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Fluoride Bioaccumulation and Toxic Effects on the Survival and Behavior of the Endangered White-Clawed Crayfish *Austropotamobius pallipes* (Lereboullet)

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Abstract Laboratory experiments were performed to examine the toxic effects of fluoride (F^-) on the survival and behavior of white-clawed crayfish (*Austropotamobius pallipes*). Body fluoride contents (bioaccumulation) of test crayfish were also examined. No significant differences between male and female crayfish regarding mortality, escape (tail-flip) response, and fluoride bioaccumulation were detected. For mortality, 48-, 72-, 96-, 120-, 144-, 168-, and 192-h median lethal concentrations (LC_{50}) were estimated to be 93.0, 55.3, 42.7, 36.5, 32.9, 30.6, and 28.9 mg F^-/l, respectively. For the escape response, 48-, 72-, 96-, 120-, 144-, 168- and 192-h median effective concentrations (EC_{50}) were estimated to be 18.4, 11.1, 8.6, 7.4, 6.7, 6.2 and 5.9 mg F^-/l, respectively. Average food consumption in test crayfish tended to decrease with increasing water fluoride concentration with a 192-h lowest–observed effect concentration of 10.7 mg F^-/l. These results indicate that the escape response was the most sensitive end point to fluoride toxicity followed by food consumption and mortality. Fluoride bioaccumulation in test crayfish increased with increasing water fluoride concentration and exposure time. The exoskeleton accumulated more fluoride than muscle. A comparison of the obtained results with previous data for other freshwater invertebrates shows that white-clawed crayfish are relatively tolerant to fluoride toxicity. We conclude that fluoride pollution in freshwater ecosystems should not be viewed as an important risk factor contributing to the catastrophic decrease of *A. pallipes* in many European countries. Our results indicate that fluoride bioaccumulation in *A. pallipes* might be used as a bioindicator of fluoride pollution in freshwater ecosystems where it is present.

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Concentrations of fluoride ion (F^-) in unpolluted freshwaters usually range from 0.01 to 0.3 mg F^-/l, whereas in unpolluted seawaters they generally range from 1.2 to 1.5 mg F^-/l (World Health Organization [WHO] 2002; Camargo 2003; Weinstein and Davison 2004). Unfortunately, fluoride must be considered as a serious pollutant because its concentration in many aquatic ecosystems is significantly increasing as a consequence of human activities. Aluminum and zinc smelters, phosphate fertilizer plants, plants producing fluoride chemicals, plants manufacturing brick, ceramics, and glass, use of fluoride-containing pesticides, and discharges of fluoridated municipal waters can considerably increase the natural background fluoride levels of surface waters (WHO 2002; Camargo 2003; Weinstein and Davison 2004). A particular case in Spain concerns the middle Duratón River, where an industrial effluent has significantly increased the natural background fluoride level of the recipient downstream reach in the last few decades (Camargo 1991; Gonzalo and Camargo 2012).

Aquatic animals, such as fish and invertebrates, can take up fluoride ions directly from the water or to a much lesser extent by way of food (Sands et al. 1998; Camargo 2003; Shi et al. 2009; Gonzalo and Camargo 2012). This uptake basically is a function of fluoride concentration in the aquatic medium, exposure time, and physiological characteristics of species (Sands et al. 1998; Camargo 2003; Shi et al. 2009; Gonzalo & Camargo 2012). Subsequently, although it may be eliminated (as F^- ions) by way of

excretory systems (Kessabi 1984), fluoride tends to be accumulated in the exoskeleton of invertebrates and in the bone tissue of fishes (Sands et al. 1998; Camargo 2003; Shi et al. 2009; Gonzalo and Camargo 2012). Fluoride accumulation can play an important role in the hardening of hard tissues (particularly the exoskeleton of marine crustaceans) due to the combination of fluoride with calcium and phosphorous yielding fluorapatite (Zhang et al. 1993; Sands et al. 1998). In addition, it has been indicated that fluoride accumulation in hard tissues may be viewed as a defense mechanism against fluoride intoxication because of the removal of fluoride from body circulation (Sigler and Neuhold 1972; Kessabi 1984). Fluoride acts as an enzymatic poison, inhibiting enzyme activity and, ultimately, interrupting essential metabolic processes (such as glycolysis and synthesis of proteins) and affecting the normal function of nervous system (Kessabi 1984; Reddy et al. 1989; Reddy and Venugopal 1990; Camargo 2003). As a result, fluoride ions can cause toxic effects on the growth, reproduction, survival, and behavior of sensitive aquatic animals (Dave 1984; Reddy and Venugopal 1990; Camargo et al. 1992, 2003; Alonso and Camargo 2011).

