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## 3 **Contrasting Effects of Heavy Metals on Sponge Cells Behavior**

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7 **Abstract** Mediterranean coastal areas are highly con- 33  
8 taminated by heavy metals, which have been reported to 34  
9 produce harmful effects in marine organisms. Sponges are 35  
10 particularly vulnerable to waterborne metals because they 36  
11 are able to process large amounts of water. Dissociated 37  
12 sponge cells can move in response to external stimuli, and 38  
13 the cell body changes shape through production of pseu- 39  
14 dopodia and phylopodia. We studied for first time the ef- 40  
15 fects of heavy metals (cadmium copper and mercury) on 41  
16 motility and aggregation of isolated sponge cells. Cell shape 42  
17 was assessed by using several shape indices. The three 43  
18 metals studied induced changes of different sign on cell 44  
19 shape. Mercury arrested movement of sponge cells, which 45  
20 tended to be rounded, without pseudopodia. In contrast, 46  
21 moderate concentrations of copper and cadmium enhanced 47  
22 pseudopodia formation and cell motility. On the other hand, 48  
23 the three metals enhanced cell aggregation at the concen- 49  
24 trations assayed. Our results show that sponge cells respond 50  
25 to metal pollution in different ways and that these responses 51  
26 can be assessed by calculating several shape indices. 52

27  
28 **Keywords** Heavy metals · Sublethal effects · 53  
29 Ecotoxicology · Sponge cells · Bioassays 54

### 30 **Introduction**

31 Coastal marine and estuarine environments have long 55  
32 been used as disposal areas for industrial and mining 56

wastes. They are also subjected to transient toxicant re- 33  
leases that may occur in the form of spills or runoff from 34  
agricultural and urban areas, as well as antifouling bio- 35  
cides. As a result, Mediterranean coastal areas are highly 36  
contaminated by heavy metals (Palanques et al. 1998; 37  
Puig et al. 1999), which produce harmful effects in 38  
marine organisms because of their toxicity and bioaccu- 39  
mulation through the trophic chains. Among heavy met- 40  
als, copper is one of the most abundant with well-known 41  
harmful effects on marine invertebrates (Ahsanullah and 42  
Florence 1984; Reichelt-Brushett and Harrison 2000; 43  
Negri and Heyward 2001). Cadmium is another relevant 44  
pollutant from industrial discharge and is highly toxic to a 45  
variety of aquatic animals (e.g., Eisler 1985; Selck 1998; 46  
Au et al. 2001), and mercury is largely known as a sig- 47  
nificant cause of mortality of several aquatic species 48  
(Cossa and Fichet 1999). 49

Sessile benthic invertebrates are especially susceptible 50  
to heavy metal pollution because of their suspension- or 51  
filter-feeding habitat and their reduced motility, which 52  
prevents them from escaping from toxicants released to a 53  
given area (Naranjo et al. 1998; Carballo and Naranjo 54  
2002; Rosenberg et al. 2004; Perez et al. 2005). Sponges 55  
are particularly vulnerable to waterborne metals because 56  
they are able to process large amounts of water (Reiswig 57  
1971; Turon et al. 1997; Ribes et al. 1999). On the other 58  
hand, sponges have been proposed as heavy metal bio- 59  
monitors because they have a high capacity for accumu- 60  
lating heavy metals (Patel et al. 1985; Olesen and Weeks 61  
1994; Hansen et al. 1995; Cebrian et al. 2003; Pérez et al. 62  
2005) and because they experience morphological, bio- 63  
logical, physiological, and biochemical responses when 64  
they are submitted to metal contamination (Agell et al. 65  
2001; Philp 1999; Schröder et al. 2000; Cebrian et al. 2003, 66  
2006; Berthet et al. 2005). 67

