Peer review history for:

Connell LJ, Beine K, Greenfield R. Cadmium bioaccumulation in two resident limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline. S Afr J Sci. 2025;121(11/12), Art. #20359. https://doi.org/10.17159/sajs.2025/20359

HOW TO CITE:
Cadmium bioaccumulation in two resident limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline [peer review history]. S Afr J Sci. 2025;121(11/12), Art. #20359. https://doi.org/10.17159/sajs.2025/20359/peerreview

---

Reviewer 1: Round 1
Date completed: 03 March 2025
Recommendation: Accept / **Revisions required** / Resubmit for review / Resubmit elsewhere / Decline / See comments
Conflicts of interest: None

Does the manuscript fall within the scope of SAJS?
Yes/No
Is the manuscript written in a style suitable for a non-specialist and is it of wider interest than to specialists alone?
Yes/No
Does the manuscript contain sufficient novel and significant information to justify publication?
Yes/No
Do the Title and Abstract clearly and accurately reflect the content of the manuscript?
Yes/No
Is the research problem significant and concisely stated?
Yes/No
Are the methods described comprehensively?
Yes/No
Is the statistical treatment appropriate?
Yes/No/Not applicable/Not qualified to judge
Are the interpretations and conclusions justified by the research results?
Yes/Partly/No
Please rate the manuscript on overall contribution to the field
Excellent/Good/Average/Below average/Poor
Please rate the manuscript on language, grammar and tone
Excellent/Good/**Average**/Below average/Poor
Is the manuscript succinct and free of repetition and redundancies?
Yes/No
Are the results and discussion confined to relevance to the objective(s)?
Yes/No
The number of tables in the manuscript is
Too few/**Adequate**/Too many/Not applicable
The number of figures in the manuscript is
Too few/Adequate/Too many/Not applicable
Is the supplementary material relevant and separated appropriately from the main document?

Yes/No/Not applicable

Please rate the manuscript on overall quality

Excellent/**Good**/Average/Below average/Poor

Is appropriate and adequate reference made to other work in the field?

Yes/No

Is it stated that ethical approval was granted by an institutional ethics committee for studies involving human subjects and non-human vertebrates?

Yes/No/Not applicable

If accepted, would you recommend that the article receives priority publication?

Yes/No

Are you willing to review a revision of this manuscript?

Yes/No

With regard to our policy on 'Publishing peer review reports', do you give us permission to publish your anonymised peer review report alongside the authors' response, as a supplementary file to the published article? Publication is voluntary and only with permission from both yourself and the author.

Yes/No

Comments to the Author:

The manuscript "Field study of cadmium bioaccumulation in two residential limpet species, Scutellastra granularis and Siphonaria capensis, along the South African coastline" is an interesting study and relevant as cadmium is toxic in low concentrations and a cause for concern with n increase in pollutants in the marine environment.

The manuscript was well written, exact and the results and discussion was focused. This said, I have a few comments and suggestions for consideration:

1) In the title it was focused on the two species names, however throughout the manuscript it is mostly referred to the genus names. I know it is difficult as both genus names start with S and the authors want to indicate and focus on the differences. Thus, I would suggest to keep it the same throughout the manuscript, either refer to S. granularis and S. capensis or change the focus to genus names if the accumulation is not species specific.
2) In the M&M section, it would be good to add a map to indicate the three sites, Marine Protected Areas, rivers that drain into the ocean as mentioned that freshwater runoff can be Cd source and other sources of Cd under land-uses
3) The statistical analyses are sound and sufficient to analyse the results, however I would suggest to add a redundancy analysis (RDA) to assess the spatial variation and natural variation in species at the sampling sites, while also assessing the associations of accumulated Cd concentrations with environmental factors, as well as biometric factors. This multivariate statistical analysis might support the discussion even further.

Other small comments is attached in the Word document.

[See Appendix 1 for Reviewer 1's comments made directly on the manuscript]

Author response to Reviewer 1: Round 1

Page 1, Line 9 – *S. granularis* and *S. capensis*

AUTHOR: *Scutellastra* and *Siphonaria* changed to *S. granularis* and *S. capensis* respectively.

Page 1, Line 10 – "l"

AUTHOR: "l" changed to "L"

Page 2, Line 39 – "due…"

AUTHOR: "due" changed to "due to"

Page 2, Line 40 – "…bioaccumulation…"

AUTHOR: "…bioaccumulation…" changed to ", and bioaccumulation ability"

Page 2, Line 63 – "metals"

AUTHOR: “metals” changed to “metal”

Page 2, Line 66 – “…coasts.”

AUTHOR: “…coasts” changed to “…coasts of South Africa.”

Page 3, Line 78 – Consistent use of indicator species, either refer to genus or *S. granularis* and *S. capensis*

AUTHOR: Both “*Scutellastra*” and “*Siphonaria*” have been cha “*S. granularis*” and “*S. capensis*”. This has been applied through the manuscript.

Page 3, Line 97 – Table 1?

AUTHOR: In this sentence, the authors talk about how organisms of similar size were collected for this study. This is why Table S3 was referenced here as it has the size and weights of the organisms – Table 1 on the other hand has the in-situ parameters for each site and therefore doesn’t apply to this sentence.

Page 3, Line 101 – were selected.

AUTHOR: “…individuals.” changed to “…individuals were selected.”

Page 3, Line 107 – Map

AUTHOR: A map has been made and included in the manuscript

Page 4, Line 127 – South Africa).

AUTHOR: Closing bracket has been included

Page 4, Line 127 – Please provide details on the stock solution

AUTHOR: A 100ppm stock solution of Cd was made up (100mg L^{-1}). This has been added to the manuscript.

Page 5, Line 158 – Tests, respectively

AUTHOR: A comma has been inserted between “tests” and “respectively”

Page 5, Line 160 – Cd

AUTHOR: cadmium has been changed to Cd

Page 7, Line 201 – Add p values on graph

AUTHOR: The p values have been added to the graph

Page 9, Line 245 - Y axis label change from bioaccumulation factor to bioconcentration factor

AUTHOR: The label has been changed from “bioaccumulation factor” to “bioconcentration factor”

Page 13, Line 354 - Maybe something to highlight is not only exposure concentration but exposure duration as larger individuals accumulated higher concentrations. Might show better in RDA

AUTHOR: This has been included in the concluding paragraph, highlighting exposure duration. Larger organisms are also shown to accumulate more Cd – as shown in the RDA included in the manuscript.

Reviewer 2: Round 1

Date completed: 18 February 2025

Recommendation: Accept / **Revisions required** / Resubmit for review / Resubmit elsewhere / Decline / See comments

Conflicts of interest: None

Does the manuscript fall within the scope of SAJS?

Yes/No

Is the manuscript written in a style suitable for a non-specialist and is it of wider interest than to specialists alone?

Yes/No

Does the manuscript contain sufficient novel and significant information to justify publication?

Yes/No

Do the Title and Abstract clearly and accurately reflect the content of the manuscript?

Yes/No

Is the research problem significant and concisely stated?

Yes/No

Are the methods described comprehensively?

Yes/No

Is the statistical treatment appropriate?

Yes/No/Not applicable/Not qualified to judge

Are the interpretations and conclusions justified by the research results?

Yes/**Partly**/No

Please rate the manuscript on overall contribution to the field

Excellent/**Good**/Average/Below average/Poor

Please rate the manuscript on language, grammar and tone

Excellent/**Good**/Average/Below average/Poor

Is the manuscript succinct and free of repetition and redundancies?

Yes/No

Are the results and discussion confined to relevance to the objective(s)?

Yes/No

The number of tables in the manuscript is

Too few/Adequate/**Too many**/Not applicable

The number of figures in the manuscript is

Too few/**Adequate**/Too many/Not applicable

Is the supplementary material relevant and separated appropriately from the main document?

Yes/**No**/Not applicable

Please rate the manuscript on overall quality

Excellent/**Good**/Average/Below average/Poor

Is appropriate and adequate reference made to other work in the field?

Yes/No

Is it stated that ethical approval was granted by an institutional ethics committee for studies involving human subjects and non-human vertebrates?

Yes/No/**Not applicable**

If accepted, would you recommend that the article receives priority publication?

Yes/**No**

Are you willing to review a revision of this manuscript?

Yes/No

With regard to our policy on 'Publishing peer review reports', do you give us permission to publish your anonymised peer review report alongside the authors' response, as a supplementary file to the published article? Publication is voluntary and only with permission from both yourself and the author.

Yes/No

Comments to the Author:

See comments in attached manuscript.

[See Appendix 2 for Reviewer 2's comments made directly on the manuscript]

Author response to Reviewer 2: Round 1

Page 3, Line 95 - Useful to include a map indicating the position of the sites.

AUTHOR: A map has been included to indicate the sites for this study

Page 4, Lines 106-107 – See comments on the table

AUTHOR: Comments on the table have been addressed

Page 4, Line 111 – What containers were used.

AUTHOR: 50ml Falcon Centrifuge Tubes were used to collect and store the water. This has been elaborated on in the manuscript.

Page 4, Line 119 - Need to indicate how soft tissues were removed from shells BEFORE drying.

AUTHOR: Soft tissues were removed from the shells using a spatula after being flash frozen in liquid nitrogen. This has been elaborated on in the manuscript.

Page 5, Lines 138-139 - Need to indicate here what certified reference material was used to ensure analytical efficiency for Cd.

AUTHOR: The standard reference material used for this study has been included in the manuscript

Page 6, Line 178 - In marine environments the salinity is already being measured as PSU - thus EC and TDS measurements are not required. Recommend to remove.

AUTHOR: EC and TDS have both been removed from the manuscript.

Page 6, Line 189 - Since you know the species names these have to be provided. Thus change all genus names to genus and species as done here.

AUTHOR: Both the genus and species have been given for both organisms. This has been done throughout the manuscript.

Page 7, Line 215 - The one-way ANOVA results (water, limpits, BCFs) do not need to be presented in Table format since the significance is indicated on the graphs. Of greater interest would be the two-way ANOVA information - site and species interactions.

AUTHOR: The authors would like to acknowledge the reviewer's comment regarding a two-way ANOVA – which was discussed during the writing of this manuscript however it was decided against. A two-way ANOVA was intentionally not performed due to the vast difference between the two species – in the vertical distribution, and their physiology. The differences observed in a two-way ANOVA will be biased and incomparable due to the differences in the two limpet species.

Page 11, Line 277 - Use the provided abbreviations consistently.

AUTHOR: The provided abbreviations have been applied throughout the manuscript

Page 11, Line 281 - To what degree are these sources of Cd present in the near vicinity of the sampling sites?

AUTHOR: These sites are marine protected areas and so these sources of Cd are minimal in the vicinity of the sampling sites – therefore showing environmental levels of Cd, which is why the levels are very low.

Page 11, Line 287 – Perna perna

AUTHOR: "Perna perna" changed to "P. perna"

Reviewer 3: Round 1

Not openly accessible under our Publishing peer review reports policy.

Reviewer 1: Round 2

Date completed: 04 June 2025

Recommendation: Accept / Revisions required / Resubmit for review / Resubmit elsewhere / Decline / See comments

Conflicts of interest: None

Have the authors adequately addressed the concerns raised in the previous review?

Yes/No

Is the manuscript publishable in its current revised form?

Yes/No

If yes, do you recommend priority publication?

Yes/No/**Not applicable**

Yes/No

Any additional comments for the authors:

The authors addressed all the comments and suggestions raised by the reviewers to satisfaction, therefore I would recommend that the manuscript can be accepted in its revised form. I would like to congratulate the authors on an interesting article.

Reviewer 2: Round 2

Date completed: 23 June 2025

Recommendation: Accept / Revisions required / Resubmit for review / Resubmit elsewhere / Decline / See comments

Conflicts of interest: None

Have the authors adequately addressed the concerns raised in the previous review?

Yes/No

Is the manuscript publishable in its current revised form?

Yes/No

If yes, do you recommend priority publication?

Yes/**No**/Not applicable

Yes/No

Any additional comments for the authors:

I am satisfied that the authors have addressed all the comments that I made.

I do however feel that the ANOVA tables are not necessary and that the information of interest in the Tables, i.e. the F value can be worked into the results text. The significance (P values) are already indicated on the graphs. Since the other reviewers did not comment on this, I leave this up to the editorial decision related to number of Tables/Figures.

Associate Editor comments to Author: Round 2

1. Move the ANOVA tables to supplementary files. You can then report the key stats in the text
2. Also, check for grammar (I have attached some suggestions for improvement)

[See Appendix 3 for Associate Editor's comments made directly on the manuscript]

Author response to Associate Editor: Round 2

The authors would like to thank the editor for the comments. We have made all the editorial changes that have been requested. We also changed the title as requested and have removed all the ANOVA tables to the supplementary material.

Appendix 1: Reviewer 1's comments on original manuscript (Round 1)

Field study of cadmium bioaccumulation in two residential limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline

Abstract

The study investigates cadmium (Cd) bioaccumulation in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline from three sites; Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP). Significant spatial variations in Cd accumulation were observed, with the highest concentrations found in limpets from NNP (*Scutellastra* – 4.17 ± 1.99 µg g^{-1}, *Siphonaria* – 2.92 ± 1.45 µg g^{-1}) despite having the lowest water Cd levels (0.13 ± 0.07 µg l^{-1}). This suggests alternative Cd uptake pathways, such as dietary intake from contaminated algae or sediments. Additionally, larger limpets exhibited higher Cd concentrations, highlighting the importance of considering organism size in biomonitoring studies. The bioconcentration factor (BCF) analysis further indicated significant site-specific differences, with the highest BCF at NNP (*Scutellastra* – 32.59 ± 15.62, *Siphonaria* – 22.81 ± 11.29). These findings highlight the relationship between environmental exposure and physiological traits in Cd bioaccumulation and the necessity for site-specific assessments and using multiple bioindicator species in environmental monitoring. The study shows the importance of continued research into long-term and spatial variations in metal bioaccumulation in marine ecosystems.

Significance:

- Highlights Cd bioaccumulation in limpets indicating potential pollution pathways.
- Demonstrates *S. granularis* and *S. capensis* as bioindicator Sp. for Cd monitoring.
- Spatial significance stresses the importance of site-specific monitoring.
- Organism size matters in pollution assessments.
- There is no correlation between water and organism metal concentrations.

Keywords: Bioconcentration factor, bioindicator, ICP-MS, intertidal, marine pollution

Supplementary data inserted at the end of the document.

Introduction

Coastal regions across the world are subjected to significant metal pollution, resulting from agricultural and industrial activities.[1] With over 70% of South Africa's coastline inhabited, elevated

Commented [A1]: *S. granularis*

Commented [A2]: *S. capensis*

Commented [A3]: L

anthropogenic pressures on the nearshore marine environment are becoming more prevalent.[2] Metals naturally occur in the marine environment at low concentrations, however, due to increased anthropogenic pressures, coastal waters are becoming progressively vulnerable to a rise in metal pollution.[3] Metals pose a considerable threat to marine ecosystems due organism toxicity at low concentrations, environmental persistence, bioaccumulation ability.[4] Bioaccumulation refers to the process of organismal toxicant uptake, such as chemicals or metals, through consumption of contaminated food or direct exposure, be it over a short (acute) or long (chronic) periods.[4,5] The degree of metal bioaccumulation is influenced by factors such as the metal bioavailability, the route of uptake, and sequestration or depuration of the toxicant.[6] The greatest threat associated with metal bioaccumulation within the tissues of organisms is biomagnification in the higher trophic levels.[7,8]

Cadmium (Cd) primarily enters waterbodies through industrial sources, including zinc (Zn) smelting, electroplating, fossil fuel combustion, plastics, pesticides, and oil refining.[9] Research on Cd has intensified globally due to its toxicity at low concentrations, and its ability to biomagnify through trophic levels, impacting humans.[1,10-18] Cadmium accumulation occurs in organisms primarily via ingestion as the metal ions are poorly soluble in water, binding to suspended particulate matter (SPM) in the ocean, which is ingested and absorbed by filter feeding organisms.[4] Local studies have demonstrated that gastropods, such as limpets, accumulate metals and can serve as potential bioindicator species.[19,20] Despite the variety of bioindicator species used in South Africa, information on the responses of limpets to metals in the marine environment is limited.

A commonly used approach for environmental biomonitoring is quantifying pollutant loads in organisms that bioaccumulate.[21] Metal concentrations in tissues depends on the uptake, excretion, and metabolic capacity to either degrade and/or transform these toxic pollutants.[21] The use of a single bioindicator species when determining metal pollution is not ideal because certain metals concentrations may differ from one species to another.[22] Therefore, different species inhabiting different coastal zones need to be assessed. Recent studies include using the invasive Mediterranean mussel (*Mytilus galloprovincialis*) and endemic Brown mussel (*Perna perna*) along the southern and east coasts.[3,23-25] Limpets were selected for this study due to their wide distribution, feeding behaviour, being reasonably sessile, and distinct ability to tolerate environmental challenges, making them a key study organism when looking at the effect of environmental changes on intertidal organisms.[19,26] The limpets *Scutellastra granularis* (Linnaeus

Commented [A4]: Due to

Commented [A5]: , and bioaccumulation ability

Commented [A6]: metal

Commented [A7]: Of South Africa

1758), a prosobranch found high on the shore, and *Siphonaria capensis* (Quoy and Gaimard 1833), a pulmonate gastropod found in pools and on emergent mid-shore rocks[27,28], were selected to investigate the variation in Cd uptake and bioaccumulation across key sites along the South African coastline. These species of limpet were used to investigate Cd bioaccumulation due to their different phylogenies (i.e., pulmonate vs patellogastropod) and associated tidal heights along their sympatric distribution in South Africa.

This study describes the spatial patterns of Cd concentrations in South African coastal waters using *Scutellastra* and *Siphonaria* as quantitative bioindicators. It includes investigating the effect of weight and size on Cd bioaccumulation. Spatial patterns for each species are used to compare Cd concentrations in water and tissue samples with inferred bioconcentration factors, discussing these in terms of background (environmental) concentrations and their sources.

Commented [A8]: Consistent use of indicator species, either refer to genus or *S. granularis* and *S. capensis*

Materials and methods

Site selection and animal collection

The study took place at three marine protected areas along a gradient from the west to east along the South African coastline. The South African coastline is a dynamic environment surrounded by two major ocean currents bringing contrasting environmental conditions.[29] The west coast is a cool temperate ecoregion influenced by the Benguela Current that transports cold nutrient-rich water, whereas the southern coast is classified as warm temperate and influenced by the warm water Agulhas Current.[27-30] Sites selected included, two from the west coast; the Namaqua National Park (NNP – 30º51'31.8" S, 17º34'31.1" E) in the Northern Cape and the West Coast National Park (WCNP – 33º8'56.2" S, 17º59'57.9" E) in the Western Cape, and the Garden Route National Park (GRNP – 34º01'14.9" S, 23º52'26.7" E) in the Eastern Cape from the southern coast ecoregion of South Africa. Field fresh samples of similar size (total shell length) were collected from all three sites (Σn = 3 sites x 2 species x 10 individuals (replicates) = 60) in January 2020 (Supplementary data, Table S3). The number of individuals was determined by both the [anonymised] animal ethics committee (number anonymised) and the SANParks permitting office (permit number anonymised). Organisms were collected during low tide (at first daylight). Organisms were carefully removed from the rock surface using either a spatula or by hand and only undamaged individuals. Individuals were classified as undamaged when there was no visible damage done to the shell or foot. Field fresh samples collected at each site were flash frozen in liquid nitrogen (-196ºC) and stored at -20ºC for baseline metal analysis. Water samples (Σn = 3 sites x 3 water samples (replicates) at 50 ml each = 9) were collected from each site and stored

Commented [A9]: Table 1?