Although a significant number of studies on fluoride toxicity to aquatic animals have been performed (see, for example, Camargo 2003), the toxic effects of fluoride on freshwater crayfish have seldom been investigated despite their important role as a keystone species in many freshwater ecosystems (Holdich 2002; Thorp and Covich 2010). In fact, as far as we know, the only published study concerns fluoride bioaccumulation in the signal crayfish *Pacifastacus leniusculus* inhabiting the middle Duratón River in Central Spain (Gonzalo and Camargo 2012). Given the scarce information on fluoride toxicity to freshwater crayfish, we performed laboratory experiments to examine the toxic effects of fluoride on the survival and behavior of the European native white-clawed crayfish *Austropotamobius pallipes* (Lereboullet). A secondary aim was to examine changes in the fluoride content (bioaccumulation) of white-clawed crayfish after their exposure to fluoride ions.

A. pallipes belongs to the family Astacidae (Decapoda, Malacostraca), and it was found long ago in most freshwater ecosystems throughout Europe, from Greece to Portugal, reaching its northerly limit in the British Isles (Holdich 2002; Souty-Grosset et al. 2006). Nevertheless, *A. pallipes* is considered to be endangered in many European countries due mainly to competition with nonnative invasive crayfish and disease from their fungal pathogen (Holdich et al. 2009; Longshaw 2011). Other factors responsible for the catastrophic decrease of *A. pallipes* may be habitat loss, overfishing, and poor water quality (Holdich 2002; Souty-Grosset et al. 2006). *A. pallipes* can, however, be quite tolerant to eutrophication, organic enrichment, and hypoxia (Demers and Reynolds 2002; Demers et al. 2006). To this respect, a third and final aim of our investigation was to compare the tolerance of *A. pallipes* to fluoride toxicity with previous published data regarding other freshwater invertebrates.

Materials and Methods

Fluoride Toxicity Experiments

White-clawed crayfish (male and female), ~6 months old, were obtained from the Rillo de Gallo crayfish farm (Guadalajara, Spain), an authorized and certified farm dedicated to the reproduction and rearing of white-clawed crayfish for repopulation purposes. Animals were transported to our research laboratory using polyethylene insulation boxes. No mortality occurred during transportation. They were stocked in glass aquaria (60 l) for acclimation for 2 weeks before the toxicity tests. Average water acclimation conditions were as follows: temperature 19.5°C, pH 7.8, dissolved oxygen 10.1 mg O_2/l, and total hardness 190 mg $CaCO_3$/l. Laboratory experiments were performed at the former Ecology Department of Alcala University in November 2011.

After acclimation, a static (with water renovation every 3 days) toxicity bioassay was performed using 35 glass aquaria (3 l) and 70 crayfish (35 male and 35 female). Sex differentiation was established by examining male gonopods. Mean values ($n = 35$; ± SD) of total body length were 57.2 ± 4.9 mm for male and 56.9 ± 5.3 mm for female crayfish with no significant (Kruskal–Wallis test: $H = 0.089$ and $P = 0.765$) difference between the sizes of both sexes. Animals were exposed to six different nominal fluoride concentrations (3, 6, 12, 24, 48, and 96 mg F^-/l), plus a control, for 8 days. The toxicity bioassay was performed with five replicates per treatment, using one male and one female per aquarium/replicate, under a natural photoperiod (12 h of light/12 h of dark). Two tubular plastic pieces were provided in each aquarium as shelters. Throughout the toxicity bioassay, crayfish were fed daily with raw carrot (changed every day), with dead animals were removed. All test crayfish were in intermoult condition.