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68 In this way, a set of recent studies has tackled the  
69 effects of heavy metals on different sponge species and at  
70 different organization levels: molecular (Agell et al. 2001;  
71 Cebrian et al. 2006), physiological (Cebrian et al. 2003),  
72 and population level (Cebrian et al. unpublished), and at  
73 different stages of the sponge life cycle (larvae, settlers,  
74 juveniles, and adults, Cebrian and Uriz 2007, Cebrian  
75 et al. unpublished). These studies have reported con-  
76 trasting effects depending on the exposure time, the spe-  
77 cies considered, and the life-cycle stage. Although heavy  
78 metals appear to be noxious for adults (Cebrian et al.  
79 2003, 2006) they seem to be innocuous or even beneficial  
80 for larvae and settlers (Cebrian and Uriz 2007). For in-  
81 stance, copper inhibited growth and reproduction in  
82 *Crambe crambe* adults, but it did not affect larval set-  
83 tlement. However, in other sponge species, such *Scopa-*  
84 *lina lophyropoda*, copper and cadmium enhanced  
85 settlement.

86 During larval settlement and metamorphosis, an exten-  
87 sive reorganization process occurs, which implies move-  
88 ment, self-recognition, and aggregation of cells. Thus,  
89 alterations in one or several aspects of the cell's behavior  
90 can have important effects on settlement processes. To  
91 understand the effects that low concentrations of heavy  
92 metals produced on *S. lophyropoda* settlement, we searched  
93 for changes in shape, motility, and aggregation in *S.*  
94 *lophyropoda* cells submitted to short pulses of copper,  
95 mercury, and cadmium.

96 Dissociated sponge cells crawl in response to external  
97 stimuli, and the cell body changes shape through produc-  
98 tion of pseudopodia and phylopodia (Stossel 1994). We  
99 used these changes on shape, assessed through shape  
100 indices, to analyze cell motility in sponge cells under the  
101 effects of copper, mercury and cadmium.

102 Moreover, heavy metals may alter  $\text{Ca}^{2+}$  homeostasis  
103 (Marchi et al. 2004; Verboost et al. 1989; Viarengo 1994)  
104 and therefore affect calcium-induced aggregation (Philp  
105 1999). Concretely, it has been reported that these con-  
106 taminants can affect the viability of the cell-to-cell  
107 aggregation in other invertebrates (Auffret and Oubella  
108 1997) and sponges (Philp 1999). Thus, we also studied  
109 the effects of heavy metals on cell aggregation by mea-  
110 suring the number and size of aggregates in metal incu-  
111 bated cells.

112 This is the first study that attempts to investigate the  
113 effects of heavy metals on sponge cell behavior as a proxy  
114 for biological effects at the settlement and early post-set-  
115 tlement stages. Although most previous studies on inver-  
116 tebrate cells have addressed extremely high concentrations  
117 that might ever be experienced by the organisms in the  
118 field, the present study attempts to analyze cellular res-  
119 sponses to metal pulses at more realistic concentrations,  
120 which could be found in polluted sites. The responses at the

cellular level may have the potential to anticipate changes  
at higher levels of biological organization (Cajaraville et al.  
2000).

## Materials and Methods

The effects of heavy metals on sponge cell behavior (*i.e.*,  
cell motility and aggregation) were assessed by shape  
indices analyses. Because cell motility generally involves  
the production of pseudopodia and phylopodia, it can be  
indirectly measured by increases in cell irregularity, which  
can be detected by cell shape indices.

The study was carried out with a suspension of cells  
isolated from *S. lophyropoda*, which is a widespread species  
with patchy distribution in the Mediterranean sublittoral,  
growing on vertical rocky walls. *S. lophyropoda* was col-  
lected from 5-m depth of Blanes sublittoral (NE Spain,  
41°40'37"N, 2°47' 30"E), placed in bowls underwater and  
transported to the laboratory. Once in the laboratory, the  
sponges were placed in sterile plastic bowls. Experiments  
were performed within 2 h of sample collection. For  
obtaining dissociated cells, a small sample of tissue was  
placed in 20 ml of filtered seawater and submitted to  
mechanical stirring for 5 min until sponge cell dissociation.

