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Impact of ageing and soil contaminants on telomere length in the land snail

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1 **Title: Impact of ageing and soil contaminants on telomere length in the land**
2 **snail**

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12

13 **Keywords:** *Cantareus aspersus*; bioindicators; cadmium; mercury; polycyclic aromatic
14 hydrocarbons

15

16 **Highlights:**

- 17 • Telomere length attrition (TLA) occurs between the juvenile and sub-adult stages.
18 • Exposure to chemical stress does not cause TLA in haemocytes of sub-adult snails.
19 • The health status of snails exposed to chemical stress was disrupted without TLA.

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29 **ABSTRACT**

30 Telomeres (TLs) are non-coding DNA sequences that are usually shortened with
31 ageing and/or chemical exposure. Bioindicators such as the land snail can be used to assess
32 the environmental risk of contaminated soils. As for most invertebrates, the evolution of TLs
33 with ageing or exposure to contaminants is unknown in this mollusc. The aims of this study
34 were to explore the relationships between ageing, contaminant exposure, sublethal effects and
35 TL length in the terrestrial gastropod *Cantareus aspersus*. TL length was investigated in
36 haemocytes from five age classes of *C. aspersus*. The impact of contaminants on sub-adult
37 snails exposed to Cd, Hg or a mixture of polycyclic aromatic hydrocarbons (PAHs) in soils
38 for one or two months was studied. Bioaccumulation, growth, sexual maturity and TLs were
39 measured. TL attrition was significant for the juvenile and sub-adult stages, but not later.
40 Exposure to Cd increased the mortality (around 30%). Exposure to polluted soils inhibited
41 growth (19 to 40%) and sexual maturity (6 to 100%). Although the health of the snails
42 exposed to Cd, Hg and PAHs was altered, TL length in haemocytes was not disturbed,
43 suggesting a high capacity of this snail species to maintain its TLs in haemocytes under
44 chemical stress. These results first address TL length in snails and reveal that the relationship
45 commonly proposed for vertebrates between TL shortening and ageing or exposure to
46 contaminants cannot be generalized.

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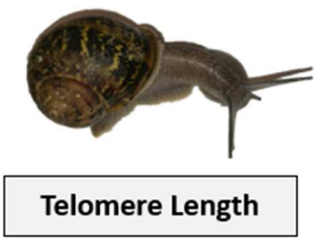
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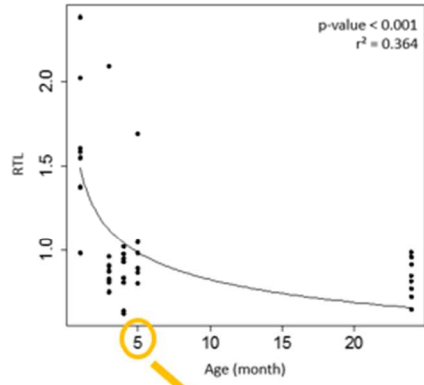
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57 **GRAPHICAL ABSTRACT**

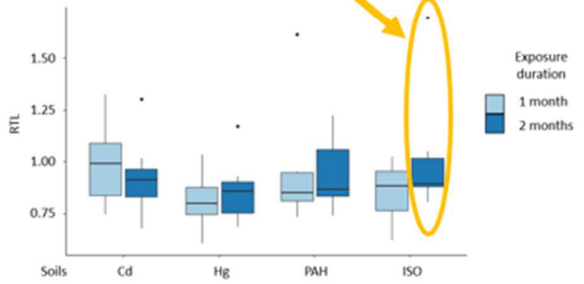
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Ageing →



Contaminants →



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70 INTRODUCTION

71 Telomeres (TLs) are highly conserved, repetitive and non-coding DNA sequences
72 located at the ends of eukaryotic chromosomes (Blackburn, 1991; Traut et al., 2007). Their
73 main function is to protect the ends of chromosomes from loss during cell division and
74 degradation (Blackburn, 2005; Oeseburg et al., 2009). Telomeric DNA is progressively lost in
75 most vertebrates during ageing due to the inability of linear chromosomes to be completely
76 replicated by DNA polymerases (Blackburn, 1991). This loss can be accelerated by
77 endogenous and exogenous factors, such as infections (Giraudeau et al., 2019) or
78 environmental contaminants (metals, polycyclic aromatic hydrocarbons (PAHs), etc.) (Perera
79 et al., 2018; Louzon et al., 2019; Matzenbacher et al., 2019). Indeed, contaminants can induce
80 TL alterations according to several mechanisms of action such as DNA strand breakage at the
81 TL level, deficiencies in DNA repair machinery, oxidative stress, mutations in genes encoding
82 proteins related to TL maintenance and disturbances in the regulation of participants in TL
83 maintenance (e.g., telomerase activity or alternative lengthening of TLs pathway) (Louzon et
84 al., 2019).

85 Data from longitudinal studies in vertebrates showed a TL loss within individuals with age
86 (Zeichner et al., 1999; Brümmendorf et al., 2002; Scott et al., 2006; Baerlocher et al., 2007)
87 with some exceptions (Ujvari and Madsen, 2009), although great variability exists depending
88 on the biological tissue (Parolini et al., 2019) and on TL length and loss between age-matched
89 individuals (Hall et al., 2004). The relationship between TL length and ageing is unknown for
90 most invertebrates as concluded following a comparative approach between biological groups
91 and the few studies on this topic have shown various patterns: either shortening, or no
92 variation or lengthening (Louzon et al., 2019). For example, for two species of *Daphnia*, two
93 different patterns have been reported: the attrition in TLs with ageing in *D. pulicaria* and no
94 variation in TL length in *D. pulex* (Schumpert et al., 2015). These differences were related to
95 the high telomerase activity that maintains TLs during the life cycle of *D. pulex* (Schumpert et
96 al., 2015). Surprisingly, for the gastropod of Baïkal Lake, *Kobeltocochlea martensiana*, which
97 has a lifespan of eight years (vs months for some *Daphnia spp.*), a lengthening of TLs with
98 ageing was shown, probably due to the high activity of TL maintenance mechanisms
99 (Maximova et al., 2017).