Except for control aquaria, in which dechlorinated tap water was only used, fluoride concentrations were made by diluting weighted amounts of sodium fluoride (minimum 99.5 % purity of NaF; Merck KGaA, Darmstadt, Germany) into dechlorinated tap water. Water fluoride levels were analyzed by the standardized colorimetric method (American Public Health Association 1998). Mean values ($n = 23$ to 35; ± SD) of measured fluoride concentrations were 0.18 ± 0.07 (control), 2.7 ± 0.5, 5.3 ± 0.8, 10.7 ± 1.4, 19.4 ± 4.0, 45.1 ± 5.5, and 84.8 ± 12.4 mg F^-/l. Because some fluoride was precipitated as insoluble calcium fluoride

(CaF_2), measured fluoride concentrations were lower than their respective nominal concentrations. Mean values of water test conditions for each fluoride treatment are listed in Table 1. There were no significant ($P > 0.05$; Kruskal–Wallis and *post hoc* Mann–Whitney tests) differences for these water test conditions between fluoride treatments. Temperature, pH, and dissolved oxygen were recorded using standard specific meters, and total hardness was determined in accordance with the standardized colorimetric method (American Public Health Association 1998).

Crayfish behavior was checked every 24 h by examining two different behavioral responses. The *escape response* was monitored in crayfish by tapping them on the abdomen three times with a glass rod. The escape or tail-flip response was defined as the animal sweeping its tail fully downward at least one time (Wigginton et al. 2010). A lack of this response in living crayfish was scored as an effect. A complete lack of response to the three taps on the abdomen (i.e., no movement of the legs, tail, and antennae) was considered as mortality. Food consumption was also monitored by weighting raw carrot pieces before and after 24 h in each aquarium and dividing the obtained difference (i.e., food consumption) by two (two crayfish per aquarium).

Fluoride Bioaccumulation Analyses

At the end of toxicity experiments, living animals were killed by freezing them at –21 °C. Fluoride content (expressed as μg F^-/g dry weight) in test crayfish was determined according to the fusion alkali method using a fluoride ion selective electrode (Gonzalo et al. 2010; Gonzalo and Camargo 2012). This fluoride content was separately analyzed in whole exoskeleton and abdominal muscle tissues in male and female crayfish. In addition, the fluoride content in diet samples (raw carrots) was also determined according to the same analytical method.

Statistical Analyses

Statistical differences in fluoride content (bioaccumulation) between sexes and between tissues were examined using Statistica 7 software (StatSoft Inc., Tulsa, Oklahoma, USA). Given the lack of normality for the whole dataset, nonparametric Kruskal–Wallis test was performed, and *post hoc* Mann–Whitney test was used for pair comparisons (Sokal and Rohlf 1995). The same methodology was used to determine statistical differences among fluoride toxicity treatments regarding food consumption in test–crayfish. A significant level of $P < 0.01$ was selected to decrease the magnitude of statistical errors through pair comparisons.

Multifactor probit analysis (MPA) software (United States Environmental Protection Agency 1991) was used to calculate 48-, 72-, 96-, 120-, 144-, 168-, and 192- h median lethal concentrations (LC_{50} for mortality) and median effective concentrations (EC_{50} for escape response) as well as their respective 95 % confidence intervals (limits). This methodology solves the concentration–time–response equation simultaneously by way of the iterative reweighed least squares technique (multiple linear regression). The dependent variable is the probit of the proportion responding to each concentration, and the independent variables are exposure time and toxicant concentration. Significant differences between male and female crayfish for mortality and escape response were accepted when 95 % confidence limits of their respective LC_{50} and EC_{50} values did not overlap.

Results

No animal was dead in the control aquaria at the end of the toxicity bioassay. No significant ($P > 0.05$; overlap test) difference between male and female *A. pallipes* was detected for mortality. Only the two highest fluoride concentrations (mean values 45.1 and 84.8 mg F^-/l) caused mortality throughout the toxicity bioassay with mortality increasing with increasing exposure time. Consequently, the no observed–effect concentration (NOEC) value for mortality after 192 h of exposure to fluoride ions was estimated to be 19.4 mg F^-/l, and the lowest observed effect concentration (LOEC) value was 45.1 mg F^-/l. Estimated LC_{50} values, and their respective 95 % confidence limits, at different exposure times for all white-clawed crayfish (male and female combined) are listed in Table 2. These LC_{50} values decreased with increasing exposure time.