For studying the effects of heavy metals, the sponge  
cells were incubated for 3 h in copper (30 and 100  $\mu\text{g}\cdot\text{l}^{-1}$ ),  
mercury (1 and 5  $\mu\text{g}\cdot\text{l}^{-1}$ ), and cadmium (5 and 10  $\mu\text{g}\cdot\text{l}^{-1}$ ).  
The metals were incorporated as solutions of cupric chlo-  
ride ( $\text{CuCl}_2$ ), mercuric chloride ( $\text{HgCl}_2$ ), and cadmium  
chloride ( $\text{CdCl}_2$ ). Controls consisted of cells incubated in  
filtered seawater from a nonpolluted area, where metal  
concentrations in the seawater were below the threshold  
levels detectable by ICP-MS. Temperature was maintained  
at 14°C during the incubation. Aliquots of 20  $\mu\text{l}$  of cell  
suspension of each treatment were dispensed onto a  
microscope slide. Slides were observed through a Leitz  
(Wild HSP 52) light microscope with a camera for image  
recording. A number of microscope fields ( $N = 30$ ) were  
photographed from each slide.

Pictures were digitalized; the number of cells was  
counted and their perimeter and area were measured with  
the program NIH image for Macintosh. Cell shape was  
approached from three shape indices, which were calcu-  
lated:

*Circularity index* (C) (Turon and Becerro 1992)

$$C = A_s/A_p \quad (1)$$

where  $A_s$  = area of sponge patch and  $A_p$  = area of a  
circle with perimeter equivalent to that of sponge patch; a  
value of 1 represents a perfect circle, whereas 0 is ap-  
proached as the outline becomes more irregular because of

169 the presence of pseudopodia, which is an indicator of cell  
170 motility.

171 *Directional index* (D) (Becerro et al. 1994)

$$D = 1 - (P/M) \quad (2)$$

173 where M = length of maximum straight line through two  
174 cell points and P = length of maximum straight line per-  
175 pendicular to M. This index measures cell elongation. A  
176 value of 0 indicates a perfect circle; 1 is approached as  
177 directionality increases, which denotes directional move-  
178 ment.

179 *Convolution index* (Cv) (Becerro et al. 1994)

$$Cv = 1 - (Pc/Ps) \quad (3)$$

181 where Pc = perimeter of an ellipse, with an area and  
182 directional growth index equivalent to those of the cell. This  
183 index is a measure of peripheral irregularity. A value of 0  
184 indicates a perfect ellipsoid; 1 is approached as irregularity  
185 of the border increases, as it happens when cells move.

186 *Aggregation Index* An index of aggregation was calcu-  
187 lated as the percentage of grouped cells among the total  
188 number of cells in a microscope field.

## 189 Data Analysis

190 Differences between treatments were analyzed by means of  
191 one-way analysis of variance (Statistica 4.1 package). The  
192 Tukey test was used for *post hoc* comparisons. Assump-  
193 tions of normality and homogeneity of variances were  
194 examined using the Kolmogorov-Smirnov and Barlett tests,  
195 respectively. Variables were rank-transformed (Conover  
196 and Iman 1981; Potvin and Roff 1993) prior to the analysis  
197 when assumptions were not fulfilled.

## 198 Results

### 199 Cadmium

200 Cadmium concentration in seawater had a significant effect  
201 on cell circularity (Table 1; Figure 1A). Circularity index  
202 was significantly lower (Tukey test,  $p < 0.05$ ) in cells at  
203  $10 \mu\text{g}\cdot\text{l}^{-1} \text{Cd}^{2+}$  than those maintained in seawater (SW). By  
204 contrast, directional growth and convolution were higher in  
205 cells cultured with cadmium (Table 1; Figure 1B and C):  
206 directional growth of cells at  $5 \mu\text{g}\cdot\text{l}^{-1} \text{Cd}^{2+}$  was signifi-  
207 cantly higher than in those at  $10 \mu\text{g}\cdot\text{l}^{-1} \text{Cd}^{2+}$  and in SW  
208 control (Tukey test,  $p < 0.05$ ), whereas convolution was  
209 significantly higher in cells at  $10 \mu\text{g}\cdot\text{l}^{-1} \text{Cd}^{2+}$  than at  
210  $5 \mu\text{g}\cdot\text{l}^{-1} \text{Cd}^{2+}$  and sea water (Tukey test,  $p < 0.05$ ). Cad-  
211 mium increased aggregation of *S. lophyropoda* cells. The

