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## Assessment of swimming behavior of the Pacific oyster D-larvae (*Crassostrea gigas*) following exposure to model pollutants

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1 Assessment of swimming behavior of Pacific oyster D-larvae (*Crassostrea gigas*)  
2 following exposure to model pollutants

3  
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15 This study describes an image analysis method that has been used to analyze early life stages behavior  
16 of native oysters (*Crassostrea gigas*) from the Arcachon Bay (SW, France). In a second time, this study  
17 evaluated the impact of Cu and S-metolachlor pollutants on D-larvae behavior and possible  
18 relationship between developmental malformations and abnormal swimming behavior. Analyses in  
19 wild and cultivated oyster D-larvae were investigated during two breeding-seasons (2014 and 2015) at  
20 different sampling sites and dates. In control condition, the average speed of larvae was 144  $\mu\text{m}\cdot\text{s}^{-1}$  and  
21 the maximum speed was 297  $\mu\text{m}\cdot\text{s}^{-1}$  while the trajectory is mainly rectilinear. In the presence of  
22 environmental concentration of copper or S-metolachlor, no significant difference in maximum or  
23 average larval speed was observed compared to the control condition but the percentage of circular  
24 trajectory increased significantly while the rectilinear swimming larvae significantly declined. The  
25 current study demonstrates that rectilinear trajectories are positively correlated to normal larvae  
26 while larvae with shell anomalies are positively correlated to circular trajectories. This aberrant  
27 behavior could impacts the survival and spread of larvae and consequently the recruitment and  
28 colonization of new habitats.

29  
30 **Key words:** early life stage; Image analysis; speed; trajectory; malformations; copper; S-  
31 metolachlor, swimming behavior.

32  
33 **Introduction**

34 Behavioral analysis is increasingly used to study the effects of chemicals and drugs on humans and  
35 other mammals (XXX). In recent years, many biological early warning systems have been developed  
36 that evaluate the behavioral responses of aquatic organisms to water quality (Melvin and Wilson,  
37 2013; Garaventa et al., 2010; Van der Schalie et al., 2001). In contrast, effects of contaminants on  
38 aquatic invertebrate behavior are less frequently studied in comparison to developmental or  
39 reproductive toxicology (Melvin and Wilson, 2013; Scott and Sloman, 2004). However, behavioral  
40 indicators of toxicity appear ideal for assessing the effects of pollutants on aquatic organisms since  
41 they link physiological function with ecological processes (Scott and Sloman, 2004). In aquatic  
42 toxicology, the link between behavioral science and impacts of toxic substances only took importance  
43 over the last decade (review in Faimali et al., 2017). The behavior can be the result of adaptations to  
44 environmental variables. Therefore, a selective response is permanently adapted by direct interaction  
45 with the physiological aspects of the chemical and physical social environment. Thus, the study of  
46 behavioral parameters now represents valuable tools to identify and assess the effects of exposure to  
47 environmental stressors. Until a few years ago, the development of tools facilitating image acquisition  
48 and behavioral endpoints, could explain this phenomenon (Melvin and Wilson, 2013). In recent years,  
49 much progress has been made in the technological tools available for quantifying behavior (Lv et al.,  
50 2013). Thus, the study of behavioral parameters now represents valuable tools to identify and assess  
51 the effects of exposure to environmental stressors. Many studies have focused on the behavioral study  
52 of fish larvae or adults (Caldwell et al., Handy et al., 1999; Kazlauskienė et al., 2010; Le Bihanic et al.,  
53 2015; Sommers et al., 2016; McCallum et al., 2017; Martin et al., 2017). Very few studies have focused  
54 on mollusks and more particularly on bivalves (review in Faimali et al., 2017). However, bivalve  
55 mollusks such as mussels, clams and oysters contribute significantly to world aquaculture production  
56 and the same species have been used as sentinel for pollution monitoring of coastal marine waters  
57 The Pacific oyster *Crassostrea gigas* (Thunberg) is ranked number one in terms of world aquaculture  
58 production with 555 913 tons estimated in 2013 (FAO, 2015). Despite living attached for most of their  
59 life stages, bivalves have a swimming larval stage, which play an important part for the colonization of  
60 the environment. In *C. gigas* embryos incubated at 20 °C, the first movements were observed in  
61 trochophore at 6.5 hours post-fertilization (hpf) and most of individual (85%) can swim at 11.5 hpf  
62 (Suquet et al., 2013). Up to now, only a few works has focused on the swimming behavior of early life  
63 stage of marine bivalves (Mileikovsky, 1973; Toost et al., 2008) and only a few ones have investigated  
64 the abiotic factors as pH, salinity (Suquet et al., 2012; Suquet et al., 2013) and pollutants (Horiguchi  
65 et al., 1998) on swimming capacities of bivalves. To our knowledge, none investigated the impact of  
66 pollutants on oyster larval swimming capacities.

67 To analyze the possible impact of pollutants on oyster larval behavior, two pesticides, copper and  
68 metolachlor, frequently occurring in the marine coastal water were tested. Copper (Cu) is among the  
69 most hazardous metal for bivalve larvae (His et al., 1999). Although Cu is an essential micronutrient for  
70 living organisms (Festa and Thiele, 2011), it can be toxic above a certain concentration depending on  
71 the organism (Flemming and Trevors, 1989). All over the world, Cu is used both as fungicide in fruit  
72 culture and as part of antifouling paint to prevent aquatic organisms from attaching themselves to the  
73 hulls of vessels (Turner, 2010). Copper concentrations in the Arcachon Bay have been increasing for  
74 several years and current levels exceed  $\mu\text{g.L}^{-1}$  (Gamain et al., 2016). The impact of copper on aquatic  
75 organism behavior has already been documented on fish (Sommers et al., 2016). Pesticide  
76 contamination in Arcachon Bay is widely dominated by the herbicide S-metolachlor at around  $10 \text{ ng.L}^{-1}$   
77 and its metabolites (Auby et al., 2007; Budzinski et al., 2011; Gamain et al., 2016). In fact, Arcachon  
78 Bay is end recipient of several rivers draining a watershed of  $4138 \text{ km}^2$  (Auby et al., 2014), which is  
79 mainly dominated by agriculture and urban areas. Metolachlor is used in agriculture to control pre-  
80 emergent and early post-emergent broadleaf and grass weeds, and is regularly detected in surface  
81 water and groundwater.

82 The main objective of this paper is to study the behavior of D-larvae of oysters, *Crassostrea gigas* in  
83 response to copper or S-metolachlor exposure. In order to do that, a method based on image analysis  
84 was developed. The open-source program ImageJ (<https://imagej.nih.gov/ij/>) and the ImageJ Plugin  
85 wrMTrck were used. This plugin was initially developed to study the motility of the nematoda  
86 *Caenorhabditis elegans* while swimming in liquids (Nussbaum-krammer et al. 2015) and it presents  
87 therefore an interesting similarity with swimming larvae. The method allows analyzing multiple  
88 parameters including the total travelled length, the distance and the speed of larvae.

89 This paper aims to: 1) describe an image analysis method that has been used to analyze D-larvae  
90 behavior, 2) apply this process to assess the impact of Cu and S-metolachlor pollutants on D-larvae  
91 behavior and 3) investigate possible relationship between developmental malformations and  
92 abnormal swimming behavior.