Commented [A10]: were selected.

at -20ºC for background metal analysis. During collection of water samples, physico-chemical parameters were recorded, including: temperature (ºC), pH, salinity (PSU), electrical conductivity (mS cm^{-1}), and total dissolved solids (ppt).

Commented [A11]: Map

Bioaccumulation analysis

Sample preparation and digestion

The water samples (50 ml), were filtered using a Millipore sintered glass filtration system (Merck, South Africa), through a gridded cellulose nitrate membrane filter (type 114, 0.45 µm). Samples were acidified to 1% with Suprapur® nitric acid – 63.01 g mol^{-1} (HNO_3) (Merck, South Africa).

Whole organism tissue was used for metal accumulation analysis in accordance with the methods outlined by Boss and Fredeen[31] and Gaines[32], and adapted from Erasmus et al.[29]. Frozen samples were weighed (± 0.01 g) to obtain a wet weight, after which the samples were freeze-dried (-50ºC at 0.61 mbar) for 48 hours using the Martin-Christ LDplus Freeze Dryer (Christ Freeze Dryers, Niedersachsen, Germany). The freeze-dried (whole organism) samples were weighed to obtain a final dry weight, in which the weight was normalised using the bioconcentration factor calculation, before being digested using the MARS 6 One Touch Technology Microwave (CEM, Charlotte, United States of America). Before digestion, the Teflon bombs used during processing were cleaned and dried in accordance with the cleaning method, using standard grade HNO_3 (Merck, South Africa) and hydrogen peroxide (H_2O_2) (Merck, South Africa). Standard reference material (SRM, Mussel Tissue – 2976, 109% recovery), as well as the freeze-dried samples, were individually added to each Teflon bomb along with 5 ml Suprapur® 65% HNO_3 (Merck, South Africa. The digestion process took a total of 70 minutes and was held at 200ºC, starting with a 15-minute warm-up period, followed by a 35-minute holding period and finally a 20-minute cool-down period. After digestion, the samples were allowed to cool, before being placed in 15 ml Falcon tubes. Samples were gravimetrically diluted using double distilled water (18.2 Ω) to a total volume of 10 ml (Millipore, United States of America).

Commented [A12]: South Africa).

ICP-MS Analysis

Water (diluted by factor of 10) and tissue samples were analysed using a PerkinElmer NexION® 300 series Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (PerkinElmer, Waltham, United States of America). The ICP-MS was calibrated using 0, 0.01, 0.1 and 1 ppb calibration

standards, made using the appropriate dilutions from a 100 ppm Cd stock solution. An internal standard (Iridium – 193) was added to each water and tissue sample before analysis for quality assurance, ensuring sufficient recovery and no drifting occurred during the analysis. Data were converted from counts per second (cps) to µg g^{-1} (tissue) and µg l^{-1} (water), standardising the sample mass using the following equations:

$$Cd\ IS\ Corrected = \frac{Cd\ 111\ (cps)}{Ir\ 193\ (cps)} \quad \text{Equation 1}$$

$$Unit\ Conversion\ (cps \rightarrow \mu g\ l^{-1}) = \frac{(Cd\ IS\ Corrected - Intercept)}{(Slope)} \quad \text{Equation 2}$$

$$Blank\ \&\ Dilution\ Correction = ((Unit\ Conversion - Blanked\ Average) * 10) \quad \text{Equation 3}$$

$$Unit\ Conversion\ (\mu g\ l^{-1} \rightarrow \mu g\ g^{-1}) = \frac{(Blank\ \&\ Dilution\ Correction * 0.01)}{(Sample\ Dry\ Weight)} \quad \text{Equation 4}$$

Bioconcentration factor (BCF)

The bioconcentration factor (BCF) was determined according to the method of Abel.[33] This method determined the ratio between the Cd concentration in organisms and water samples ($C_{Organism\ dry\ weight}/C_{Water}$), where the $C_{Organism\ dry\ weight}$ (µg g^{-1} dry weight) and C_{Water} (µg l^{-1}) are the fractions of the element within the organism and the environment (water). The median concentration values of the element present in each organism and the water were used to calculate the BCF.

Statistical analysis

Statistical analyses were conducted using RStudio (v. 4.0.3). All data met the assumptions of normality and homogeneity of variance, following Levene's and Shapiro-Wilk tests respectively.[34] Following testing of assumptions, one-way Analysis of Variances (ANOVAs) were performed on cadmium concentrations in water samples (factors site, three levels and fixed, significance set at $p < 0.05$), tissue samples (factors site, three levels and fixed, significance set at $p < 0.05$), and bioconcentration factor (factors site, three levels and fixed, significance set at $p < 0.05$) for each species respectively. Where there were significant factors, a pairwise comparison was performed using Tukey's HSD.[34] All statistical analyses were performed using the *car* and *tidyverse*

Commented [A13]: Please provide details on the stock solution

Commented [A14]: Tests, respectively

Commented [A15]: Cd

packages in RStudio.[35,36] Barplots (±SEM) were created using the *ggplot2* and *plyr* packages. in RStudio.[37,38]

Results

Environmental water quality parameters

Physico-chemical parameters; temperature (ºC), pH, salinity (PSU), electrical conductivity (mS cm^{-1}), and total dissolved solids (ppt) measured *in situ* are represented in Table 1. The measured parameters across the three sites showed great variation, between sampling periods at each site (high SD values) as well as among the sites themselves.

Table 1: Mean (±SD) water quality measures taken in situ at each sampling site (NNP – Namaqua National Park, WCNP – West Coast National Park, GRNP – Garden Route National Park) during each day of sampling (n=3). Common superscripts indicate statistically significant differences in water quality parameters across sites ($p<0.05$)

	NNP	WCNP	GRNP
Temperature (ºC)	**16.18 ± 2.01^{ab}**	**16.47 ± 0.06^{ac}**	**19.40 ± 0.17^{bc}**
pH	8.01 ± 0.10	7.06 ± 0.69	7.71 ± 0.31
Salinity (PSU)	33.17 ± 1.17	38.33 ± 2.52	35.33 ± 3.21
Electrical conductivity (mS cm^{-1})	60.77 ± 1.70	68.63 ± 4.53	66.50 ± 5.22
Total dissolved solids (ppt)	37.68 ± 1.05	42.67 ± 2.74	38.73 ± 3.06

Temperature showed significant variation across sites ($F_{(2,\ 8)}$ = 91.5, p = 3.2e^{-05}). Water temperature at GRNP was significantly higher than both west coast sites (NNP and WCNP, Table 1). Water temperature also varied significantly between the two west coast sites (NNP and WCNP), with significantly lower temperatures recorded at NNP. All other water quality parameters (pH, salinity, electrical conductivity, and total dissolved solids) showed statistically non-significant differences across the three sites ($p>0.05$, Table 1).

Cadmium concentrations versus weight and size

Cadmium concentrations in whole body tissues (without shell) were plotted against the wet weight (g) and size (mm) for both species of limpets, *Scutellastra* and *Siphonaria*; with results being illustrated in Figure 1A and B, respectively. Concentrations of Cd in whole body limpets showed no significant change with increasing weight of either species, $F_{(3,\ 56)}$ = 0.0482, p = 0.986 (Figure 1A). With increasing weight, both *Scutellastra* and *Siphonaria* had non-significant changes in their

individual Cd concentrations, with R^2 values of 0.001 and 0.002, respectively. In contrast, concentrations of Cd in whole body limpets showed a significant change with increasing size, $F_{(3, 56)} = 5.347$, p = 0.003 (Figure 1B). *Siphonaria* showed a greater increase in Cd concentrations with an increase in size ($R^2 = 0.26$), whereas the larger *Scutellastra* showed a similar increase in Cd concentrations with an increase in size ($R^2 = 0.20$).

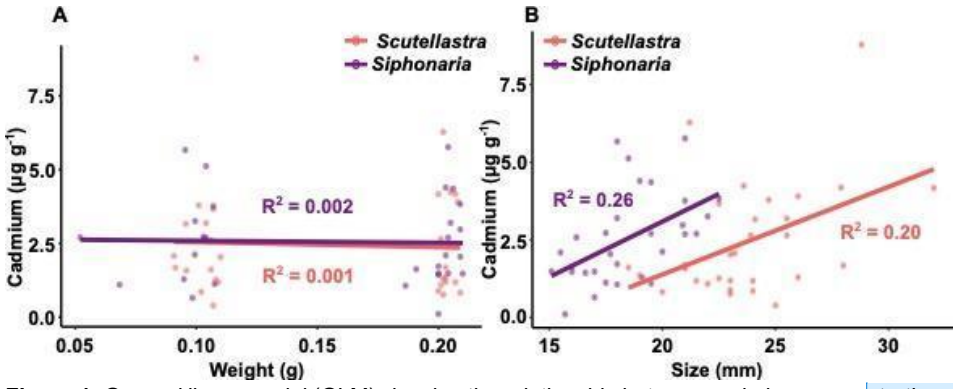

Figure 1: General linear model (GLM) showing the relationship between cadmium concentrations (µg g^{-1}) and the weight (A) and size (B) of *Scutellastra* and *Siphonaria*

Commented [A16]: Add p values on graph

Cadmium concentration in field-fresh water samples from the southern and west coasts

Cadmium concentrations within water samples collected at each respective site during the sampling of *Scutellastra* and *Siphonaria* are represented in Figure 2. No statistically significant differences in cadmium concentrations were present in water samples between the three sites (Figure 2, Table 2). Water samples from the southern coast (GRNP) had the greatest Cd concentrations (0.24 µg l^{-1}), whilst samples from the west coast at NNP had the lowest concentrations (0.13 µg l^{-1}).

Table 2: One-way ANOVA results comparing Cd levels in field-fresh water samples (n=3) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh water samples ($F_{(2, 6)} = 3.221$, p = 0.112)

	Df	Sum Sq	Mean Sq	*F*	p

Sites	2	0.0219	0.0110	1.8370	0.2386
Residuals	6	0.0358	0.0060		
Total	8	0.0578			

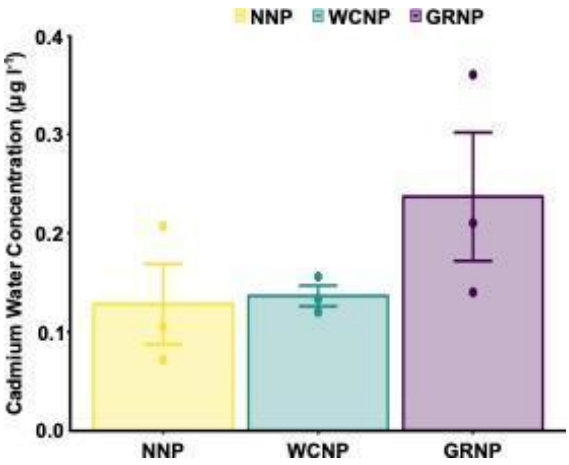

Figure 2: Mean (±SEM) levels of cadmium in water samples (n=3 per site) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP)

Cadmium concentrations in field-fresh Scutellastra and Siphonaria from the southern and west coasts

The measured concentrations of Cd in both *Scutellastra* and *Siphonaria* are presented in Table 3 and Figure 3A. Spatial differences in Cd concentrations from whole body *Scutellastra* tissue showed significance, $F_{(2, 29)}$ = 11.978, p = 0.0002 (Table 3). At NNP, *Scutellastra* accumulated significantly higher concentrations of Cd than both remaining sites, WCNP and GRNP (Figure 3A. A similar trend was observed in *Siphonaria*, with spatial variances in Cd concentrations across all three sites, $F_{(2, 29)}$ = 9.522, p = 0.0007 (Table 3). Limpets collected from NNP having significantly higher Cd concentrations (p<0.01) to those collected and analysed from the WCNP (Figure 3A). Dissimilarly, along the southern coast at GRNP, *Siphonaria* had the highest Cd concentrations out of the three sites, being significantly higher than WCNP (p<0.001).

Table 3: One-way ANOVA results comparing Cd levels in field-fresh *Scutellastra* and *Siphonaria* (n=10) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh *Scutellastra* ($F_{(2, 27)}$ = 2.260, p = 0.124) and *Siphonaria* ($F_{(2, 27)}$ = 1.961, p = 0.160)

Scutellastra					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**46.355**	**23.178**	**11.978**	**0.0002**
Residuals	27	52.244	1.935		
Total	29	98.599			

Siphonaria					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**25.640**	**12.820**	**9.522**	**0.0007**
Residuals	27	36.353	1.346		
Total	29	61.993			

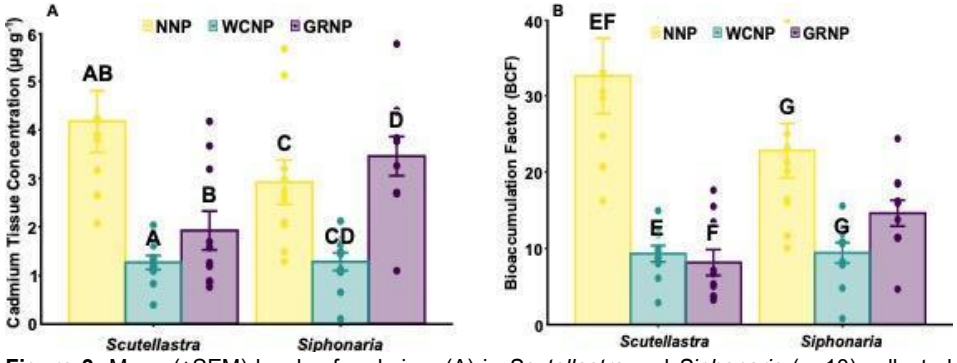

Figure 3: Mean (±SEM) levels of cadmium (A) in *Scutellastra* and *Siphonaria* (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP). Bioconcentration factor (B) variation in *Scutellastra* and *Siphonaria* (n=10) using cadmium concentrations in water and tissue samples collected from each site (NNP, WCNP, and GRNP)

Commented [A17]: Y axis label change from bioaccumulation factor to bioconcentration factor

Bioconcentration factor of cadmium in field-fresh Scutellastra and Siphonaria from the southern and west coasts

The results obtained show the bioconcentration factor (BCF) of Cd in both *Scutellastra* and *Siphonaria* and are presented in Table 4 and Figure 3B. Spatial differences in the BCF of Cd in *Scutellastra* showed significance, $F_{(2, 29)} = 20.120$, $p<0.001$. As seen in the Cd concentrations of *Scutellastra,* the BCF spatial variance showed the same pattern. *Scutellastra* from NNP showed the greatest BCF, differing significantly from both WCNP and GRNP ($p<0.001$, Figure 3B). No difference was observed between the BCF of *Scutellastra* at WCNP and GRNP ($p>0.05$). Unlike in the Cd concentrations observed in *Siphonaria* (Figure 3A), a dissimilar trend was observed in the BCF values across sites, although significantly different spatial differences were observed, $F_{(2, 29)} = 7.86$, $p = 0.002$ (Figure 3B, Table 4). *Siphonaria* from NNP exhibited the greatest BCF, differing significantly with limpets sampled from WCNP ($p<0.01$, Figure 3B). No other significant spatial differences were observed in *Siphonaria* between NNP and GRNP, nor between NNP and GRNP ($p>0.05$, Figure 3B).

Table 4: One-way ANOVA results comparing the Bioconcentration Factor (BCF) in field-fresh *Scutellastra* and *Siphonaria* (n=10) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed BCF variations in field-fresh *Scutellastra* ($F_{(2, 27)} = 3.259$, $p = 0.055$) and *Siphonaria* ($F_{(2, 27)} = 2.508$, $p = 0.100$)

Scutellastra					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**3805.382**	**1902.691**	**20.120**	**$4.472e^{-06}$**
Residuals	27	2553.365	94.569		
Total	29	6358.747			
Siphonaria					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**912.587**	**456.293**	**7.86**	**0.002**

Residuals	27	1566.557	58.021
Total	29	2479.144	

Discussion

While metal contaminants occur naturally in the marine environment at low concentrations, anthropogenic influence on the marine environment has exacerbated the situation.[39,40] The results from this study reveal significant bioaccumulation of cadmium (Cd) in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, collected from different coastal regions in South Africa. Comparing Cd concentrations in both limpet species to that found in water samples analysed indicates that Cd is strongly accumulated in both species from their respective surrounding environments. Potential sources of Cd in the marine environment come from the emissions of industrial combustion processes, metallurgic industries, road transport, and waste streams, all of which occur along the west and southern coasts of South Africa.[20,29,41,42] Water samples analysed for Cd showed variation between the southern coast site (GRNP) and the two west coast sites (NNP and WCNP). Studies conducted by Reinecke et al.[19,43] indicated Cd concentrations along the South African coastline varied considerably spatially and temporally. Degger et al.[40], measured unexpectedly high metal concentrations (Cd and Zn) in brown mussels (*Perna perna*) along the southern coast at Tsitsikamma National Park (within GRNP) compared to localities with known anthropogenic activities and influence (such as WCNP). High concentrations of metals in the study by Degger et al.[40] follow a similar pattern shown in data from the present study (both the water and organisms). This is indicative of long-term exposure, resulting from freshwater input through geogenic weathering and frequent upwelling that occur along South Africa's southern coast.[29] *Siphonaria* showed the greatest Cd accumulation at Namaqua National Park (NNP), with both species having the greatest metal uptake at this site despite lower Cd concentrations in the corresponding water samples. This suggests that these limpets are accumulating Cd from a different source, such as dietary intake from contaminated algae or sediments.[4,20] Additionally, they possess physiological traits that enhance Cd retention, such as slower excretion rates or higher binding affinities in tissue proteins.[21,44] This finding suggests similarity with previous studies, indicating bioaccumulation in marine organisms occuring through various pathways, including dietary intake and sediment interaction.[4,29]

The bioconcentration factor (BCF) indicated Cd had been accumulated in whole tissue samples of *Scutellastra* and *Siphonaria* in comparison to the concentration of Cd in the environment.[33] Whole tissue samples of both species varied significantly in their accumulation of Cd in relation to their sampling site. This however does not correlate with Cd concentrations from water sampled. Interestingly, despite higher water Cd levels at GRNP, the bioconcentration factor (BCF) was highest at NNP for *Scutellastra*, suggesting species-specific and site-specific bioaccumulation dynamics, seasonal and exposure level variation.[45] High Cd concentrations at GRNP, as discussed previously, are a direct result of both the freshwater input from geogenic weathering in the region and from upwelling events that are known to occur along the southern coast of South Africa at GRNP.[29,40] The significantly high BCFs at NNP are a direct result of the significantly high concentrations of Cd detected in whole tissue samples of *Scutellastra* and *Siphonaria*, despite the lower concentrations of Cd in the water. This result is indicative of either a short-term but high-dose exposure to Cd or a different means of uptake in both limpet species other than directly from the water column.[40,46] Elevated metal and pollutant levels along the west coast, observed at NNP and WCNP, are a direct result of the mining and port activities, as well as the iron and crude oil facilities in the surrounding areas, which lead to elevated pollution of local coastal waters affecting the inhabitants of these waters.[20,29,42]

The anatomy and physiology of the limpets is also known to play a primary role in the uptake and accumulation of metals. Differences in body sizes of individuals of the same species has been shown to result in varying metal tissue concentrations.[22,29,47,48] The size of marine ectotherms is related to their metabolic activities in relation to their life history stage.[8] Various metals such as copper (Cu), iron (Fe), and zinc (Zn) in moderate concentrations are essential in an organism's growth and development, however non-essential metals such as Cd lead to reduced growth and development, even at low concentrations.[8] Limpet size was positively correlated with tissue Cd concentration, indicating that larger individuals tend to accumulate more Cd, potentially due to prolonged exposure and greater overall uptake over time.[8,48] This size-related bioaccumulation trend has been observed in other marine invertebrates and highlights the importance of considering organism size in biomonitoring studies.[19,49]

The physiological and anatomical differences between *Scutellastra* and *Siphonaria* likely contribute to the observed differences in Cd bioaccumulation. *Scutellastra*, a prosobranch, and *Siphonaria*, a pulmonate gastropod, exhibit distinct respiratory and excretory mechanisms that influence metal uptake and storage.[44] Pulmonate gastropods, such as *Siphonaria*, exhibit higher

rates of metal accumulation due to their ability to absorb metals through their foot and mantle cavity, in addition to dietary intake.[21,44] Comparative studies have shown that bioaccumulation patterns in marine organisms can vary widely based on local environmental conditions and the species involved. Research on the Brown mussel (*Perna perna*) has demonstrated significant spatial variation in metal concentrations along the South African coastline, similar to the patterns observed in this study.[40] These findings underscore the importance of site-specific assessments and the use of multiple bioindicator species to accurately gauge environmental metal pollution levels.[20,21]

Conclusion

This study provides insight into the spatial patterns of Cd bioaccumulation in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline. The findings from both limpet species showed significant spatial variation in Cd accumulation, with *Scutellastra* having the highest Cd levels at NNP despite lower Cd concentrations in corresponding water samples. This suggests alternative pathways for Cd uptake, such as dietary intake from contaminated algae or sediments. Additionally, larger individuals from both species exhibited higher Cd concentrations, emphasising the importance of considering organism size in biomonitoring studies. The bioconcentration factor (BCF) analysis further indicated that the BCF of Cd in *Scutellastra* and *Siphonaria* was highest at NNP, highlighting the complex interplay between environmental exposure and physiological traits in Cd bioaccumulation. These findings emphasise the need for site-specific assessments and the use of multiple bioindicator species to effectively assess environmental metal pollution levels in marine ecosystems. Future research should focus on the long-term monitoring of these species across a greater seasonal and spatial gradient, and the exploration of additional environmental factors influencing metal bioaccumulation.