**Table 1** One-way analysis of variance for treatments (cadmium, copper and mercury) on shape indices and aggregation of *Scopalina lophyropoda* cells

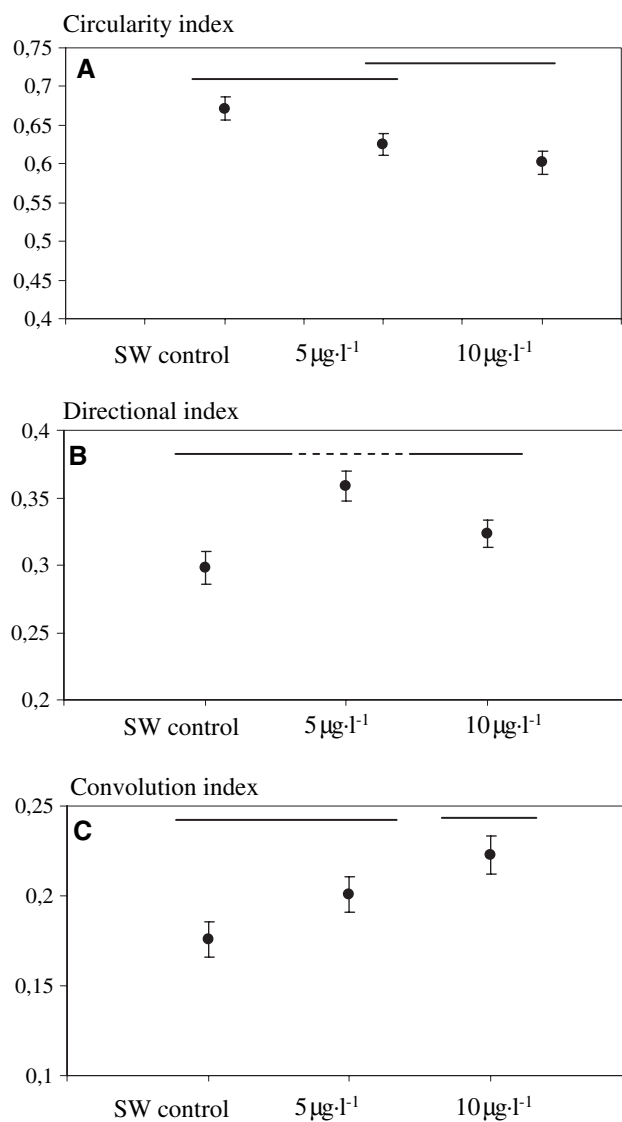
Treatment	Variable	Factor	DF	F	p
Cadmium ( $\mu\text{g}\cdot\text{l}^{-1}$ )	Circularity	Treatment	2	5.61	0.004
		Error	307		
	Directionality	Treatment	2	7.09	0.000
		Error	307		
	Convolution	Treatment	2	5.34	0.005
		Error	307		
	Aggregation	Treatment	2	56.39	0.000
		Error	220		
Copper ( $\mu\text{g}\cdot\text{l}^{-1}$ )	Circularity	Treatment	2	3.77	0.025
		Error	149		
	Directionality	Treatment	2	4.76	0.009
		Error	149		
	Convolution	Treatment	2	1.62	0.200
		Error	149		
	Aggregation	Treatment	2	5.22	0.007
		Error	80		
Mercury ( $\mu\text{g}\cdot\text{l}^{-1}$ )	Circularity	Treatment	2	7.93	0.000
		Error	279		
	Directionality	Treatment	2	5.86	0.003
		Error	279		
	Convolution	Treatment	2	5.74	0.003
		Error	279		
	Aggregation	Treatment	2	5.22	0.000
		Error	179	17.15	

aggregation index was significantly higher in cells incu- 212  
213 bated under the two cadmium treatments than in SW  
214 controls (Table 1; Figure 2).