The number of affected crayfish regarding escape response increased with increasing fluoride concentration

Table 1 Mean ($n = 16$ to 40; ± SD) values of water test conditions for each fluoride treatment (as mg F^-/l) including the control (0.18 mg F^-/l)

Parameters	0.18	2.7	5.3	10.7	19.4	45.1	84.8
Temperature (°C)	19.5 ± 0.8	19.6 ± 0.9	19.4 ± 1.0	19.3 ± 0.9	19.6 ± 0.9	19.5 ± 0.7	19.4 ± 0.5
Dissolved oxygen (mg O_2/l)	9.8 ± 0.5	10.0 ± 0.7	10.2 ± 0.7	10.3 ± 0.5	10.1 ± 0.6	9.9 ± 0.1	10.3 ± 0.3
pH	7.9 ± 0.1	8.1 ± 0.2	8.1 ± 0.2	8.1 ± 0.3	8.1 ± 0.3	7.8 ± 0.1	7.7 ± 0.1
Total hardness (mg $CaCO_3$/l)	194 ± 10	195 ± 3	193 ± 4	191 ± 3	188 ± 4	182 ± 9	180 ± 5

Table 2 Estimated LC_{50} (mortality) and EC_{50} (escape response) values at different exposure times for white-clawed crayfish (*A. pallipes*; male and female combined)

Exposure times (h)	LC_{50} (mg F^-/l)	EC_{50} (mg F^-/l)
48	93.0 (68.3–130.5[a])	18.4 (15.4–22.3)
72	55.3 (46.9–65.9)	11.1 (9.3–13.3)
96	42.7 (37.1–49.0)	8.6 (7.2–10.5)
120	36.5 (31.3–42.2)	7.4 (6.1–9.1)
144	32.9 (27.7–38.7)	6.7 (5.4–8.3)
168	30.6 (25.2–36.5)	6.2 (5.0–7.8)
192	28.9 (23.5–35.0)	5.9 (4.7–7.4)

[a] 95 % confidence limits are in parentheses

and exposure time. However, no significant ($P > 0.05$; overlap test) difference between male and female *A. pallipes* was detected for this behavioral end point. The NOEC value for escape response was considered to be 0.18 mg F^-/l (i.e., the measured fluoride concentration in control aquaria) because no control crayfish manifested effects for escape response throughout the toxicity bioassay. The LOEC value was estimated to be 2.7 mg F^-/l. Estimated EC_{50} values, and their respective 95 % confidence limits, at different exposure times for all white-clawed crayfish (male and female combined) are listed in Table 2. These EC_{50} values decreased with increasing exposure time. They were significantly ($P < 0.05$) lower than the estimated LC_{50} values because their 95 % confidence limits did not overlap in any case (Table 2).

Mean daily food consumption in test crayfish throughout the toxicity bioassay tended to decrease with increasing the water fluoride concentration (Fig. 1): the highest food consumption occurred in control crayfish (0.18 mg F^-/l), whereas the lowest food consumption occurred in crayfish exposed to the highest fluoride concentration (84.8 mg F^-/l). Except for the two lowest mean fluoride concentrations (2.7 and 5.3 mg F^-/l), significant ($P < 0.01$; Kruskal–Wallis and *post hoc* Mann–Whitney tests) differences in food consumption between the control and fluoride treatments were found. Therefore, the NOEC value for this behavioural end point was estimated to be 5.3 mg F^-/l, and the LOEC value was estimated to be 10.7 mg F^-/l.

Regarding fluoride bioaccumulation, there were no significant ($P > 0.05$; Kruskal–Wallis and *post hoc* Mann–Whitney tests) differences in fluoride content between male and female crayfish at the same water fluoride concentrations. Both sexes were hence analyzed together. Results of this analysis are listed in Table 3. As expected, in both tissues (exoskeleton and muscle), the fluoride content increased with increasing water fluoride concentration and exposure time. However, exoskeleton accumulated significantly ($P < 0.01$; Kruskal–Wallis and *post hoc* Mann–Whitney tests) more fluoride than muscle, except in the case of crayfish exposed to 84.8 mg F^-/l during the first 48 h (Table 3).