## 93 **2. Materials and methods**

94

### 95 **2.1 Method based on imagery**

#### 96 2.1.1 Image acquisition

97 To analyze the behavior of the native oyster, wild and cultivated oysters were collected during oyster  
98 breeding period (July and August 2014 and 2015) from three different sites in the Arcachon Bay:  
99 Comprian, Les Jacquets and Banc d'Arguin (Fig. 1). Comprian is located close to the River L'Eyre and

100 therefore is under the influence of fresh water inputs in contrast to Les Jacquets or to Arguin. The  
101 reference water was collected near the Banc d'Arguin. Adult oysters were brought back to the  
102 laboratory in tanks filled with seawater from the sampled site and were kept overnight in this water  
103 continuously aerated and maintained at 12 °C.

104 Mature oysters (male and female) were induced to spawn by thermal stimulation, alternating  
105 immersion in FSW of 15 °C and 28 °C for 30 min. Spawning males and females were individually isolated  
106 in beakers containing 500 mL of 0.2 µm FSW at the spawning temperature. They were left undisturbed  
107 for 15 min and were then removed from beakers. Eggs and sperm from two individuals were selected  
108 to give a single pairing. Sperms and eggs were sieved separately through a 50 µm and 100 µm meshes  
109 (Sefar Nitex), respectively to eliminate debris and feces. Sperm mobility was checked and the number  
110 of eggs was counted under microscope (LEICA DME) at a magnification of 100. Eggs were fertilized with  
111 sperm in ratio of 1:10 (egg:sperm) homogenizing with an agitator to prevent polyspermy. Fertilization  
112 success was verified under microscope, and embryos were then counted and transferred to 24-well  
113 microplate (Greiner Bio-One, CellStar free of detectable DNase, RNase, human DNA and pyrogens) for  
114 embryotoxicity assays. The embryotoxicity assay has been described in details by His et al. (1999) and  
115 Quiniou et al. (2005) and normalized (AFNOR 2009). Fertilized eggs (around 300 eggs) were exposed  
116 in wells containing 2 ml of toxicant solution. The eggs density was slightly changed compare to the  
117 recommended AFNOR document specifying densities between 20 000 and 50 000 embryos per liter.  
118 These microplates were incubated in climatic chamber at 24 °C for 24 h in the dark.

119 After 24h incubation, a 2-minute film was recorded by means of a microscope (Nikon Inverted  
120 Microscope Eclipse TS 100 / TS100-F, TS100LED MV / MV F-TS100 LED) and an acquisition software  
121 (NIS Element D). Recorded films were on a MPEG format and included 100 frames per second.

122

### 123 2.1.2 Description of the image analysis process

124 A freeware (VirtualDub) for video conversion has been used to subsample the film to 4 fps and convert  
125 it into AVI format. The AVI format is used within ImageJ. The entire flow of analysis as well as the  
126 expected results are schematized on Fig. 2. The films (.avi) are opened as a stack of images (Fig. 2A)  
127 and converted into grayscale (Fig.2B). The entire stack of images is then converted into a binary stack  
128 of images using the maximum entropy (Kapur et al., 1985) of their histogram to establish a threshold  
129 (Fig. 2C). When running the wrMTrck plugin, proper settings to track oyster larvae were selected (Table  
130 1).

131 As a result, each tracked larvae had a number assigned, each number is used to identify the tracked  
132 larvae in the result file. The result file (Fig. 2E) include: the track number, the length which is the sum  
133 of all movement vectors (pixel), the distance which is the distance between the start and the finish

134 position (pixel), the number of frames, the first frame, the total time (seconds), the maximum speed  
 135 (pixel/second), the mean and the standard deviation of the surface of a larva during the tracked period  
 136 (pixel<sup>2</sup>), the mean and the standard deviation of the perimeter of a larva during the tracked period  
 137 (pixel), the average speed, the body length per second, the average X position of the larva during the  
 138 tracked period and the average Y position of the larva during the tracked period. A drawing gathering  
 139 all tracked larval paths detected throughout the video (Fig. 2D) is also obtained as a result.

140 The gathering drawing allowed to identify three different types of traces of larval path: (1) Rectilinear,  
 141 defined as the trajectory in which the length and the distance are quite similar (Track 1 Fig. 3), (2)  
 142 Circular, defined as the trajectory in which the length is a lot bigger than the distance (Track 2 Fig. 3),  
 143 (3) motionless, in which the length has a low value (Track 3 from Fig. 3). It is important to note that a  
 144 single larva can be detected multiple times since they can exit and enter the field of view (Track 4 from  
 145 Fig. 3). Furthermore, two larvae may collide, if it is the case, their larval path may be altered (Track 5  
 146 from Fig. 3).

147

148 **Table 1:** Optimized settings for wrMTrck plugin

149

<b>Parameters for tracking oyster D-larvae in 'wrMTrck'</b>	<b>Value</b>
Minimum size (pixel <sup>2</sup> )	400
Maximum size (pixel <sup>2</sup> )	1000
Maximum velocity (pixels/frame)	100
Maximum area change (%)	50
Minimum track length	20
Threshold for turn	2
Size of bin for speed histogram (pixel frame)	0
Show Path Length	Yes
Show Labels	Yes
Show Positions	Yes
Show Paths	Yes
Show summary	Yes
Smoothing	Yes
Raw Data (0:off, 5:bendcalc)	0
Ben detection (0:off, 3:AR+Histogram)	0
Frame per second	4
Background subtraction (0:off)	0

Threshold method	Otsu
Size of labeling font	18

---

150

## 151 **2.2 Developmental abnormalities**

152

153 At the end of the 24 h incubation, 25  $\mu\text{L}$  of 1% buffered formalin were added in each well and the  
154 percentage of abnormal larvae was recorded. Hundred individuals per well were directly observed  
155 under inverted microscope (Nikon eclipse TS100/TS100-F; TS100 LED MV/ TS100 LED-F MV) to  
156 determine the number of abnormal D-shell larvae according to the criteria described in His et al. (1999)  
157 and Quiniou et al. (2005). An important prerequisite for this test is the presence, in control condition  
158 (24 °C in the absence of contamination) of less than 20 % of abnormal larvae. In this experimentation,  
159 four different couples were used and four replicates were performed for each condition.

160

## 161 **2.3 Chemical analyses**

162 Reference toxicants ( $\text{CuSO}_4$  and S-metolachlor) and formalin were purchased from Sigma–Aldrich  
163 Chemical (St. Quentin Fallavier, France). Seawater was collected outside the Arcachon Bay (SW France)  
164 near the Banc d'Arguin. Immediately after sampling, seawater was filtered using membrane filter of  
165 0.45  $\mu\text{m}$  and then 0.2  $\mu\text{m}$  (Xilab) to eliminate debris and microorganisms. FSW was stored at 4 °C in  
166 the dark and was used within 3 days. A few hours before experiment, FSW was filtered again at 0.2  
167  $\mu\text{m}$ . This reference water was chemically analyzed for pollutant concentration determination.

168 The range of test concentrations was chosen on the basis of preliminary studies (Gamain et al., 2016).  
169 The metal and pesticide solutions were made up from analytical grade copper sulfate ( $\text{CuSO}_4, 5\text{H}_2\text{O}$ )  
170 and S-metolachlor. Working solutions were obtained diluting the stock solutions (100  $\text{mg}\cdot\text{L}^{-1}$  for copper  
171 and 250  $\text{mg}\cdot\text{L}^{-1}$  for S-metolachlor) in FSW and were chemically analyzed. Two concentrations of  
172 exposure were selected for copper: 1 and 10  $\mu\text{g}\cdot\text{L}^{-1}$  and three for -S-metolachlor: 10, 100 and 1000  
173  $\text{ng}\cdot\text{L}^{-1}$ . The working solutions were chemically analyzed to confirm pollutant concentrations.

