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Tese de doutoramento

Potencial das algas pardas como biomonitores de elementos contaminantes: importancia dos mecanismos de carga

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POTENCIAL DAS ALGAS PARDAS COMO BIOMONITORES DE ELEMENTOS CONTAMINANTES: IMPORTANCIA DOS MECANISMOS DE CARGA

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Declaración de conflitos de interese

Eu, Antón Vázquez Arias, presento a miña tese titulada "Potencial das algas pardas como biomonitores de elementos contaminantes: importancia dos mecanismos de carga" e, seguindo o procedemento adecuado ó regulamento, declaro que:

1) A tese abarca os resultados da elaboración do meu traballo.
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3) Teño o permiso tanto dos coautores como das revistas para empregar os traballos publicados incluídos na tese.
4) Todas as figuras son de elaboración propia.

En Santiago de Compostela a 29 de novembro de 2024.

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Índice

1. **Abstract** 4
2. **Resumo** 6
3. **Introdución** 8
   - 3.1. Contaminación ambiental 8
   - 3.2. PTEs como contaminantes 8
   - 3.3. Contaminación no medio mariño 9
   - 3.4. Control da contaminación 10
   - 3.5. Biomonitorización 11
   - 3.6. Biomonitorización con algas e a súa importancia 11
   - 3.7. Avances e ocos 12
   - 3.8. Unidade da tese 14
4. **Obxectivos** 16
5. **Metodoloxía** 18
   - 5.1. Recollida de mostras 18
   - 5.2. Transplantes naturais 18
   - 5.3. Transplantes en bolsas 19
   - 5.4. Desvitalización 19
   - 5.5. Recollida de auga 19
   - 5.6. Determinacións elementais 20
   - 5.7. Extracción secuencial 20
   - 5.8. Criofixación 20
   - 5.9. Microscopía 21
   - 5.10. Obtención de mapas de distribución elemental 21
   - 5.11. Busca bibliográfica 22
   - 5.12. Análise de datos 23
   - 5.13. Procesado de imaxes 23
6. **Capítulos publicados** 24
   - 6.1. Capítulo 1: What dead seaweeds can tell us about metal uptake and their application to control marine pollution 24
   - 6.2. Capítulo 2: Assessing the effectiveness of seaweed transplants in reflecting seawater pollution levels 34

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

6.3. Capítulo 3: Do we know the cellular location of heavy metals in seaweed? An up-to-date review of the techniques 62
6.4. Capítulo 4: Nanoscale distribution of potentially toxic elements in seaweeds revealed by synchrotron X-ray fluorescence 71
7. **Capítulo sen publicar** 85
7.1. Capítulo 5: Deciphering uptake mechanisms of potentially toxic elements in seaweeds using high resolution imaging analysis 85
7.1.1. Abstract 85
7.1.2. Introduction 85
7.1.3. Material and methods 88
7.1.4. Results 92
7.1.5. Discussion 97
7.1.6. Conclusion 102
7.1.7. Acknowledgements 102
7.1.8. References 103
8. **Discusión xeral** 108
8.1. Emprego das algas como biomonitores 108
8.2. Localización celular dos PTEs 113
9. **Conclusións** 123
10. **Referencias xerais** 124
11. **Anexos** 134
11.1. Anexo I: Material suplementario do capítulo 2 134
11.2. Anexo II: Material suplementario do capítulo 5 138
11.3. Anexo III: Permiso das revistas 143

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

1. Abstract

Pollution by potentially toxic elements (PTEs) is one of the main threats to coastal marine ecosystems. One proposed technique for monitoring PTE concentrations is biomonitoring using seaweeds, which involves measuring pollutant concentrations in seaweeds to assess pollution in their environment. However, the lack of a standardized protocol has limited the applicability of this technique, and the lack of understanding of how algae take up contaminants has hindered the interpretation of the results. Recent studies have recommended using transplants of the brown seaweed *Fucus vesiculosus* for this purpose, but its implementation and the underlying mechanisms are still unknown.

This thesis comprises five studies addressing these two issues. The first two studies investigate the application of the technique, focusing on the use of pre-exposure treatments for transplant material and the relationship between PTE concentrations in seaweeds and in water. These studies reveal that algae exhibit highly complex mechanisms and dynamics of PTE uptake, complicating the interpretation of their results for assessing environmental pollution. The next three chapters focus on unraveling this complexity. This is achieved by investigating the subcellular localization of PTEs in seaweeds, as understanding which cellular compartments accumulate contaminants under various conditions can shed light on the mechanisms through which they are absorbed. The last two studies reveal that seaweeds have strict and precise PTE regulation mechanisms, avoiding the incorporation of PTEs without cellular functions while internalizing metabolically important elements and sequestering them in physodes, specialized structures that mitigate their toxicity. Additionally, most of the PTE concentrations detected in seaweeds are found outside the cells, either in mineral particles deposited on the surface or bound to cell walls through electrostatic interactions. This suggests that these concentrations may not reflect the potential toxicity or bioavailability of PTEs in the environment, raising questions about the interpretation of biomonitoring studies.