Commented [A18]: Maybe something to highlight is not only exposure concentration but exposure duration as larger individuals accumulated higher concentrations. Might show better in RDA

This work appears in whole or in part in a thesis/dissertation.

References

1. Boudjema K, Kourdali S, Bounakous N, Meknachi A, Badis A. Catalase activity in Brown Mussels (*Perna perna*) under acute cadmium, lead, and copper exposure and depuration tests. J Mar Biol. 2014:1–9. https://doi.org/10.1155/2014/830657
2. Sink K, Holness S, Harris L, Majiedt P, Atkinson L, Robinson T, Kirkman S, Hutchings L, Leslie R, Lamberth S, Kerwath S, Von der Heyden S, Lombard A, Attwood C, Branch G, Fairweather T, Taljaard S, Weerts S, Cowley P, Awad A, Halpern B, Grantham H, Wolf T. National Biodiversity Assessment 2011: technical report. In: Marine and Coastal Component, 4. South African National Biodiversity Institute; 2012. p. 325.
3. Greenfield R, Brink K, Degger N, Wepener V. The usefulness of transplantation studies in monitoring of metals in the marine environment: South African experience. Mar Pollut Bull. 2014;85:566–573. https://doi.org/10.1016/j.marpolbul.2014.03.032
4. Jakimska A, Konieczka P, Skóra K, Namieśnik J. Bioaccumulation of metals in tissues of marine animals, Part I: The role and impact of heavy metals on organisms. Pol J Environ Stud. 2011a; 20:1117–1125.
5. Perera PACT, Kodithuwakku SP, Sundarabarathy TT, Edirisinghe U. Bioaccumulation of cadmium in freshwater fish: An environmental perspective. Insight Ecology. 2015;4:1–12. https://doi.org/10.5567/ECOLOGY-IK.2015.1.12
6. Valavanidis A, Vlachogianni T. Metal pollution in ecosystems: ecotoxicology studies and risk assessment in the marine environment. 2010.
7. Fang T-H, Hwang J-S, Hsiao S-H, Chen H-Y. Trace metals in seawater and copepods in the ocean outfall area off the northern Taiwan coast. Mar Environ Res. 2006;61:224–243. https://doi.org/10.1016/j.marenvres.2005.10.002
8. Jakimska A, Konieczka P, Skóra K, Namieśnik J. Bioaccumulation of metals in tissues of marine animals, part II: Metal concentrations in animal tissues. Pol J Environ Stud. 2011b;20:1127–1146.
9. Chandrudu M, Radhakrishnaiah K. A study on the toxicity of cadmium on certain aspects of protein metabolism of the freshwater mussel *Lamellidens marginalis* (Lamarck) and freshwater fish *Labeo rohita* (Hamilton). Int J Environ Sci. 2013;4:15–27. https://doi.org/10.6088/ijes.2013040100003
10. CCME (Canadian Council of Ministers of the Environment). Canadian water quality guidelines for the protection of aquatic life: Cadmium. Winnipeg. 2014.
11. Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Nair AR, Munters E, Artois TJ, Nawrot T, Vangronsveld J, Smeets K. Cadmium stress: An oxidative challenge. BioMetals. 2010;23:927–940. https://doi.org/10.1007/s10534-010-9329-x
12. Environmental Protection Agency (EPA). Fact sheet: Aquatic life ambient water quality criteria update for cadmium. 2016.
13. Legeay A, Achard-Joris M, Baudrimont M, Massabuau J-C, Bourdineaud J-P. Impact of cadmium contamination and oxygenation levels on biochemical responses in the Asiatic clam *Corbicula fluminea*. Aquat Toxicol. 2005;74:242–253. https://doi.org/10.1016/j.aquatox.2005.05.015
14. Neff JM. 2002. Cadmium in the ocean, in: Bioaccumulation in Marine Organisms. Elsevier pp. 89–102. https://doi.org/10.1016/B978-008043716-3/50006-3

15. Phillips DJH. The Common Mussel *Mytilus edulis* as an Indicator of Pollution by Zinc, Cadmium, Lead and Copper. I. Effects of Environmental Variables on Uptake of Metals. Mar Biol. 1976;38:59–69. https://doi.org/10.1007/bf00391486
16. Ray S. Bioaccumulation of cadmium in marine organisms. Experientia. 1984;40:14–23. https://doi.org/10.1007/bf01959097
17. Scholz N. Accumulation, loss and molecular distribution of cadmium in *Mytilus edulis*. Helgoländer Meeresunters. 1980;33:68–78. https://doi.org/10.1007/bf02414736
18. Yamamura M, Suzuki KT, Hatakeyama S, Kubota K. Tolerance to cadmium and cadmium-binding proteins induced in the midge larvae, *Chironomus yoshimatsui* (Diptera, Chironimidae). Comp Biochem Physiol C Toxicol Pharmacol. 1983;75:21–24. https://doi.org/10.1016/0742-8413(83)90005-1
19. Reinecke AJ, Mdzeke NP, Reinecke SA. Spatial and temporal variation in cadmium body loads of four intertidal invertebrates from False Bay, South Africa. Afr Zool. 2012;47:12–25. https://doi.org/10.1080/15627020.2012.11407518
20. Erasmus A, Ikenaka Y, Nakayama SMM, Ishizuka M, Smit NJ, Wepener V. Trophic transfer of pollutants within two intertidal rocky shore ecosystems in different biogeographic regions of South Africa. Mar Pollut Bull. 2020;157:article 111309. https://doi.org/10.1016/j.marpolbul.2020.111309
21. Reguera P, Couceiro L, Fernández N. A review of the empirical literature on the use of limpets *Patella* spp. (Mollusca: Gastropoda) as bioindicators of environmental quality. Ecotoxicol Environ Saf. 2018;148:593-600. https://doi.org/10.1016/j.ecoenv.2017.11.004
22. Mbandzi N, Nakin MDV, Saibu GM, Oyedeji AO. Heavy metal profiles in limpets and algae on the Eastern Cape coast of South Africa. Afr J Mar Sci. 2021;43:293–308. https://doi.org/10.2989/1814232X.2021.1948918
23. Sparks C, Odendaal J, Snyman R. An analysis of historical Mussel Watch Programme data from the west coast of the Cape Peninsula, Cape Town. Mar Pollut Bull. 2014;87:374–380. https://doi.org/10.1016/j.marpolbul.2014.07.047
24. Greenfield R, Wepener V, Degger N, Brink K. Richards Bay Harbour: metal exposure monitoring over the last 34 years. Mar Pollut Bull. 2011;62:1926–1931. https://doi.org/10.1016/j.marpolbul.2011.04.026
25. Wepener V, Degger N. Monitoring of metals in South African harbours using resident brown mussels (*Perna perna*) as indicator organisms. Afr Zool. 2020;55:267–277. https://doi.org/10.1080/15627020.2020.1799720
26. Kankondi SL, McQuaid CD, Tagliarolo M. Influence of respiratory mode on the thermal tolerance of intertidal limpets. PLoS One. 2018;13:1–15. https://doi.org/10.1371/journal.pone.0203555
27. Branch G, Griffiths C, Branch M, Beckley L.. Two oceans: A guide to the marine life of southern Africa, 4th Edition. Cape Town: Struik Nature; 2016.
28. Branch G, Branch M. Living Shores: Interacting with southern Africa's marine ecosystems. Cape Town: Struik Nature. 2018.
29. Erasmus A, Smit NJ, Zimmermann S, Nachev M, Sures B, Wepener V. Metal and metalloid concentrations in the southern African endemic inter- and infratidal super klipfish, *Clinus superciliosus*, from the west and south coasts of South Africa. Mar Pollut Bull. 2021;172:112852. https://doi.org/10.1016/j.marpolbul.2021.112852

30. Bustamante RH, Branch GM, Eekhout S, Robertson B, Zoutendyk P, Schleyer M, Dye A, Hanekom N, Keats D, Jurd M, McQuaid C. Gradients of intertidal primary productivity around the coast of South Africa and their relationships with consumer biomass. Oecologia. 1995;102:189–201. https://doi.org/10.1007/BF00333251
31. Boss CB, Fredeen KJ. Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectroscopy. 3rd Edition. Connecticut: PerkinElmer; 2004.
32. Gaines PR. ICP Operations Guide: A Guide for using ICP-OES and ICP-MS. Inorganic Ventures; 2011.
33. Abel PD. Pollutant toxicity to aquatic animals - methods of study and their applications. Rev Environ Health. 1989;8. https://doi.org/10.1515/reveh-1989-1-407
34. Field A, Miles J, Field Z. Discovering statistics using R. London: SAGE Publications Ltd; 2012. https://doi.org/10.1111/insr.12011_21
35. Fox J, Weisberg S. 2019. An R Companion to Applied Regression, Third edition. California: Sage; 2019.
36. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H. Welcome to the tidyverse. J Open Source Softw. 2019;4(43):1686. https://doi.org/10.21105/joss.01686
37. Wickham H. The Split-Apply-Combine Strategy for Data Analysis. J Stat Softw. 2011;40(1):1–29. https://doi.org/10.18637/jss.v040.i01
38. Wickham H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
39. Ansari TM, Marr IL, Tariq N. Heavy metals in marine pollution perspective – A mini review. J Appl Sci. 2004;4:1-20. https://doi/org/10.3923/jas.2004.1.20
40. Degger N, Wepener V, Richardson BJ, Wu RSS. Application of artificial mussels (AMs) under South African marine conditions: A validation study. Mar Pollut Bull. 2011;63: 108–118. https://doi.org/10.1016/j.marpolbul.2011.04.040
41. OSPAR (Oslo and Paris Conventions). 2010. Quality Status Report 2010: Status and Trend of marine chemical pollution.
42. Clark B, Massie V, Hutchings K, Brown E, Biccard A, Laird M, Harmer R, Makhosonke A, Wright A, Turpie J. 2017. The state of Saldanha Bay and Langebaan Lagoon 2017, technical report.
43. Reinecke AJ, Schoeman W, Reinecke SA, Reinecke S, Schoeman W, Reinecke K. Cellular biomarker responses of limpets (Mollusca) as measure of sensitivity to cadmium contamination. Suid-Afrikaanse Tydskrif vir Natuur-wetenskap en Tegnologie. 2008;27:123–142. https://doi.org/10.4102/satnt.v27i2.86
44. Marchán S, Davies MS, Fleming S, Jones HD. Effects of copper and zinc on the heart rate of the limpet *Patella vulgata* L. Comp Biochem Physiol A Mol Integr Physiol. 1999;123:89 – 93. https://doi.org/10.1016/s1095-6433(99)00043-4
45. Nakhlé KF, Cossa D, Khalaf G, Beliaeff B. *Brachidontes variablilis* and *Patella* sp. as quantitative biological indicators for cadmium, lead and mercury in the Lebanese coastal waters. Environmental Pollution. 2006;142:73-82. https://doi.org/10.1016/j.envpol.2005.09.016

494 46. Palladino G, Rampelli S, Scicchitano D, Musella M, Quero GM, Prada F, Mancuso A,
495 Seyfarth AM, Turroni S, Candela M, Biagi E. Impact of marine aquaculture on the
496 microbiome associated with nearby Holobionts: The case of *Patella caerulea* living in
497 proximity of Sea Bream aquaculture cages. Microorganisms. 2021;9:455.
498 https://doi.org/10.3390/microorganisms9020455
499 47. Mubiana VK, Vercauteren K, Blust R. The influence of body size, condition index and tidal
500 exposure on the variability in metal bioaccumulation in *Mytilus edulis*. Environ Pollut.
501 2006;144:272–279. https://doi.org/10.1016/j.envpol.2005.12.017
502 48. Sánchez-Marín P, Schultze F, Besada V. Use of limpets as alternative to mussels in metal
503 pollution monitoring; application in the Canary Islands. Environ Pollut. 2022;308:119614.
504 https://doi.org/10.1016/j.envpol.2022.119614
505 49. Spann N, Aldridge DC, Griffin JL, Jones OAH. Size-dependent effects of low level
506 cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*.
507 Aquatic Toxicology. 2011;105:589-599. https://doi.org/10.1016/j.aquatox.2011.08.010
508

Supplementary data

Table S1: Physico-chemical variables: temperature, pH, salinity, electrical conductivity, and total dissolved solids measured in situ (n=3) at each site during sampling of field-fresh *Scutellastra* and *Siphonaria*. Sites include Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP)

Site	Temperature (ºC)	pH	Salinity (PSU)	Electrical conductivity (mS cm^{-1})	Total dissolved solids (ppt)
NNP	18.5	7.84	33	60.1	37.4
	17.7	8.06	31	57.6	35.7
	17.8	8.12	34	61.3	38
WCNP	16.4	6.28	36	63.6	39.7
	16.5	7.31	38	69.9	43.2
	16.5	7.58	41	72.4	45.1
GRNP	19.5	7.8	33	60.6	35.2
	19.5	7.96	39	68.4	40.4
	19.9	7.89	45	80.3	46.7

Table S2: Cadmium (Cd) values in field-fresh water samples (n=3) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh water samples ($F_{(2, 6)}$ = 3.221, p = 0.112)

	NNP	WCNP	GRNP

	0.105	0.133	0.140
Cd Concentration in water samples (µg l^{-1})	0.207	0.156	0.120
	0.072	0.120	0.360

Table S3: Morphometric measurements of *Scutellastra* and *Siphonaria* collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Measurements include weight (g) and size (mm)

	NNP		WCNP		GRNP	
Species	**Size (mm)**	**Weight (g)**	**Size (mm)**	**Weight (g)**	**Size (mm)**	**Weight (g)**
Scutellastra	24.10	0.0957	22.00	0.201	25.5	0.104
	28.80	0.1	20.00	0.209	22.5	0.108
	24.70	0.101	26.00	0.202	24	0.102
	23.20	0.0908	19.00	0.203	21	0.0959
	25.50	0.103	23.00	0.203	22.5	0.107
	27.90	0.207	23.00	0.11	23	0.202
	23.60	0.205	25.00	0.107	23	0.2
	21.20	0.202	24.00	0.109	32	0.2
	26.00	0.208	28.00	0.0913	21.5	0.207
	23.90	0.201	8.50	0.106	21	0.203
Siphonaria	18.00	0.0954	15.70	0.20	19.5	0.0683
	19.10	0.0949	16.60	0.20	21	0.104
	18.50	0.104	18.00	0.20	19.5	0.0521
	16.20	0.106	17.00	0.20	21	0.107
	17.50	0.103	16.00	0.21	22	0.0995
	20.90	0.209	16.00	0.908	21.5	0.204

	18.00	0.205	18.00	0.864	22.5	0.209
	15.10	0.204	17.50	0.1075	19.5	0.206
	15.50	0.203	20.00	0.0994	21	0.204
	17.70	0.209	17.00	0.0984	19	0.203

Table S4: Raw accumulation values of Cd measured in field-fresh Siphonaria capensis (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh *Scutellastra* ($F_{(2, 27)}$ = 2.260, p = 0.124) and *Siphonaria* ($F_{(2, 27)}$ = 1.961, p = 0.160)

	Cadmium (Cd) accumulation (µg g^{-1})		
Species	**NNP**	**WCNP**	**GRNP**
Scutellastra	3.16514376	1.08869686	3.18878959
	8.78170934	0.83019095	1.26444493
	3.79152699	1.29859698	0.85820639
	2.07593463	1.35995523	1.58030948
	2.64683204	1.19451389	3.66738478
	4.18513177	2.03684873	0.76091392
	4.2363977	0.39318765	0.88856608
	6.27833467	1.18389903	4.17147066
	3.9026208	1.67768911	1.18919232
	2.64978426	1.61210104	1.69226698
Siphonaria	5.66927395	0.10923433	1.09878025
	1.28964454	1.4431792	2.68499889
	5.12052839	1.72352601	2.70568566
	2.58363282	1.48040272	3.76164186
	2.72354819	1.4751596	3.25858026
	2.97504533	1.62800252	2.69910768

	3.19954686	1.06711348	3.8320237
	1.48778817	1.12698442	4.35968629
	2.09607349	2.12176087	5.76793155
	2.04874289	0.65328106	4.39765685

Table S5: Bioconcentration factor (BCF) values of Cd measured in field-fresh *Scutellastra granularis* and *Siphonaria capensis* (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed BCF variations in field-fresh *Scutellastra* ($F_{(2, 27)}$ = 3.259, p = 0.055) and *Siphonaria* ($F_{(2, 27)}$ = 2.508, p = 0.100)

	Bioconcentration factor (BCF)		
Species	**NNP**	**WCNP**	**GRNP**
Scutellastra	24.7276857	7.98555153	13.4737588
	68.6071042	6.08942019	5.34272507
	29.6213046	9.52516124	3.62622417
	16.2182393	9.97522174	6.67736402
	20.6783753	8.7617156	15.495992
	32.696342	14.9402107	3.21512922
	33.096857	2.88401702	3.75450454
	49.0494896	8.68385597	17.6259324
	30.489225	12.3057881	5.02475626
	20.7014395	11.824702	7.15042388
Siphonaria	44.2912027	0.80122983	4.64273347
	10.075348	10.5856665	11.3450657
	40.004128	12.6420001	11.4324746
	20.1846314	10.8586997	15.8942614
	21.2777202	10.8202416	13.768649
	23.2425417	11.9413388	11.4046803

	24.9964599	7.8272382	16.1916494
	11.623345	8.26638936	18.4212097
	16.3755742	15.5630382	24.3715418
	16.0058039	4.79179263	18.5816487

543

Appendix 2: Reviewer 2's comments on original manuscript (Round1)

Field study of cadmium bioaccumulation in two residential limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline

Formatted: Strikethrough

Abstract

The study investigates cadmium (Cd) bioaccumulation in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline from three sites; Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP). Significant spatial variations in Cd accumulation were observed, with the highest concentrations found in limpets from NNP (*Scutellastra* – 4.17 ± 1.99 µg g^{-1}, *Siphonaria* – 2.92 ± 1.45 µg g^{-1}) despite having the lowest water Cd levels (0.13 ± 0.07 µg l^{-1}). This suggests alternative Cd uptake pathways, such as dietary intake from contaminated algae or sediments. Additionally, larger limpets exhibited higher Cd concentrations, highlighting the importance of considering organism size in biomonitoring studies. The bioconcentration factor (BCF) analysis further indicated significant site-specific differences, with the highest BCF at NNP (*Scutellastra* – 32.59 ± 15.62, *Siphonaria* – 22.81 ± 11.29). These findings highlight the relationship between environmental exposure and physiological traits in Cd bioaccumulation and the necessity for site-specific assessments and using multiple bioindicator species in environmental monitoring. The study shows the importance of continued research into long-term and spatial variations in metal bioaccumulation in marine ecosystems.