174

### 175 **2.3.1 Copper analysis**

176 For chemical analysis of copper at 1 and 10  $\mu\text{g}\cdot\text{L}^{-1}$ , each seawater sample was acidified with 5% final of  
177 nitric acid (Nitric acid 65%, Fluka). Samples were then analyzed by Inductively-Coupled Plasma Optic  
178 Emission Spectrometry (ICP-OES, Vista Pro, Agilent Technologies) and by Inductively-Coupled Plasma  
179 Mass Spectrometry (ICP-MS, Xseries2, Thermofisher Scientific). The standards solutions were prepared  
180 from a multi-elementary calibration solution (Astasol-Mix M010, Analytika, Czech Republic), in a  
181 seawater certified solution (NASS-6 from NRCC-CNRC, Ottawa, Canada). The samples were diluted in a

182 3% final nitric acid solution (made from a nitric acid 65% FisherScientific Trace Metal Grade solution)  
183 1:2 (v/v) for ICP-OES analysis and 1:3 (v/v) for ICP-MS analysis. Quantification limit were  $10 \mu\text{g}\cdot\text{L}^{-1}$  (ICP-  
184 OES) and  $0.3 \mu\text{g}\cdot\text{L}^{-1}$  (ICP-MS).

185

### 186 2.3.2 Metolachlor analysis

#### 187 *Chemical extraction*

188 50-mL water samples (pH adjusted to  $7.0 \pm 0.1$  with HCl 0.1 N) were filtered using GF/F glass microfiber  
189 filters ( $0.7 \mu\text{m}$  pore size). Before analysis, pre-concentration of the analytes was performed using Solid-  
190 Phase Extraction (SPE) with Oasis HLB cartridges (Waters), according to the method described by  
191 Lissalde et al. (2011). SPE was conducted using a Visiprep 12-port manifold (Supelco, France). The  
192 conditioning, extraction and rising steps were carried out under a 53.33 kPa vacuum. The SPE cartridges  
193 were successively washed with 5 mL of methanol, conditioned with 5 mL of ultrapure water, loaded  
194 with 50 mL water samples, then rinsed with 5 mL of UPW containing 15% HPLC grade methanol.  
195 Cartridges were then dried under a nitrogen stream for 30 min. Elutions were achieved with 3 mL of  
196 methanol, followed by 3 mL of a mix of methanol: ethyl acetate (75:25 v/v).  $2.5 \mu\text{L}$  of a solution of  
197 internal standard (metolachlor d6) at  $1 \text{ ng } \mu\text{L}^{-1}$  was then added to the 6-mL extracts, followed by a  
198 solvent evaporation under a gentle stream of nitrogen and then dissolved in  $250 \mu\text{L}$  of UPW containing  
199 10% HPLC grade acetonitrile prior to analysis.

200

#### 201 *Instrumentation and data treatment*

202 Metolachlor analyses were performed by liquid chromatography ACQUITY UPLC H-Class coupled to a  
203 Xevo G2-S TOF-MS (Waters). The electrospray source was operated in positive mode at 0.7 kV and the  
204 sample cone voltage set at 30 V. Nitrogen was used as nebulizer (flow rate  $50 \text{ L h}^{-1}$ ,  $150 \text{ }^\circ\text{C}$ ) and  
205 desolvation gas (flow rate  $1200 \text{ L h}^{-1}$ , temperature  $600 \text{ }^\circ\text{C}$ ). Data was acquired in the range from 50 to  
206  $1200 \text{ m/z}$  and acquisition speed was set to 0.2 s. The resolving power full width at half maximum  
207 (FWHM) was 30,000 at  $\text{m/z}$  556.2771 (leucine enkephalin used as lockmass compound). Data was  
208 acquired using MSE in order to obtain both protonated molecular ions at low collision energy (CE=6  
209 eV) and/or adducts and fragment ions with a collision energy ramp (CE=10-30 eV). Separation was  
210 performed on an ACQUITY BEH C18 column ( $100 \times 2.1 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) from Waters (Milford, MA, USA)  
211 with a column temperature of  $45 \text{ }^\circ\text{C}$  and using a binary gradient of water (A) and methanol (B) both  
212 containing ammonium acetate (10 mM) at pH 5.0. A flow rate of  $0.45 \text{ mL min}^{-1}$  was used and the  
213 gradient ranged as follows: 98% A (0–0.25 min), 1% A (12.25–13 min), 98% A (13.01–17.00). Injection  
214 volume was  $20 \mu\text{L}$ . Data treatment was performed with MassLynx v4.1.

215

#### 216 *Method validation and quality controls*



217 Analytical method was validated in terms of calibration linearity, extraction recoveries, and limits of  
 218 quantifications (LOQ) according to the French standard NF T90-210. Recovery, LOQ and LOD are  
 219 showed in Table for the metolachlor into the different samples. For the quality controls, SPE was  
 220 carried out routinely controlled, and the recoveries of two levels of spiked mineral water (e.g. 40 and  
 221 200 ng L<sup>-1</sup>) were evaluated for each batch. The periodic control of two calibrating standards (e.g. 2 and  
 222 25 µg L<sup>-1</sup>, every 10 samples) and analytical blanks were performed as well.

223

224

225

**Table 2.** Acquisition and validation data for the metolachlor.

Compound	Retention time (min)	Quantification ion (m/z)	Confirmation ion (m/z)	Internal standard	% Recovery (±1 SD)	LOQ <sup>a</sup> (ng.L <sup>-1</sup> )	LOD <sup>a</sup> (ng.L <sup>-1</sup> )
Metolachlor	9.52	284.142	176.144	Metolachlor d6	110 (22)	0.4	0.1
Metolachlor d6	9.49	289.172	182.181	N/A	N/A	N/A	N/A

226 <sup>a</sup> Limits of quantification and detection, respectively.

227

228

229

## 230 2.4 Statistical analysis

231

232 All data is expressed as means ± standard deviation (S.D.). Data was first processed using the  
 233 transformation:  $p' = \arcsin \sqrt{p}$ . P corresponds to the raw data (frequency of abnormalities)  
 234 specified in p values from 0 to 1 (Legendre and Legendre 1998). Homogeneity of variance (Levene's  
 235 test) was verified and statistical analysis was performed by the Kruskal-Wallis tests. Differences among  
 236 data from different conditions were tested using Kruskal post hoc test (equivalent to the Tuckey HSD  
 237 test for non-parametric data).

238 Principal component analysis (PCA) was performed using XXX to analysis both the spatial distribution  
 239 pattern of the different studied parameters and the relative relationship between treatment groups.

240

## 241 3. Results

242

### 243 3.1 Chemical analysis

244

245 The copper and S-metolachlor concentrations measured in the reference seawater and the different  
 246 contamination solutions are shown in Table 3. Analyzes revealed the presence of copper in the  
 247 reference seawater at concentrations of 3.6  $\mu\text{g.L}^{-1}$ . At the nominal concentration of 1  $\mu\text{g.L}^{-1}$ , the  
 248 measured concentration was closed to that of control (3.75  $\mu\text{g.L}^{-1}$ ). At 10  $\mu\text{g.L}^{-1}$ , the measured  
 249 concentration was 18% above expected concentration. Analyzes revealed the presence of S-  
 250 metolachlor in the reference seawater at concentrations lower than 5  $\text{ng.L}^{-1}$ . At the nominal  
 251 concentration of 10  $\text{ng.L}^{-1}$ , measured concentration was 18  $\text{ng.L}^{-1}$ . At 100  $\text{ng.L}^{-1}$  and 1000  $\text{ng.L}^{-1}$   
 252 measured concentrations were closed to expected one with a range of variation of the order of 8.1%  
 253 and 8.4% respectively.