100 For terrestrial snails, the relationships between TL dynamics and ageing are unknown,
101 especially for the soil quality bioindicator, *Cantareus aspersus*, which have an average life of

102 five years and which belongs to various food chains at the air-soil-plant interface. It is
103 consumed by predators and integrates various routes of exposure of contaminants in soils
104 (digestive, cutaneous, respiratory) (Comfort, 1957; Scheifler et al., 2002; Scheifler et al.,
105 2006). For these ecological reasons, this ubiquitous species is often used in environmental risk
106 assessment of contaminated soils (Pauget and de Vaufleury, 2015) by combining assessments
107 of bioavailability (Pauget et al., 2013) and health effects (Baurand et al., 2014).

108 Based on data showing that chemicals are able to induce premature ageing and decrease the
109 lifespan of humans (Chen et al., 2013; Puri et al., 2017), it is relevant to explore the
110 relationships among TL dynamics, health effects and contaminant exposure in *C. aspersus*.
111 This could contribute to filling the gap between the molecular and individual effects of
112 contaminants. Considering this objective, the first aim is to describe how TL dynamics in
113 haemocytes is affected by ageing in snails living in a safe environment. This study also aimed
114 to investigate the relationships between the exposure to contaminants in soils (cadmium,
115 mercury or PAHs), the alteration in health status (by monitoring survival, growth and the
116 attainment of sexual maturity) and TL length in *C. aspersus*.

117 MATERIAL AND METHODS

118 SNAILS

119 Breeding snails from a snail farm (CFPPA of Châteaufarine, 25000 Besançon, France)
120 were used to produce a F1 laboratory generation. The species *C. aspersus* was verified by
121 DNA barcoding (Allgenetics, 15008 A Coruña, Spain). After incubating eggs, hatchlings were
122 reared in the laboratory under controlled conditions (20°C, 80% relative humidity,
123 photoperiod of 16 h of light and 8 h of dark) (Gomot-de Vaufleury and Pihan, 2000) for two
124 years. Snails reared of 1 month old were classified as juveniles, 3 months old as sub-adults, 4
125 months old as young adults, 5 months old as young reproducers and 2 years old as old
126 reproducers (**Figure 1**). Eight snails of each age class were selected to investigate the
127 relationships between ageing and TL length.

128 To study the effect of exposure to contaminants, only sub-adult snails were used to avoid
129 confounding factors related to growth and reproduction (Gimbert et al., 2008). Two groups of
130 sub-adults were exposed in quadruplicate to different contaminants for 1 and 2 months. Snails
131 were fed with uncontaminated commercial food (**Table 1**) Helixal® (Berthon S.A., France) *ad*
132 *libitum* during growth and exposure.

133 SOILS

134 Snails were exposed to four different soil conditions (ISO, Cd, Hg and PAHs). An
135 artificial ISO soil was prepared according to the [OECD \(1984\)](#) guidelines (mixture of 70%
136 fine sand, 20% kaolin clay, and 10% coarse ground sphagnum peat, and the pH was adjusted
137 to 6.7 ± 0.1 with powdered calcium carbonate, 99.5%, Sigma Aldrich, Lyon, France). The
138 cation exchange capacity (CEC) was $13.5 \text{ cmol kg}^{-1}$, and the organic matter content
139 (estimated on the basis of the loss of ignition) was 12%. The ISO substrate was spiked at
140 $13\,000 \text{ mg.kg Hg.kg}^{-1}$ dry weight (dw) with a mercury chloride solution (HgCl_2 , 99.5%,
141 Sigma Aldrich) or at 1500 mg.kg^{-1} dw with a cadmium chloride solution (CdCl_2 , 99.9%,
142 Sigma Aldrich), in both cases diluted in a volume of osmotic water needed to achieve 50% of
143 the soil water holding capacity ([Pauget et al., 2012](#)). The soils were then allowed to stabilize
144 for 5 days prior to the experiment. An anthropogenic French soil (Vieux Charmont, France)
145 contaminated with PAHs **due to past industrial activities of the automobile sector (BASOL**
146 **25.0020, 2015)** was used under the same preparation conditions (except spiking).
147 Concentrations were measured by ICP-MS for Cd and Hg ([Mariet et al., 2017](#)) and by GC-
148 MS/MS for the 16 PAHs listed by the US EPA ([Morin-Crini et al., 2014](#)) (**Table 1**).

149 EXPOSURE MODALITIES

150 Sub-adult snails (3-months old snails) were exposed to Cd or Hg contaminated substrate,
151 PAHs contaminated soils and or uncontaminated ISO substrate for 1 and 2 months. For each
152 of the 4 substrates/soils, six snails were exposed in quadruplicate in transparent polystyrene
153 containers (4032 cm^3) ([Gomot-de Vaufleury, 2000](#)). Two snails per replicate ($n=8$ individuals
154 per exposure conditions) were sampled randomly after 1 and 2 months for analysis of the
155 visceral concentrations of contaminants and assessment of TL length. Briefly, Helixal® food
156 was provided in a petri dish left on the bottom of the container, the day/night cycle
157 photoperiod was 18/6 h, the temperature was $20^\circ\text{C} \pm 1$, and the air relative humidity was $80 \pm$
158 10% . Three times a week, the containers were cleaned, the faeces were removed, survival was
159 monitored, and the food was renewed. At the beginning of exposure (t_0), sub-adult snails
160 weighed $5.22 \text{ g} \pm 0.58$, and the internal concentrations of Cd, Hg and PAHs were measured in
161 the visceral mass from six sub-adult snails (**Table 1**).

162 GROWTH AND ATTAINMENT OF SEXUAL MATURITY ASSESSMENTS

163 Growth was assessed monthly by weighing the fresh mass (whole body) of snails. The
164 growth inhibition (GI) was calculated as follows (after validation of the absence of abnormal

165 mortality of snails exposed to the ISO soil): $GI = 100 - (\text{mean mass of exposed snails} / \text{mean}$
166 $\text{mass of snails exposed to uncontaminated ISO soil}) * 100$. Sexual maturity was assessed
167 monthly by counting the snails exhibiting a shell with a curled edge, which can be used as an
168 indicator of the attainment of sexual maturity in snails (Lucarz and Gomot, 1985; Gimbert et
169 al., 2008; Gimbert et al., 2016). The inhibition of the attainment of sexual maturity was
170 calculated by the following formula: $100 - (\text{number of exposed snails having acquired sexual}$
171 $\text{maturity} / \text{number of snails exposed to uncontaminated ISO substrate having acquired sexual}$
172 $\text{maturity}) * 100$.