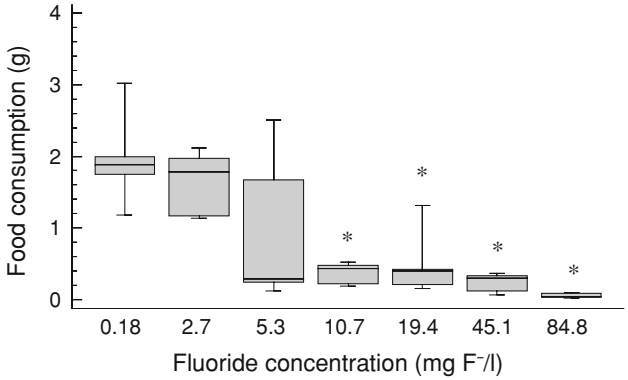

Fig. 1 Median comparison of food consumption (g) by test crayfish (male and female combined) exposed to six different mean fluoride concentrations, plus a control (0.18 mg F^-/l), for 8 days. *Bars* indicate wide ranges of data. *Asterisks* indicate significant ($P < 0.01$) differences between the control and fluoride treatments

Discussion

Fluoride ions are mainly taken up directly from water by aquatic animals (primarily by way of their gills) far less than from food (Sands et al. 1998; Camargo 2003; Shi et al. 2009; Gonzalo and Camargo 2012). Subsequently, fluoride tends to be eliminated by way of excretory systems or accumulated in the body of aquatic animals, particularly in hard tissues (Kessabi 1984; Sands et al. 1998; Camargo 2003; Shi et al. 2009; Gonzalo and Camargo 2012). However, before it is either eliminated or bioaccumulated, fluoride can cause significant adverse effects on the survival and behavior of sensitive aquatic animals (Dave 1984; Reddy and Venugopal 1990; Camargo et al. 1992; 2003; Alonso and Camargo 2011). Results of our research with white-clawed crayfish clearly concur with those previous findings.

Mortality increased, but escape response and food consumption decreased, with increasing water fluoride concentration and exposure time. Furthermore, our results indicate that escape (tail-flip) response was the most sensitive end point to fluoride toxicity followed by food consumption and mortality. It should be evident that the escape response is a relevant end point because a significant decrease in this behavioral response can increase the vulnerability of crayfish to predators and other environmental stressors. Similarly, food consumption is an important end point because a negative change in this behavioral response can decrease the survival of affected animals. Other studies have reported similar findings. Alonso and Camargo (2004), after exposing the freshwater gammarid *Eulimnogammarus*

Table 3 Mean (± SD) fluoride contents (as μg F^-/g dry weight) in whole exoskeleton and abdominal muscle tissues of white-clawed crayfish (*A. pallipes*; male and female combined) exposed to six different mean fluoride concentrations (mg F^-/l), plus a control (0.18 mg F^-/l), for 8 days

Tissue	Exposure time (h)	0.18	2.7	5.3	10.7	19.4	45.1	84.8
Exoskeleton	192	43 ± 31	88 ± 42	141 ± 55	208 ± 67	499 ± 250	2837 ± 0	–
	168	–	–	–	–	–	–	–
	144	–	–	–	–	–	–	–
	120	–	–	–	–	–	1135 ± 329	–
	96	–	–	–	–	–	778 ± 35	3057 ± 686
	72	–	–	–	–	–	746 ± 0	2151 ± 1050
	48	–	–	–	–	–	–	314 ± 0
Muscle	192	16 ± 8	29 ± 13	48 ± 13	76 ± 21	104 ± 66	728 ± 0	–
	168	–	–	–	–	–	–	–
	144	–	–	–	–	–	–	–
	120	–	–	–	–	–	386 ± 87	–
	96	–	–	–	–	–	354 ± 200	1195 ± 656
	72	–	–	–	–	–	261 ± 0	778 ± 467
	48	–	–	–	–	–	–	567 ± 0

– = Dashes indicate no dead crayfish on which to perform bioaccumulation analyses

toletanus to ammonia toxicity, found that feeding activity exhibited a faster response than mortality. Wigginton et al. (2010), after exposing several species of freshwater crayfish to cadmium toxicity, found that the escape (tail-flip) response was a much more sensitive end point than mortality.