254

255 **Table 3:** Nominal and measured copper and S-metolachlor concentrations (mean values  $\pm$  SD) at the  
 256 beginning of the embryotoxicity test

257

	Nominal concentrations	Control (FSW)	1	10	100	1000
Measured concentration	Copper ( $\mu\text{g L}^{-1}$ )	3.63	3.75	11.8	-	-
	S-metolachlor ( $\text{ng L}^{-1}$ )	4.6	-	18	108.1	916

258

259

260

### 261 **3.2 Swimming activity**

262

263 In the control, the average speed of larvae was  $144.4 \pm 34.6 \mu\text{m.s}^{-1}$  and the maximum speed was  
 264  $297.3 \pm 85.2 \mu\text{m.s}^{-1}$  (Fig. 4) while the trajectory is mainly rectilinear ( $80.8 \pm 15.6$  % of larvae) and to a  
 265 lesser extent circular ( $17.5 \pm 13.3$  %). (Fig. 5). Motionless larvae are poorly observed in the control group  
 266 ( $1.9 \pm 3.1$  %). In the presence of copper, no significant difference in maximum or average larval speed  
 267 was observed at the concentration of 1  $\mu\text{g.L}^{-1}$  or 10  $\mu\text{g.L}^{-1}$  compared to the control condition (Fig. 4A).  
 268 After exposure to S-metolachlor, no significant difference in maximum or average larval speed was  
 269 observed at the concentrations of 1  $\mu\text{g.L}^{-1}$  and 10  $\mu\text{g.L}^{-1}$  compared to the control condition (Fig. 4B).  
 270 Following copper exposure at both concentrations, the percentage of circular trajectory increased  
 271 significantly while the rectilinear swimming larvae significantly declined and a dose-dependent effect  
 272 was observed (Fig. 5A). In the presence of S-metolachlor, the percentage of circular trajectory tends  
 273 to increase with increasing S-metolachlor concentrations but it was only significant at 100  $\text{ng.L}^{-1}$  (Fig.  
 274 5B). In the meanwhile, rectilinear trajectories were reduced at all concentrations tested but it was only  
 275 significant at 10 and 1000  $\text{ng.L}^{-1}$ .

276

### 277 3.3 Malformations

278

279 **Table 3: Abnormal larvae and malformation frequency in oyster larvae exposed to copper or S-**  
280 **Metolachlore**

Conditions	Abnormal larvae	Development arrest	Mantle anomalies	Shell anomalies
Control	17.0 ± 2.42	4.05 ± 1.85	7.4 ±	5.55 ±
Cu 1	36.98 ± 3.30	10.94 ± 2.49	14.5 ±	11.79 ±
Cu 10	60.31 ± 6.6	19.67 ± 1.61	25.89 ±	14.75 ±
Met 10	32.48 ± 11.51	14.38 ± 3.21	12.58 ±	5.52 ±
Met 100	47.75 ± 12.58	18.70 ± 5.52	12.15 ±	16.9 ±
Met 1000	59.15 ± 5.24	28.60 ± 9.89	16.20 ±	14.35 ±

281

282 Abnormal larvae average frequency was  $17.0 \pm 2.4$  % in control condition (Fig. 6 and Table 3). The  
283 percentage of developmental arrest increase significantly at  $10 \mu\text{g.L}^{-1}$  of copper while a dose-  
284 dependent increase of malformed larvae was observed at all copper concentration tested (Fig. 6A and  
285 Table 3). After exposure to S-metolachlor the percentage of developmental arrest was significantly  
286 increased from  $100 \text{ ng.L}^{-1}$  whereas the percentage of abnormalities increased significantly only at  $1000$   
287  $\text{ng.L}^{-1}$  (Fig. 6B and Table 3).

288

### 289 3.4 Relationship between malformations and swimming behavior

290 PCA featured 60.88 % of the selected variables considering the two first axes. The first axis accounted  
291 for 38.23% while the second axis accounted for 22.65% of the variability.

292 For the plot of variables (Fig 7A), the PC1 was positively loaded by both circular trajectories and shell  
293 abnormalities, and negatively loaded by rectilinear trajectories. PC2 was negatively loaded by  
294 maximum and average speeds. Rectilinear trajectories appeared positively correlated to normal larvae  
295 ( $r=0.47$ ,  $p=0.012$ ) while larvae with shell anomalies were positively correlated to circular trajectories  
296 ( $r=0.60$ ,  $p=0.001$ ). In addition, motionless larvae were obviously anti-correlated to maximum and  
297 average speeds.

298 For the plot of treatment groups (Fig. 7B), the PC1 and PC2 allowed a good separation of the control  
299 group versus copper  $1 \mu\text{g/L}$  and copper  $10 \mu\text{g/L}$  groups. The separation of the control group and

300 metolachlor 100 ng/L and metolachlor 1000 ng/L groups was well defined. In contrast, the metolachlor  
301 10 ng/L group was partly overlapping with the control, metolachlor 100 and 1000 ng/L groups.

302 Le groupe contrôle est bien associée à des larves normales et à des trajectoires rectilignes (Figure 7  
303 A&B). Les malformations du manteau sont corrélées principalement à l'exposition au cuivre à 10 µg/L  
304 et au S-metachlor à 1000 ng/L.

305

## 306 **4. Discussion**

### 307 **4.1 Normal swimming behavior of oyster D-larvae**

308 The herein described video tracking system represents an operational tool to evaluate different  
309 swimming parameters of D-larvae of the Pacific oyster *Crassostrea gigas*. A lot of others automated  
310 systems have been described to analyze swimming activity, motility or frequency of pulsation in  
311 numerous invertebrates (for review see Faimali et al., 2017) but to our knowledge, this study is the  
312 only one analyzing both swimming speed and trajectory.

313 Our semi-automated video tracking system was able to identified three major types of traces of larval  
314 path: rectilinear, circular and motionless. It is to be noted that the identified three traces of larval path  
315 do only consider two dimensions and that larvae oysters moving in their environment can move in  
316 three dimensions. A study on three dimensions would not affect rectilinear paths but circular paths  
317 might split between real circles and 3D spirals.

318 In our experiment, under optimal development conditions (24 °C, salinity of 33 usi), oyster larvae  
319 mainly adopted rectilinear trajectories (81% ± 15). Thus, the most common behavior of the larvae  
320 would correspond to rectilinear swimming. These results confirm the early work of His et al. in 1989  
321 on the mussel *Mytilus galloprovincialis*, which suggested that a circular or spiral swimming denoted an  
322 erratic behavior of the larvae.

323 In the same laboratory conditions (24 °C, salinity of 33 usi), the average speed and the mean maximum  
324 velocity of oyster D-larvae were 144 and 297 µm.s<sup>-1</sup>, respectively. Suquet et al., (2012) reported an  
325 average swimming speed of 105 µm.s<sup>-1</sup> for *C. gigas* D-larvae in the absence of contamination. This  
326 value is slight lower than the one calculated in our study. This difference can be explained, at least in  
327 part, by the temperature difference between the two experiments, e.g. 19 °C for Suquet's study and  
328 24°C for this study. 24 °C correspond to the ideal temperature of development for *C. gigas* (AFNOR,  
329 2009, Gamain et al., 2016). Incubation of *C. gigas* embryos at temperature less than or equal to 20 °C  
330 give rise to significant increase of developmental abnormalities (Gamain et al., 2016). Those  
331 abnormalities have likely consequences on the behavior and the speed of swimming of larvae. Indeed,

332 Horiguchi et al., (1998) reported unusual swimming behavior, low swimming activity and irregular  
333 movement of cilia due to atrophy of velum in larvae of different mollusk species exposed to organotin  
334 compounds.