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This information not only provides context for the results of biomonitoring studies, but also enhances our understanding of the impact of these pollutants on seaweed health and helps predict the effects of pollution on the ecosystems they support.

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2. Resumo

A contaminación por elementos potencialmente tóxicos (PTEs) é unha das principais ameazas para os ecosistemas mariños costeiros. Unha técnica que se propuxo para monitorizar as concentracións de PTEs é a biomonitorización con algas, que consiste en medir as concentracións de contaminantes nas algas para coñecer a contaminación do seu ambiente. Non obstante, a falta dun protocolo estandarizado limitou a utilidade da súa aplicación, e o descoñecemento de como as algas captan os contaminantes dificultou a interpretación dos resultados. Estudos recentes recomendaron o emprego de transplantes da alga parda *Fucus vesiculosus* para este fin, pero o seu modo de aplicación e o funcionamento subxacente aínda non foran descritos.

Esta tese está constituída por cinco traballos que abordan estes dous problemas. Os dous primeiros traballos estudan a aplicación da técnica, centrándose no uso de tratamentos pre-exposición do material dos transplantes, e na relación existente entre as concentracións de PTEs nas algas e na auga. Nestes estudos descóbrese que as algas teñen mecanismos e dinámicas de captación de PTEs moi complexas, que complican a interpretación dos seus resultados para obter información da contaminación do medio. Os tres seguintes capítulos céntranse en desentrañar esta complexidade. Isto faise investigando a localización dos PTEs nas algas a escala subcelular, xa que coñecer en que compartimentos celulares se acumulan os contaminantes nunha diversidade de condicións permite inferir cales son os mecanismos polos que están chegando aí. Así, os dous últimos estudos revelan que as algas regulan a captación de PTEs dunha maneira estrita e precisa, sendo capaces de evitar introducir nas células aqueles PTEs que non teñen funcións celulares, mentres que aqueles de importancia metabólica son internalizados, pero secuéstranos en fisoides, estruturas especializadas que permiten evitar a súa toxicidade. Ademais, vese que a maior parte das concentracións de PTEs que se detectan nas algas están fóra das células, ben en partículas minerais depositadas na superficie ou asociadas á parede celular por enlaces electrostáticos. Isto implica que estas concentracións non teñen por que reflexar a potencial toxicidade

nin a dispoñibilidade dos PTEs no ambiente, chamando a cuestionar a interpretación dos estudos de biomonitorización.

Esta información non só axuda a contextualizar os resultados dos estudos de biomonitorización, senón que tamén axuda a entender o impacto destes contaminantes sobre a saúde das algas e a predicir o efecto da contaminación sobre os ecosistemas que sustentan.

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3. Introdución

3.1. Contaminación ambiental

A contaminación, definida como a "presenza de substancias ou calor nos medios ambientais (aire, auga e terra) cuxa natureza, localización e cantidade produce efectos ambientais indesexables" (Nacións Unidas, 2016), provoca a morte de aproximadamente nove millóns de persoas ó ano, sendo unha das maiores ameazas para a vida do ser humano no planeta (Fuller *et al.*, 2022). Ademais, representa un grave perigo para a vida do resto de organismos, causando grandes perdas de biodiversidade (Sigmund *et al.*, 2023). Hai unha ampla variedade de substancias que poden actuar como contaminantes ambientais, que en base á súa natureza química se poden dividir en contaminantes inorgánicos (metais, gases de xofre, ozono, etc) e orgánicos (hidrocarburos aromáticos policíclicos, bifenilos policlorados, etc) (USEPA, 2024a).

3.2. PTEs como contaminantes

Entre os contaminantes inorgánicos destacan os metais pesados, xa que algúns deles son os contaminantes que historicamente tiveron un maior impacto global. Por exemplo, o chumbo (Pb) é un metal abundante e fácil de traballar, polo que foi un dos primeiros metais en ser empregados polo ser humano hai uns 7000 anos (Yahalom-Mack *et al.*, 2015). Desde entón o Pb lévase extraendo e empregando para facer canos, conservantes para o viño, pinturas, e máis recentemente aditivos para a gasolina e baterías eléctricas, entre outros moitos usos (UNICEF e Pure Earth, 2020). Isto incrementou de forma dramática o Pb ó que estamos expostos, sobre todo en tempos recentes, facendo que as concentracións de Pb en sangue hoxe en día superen en centos de veces as pre-industriais (Tong *et al.*, 2000). O motivo polo que isto é un problema é que o Pb é extremadamente tóxico: incluso en concentracións baixas, a exposición a este elemento causa danos cardiovasculares, renais, e reprodutivos (USEPA, 2024b). Ademais, o impacto do Pb sobre a saúde é moito maior nos nenos, xa que afecta ó desenvolvemento do

