012; Portocali et al., 1997). Differences in habitat and feeding regimes might explain the discrepancies in sensitivity to pollutants between these two species (e.g. Auernheimer and Chinchon, 1997). In addition, within a single organism different skeletal parts and tissues show different degrees of sensitivity to pollutants with plates concentrating more pollutants than spines. This might be related to differences in the magnesian calcite crystalline structure among the parts of the corona causing some skeletal parts to be more prone to incorporate specific heavy metals as well as other chemicals (e.g. Chinchon et al., 2000). A higher release of heavy metals at Cortiou could consequently lead to an increased assimilation of these elements in the measured skeletal structures of both species. Our results support the analysis of FA in sea urchins as a fast, inexpensive, and reliable approach, complementing standard chemical studies, and enriching the biomonitoring toolkit for environmental and pollution surveys.

There are still debates concerning the actual beneficial effect of the treatment plants installed at Cortiou and Niolon on the biological communities of the Mediterranean Sea. Even if studies have pointed out their efficiency in filtering out most of the chemicals from the Cortiou sewage, some heavy metal contaminants were still measured in high proportions in *P. lividus* several years after the installation of the Cortiou treatment plant (e.g. Warnau et al., 1998). Nevertheless, important changes in the composition of the biological communities have also been noticed and some species that faded out prior to the installation of the treatment plant now flourish again on the rocky seafloor (Perez et al., 2005). For instance, the alga *Cystoseira amantacea* that grows in fairly healthy environments has reappeared in the East sector, suggesting a substantial decrease in concentrations of heavy metals and other contaminants (Perez et al., 2005). In 2008, a section of the Géolide wastewater treatment plant was installed to specifically filter out all biological waste from the Cortiou sewage (SERAM, 2008). Every year, 85 million $m^3$ of wastewater (225 000 $m^3$ per day) coming from Marseilles and 16 surrounding towns go through the Géolide Station to be cleaned from different sources of pollution before being released

into the sea (SERAM, 2008, 2012). The efficiency of the newly installed Géolide plant on marine communities is still relatively unknown and has not been properly tested yet, but a study claims it does not filter organochlorine pesticides or PCBs (Syakti et al., 2012). Our sampling was carried out before the installation of the Géolide plant and therefore provides a benchmark for future studies to evaluate whether or not this new treatment plant has indeed improved the quality of the seawater.

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## Data

Data have been archived at Pangaea (http://doi.pangaea.de/10.1594/PANGAEA.842844).

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