Significance:

- Highlights Cd bioaccumulation in limpets indicating potential pollution pathways.
- Demonstrates *S. granularis* and *S. capensis* as bioindicator Sp. for Cd monitoring.
- Spatial significance stresses the importance of site-specific monitoring.
- Organism size matters in pollution assessments.
- There is no correlation between water and organism metal concentrations.

Keywords: Bioconcentration factor, bioindicator, ICP-MS, intertidal, marine pollution

Supplementary data inserted at the end of the document.

Introduction

Coastal regions across the world are subjected to significant metal pollution, resulting from agricultural and industrial activities.[1] With over 70% of South Africa's coastline inhabited, elevated

anthropogenic pressures on the nearshore marine environment are becoming more prevalent.[2] Metals naturally occur in the marine environment at low concentrations, however, due to increased anthropogenic pressures, coastal waters are becoming progressively vulnerable to a rise in metal pollution.[3] Metals pose a considerable threat to marine ecosystems due organism toxicity at low concentrations, environmental persistence, bioaccumulation ability.[4] Bioaccumulation refers to the process of organismal toxicant uptake, such as chemicals or metals, through consumption of contaminated food or direct exposure, be it over a short (acute) or long (chronic) periods.[4,5] The degree of metal bioaccumulation is influenced by factors such as the metal bioavailability, the route of uptake, and sequestration or depuration of the toxicant.[6] The greatest threat associated with metal bioaccumulation within the tissues of organisms is biomagnification in the higher trophic levels.[7,8]

Cadmium (Cd) primarily enters waterbodies through industrial sources, including zinc (Zn) smelting, electroplating, fossil fuel combustion, plastics, pesticides, and oil refining.[9] Research on Cd has intensified globally due to its toxicity at low concentrations, and its ability to biomagnify through trophic levels, impacting humans.[1,10-18] Cadmium accumulation occurs in organisms primarily via ingestion as the metal ions are poorly soluble in water, binding to suspended particulate matter (SPM) in the ocean, which is ingested and absorbed by filter feeding organisms.[4] Local studies have demonstrated that gastropods, such as limpets, accumulate metals and can serve as potential bioindicator species.[19,20] Despite the variety of bioindicator species used in South Africa, information on the responses of limpets to metals in the marine environment is limited.

A commonly used approach for environmental biomonitoring is quantifying pollutant loads in organisms that bioaccumulate.[21] Metal concentrations in tissues depends on the uptake, excretion, and metabolic capacity to either degrade and/or transform these toxic pollutants.[21] The use of a single bioindicator species when determining metal pollution is not ideal because certain metals concentrations may differ from one species to another.[22] Therefore, different species inhabiting different coastal zones need to be assessed. Recent studies include using the invasive Mediterranean mussel (*Mytilus galloprovincialis*) and endemic Brown mussel (*Perna perna*) along the southern and east coasts.[3,23-25] Limpets were selected for this study due to their wide distribution, feeding behaviour, being reasonably sessile, and distinct ability to tolerate environmental challenges, making them a key study organism when looking at the effect of environmental changes on intertidal organisms.[19,26] The limpets *Scutellastra granularis* (Linnaeus

1758), a prosobranch found high on the shore, and *Siphonaria capensis* (Quoy and Gaimard 1833), a pulmonate gastropod found in pools and on emergent mid-shore rocks[27,28], were selected to investigate the variation in Cd uptake and bioaccumulation across key sites along the South African coastline. These species of limpet were used to investigate Cd bioaccumulation due to their different phylogenies (i.e., pulmonate vs patellogastropod) and associated tidal heights along their sympatric distribution in South Africa.

This study describes the spatial patterns of Cd concentrations in South African coastal waters using *Scutellastra* and *Siphonaria* as quantitative bioindicators. It includes investigating the effect of weight and size on Cd bioaccumulation. Spatial patterns for each species are used to compare Cd concentrations in water and tissue samples with inferred bioconcentration factors, discussing these in terms of background (environmental) concentrations and their sources.

Materials and methods

Site selection and animal collection

The study took place at three marine protected areas along a gradient from the west to east along the South African coastline. The South African coastline is a dynamic environment surrounded by two major ocean currents bringing contrasting environmental conditions.[29] The west coast is a cool temperate ecoregion influenced by the Benguela Current that transports cold nutrient-rich water, whereas the southern coast is classified as warm temperate and influenced by the warm water Agulhas Current.[27-30] Sites selected included, two from the west coast; the Namaqua National Park (NNP – 30º51'31.8" S, 17º34'31.1" E) in the Northern Cape and the West Coast National Park (WCNP – 33º8'56.2" S, 17º59'57.9" E) in the Western Cape, and the Garden Route National Park (GRNP – 34º01'14.9" S, 23º52'26.7" E) in the Eastern Cape from the southern coast ecoregion of South Africa. Field fresh samples of similar size (total shell length) were collected from all three sites (Σn = 3 sites x 2 species x 10 individuals (replicates) = 60) in January 2020 (Supplementary data, Table S3). The number of individuals was determined by both the [anonymised] animal ethics committee (number anonymised) and the SANParks permitting office (permit number anonymised). Organisms were collected during low tide (at first daylight). Organisms were carefully removed from the rock surface using either a spatula or by hand and only undamaged individuals. Individuals were classified as undamaged when there was no visible damage done to the shell or foot. Field fresh samples collected at each site were flash frozen in liquid nitrogen (-196ºC) and stored at -20ºC for baseline metal analysis. Water samples (Σn = 3 sites x 3 water samples (replicates) at 50 ml each = 9) were collected from each site and stored

Commented [A1]: Useful to include a map indicating the position of the sites.

at -20ºC for background metal analysis. During collection of water samples, physico-chemical parameters were recorded, including: temperature (ºC), pH, salinity (PSU), electrical conductivity (mS cm^{-1}), and total dissolved solids (ppt).

Bioaccumulation analysis

Sample preparation and digestion

The water samples (50 ml), were filtered using a Millipore sintered glass filtration system (Merck, South Africa), through a gridded cellulose nitrate membrane filter (type 114, 0.45 µm). Samples were acidified to 1% with Suprapur® nitric acid – 63.01 g mol^{-1} (HNO_3) (Merck, South Africa).

Whole organism tissue was used for metal accumulation analysis in accordance with the methods outlined by Boss and Fredeen[31] and Gaines[32], and adapted from Erasmus et al.[29]. Frozen samples were weighed (± 0.01 g) to obtain a wet weight, after which the samples were freeze-dried (-50ºC at 0.61 mbar) for 48 hours using the Martin-Christ LDplus Freeze Dryer (Christ Freeze Dryers, Niedersachsen, Germany). The freeze-dried (whole organism) samples were weighed to obtain a final dry weight, in which the weight was normalised using the bioconcentration factor calculation, before being digested using the MARS 6 One Touch Technology Microwave (CEM, Charlotte, United States of America). Before digestion, the Teflon bombs used during processing were cleaned and dried in accordance with the cleaning method, using standard grade HNO_3 (Merck, South Africa) and hydrogen peroxide (H_2O_2) (Merck, South Africa). Standard reference material (SRM, Mussel Tissue – 2976, 109% recovery), as well as the freeze-dried samples, were individually added to each Teflon bomb along with 5 ml Suprapur® 65% HNO_3 (Merck, South Africa. The digestion process took a total of 70 minutes and was held at 200ºC, starting with a 15-minute warm-up period, followed by a 35-minute holding period and finally a 20-minute cool-down period. After digestion, the samples were allowed to cool, before being placed in 15 ml Falcon tubes. Samples were gravimetrically diluted using double distilled water (18.2 Ω) to a total volume of 10 ml (Millipore, United States of America).

ICP-MS Analysis

Water (diluted by factor of 10) and tissue samples were analysed using a PerkinElmer NexION® 300 series Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (PerkinElmer, Waltham, United States of America). The ICP-MS was calibrated using 0, 0.01, 0.1 and 1 ppb calibration

Commented [A2]: See comments on the Table.

Commented [A3]: What containers were used.

Commented [A4]: Need to indicate how soft tissues were removed from shells BEFORE drying.

Formatted: Highlight

Commented [A5]: This is unclear (and very unusual). Why normalise with BCF? This does not make sense since the BCF assumes that the Cd is taken up from water. It could be from the algae that limpets feed on. The normalisation is thus based on reporting concentration based on g dry weight limpit.

standards, made using the appropriate dilutions from a 100 ppm Cd stock solution. An internal standard (Iridium – 193) was added to each water and tissue sample before analysis for quality assurance, ensuring sufficient recovery and no drifting occurred during the analysis. Data were converted from counts per second (cps) to µg g^{-1} (tissue) and µg l^{-1} (water), standardising the sample mass using the following equations:

Commented [A6]: Need to indicate here what certified reference material was used to ensure analytical efficiency for Cd.

$$Cd\ IS\ Corrected = \frac{Cd\ 111\ (cps)}{Ir\ 193\ (cps)} \quad \text{Equation 1}$$

$$Unit\ Conversion\ (cps\ \rightarrow\ \mu g\ l^{-1}) = \frac{(Cd\ IS\ Corrected - Intercept)}{(Slope)} \quad \text{Equation 2}$$

$$Blank\ \&\ Dilution\ Correction\ = ((Unit\ Conversion\ - Blanked\ Average) * 10) \quad \text{Equation 3}$$

$$Unit\ Conversion\ (\mu g\ l^{-1} \rightarrow \mu g\ g^{-1}) = \frac{(Blank\ \&\ Dilution\ Correction * 0.01)}{(Sample\ Dry\ Weight)} \quad \text{Equation 4}$$

Bioconcentration factor (BCF)

The bioconcentration factor (BCF) was determined according to the method of Abel.[33] This method determined the ratio between the Cd concentration in organisms and water samples ($C_{Organism\ dry\ weight}/C_{Water}$), where the $C_{Organism\ dry\ weight}$ (µg g^{-1} dry weight) and C_{Water} (µg l^{-1}) are the fractions of the element within the organism and the environment (water). The median concentration values of the element present in each organism and the water were used to calculate the BCF.

Statistical analysis

Statistical analyses were conducted using RStudio (v. 4.0.3). All data met the assumptions of normality and homogeneity of variance, following Levene's and Shapiro-Wilk tests respectively.[34] Following testing of assumptions, one-way Analysis of Variances (ANOVAs) were performed on cadmium concentrations in water samples (factors site, three levels and fixed, significance set at $p < 0.05$), tissue samples (factors site, three levels and fixed, significance set at $p < 0.05$), and bioconcentration factor (factors site, three levels and fixed, significance set at $p < 0.05$) for each species respectively. Where there were significant factors, a pairwise comparison was performed using Tukey's HSD.[34] All statistical analyses were performed using the *car* and *tidyverse*

packages in RStudio.[35,36] Barplots (±SEM) were created using the *ggplot2* and *plyr* packages. in RStudio.[37,38]

Results

Environmental water quality parameters

Physico-chemical parameters; temperature (ºC), pH, salinity (PSU), electrical conductivity (mS cm^{-1}), and total dissolved solids (ppt) measured *in situ* are represented in Table 1. The measured parameters across the three sites showed great variation, between sampling periods at each site (high SD values) as well as among the sites themselves.

Table 1: Mean (±SD) water quality measures taken in situ at each sampling site (NNP – Namaqua National Park, WCNP – West Coast National Park, GRNP – Garden Route National Park) during each day of sampling (n=3). Common superscripts indicate statistically significant differences in water quality parameters across sites (p<0.05)

	NNP	WCNP	GRNP
Temperature (ºC)	**16.18 ± 2.01^{ab}**	**16.47 ± 0.06^{ac}**	**19.40 ± 0.17^{bc}**
pH	8.01 ± 0.10	7.06 ± 0.69	7.71 ± 0.31
Salinity (PSU)	33.17 ± 1.17	38.33 ± 2.52	35.33 ± 3.21
Electrical conductivity (mS cm^{-1})	60.77 ± 1.70	68.63 ± 4.53	66.50 ± 5.22
Total dissolved solids (ppt)	37.68 ± 1.05	42.67 ± 2.74	38.73 ± 3.06

Commented [A7]: In marine environments the salinity is already being measured as PSU - thus EC and TDS measurements are not required. Recommend to remove.

Temperature showed significant variation across sites ($F_{(2, 8)}$ = 91.5, p = 3.2e^{-05}). Water temperature at GRNP was significantly higher than both west coast sites (NNP and WCNP, Table 1). Water temperature also varied significantly between the two west coast sites (NNP and WCNP), with significantly lower temperatures recorded at NNP. All other water quality parameters (pH, salinity, electrical conductivity, and total dissolved solids) showed statistically non-significant differences across the three sites (p>0.05, Table 1).

Cadmium concentrations versus weight and size

Cadmium concentrations in whole body tissues (without shell) were plotted against the wet weight (g) and size (mm) for both species of limpets, ~~*Scutellastra*~~ *S. granularis* and ~~*Siphonaria*~~*S. capensis*; with results being illustrated in Figure 1A and B, respectively. Concentrations of Cd in whole body limpets showed no significant change with increasing weight of either species, $F_{(3, 56)}$ = 0.0482, p = 0.986 (Figure 1A). With increasing weight, both *Scutellastra* and *Siphonaria* had

Commented [A8]: Since you know the species names these have to be provided. Thus change all genus names to genus and species as done here.

non-significant changes in their individual Cd concentrations, with R^2 values of 0.001 and 0.002, respectively. In contrast, concentrations of Cd in whole body limpets showed a significant change with increasing size, $F_{(3, 56)} = 5.347$, p = 0.003 (Figure 1B). *Siphonaria* showed a greater increase in Cd concentrations with an increase in size ($R^2 = 0.26$), whereas the larger *Scutellastra* showed a similar increase in Cd concentrations with an increase in size ($R^2 = 0.20$).

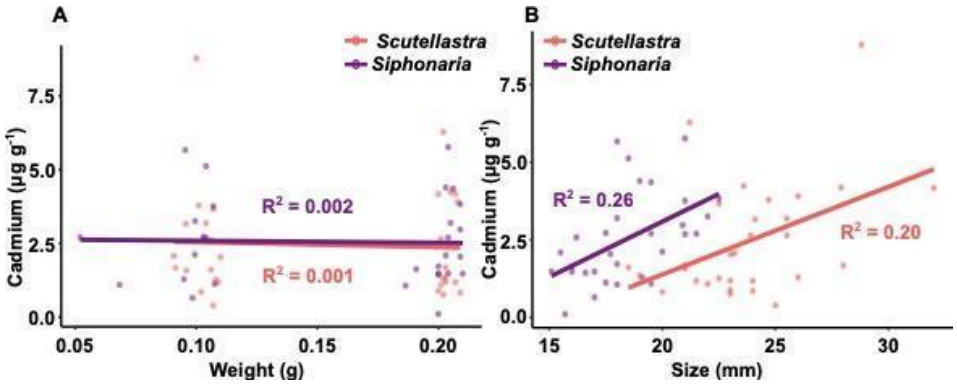

Figure 1: General linear model (GLM) showing the relationship between cadmium concentrations (µg g^{-1}) and the weight (A) and size (B) of *Scutellastra* and *Siphonaria*

Cadmium concentration in field-fresh water samples from the southern and west coasts

Cadmium concentrations within water samples collected at each respective site during the sampling of *Scutellastra* and *Siphonaria* are represented in Figure 2. No statistically significant differences in cadmium concentrations were present in water samples between the three sites (Figure 2, Table 2). Water samples from the southern coast (GRNP) had the greatest Cd concentrations (0.24 µg l^{-1}), whilst samples from the west coast at NNP had the lowest concentrations (0.13 µg l^{-1}).

Table 2: One-way ANOVA results comparing Cd levels in field-fresh water samples (n=3) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh water samples ($F_{(2, 6)} = 3.221$, p = 0.112)

	Df	Sum Sq	Mean Sq	*F*	p

Commented [A9]: The one-way ANOVA results (water, limpits, BCFs) do not need to be presented in Table format since the significance is indicated on the graphs. Of greater interest would be the two-way ANOVA information - site and species interactions.

Sites	2	0.0219	0.0110	1.8370	0.2386
Residuals	6	0.0358	0.0060		
Total	8	0.0578			

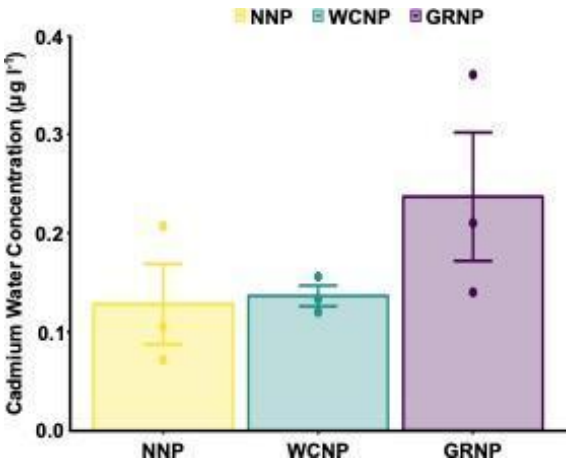

Figure 2: Mean (±SEM) levels of cadmium in water samples (n=3 per site) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP)

Cadmium concentrations in field-fresh Scutellastra and Siphonaria from the southern and west coasts

The measured concentrations of Cd in both *Scutellastra* and *Siphonaria* are presented in Table 3 and Figure 3A. Spatial differences in Cd concentrations from whole body *Scutellastra* tissue showed significance, $F_{(2, 29)}$ = 11.978, p = 0.0002 (Table 3). At NNP, *Scutellastra* accumulated significantly higher concentrations of Cd than both remaining sites, WCNP and GRNP (Figure 3A. A similar trend was observed in *Siphonaria*, with spatial variances in Cd concentrations across all three sites, $F_{(2, 29)}$ = 9.522, p = 0.0007 (Table 3). Limpets collected from NNP having significantly higher Cd concentrations (p<0.01) to those collected and analysed from the WCNP (Figure 3A). Dissimilarly, along the southern coast at GRNP, *Siphonaria* had the highest Cd concentrations out of the three sites, being significantly higher than WCNP (p<0.001).

