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Differential Elemental Accumulation of the Signal Crayfish (*Pacifastacus leniusculus*) Along an Invasion Gradient

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Abstract

The non-native signal crayfish (*Pacifastacus leniusculus*) may alter ecosystems and affect local biota by changing the availability of chemical elements that accumulate and may act as contaminants. This elemental availability may change spatially. In this study, we investigate the differences in elemental exposure in signal crayfish in the Rabaçal River, Portugal, focusing on possible intrapopulation differences between individuals from the core and front (n=30 each) of an invasion gradient. We examined 57 elements, including essential (EEs): Mn, Co, Ni, Cu, Zn, Mg, Ca, Fe, Se, V, S; potentially toxic (PTEs): Cd, Hg, Pb, U, As, Sr, Ba, Cr, Zr, Cs, Tl; and technology-critical elements (TCEs): Ti, Rb, La, Ce, Pr, Gd, Dy, Ho, Er, Yb, Ga, Ge, Hf, Ta, In, Re, Te, Pt, and explored their relationship with signal crayfish diet, behaviour, and parasitic load. Significant differences in element concentrations were found between individuals from the core and front (mean $\mu\text{g}/\text{kg}$, dry weight). Individuals from the front showed higher levels of Co: (476 vs. 297), V: (390 vs. 262), Mn: (2.6×10^4 vs. 1.0×10^4), Hg: (2526 vs. 1658), and Ta: (21 vs. 11). Conversely, individuals from the core had higher levels of Pb: (361 vs. 234), and Sr: (39772 vs. 20018). These differences likely reflect dietary and behavioural variations since front individuals have higher $\delta^{15}\text{N}$ values and more exploratory behaviour, possibly relying more on higher trophic levels such as macroinvertebrates. In contrast, core individuals, with higher parasitic load, likely experienced reduced metabolic efficiency and their diet relied more on lower trophic levels such as plant detritus. This study increases our understanding of contaminant accumulation along an invasion gradient, offering insights for management practices to minimise ecological impacts to other trophic levels.

Keywords: Non-native species; Invasion gradient; Freshwater ecosystems; Behaviour; Diet; Parasitic load; Stable isotopes; Environmental contaminants

Introduction

Freshwater ecosystems are crucial biodiversity hotspots, providing essential ecosystem services such as water purification and nutrient cycling (Dudgeon, 2019). Despite their ecological significance, these systems are among the most threatened globally, facing biodiversity declines at a rate surpassing terrestrial and marine ecosystems (WWF, 2024; Sayer et al. 2025). For example, the abundance of monitored freshwater vertebrate populations has decreased by 85% since 1970 (WWF, 2024), many times driven by the introduction and establishment of invasive species, which disrupt ecological balances, modify habitats and alter trophic dynamics (Gurevitch & Padilla, 2004; Simberloff, 2010; Gutiérrez et al., 2014; Li et al., 2023; Dudgeon, 2019; Vörösmarty et al., 2010).

The signal crayfish (*Pacifastacus leniusculus*), native to North America, exemplifies a highly invasive species given its ecological plasticity, rapid growth, early maturity, and production of high numbers of offspring (Geraldes & Teixeira, 2013; Capurro et al, 2015; Vedia, 2018). This species was first detected in Portugal in 1997 in the Maçãs River (Costa et al., 2010), having invaded adjacent river basins, such as the Rabaçal River, in 2013 (Sousa et al., 2015, 2019; Carvalho et al., 2022). Beyond its ecological impacts, the signal crayfish also serves as a sentinels for accumulating macroelements and trace elements in aquatic ecosystems (Nędzarek et al., 2020). Pollution is a factor that can be allied to invasion impacts, for example, contaminants such as trace elements can interact significantly with invasive species, potentially amplifying the negative impacts on biodiversity and trophic chains (Li et al., 2023).

Trace elements play dual roles in ecosystems, with essential elements (EEs) such as Zn, Cu, and Fe being vital for physiological functions at low concentrations but toxic at elevated levels (Islam et al., 2023). Non-essential and potentially toxic elements (PTEs), such as Pb, Cd, and As,

pose additional risks to organisms even at low concentrations (Zoroddu et al., 2019; Saad et al., 2014). Technology-critical elements (TCEs) are increasingly generating environmental concern due to their growing use, especially in technological devices, and are considered emerging contaminants (Gwenzi et al., 2018; Balaram, 2019). The accumulation of these compounds in nature, in high concentrations, results in negative environmental impacts, especially for aquatic organisms, which absorb and bioaccumulate the compounds, compromising the normal functioning of organs and reproduction (Picone et al. 2022; Espejo et al. 2023). These elements have not been the subject of many studies, so their increasing use makes them an uncertain threat to the environment (Adeel et al., 2019; Malhotra et al., 2020). The ability of *P. leniusculus* to bioaccumulate these elements, particularly in the hepatopancreas and exoskeleton, highlights its role in both ecosystem and human health (Nędzarek et al., 2020). This duality underscores the need to monitor invasive species not only for their ecological impact but also for human health since these invasive species may be consumed by humans.

Invasion gradients offer a unique opportunity to investigate how environmental pressures shape the traits of invasive species (Sousa et al., 2024). Theoretically, individuals at the invasion front face distinct selective pressures, such as distinct exposure to predators, enhanced resource availability, and reduced intraspecific competition, compared to those in the invasion core (Sousa et al., 2024; Alves et al., in press). These differences can drive phenotypic and behavioural adaptations, such as heightened exploratory behaviour, aggression, and risk-taking, which may enhance the success of individuals in colonizing new habitats (Phillips et al., 2010; Gruber et al., 2018; Yagound et al., 2022). Conversely, individuals in the core often experience higher intraspecific competition, and potentially greater parasitic loads, which can influence their

behaviour and contaminant accumulation patterns (Rebrina et al., 2015; Herse et al., 2018; Sousa et al., 2024).

Despite advances in understanding the ecological impacts of non-native species, intra-population differences along invasion gradients remain poorly explored, particularly regarding trace element accumulation and its interactions with diet, behaviour, and parasitism (Sousa et al., 2024). While previous studies have focused on species-level impacts (Nędzarek et al., 2020; Li et al., 2023), few have delved into population-level dynamics, which are critical for elucidating the mechanisms underlying invasion success and to better understanding environmental contamination at meaningful spatial scales (Morales, 2004). Our study is pioneering, since few studies analysed elements such as rare earths, with the majority focusing on studying elements such as arsenic (As), mercury (Hg) and lead (Pb) (Suárez et al., 2010; Bellante et al., 2015; Gedik et al., 2017; Zhang et al., 2023). This gap is critical, as intra-population variation can reveal how local environmental pressures, diet, behaviour, and ecological interactions shape contaminant bioaccumulation and subsequent ecological impacts (Herse et al., 2018). For example, previous research on invasive *Procambarus clarkii* has demonstrated population-level differences in trophic interactions (Li et al., 2023). However, these studies often overlook behavioural traits, such as risk-taking and aggression, which may drive differential exposure to contaminants along invasion gradients.

This study addresses this gap by analysing trace element bioaccumulation, the influence of diet through stable isotope analysis ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$), and assessing differences in behaviour and parasitic load in *P. leniusculus* along the invasion gradient in the Rabaçal River, Portugal. We hypothesize that individuals at the invasion front will exhibit higher exploratory behaviour and risk-taking, correlating with increased accumulation of essential trace elements due to greater

metabolic demands. As previous studies, such as Alves et al. (in press), have indicated, the diet differs between individuals at the core and front of the invasion gradient, which might influence their accumulation of elements. Meanwhile, individuals in the invasion core will show a greater accumulation of toxic elements due to prolonged exposure and higher parasitic loads. Understanding these dynamics provides critical insights into how invasive species interact with contaminants, adapt to ecological pressures, and influence ecosystem health. This knowledge is essential for developing effective management strategies to mitigate their impacts and preserve freshwater biodiversity.