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sistema nervioso, causando problemas cognitivos e de conduta permanentes (UNICEF e Pure Earth, 2020). Este efecto é tan marcado que nos Estados Unidos apréciase unha forte correlación entre as concentracións de Pb en sangue e o coeficiente intelectual dos nenos (Schwartz, 1994). Debido a esta elevada toxicidade considérase que ningunha concentración de Pb no sangue é segura (Vorvolakos *et al.*, 2016), algo único entre os contaminantes. Outros metais pesados tamén supoñen graves ameazas sanitarias e ambientais; por exemplo, o mercurio (Hg) é tóxico a concentracións extremadamente baixas, e tende a biomagnificarse nas cadeas tróficas. Isto supón un risco para depredadores, e para nós ó comer animais situados na cima das redes tróficas como é o atún (Kumar, 2018). Outro exemplo é a prata (Ag), que foi collendo peso como contaminante nas últimas décadas debido á utilización de nanopartículas deste elemento en numerosos produtos comerciais, incluído pinturas, roupa e obxectos de plástico (McGillicuddy *et al.*, 2017).

Con todo, hai moitos máis elementos cuxas emisións antropoxénicas danan o medio, non todos eles metais. Para agrupalos, nos últimos anos é común o uso do termo “elementos potencialmente tóxicos” (ou PTEs, polas súas siglas en inglés), que inclúe semimetais como o arsénico (As) e non-metais como o selenio (Se) (Pourret e Hursthouse, 2019). Moitos destes PTEs son nutrientes esenciais para os seres vivos, que aínda así supoñen unha ameaza en condicións elevadas. Este é o caso do cobre (Cu), que ó empregarse no transporte de electróns da mitocondria é necesario para a obtención de enerxía en todos os organismos eucariotas. Non obstante, actividades mineiras, agrarias, e industriais poden subir as súas concentracións ambientais en varias ordes de magnitude (Ballabio *et al.*, 2018). En concentracións así de altas pode ser letal para moitos organismos, especialmente no medio mariño (Cui *et al.*, 2024).

3.3. Contaminación no medio mariño

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Os ecosistemas mariños costeiros son particularmente vulnerables á contaminación por PTEs, xa que ademais de que os organismos que viven neles se

ven moi afectados pola toxicidade dos PTEs, a súa situación xeográfica fai que reciban grandes afluentes destes contaminantes (Nunes e Leston, 2022). Isto débese a que as emisións de PTEs en terra pasan por escorrentía ós ríos, que os acaban vertendo á costa. A costa ten un gran valor para a nosa sociedade, xa que é unha fonte importante de recursos como alimento e produtos químicos, e alberga todo tipo de actividades recreativas e industriais. Por esta razón, nas últimas décadas, a protección destes ambientes foi collendo relevancia (Islam e Tanaka, 2004), o que levou a moitas entidades políticas a promulgar lexislación impoñendo límites ás concentracións de PTEs en augas costeiras. Exemplos disto son a Directiva Marco da Auga na Unión Europea (Parlamento Europeo, 2010), a Lei Federal de Augas Limpas nos Estados Unidos (US EPA, 1972), a Lei de Prevención e Control da Contaminación Acuática en China (República Popular China, 2008), e o Convenio de Minamata sobre o Mercurio (Nacións Unidas, 2013).

3.4. Control da contaminación

Para protexer os ecosistemas costeiros da contaminación por metais pesados é fundamental identificar os sitios contaminados, o que permite intervir cando é necesario. A maneira máis fácil de facelo é medindo as concentracións de PTEs directamente na auga do medio, un sistema moi estendido, que é o que se emprega para establecer límites na lexislación nomeada na sección anterior. Non obstante, está técnica ten limitacións importantes. A principal é que as concentracións na auga son variables ó longo do tempo, de modo que unha medición puntual pode non ser representativa da contaminación á que están expostos os organismos dese ecosistema (Elbaz-Poulichet *et al.*, 2006). Isto é especialmente grave no caso de sitios expostos a contaminación puntual ou intermitente, que pode danar ós organismos, pero que adoita pasar inadvertida en medicións directas posteriores. Ademais disto, as determinacións de concentracións totais na auga do mar, aínda que se fixesen coa periodicidade necesaria, non teñen por que ser representativas dos niveis de PTEs ós que os organismos están realmente expostos, xa que estes dependen da especie química, e de se están unidos a partículas orgánicas ou

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minerais (de Paiva Magalhães *et al.*, 2015). As determinacións analíticas en auga de mar tamén presentan dificultades técnicas, xa que as concentracións de moitos elementos son moi baixas, dificultando a súa detección, e as altas concentracións de cloruro sódico poden afectar ós equipos analíticos (Bulska e Ruszczyńska, 2017).

3.5. Biomonitorización

Para combater os problemas das medicións directas en matrices ambientais, propúxose a técnica da biomonitorización, que consiste en facer medicións nos organismos que viven nun ambiente para obter información do estado ecolóxico deste. Aplicado ós PTEs, mídense as súas concentracións en organismos para coñecer o estado de contaminación do ecosistema (Zhou *et al.*, 2008). Facer isto aporta información dun período máis longo de tempo, xa que as concentracións de contaminantes dos organismos vense afectadas polas condicións do medio ó longo do tempo, e só daqueles contaminantes que lles chegan. Ademais, hai organismos que alcanzan concentracións de PTEs moito máis altas que as ambientais, facilitando a súa determinación analítica (Ravera *et al.*, 2003). Esta técnica tense aplicado en distintas matrices ambientais empregando moitos organismos distintos. Por exemplo, para contaminación atmosférica adoitan empregarse liques e musgos (Szczepaniak e Biziuk, 2003), e para a fluvial musgos acuáticos (Debén *et al.*, 2017).