Table 3: One-way ANOVA results comparing Cd levels in field-fresh *Scutellastra* and *Siphonaria* (n=10) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh *Scutellastra* ($F_{(2, 27)}$ = 2.260, p = 0.124) and *Siphonaria* ($F_{(2, 27)}$ = 1.961, p = 0.160)

Scutellastra					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**46.355**	**23.178**	**11.978**	**0.0002**
Residuals	27	52.244	1.935		
Total	29	98.599			

Siphonaria					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**25.640**	**12.820**	**9.522**	**0.0007**
Residuals	27	36.353	1.346		
Total	29	61.993			

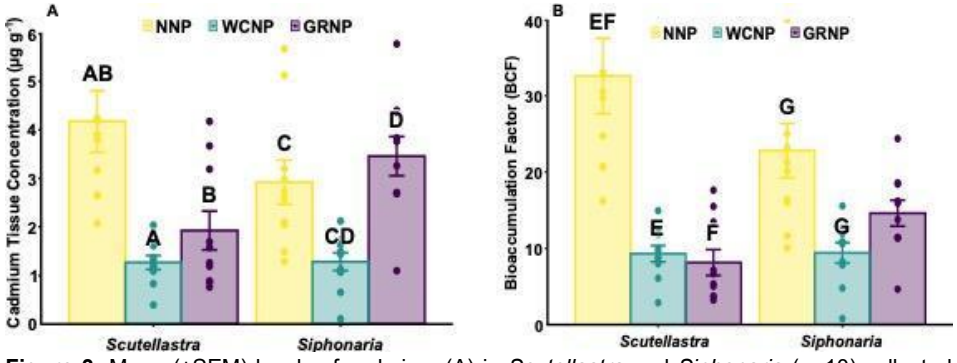

Figure 3: Mean (±SEM) levels of cadmium (A) in *Scutellastra* and *Siphonaria* (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP). Bioconcentration factor (B) variation in *Scutellastra* and *Siphonaria* (n=10) using cadmium concentrations in water and tissue samples collected from each site (NNP, WCNP, and GRNP)

Bioconcentration factor of cadmium in field-fresh Scutellastra and Siphonaria from the southern and west coasts

The results obtained show the bioconcentration factor (BCF) of Cd in both *Scutellastra* and *Siphonaria* and are presented in Table 4 and Figure 3B. Spatial differences in the BCF of Cd in *Scutellastra* showed significance, $F_{(2, 29)} = 20.120$, $p<0.001$. As seen in the Cd concentrations of *Scutellastra,* the BCF spatial variance showed the same pattern. *Scutellastra* from NNP showed the greatest BCF, differing significantly from both WCNP and GRNP ($p<0.001$, Figure 3B). No difference was observed between the BCF of *Scutellastra* at WCNP and GRNP ($p>0.05$). Unlike in the Cd concentrations observed in *Siphonaria* (Figure 3A), a dissimilar trend was observed in the BCF values across sites, although significantly different spatial differences were observed, $F_{(2, 29)} = 7.86$, $p = 0.002$ (Figure 3B, Table 4). *Siphonaria* from NNP exhibited the greatest BCF, differing significantly with limpets sampled from WCNP ($p<0.01$, Figure 3B). No other significant spatial differences were observed in *Siphonaria* between NNP and GRNP, nor between NNP and GRNP ($p>0.05$, Figure 3B).

Table 4: One-way ANOVA results comparing the Bioconcentration Factor (BCF) in field-fresh *Scutellastra* and *Siphonaria* (n=10) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed BCF variations in field-fresh *Scutellastra* ($F_{(2, 27)} = 3.259$, $p = 0.055$) and *Siphonaria* ($F_{(2, 27)} = 2.508$, $p = 0.100$)

Scutellastra					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**3805.382**	**1902.691**	**20.120**	**$4.472e^{-06}$**
Residuals	27	2553.365	94.569		
Total	29	6358.747			
Siphonaria					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	**2**	**912.587**	**456.293**	**7.86**	**0.002**

Residuals	27	1566.557	58.021
Total	29	2479.144	

Discussion

While metal contaminants occur naturally in the marine environment at low concentrations, anthropogenic influence on the marine environment has exacerbated the situation.[39,40] The results from this study reveal significant bioaccumulation of ~~cadmium (~~Cd~~)~~ in two limpet species, ~~*Scutellastra*~~ *S. granularis* and ~~*Siphonaria*~~ *S. capensis*, collected from different coastal regions in South Africa. Comparing Cd concentrations in both limpet species to that found in water samples analysed indicates that Cd is strongly accumulated in both species from their respective surrounding environments. Potential sources of Cd in the marine environment come from the emissions of industrial combustion processes, metallurgic industries, road transport, and waste streams, all of which occur along the west and southern coasts of South Africa.[20,29,41,42] Water samples analysed for Cd showed variation between the southern coast site (GRNP) and the two west coast sites (NNP and WCNP). Studies conducted by Reinecke et al.[19,43] indicated Cd concentrations along the South African coastline varied considerably spatially and temporally. Degger et al.[40], measured unexpectedly high metal concentrations (Cd and Zn) in brown mussels (~~*Perna*~~ *P. perna*) along the southern coast at Tsitsikamma National Park (within GRNP) compared to localities with known anthropogenic activities and influence (such as WCNP). High concentrations of metals in the study by Degger et al.[40] follow a similar pattern shown in data from the present study (both the water and organisms). This is indicative of long-term exposure, resulting from freshwater input through geogenic weathering and frequent upwelling that occur along South Africa's southern coast.[29] *Siphonaria* showed the greatest Cd accumulation at Namaqua National Park (NNP), with both species having the greatest metal uptake at this site despite lower Cd concentrations in the corresponding water samples. This suggests that these limpets are accumulating Cd from a different source, such as dietary intake from contaminated algae or sediments.[4,20] Additionally, they possess physiological traits that enhance Cd retention, such as slower excretion rates or higher binding affinities in tissue proteins.[21,44] This finding suggests similarity with previous studies, indicating bioaccumulation in marine organisms occuring through various pathways, including dietary intake and sediment interaction.[4,29]

Commented [A10]: Use the provided abbreviations consistently.

Commented [A11]: To what degree are these sources of Cd present in the near vicinity of the sampling sites?

The bioconcentration factor (BCF) indicated Cd had been accumulated in whole tissue samples of *Scutellastra* and *Siphonaria* in comparison to the concentration of Cd in the environment.[33] Whole tissue samples of both species varied significantly in their accumulation of Cd in relation to their sampling site. This however does not correlate with Cd concentrations from water sampled. Interestingly, despite higher water Cd levels at GRNP, the bioconcentration factor (BCF) was highest at NNP for *Scutellastra*, suggesting species-specific and site-specific bioaccumulation dynamics, seasonal and exposure level variation.[45] High Cd concentrations at GRNP, as discussed previously, are a direct result of both the freshwater input from geogenic weathering in the region and from upwelling events that are known to occur along the southern coast of South Africa at GRNP.[29,40] The significantly high BCFs at NNP are a direct result of the significantly high concentrations of Cd detected in whole tissue samples of *Scutellastra* and *Siphonaria*, despite the lower concentrations of Cd in the water. This result is indicative of either a short-term but high-dose exposure to Cd or a different means of uptake in both limpet species other than directly from the water column.[40,46] Elevated metal and pollutant levels along the west coast, observed at NNP and WCNP, are a direct result of the mining and port activities, as well as the iron and crude oil facilities in the surrounding areas, which lead to elevated pollution of local coastal waters affecting the inhabitants of these waters.[20,29,42]

The anatomy and physiology of the limpets is also known to play a primary role in the uptake and accumulation of metals. Differences in body sizes of individuals of the same species has been shown to result in varying metal tissue concentrations.[22,29,47,48] The size of marine ectotherms is related to their metabolic activities in relation to their life history stage.[8] Various metals such as copper (Cu), iron (Fe), and zinc (Zn) in moderate concentrations are essential in an organism's growth and development, however non-essential metals such as Cd lead to reduced growth and development, even at low concentrations.[8] Limpet size was positively correlated with tissue Cd concentration, indicating that larger individuals tend to accumulate more Cd, potentially due to prolonged exposure and greater overall uptake over time.[8,48] This size-related bioaccumulation trend has been observed in other marine invertebrates and highlights the importance of considering organism size in biomonitoring studies.[19,49]

The physiological and anatomical differences between *Scutellastra* and *Siphonaria* likely contribute to the observed differences in Cd bioaccumulation. *Scutellastra*, a prosobranch, and *Siphonaria*, a pulmonate gastropod, exhibit distinct respiratory and excretory mechanisms that influence metal uptake and storage.[44] Pulmonate gastropods, such as *Siphonaria*, exhibit higher

rates of metal accumulation due to their ability to absorb metals through their foot and mantle cavity, in addition to dietary intake.[21,44] Comparative studies have shown that bioaccumulation patterns in marine organisms can vary widely based on local environmental conditions and the species involved. Research on the Brown mussel (*Perna perna*) has demonstrated significant spatial variation in metal concentrations along the South African coastline, similar to the patterns observed in this study.[40] These findings underscore the importance of site-specific assessments and the use of multiple bioindicator species to accurately gauge environmental metal pollution levels.[20,21]

Conclusion

This study provides insight into the spatial patterns of Cd bioaccumulation in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline. The findings from both limpet species showed significant spatial variation in Cd accumulation, with *Scutellastra* having the highest Cd levels at NNP despite lower Cd concentrations in corresponding water samples. This suggests alternative pathways for Cd uptake, such as dietary intake from contaminated algae or sediments. Additionally, larger individuals from both species exhibited higher Cd concentrations, emphasising the importance of considering organism size in biomonitoring studies. The bioconcentration factor (BCF) analysis further indicated that the BCF of Cd in *Scutellastra* and *Siphonaria* was highest at NNP, highlighting the complex interplay between environmental exposure and physiological traits in Cd bioaccumulation. These findings emphasise the need for site-specific assessments and the use of multiple bioindicator species to effectively assess environmental metal pollution levels in marine ecosystems. Future research should focus on the long-term monitoring of these species across a greater seasonal and spatial gradient, and the exploration of additional environmental factors influencing metal bioaccumulation.

References

1. Boudjema K, Kourdali S, Bounakous N, Meknachi A, Badis A. Catalase activity in Brown Mussels (*Perna perna*) under acute cadmium, lead, and copper exposure and depuration tests. J Mar Biol. 2014:1–9. https://doi.org/10.1155/2014/830657
2. Sink K, Holness S, Harris L, Majiedt P, Atkinson L, Robinson T, Kirkman S, Hutchings L, Leslie R, Lamberth S, Kerwath S, Von der Heyden S, Lombard A, Attwood C, Branch G, Fairweather T, Taljaard S, Weerts S, Cowley P, Awad A, Halpern B, Grantham H, Wolf T. National Biodiversity Assessment 2011: technical report. In: Marine and Coastal Component, 4. South African National Biodiversity Institute; 2012. p. 325.
3. Greenfield R, Brink K, Degger N, Wepener V. The usefulness of transplantation studies in monitoring of metals in the marine environment: South African experience. Mar Pollut Bull. 2014;85:566–573. https://doi.org/10.1016/j.marpolbul.2014.03.032
4. Jakimska A, Konieczka P, Skóra K, Namieśnik J. Bioaccumulation of metals in tissues of marine animals, Part I: The role and impact of heavy metals on organisms. Pol J Environ Stud. 2011a; 20:1117–1125.
5. Perera PACT, Kodithuwakku SP, Sundarabarathy TT, Edirisinghe U. Bioaccumulation of cadmium in freshwater fish: An environmental perspective. Insight Ecology. 2015;4:1–12. https://doi.org/10.5567/ECOLOGY-IK.2015.1.12
6. Valavanidis A, Vlachogianni T. Metal pollution in ecosystems: ecotoxicology studies and risk assessment in the marine environment. 2010.
7. Fang T-H, Hwang J-S, Hsiao S-H, Chen H-Y. Trace metals in seawater and copepods in the ocean outfall area off the northern Taiwan coast. Mar Environ Res. 2006;61:224–243. https://doi.org/10.1016/j.marenvres.2005.10.002
8. Jakimska A, Konieczka P, Skóra K, Namieśnik J. Bioaccumulation of metals in tissues of marine animals, part II: Metal concentrations in animal tissues. Pol J Environ Stud. 2011b;20:1127–1146.
9. Chandrudu M, Radhakrishnaiah K. A study on the toxicity of cadmium on certain aspects of protein metabolism of the freshwater mussel *Lamellidens marginalis* (Lamarck) and freshwater fish *Labeo rohita* (Hamilton). Int J Environ Sci. 2013;4:15–27. https://doi.org/10.6088/ijes.2013040100003
10. CCME (Canadian Council of Ministers of the Environment). Canadian water quality guidelines for the protection of aquatic life: Cadmium. Winnipeg. 2014.
11. Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Nair AR, Munters E, Artois TJ, Nawrot T, Vangronsveld J, Smeets K. Cadmium stress: An oxidative challenge. BioMetals. 2010;23:927–940. https://doi.org/10.1007/s10534-010-9329-x
12. Environmental Protection Agency (EPA). Fact sheet: Aquatic life ambient water quality criteria update for cadmium. 2016.
13. Legeay A, Achard-Joris M, Baudrimont M, Massabuau J-C, Bourdineaud J-P. Impact of cadmium contamination and oxygenation levels on biochemical responses in the Asiatic clam *Corbicula fluminea*. Aquat Toxicol. 2005;74:242–253. https://doi.org/10.1016/j.aquatox.2005.05.015
14. Neff JM. 2002. Cadmium in the ocean, in: Bioaccumulation in Marine Organisms. Elsevier pp. 89–102. https://doi.org/10.1016/B978-008043716-3/50006-3

15. Phillips DJH. The Common Mussel *Mytilus edulis* as an Indicator of Pollution by Zinc, Cadmium, Lead and Copper. I. Effects of Environmental Variables on Uptake of Metals. Mar Biol. 1976;38:59–69. https://doi.org/10.1007/bf00391486
16. Ray S. Bioaccumulation of cadmium in marine organisms. Experientia. 1984;40:14–23. https://doi.org/10.1007/bf01959097
17. Scholz N. Accumulation, loss and molecular distribution of cadmium in *Mytilus edulis*. Helgoländer Meeresunters. 1980;33:68–78. https://doi.org/10.1007/bf02414736
18. Yamamura M, Suzuki KT, Hatakeyama S, Kubota K. Tolerance to cadmium and cadmium-binding proteins induced in the midge larvae, *Chironomus yoshimatsui* (Diptera, Chironimidae). Comp Biochem Physiol C Toxicol Pharmacol. 1983;75:21–24. https://doi.org/10.1016/0742-8413(83)90005-1
19. Reinecke AJ, Mdzeke NP, Reinecke SA. Spatial and temporal variation in cadmium body loads of four intertidal invertebrates from False Bay, South Africa. Afr Zool. 2012;47:12–25. https://doi.org/10.1080/15627020.2012.11407518
20. Erasmus A, Ikenaka Y, Nakayama SMM, Ishizuka M, Smit NJ, Wepener V. Trophic transfer of pollutants within two intertidal rocky shore ecosystems in different biogeographic regions of South Africa. Mar Pollut Bull. 2020;157:article 111309. https://doi.org/10.1016/j.marpolbul.2020.111309
21. Reguera P, Couceiro L, Fernández N. A review of the empirical literature on the use of limpets *Patella* spp. (Mollusca: Gastropoda) as bioindicators of environmental quality. Ecotoxicol Environ Saf. 2018;148:593-600. https://doi.org/10.1016/j.ecoenv.2017.11.004
22. Mbandzi N, Nakin MDV, Saibu GM, Oyedeji AO. Heavy metal profiles in limpets and algae on the Eastern Cape coast of South Africa. Afr J Mar Sci. 2021;43:293–308. https://doi.org/10.2989/1814232X.2021.1948918
23. Sparks C, Odendaal J, Snyman R. An analysis of historical Mussel Watch Programme data from the west coast of the Cape Peninsula, Cape Town. Mar Pollut Bull. 2014;87:374–380. https://doi.org/10.1016/j.marpolbul.2014.07.047
24. Greenfield R, Wepener V, Degger N, Brink K. Richards Bay Harbour: metal exposure monitoring over the last 34 years. Mar Pollut Bull. 2011;62:1926–1931. https://doi.org/10.1016/j.marpolbul.2011.04.026
25. Wepener V, Degger N. Monitoring of metals in South African harbours using resident brown mussels (*Perna perna*) as indicator organisms. Afr Zool. 2020;55:267–277. https://doi.org/10.1080/15627020.2020.1799720
26. Kankondi SL, McQuaid CD, Tagliarolo M. Influence of respiratory mode on the thermal tolerance of intertidal limpets. PLoS One. 2018;13:1–15. https://doi.org/10.1371/journal.pone.0203555
27. Branch G, Griffiths C, Branch M, Beckley L.. Two oceans: A guide to the marine life of southern Africa, 4th Edition. Cape Town: Struik Nature; 2016.
28. Branch G, Branch M. Living Shores: Interacting with southern Africa's marine ecosystems. Cape Town: Struik Nature. 2018.
29. Erasmus A, Smit NJ, Zimmermann S, Nachev M, Sures B, Wepener V. Metal and metalloid concentrations in the southern African endemic inter- and infratidal super klipfish, *Clinus superciliosus*, from the west and south coasts of South Africa. Mar Pollut Bull. 2021;172:112852. https://doi.org/10.1016/j.marpolbul.2021.112852

30. Bustamante RH, Branch GM, Eekhout S, Robertson B, Zoutendyk P, Schleyer M, Dye A, Hanekom N, Keats D, Jurd M, McQuaid C. Gradients of intertidal primary productivity around the coast of South Africa and their relationships with consumer biomass. Oecologia. 1995;102:189–201. https://doi.org/10.1007/BF00333251
31. Boss CB, Fredeen KJ. Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectroscopy. 3rd Edition. Connecticut: PerkinElmer; 2004.
32. Gaines PR. ICP Operations Guide: A Guide for using ICP-OES and ICP-MS. Inorganic Ventures; 2011.
33. Abel PD. Pollutant toxicity to aquatic animals - methods of study and their applications. Rev Environ Health. 1989;8. https://doi.org/10.1515/reveh-1989-1-407
34. Field A, Miles J, Field Z. Discovering statistics using R. London: SAGE Publications Ltd; 2012. https://doi.org/10.1111/insr.12011_21
35. Fox J, Weisberg S. 2019. An R Companion to Applied Regression, Third edition. California: Sage; 2019.
36. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H. Welcome to the tidyverse. J Open Source Softw. 2019;4(43):1686. https://doi.org/10.21105/joss.01686
37. Wickham H. The Split-Apply-Combine Strategy for Data Analysis. J Stat Softw. 2011;40(1):1–29. https://doi.org/10.18637/jss.v040.i01
38. Wickham H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
39. Ansari TM, Marr IL, Tariq N. Heavy metals in marine pollution perspective – A mini review. J Appl Sci. 2004;4:1-20. https://doi/org/10.3923/jas.2004.1.20
40. Degger N, Wepener V, Richardson BJ, Wu RSS. Application of artificial mussels (AMs) under South African marine conditions: A validation study. Mar Pollut Bull. 2011;63: 108–118. https://doi.org/10.1016/j.marpolbul.2011.04.040
41. OSPAR (Oslo and Paris Conventions). 2010. Quality Status Report 2010: Status and Trend of marine chemical pollution.
42. Clark B, Massie V, Hutchings K, Brown E, Biccard A, Laird M, Harmer R, Makhosonke A, Wright A, Turpie J. 2017. The state of Saldanha Bay and Langebaan Lagoon 2017, technical report.
43. Reinecke AJ, Schoeman W, Reinecke SA, Reinecke S, Schoeman W, Reinecke K. Cellular biomarker responses of limpets (Mollusca) as measure of sensitivity to cadmium contamination. Suid-Afrikaanse Tydskrif vir Natuur-wetenskap en Tegnologie. 2008;27:123–142. https://doi.org/10.4102/satnt.v27i2.86
44. Marchán S, Davies MS, Fleming S, Jones HD. Effects of copper and zinc on the heart rate of the limpet *Patella vulgata* L. Comp Biochem Physiol A Mol Integr Physiol. 1999;123:89 – 93. https://doi.org/10.1016/s1095-6433(99)00043-4
45. Nakhlé KF, Cossa D, Khalaf G, Beliaeff B. *Brachidontes variablilis* and *Patella* sp. as quantitative biological indicators for cadmium, lead and mercury in the Lebanese coastal waters. Environmental Pollution. 2006;142:73-82. https://doi.org/10.1016/j.envpol.2005.09.016

494 46. Palladino G, Rampelli S, Scicchitano D, Musella M, Quero GM, Prada F, Mancuso A,
495 Seyfarth AM, Turroni S, Candela M, Biagi E. Impact of marine aquaculture on the
496 microbiome associated with nearby Holobionts: The case of *Patella caerulea* living in
497 proximity of Sea Bream aquaculture cages. Microorganisms. 2021;9:455.
498 https://doi.org/10.3390/microorganisms9020455
499 47. Mubiana VK, Vercauteren K, Blust R. The influence of body size, condition index and tidal
500 exposure on the variability in metal bioaccumulation in *Mytilus edulis*. Environ Pollut.
501 2006;144:272–279. https://doi.org/10.1016/j.envpol.2005.12.017
502 48. Sánchez-Marín P, Schultze F, Besada V. Use of limpets as alternative to mussels in metal
503 pollution monitoring; application in the Canary Islands. Environ Pollut. 2022;308:119614.
504 https://doi.org/10.1016/j.envpol.2022.119614
505 49. Spann N, Aldridge DC, Griffin JL, Jones OAH. Size-dependent effects of low level
506 cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*.
507 Aquatic Toxicology. 2011;105:589-599. https://doi.org/10.1016/j.aquatox.2011.08.010
508

Supplementary data

Commented [A12]: Supplementary data are usually the information that cannot form part of the main body - NOT the raw data on which the bar graphs are based.