The body fluoride content in *A. pallipes* increased with increasing water fluoride concentration and exposure time. Bioaccumulation of fluoride was significantly ($P < 0.01$) greater in exoskeleton than in muscle. Similar results were obtained by Gonzalo and Camargo (2012) after exposing signal crayfish (*Pacifastacus leniusculus*) to fluoride ions. The fact that no significant ($P > 0.05$) difference in fluoride bioaccumulation between male and female *A. pallipes* was detected also agrees with previous data on fluoride bioaccumulation in male and female *P. leniusculus* (Gonzalo and Camargo 2012). In this regard, it is worth noting that Kouba et al. (2010), after reviewing field and laboratory studies concerning bioaccumulation of heavy metals in freshwater crayfish, concluded that, in general, significant differences between sexes are exceptional.

A comparison of the estimated 96-h LC_{50} value of 42.7 mg F^-/l with previous 96-h LC_{50} values for other freshwater invertebrate species shows that *A. pallipes* is relatively tolerant to fluoride toxicity. For example, 96-h LC_{50} values for the trichopterans *Hydropsyche bronta*, *H. bulbifera*, *H. exocellata*, *H. occidentalis*, *H. pellucidula*, *Cheumatopsyche pettiti*, and *Chimarra marginata* were estimated to be 17.0, 26.3, 26.5, 34.7, 38.5, 42.5 and 44.9 mg F^-/l, respectively (Camargo and Tarazona 1990; Camargo et al. 1992); 96-h LC_{50} values for the amphipod *Hyalella azteca*, the ephemeropteran *Hexagenia limbata*, and the dipteran *Chironomus tentans* were estimated to be 14.6, 32.3, and 124.1 mg F^-/l, respectively (Metcalfe-Smith et al. 2003); the 96-h LC_{50} value for the amphipod *Dikerogammarus villosus* was estimated to be 5.8 mg F^-/l (Gonzalo et al. 2010); and the 96-h LC_{50} value for the snail *Potamopyrgus antipodarum* ranged between 58.5 and 69.9 mg F^-/l (Aguirre-Sierra et al. 2011; Alonso and Camargo 2011).

The relatively high tolerance of *A. pallipes* to fluoride toxicity is also evidenced by comparing fluoride contents in this crayfish species with those in the amphipod *Dikerogammarus villosus*. This invertebrate species is sensitive to fluoride toxicity, and it exhibited a whole-body fluoride content of 3637 μg F^-/g dry weight after an exposure of 96 h to 4.2 mg F^-/l (Gonzalo et al. 2010). In contrast, the exoskeleton of *A. pallipes* exhibited a fluoride content of 141 μg F^-/g dry weight after an exposure of 192 h to 5.3 mg F^-/l. This indicates that the sensitivity of freshwater invertebrates to fluoride toxicity can increase with their capability to take up and retain fluoride during short-term exposures, which is consistent with the ecotoxicological principle that the toxicity of pollutants depends greatly on their uptake and retention by organisms (Walker et al. 2005; Newman 2010).

Because white-clawed crayfish (both male and female, indistinctly) seem to be relatively tolerant to fluoride toxicity, fluoride pollution in freshwater ecosystems should not be viewed as an important risk factor contributing to the catastrophic decrease of *A. pallipes* in many European countries. Other environmental factors, particularly the presence of nonnative invasive crayfish species (mainly

Pacifastacus leniusculus and *Procambarus clarkii*), as well as their fungal pathogen *Aphanomyces astaci* (Holdich et al. 2009; Longshaw 2011), are the major causes for their decrease. This could be the particular case in the middle Duratón River (Central Spain), where an industrial effluent has significantly increased the natural background fluoride levels of the recipient downstream reach in the last few decades (Camargo 1991; Gonzalo and Camargo 2012). Nevertheless, despite increased fluoride levels, net-spinning caddisflies (*Hydropsyche bulbifera*, *H. exocellata*) and signal crayfish (*Pacifastacus leniusculus*) were collected downstream from the industrial effluent, whereas white-clawed crayfish were never detected (Camargo 1991; Gonzalo and Camargo 2012).

Finally, because white-clawed crayfish (regardless the sex) significantly accumulated fluoride in their tissues, particularly in their exoskeleton, we can conclude that fluoride bioaccumulation in *A. pallipes* might be used as a suitable bioindicator of fluoride pollution in those freshwater ecosystems where this crayfish species still is present. A similar conclusion was reached by Gonzalo and Camargo (2012) regarding *Pacifastacus leniusculus* after determining the fluoride content in the tissues of signal crayfish exposed to fluoride pollution conditions.

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