### 215 Copper

216 Copper at the higher concentration assayed ( $100 \mu\text{g}\cdot\text{l}^{-1}$  216  
217  $\text{Cu}^{2+}$ ) (Tukey test,  $p < 0.05$ ) had a significant effect on the  
218 cells circularity index, which was significantly lower than  
219 that of cells in seawater and at  $30 \mu\text{g}\cdot\text{l}^{-1} \text{Cu}^{2+}$  (Table 1;  
220 Figure 3A). Copper at the lowest concentration assayed  
221 ( $30 \mu\text{g}\cdot\text{l}^{-1} \text{Cu}^{2+}$ ) (Tukey test,  $p < 0.05$ ) had a significant  
222 effect on the directional indices (D) of the *S. lophyropoda*  
223 cells, being significantly higher (Tukey test,  $p < 0.05$ ) in  
224 cells at  $30 \mu\text{g}\cdot\text{l}^{-1} \text{Cu}^{2+}$  than in cells at  $100 \mu\text{g}\cdot\text{l}^{-1} \text{Cu}^{2+}$  and in  
225 seawater control (Table 1; Figure 3B). No effects of cop-  
226 per concentration were observed on the convolution index  
227 for all concentrations assayed (Table 1; Figure 3C).

228 Cell aggregation was significantly enhanced in cells  
229 cultured at  $100 \mu\text{g}\cdot\text{l}^{-1} \text{Cu}^{2+}$  (Table 1; Figure 4) with respect  
230 to control cells. But no differences were found between

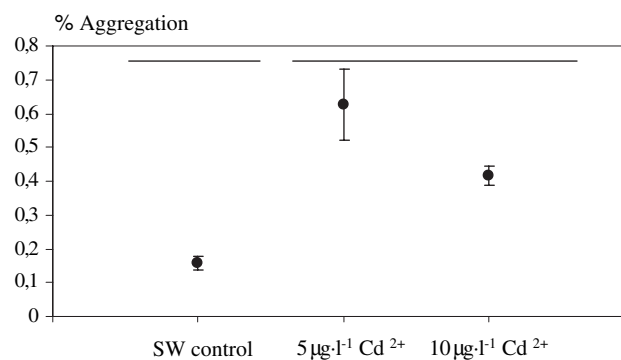


**Fig. 1** Shape indices of *Scalpinia lophyropoda* cells incubated in cadmium and SW control: circularity (A), directionality (B), and convolution (C). Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines

231 treatments and between cells at the lower concentration of  
232 copper and seawater control.

233 Mercury

234 Mercury concentration had a significant effect on circu-  
235 larity, directional growth, and convolution of the cells  
236 cultured at the highest concentration assayed (5 µg·l<sup>-1</sup>  
237 Hg<sup>2+</sup>) (Table 1; Figure 5A–C): circularity significantly  
238 increased, whereas directional growth and convolution  
239 decreased compared with SW control. But no differences  
240 were found between treatments and between cells at the  
241 lower concentration (1 µg·l<sup>-1</sup>Hg<sup>2+</sup>) and seawater control.