Table S1: Physico-chemical variables: temperature, pH, salinity, electrical conductivity, and total dissolved solids measured in situ (n=3) at each site during sampling of field-fresh *Scutellastra* and *Siphonaria*. Sites include Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP)

Site	Temperature (ºC)	pH	Salinity (PSU)	Electrical conductivity (mS cm^{-1})	Total dissolved solids (ppt)
NNP	18.5	7.84	33	60.1	37.4
	17.7	8.06	31	57.6	35.7
	17.8	8.12	34	61.3	38
WCNP	16.4	6.28	36	63.6	39.7
	16.5	7.31	38	69.9	43.2
	16.5	7.58	41	72.4	45.1
GRNP	19.5	7.8	33	60.6	35.2
	19.5	7.96	39	68.4	40.4
	19.9	7.89	45	80.3	46.7

Table S2: Cadmium (Cd) values in field-fresh water samples (n=3) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh water samples ($F_{(2, 6)}$ = 3.221, p = 0.112)

	NNP	WCNP	GRNP

	0.105	0.133	0.140
Cd Concentration in water samples (µg l^{-1})	0.207	0.156	0.120
	0.072	0.120	0.360

Table S3: Morphometric measurements of *Scutellastra* and *Siphonaria* collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Measurements include weight (g) and size (mm)

	NNP		WCNP		GRNP	
Species	**Size (mm)**	**Weight (g)**	**Size (mm)**	**Weight (g)**	**Size (mm)**	**Weight (g)**
Scutellastra	24.10	0.0957	22.00	0.201	25.5	0.104
	28.80	0.1	20.00	0.209	22.5	0.108
	24.70	0.101	26.00	0.202	24	0.102
	23.20	0.0908	19.00	0.203	21	0.0959
	25.50	0.103	23.00	0.203	22.5	0.107
	27.90	0.207	23.00	0.11	23	0.202
	23.60	0.205	25.00	0.107	23	0.2
	21.20	0.202	24.00	0.109	32	0.2
	26.00	0.208	28.00	0.0913	21.5	0.207
	23.90	0.201	8.50	0.106	21	0.203
Siphonaria	18.00	0.0954	15.70	0.20	19.5	0.0683
	19.10	0.0949	16.60	0.20	21	0.104
	18.50	0.104	18.00	0.20	19.5	0.0521
	16.20	0.106	17.00	0.20	21	0.107
	17.50	0.103	16.00	0.21	22	0.0995
	20.90	0.209	16.00	0.908	21.5	0.204

	18.00	0.205	18.00	0.864	22.5	0.209
	15.10	0.204	17.50	0.1075	19.5	0.206
	15.50	0.203	20.00	0.0994	21	0.204
	17.70	0.209	17.00	0.0984	19	0.203

Table S4: Raw accumulation values of Cd measured in field-fresh Siphonaria capensis (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh *Scutellastra* ($F_{(2, 27)}$ = 2.260, p = 0.124) and *Siphonaria* ($F_{(2, 27)}$ = 1.961, p = 0.160)

	Cadmium (Cd) accumulation (µg g^{-1})		
Species	**NNP**	**WCNP**	**GRNP**
Scutellastra	3.16514376	1.08869686	3.18878959
	8.78170934	0.83019095	1.26444493
	3.79152699	1.29859698	0.85820639
	2.07593463	1.35995523	1.58030948
	2.64683204	1.19451389	3.66738478
	4.18513177	2.03684873	0.76091392
	4.2363977	0.39318765	0.88856608
	6.27833467	1.18389903	4.17147066
	3.9026208	1.67768911	1.18919232
	2.64978426	1.61210104	1.69226698
Siphonaria	5.66927395	0.10923433	1.09878025
	1.28964454	1.4431792	2.68499889
	5.12052839	1.72352601	2.70568566
	2.58363282	1.48040272	3.76164186
	2.72354819	1.4751596	3.25858026
	2.97504533	1.62800252	2.69910768

	3.19954686	1.06711348	3.8320237
	1.48778817	1.12698442	4.35968629
	2.09607349	2.12176087	5.76793155
	2.04874289	0.65328106	4.39765685

Table S5: Bioconcentration factor (BCF) values of Cd measured in field-fresh *Scutellastra granularis* and *Siphonaria capensis* (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed BCF variations in field-fresh *Scutellastra* ($F_{(2, 27)}$ = 3.259, p = 0.055) and *Siphonaria* ($F_{(2, 27)}$ = 2.508, p = 0.100)

	Bioconcentration factor (BCF)		
Species	**NNP**	**WCNP**	**GRNP**
Scutellastra	24.7276857	7.98555153	13.4737588
	68.6071042	6.08942019	5.34272507
	29.6213046	9.52516124	3.62622417
	16.2182393	9.97522174	6.67736402
	20.6783753	8.7617156	15.495992
	32.696342	14.9402107	3.21512922
	33.096857	2.88401702	3.75450454
	49.0494896	8.68385597	17.6259324
	30.489225	12.3057881	5.02475626
	20.7014395	11.824702	7.15042388
Siphonaria	44.2912027	0.80122983	4.64273347
	10.075348	10.5856665	11.3450657
	40.004128	12.6420001	11.4324746
	20.1846314	10.8586997	15.8942614
	21.2777202	10.8202416	13.768649
	23.2425417	11.9413388	11.4046803

	24.9964599	7.8272382	16.1916494
	11.623345	8.26638936	18.4212097
	16.3755742	15.5630382	24.3715418
	16.0058039	4.79179263	18.5816487

543

Appendix 3: Associate Editor's comments on revised manuscript (Round 2)

Field study of cadmium bioaccumulation in two resident limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline

Commented [A1]: Delete and just change your title to "Cadmium bioaccumulation in two resident limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline"

Abstract

The study investigates cadmium (Cd) bioaccumulation in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline from three sites~~,~~: Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP). Significant spatial variations in Cd accumulation were observed, with the highest concentrations found in limpets from NNP (*S. granularis* – 4.17 ± 1.99 µg g^{-1}, *S. capensis* – 2.92 ± 1.45 µg g^{-1}) despite having the lowest water Cd levels (0.13 ± 0.07 µg L^{-1}). This suggests alternative Cd uptake pathways, such as dietary intake from contaminated algae or sediments. Additionally, larger limpets exhibited higher Cd concentrations, highlighting the importance of considering organism size in biomonitoring studies. The bioconcentration factor (BCF) analysis further indicated significant site-specific differences, with the highest BCF at NNP (*S. granularis* – 32.59 ± 15.62, *S. capensis* – 22.81 ± 11.29). These findings highlight the relationship between environmental exposure and physiological traits in Cd bioaccumulation and the necessity for site-specific assessments and using multiple bioindicator species in environmental monitoring. The study shows the importance of continued research into long-term and spatial variations in metal bioaccumulation in marine ecosystems.

Significance:

- Highlights Cd bioaccumulation in limpets indicating potential pollution pathways.
- Demonstrates *S. granularis* and *S. capensis* as bioindicators ~~Sp.~~ for Cd monitoring.
- Spatial significance stresses the importance of site-specific monitoring.
- Organism size matters in pollution assessments.
- There is no correlation between water and ~~organism~~ the metal concentrations of organisms.

Keywords: Bioconcentration factor, bioindicator, ICP-MS, intertidal, marine pollution

Supplementary data inserted at the end of the document.

Introduction

Coastal regions across the world are subjected to significant metal pollution, resulting from agricultural and industrial activities.[1] With over 70% of South Africa's coastline inhabited, elevated anthropogenic pressures on the nearshore marine environment are becoming more prevalent.[2] Metals naturally occur in the marine environment at low concentrations~~,~~ ; however, due to increased anthropogenic pressures, coastal waters are becoming progressively vulnerable to a rise in metal pollution.[3] Metals pose a considerable threat to marine ecosystems due to ~~organism~~their toxicity at low concentrations, environmental persistence, and bioaccumulation ability.[4] Bioaccumulation refers to the process of organismal toxicant uptake, such as chemicals or metals, through consumption of contaminated food or direct exposure, be it over a short (acute) or long (chronic) periods.[4,5] The degree of metal bioaccumulation is influenced by factors such as the metal bioavailability, the route of uptake, and sequestration or depuration of the toxicant.[6] The greatest threat associated with metal bioaccumulation within the tissues of organisms is biomagnification in the higher trophic levels.[7,8]

Cadmium (Cd) primarily enters waterbodies through industrial sources, including zinc (Zn) smelting, electroplating, fossil fuel combustion, plastics, pesticides, and oil refining.[9] Research on Cd has intensified globally due to its toxicity at low concentrations, and its ability to biomagnify through trophic levels, impacting humans.[1,10-18] Cadmium accumulation occurs in organisms primarily via ingestion as the metal ions are poorly soluble in water, binding to suspended particulate matter (SPM) in the ocean, which is ingested and absorbed by ~~filter~~ filter-feeding organisms.[4] Local studies have demonstrated that gastropods, such as limpets, accumulate metals and can serve as potential bioindicator species.[19,20] Despite the variety of bioindicator species used in South Africa, information on the responses of limpets to metals in the marine environment is limited.

A commonly used approach for environmental biomonitoring is quantifying pollutant loads in organisms that bioaccumulate.[21] Metal concentrations in tissues depend~~s~~ on the uptake, excretion, and metabolic capacity to either degrade and/or transform these toxic pollutants.[21] The use of a single bioindicator species when determining metal pollution is not ideal because ~~certain~~specific metal concentrations may differ from one species to another.[22] Therefore, different species inhabiting different coastal zones need to be assessed. Recent studies include using the invasive Mediterranean mussel (*Mytilus galloprovincialis*) and endemic Brown mussel (*Perna perna*) along the southern and east coasts of South Africa.[3,23-25] Limpets were selected for this study due to their wide distribution, feeding behaviour, being reasonably sessile, and distinct

ability to tolerate environmental challenges, making them a key study organism when looking at the effect of environmental changes on intertidal organisms.[19,26] The limpets *Scutellastra granularis* (Linnaeus 1758), a prosobranch found high on the shore, and *Siphonaria capensis* (Quoy and Gaimard 1833), a pulmonate gastropod found in pools and on emergent mid-shore rocks[27,28], were selected to investigate the variation in Cd uptake and bioaccumulation across key sites along the South African coastline. These species of limpet were used to investigate Cd bioaccumulation due to their different phylogenies (i.e., pulmonate vs patellogastropod) and associated tidal heights along their sympatric distribution in South Africa.

This study describes the spatial patterns of Cd concentrations in South African coastal waters using *S. granularis* and *S. capensis* as quantitative bioindicators. It includes investigating the effect of weight and size on Cd bioaccumulation. Spatial patterns for each species are used to compare Cd concentrations in water and tissue samples with inferred bioconcentration factors, discussing these in terms of background (environmental) concentrations and their sources.

Materials and methods

Site selection and animal collection

The study took place ~~at~~in three marine protected areas~~,~~ chosen in a west-east direction along the South African coastline. The South African coastline is a dynamic environment surrounded by two major ocean currents bringing contrasting environmental conditions.[29] The west coast is a cool temperate ecoregion influenced by the Benguela Current that transports cold nutrient-rich water, whereas the southern coast is classified as warm temperate and influenced by the warm water Agulhas Current.[27-30] Sites selected included, two from the west coast; the Namaqua National Park (NNP – 30º51'31.8" S, 17º34'31.1" E) in the Northern Cape and the West Coast National Park (WCNP – 33º8'56.2" S, 17º59'57.9" E) in the Western Cape, and the Garden Route National Park (GRNP – 34º01'14.9" S, 23º52'26.7" E) in the Eastern Cape from the southern coast ecoregion of South Africa (Figure 1). Field fresh samples of similar size (total shell length) were collected from all three sites (Σn = 3 sites x 2 species x 10 individuals (replicates) = 60) in January 2020 (Supplementary data, Table S3). The number of individuals was determined by both the [anonymised] animal ethics committee (number anonymised) and the SANParks permitting office (permit number anonymised). Organisms were collected during low tide (at first daylight). Organisms were carefully removed from the rock surface using either a spatula or by hand, and only undamaged individuals were selected. Individuals were classified as undamaged when there was no visible damage ~~done~~ to the shell or foot. Field fresh samples collected at each site were

flash frozen in liquid nitrogen (-196ºC) and stored at -20ºC for baseline metal analysis. Water samples (Σn = 3 sites x 3 water samples (replicates) at 50 ml each = 9) were collected from each site (1 sample per day of sampling) and stored at -20ºC for background metal analysis. During collection of water samples, physico-chemical parameters were recorded using a Thermo Scientific Eutech Elite PCTS multi-parameter meter, including: temperature (ºC), pH, and salinity (PSU).

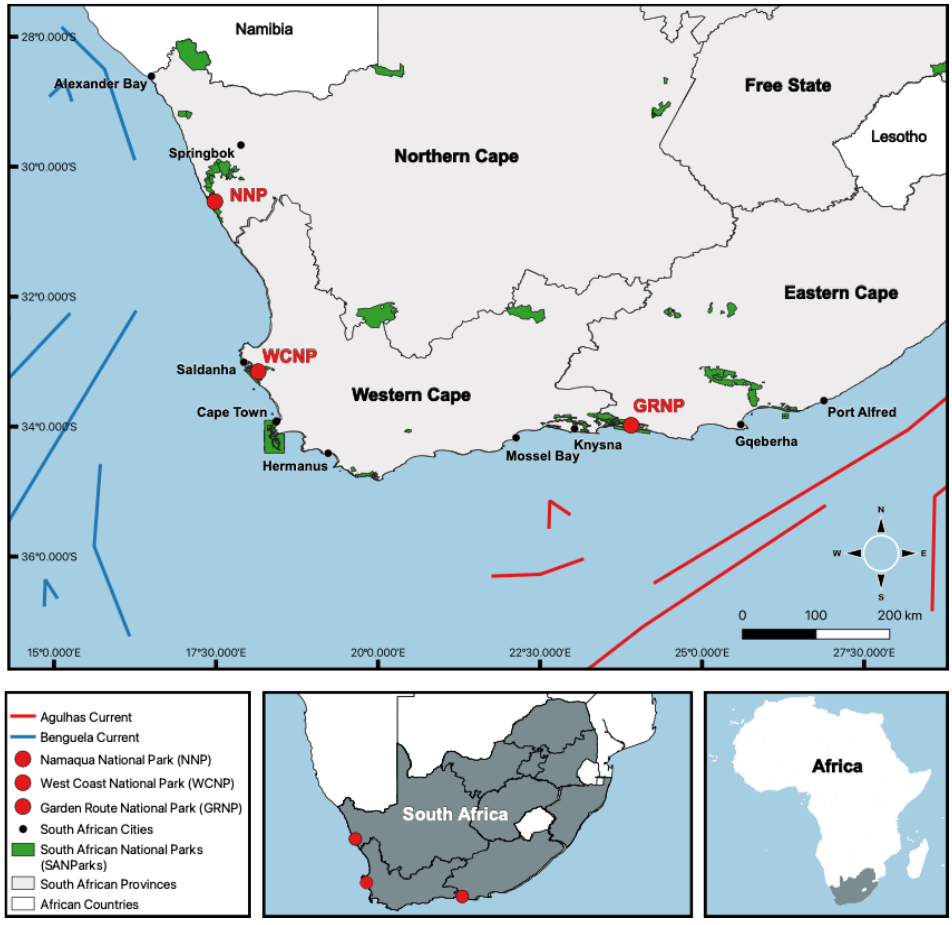

Figure 1: Map of sampling localities along the West and Southern coasts of South Africa. Localities include Namaqua National Park (NNP) and the West Coast National Park (WCNP) along the West coast, and the Garden Route National Park (GRNP) along the Southern coast.

Bioaccumulation analysis

Sample preparation and digestion

The water samples (50 ml) stored in 50ml Falcon Centrifuge Tubes, were filtered using a Millipore sintered glass filtration system (Merck, South Africa), through a gridded cellulose nitrate membrane filter (type 114, 0.45 µm). Samples were acidified to 1% with Suprapur® nitric acid – 63.01 g mol^{-1} (HNO_3) (Merck, South Africa).

Whole organism tissue was removed from the shell using a spatula before being used for metal accumulation analysis in accordance with the methods outlined by Boss and Fredeen[31] and Gaines[32], and adapted from Erasmus et al.[29] Frozen samples were weighed (± 0.01 g) to obtain a wet weight, after which the samples were freeze-dried (-50ºC at 0.61 mbar) for 48 hours using the Martin-Christ LDplus Freeze Dryer (Christ Freeze Dryers, Niedersachsen, Germany). The freeze-dried (whole organism) samples were weighed to obtain a final dry weight, in which the weight was normalised using the bioconcentration factor calculation, before being digested using the MARS 6 One Touch Technology Microwave (CEM, Charlotte, United States of America). Before digestion, the Teflon bombs used during processing were cleaned and dried in accordance with the cleaning method, using standard grade HNO_3 (Merck, South Africa) and hydrogen peroxide (H_2O_2) (Merck, South Africa). Standard reference material (SRM, Mussel Tissue – 2976, 109% recovery), as well as the freeze-dried samples, were individually added to each Teflon bomb along with 5 ml Suprapur® 65% HNO_3 (Merck, South Africa). After digestion, the samples were allowed to cool, before being placed in 15 ml Falcon tubes. Samples were gravimetrically diluted using double distilled water (18.2 Ω) to a total volume of 10 ml (Millipore, United States of America).

ICP-MS Analysis

Water (diluted by factor of 10) and tissue samples were analysed using a PerkinElmer NexION® 300 series Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (PerkinElmer, Waltham, United States of America). The ICP-MS was calibrated using 0, 0.01, 0.1 and 1 ppb calibration standards, made using the appropriate dilutions from a 100 ppm Cd stock solution (100 mg L^{-1}). An internal standard (Iridium – 193) was added to each water and tissue sample (100ppm) before analysis for quality assurance, along with a Standard Reference Material (Mussel Tissue 2976), ensuring sufficient recovery and no drifting occurred during the analysis.