Material and Methods

Study Area and Sampling

This study was conducted on the Rabaçal River (Figure 1), located in northeastern Portugal, with a length of 88 km and its source in Spain (Sousa et al., 2018). The river experiences low anthropogenic pressure, and part of it lies within the Montesinho Natural Park, one of Portugal's protected areas, established in 1979 to preserve the rich biodiversity across an area of 748 km² (Pereira et al., 2007; Castro et al., 2010).

On the Rabaçal River, eight sampling sites (R1–R8) were selected to delineate the invasion gradient of the signal crayfish. R1, located in Edroso (41.92053, -7.1224), represents the invasion core, while R7, in Candedo (41.86436, -7.11961), marks the current invasion front. The selection of these sites allowed us to determine the extent of the invasion and establish a reference framework. However, we focused on individuals from the invasion core and front, as they best represent different invasion stages. All signal crayfish individuals were captured using coarse-mesh trap nets during the summer of 2023 for elemental, diet, and behavioural tests, as well as

parasite analyses. The traps were submerged for 24-hour periods and subsequently removed from the river. All individuals were measured (carapace length in cm), and their sex was recorded.

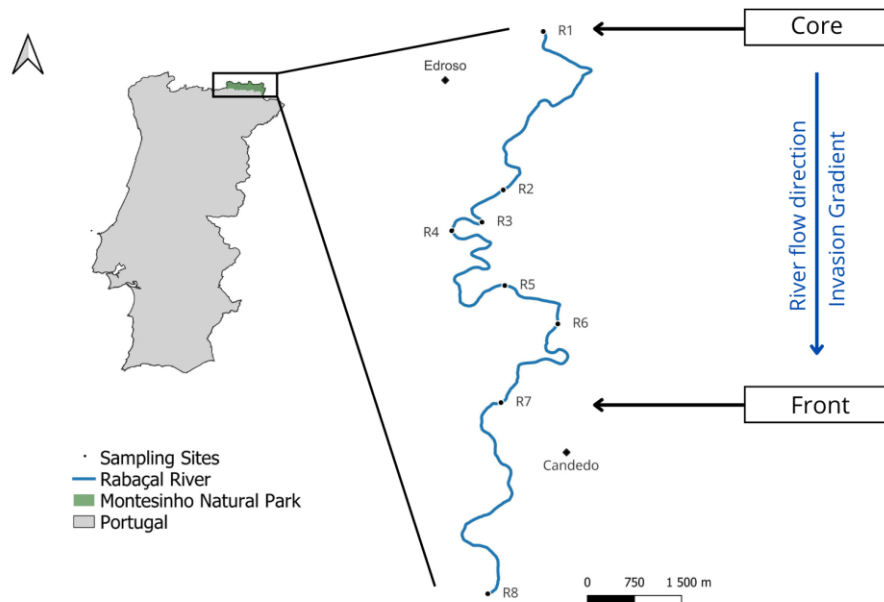


Figure 1. Sampled sites (R1-R8) along the Rabaçal River. Several sites (R1 to R6) are within Montesinho Natural Park.

Trace element analysis

Sample preparation began with the freezing sacrifice of 15 males and 15 females randomly collected from each site (core vs. front of invasion). Specimens were stored at -20°C until analysis at IB-S (Institute of Science and Innovation for Bio-Sustainability, Braga, Portugal). The lengths of the individuals from the core ranged from 6.5 cm to 10.5 cm while those from the front ranged from 7.0 cm to 11.8 cm. Muscle samples were dried at 40°C for 24 hours in an Infors HT, Multitron Pro, ground in a mortar to achieve a homogeneous powder consistency, and subsequently sent to the Department of Analytical Chemistry, in Poznań, Poland. Approximately, 0.8 ± 0.02 mg of each sample was digested with 3 mL 65% HNO_3 using a microwave oven (Mars 6, CEM Corporation, Austria). The samples were then made up to 10 mL with Milli-Q water (Direct-Q system,

Millipore, Germany). Immediately before the elemental analysis, the samples were diluted 20 times with milli-Q water. A total of 57 elements (Al, V, Mn, Co, Ni, Cu, Zn, Cd, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Er, Hg, Na, Mg, P, K, Ca, Fe, B, Rb, Sr, Ba, Pb, U, Cr, As, Se, S, Ti, Ga, Ge, Yb, Lu, Hf, Ta, Be, Zr, Nb, Ru, Pd, In, Te, Cs, W, Re, Os, Pt, Au, Tl, Si) were analysed using inductively coupled plasma mass spectrometry (ICP MS, PlasmaQuant® MS Q, Analytik Jena, Germany).

For quality control, analytical blanks ($n = 4$) and certified reference materials – DORM 5: (fish protein; $n = 3$; National Research Council, Canada) and BCR 668 (mussel tissue; $n = 4$; Institute for Reference Materials and Measurements, Belgium) were submitted to the same procedures as the samples. Detection limits ranged from 0,00017 $\mu\text{g}/\text{kg}$ to 1203 $\mu\text{g}/\text{kg}$ for S. The recovery rates for certified materials were according to international QA/QC criteria being between 80 and 120%. For non-certified elements, accuracy was monitored using a standard solution. To ensure optimal precision and accuracy in the analyses, a solution containing – Bi, Ir, Li, Rh, Sc, and Y – was used as internal standards. For multi-element determination, the following conditions were standardized: nebulizer gas flow at 1.02 L/min, auxiliary gas flow at 1.5 L/min, plasma gas flow at 9.0 L/min, and Radio Frequency (RF) power set to 1.20 kW. Signal measurements were conducted over 20 scans in 5 replicates, using three sequential modes: without gas addition, and with the integrated Collision Reaction Cell (iCRC), utilizing hydrogen as the reaction gas and helium as the collision gas to minimize mass interference.

Diet analysis

The sample preparation procedure was the same as that described for analysing trace elements. The elemental and isotopic composition analyses were carried out on a Thermo Scientific Flash 2000 organic elemental analyser (EA), Organic Elemental Analyser, connected to an isotope ratio mass spectrometer (IRMS) Delta V Advantage via Conflo IV, at Ciimar (Centro Interdisciplinar de Investigação Marinha e Ambiental) in Porto. Stable carbon and nitrogen isotope values were calculated using the following formula:

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000,$$

where X is the isotope of carbon or nitrogen, and R is the ratio between the heavy and light isotopes of carbon or nitrogen.

The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of the samples and standards were normalised with reference materials for each element (IAEA-N-1, IAEA-N-2 and IAEA-NO3 for nitrogen; USGS-24 and USGS-40 for carbon) with a margin of analytical error of approximately 0.1‰. Using an internal standard from the sea bass *Dicentrarchus labrax* (read after 12 analyses to have analytical control), the isotopic composition of the samples present in the extracted abdominal tissue was accurately determined. Corrections were also made to the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values according to a calibration curve derived from the delta values of N and C, based on the mass amplitudes of a caffeine standard. The elemental composition was also determined using the Chlorella K factor, and the samples were analysed twice to keep the coefficient of variation below 10%.

Behavioural tests

Three behavioural tests were carried out, in which the individuals collected in the field in the core and front sites underwent a period of acclimatisation in tanks at 18°C, without food for 3 days. The tests were carried out in a laboratory room, in a 60-litre tank (46 x 63 x 32 cm).