173 **BIOACCUMULATION MEASUREMENT**

174 Internal concentrations of Cd and Hg were measured according to the
175 methodology detailed in Pauget and de Vaufleury (2015). Snails were frozen at -80°C. After
176 thawing, the visceral mass was dissected for each snail and lyophilized for two days before
177 Cd and Hg analyses. Viscera were digested between 47°C and 98°C for 265 min (DigiPREP
178 MS, SCP Science, Courtaboeuf, France) in 7 mL of nitric acid (HNO₃, 65%, ultratrace
179 Optima[®], FisherScientific, Illkirch, France) that was diluted with 43 mL of Milli-Q water.
180 Then, samples were filtered through a 1 µm filter and diluted as a function of concentration
181 between 10 to 100x for Cd and 10 to 10000x for Hg analyses in ICP-MS (Thermo Scientific
182 X Series II, Courtaboeuf, France). Analyses were validated with a certified material (TORT2,
183 lobster hepatopancreas, Canada). The recovery rates were 98% and 116% for Cd and Hg,
184 respectively. For the PAHs analysis, two fresh visceral masses per replicate were dissected
185 and pooled. Then, pooled samples were mashed with 25 ceramic sphere mills (FastPrep, MP
186 Biomedicals, Illkirch, France) for two cycles (4.0 m/s, 20 s) interrupted by a pause time of
187 180 s. PAHs were extracted with QuEChERS methodology from RESTEK (Lisse, France),
188 and concentrations of the 16 PAHs (Sun et al., 1998) were measured by GC-MS/MS (7890A
189 GC system, 7000 GC triple quadrupole mass spectrometer, HP5MS column, Agilent, Massy,
190 France). The bioaccumulated concentrations in the snails and the soil concentrations were
191 used to calculate the bioaccumulation factors (BAF).

192 **DNA EXTRACTION FROM HAEMOCYTES**

193 The haemolymph of snails was collected by individually puncturing the heart with a
194 needle (18 G) and was frozen at -20°C in sterile 1.5 mL microtubes (DNase, RNase,
195 endotoxin free, VWR). After quick thawing at 4°C, DNA extractions were performed using a
196 preliminary optimized method to isolate DNA with a sufficient amount and quality from

197 haemolymph. Salting out was the selected methodology (Gentra Puregene Blood Kit, Qiagen,
198 Courtaboeuf, France). The concentration and purity of extracted DNA were checked with a
199 NanoDrop 1000 spectrophotometer (ThermoScientific, Illkirch, France) and by fluorescence
200 (Quantifluor® ONE dsDNA System, Promega, Charbonnière-les-Bains, France). The quality
201 and the integrity of DNA were confirmed by electrophoresis on 1% agarose gels stained with
202 ethidium bromide.

203 **TELOMERE LENGTH MEASUREMENT**

204 For quickness and robustness (Nussey et al., 2014), TLs were measured by quantitative
205 real-time PCR amplification (qPCR) (Cawthon, 2002) using the method described by
206 Criscuolo et al. (2009) and adapted to land snails. This method amplified telomeric DNA and
207 expressed its quantity as a T/S ratio, where T was the quantity of telomeric DNA and S was
208 the quantity of a gene used as a reference (gene that is non-variable in copy number in the
209 study species) (Reichert et al., 2017). Primers used for the telomeric DNA of snails were the
210 same as those used for humans (forward: 5'-
211 CGGTTTGGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3', reverse: 5'-
212 GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') because the TL
213 sequences of *C. aspersus* are the same as those of humans (Vitturi et al., 2005). To identify
214 the most appropriate reference gene, we conducted preliminary analyses considering a variety
215 of candidate reference genes and primer pairs. The most consistent amplification profile and
216 cleanest melting curve was obtained using Eurofins Genomics primers targeting the β -actin
217 gene, which we selected as our reference gene (forward: 5'-
218 GCTGGTCGTGACCTCACAGAT-3', reverse: 5'-AGCTTCTCCTTGATGTACGAAC-3').
219 The TL and β -actin primers were used at a concentration of 4 pmol for a final volume of 10
220 μ L containing 5 μ L of GoTaq® qPCR master mix (Promega, Charbonnière-les-Bains, France).
221 Real-time amplification of TL and β -actin sequences was performed in separate 384 well
222 plates filled by an automated liquid handling workstation (epmotion, Eppendorf, Montesson,
223 France). The TL thermal profile was 2 min at 95°C, followed by 27 cycles of 15 s at 95°C, 30
224 s at 56°C and 30 s at 72°C. The β -actin thermal profile was 2 min at 95°, followed by 40
225 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. Both assays were followed by melting
226 curve analysis (60-95°C; 1°C/5 s ramp). Due to the different thermal profiles between TL and
227 β -actin, two 384 well plates were used in our study to measure TL length of all our samples.
228 The first is for the amplification of TL and the second is for the amplification of the β -actin
229 gene. Each DNA extract was tested in duplicate due to the use of the calibrated automated

230 liquid handling workstation and to the fact that mean coefficient of variation intra-run were
231 $0.51\% \pm 0.05$ and $1.07\% \pm 0.09$ for Cq- β -actin and Cq-TL, respectively. Moreover, the
232 intraclass correlation coefficient intra plate for the T/S ratio was ranked excellent, 0.949
233 (Cicchetti, 1994; Nettle et al., 2019). Relative TL length (RTL) was calculated for each
234 sample by the ratio of T/S. DNA from the same reference snail was used as a golden sample
235 and to generate a reference curve to control for the amplifying efficiency of the qPCR
236 between various 384 well plates (based on a standard range of 7 points, the amplification
237 efficiencies for TL and β -actin amplification were 99.3% and 99.7%, respectively).