335

#### 336 **4.2. Effect of model pollutants on swimming behavior of oyster D-larvae**

337 In our experimental setup (24 °C, salinity of 33 usi), there are no significant differences of the average  
338 and maximum swimming speeds up to 10 µg/L of copper and 1 µg/L of S-metolachlor. In contrast, the  
339 percentage of rectilinear larval paths decreased with the concentration of copper and S-metolachlor  
340 (except for the 1000 ng/L) while the percentage of circular larval paths increases with the  
341 concentration of copper and S-metolachlor (except for the 1000 ng/L). Absence of effect on swimming  
342 speed means that the two compounds did not targeted the metabolism and/or the neuro-muscular  
343 system of oyster D-larvae. Besides, a significant change of the larval trajectory was observed at the  
344 lowest tested concentrations of copper (1 µg/L) and S-metolachlor (10 ng/L) with a striking shift of  
345 rectilinear paths to circular ones. This trajectory shift was likely associated to the appearance of  
346 morphological anomalies in developing oyster embryos since a significant positive correlation was  
347 noted between circular trajectory and shell abnormality of D-larvae.

348 No data is available about effect of S-metolachlor on swimming activity of aquatic organisms. Besides,  
349 a few works have documented effects of copper on fish and invertebrates. LaBretech et al. (2002)  
350 observed a decrease of motility in larvae of *Mercenaria mercenaria* exposed to concentration of Cu  
351 above 10 µg/L. Kwok and Ang (2013) also reported a significant inhibition of swimming activity of larvae  
352 of the coral *Platigrya acuta* at concentration above 40 µg/L. After 48h of depuration the larval motility  
353 was not recovered indicating a possible persistent effect of copper. In zebrafish larvae exposed to Cu  
354 concentrations from 9-20 µg/L, the mean and the maximal velocity were significantly increased, but  
355 not effect was observed at higher concentrations (Da Silva Acosta et al., 2016).

356 Toxicity of copper and S-metolachlor on early life stage of aquatic species is well document. It has been  
357 shown that both compounds induced significant developmental anomalies and DNA damage in  
358 bivalves at environmentally realistic concentrations (Mai et al., 2012; 2013; Gamain et al., 2017).  
359 Behavior is an integrated and whole-organism response (Faimali et al., 2017). Punctual and/or limited  
360 modifications of this behavior is assume to be an immediate and adaptive response to stress. At the  
361 contrary, permanent and deep perturbations of behavior are the results of irreversible changes at the  
362 sub-individual level that can have dramatically impact on fitness and survival of individuals. The  
363 significant positive correlation between circular trajectory and anomalies of the shell in oyster larvae  
364 exposed to copper or S-metholachlor demonstrate the deep impact of these molecules on the embryo-

365 larval development of this specie. This study demonstrates, once again, the very high sensitivity of the  
366 early development stages of oyster to chemical contamination.

367

#### 368 **4.3. Application of swimming activity monitoring of oyster larvae**

369 Since a decade, researchers are using swimming activity in model vertebrates (Le Bihanic et al., 2015;  
370 XXX) and invertebrates (Chevalier et al., 2015; XXXX) as new endpoints to evaluate toxicity of  
371 chemicals. This kind of endpoint is increasingly used for environmental risk or water quality assessment  
372 in non-model organism such as crustacean and sea urchin (Garaventa et al., 2010; Morgana et al.,  
373 2016). Although mussels and oysters are widely used as sentinel species for pollution monitoring, to  
374 date very few works focused on swimming behavior of bivalves exposed to pollutants (Horiguchi et al.,  
375 1998).

376 In the present study, effect of pollutants was clearly detected on swimming behavior of oyster larvae  
377 at concentrations as low as 1 µg/L of copper and 10 ng/L of S-metolachlor. These levels of  
378 contamination are environmentally relevant and are currently detected in seawater of numerous  
379 European coastal areas (XXXX) and notably in the Arcachon Bay (Gamain et al., 2017b). The swimming  
380 activity of oyster larvae could be used as an early warning indicator of the quality of water and/or of  
381 the toxicity of pollutants.

382 Abnormal swimming behavior of D-larvae can directly affect their survival or fitness. In addition,  
383 abnormal trajectories can impair dispersion of larvae and colonization of new habitats. Both  
384 phenomenon could impair recruitment and at terms impact survival of wild population and viability of  
385 production of oysters. In this respect, swimming behavior could be used as a suitable tool to analyze  
386 the health status of larvae from wild or farmed oyster populations.

387 In the future, we can expect the use of video-tracking of oyster larvae as a convenient tool to guide  
388 the shellfish farmers towards the choice of the best batch of larvae and environmental agencies for  
389 the assessment of coastal water quality.

390

#### 391 **Conclusion**

392 This study describes a semi-automated image analysis tool to analyze D-larvae swimming behavior of  
393 bivalves. Oyster larvae exposed to environmental concentrations of copper or S-metolachlor showed  
394 aberrant swimming trajectories but average and maximum speeds were not impacted. Rectilinear  
395 trajectories appeared positively correlated to normal larvae while larvae with shell anomalies were  
396 positively correlated to circular trajectories. This study showed that video-tracking of oyster swimming

397 behavior is a sensitive and easy to perform tool for ecotoxicological testing. The use of this tool at  
398 large scale could need **some improvements** in particular for temperature control and automating of  
399 trajectory reading.

400

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405 adaptability and governance”.

406

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## 462 **Figures**

463 **Figure 1:** sampling site for mature oysters in the Arcachon Bay (SX France): 1) Les Jacquets, 2) Comprian  
464 and 3) Banc d'Arguin.

465 **Figure 2:** Image Analysis: A) Original image, B) Grayscale image, C) 'Binarized' image, D) Traces of larval  
466 path, E) Quantified parameters.



467 **Figure 3:** Gathering drawing with different traces of larval paths: 1) Rectilinear, 2) Circular, 3)  
468 Motionless, 4) larval enters and exits field of view, 5) collision between two larvae.

469 **Figure 4:** Average and maximum speed ( $\mu\text{m}\cdot\text{s}^{-1}$ ) of D-larvae exposed to different concentrations of (A)  
470 copper ( $\mu\text{g}\cdot\text{L}^{-1}$ ) or (B) S-metolachlor ( $\text{ng}\cdot\text{L}^{-1}$ ). Data points represent mean  $\pm$  SD with 3-5 replicates per  
471 concentration and 12-74 larvae for each replicate.

472 **Figure 5:** Trajectories of D-larvae exposed to different concentrations of (A) copper ( $\mu\text{g}\cdot\text{L}^{-1}$ ) or (B) S-  
473 metolachlor ( $\text{ng}\cdot\text{L}^{-1}$ ). Data points represent mean  $\pm$  SD with 3-5 replicates per concentration and 12-74  
474 larvae for each replicate. Different letters indicated significant differences between exposure  
475 conditions ( $p<0.05$ ).