3.6. Biomonitorización con algas e a súa importancia

No medio mariño, a Directiva Marco da Auga europea recolle o uso de distintos organismos como biomonitores, especificamente algas, plantas acuáticas, crustáceos do xénero *Daphnia*, e peixes (Parlamento Europeo, 2010). De entre estas, as algas teñen unha serie de vantaxes sobre outros organismos: son xeograficamente abundantes, sésiles, están dispoñibles todo o ano, son resistentes a unha gran variedade de condicións ambientais, e acumulan niveis altos de PTEs (García-Seoane *et al.*, 2018b). Debido a isto, foron dos organismos

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máis comúns na bibliografía, con centos de traballos científicos que as usan para biomonitorización de contaminantes (García-Seoane *et al.*, 2018b, 2018a).

O interese en coñecer a relación entre as algas mariñas e os PTEs non se limita á súa potencial utilidade como biomonitores, xa que son organismos de gran importancia tanto para os ecosistemas costeiros como para a nosa sociedade. O seu valor ecolóxico débese a que as macroalgas mariñas son organismos fundacionais en augas costeiras, sendo fontes importantes de alimento e refuxio para todo o ecosistema (Pessarrodona *et al.*, 2022; Umanzor *et al.*, 2019). Así, coñecer o impacto da contaminación por PTEs sobre elas permite entender o efecto destes elementos sobre os ecosistemas mariños, e predicir como evolucionarán ante o cambio global. Socialmente, ademais de ser consumidas directamente por moitas sociedades, produtos como o agar-agar e os alxinatos extráense das algas, e teñen moitas aplicacións na industria alimentaria e farmacéutica (Catarino *et al.*, 2018; Lomartire e Gonçalves, 2022). Entón, coñecer se os PTEs van pasar a estes produtos e en que cantidades é clave para poder garantir a súa calidade e seguridade.

3.7. Avances e ocos

Un problema que limita a utilidade dos traballos de biomonitorización con algas é a variabilidade dos protocolos entre estudos (García-Seoane *et al.*, 2018b). Téñense empregado unha gran diversidade de especies dos tres principais grupos de macroalgas (verdes, vermellas e pardas). Ademais, aínda que a maior parte dos estudos son de biomonitorización pasiva, que consiste en medir as concentracións de contaminantes en organismos que se encontran naturalmente no medio, tamén hai decenas de traballos de biomonitorización activa, que fan transplantes de algas con material homoxéneo, e expóñeno durante un período coñecido na zona de estudo (García-Seoane *et al.*, 2018a). Entre estes traballos hai distintos protocolos de exposición, empregando distintos transplantes e expoñéndoos durante distintos tempos. Toda esta variedade imposibilita comparar os resultados de distintos estudos, o que diminúe a utilidade da técnica. Para combater este problema, nos

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últimos anos publicáronse algúns estudos que buscan estandarizar e optimizar a biomonitorización costeira con algas. Así, García-Seoane et al. (2018b) recomendan o uso de algas pardas do xénero *Fucus*, como *Fucus vesiculosus* L., como candidatas para empregárense de forma estendida na súa área de distribución do Atlántico Norte. Os motivos para seleccionar *F. vesiculosus* son que é unha especie do orde das Fucales moi abundante en ambientes intermareais, o que facilita a súa mostraxe, e é unha das algas máis empregadas en estudos de biomonitorización, polo que hai bastante información xa dispoñible. Noutro traballo, García-Seoane et al. (2018a) recomenda facer biomonitorización activa para biomonitorizar con *F. vesiculosus*, xa que o uso de transplantes permite estudar lugares onde a especie non está naturalmente presente, permite controlar o período de exposición, e reduce a variación causada pola diversidade xenética.

A pesar destes avances, a biomonitorización de PTEs en augas costeiras con *F. vesiculosus* está lonxe de poderse considerar asentada, xa que hai moitos aspectos do protocolo experimental que aínda non están estandarizados. Ademais, a maior limitación da técnica é que o proceso de carga de PTEs das algas é descoñecido. Unha das características que debe ter un biomonitor é que as súas concentracións de contaminantes teñan unha relación lineal coas do medio (Markert *et al.*, 1999), pero isto aínda non foi testado, polo que os traballos da bibliografía asumen que esta relación existe. Así a todo, a relación entre as concentracións na auga e nas algas pode ser non-lineal debido á complexidade de estruturas ás que se poden cargar. As células de *F. vesiculosus* teñen unha parede celular formada por polisacáridos estruturais como celulosas, alxinatos e fucoidanos. Estas macromoléculas teñen grupos funcionais que no mar poden estar cargados negativamente, o que fai que se lles poidan asociar PTEs en forma catiónica (Rey-Castro *et al.*, 2004). Polo tanto, o primeiro nivel de complexidade depende de se os PTEs cargados están dentro das células ou asociados a cargas negativas no exterior. Disto non só van depender as dinámicas de carga, senón tamén o impacto sobre a saúde das algas que van ter estes elementos. Os PTEs que entren nas células poden estar libres no citoplasma, precipitados en cristais, asociados a membranas, ou dentro de orgánulos concretos, o que tamén pode afectar á carga. Fóra das células,