In two tests, 16 individuals (8 females and 8 males, ranging from 8 to 11 cm in length) were randomly selected from both the core and the front of the invasion gradient. These individuals underwent a 5-minute acclimatization period in a shelter before being tested for 15 minutes. The objectives were to assess the individuals' risk-taking ability (measuring the time, in seconds, to leave the shelter) and neophilia (measuring the time, in seconds, to leave the shelter, approach the object, and touch it). For the neophilia test, a fish bait that simulated an insect was used as the object, and it was placed in motion to stimulate interaction. For the behavioural tests, the individuals were placed in a controlled room at 18°C. The experimental tank was 46 x 63 x 32 cm (total volume: 60 L).

Aggression tests were also carried out, comparing the following combat groups: core female vs. front female, core male vs. front male, core female vs. front male, and front female vs. core male. No acclimatization period was given before these tests. In each combat test, individuals of similar sizes and with no shell deformities were selected and positioned face-to-face to fight over food (cat food) and space, with the duration of the fight recorded in seconds. Each of these tests was repeated eight times with different individuals.

Parasite Analysis

The abundance of external parasites (*Branchiobdella astaci*) was analysed *in situ* along the invasion. The individuals were assessed for parasites in the claws and classified on a 4-value

qualitative scale (0-absence; 1-few; 2- some; 3- many; see Figure S1 for details about the categorization).

Data Analysis

All data analysis was carried out in the R Studio software (R Core Team, 2022), using various packages such as readxl, ggplot2, devtools, dplyr, rjags, SIBER and multcomp. The results for the 57 trace elements were filtered to select the compounds that could be analysed, removing those whose value was not within the detection limits. A total of 49 elements were analysed and the mean and standard deviation were calculated for each group (front female, front male, core female and core male). The statistical analysis consisted of a two-way ANOVA, one to check for possible differences between the 4 groups (front female, front male, core female and core male) and a Tukey test. Boxplots were made for all the elements whose results were significant in both analyses. A Pearson correlation analysis was conducted between the concentrations of 49 trace elements and individual weight to evaluate potential relationships.

A Pearson correlation was also made between the 49 trace elements analysed and the stable isotopes $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ to identify the possible influence of diet on the concentration of the trace elements analysed. We consider there to be a weak relationship when the correlation is up to 0.3, moderate from 0.3-0.7 and strong from 0.7-1, as is traditionally accepted (Schober et al., 2018).

For the behavioural tests, the data was analysed for normality using the Shapiro-Wilk test and normalised with log10 for the risk-taking and neophilia tests. For these tests, ANOVAs were used to determine the significance of the invasion site and sex. For the aggressiveness test, chi-squared tests were performed to assess whether individuals at the front or were more aggressive and to evaluate differences in behaviour between sexes. This test is appropriate for categorical data

identifying which individual reached the food first or claimed the space. Fisher's exact tests were also conducted due to the small sample size.

To analyze parasite data, a chi-square test was carried out to evaluate the influence of location and sex on parasitism. Pairwise comparisons were also explored to assess differences between groups.

Results

Elemental analysis

The concentration values (mean \pm standard deviation) for 49 elements analysed plus the stable isotopes of carbon and nitrogen are shown in Table 1, in $\mu\text{g}/\text{kg}$ (dry weight). Eight elements were not considered in the statistical tests (lutetium, niobium, ruthenium, palladium, indium, rhenium, osmium and gold) because they were below the detection limit.

Table 1. Mean values \pm standard deviation obtained for the trace elements under study ($\mu\text{g}/\text{kg}$, dry weight) and stable isotopes for the signal crayfish (*Pacifastacus leniusculus*).

n	Front		Core	
	Female	Male	Female	Male
	14	17	13	15
Al	104902 ± 73604	129438 ± 55905	51298 ± 69884	42411 ± 28330
V	401 ± 298	380 ± 166	268 ± 163	256 ± 102
Mn	17893 ± 11502	35156 ± 44898	7238 ± 5150	13911 ± 12406
Co	489 ± 459	463 ± 373	293 ± 162	301 ± 157
Ni	453 ± 571	397 ± 466	397 ± 300	1162 ± 1989
Cu	78762 ± 65501	63478 ± 28898	64864 ± 28095	81726 ± 43015
Zn	108026 ± 74333	89012 ± 39519	76690 ± 16325	110156 ± 80689
Cd	211 ± 163	171 ± 100	150 ± 72	192 ± 129
La	53 ± 108	50 ± 62	28 ± 29	153 ± 273
Ce	115 ± 200	125 ± 122	75 ± 59	123 ± 248
Pr	16 ± 26	13 ± 15	8,62 ± 8,49	14 ± 36
Nd	62 ± 95	63 ± 58	41 ± 25	68 ± 128
Sm	22 ± 22	26 ± 17	19 ± 8,06	28 ± 24
Eu	1,8 ± 1,3	2,35 ± 2,49	2,75 ± 3,29	2,61 ± 1,66
Gd	11 ± 13	15 ± 11	10 ± 5,02	14 ± 12
Dy	7 ± 6,92	9,32 ± 7,53	7,56 ± 3,82	8,10 ± 3,19
Ho	3,33 ± 3,67	4,16 ± 3,35	2,80 ± 2,56	3,53 ± 3, 86
Er	3,67 ± 3,74	5,43 ± 4,27	4,49 ± 3,25	4,38 ± 1,77
Hg	2858 ± 1956	2195 ± 918	1647 ± 942	1670 ± 964
Na	13173834 ± 9124346	9809414 ± 4025267	10650455 ± 4306602	10689730 ± 6460304
Mg	1815545 ± 1243487	1289553 ± 496082	1750486 ± 534711	1761397 ± 694511
P	13367983 ± 9453734	11581499 ± 5068599	7737853 ± 1327441	9614962 ± 4709918
K	22432189 ± 1653791	18189038 ± 8103566	1430104 ± 3945718	20983848 ± 18855258
Ca	6942514 ± 5696204	3432673 ± 1734778	6934692 ± 3239298	11854283 ± 7933547
Fe	157276 ± 106304	156705 ± 88189	153796 ± 172209	133133 ± 52397
B	29865 ± 26558	31606 ± 11562	14768 ± 10544	38502 ± 13350
Rb	58228 ± 42622	41813 ± 17649	43008 ± 17426	81837 ± 119259
Sr	26046 ± 20521	13990 ± 6519	33194 ± 13393	46351 ± 27108
Ba	3839 ± 3028	3087 ± 1842	4948 ± 2480	7320 ± 4001
Pb	411 ± 612	58 ± 52	547 ± 593	176 ± 56
U	25 ± 33	16 ± 11	15 ± 11	15 ± 10
Cr	902 ± 908	814 ± 695	437 ± 201	795 ± 744
As	1190 ± 1174	773 ± 325	1202 ± 1112	814 ± 538
Se	907 ± 638	686 ± 289	670 ± 244	542 ± 436
S	15811652 ± 10396139	12121667 ± 4554600	12197292 ± 2917404	12678303 ± 5839739
Ti	4893 ± 6529	3635 ± 3917	2134 ± 2553	2084 ± 1308
Ga	158 ± 130	138 ± 78	219 ± 111	311 ± 171
Ge	44 ± 40	58 ± 32	46 ± 17	62 ± 25
Yb	5,33 ± 5,51	7,11 ± 5,19	6,04 ± 3,39	6,75 ± 3,22
Hf	9,16 ± 7,61	9,35 ± 4,70	6,74 ± 2,05	29 ± 68
Ta	25 ± 22	16 ± 7,24	9,55 ± 2,98	13 ± 4,18
Be	10 ± 7,99	14 ± 7,17	14 ± 11	18 ± 8,25
Zr	73 ± 68	49 ± 33	26 ± 10	550 ± 1811
Te	32 ± 22	34 ± 11	29 ± 5,87	51 ± 29
Cs	2361 ± 1586	1852 ± 684	2148 ± 877	3592 ± 4983
W	27 ± 24	22 ± 17	8,25 ± 6,79	62 ± 90
Pt	10 ± 7,94	7,37 ± 2,71	1,42 ± 1,01	4,55 ± 1,79
Tl	34 ± 26	26 ± 12	24 ± 10	30 ± 29
Si	91945 ± 56399	97099 ± 67142	71535 ± 54459	72719 ± 50208
δ ¹⁵ N	8,58 ± 0,55	8,56 ± 0,38	8,09 ± 0,32	8,31 ± 0,39
δ ¹³ C	-23 ± 0,84	-23 ± 0,66	-23 ± 0,67	-23 ± 0,55

Figures 2 and 3 illustrate the results for the elements that showed significant differences (ANOVA) between the groups. The results are divided into essential elements, potentially toxic elements, and technology-critical elements (TCEs).