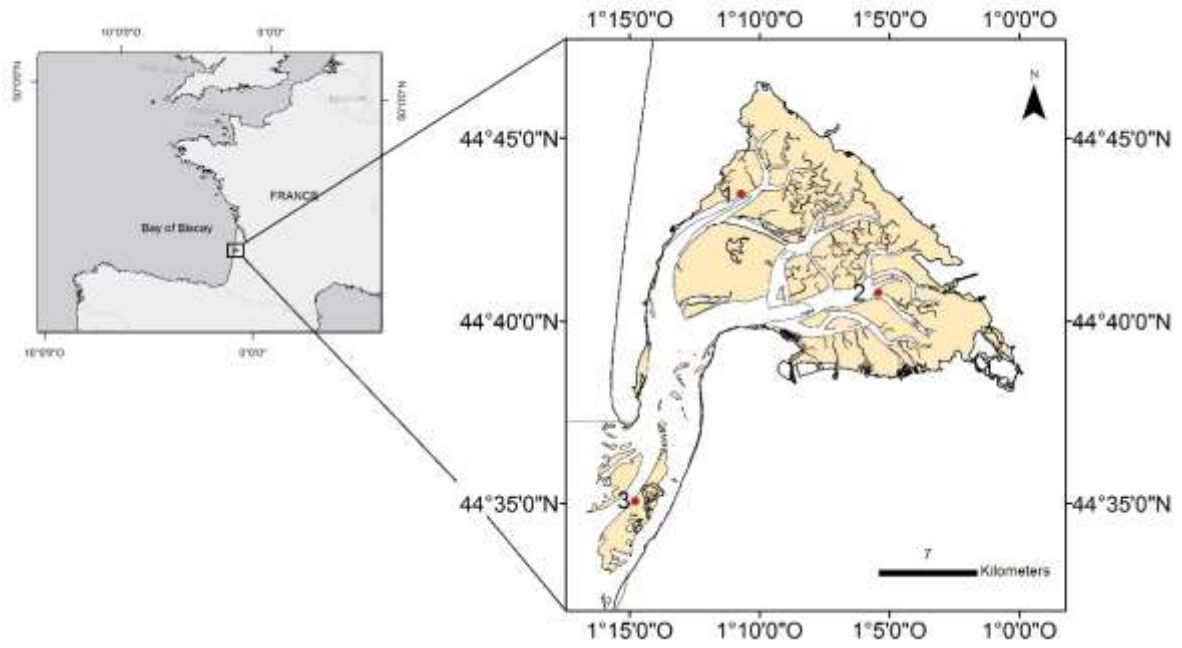
476 **Figure 6:** Percentages of developmental arrest (DA) and abnormal D-larvae exposed to different  
477 concentrations of (A) cooper ( $\mu\text{g}\cdot\text{L}^{-1}$ ) or (B) S-metolachlor ( $\text{ng}\cdot\text{L}^{-1}$ ). Data points represent mean  $\pm$  SD.  
478 N=4 replicates per condition, and 100 larvae for each replicate. Different letters indicated significant  
479 differences between exposure conditions ( $p<0.05$ ).

480 **Figure 7:** Principal component analysis of the different types of abnormalities and trajectories of D-  
481 larvae. Analysis represents normalized coefficients on the first two axes (axis 1: 38.23 %; axis 2: 22.65%)  
482 for three D-larvae abnormalities (normal, shell abnormalities, mantle abnormalities), three trajectories  
483 (R : Rectilinear ; S: Motionless, C: Circular) two speed (Vit Moy: average speed, Vit Max: maximum  
484 speed). The variables factor map is shown in (A) and the individual factor map in (B). Pearson  
485 correlation analysis between shell abnormalities and circular trajectories (C), and rectilinear  
486 trajectories and normal D-larvae (D).

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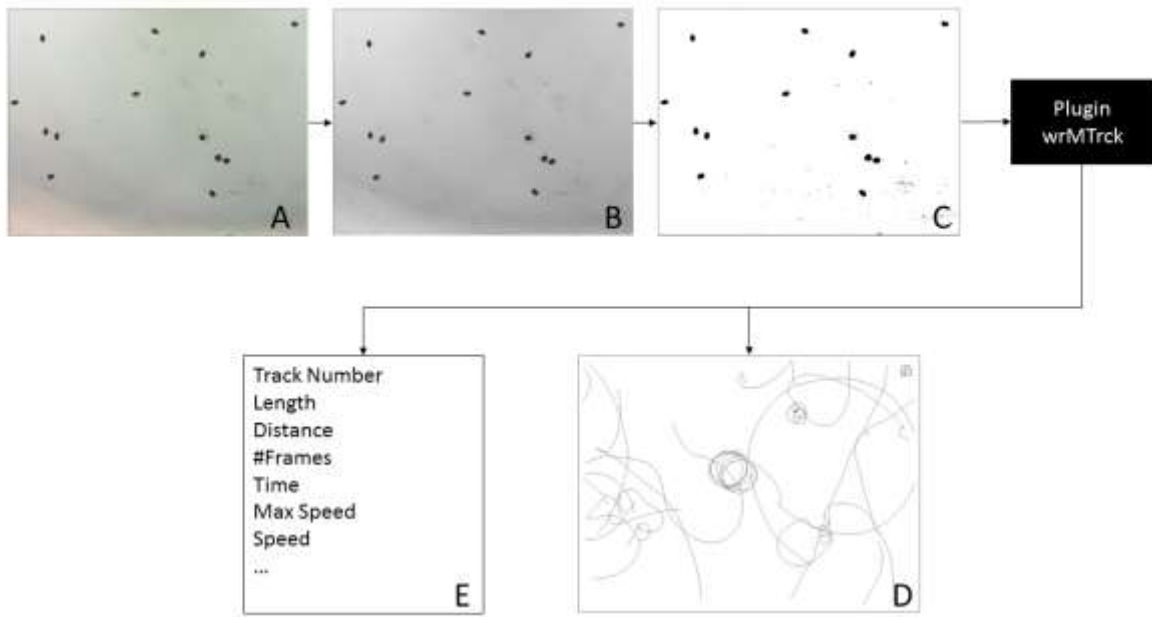
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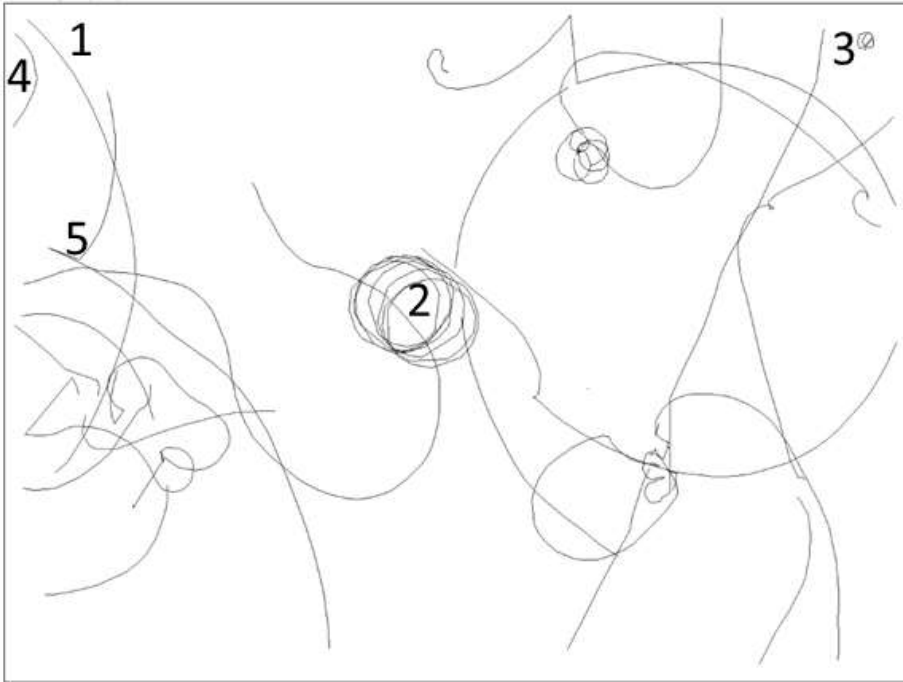
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491 Figure 1