238 DATA ANALYSIS

239 All statistical analyses were performed with R (version 3.4.2.). Normality and
240 homogeneity of variances were checked with Shapiro-Wilk and Bartlett tests, respectively.
241 The relationship between ageing and TL length was fitted with a non-linear model using the
242 nlme (nonlinear mixed effect) procedure (Lindstrom and Bates, 1990) (package nlme). The
243 adequacy and quality of the model were compared (package pgirmess) according to the
244 Akaike information criterion (AICc) (Akaike, 1998) to select the best model. Then,
245 differences in TL length between age classes or control (ISO) vs contaminated soils were
246 assessed with a non-parametric Kruskal-Wallis test followed by the multiple pairwise
247 comparison test (Magniez et al., 2018). The Wilcoxon test was used to distinguish significant
248 differences of mortality, growth inhibition and the inhibition of the attainment of the sexual
249 maturity between the different soils (control ISO vs contaminated) after transforming data
250 with $\log_{10}(x+1)$.

251 RESULTS AND DISCUSSION

252 RELATIONSHIPS BETWEEN AGEING AND TELOMERE LENGTH

253 TLs were studied in the haemocytes of a *C. aspersus* population because they are involved
254 in immune functions of gastropods (Pila et al., 2016) and haemolymph sampling is a non-
255 lethal way in snails. The relationship between TL length in haemocytes and ageing in *C.*
256 *aspersus* was studied for five age classes, from juveniles to old adults (Figure 1). The attrition
257 in TLs in haemocytes (p-value < 0.001, r^2 adjusted = 0.364) (Figure 2a) was significant
258 between juveniles and all other stages (p-values < 0.05) (Figure 2b). Indeed, TL length
259 decreased rapidly in *C. aspersus* haemocytes during the first phase of growth before the
260 maturation of the genital tract (Gimbert et al., 2008) and then, was stable from sub-adults to
261 old reproducers (Figure 2b). TL attrition is one of the nine hallmarks of ageing identified in

262 most organisms (Lopez-Otin et al., 2013) because chromosomes lose telomeric patterns
263 (Harley et al., 1992) at each cell division. This attrition in TLs was detected at a different
264 magnitude in the foot of the freshwater mollusc *Benedictia fragilis*, in which TL length
265 decreased progressively during approximately 0 to 5 years for males (studied over 8 years)
266 (Maximova et al., 2017). However, for two other long-lived freshwater molluscs studied from
267 0 to 8 years, *Benedictia baicalensis* and *K. martensiana*, the patterns were different and sex-
268 specific, with an increase in TL length in the foot with ageing (Maximova et al., 2017).
269 Notably, the influence of gender is irrelevant to study for *C. aspersus* due to its protandrous
270 hermaphroditic reproductive system (Bride and Gomot, 1991). These various patterns can
271 possibly be explained by a higher telomerase activity in tissues that maintain TL length
272 (Greider and Blackburn, 1985; Greider and Blackburn, 1989) during ageing (Goyns and
273 Lavery, 2000; Shay, 2016), as demonstrated by Schumpert et al. (2015) for the aquatic
274 arthropod *Daphnia pulex* and for the marine bivalve *Arctica islandica* (Gruber et al., 2014).
275 Although rapid attrition during growth seems not to be a general rule for invertebrates
276 excepted for some organisms such as the Sydney rock oyster (*Saccostrea glomerata*) (Godwin
277 et al., 2012), this pattern is often found in vertebrate species (Angelier et al., 2018; Dugdale et
278 al., 2018) (e.g., *Acrocephalus sechellensis*, Spurgin et al. (2018)). The stability of TL length
279 after the initial growth of land snails is similar to the TL dynamics described in invertebrates
280 that have a long lifespan and high telomerase activity, such as lobster (Klapper et al., 1998).
281 This finding is also consistent with the hypothesis of Schumpert et al. (2015) who assumed
282 that the differences in TL length dynamics with ageing between the short and long lifespans
283 of *Daphnia* spp. were associated with an enhanced ability to protect individuals from
284 proteotoxicity with chaperone HSP70 (Schumpert et al., 2014).

285 LETHAL AND SUBLETHAL EFFECTS OF CONTAMINANTS

286 The mortality rates we measured in the snails exposed to the uncontaminated ISO
287 substrate, 4.25% and 8.50% after one and two months of experiment, respectively, were
288 closed to that reported for snails reared on uncontaminated ISO substrates for 168 days (i.e.,
289 4%) in the study of Gimbert et al. (2016). For the soils contaminated with Hg and PAHs
290 (Table 1), the survival rates were similar to those of the ISO groups (p-values > 0.05), while
291 it was lower for Cd-exposed snails compared to controls (p-value = 0.011), reaching 20.5%
292 after two months of exposure (Figure 3a). This high mortality of Cd exposed snails is in
293 accordance with the literature, notably after Cd ingestion (Gomot, 1997). Snail growth was
294 inhibited after one and two months of exposure to all of the contaminated soils, with

295 inhibitions of $34.4\% \pm 10.5$, $39.3\% \pm 4.95$, and $18.5\% \pm 5.04$ for snails in Cd, Hg and PAHs
296 substrates/soils, respectively (**Figure 3b**). Such inhibition in the sub-adults was in the same
297 range as that reported for juvenile *C. aspersus* (Coourdassier et al., 2002; Gimbert et al.,
298 2016). Some contaminants, such as Cd or Hg, are also known to cause reproductive disorders
299 in snails (de Vaufleury and Kerhoas, 2000; Gimbert et al., 2016), especially Cd, which is an
300 endocrine disruptor (Sabir et al., 2019) for pulmonate molluscs (Lagadic et al., 2007). All the
301 studied contaminants inhibited sexual maturity after one and two months of exposure,
302 whereas 100% of snails were sexually mature after one month of exposure on the control
303 substrate (ISO). No snail exposed to Cd or Hg was mature after one month of exposure, while
304 only a few individuals matured (12.5%) on Cd substrate after two months of exposure (**Figure**
305 **3c**). In PAHs soil, maturity was also strongly inhibited after one and two months of exposure
306 but was significantly lower than the inhibition of maturity in the snails exposed to the Cd and
307 Hg soils (**Figure 3c**). The disruption of growth and sexual maturity can be the result of the
308 reallocation of energy to excretion and repair systems, a decrease in metabolic performance,
309 behaviour alterations for food intake and neuro-endocrine disruption (Gomot et al., 1992;
310 Gomot and Gomot, 1995; Matthiessen and Gibbs, 1998; Lagadic et al., 2007; Druart et al.,
311 2017; Sabir et al., 2019). Because the concentration of Hg-contaminated substrate reflects
312 those of contaminated soils found in anthropic areas as the mining district of Almaden (Spain)
313 (Molina et al., 2006), the inhibition of growth and sexual maturity can impact the snail
314 population size and dynamic. The sub-lethal effects we measured showed that the health
315 status of snails exposed to contaminated substrates/soils in this study was strongly altered.