For the Essential Elements (EEs) calcium, boron, manganese and strontium ($p < 0.001$; $p = 0.002$; $p = 0.03$; $p < 0.001$, respectively, Figure 2) significant differences between the sexes for each location were detected. For calcium, males from the core presented the highest concentrations than males from the front. In the case of boron, core females exhibited the lowest concentrations, whereas males, both from the core and the front, had significantly higher values. Manganese concentrations were highest in males from the front when compared to females from the core. For strontium, males from the core had the highest concentrations overall, followed by females from the front. Conversely, males from the front and core females exhibited lower values, underscoring the influence of both sex and invasion location on strontium levels.

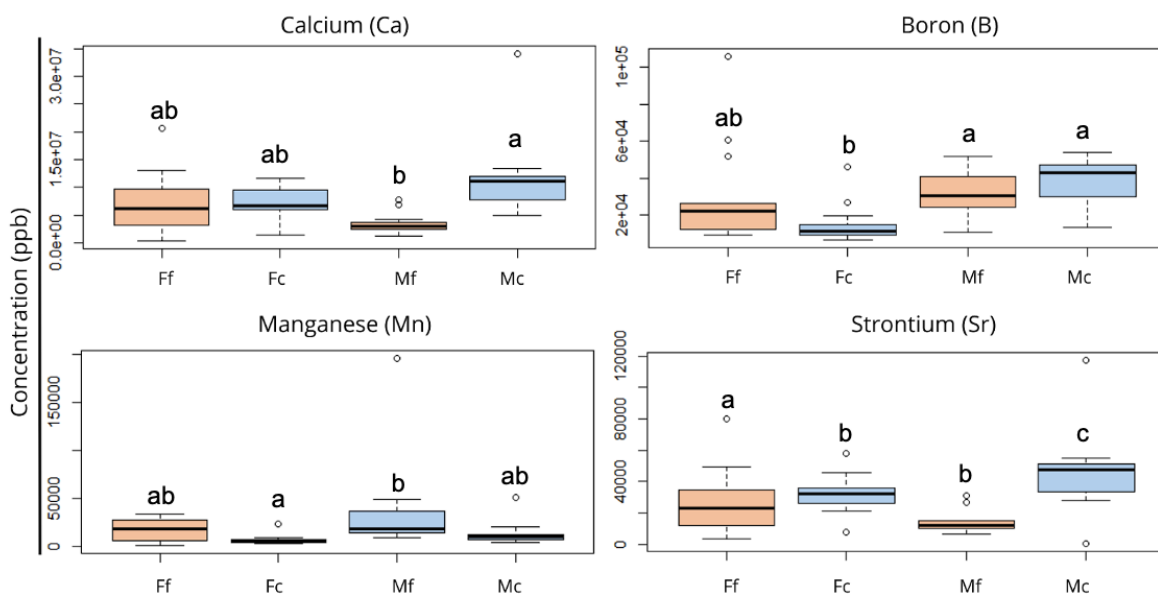


Figure 2. Essential elements with significant differences between front female (Ff), front male (Fm), core female (Cf) and core male (Cm): calcium, boron, manganese and strontium for signal crayfish (*Pacifastacus leniusculus*).

For potential toxic elements (PTEs) and technology-critical elements (TCEs), significant differences were found for aluminium, tellurium, platinum, lead, gallium, tantalum and tungsten ($p < 0.001$; $p = 0.02$; $p = 0.001$; $p = 0.005$; $p = 0.003$; $p = 0.01$; $p = 0.03$, respectively). Aluminium levels were significantly higher in front males compared to core females and males. For lead and gallium, core males exhibited higher concentrations than front males and females. Platinum showed significantly higher values in front of females compared to core females and males. Tantalum concentrations were higher in front females compared to core females. Finally, differences in tungsten and tellurium were observed between core females and core males, with core females generally exhibiting lower concentrations of these elements (Figure 3).

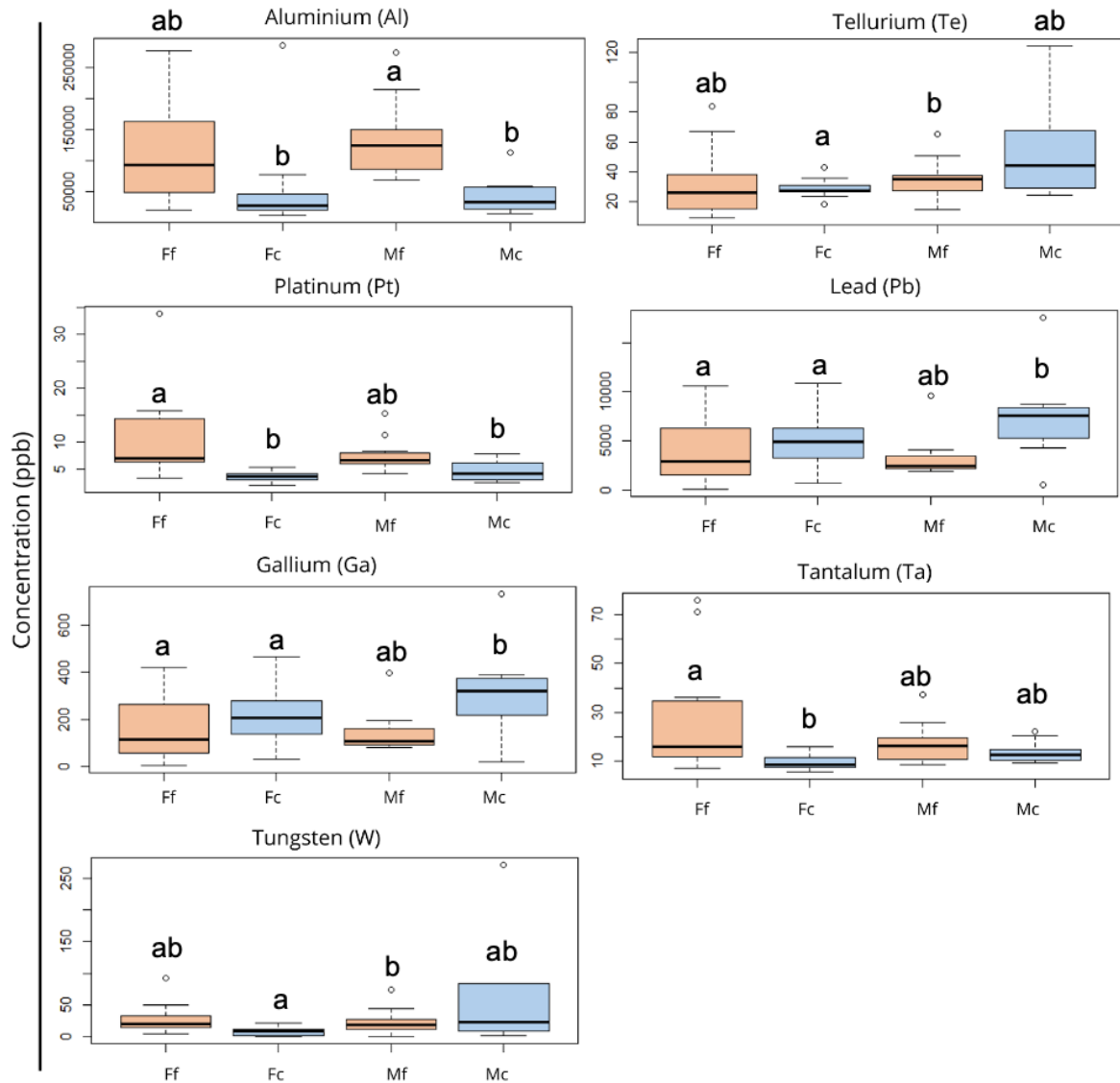


Figure 3. Potential toxic elements and technology-critical elements (TCEs) with significant differences between front female (Ff), front male (Fm), core female (Cf) and core male (Cm): aluminium, tellurium, platinum, lead, gallium, tantalum and tungsten for signal crayfish (*Pacifastacus leniusculus*).