ademais dos enlaces iónicos a macromoléculas, tamén pode haber partículas minerais que conteñan PTEs, contribuíndo ás concentracións totais na alga sen estar biodispoñibles. Outro nivel de complexidade vén dado pola organización celular, xa que esta especie ten tres tipos celulares organizados en capas. A máis externa é o meristodermo, unha capa de células en estacada que forman a fronteira co exterior; por debaixo está o córtex, formado por entre unha e catro capas de células grandes globulares; e no centro do talo está a medula, un tecido con células alongadas que están raras nunha matriz de polisacáridos. Dependendo das estruturas celulares ás que se una cada elemento, as dinámicas e mecanismos de carga poden ser distintos, o que podería complicar a interpretación dos mecanismos de captación.

Noutros biomonitores téñense proposto dous posibles funcionamentos. Unha posibilidade é que o biomonitor capte contaminantes ó longo do tempo dunha maneira acumulativa, de tal maneira que vaia integrando a contaminación co paso do tempo (Harmens *et al.*, 2008). A outra é que as concentracións de PTEs estean en equilibrio coas do medio, de tal maneira que ante condicións ambientais variables as concentracións no biomonitor flutúen en vez de acumularse indefinidamente (van Straalen, 2008). Ata agora toda esta complexidade referida ao proceso de carga non fora contemplada na bibliografía, o que limita a fiabilidade dos estudos previos con esta técnica.

3.8. Unidade da tese

Todos os traballos incluídos nesta tese están enfocados a un obxectivo global común: coñecer o potencial das algas pardas para a biomonitorización activa de PTEs en augas costeiras. O capítulo un avanza este obxectivo centrándose en como tratar os transplantes antes de expoñelos. O capítulo dous investiga se os transplantes de augas cumpren un dos requisitos máis importantes para funcionar como biomonitor: ter relacións lineares coas concentracións ambientais. En base ós resultados dos dous primeiros capítulos vese que os mecanismos e factores que controlan a captación de PTEs por algas son descoñecidos. Este coñecemento é

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necesario para poder entender a carga dos transplantes, polo que os tres últimos traballos están centrados en entender os mecanismos de captación, comezando por coñecer a que compoñentes celulares están asociados os PTEs. O capítulo tres fai unha revisión crítica da información dispoñible sobre a localización dos PTEs nas algas, no catro descríbese a localización subcelular destes elementos, e no cinco profundízase nas dinámicas e mecanismos de captación. Estes tres capítulos mostran a complexidade da carga de PTEs por parte das algas, con importantes implicacións para a biomonitorización.

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4. Obxectivos

Cada un dos traballos incluídos a continuación ten un obxectivo xeral dentro do marco da tese, que se subdivide nos obxectivos específicos de cada traballo. A lista de obxectivos é a seguinte:

1) Comparar a idoneidade de transplantes de *F. vesiculosus* sometidos a distintos tratamentos para a biomonitorización de PTEs en augas costeiras
    a. Identificar o mellor tipo de transplante para a biomonitorización en base á súa durabilidade e carga de PTEs
    b. Estudar o efecto do metabolismo e a desvitalización sobre a captación de PTEs
2) Estudar a relación entre as concentracións de PTEs en transplantes de *F. vesiculosus* e nunha mostra de auga representativa do seu período de exposición, e comparar esta relación coa existente entre algas nativas e auga
    a. Modelizar a relación entre as concentracións de PTEs nos transplantes e na auga
    b. Comprobar se as ratios de enriquecemento dos transplantes teñen unha mellor correlación coas concentracións na auga que os datos brutos de concentración
    c. Comprobar se os transplantes reflicten as concentracións na auga mellor que as algas nativas
    d. Estudar o efecto da fisioloxía sobre a relación das concentracións dos transplantes coas da auga
    e. Ver o efecto dos parámetros fisicoquímicos da auga sobre esta relación
3) Revisar a bibliografía dispoñible sobre a localización celular de PTEs en algas
    a. Comparar os resultados para distintos elementos e especies para analizar os seus patróns de localización
    b. Facer unha avaliación crítica das metodoloxías empregadas
4) Explorar a localización de PTEs en *F. vesiculosus* a escala nanométrica

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

   a. Describir os patróns de distribución dunha ampla selección de elementos
   b. Estudar se diferentes compoñentes ultraestruturais, capas celulares, e mostras expostas a distintos niveis de contaminación teñen concentracións distintas de PTEs

5) Estudar as dinámicas de captación de elementos en mostras de *F. vesiculosus* transplantadas entre un sitio con altos niveles de contaminación e outro con niveles baixos
   a. Estudar a localización celular de Pb, que foi indetectable en estudos previos
   b. Ver os compoñentes celulares responsables da captación e liberación de PTEs en resposta a concentracións celulares distintas

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5. Metodoloxía

Debido á diversidade dos experimentos desta tese e dos seus obxectivos, empregouse unha gran variedade de técnicas experimentais, analíticas, e de tratamento de datos. Estas están detalladas en cada capítulo, pero aquí preséntase un resumo das máis salientables.