316 **BIOACCUMULATION**

317 Bioaccumulation measurements at 0, 1 and 2 months are presented in **Table 1**. The
318 results showed high internal concentrations of Cd, Hg and PAHs after exposure to
319 contaminated substrates/soils, while they remained low in the control snails (**Table 1**).
320 Bioaccumulation factors (BAFs) at 1 and 2 months for Cd- and Hg-contaminated soils were
321 0.434, 0.518, 0.159, and 0.183, respectively. These results were in accordance with those
322 obtained by Coourdassier et al. (2002) after exposing juvenile snails to 1000 mg kg^{-1} Cd for
323 28 days in artificial ISO substrate (BAF = 0.290). BAFs were lower for PAHs (for one and
324 two months: 0.004 and 0.003, respectively) than for Cd and Hg, probably due to the low
325 contamination of the soil used (9.36 mg kg^{-1}) and possible metabolization of PAHs (Ismert et
326 al., 2002; Sverdrup et al., 2006). Measured internal concentrations may result of the digestive
327 and cutaneous exposure of snails to metals and PAHs in soils (Gomot et al., 1989; Gomot-de

328 [Vaufleury and Pihan, 2000](#); [de Vaufleury, 2015](#); [Bamze Attoumani et al., 2020](#)). The results
329 reveal bioaccumulation of the studied compounds associated to damage on some
330 physiological endpoints (survival, growth).

331 EFFECTS OF CONTAMINANTS ON TELOMERE LENGTH IN HAEMOCYTES

332 Based on the bioaccumulation level and the sub-lethal effects we measured, the health
333 status of snails exposed to contaminated substrates/soils was altered. Additionally, some
334 molecular markers can be used as early indicators of the alteration in health status ([Mleiki et
335 al., 2018](#)), TLs are commonly proposed as good candidates to reveal exposure and deleterious
336 effects of environmental contaminants on individuals and their populations ([Louzon et al.,
337 2019](#)). However, no significant difference (p-value > 0.05, **Figure 4**) in RTLs was detected
338 between the snails exposed to contaminated substrates/soils and the snails exposed to
339 uncontaminated substrates for one or two months. **Snails have been previously described as
340 sensitive to oxidative stress and have exhibited genotoxic damages notably on haemocytes
341 when exposed to soils contaminated with inorganic and organic compounds, such as Cd,
342 glyphosate, and PAHs (Regoli et al., 2006; Itziou et al., 2011; Abdel-Halim et al., 2013;
343 Angeletti et al., 2013; Baurand et al., 2014; Baurand et al., 2015).** However, *C. aspersus*
344 seems to have a strong ability to maintain its TL length in haemolymph even under chemical
345 stress. This can possibly be linked to **telomerase activity and alternative lengthening of TLs
346 pathway** activities or to the induction of other mechanisms to protect and repair TLs, such as
347 DNA repair mechanisms. In any case, the mechanisms underlying sublethal effects observed
348 on the growth or sexual maturation of snails exposed to Cd, Hg and PAHs (at least at the
349 tested concentrations and durations of exposure) do not indicate TL alteration in haemocytes
350 of surviving snails. **Moreover, although the lifespan and TL length of vertebrates can be
351 modified (shortened or lengthened) by chemicals (Louzon et al., 2019), some studies have
352 shown that TL length is not altered by pollutants, despite cytotoxic and genotoxic damages
353 occurred, notably for soybean farmers exposed to various pesticides (de Oliveira et al., 2019).**
354 Nevertheless, as it has been shown that TL length varied across somatic tissues suggesting
355 independent TL dynamics ([Parolini et al., 2019](#)), further measurements of TLs on other
356 biological tissues may be considered in the future.

357 CONCLUSIONS

358 **This study provides the first results on the TL length dynamic in terrestrial gastropods
359 according to ageing or chemical exposure to contaminated soils. TL attrition was observed**

360 only during the first phase of growth suggesting that TL length in the haemocytes of *C.*
361 *aspersus* is not a specific marker of ageing for this invertebrate. The health of the snails
362 exposed to Cd, Hg or PAHs was altered (survival, growth and sexual maturity) but TL length
363 in haemocytes was not changed, suggesting the high capacity of this snail species to maintain
364 its TLs in haemocytes under chemical stress. Present results first address TL length in land
365 snails and reveal that the relationship proposed for vertebrates between TL shortening and
366 ageing or exposure to contaminants cannot be generalized to the terrestrial gastropods.

367

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369 The authors declare that they have no competing financial interests.

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Table of figures






Figure 1: The investigated *C. aspersus* age classes.

Figure 2: A: Relationship between the relative telomere length (RTL) in haemocytes and age of *C. aspersus* (n=8 snails per age class represented by black dots); **B:** RTL in haemocytes of *C. aspersus* of different age classes (statistically significant differences are indicated by letters when p-values < 0.05).

Figure 3: A: Percentage of mortality of snails (mean and standard deviation), **B:** percentage of growth and **C:** sexual maturity inhibition (mean and standard deviation) after exposure to contaminated (Cd, Hg, PAHs) or uncontaminated soils (ISO) for 1 and 2 months (statistically significant differences are indicated by letters with p-value < 0.05).

Figure 4: Relative telomere length (RTL) in the haemocytes of snails (n=8 per condition) exposed to contaminated (Cd, Hg, PAHs) or uncontaminated soils (ISO) for 1 and 2 months (no statistically significant differences between soils and/or exposure durations).