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 494 Figure 2  
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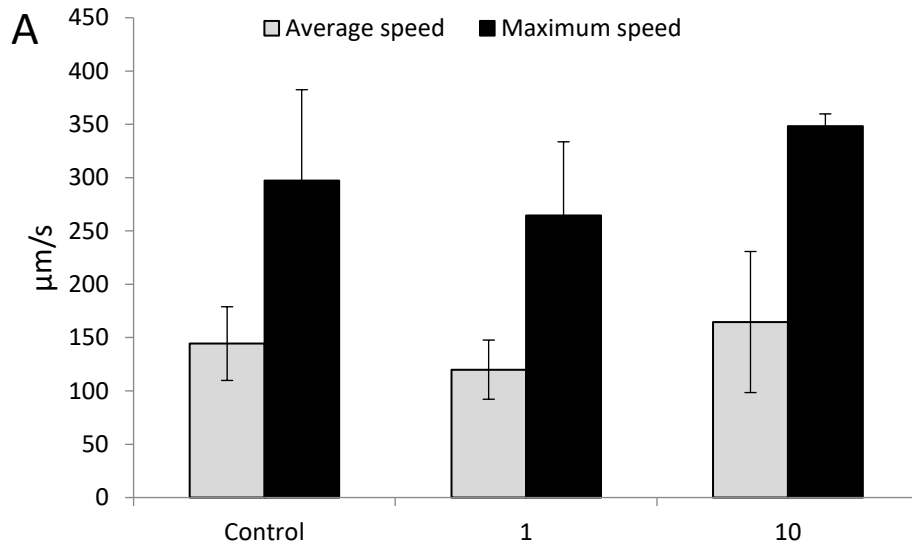


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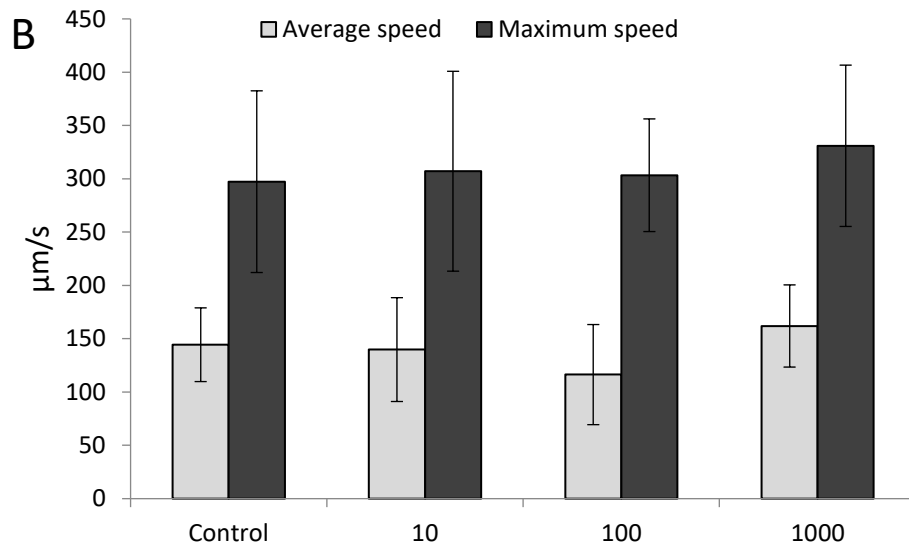
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Figure 3



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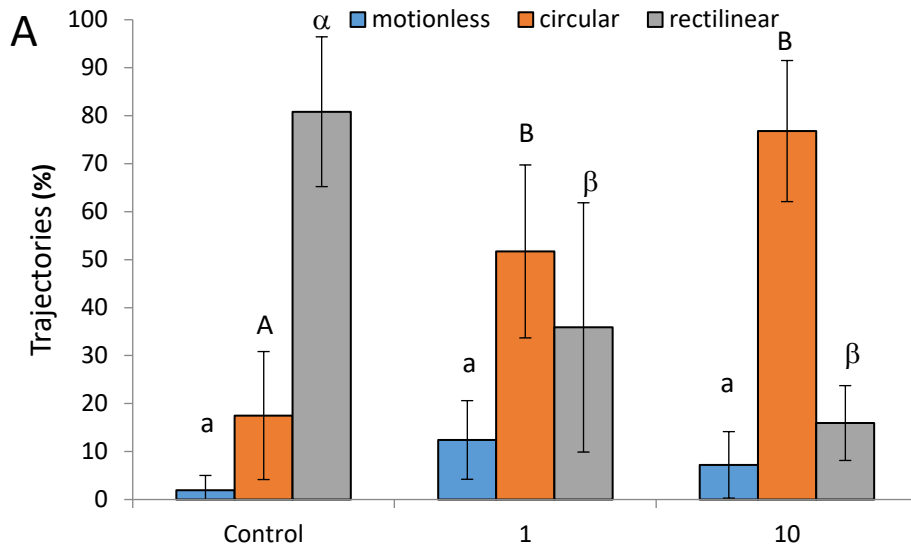
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Figure 4

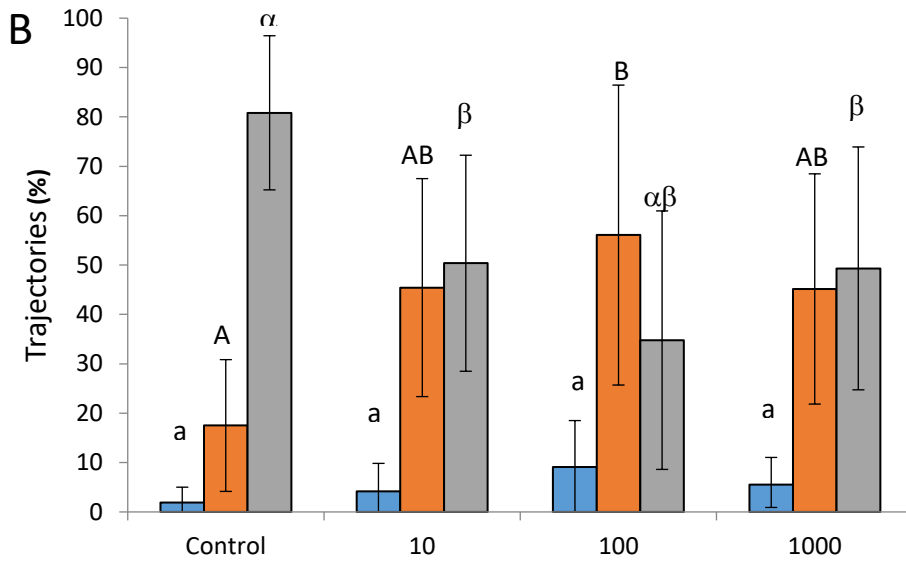
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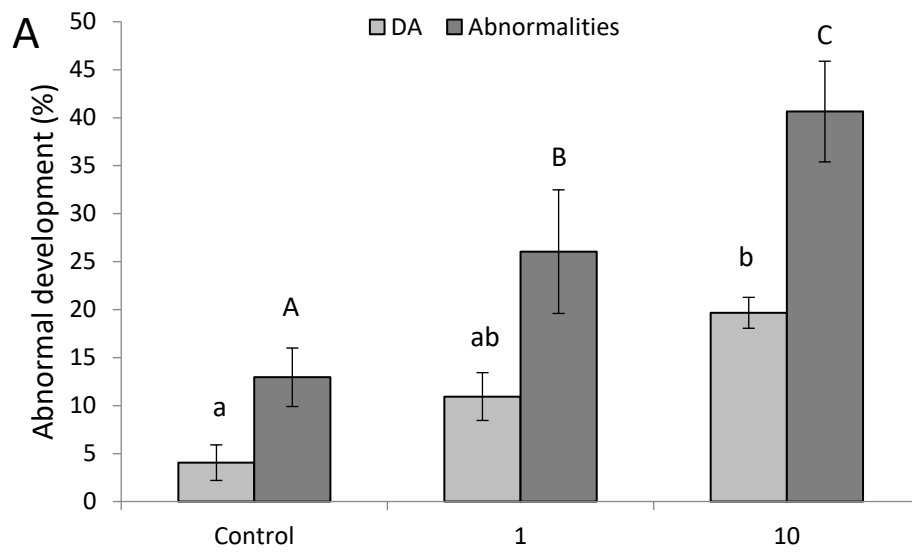
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Figure 5