5.1. Recollida de mostras

Os traballos desta tese realizáronse coa especie *Fucus vesiculosus* L., unha alga parda común na costa Atlántica europea. O material desta especie necesario para os experimentos recolleuse de diversos puntos da costa galega. A maior parte de material recolleuse da praia do Testal de Noia (42°47'23.0"N 8°55'03.5"W, WGS84), un punto con concentracións baixas da maior parte de elementos potencialmente tóxicos. Outros experimentos que requirían algas expostas a distintos niveis de contaminación recolléronse por todo o litoral galego. Ó ser unha alga intermareal, as mostraxes realizáronse durante a marea baixa, seleccionando manualmente talos sans, axitándoos brevemente na auga do arredor para quitar o sedimento depositado sobre eles, e gardándoos en bolsas de polietileno para levalos ó laboratorio. Logo a este material déronselle os distintos usos requiridos por cada experimento.

5.2. Transplantes naturais

Os experimentos dos capítulos un e cinco fixéronse transplantando material dun punto da costa a outro, procurando que as condicións de exposición no novo sitio imitasen condicións naturais. Estas exposicións fixéronse collendo pedras planas do sitio onde se ía facer a exposición, e pegándolles os talos frescos pola súa base empregando silicona de selado resistente á auga. Logo as pedras cos transplantes envolvéronse cunha malla de plástico cunha luz de 2 cm, para evitar que se soltasen os transplantes, pero permitindo o fluxo de auga. Unha vez seca a silicona,

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estes transplantes leváronse ó punto de exposición, onde se depositaron nunha zona na que tamén houbese exemplares de *F. vesiculosus* nativos.

5.3. Transplantes en bolsas

Nos capítulos un e dous empregáronse tamén transplantes que non imitaban condicións naturais, senón que intentaban optimizar a exposición da alga ó medio. Isto fíxose imitando a técnica da "bolsa de musgo", que consiste en facer transplantes poñendo o material dentro de bolsas de malla. As bolsas fixéronse con malla de fibra de vidro cunha luz de 2 mm, confeccionando bolsas planas de 9 x 9 cm. Para encher estas bolsas seleccionáronse partes apicais de talos de *F. vesiculosus* sans e sen estruturas reprodutivas, coa intención de ter un material homoxéneo.

5.4. Desvitalización

Nos capítulos un e dous empregáronse algas desvitalizadas, é dicir, algas que se mataron de tal maneira que non perdesen as súas propiedades para a biomonitorización. Isto fíxose de dúas maneiras distintas. En ambos os experimentos fíxose secándoas, para o que se expuxeron a unha rampla de temperatura de entre 50 e 100°C ó longo dun mínimo de 24h. Ademais, no capítulo un tamén se desvitalizaron cocéndoas durante cinco minutos.

5.5. Recollida de auga

Para o capítulo dous, ademais de coller mostras de algas, tamén se colleron mostras de auga. Isto fíxose coa axuda de automostreadores, aparellos que contan con botellas e unha bomba, e que poden ser programados para coller auga periodicamente. Neste experimento programáronse para coller 50 mL de auga cada

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hora durante dúas semanas. Ó chegar ó laboratorio mesturouse a auga destas botellas para ter unha mostra composta representativa do período de recollida.

5.6. Determinacións elementais

A principal técnica utilizada para determinar as concentracións dos elementos nas mostras de algas e auga foi a espectrometría de masas con plasma acoplado inductivamente (ICP-MS). Esta é unha técnica que permite facer determinacións analíticas dunha gran cantidade de elementos cunha sensibilidade moi alta.

Tamén se empregou un analizador directo de mercurio (DMA) para medir as concentracións deste elemento, que ten niveles moi baixos difíciles de detectar con equipamento menos especializado.

5.7. Extracción secuencial

A primeira aproximación para entender a captación de elementos por parte das algas fíxose coa técnica da extracción secuencial, incluída no material suplementario tres do capítulo tres. Esta técnica consiste en empregar axentes extractantes como o EDTA coa intención de separar os elementos asociados a distintos compoñentes das células das algas. Logo fanse determinacións elementais dos extractos obtidos cos distintos axentes coa intención de coñecer que fracción de cada elemento está asociada a cada compoñente.