The Pearson correlation analysis between individual weight and element concentrations revealed several significant relationships (Figure 4). Sodium (Na) exhibited a negative correlation with weight ($r = -0.278$, $p = 0.038$), as did Strontium (Sr, $r = -0.277$, $p = 0.039$) and Arsenic (As, $r = -0.286$, $p = 0.033$). Conversely, Rubidium (Rb, $r = 0.292$, $p = 0.029$), Hafnium (Hf, $r = 0.411$, $p = 0.0016$), Zirconium (Zr, $r = 0.401$, $p = 0.0022$), Niobium (Nb, $r = 0.475$, $p = 0.016$), and Cesium (Cs, $r = 0.302$, $p = 0.023$) exhibited positive correlations. Among them, Hafnium (Hf) and Zirconium (Zr) displayed the strongest positive correlations, whereas Arsenic (As) and Strontium (Sr) had the most pronounced negative associations. For all the other elements no correlation was found (data not shown).

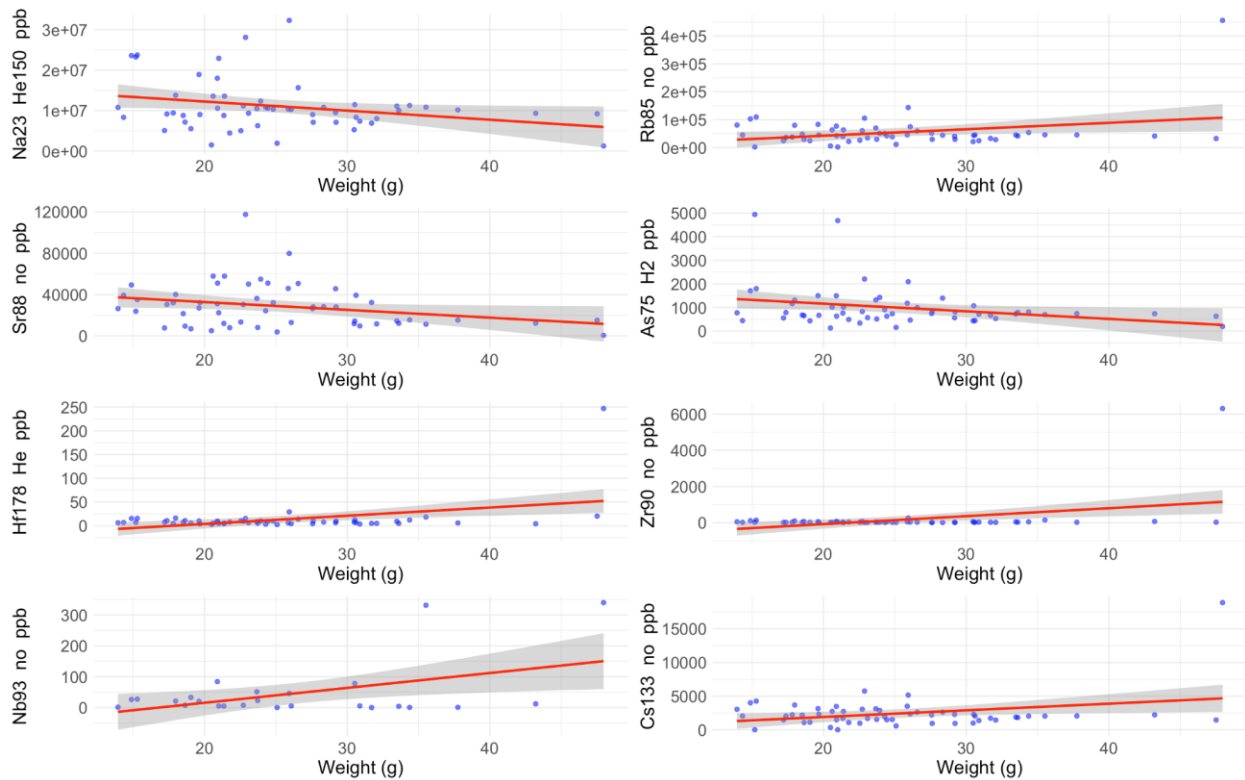


Figure 4. Scatterplots showing the relationship between individual weight and the concentrations of significant metals (Na, Rb, Sr, As, Hf, Zr, Nb, and Cs). Each plot includes a fitted linear regression line (red) with a 95% confidence interval (grey shading). Negative correlations were observed for Na, Sr, and As, while positive correlations were found for Rb, Hf, Zr, Nb, and Cs.

Behavioural tests

The results of the behavioural tests (Figure S2) showed no significant differences in the time it took for signal crayfish to leave the shelter during the risk-taking test (S2A) between invasion sites ($p = 0.25$) or between sexes ($p = 0.49$). Similarly, no significant differences were found for the time to leave the shelter (S2B), spent near the object in the neophilia test (S2C), or for the time taken to touch the object (S2D), with no variation between invasion sites ($p = 0.32$, $p = 0.84$, respectively) or between sexes ($p = 0.54$, $p = 0.79$, respectively). These results suggest that both risk-taking and neophilia behaviours were not significantly influenced by the invasion site or sex.

Figure 5 (A) shows, as a percentage (%), the victories for whoever got to the food first for each fight: core female vs. front female, core male vs. front male, core female vs. front male and front female vs. core male. Regarding the front male vs. core male, front female vs. core female and core female vs. front male fights, there were no significant differences in who got to the food first ($p = 0.62$; $p = 0.13$; $p = 0.13$, respectively). However, in the front female vs. core male fight, there were significant differences ($p = 0.01$). Figure 5 (B) shows, as a percentage, who won by space for each fight (as above). Significant differences were found in the front male vs. core male and front female vs. core female fights for space ($p < 0.01$; $p = 0.03$, respectively). However, in

the fight between female vs. male in front and female in front vs. male in front, there were no significant differences ($p = 0.28$; $p = 0.28$, respectively).