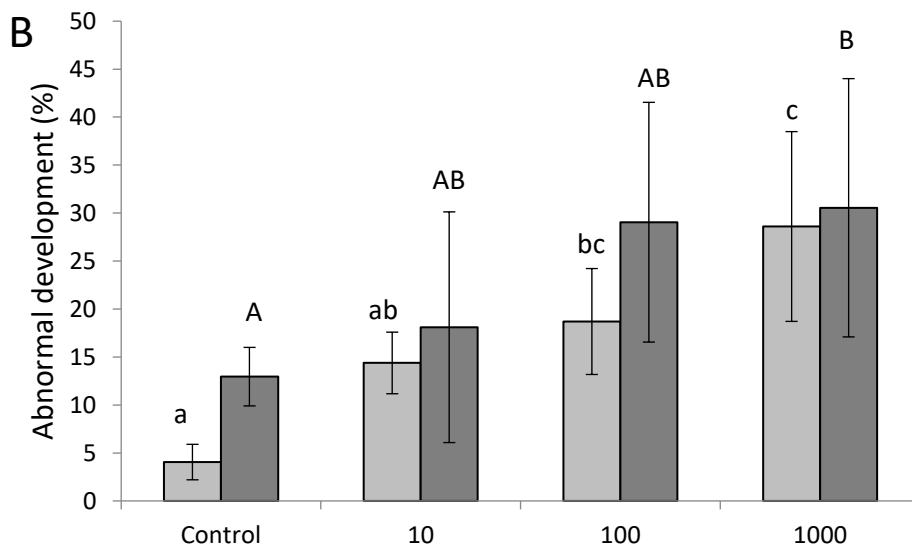
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515 Figure 6

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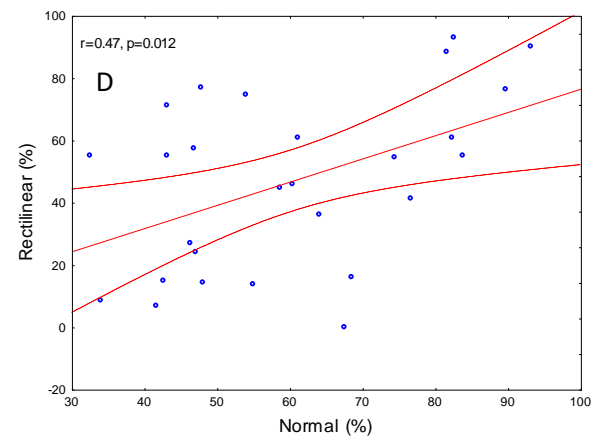
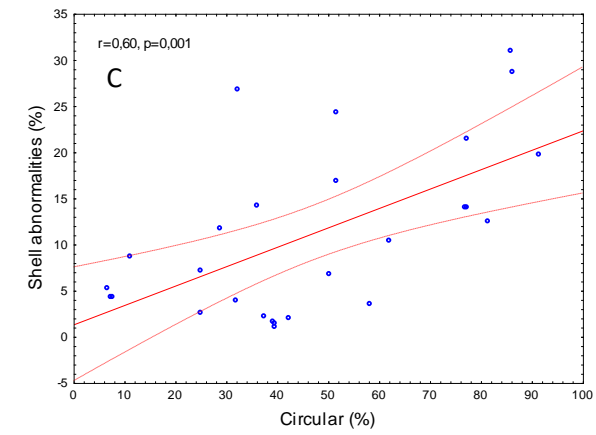
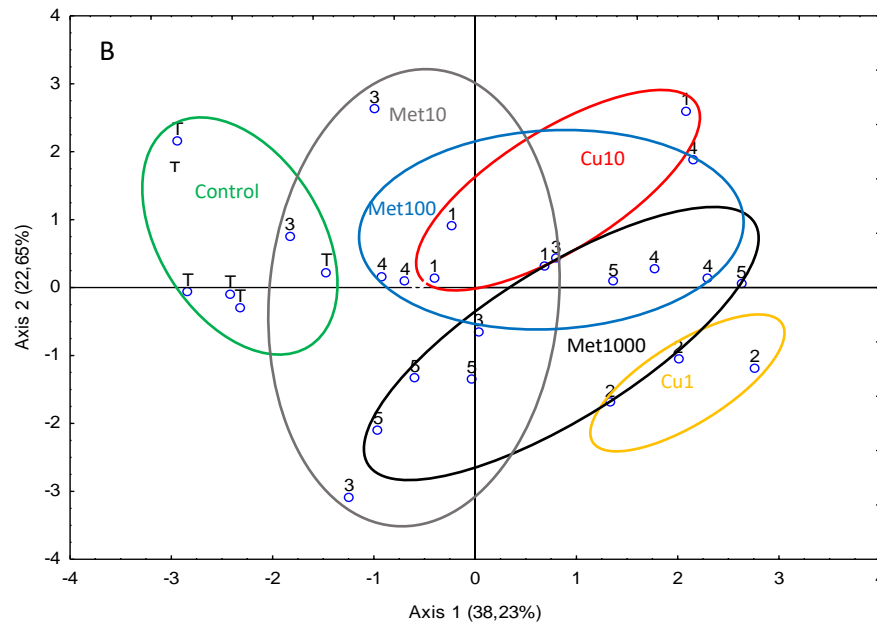
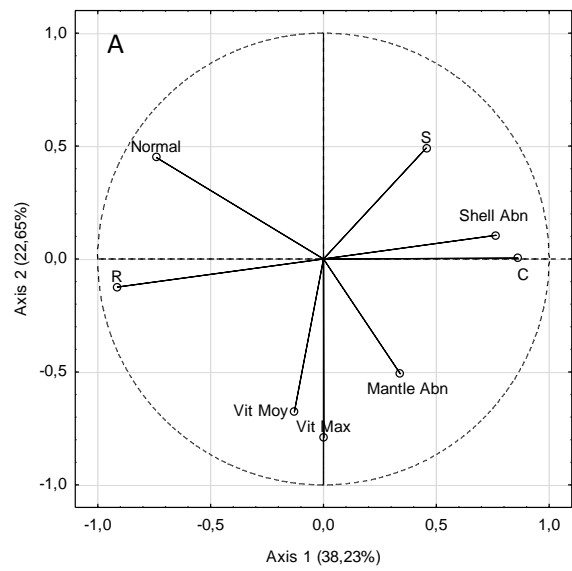


Figure 7