Figure 1: The investigated *C. aspersus* age classes.

Juveniles ~1 g – 1 month	Sub-adults (t0) ~5 g – 3 months	Young adults (t1) ~9 g – 4 months	Young reproducers (t2) ~11 g – 5 months	Old reproducers ~14 g – 24 months
				

1 cm

Figure 2: **A:** Relationship between the relative telomere length (RTL) in haemocytes and age of *C. aspersus* (n=8 snails per age class represented by black dots); **B:** RTL in haemocytes of *C. aspersus* of different age classes (statistically significant differences are indicated by letters when p-values < 0.05).

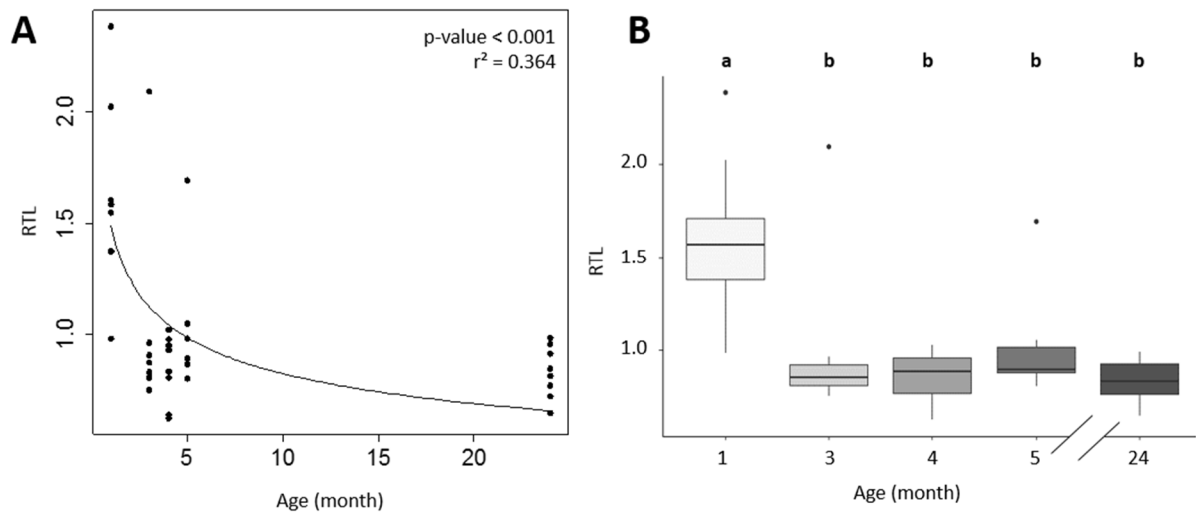


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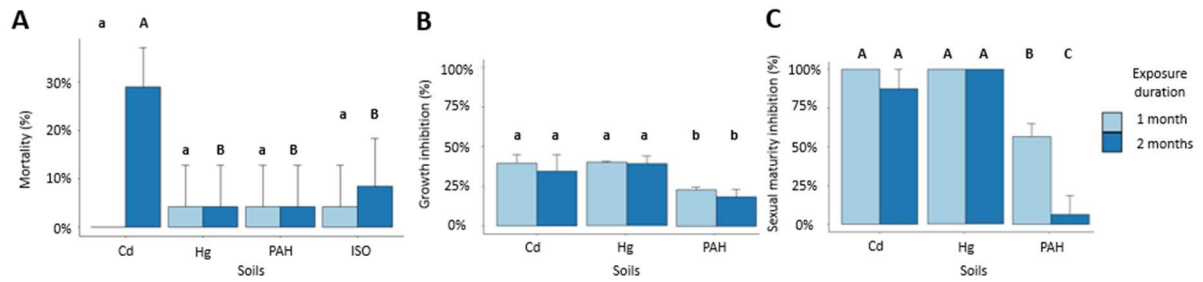
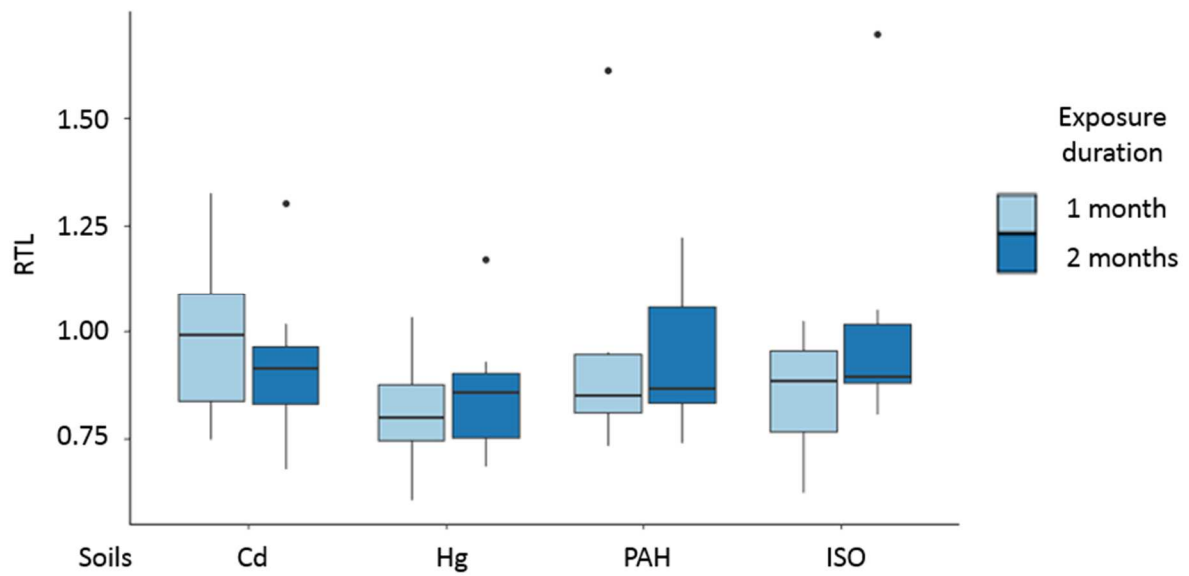


Figure 4: Relative telomere length (RTL) in the haemocytes of snails (n=8 per condition) exposed to contaminated (Cd, Hg, PAHs) or uncontaminated soils (ISO) for 1 and 2 months (no statistically significant differences between soils and/or exposure durations).