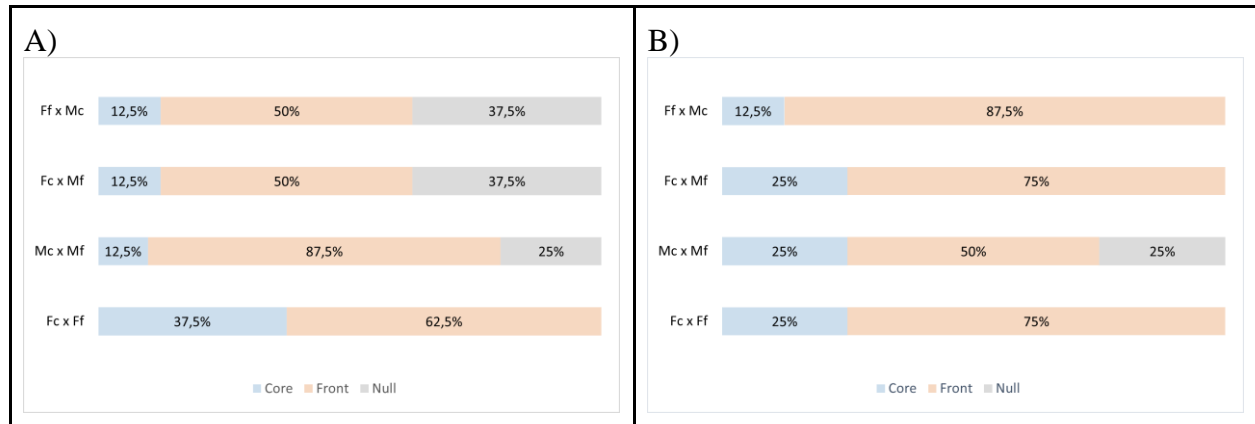


Figure 5. (A) Victories for space in the aggressiveness test; (B) Victories for who got to the food first in the aggressiveness test, for each of the four fights with signal crayfish (*Pacifastacus leniusculus*).

Diet analysis

The ANOVA results for stable isotope values revealed a significant difference between the core and front groups ($F(1, 58) = 11.27$, $p = 0.001$). Post hoc analysis using the Tukey HSD test confirmed this finding, with $\delta^{15}\text{N}$ values being significantly lower in the individuals from the core group compared to the front (95% CI: -0.58 to -0.15, $p = 0.001$).

To observe the influence of diet on the exposure of signal crayfish to the trace elements being analysed, a Pearson correlation was carried out between the 49 elements and the stable isotopes $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, as can be seen in Figure 6. There is a positive and strong correlation

between the $\delta^{13}\text{C}$ isotope and the elements Silicon (Si), Caesium (Cs), Titanium (Ti), Rubidium (Rb), Neodymium (Nd), Praseodymium (Pr), Cerium (Ce), Lanthanum (La), Copper (Cu), and strongly negative with Arsenic (As), Uranium (U) and Lead (Pb). There was also a strong positive correlation between $\delta^{15}\text{N}$ and the elements Silicon (Si), Zirconium (Zr), Tantalum (Ta), Hafnium (Hf), Germanium (Ge), Titanium (Ti), Strontium (Sr), Iron (Fe), Dysprosium (Dy), Gadolinium (Gd), Samarium (Sm), Neodymium (Nd), Praseodymium (Pr), Cerium (Ce), Lanthanum (La), Cadmium (Cd) and Aluminium (Al), and strongly negative with Strontium (Sr).

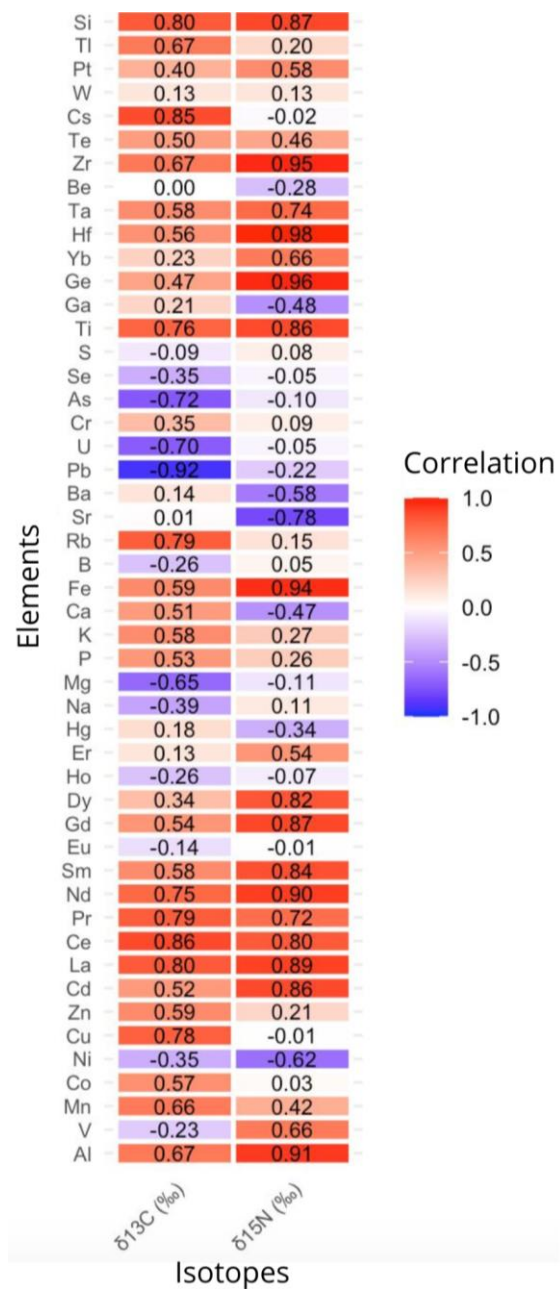


Figure 6. Pearson's correlation between 49 trace elements and the stable isotopes of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ for signal crayfish (*Pacifastacus leniusculus*). 0-0.3 is a weak correlation, 0.3-0.7 is a medium correlation and 0.7-1 is a strong correlation. A reddish colour indicates a positive correlation and a bluish colour a negative correlation.

Parasite Analysis

The results of the classification of external parasites by location and sex are shown in Figure 7. The chi-square tests revealed a highly significant difference between location and score categories (p -value < 0.001), suggesting that the distribution of scores differs strongly between the core and front groups. Additionally, when analyzing the relationship between sex and score categories within each location, no significant associations were observed for either the core (p -value = 0.57) or the front (p -value = 0.29) groups.

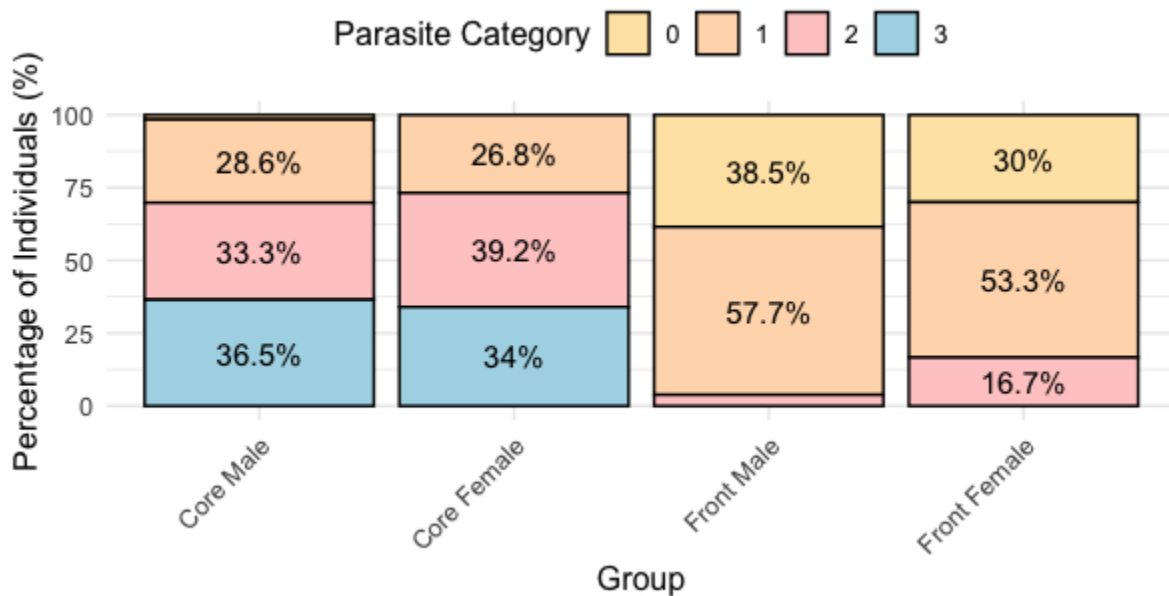


Figure 7. Percentage distribution of parasite categories by site (Core and Front) and sex (Female and Male) of signal crayfish (*Pacifastacus leniusculus*). The bars represent the percentage of individuals in each parasite category (0, 1, 2, and 3).