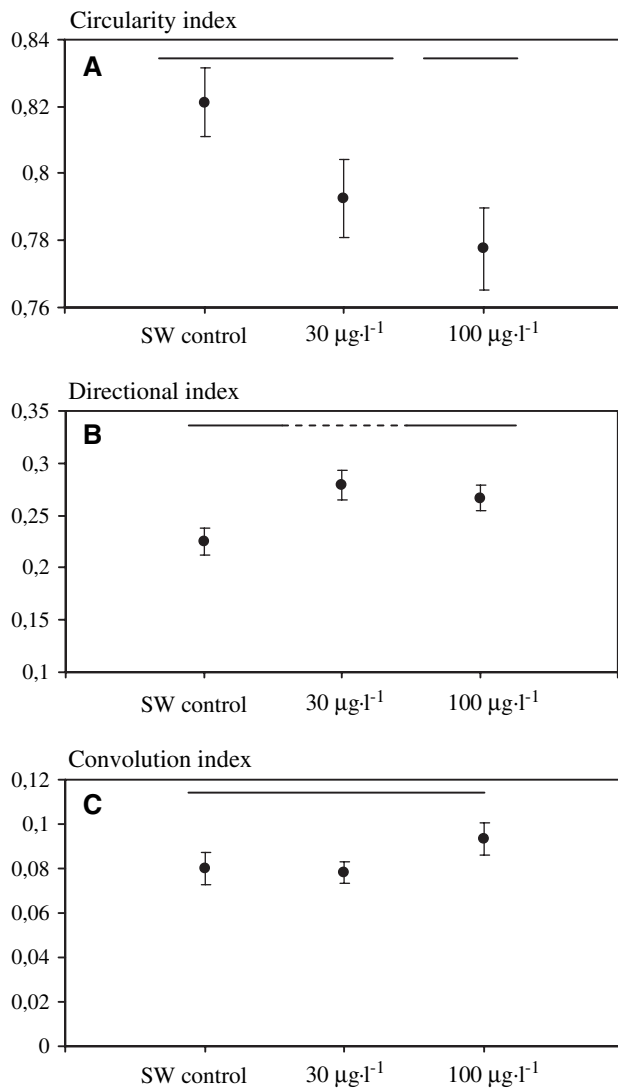
**Fig. 2** Aggregation index of *Scalpinia lophyropoda* cells, incubated in cadmium and SW control. Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines

The cell aggregation index was significantly higher in 242  
243 cells cultured with mercury at both concentrations (1 and  
244 5 µg·l<sup>-1</sup>Hg<sup>2+</sup>) (Tukey test,  $p < 0.05$ ) than in seawater  
245 control cells (Table 1; Figure 6).

## 246 Discussion

247 Sponge populations in metal polluted environments can  
248 bioaccumulate metals (e.g., Cebrian et al. 2003, 2006),  
249 which may impair cell functions. In the present study, we  
250 have shown that copper, cadmium, and mercury cause  
251 morphological cellular changes and affect cell aggregation  
252 in *S. lophyropoda* after 3 h of metal incubation. Changes in  
253 cell shape and motility, which are likely related with  
254 changes in the cytoskeleton (White 1974; Syversen et al.  
255 1984), have also been reported to occur in cells of other  
256 invertebrates submitted to heavy metals (Burlando et al.  
257 2000, 2003).

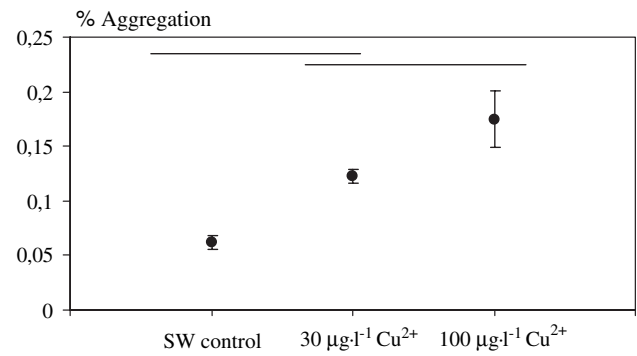
258 *S. lophyropoda* cells did not respond in the same manner  
259 to the different metal pollutants assayed. Cadmium and  
260 copper caused an increase of the irregularity and direc-  
261 tional shape of sponge cells by enhancing pseudopodia/  
262 phylopodia formation, which indicate cell movement. In  
263 line with our results, it has also been reported that cadmium  
264 and copper at low concentrations significantly increase  
265 shape irregularity (Gómez-Mendikute and Cajaraville  
266 2003) and pseudopodia production in other invertebrate  
267 cells by promoting phagocytosis (Cheng and Sullivan  
268 1984; Coles et al. 1995; Pipe et al. 1999; Olabarrieta et al.  
269 2001). Conversely, at higher concentrations or during  
270 longer incubation times (e.g., at 100 ppm Cd<sup>2+</sup> during 24 h  
271 or at 5 ppm Cu<sup>2+</sup> for 1 month), the effects of copper and  
272 cadmium appear to be harmful for invertebrate cells be-  
273 cause they inhibit cytoskeleton production and the cells  
274 adopt a round shape with no cell extensions (Cheng and  
275 Sullivan 1984).