Juveniles
~1 g – 1 month



Sub-adults (t0)
~5 g – 3 months



Young adults (t1)
~9 g – 4 months



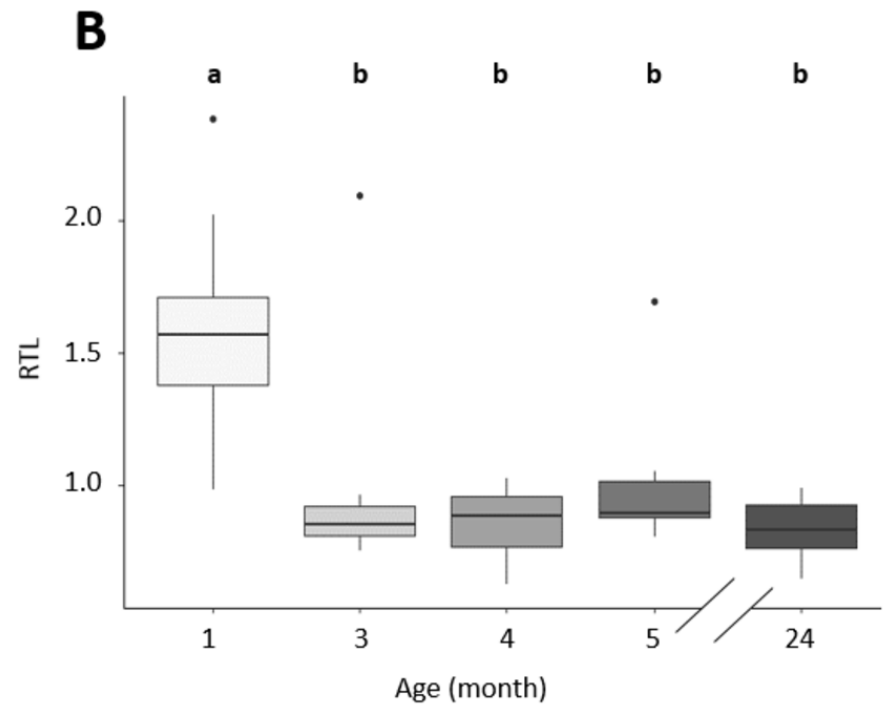
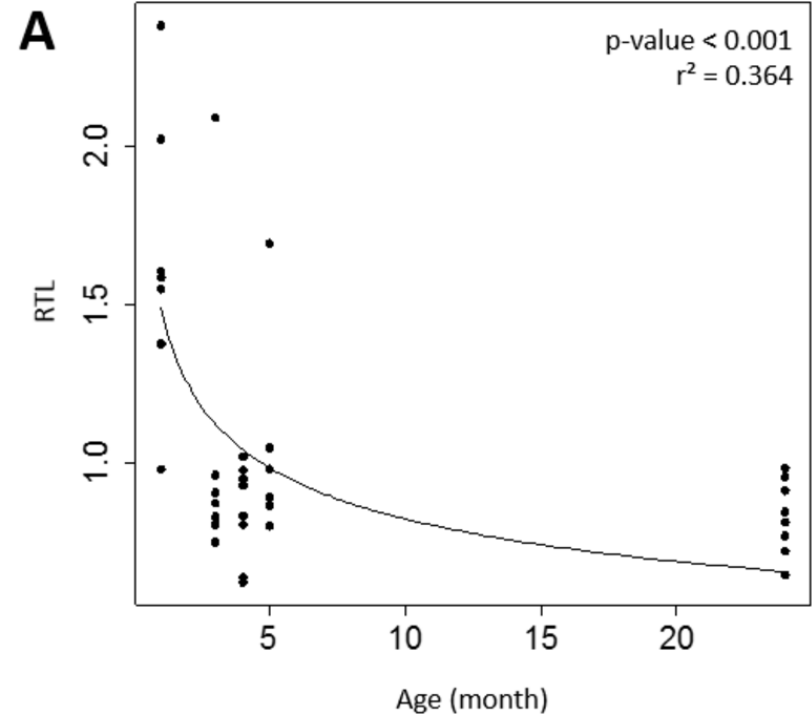
Young reproducers (t2)
~11 g – 5 months

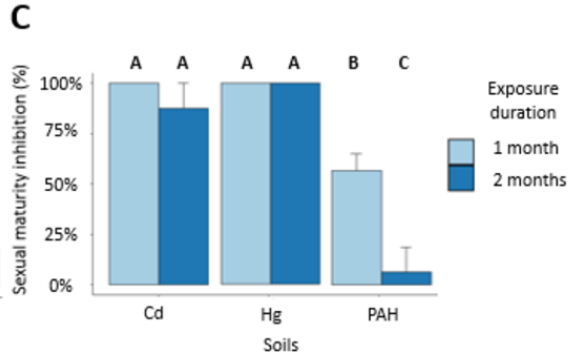
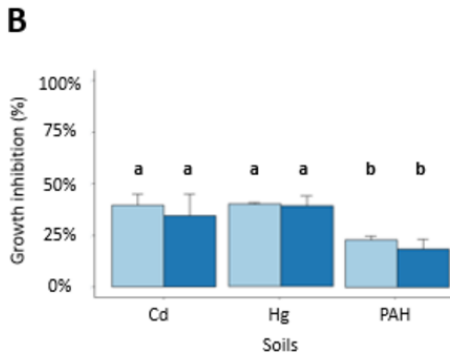
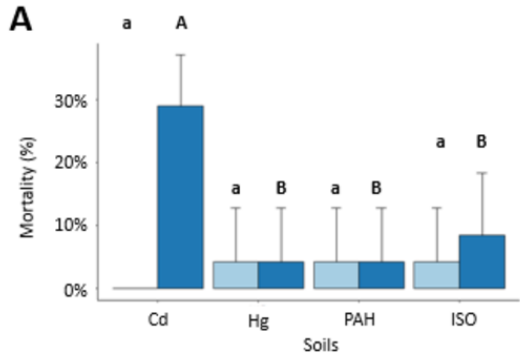