Discussion

This study investigates differences in elements bioaccumulation in the signal crayfish (*Pacifastacus leniusculus*) along an invasion gradient and how these differences may be linked to factors such as diet, behaviour, and parasitic load. We assume, based on recent studies (Alves et al., in press; Carvalho et al., 2025; Sousa et al., 2015, 2018, 2019, 2020; Nogueira et al., 2021a,b), that the environmental conditions in the two sites are very similar, with the Rabaçal River being less disturbed by human actions, so their contribution to the possible differences is minimal. We observed that individuals at the invasion front exhibit higher concentrations of certain potentially toxic, technology-critical, and essential elements, which may be associated with their higher trophic position, more exploratory behaviour, and lower parasite load. Conversely, individuals from the invasion core tend to accumulate elements such as lead and strontium, potentially due to prolonged exposure and a diet based on benthic food sources such as plant detritus. These findings are particularly relevant for understanding how invasive species influence contaminant transfer within ecosystems.

Elemental accumulation

Crustaceans can accumulate elements through bioaccumulation via water absorption, sediment uptake, and food ingestion (Nędzarek et al., 2020; Li et al., 2023). Due to their omnivorous diet, the signal crayfish is particularly susceptible to accumulating toxic elements, which can affect growth and development (Li et al., 2023). Elemental analysis indicates a higher tendency for crayfish at the invasion front to accumulate more elevated concentrations of essential elements such as cobalt, phosphorus, vanadium, and manganese, as well as non-essential elements

including aluminium, mercury, platinum, and tantalum. The higher nitrogen values observed in individuals at the invasion front may suggest a biomagnification process, indicating that signal crayfish could act as vectors for increased exposure to potentially harmful elements, such as mercury and tantalum, for their predators in invaded sites, such as the Eurasian otter *Lutra lutra* and brown trout *Salmo trutta* (Espejo et al., 2018; Rodrigues et al., 2019).

Conversely, elements such as calcium (an essential element for crustaceans), lead, strontium, and gallium (non-essential elements) exhibited higher concentrations in core individuals. These differences in elemental concentrations in the muscle tissue of individuals from the core and the front of the invasion gradient can likely be attributed to variations in diet between the two sites (see further discussion below). Some elements, such as technology-critical elements (TCEs), pose a threat due to their high toxicity, long environmental persistence, and potential for bioaccumulation and biomagnification in the food chain (Mistri et al., 2020). TCEs, including tantalum and platinum, showed higher concentrations in individuals from the invasion front. These elements are increasingly used in advanced technological applications, such as electronics, solar panels, and medical devices, raising concerns about their potential negative ecological impacts (Balaram, 2019; Gwenzi et al., 2018). Tantalum, for example, may be linked to biomagnification processes, as this element has been reported to accumulate in aquatic food webs (Espejo et al., 2018). The presence of platinum, commonly associated with catalytic converters and industrial emissions, suggests possible contamination from atmospheric deposition or local runoff sources (Picone et al., 2022). The observed patterns reinforce the idea that invasive species such as the signal crayfish can serve as sentinels of emerging contaminants, highlighting the need for further research on the ecological and toxicological implications of TCE accumulation in freshwater ecosystems.

When compared with other studies (Table 2), it was found that the concentrations of lead, mercury and arsenic appear to fall within values found in the literature, while the concentration of copper is higher than that obtained in other studies with crayfish. The concentration of cadmium is similar to that obtained in the study by Rowe et al. (2001) but higher than the values obtained in the other studies.

Table 2. Elemental concentration ($\mu\text{g}/\text{kg}$, dry weight) in studies with crayfish species. Rowe et al. (2001) analysed *Procambarus acutus*; Suárez et al. (2010), Bellante et al. (2015), Gedik et al. (2017) and Zhang et al. (2023) used the species *Procambarus clarkii*.

	($\mu\text{g}/\text{kg}$, dry weight)	Pb	Hg	Cu	Cd	As
<i>P. leniusculus</i>	This study	58-547	1647- 2858	63478- 81726	150- 211	773- 1203
<i>P. acutus</i>	Rowe et al. (2001)	-	-	46990	290	950
<i>P. clarkii</i>	Suárez et al. (2010)	410-4200	220- 3100	12000- 82300	-	-
<i>P. clarkii</i>	Bellante et al. (2015)	180	-	17300	10	1790
<i>P. clarkii</i>	Gedik et al. (2017)	2440- 4490	-	23900- 34300	60	150- 3670
<i>P. clarkii</i>	Zhang et al. (2023)	139	500	16000	5	700

There appear to be some differences in some of the elements studied concerning sex, such as B and Sr. For example, in the study by Nędzarek (2020), for the elements Se, Ni, Cd and Pb

there were significant differences in the concentrations present in females and males. A possible explanation is that female crayfish release eggs, which could serve as a means of excreting certain elements, leading to different accumulation patterns compared to males (Güner, 2010). However, this remains speculative and only further studies can shed some light on this topic.

A positive correlation of the elements with $\delta^{15}\text{N}$ indicates that there is an increase in the concentrations of the element with increasing position in the trophic chain, which may suggest biomagnification processes, as higher trophic levels accumulate more of these elements through dietary exposure (Dung et al., 2023). Conversely, a negative correlation indicates that there is a higher concentration of the element as it moves down the trophic chain, which could be indicative of biodilution, where lower trophic levels accumulate more of the element from the environment (Dung et al., 2023). The results showed a strong positive correlation with the elements Si, Zr, Ta, Ti, Hf, Ge, Sr, Fe, Nd, Pr, Ce, La, Dy, Gd, Sm, Cd and Al, which means that along the trophic chain, species that are higher up the chain, such as in the case of the Eurasian otter or brown trout may be more exposed to a higher concentration than species lower down the food chain (Esposito et al., 2020). On the other hand, species at the top of the chain will accumulate a lower concentration of Sr, as this element was negatively correlated, and in this case, the process of biodilution occurs (Dung et al., 2023).

Possible mechanisms explaining different elemental accumulation along the invasion gradient

The signal crayfish's omnivorous diet reflects high dietary plasticity, enabling them to consume a wide variety of food sources such as fish, invertebrates, aquatic plants, and detritus (Jackson & Britton, 2014; Olsson et al., 2009). Food availability influences their trophic position,

and as Alves et al. (in press) observed using the SIBER model, individuals at the invasion front in the Rabaçal River occupy a different trophic niche, benefiting from the higher availability of higher trophic-level prey, such as macroinvertebrates, compared to core individuals who rely more on plants due to increased competition and resource depletion given their higher abundance in this site (Galib et al., 2022). It is known that individuals who feed on what is at the bottom of the water column (e.g. algae, organic matter such as leaf litter) may have higher C^{13} values when compared to individuals feeding on organisms in the water column (e.g. macroinvertebrates and fish) (Pacioglu et al., 2019). These results corroborate our findings, indicating that the individuals in the core opt for a more benthic diet than those in the front and tend to accumulate higher concentrations of elements like lead and strontium, which are associated with lower trophic levels (Pacioglu et al., 2019).