Bioconcentration factor (BCF)

The bioconcentration factor (BCF) was determined according to the method of Abel.[33] This method determined the ratio between the Cd concentration in organisms and water samples ($C_{Organism\ dry\ weight}/C_{Water}$), where the $C_{Organism\ dry\ weight}$ (µg g^{-1} dry weight) and C_{Water} (µg L^{-1}) are the fractions of the element within the organism and the environment (water). The median concentration values of the element present in each organism and the water were used to calculate the BCF.

Statistical analysis

Statistical analyses were conducted using RStudio (v. 4.0.3). All data met the assumptions of normality and homogeneity of variance, following Levene's and Shapiro-Wilk tests, respectively.[34] Following testing of assumptions, one-way Analysis of Variances (ANOVAs) were performed on Cd concentrations in water samples (factors site, three levels and fixed, significance set at $p < 0.05$), tissue samples (factors site, three levels and fixed, significance set at $p < 0.05$), and bioconcentration factor (factors site, three levels and fixed, significance set at $p < 0.05$) for each species respectively. Where there were significant factors, a pairwise comparison was performed using Tukey's HSD.[34] All statistical analyses were performed using the *car* and *tidyverse* packages in RStudio.[35,36] Barplots (±SEM) were created using the *ggplot2* and *plyr* packages in RStudio.[37,38]

Results

Environmental water quality parameters

Physico-chemical parameters; temperature (ºC), pH, and salinity (PSU) measured *in situ* are represented in Table 1. The measured parameters across the three sites showed ~~grea~~significant variation, between sampling periods at each site (high SD values) as well as among the sites themselves. Temperature showed significant variation across sites ($F_{(2, 8)} = 91.5$, $p = 3.2e^{-05}$). Water temperature at GRNP was significantly higher than both west coast sites (NNP and WCNP, Table 1). Water temperature also varied ~~significant~~considerably between the two west coast sites (NNP and WCNP), with significantly lower temperatures recorded at NNP. All other water quality parameters (pH and salinity) showed non-significant differences across the three sites ($p > 0.05$, Table 1).

Table 1: Mean (±SD) water quality measures taken in situ at each sampling site (NNP – Namaqua National Park, WCNP – West Coast National Park, GRNP – Garden Route National Park) during each day of sampling (n=3). Common superscripts indicate statistically significant differences in water quality parameters across sites ($p<0.05$)

	NNP	WCNP	GRNP
Temperature (ºC)	16.18 ± 2.01^{ab}	16.47 ± 0.06^{ac}	19.40 ± 0.17^{bc}
pH	8.01 ± 0.10	7.06 ± 0.69	7.71 ± 0.31
Salinity (PSU)	33.17 ± 1.17	38.33 ± 2.52	35.33 ± 3.21

Cadmium concentrations versus weight and size

Cadmium concentrations in whole body tissues (without shell) were plotted against the wet weight (g) and size (mm) for both species of limpets, *S. granularis* and *S. capensis*; with results being illustrated in Figure 1A and B, respectively. Concentrations of Cd in whole body limpets showed no significant change with increasing weight of either species, $F_{(3, 56)} = 0.0482$, $p = 0.986$ (Figure 2A). With increasing weight, both *S. granularis* and *S. capensis* had non-significant changes in their individual Cd concentrations, with R^2 values of 0.001 and 0.002, respectively. In contrast, concentrations of Cd in whole body limpets showed a significant change with increasing size, $F_{(3, 56)} = 5.347$, $p = 0.003$ (Figure 2B). *S. capensis* showed a greater increase in Cd concentrations with an increase in size ($R^2 = 0.26$), whereas the larger *S. granularis* showed a similar increase in Cd concentrations with an increase in size ($R^2 = 0.20$).

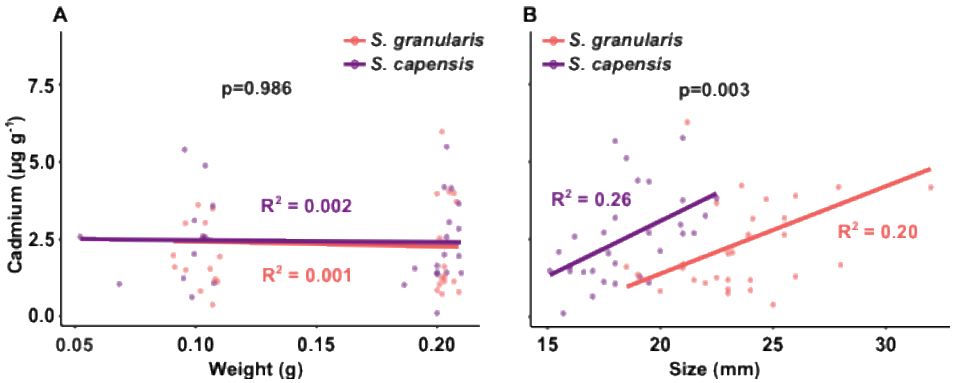

Figure 2: General linear model (GLM) showing the relationship between cadmium concentrations (µg g^{-1}) and the weight (A) and size (B) of *S. granularis* and *S. capensis*

Cadmium concentration in field-fresh water samples from the southern and west coasts

Cadmium concentrations within water samples collected at each respective site during the sampling of *S. granularis* and *S. capensis* are represented in Figure 3. ~~N~~There were no statistically significant differences in cadmium concentrations ~~were present~~ in water samples between the three sites (Figure 3, Table 2). Water samples from the southern coast (GRNP) had the greatest Cd concentrations (0.24 µg L^{-1}), whilst samples from the west coast at NNP had the lowest concentrations (0.13 µg L^{-1}).

Table 2: One-way ANOVA results comparing Cd levels in field-fresh water samples (n=3) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh water samples ($F_{(2, 6)}$ = 3.221, p = 0.112)

	Df	Sum Sq	Mean Sq	*F*	p
Sites	2	0.0219	0.0110	1.8370	0.2386
Residuals	6	0.0358	0.0060		
Total	8	0.0578			

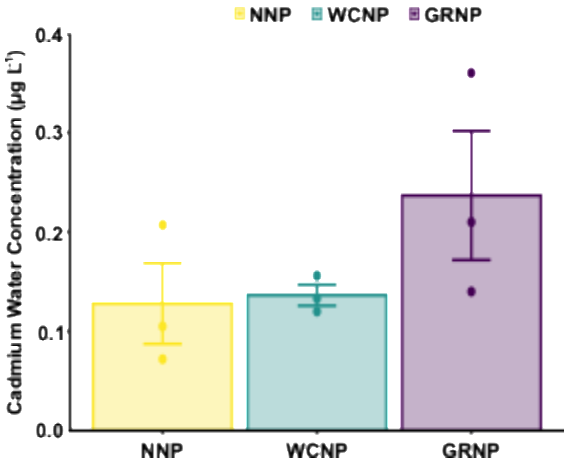

Figure 3: Mean (±SEM) levels of cadmium in water samples (n=3 per site) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP)

Commented [A2]: Delete this table. You do not need to have this table this could just be reported in text since you only have a single one-way ANOVA

Commented [A3R2]: You have so many ANOVA tables - you could just report the ANOVAs in the text and move all these tables to your supplementary.

Cadmium concentrations in field-fresh S. granularis and S. capensis from the southern and west coasts

The measured concentrations of Cd in both *S. granularis* and *S. capensis* are presented in Table 3 and Figure 4A. Spatial differences in Cd concentrations from whole body *S. granularis* tissue showed significance, $F_{(2, 29)}$ = 11.978, p = 0.0002 (Table 3). At NNP, *S. granularis* accumulated significantly higher concentrations of Cd than both remaining sites, WCNP and GRNP (Figure 4A. A similar trend was observed in *S. capensis*, with spatial variances in Cd concentrations across all three sites, $F_{(2, 29)}$ = 9.522, p = 0.0007 (Table 3). Limpets collected from NNP having significantly higher Cd concentrations (p<0.01) to those collected and analysed from the WCNP (Figure 4A). Dissimilarly, along the southern coast at GRNP, *S. capensis* had the highest Cd concentrations out of the three sites, being significantly higher than WCNP (p<0.001).

Table 3: One-way ANOVA results comparing Cd levels in field-fresh *S. granularis* and *S. capensis* (n=10) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh *S. granularis* ($F_{(2, 27)}$ = 2.260, p = 0.124) and *S. capensis* ($F_{(2, 27)}$ = 1.961, p = 0.160)

S. granularis					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	2	46.355	23.178	11.978	0.0002
Residuals	27	52.244	1.935		
Total	29	98.599			
S. capensis					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	2	25.640	12.820	9.522	0.0007
Residuals	27	36.353	1.346		
Total	29	61.993			

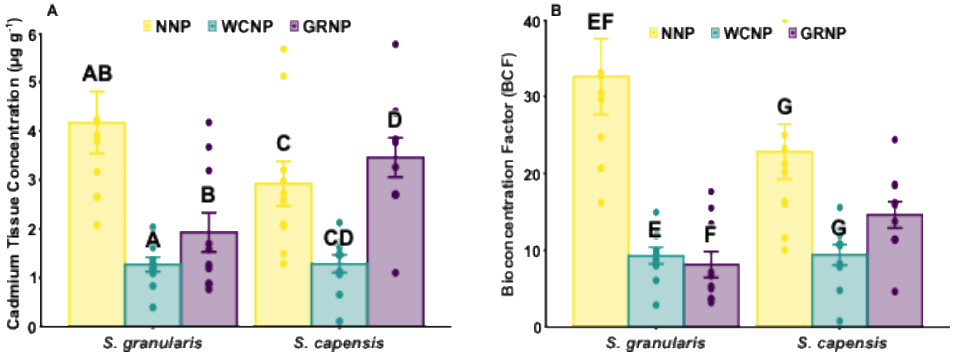

Figure 4: Mean (±SEM) levels of cadmium (A) in *S. granularis* and *S. capensis* (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and Garden Route National Park (GRNP). Bioconcentration factor (B) variation in *S. granularis* and *S.* capensis (n=10) using cadmium concentrations in water and tissue samples collected from each site (NNP, WCNP, and GRNP)

A Redundancy Analysis (RDA) triplot is shown in Figure 5. Individual *S. granularis* and *S. capensis* from each site are represented by ellipses (encompassing 95% of the sample area), with environmental variables depicted as arrows indicating direction and strength of influence. The RDA explains 62.58% of the total variation in Cd bioaccumulation attributable to environmental variables, with RDA 1 (x-axis) accounting for 37.20%, and RDA 2 (y-axis) for 25.38% of the variance in bioaccumulation.

The plot indicates that temperature, pH, and limpet size (length) in GRNP are the strongest positive drivers of Cd bioaccumulation in both species along the southern coast. Both *S. granularis* and *S. capensis* from GRNP exhibit the largest ellipses, indicating greater individual variation in bioaccumulation levels. In contrast, individuals from WCNP show tighter ellipses, reflecting less variation in bioaccumulation. For these populations, weight and Cd concentration in water are the most influential positive drivers, suggesting a strong relationship between individual weight and Cd bioaccumulation. Finally, both species from NNP display the tightest ellipses, indicating minimal intra-site variation in bioaccumulation. Bioaccumulation is also positively influenced by pH and size (length) and negatively influenced by salinity and Cd levels in water.

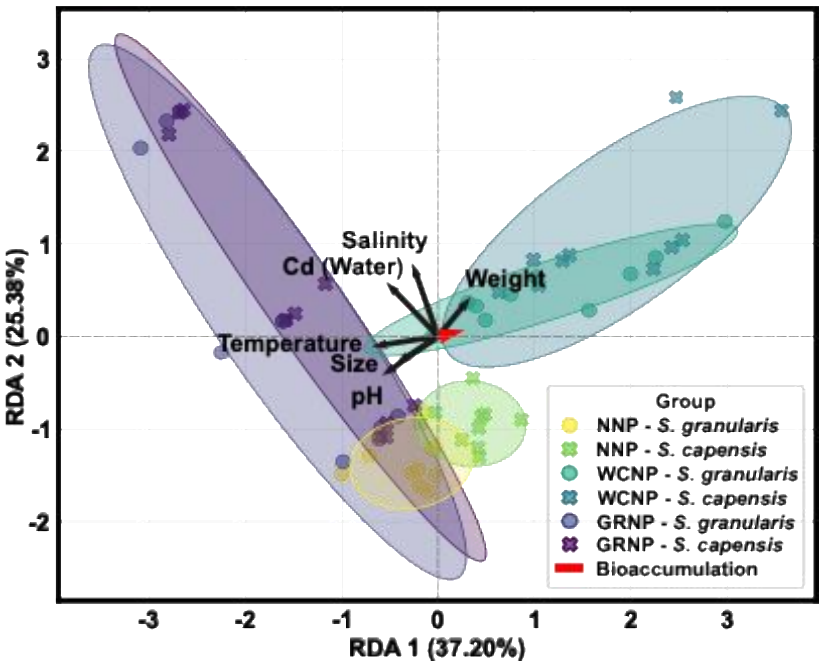

Figure 5: Redundancy Analysis (RDA) triplot showing bioaccumulation of Cd in *S. granularis* and *S. capensis* and the effect that environmental variables (drivers) have. Sites and species are represented by ellipses, and drivers (environmental variables) by arrows. Ellipses for each group are set to 95% inclusion. The triplot explains 62.58% of the variation, with 37.20% on the x-axis (RDA 1) and 25.38% on the y-axis (RDA 2).

Bioconcentration factor of cadmium in S. granularis and S. capensis from the southern and west coasts

The results obtained show the bioconcentration factor (BCF) of Cd in both *S. granularis* and *S. capensis* and are presented in Table 4 and Figure 4B. Spatial differences in the BCF of Cd in *S. granularis* showed significance, $F_{(2, 29)}$ = 20.120, p<0.001. As seen in the Cd concentrations of *S. granularis,* the BCF spatial variance showed the same pattern. *S. granularis* from NNP showed the greatest BCF, differing significantly from ~~both~~ WCNP and GRNP (p<0.001, Figure 4B). No difference was observed between the BCF of *S. granularis* at WCNP and GRNP (p>0.05). Unlike in the Cd concentrations observed in *S. capensis* (Figure 4A), a dissimilar trend was observed in the BCF values across sites, although significantly different spatial differences were observed, $F_{(2, 29)}$ = 7.86, p = 0.002 (Figure 4B, Table 4). *S. capensis* from NNP exhibited the greatest BCF, differing significantly with limpets sampled from WCNP (p<0.01, Figure 4B). No other significant

spatial differences were observed in *S. capensis* between NNP and GRNP, nor between NNP and GRNP (p>0.05, Figure 4B).

Table 4: One-way ANOVA results comparing the Bioconcentration Factor (BCF) in field-fresh *S. granularis* and *S. capensis* (n=10) from three sites (NNP, WCNP, and GRNP). Levene's test indicated equal variances for untransformed BCF variations in field-fresh *S. granularis* ($F_{(2, 27)}$ = 3.259, p = 0.055) and *S. capensis* ($F_{(2, 27)}$ = 2.508, p = 0.100)

S. granularis					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	2	3805.382	1902.691	20.120	$4.472e^{-06}$
Residuals	27	2553.365	94.569		
Total	29	6358.747			
S. capensis					
	Df	**Sum Sq**	**Mean Sq**	***F***	**p**
Sites	2	912.587	456.293	7.86	0.002
Residuals	27	1566.557	58.021		
Total	29	2479.144			

Discussion

While metal contaminants occur naturally in the marine environment at low concentrations, anthropogenic influence on the marine environment has exacerbated the situation.[39,40] The results from this study reveal significant bioaccumulation of Cd in two limpet species, *S. granularis* and *S. capensis*, collected from different coastal regions in South Africa. Comparing Cd concentrations in both limpet species to that found in water samples analysed indicates that Cd is strongly accumulated in both species from their respective surrounding environments, as indicated from the BCF results. Cadmium is known as a toxic metal, even at low concentrations, and as such is considered non-essential for biological function. Metalloproteins such as Metallothionein (MT) are produced and upregulated in limpets as a response to cadmium-induced oxidative stress, serving as a protective mechanism against metal toxicity.[11,13,43] Potential sources of Cd in the marine environment come from the emissions of industrial combustion processes,

metallurgic industries, road transport, and waste streams, all of which occur along the west and southern coasts of South Africa.[20,29,41,42] Water samples analysed for Cd showed variation between the southern coast site (GRNP) and the two west coast sites (NNP and WCNP). Studies conducted by Reinecke et al.[19,43] indicated Cd concentrations along the South African coastline varied considerably spatially and temporally. Degger et al.[40], measured unexpectedly high metal concentrations (Cd and Zn) in brown mussels (*P. perna*) along the southern coast at Tsitsikamma National Park (within GRNP) compared to localities with known anthropogenic activities and influence (such as WCNP). High concentrations of metals in the study by Degger et al.[40] follow a similar pattern shown in data from the present study (both the water and organisms). This i~~s indicative of~~ndicates long-term exposure, resulting from freshwater input through geogenic weathering and frequent upwelling ~~that occur~~ along South Africa's southern coast.[29] *S. capensis* showed the greatest Cd accumulation at Namaqua National Park (NNP), with both species having the greatest metal uptake at this site despite lower Cd concentrations in the corresponding water samples. Additionally, these limpets possess physiological traits that enhance Cd retention, such as slower excretion rates or higher binding affinities in tissue proteins.[21,44] This finding suggests similarity with previous studies, indicating bioaccumulation in marine organisms occurring through various pathways, including dietary intake and sediment interaction.[4,29]

The bioconcentration factor (BCF) indicated Cd had been accumulated in whole tissue samples of *S. granularis* and *S. capensis* in comparison to the concentration of Cd in the environment.[33] Whole tissue samples of both species varied significantly in their accumulation of Cd in relation to their sampling site. This however does not correlate with Cd concentrations from water sampled. Interestingly, despite higher water Cd levels at GRNP, the bioconcentration factor (BCF) was highest at NNP for *S. granularis*, suggesting species-specific and site-specific bioaccumulation dynamics, seasonal and exposure level variation.[45] High Cd concentrations at GRNP, as discussed previously, are a direct result of both the freshwater input from geogenic weathering in the region and from upwelling events that are known to occur along the southern coast of South Africa at GRNP.[29,40] The significantly high BCFs at NNP are a direct result of the significantly high concentrations of Cd detected in whole tissue samples of *S. granularis* and *S. capensis*, despite the lower concentrations of Cd in the water. This result is indicative of either a short-term but high-dose exposure to Cd or a different means of uptake in both limpet species other than directly from the water column.[40,46] Elevated metal levels along the west coast, observed at NNP and WCNP, are a direct result of the mining and port activities, as well as the

iron and crude oil facilities in the surrounding areas, which lead to elevated pollution of local coastal waters affecting the inhabitants of these waters.[20,29,42]

The anatomy and physiology of the limpets is also known to play a primary role in the uptake and accumulation of metals. Differences in body sizes of individuals of the same species has been shown to result in varying metal tissue concentrations.[22,29,47,48] The size of marine ectotherms is related to their metabolic activities in relation to their life history stage.[8] Various metals such as copper, iron, and zinc in moderate concentrations are essential in an organism's growth and development, however non-essential metals such as Cd lead to reduced growth and development, even at low concentrations.[8] Limpet size was positively correlated with tissue Cd concentration, indicating that larger individuals tend to accumulate more Cd, potentially due to prolonged exposure and greater overall uptake over time.[8,48] This size-related bioaccumulation trend has been observed in other marine invertebrates and highlights the importance of considering organism size in biomonitoring studies.[19,49]

The physiological and anatomical differences between *S. granularis* and *S. capensis* likely contribute to the observed differences in Cd bioaccumulation. *S. granularis*, a prosobranch, and *S. capensis*, a pulmonate gastropod, exhibit distinct respiratory and excretory mechanisms that influence metal uptake and storage.[44] Pulmonate gastropods, such as *S. capensis*, exhibit higher rates of metal accumulation due to their ability to absorb metals through their foot and mantle cavity, in addition to dietary intake.[21,44] Comparative studies have shown that bioaccumulation patterns in marine organisms can vary widely based on local environmental conditions and the species involved. Research on the Brown mussel (*P. perna*) has demonstrated significant spatial variation in metal concentrations along the South African coastline, similar to the patterns observed in this study.[40] These findings underscore the importance of site-specific assessments and the use of multiple bioindicator species to accurately gauge environmental metal pollution levels.[20,21]

Conclusion

This study provides insight into the spatial patterns of Cd bioaccumulation in two limpet species, *Scutellastra granularis* and *Siphonaria capensis*, along the South African coastline. The findings from both limpet species showed significant spatial variation in Cd accumulation, with *S. granularis* having the highest Cd levels at NNP despite lower Cd concentrations in corresponding water samples. This suggests alternative pathways for Cd uptake, such as dietary intake from

contaminated algae or sediments. Additionally, larger individuals from both species exhibited higher Cd concentrations, likely due to greater exposure duration, emphasising the importance of considering organism size in biomonitoring studies. The bioconcentration factor (BCF) analysis further indicated that the BCF of Cd in *S. granularis* and *S. capensis* was highest at NNP, highlighting the complex interplay between environmental exposure and physiological traits in Cd bioaccumulation. These findings emphasise the need for site-specific assessments and the use of multiple bioindicator species to effectively assess environmental metal pollution levels in marine ecosystems. Future research should focus on the long-term monitoring of these species across a greater seasonal and spatial gradient, and the exploration of additional environmental factors influencing metal bioaccumulation.