**Fig. 3** Shape indices of *Scopalina lophyropoda* cells incubated in copper and SW control: circularity (A), directionality (B), and convolution (C). Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines

276 In *S. lophyropoda*, low concentrations of mercury, even  
 277 after a short period of time, caused impairments on the  
 278 sponge cell activity that were not evidenced with copper  
 279 and cadmium. Incubated cells became more spherical and  
 280 without irregularities and cytoplasmic pseudopodia (in-  
 281 volved in phagocytosis and cell movement). Thus, among  
 282 the three metals studied, mercury seems to be by far the  
 283 most toxic as for cytoskeleton alteration and the subsequent  
 284 decrease in cell motility.

285 Heavy metals have been reported to modify cell calcium  
 286 homeostasis, which results in a series of cytotoxic mech-  
 287 anisms that affect the cytoskeleton (Cima et al. 1998)  
 288 causing a spherical shape of cells. A similar process may



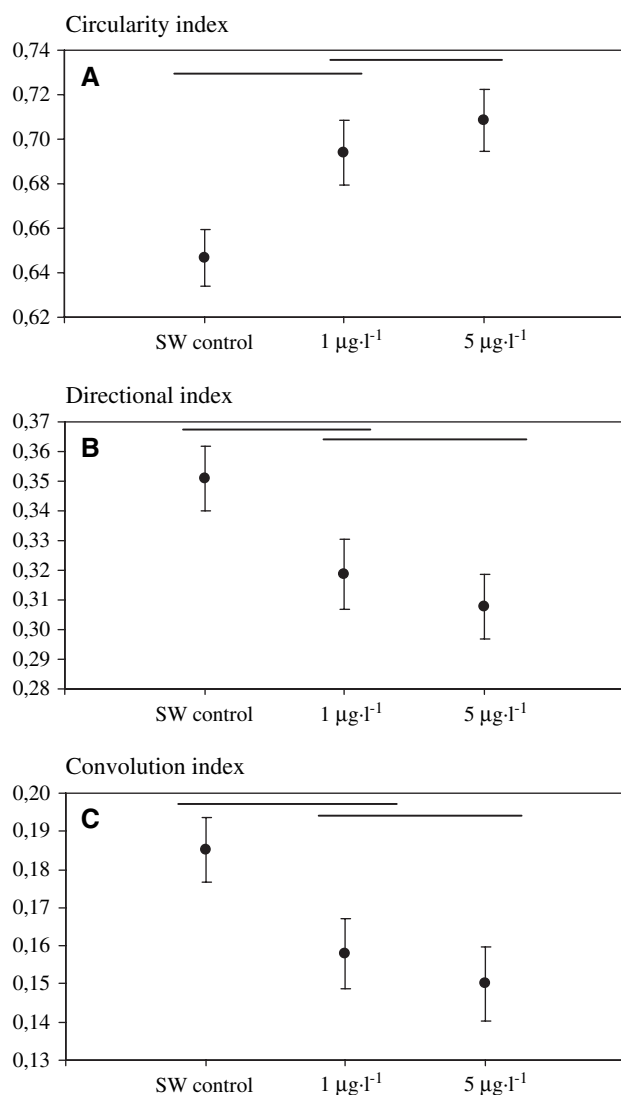
**Fig. 4** Aggregation indices of *Scopalina lophyropoda* incubated in copper and SW control. Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines

also be responsible for the spherical shape of *S. lophyropoda* cells submitted to mercury, but it does not seem to occur in sponge cells under copper and cadmium, likely because of the low metal concentrations or the short exposition time used in our experiments. It seems that mercury is more toxic to the sponge cells than copper and cadmium at the concentrations assayed.