Many organisms rapidly adapt their behavioural traits to expand their range and make risk-related decisions, driven by environmental challenges (Yagound et al., 2022; Biro & Stamps, 2008). In signal crayfish, the behavioural adaptations observed at the invasion front, including higher aggression and risk-taking, likely interact with trophic ecology and trace element accumulation. Studies by Groen et al. (2012) and Myles et al. (2015) support this, showing that front individuals take more risks and move faster, as observed in our trials where they were the first to reach the food and dominate the space. This greater exploration and aggression, combined with their elevated $\delta^{15}N$ values likely contribute to higher bioaccumulation of elements prone to biomagnification, such as cobalt, mercury, and manganese, in front of individuals (Kouba et al., 2010; Johnson et al., 2014). The reduced aggression observed in core individuals during behavioural tests further supports the hypothesis that competition may constrain their ability to exploit higher trophic levels, influencing elemental accumulation (Galib et al., 2022); however,

some studies show the opposite. For example, in the study by Hudina et al. (2015), where they observed that the signal crayfish (*Pacifastacus leniusculus*) in a Croatian population had higher rates of aggression in the core, even though they had better physical conditions. One possible explanation for this difference is that aggression in the core may not necessarily provide an immediate dispersal advantage but could instead help maintain dominance in established populations where competition is intense. These variations highlight the importance of studying behavioural traits at the population level rather than assuming uniform patterns across the species as a whole (Sousa et al., 2024). The dynamics of invasion can be influenced by multiple ecological factors, including resource availability, predation pressure, and habitat structure, which may shape different aggression strategies depending on the specific environmental context (Haubrock et al., 2024; Sousa et al., 2024). Understanding these behavioural differences is essential for predicting invasion success and developing targeted management strategies.

These findings emphasize the importance of considering the interplay between behaviour and trophic ecology in understanding contaminant dynamics along invasion gradients. Behavioural syndromes, such as increased boldness and aggression at the front, not only facilitate dispersal but also drive dietary and ecological shifts that influence exposure to trace elements. Anyway, it should be noted that this behavioural study was carried out in a laboratory and for this reason the results may not represent reality due to the lack of influence from natural river processes and biotic relationships, so further studies on this topic are essential. Intraspecific variations in behaviour are important for the species' ecological and evolutionary processes of the species and are an important factor in its stability, resilience and persistence (Gruber et al, 2018).

Interestingly, parasitic load appears to interact with these behavioural traits, as individuals in the core exhibited higher parasitic loads, which may further limit their ecological flexibility and

overall fitness (DeWitt et al., 2013). Additionally, many studies show that parasitized animals tend to eat less, which can also affect their overall performance. Sargent et al. (2014) demonstrated that a trematode parasite alters growth, feeding behaviour, and demographic success in invasive rusty crayfish (*Orconectes rusticus*), further linking parasitism with changes in feeding behaviour and fitness.

The abundance of these parasites may also be linked to host density, as higher host densities can lead to increased parasite transmission and proliferation, resulting in a direct correlation between higher parasite levels and greater host density (DeWitt et al., 2013). However, this relationship can be more complex, as in some cases, a higher host density can lead to a dilution effect, where the parasitic load is distributed among more individuals, reducing the pressure on any specific host and consequently decreasing the prevalence of parasitism. Creed et al. (2022) highlight this dilution effect in the context of mutualisms in freshwater environments, noting that the introduction of host species can affect parasite abundance in native species, showing that both accumulation and dilution of parasites are possible, depending on the ecological conditions. The signal crayfish is host to several parasites, including *Branchiobdella astaci*, an annelid commonly known to parasitise crayfish (Rosewarne et al., 2012). This species mainly parasitise the gills of crayfish, affecting the growth, survival and health of crayfish by consuming tissues and blood and damaging these organs, sometimes leading to a higher mortality rate (Rosewood et al., 2012; DeWitt et al., 2013; James et al., 2015; Subchev et al., 2020). In addition, they are easily found in their cheliped, where they attach their cocoons and can compromise the crayfish's physical and physiological activities (Olifirenko et al., 2023). Parasites, such as *Branchiobdella astaci*, may impair the crayfish's ability to acquire nutrients and metabolize effectively, with the higher parasitic load at the core acting as an additional stressor that limits the individuals' ability to exploit

higher trophic levels, impacting their metabolic efficiency and elemental bioaccumulation (DeWitt et al., 2013). Parasites can influence host physiology, behaviour and metabolism, potentially contributing to the observed differences in elemental concentrations, as seen in the higher levels of lead and strontium in core individuals, which may be linked to altered feeding behaviour or reduced metabolic efficiency due to parasitic stress (Overstreet, 1997).

Overall, the interplay between elemental accumulation and ecological traits such as diet (trophic position), behaviour and parasitism highlights the complexity of invasion dynamics. Reduced parasitism at the invasion front may confer an advantage, allowing individuals to allocate more energy to behaviours that enhance dispersal and resource acquisition. In contrast, higher parasitic loads at the core may constrain ecological flexibility, reinforcing the observed differences in diet and element accumulation between populations. Future studies should further explore these relationships to understand better how parasitism interacts with elemental accumulation in invasive species. This information may be crucial for better understanding the dynamics of invasive species and possible impacts in the bioaccumulation and biomagnification or biodilution of contaminants and should be considered when designing management strategies.

Management implications

Management strategies could consider approaches to limit the transfer of contaminants to higher trophic levels, such as predators like the Eurasian otter, brown trout, and other species, which are exposed to bioaccumulated elements through their diet (Nędzarek et al., 2020). One possible approach is targeted removal programs, prioritizing population control at invasion fronts where individuals have higher concentrations of toxic elements, such as Hg, thereby reducing their availability to predators (Ficetola et al., 2012; Johnson et al., 2014). These removal programs may

involve public awareness (Gherardi et al., 2011). However, to make these strategies more effective, it is crucial to incorporate an understanding of individual and population-level traits, such as trophic ecology, behavior, and parasitic load, as these factors can influence contaminant accumulation and dispersal dynamics (Sousa et al., 2024). These aspects are often neglected in management studies, yet they may play a key role in determining the success of mitigation strategies (Haubrock et al., 2024; Sousa et al., 2024). Finally, long-term contaminant monitoring is essential to track bioaccumulation trends across different invasion stages, allowing for adaptive management strategies that respond to changes in environmental conditions and invasion dynamics (Nędzarek et al., 2020).

Conclusion

This study provides a detailed analysis of differential element accumulation in the signal crayfish (*Pacifastacus leniusculus*) along an invasion gradient in the Rabaçal River. Our findings reveal significant intra-population variations in elemental concentrations, likely driven by dietary differences, behaviour, and degree of parasitism associated with the invasion front and core.

Signal crayfish from the invasion front exhibited higher concentrations of certain essential and potentially toxic elements, such as cobalt, vanadium, manganese, mercury, and tantalum, correlating with a diet richer in macroinvertebrates and higher metabolic demands. Conversely, individuals from the core accumulated higher levels of elements like lead and strontium, potentially reflecting longer exposure and a diet more reliant on plant detritus. The strong positive correlation between $\delta^{15}\text{N}$ and several elements highlights the role of trophic positioning in shaping contaminant bioaccumulation.

Behavioural tests confirmed that individuals at the invasion front exhibited greater aggression, traits that may facilitate range expansion and resource acquisition. Additionally, the reduced parasitic load observed in front populations underscores the potential adaptive benefits of lower host densities and novel environments leading to higher consumption and potentially higher accumulation of certain elements.

These findings underscore the complex interactions between invasion dynamics, environmental contaminants, and ecological traits. By combining elemental analysis, stable isotope data, and behavioural observations, this study contributes to a better understanding of the ecology of a highly invasive species with potential payoffs on their future management.

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