Old reproducers
~14 g – 24 months



—
1 cm





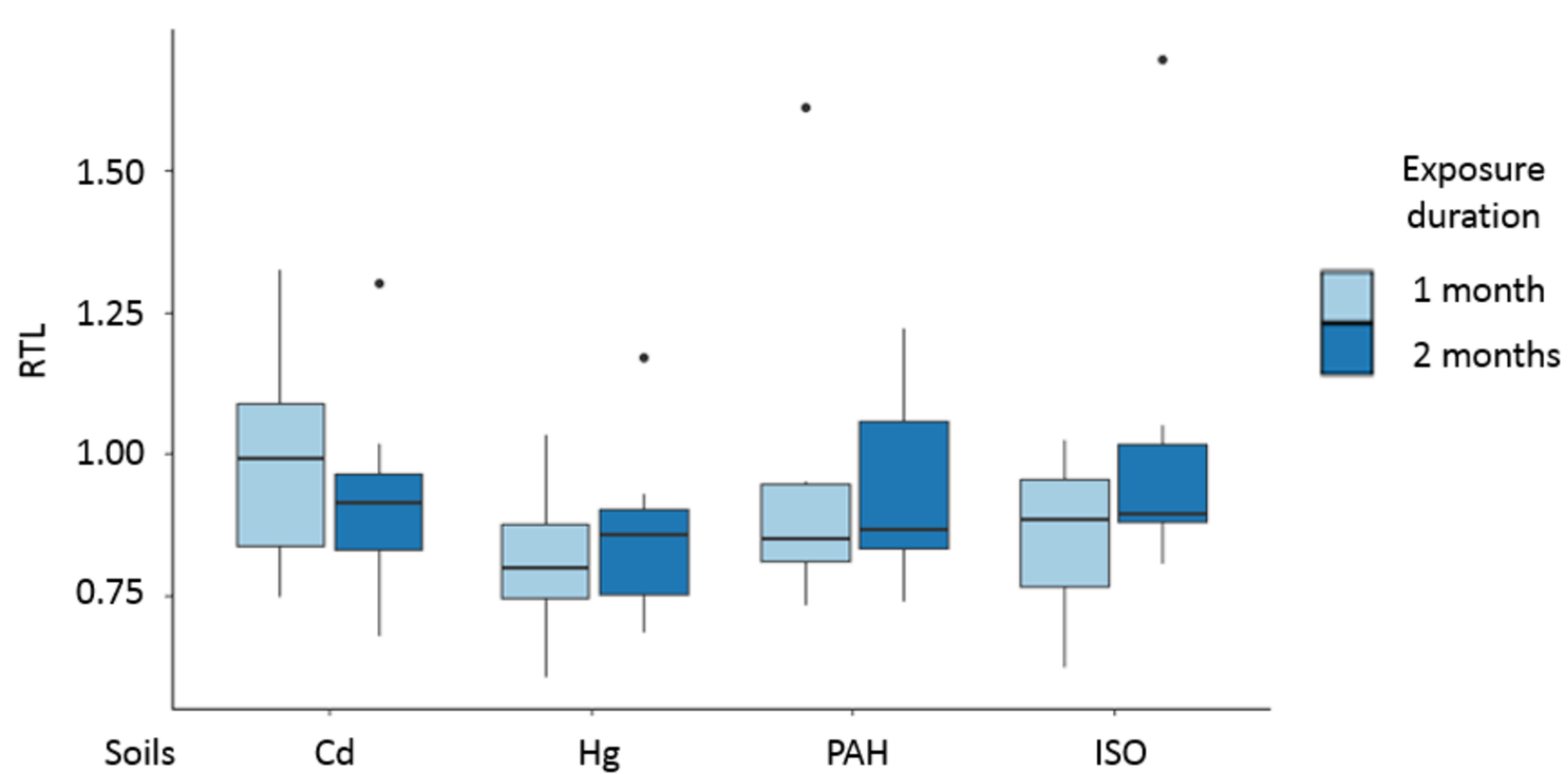


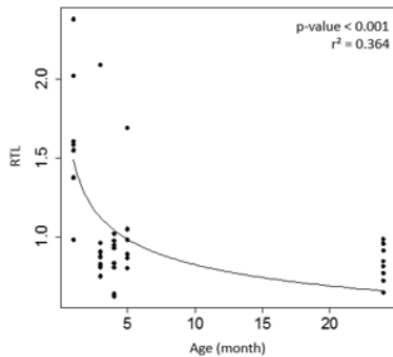
Table 1: Concentration of Cd, Hg and sum of the 16 PAHs in uncontaminated commercial food (Helixal®), soils and snails before exposure (t0) and after 1 month (t1) and 2 months (t2) exposure to Cd, Hg or PAHs in soils (mean and standard deviation for Cd, Hg and PAHs concentrations, expressed in mg kg⁻¹ dry weight (dw) for Cd or Hg in the soils, food and snails; while PAHs are expressed in mg kg⁻¹ fresh weight (fw) of snails and dw of the food and soils).

		Cd (mg kg ⁻¹ dw)	Hg (mg kg ⁻¹ dw)	ΣPAHs (mg kg ⁻¹)	
Food	Helixal®	0.430	0.490	< 1.00	
	ISO soil	0.315	0.029	0.044	
Soil	Cd soil	1508	-	-	
	Hg soil	-	13410	-	
	PAHs soil	-	-	9.36	
Snails	T0	t0	3.57 ± 4.83	0.017 ± 0.014	0.016 ± 0.003
	ISO	t1	1.84 ± 0.021	0.009 ± 0.000	0.017 ± 0.002
		t2	4.08 ± 1.72	0.009 ± 0.000	0.017 ± 0.002
	Cd	t1	654 ± 275	-	-
		t2	781 ± 243	-	-
	Hg	t1	-	2136 ± 754	-
		t2	-	2458 ± 714	-
	PAHs	t1	-	-	0.035 ± 0.021
		t2	-	-	0.026 ± 0.011



Telomere Length

Ageing



Contaminants