5.8. Criofixación

Os capítulos catro e cinco céntranse na visualización directa dos PTEs nas células das algas a través de técnicas de determinación analítica con alta resolución espacial. A aplicación destas técnicas, así como a visualización dos tecidos para microscopía, require que as mostras estean fixadas. Nos nosos experimentos isto fíxose mediante criofixación, unha técnica alternativa á fixación química, que é a

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máis habitual, que minimiza a mobilización dos elementos na mostra. O primeiro paso para levala a cabo foi a obtención de fragmentos finos transversais da alga, o que se fixo mediante un vibrátomo que permitiu a obtención de fragmentos de 150 µm. Estes fragmentos foron sometidos a conxelación de alta presión (HPF). Esta técnica baixa a temperatura da alga a -196°C cunha velocidade e presión tan altas que vitrifica a mostra, é dicir, conxélaa sen permitir que se formen cristais de xeo que poderían romper as membranas celulares. As mostras vitrificadas logo criosubstitúense, un proceso que consiste en substituír a auga da mostra por unha solución fixadora a moi baixa temperatura. Isto fíxose empregando unha solución de tetraóxido de osmio en acetona, o que fixa a estrutura da mostra. A continuación a mostra deshidrátase completamente con acetona, e finalmente embébese en resina, obtendo bloques sólidos nos que hai fragmentos de alga que conservan a súa estrutura e composición. Logo usouse un ultramicrotomo para cortar estes bloques, obtendo seccións de distintos grosores en función do seu uso posterior.

5.9. Microscopía

Para visualizar a conservación e estrutura das mostras fixadas dos capítulos catro e cinco empregouse microscopía óptica, electrónica de barrido (SEM), e electrónica de transmisión (TEM). Para microscopía óptica obtivéronse seccións de 500 nm, que se puxeron sobre portaobxectos, e que se tinguiron con azul de toluidina. Para SEM cortáronse seccións de 200 nm de grosor que se colocaron sobre obleas de silicio, e que se tinguiron con acetato de uranilo. Finalmente para TEM cortáronse seccións de 70 nm, que se colocaron sobre gradiñas de níquel e que se tinguiron con acetato de uranilo e citrato de chumbo. Unha vez fixadas, cada unha destas preparacións observáronse co equipo correspondente.

5.10. Obtención de mapas de distribución elemental

No capítulo catro analízase a composición das mostras a escala nanométrica empregando un analizador de fluorescencia de raios X asociado a sincrotrón (S-XRF). Un sincrotrón é un acelerador de partículas que se emprega como fonte de

radiación electromagnética para equipamento científico da máxima precisión. O XRF é unha técnica analítica que bombardea unha mostra con raios X e mide a fluorescencia emitida. Como o espectro de emisión de fluorescencia dos átomos de cada elemento é distinto, o espectro da mostra permite cuantificar a concentración dos elementos presentes nela. Ó empregar o sincrotrón como fonte de enerxía, esta técnica adquire a sensibilidade e resolución necesarias para obter mapas de distribución de elementos en mostras cunha resolución de ata 50 nm.

No noso caso, aplicamos esta técnica a seccións dos bloques de resina con fragmentos de *F. vesiculosus*. Para esta técnica obtivéronse seccións de 200 nm de grosor, que se colocaron sen tinguir sobre membranas de silicio. Estas mostras analizáronse na Instalación Europea de Radiación Sincrotrón (ESRF), en Grenoble, Francia,

No capítulo cinco tamén se obteñen mapas de distribución de elementos con escala nanométrica, neste caso empregando Espectrometría de Masas de Ións Secundarios a Nanoescala (NanoSIMS). Esta técnica emprega un raio de ións (que poden ser positivos ou negativos segundo os elementos a analizar) que fai que os átomos da superficie da mostra saian despedidos. Estes elementos logo analízanse por espectrometría de masas.

As seccións dos bloques que se analizaron mediante esta técnica foron os mesmos que os que se analizaron con SEM, xa que as obleas de silicio son un soporte adecuado para NanoSIMS. A análise fíxose no Instituto para a Investigación de Mostras de Sedimento de Kochi, Xapón.

5.11. Busca bibliográfica

Aínda que todos os capítulos requiriron o manexo de bibliografía, a maior parte deles foron traballos experimentais. Non obstante, para o capítulo tres fíxose unha revisión crítica da bibliografía existente sobre a localización de elementos potencialmente tóxicos en algas. Para isto levouse a cabo unha busca bibliográfica exhaustiva, principalmente a través do portal de busca de Scopus.

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5.12. Análise de datos

Na análise e visualización dos datos dos distintos capítulos empregáronse distintos tests, modelos e gráficos. Non obstante, todos eles se realizaron empregando o software estatístico R. A visualización dos resultados fíxose co paquete ggplot2 dentro deste software.

5.13. Procesado de imaxes

O procesamento dos datos dos mapas de distribución elemental nos capítulos catro e cinco requiriu o emprego de software especializado. No caso dos datos de S-XRF este foi o software PyMca, que se empregou para exportar os mapas como arquivos TIFF. Os datos de NanoSIMS foron procesados co *plugin* OpenMIMS dentro do software ImageJ, que permitiu exportalos no mesmo formato. Logo os arquivos TIFF exportados procesáronse e visualizáronse empregando ImageJ.