References

1. Boudjema K, Kourdali S, Bounakous N, Meknachi A, Badis A. Catalase activity in Brown Mussels (*Perna perna*) under acute cadmium, lead, and copper exposure and depuration tests. J Mar Biol. 2014:1–9. https://doi.org/10.1155/2014/830657
2. Sink K, Holness S, Harris L, Majiedt P, Atkinson L, Robinson T, Kirkman S, Hutchings L, Leslie R, Lamberth S, Kerwath S, Von der Heyden S, Lombard A, Attwood C, Branch G, Fairweather T, Taljaard S, Weerts S, Cowley P, Awad A, Halpern B, Grantham H, Wolf T. National Biodiversity Assessment 2011: technical report. In: Marine and Coastal Component, 4. South African National Biodiversity Institute; 2012. p. 325.
3. Greenfield R, Brink K, Degger N, Wepener V. The usefulness of transplantation studies in monitoring of metals in the marine environment: South African experience. Mar Pollut Bull. 2014;85:566–573. https://doi.org/10.1016/j.marpolbul.2014.03.032
4. Jakimska A, Konieczka P, Skóra K, Namieśnik J. Bioaccumulation of metals in tissues of marine animals, Part I: The role and impact of heavy metals on organisms. Pol J Environ Stud. 2011a; 20:1117–1125.
5. Perera PACT, Kodithuwakku SP, Sundarabarathy TT, Edirisinghe U. Bioaccumulation of cadmium in freshwater fish: An environmental perspective. Insight Ecology. 2015;4:1–12. https://doi.org/10.5567/ECOLOGY-IK.2015.1.12
6. Valavanidis A, Vlachogianni T. Metal pollution in ecosystems: ecotoxicology studies and risk assessment in the marine environment. 2010.
7. Fang T-H, Hwang J-S, Hsiao S-H, Chen H-Y. Trace metals in seawater and copepods in the ocean outfall area off the northern Taiwan coast. Mar Environ Res. 2006;61:224–243. https://doi.org/10.1016/j.marenvres.2005.10.002
8. Jakimska A, Konieczka P, Skóra K, Namieśnik J. Bioaccumulation of metals in tissues of marine animals, part II: Metal concentrations in animal tissues. Pol J Environ Stud. 2011b;20:1127–1146.
9. Chandrudu M, Radhakrishnaiah K. A study on the toxicity of cadmium on certain aspects of protein metabolism of the freshwater mussel *Lamellidens marginalis* (Lamarck) and

freshwater fish *Labeo rohita* (Hamilton). Int J Environ Sci. 2013;4:15–27. https://doi.org/10.6088/ijes.2013040100003
10. CCME (Canadian Council of Ministers of the Environment). Canadian water quality guidelines for the protection of aquatic life: Cadmium. Winnipeg. 2014.
11. Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Nair AR, Munters E, Artois TJ, Nawrot T, Vangronsveld J, Smeets K. Cadmium stress: An oxidative challenge. BioMetals. 2010;23:927–940. https://doi.org/10.1007/s10534-010-9329-x
12. Environmental Protection Agency (EPA). Fact sheet: Aquatic life ambient water quality criteria update for cadmium. 2016.
13. Legeay A, Achard-Joris M, Baudrimont M, Massabuau J-C, Bourdineaud J-P. Impact of cadmium contamination and oxygenation levels on biochemical responses in the Asiatic clam *Corbicula fluminea*. Aquat Toxicol. 2005;74:242–253. https://doi.org/10.1016/j.aquatox.2005.05.015
14. Neff JM. 2002. Cadmium in the ocean, in: Bioaccumulation in Marine Organisms. Elsevier pp. 89–102. https://doi.org/10.1016/B978-008043716-3/50006-3
15. Phillips DJH. The Common Mussel *Mytilus edulis* as an Indicator of Pollution by Zinc, Cadmium, Lead and Copper. I. Effects of Environmental Variables on Uptake of Metals. Mar Biol. 1976;38:59–69. https://doi.org/10.1007/bf00391486
16. Ray S. Bioaccumulation of cadmium in marine organisms. Experientia. 1984;40:14–23. https://doi.org/10.1007/bf01959097
17. Scholz N. Accumulation, loss and molecular distribution of cadmium in *Mytilus edulis*. Helgoländer Meeresunters. 1980;33:68–78. https://doi.org/10.1007/bf02414736
18. Yamamura M, Suzuki KT, Hatakeyama S, Kubota K. Tolerance to cadmium and cadmium-binding proteins induced in the midge larvae, *Chironomus yoshimatsui* (Diptera, Chironimidae). Comp Biochem Physiol C Toxicol Pharmacol. 1983;75:21–24. https://doi.org/10.1016/0742-8413(83)90005-1
19. Reinecke AJ, Mdzeke NP, Reinecke SA. Spatial and temporal variation in cadmium body loads of four intertidal invertebrates from False Bay, South Africa. Afr Zool. 2012;47:12–25. https://doi.org/10.1080/15627020.2012.11407518
20. Erasmus A, Ikenaka Y, Nakayama SMM, Ishizuka M, Smit NJ, Wepener V. Trophic transfer of pollutants within two intertidal rocky shore ecosystems in different biogeographic regions of South Africa. Mar Pollut Bull. 2020;157:article 111309. https://doi.org/10.1016/j.marpolbul.2020.111309
21. Reguera P, Couceiro L, Fernández N. A review of the empirical literature on the use of limpets *Patella* spp. (Mollusca: Gastropoda) as bioindicators of environmental quality. Ecotoxicol Environ Saf. 2018;148:593-600. https://doi.org/10.1016/j.ecoenv.2017.11.004
22. Mbandzi N, Nakin MDV, Saibu GM, Oyedeji AO. Heavy metal profiles in limpets and algae on the Eastern Cape coast of South Africa. Afr J Mar Sci. 2021;43:293–308. https://doi.org/10.2989/1814232X.2021.1948918
23. Sparks C, Odendaal J, Snyman R. An analysis of historical Mussel Watch Programme data from the west coast of the Cape Peninsula, Cape Town. Mar Pollut Bull. 2014;87:374–380. https://doi.org/10.1016/j.marpolbul.2014.07.047

24. Greenfield R, Wepener V, Degger N, Brink K. Richards Bay Harbour: metal exposure monitoring over the last 34 years. Mar Pollut Bull. 2011;62:1926–1931. https://doi.org/10.1016/j.marpolbul.2011.04.026
25. Wepener V, Degger N. Monitoring of metals in South African harbours using resident brown mussels (*Perna perna*) as indicator organisms. Afr Zool. 2020;55:267–277. https://doi.org/10.1080/15627020.2020.1799720
26. Kankondi SL, McQuaid CD, Tagliarolo M. Influence of respiratory mode on the thermal tolerance of intertidal limpets. PLoS One. 2018;13:1–15. https://doi.org/10.1371/journal.pone.0203555
27. Branch G, Griffiths C, Branch M, Beckley L.. Two oceans: A guide to the marine life of southern Africa, 4th Edition. Cape Town: Struik Nature; 2016.
28. Branch G, Branch M. Living Shores: Interacting with southern Africa's marine ecosystems. Cape Town: Struik Nature. 2018.
29. Erasmus A, Smit NJ, Zimmermann S, Nachev M, Sures B, Wepener V. Metal and metalloid concentrations in the southern African endemic inter- and infratidal super klipfish, *Clinus superciliosus*, from the west and south coasts of South Africa. Mar Pollut Bull. 2021;172:112852. https://doi.org/10.1016/j.marpolbul.2021.112852
30. Bustamante RH, Branch GM, Eekhout S, Robertson B, Zoutendyk P, Schleyer M, Dye A, Hanekom N, Keats D, Jurd M, McQuaid C. Gradients of intertidal primary productivity around the coast of South Africa and their relationships with consumer biomass. Oecologia. 1995;102:189–201. https://doi.org/10.1007/BF00333251
31. Boss CB, Fredeen KJ. Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectroscopy. 3rd Edition. Connecticut: PerkinElmer; 2004.
32. Gaines PR. ICP Operations Guide: A Guide for using ICP-OES and ICP-MS. Inorganic Ventures; 2011.
33. Abel PD. Pollutant toxicity to aquatic animals - methods of study and their applications. Rev Environ Health. 1989;8. https://doi.org/10.1515/reveh-1989-1-407
34. Field A, Miles J, Field Z. Discovering statistics using R. London: SAGE Publications Ltd; 2012. https://doi.org/10.1111/insr.12011_21
35. Fox J, Weisberg S. 2019. An R Companion to Applied Regression, Third edition. California: Sage; 2019.
36. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H. Welcome to the tidyverse. J Open Source Softw. 2019;4(43):1686. https://doi.org/10.21105/joss.01686
37. Wickham H. The Split-Apply-Combine Strategy for Data Analysis. J Stat Softw. 2011;40(1):1–29. https://doi.org/10.18637/jss.v040.i01
38. Wickham H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
39. Ansari TM, Marr IL, Tariq N. Heavy metals in marine pollution perspective – A mini review. J Appl Sci. 2004;4:1-20. https://doi/org/10.3923/jas.2004.1.20

40. Degger N, Wepener V, Richardson BJ, Wu RSS. Application of artificial mussels (AMs) under South African marine conditions: A validation study. Mar Pollut Bull. 2011;63: 108–118. https://doi.org/10.1016/j.marpolbul.2011.04.040
41. OSPAR (Oslo and Paris Conventions). 2010. Quality Status Report 2010: Status and Trend of marine chemical pollution.
42. Clark B, Massie V, Hutchings K, Brown E, Biccard A, Laird M, Harmer R, Makhosonke A, Wright A, Turpie J. 2017. The state of Saldanha Bay and Langebaan Lagoon 2017, technical report.
43. Reinecke AJ, Schoeman W, Reinecke SA, Reinecke S, Schoeman W, Reinecke K. Cellular biomarker responses of limpets (Mollusca) as measure of sensitivity to cadmium contamination. Suid-Afrikaanse Tydskrif vir Natuur-wetenskap en Tegnologie. 2008;27:123–142. https://doi.org/10.4102/satnt.v27i2.86
44. Marchán S, Davies MS, Fleming S, Jones HD. Effects of copper and zinc on the heart rate of the limpet *Patella vulgata* L. Comp Biochem Physiol A Mol Integr Physiol. 1999;123:89 – 93. https://doi.org/10.1016/s1095-6433(99)00043-4
45. Nakhlé KF, Cossa D, Khalaf G, Beliaeff B. *Brachidontes variablilis* and *Patella* sp. as quantitative biological indicators for cadmium, lead and mercury in the Lebanese coastal waters. Environmental Pollution. 2006;142:73-82. https://doi.org/10.1016/j.envpol.2005.09.016
46. Palladino G, Rampelli S, Scicchitano D, Musella M, Quero GM, Prada F, Mancuso A, Seyfarth AM, Turroni S, Candela M, Biagi E. Impact of marine aquaculture on the microbiome associated with nearby Holobionts: The case of *Patella caerulea* living in proximity of Sea Bream aquaculture cages. Microorganisms. 2021;9:455. https://doi.org/10.3390/microorganisms9020455
47. Mubiana VK, Vercauteren K, Blust R. The influence of body size, condition index and tidal exposure on the variability in metal bioaccumulation in *Mytilus edulis*. Environ Pollut. 2006;144:272–279. https://doi.org/10.1016/j.envpol.2005.12.017
48. Sánchez-Marín P, Schultze F, Besada V. Use of limpets as alternative to mussels in metal pollution monitoring; application in the Canary Islands. Environ Pollut. 2022;308:119614. https://doi.org/10.1016/j.envpol.2022.119614
49. Spann N, Aldridge DC, Griffin JL, Jones OAH. Size-dependent effects of low level cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*. Aquatic Toxicology. 2011;105:589-599. https://doi.org/10.1016/j.aquatox.2011.08.010

Supplementary data

Table S1: Physico-chemical variables: temperature, pH, salinity, electrical conductivity, and total dissolved solids measured in situ (n=3) at each site during sampling of field-fresh *S. granularis* and *S. capensis*. Sites include Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP)

Site	Temperature (ºC)	pH	Salinity (PSU)	Electrical conductivity (mS cm^{-1})	Total dissolved solids (ppt)
NNP	18.5	7.84	33	60.1	37.4
	17.7	8.06	31	57.6	35.7
	17.8	8.12	34	61.3	38
WCNP	16.4	6.28	36	63.6	39.7
	16.5	7.31	38	69.9	43.2
	16.5	7.58	41	72.4	45.1
GRNP	19.5	7.8	33	60.6	35.2
	19.5	7.96	39	68.4	40.4
	19.9	7.89	45	80.3	46.7

Table S2: Cadmium (Cd) values in field-fresh water samples (n=3) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh water samples ($F_{(2, 6)}$ = 3.221, p = 0.112)

	NNP	WCNP	GRNP
Cd Concentration in water samples (µg l^{-1})	0.105	0.133	0.140
	0.207	0.156	0.120
	0.072	0.120	0.360

Table S3: Morphometric measurements of *S. granularis* (Scutellastra) and *S. capensis* (Siphonaria) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Measurements include weight (g) and size (mm)

	NNP		WCNP		GRNP	
Species	**Size (mm)**	**Weight (g)**	**Size (mm)**	**Weight (g)**	**Size (mm)**	**Weight (g)**
Scutellastra	24.10	0.0957	22.00	0.201	25.5	0.104
	28.80	0.1	20.00	0.209	22.5	0.108
	24.70	0.101	26.00	0.202	24	0.102
	23.20	0.0908	19.00	0.203	21	0.0959
	25.50	0.103	23.00	0.203	22.5	0.107
	27.90	0.207	23.00	0.11	23	0.202
	23.60	0.205	25.00	0.107	23	0.2
	21.20	0.202	24.00	0.109	32	0.2
	26.00	0.208	28.00	0.0913	21.5	0.207
	23.90	0.201	8.50	0.106	21	0.203
Siphonaria	18.00	0.0954	15.70	0.20	19.5	0.0683
	19.10	0.0949	16.60	0.20	21	0.104
	18.50	0.104	18.00	0.20	19.5	0.0521
	16.20	0.106	17.00	0.20	21	0.107
	17.50	0.103	16.00	0.21	22	0.0995
	20.90	0.209	16.00	0.908	21.5	0.204

	18.00	0.205	18.00	0.864	22.5	0.209
	15.10	0.204	17.50	0.1075	19.5	0.206
	15.50	0.203	20.00	0.0994	21	0.204
	17.70	0.209	17.00	0.0984	19	0.203

544
545

Table S4: Raw accumulation values of Cd measured in field-fresh *S. capensis* (Siphonaria) (n=10) collected from Namaqua National Park (NNP), West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test indicated equal variances for untransformed Cd levels in field-fresh *S. capensis* (Scutellastra) ($F_{(2, 27)}$ = 2.260, p = 0.124) and *S. granularis* ($F_{(2, 27)}$ = 1.961, p = 0.160)

	Cadmium (Cd) accumulation (µg g^{-1})		
Species	**NNP**	**WCNP**	**GRNP**
Scutellastra	3.16514376	1.08869686	3.18878959
	8.78170934	0.83019095	1.26444493
	3.79152699	1.29859698	0.85820639
	2.07593463	1.35995523	1.58030948
	2.64683204	1.19451389	3.66738478
	4.18513177	2.03684873	0.76091392
	4.2363977	0.39318765	0.88856608
	6.27833467	1.18389903	4.17147066
	3.9026208	1.67768911	1.18919232
	2.64978426	1.61210104	1.69226698
Siphonaria	5.66927395	0.10923433	1.09878025
	1.28964454	1.4431792	2.68499889
	5.12052839	1.72352601	2.70568566
	2.58363282	1.48040272	3.76164186
	2.72354819	1.4751596	3.25858026
	2.97504533	1.62800252	2.69910768

	3.19954686	1.06711348	3.8320237
	1.48778817	1.12698442	4.35968629
	2.09607349	2.12176087	5.76793155
	2.04874289	0.65328106	4.39765685

553 Table S5: Bioconcentration factor (BCF) values of Cd measured in field-fresh *S. granularis*
554 (Scutellastra) and *S. capensis* (Siphonaria) ((n=10) collected from Namaqua National Park (NNP),
555 West Coast National Park (WCNP), and the Garden Route National Park (GRNP). Levene's test
556 indicated equal variances for untransformed BCF variations in field-fresh *S. granularis* ($F_{(2, 27)}$ =
557 3.259, p = 0.055) and *S. capensis* ($F_{(2, 27)}$ = 2.508, p = 0.100)

	Bioconcentration factor (BCF)		
Species	**NNP**	**WCNP**	**GRNP**
Scutellastra	24.7276857	7.98555153	13.4737588
	68.6071042	6.08942019	5.34272507
	29.6213046	9.52516124	3.62622417
	16.2182393	9.97522174	6.67736402
	20.6783753	8.7617156	15.495992
	32.696342	14.9402107	3.21512922
	33.096857	2.88401702	3.75450454
	49.0494896	8.68385597	17.6259324
	30.489225	12.3057881	5.02475626
	20.7014395	11.824702	7.15042388
Siphonaria	44.2912027	0.80122983	4.64273347
	10.075348	10.5856665	11.3450657
	40.004128	12.6420001	11.4324746
	20.1846314	10.8586997	15.8942614
	21.2777202	10.8202416	13.768649
	23.2425417	11.9413388	11.4046803

	24.9964599	7.8272382	16.1916494
	11.623345	8.26638936	18.4212097
	16.3755742	15.5630382	24.3715418
	16.0058039	4.79179263	18.5816487

558