Actually, most pollutants enter marine waters in short pulses at moderate concentrations, conditions that we aimed to reproduce in the present study. However, a noxious effect of copper and cadmium at higher concentrations or longer exposition cannot be ruled out.

Copper, cadmium, and mercury enhanced cell aggregation in *S. lophyropoda* at the concentrations assayed (30 and 100 µg·l<sup>-1</sup> copper, 5 and 10 µg·l<sup>-1</sup> cadmium, and 1 and 5 µg·l<sup>-1</sup> mercury). We suggest that an increment of the cytosolic calcium concentration, which is required for cell aggregation in sponges (Dunham et al. 1983; Weissmann et al. 1985), was induced by the heavy metals through an alteration of calcium homeostasis (e.g., Burlando et al. 2000; Pourahmad and O'Brien 2000) and an increase in the intracellular calcium concentration. However, much higher concentrations (10 ppm) of copper and cadmium may be toxic, because they have been reported to inhibit cell aggregation in oyster hemocytes, whereas intermediate concentrations (1 ppm) did not affect cell aggregation at all (Auffret and Oubella 1997).

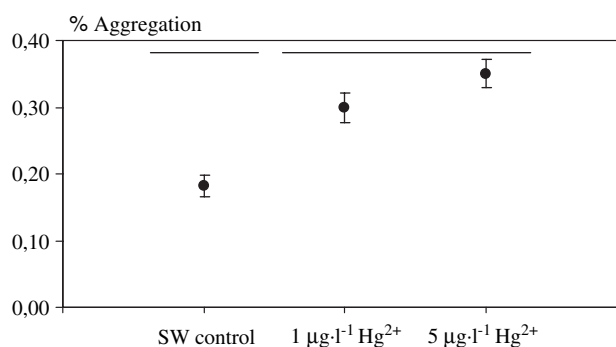
To summarize, the three metals studied induced changes of different sign on cell shape. Concentrations of 1 or 5 µg·l<sup>-1</sup> Hg<sup>2+</sup> impaired normal behavior of sponge cells, which tended to be rounded, without pseudopodia, which impairs several cellular functions, such as motility and phagocytosis. In contrast, moderate concentrations of copper and cadmium enhanced pseudopodia formation and cell motility. On the other hand, the three metals enhanced cell aggregation at the concentrations assayed.



**Fig. 5** Shape indices of *Scopalina lophyropoda* cells incubated in mercury and SW control: circularity (A), directionality (B), and convolution (C). Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines

325 Our results show that sponge cells respond to metal  
 326 pollution in different ways and that these responses can be  
 327 detected by calculating several shape indices. These effects  
 328 at a cellular level may anticipate changes that would occur  
 329 at higher levels of biological organisation, such as organ-  
 330 isms and populations. The study of effects at the cellular  
 331 level add to the available methods for early detection for  
 332 harmful pollution-related effects, which is desirable for  
 333 designing adequate management strategies and to prevent  
 334 future outbreaks in marine ecosystems.

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 337 funded by grants from the CE (SPONGES project COOP-CT-205-



**Fig. 6** Aggregation indices of *Scopalina lophyropoda* cells incubated in mercury and SW control. Vertical bars are standard errors. Mean concentrations, which were not significantly different in a Tukey test, are joined by horizontal lines

017800) and the CICYT (Spain) (INTERGEN, CTM2004-05265/MAR).

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