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6. Capítulos publicados

6.1. Capítulo 1: What dead seaweeds can tell us about metal uptake and their application to control marine pollution

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USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

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What dead seaweeds can tell us about metal uptake and their application to control marine pollution

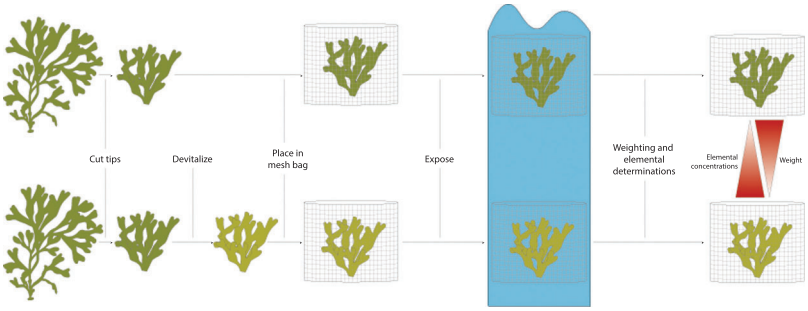
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HIGHLIGHTS

- We compared the elemental uptake by fresh and devitalized seaweed transplants.
- Most of the material in devitalized transplants was lost during the exposure period.
- Devitalized samples had higher elemental uptake for most trace elements.
- Physicochemical changes on the seaweeds' surface contribute to the increased uptake.
- Extracellular chemical binding and physical adsorption are relevant uptake pathways.

GRAPHICAL ABSTRACT



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ABSTRACT

The mechanisms of trace element uptake by seaweeds are still unknown, despite being key to understand the impact of pollution in coastal environments. This knowledge gap, in addition to the lack of standardization, have also hindered the use of seaweeds to monitor seawater pollution. To address these shortcomings, we tested the use of devitalization as a pre-exposure treatment for brown seaweed transplants, and we compared devitalized and fresh transplants to gain some insights into the mechanisms of element uptake. We exposed four types of *Fucus vesiculosus* transplants in 6 sites for 4, 8 and 20 days: fresh and devitalized (dried or boiled) algal segments held in mesh bags, and whole algal thalli imitating natural conditions. We then determined he concentrations of 11 trace elements in the algal tissues. The element concentrations were highest in the devitalized transplants, but the material lost consistency and weight throughout the exposure period, limiting their use to short periods. We proposed several factors that may contribute to the different accumulation patterns between treatments, and examined the implications for the uptake mechanisms, revealing that two of the most important are surface adsorption of sediment particles and chemical bounds to extracellular components.

1. Introduction

Pollution is one of the main ways by which human activities damage the environment [6] and is also harmful to human health, being responsible for up to 9 million deaths a year [29]. Many countries and organizations have regulatory agencies and legislation intended to

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control pollution levels and prevent the impact of pollutants on ecosystems and human health. Aquatic environments are particularly sensitive to pollution [23] and contamination is strictly regulated by legislation worldwide, such as the Clean Water Act in the United States [40], the European Water Framework Directive [13] and the Marine Environment Protection Law in China [34]. The regulations apply to coastal waters, which are important for food production, and also to marine ecosystems in general, owing to their importance in primary production and as nurseries [37]. Nonetheless, aquatic environments are severely affected by human activities, as water has been historically used for waste disposal, and persistent pollutants such as potentially toxic elements (PTEs) frequently reach coastal environments [4,42].

The first step in controlling pollution is to obtain reliable information about pollutant levels. The classical approach of measuring the concentration of pollutants directly in seawater has some disadvantages: the analytical determinations can be difficult and costly [28]; there is no temporal representativeness, owing to the rapid rate of replacement of coastal water; and the measurements do not reflect the impact of the pollutants on organisms, which may depend on the chemical species involved. Biomonitoring is an alternative approach that solves these problems by measuring the concentrations of pollutants in organisms exposed to the water rather than directly in the water. This approach is useful for providing information about the environment where the organisms live and about the organisms themselves, which may make it especially valuable for environmental impact assessments. However, this technique also has some disadvantages, such as the lack of comparability of different studies owing to the use of different organisms and methods [15,17].

To standardize the organism used for such studies, the taxonomic group that has attracted the most attention is seaweed. They have been widely used in the past in coastal biomonitoring because they are abundant in most regions throughout the year [17]. Not only this, but measuring the concentration of pollutants in seaweed tissues is also interesting due to their great importance; in coastal ecosystems they play a role as ecosystem engineers [39], and provide food and shelter for many other organisms; and directly for society as we extract countless products from them, including food, biofuels, and substances used in the food and pharmaceutical industries [5,31]. Thus, understanding how seaweed interact with PTEs in the environment is key to predicting how pollution will affect their ecosystem functions, the properties of seaweed-derived products, and to interpreting the results of biomonitoring studies. However, the accumulation mechanisms and factors affecting it are barely understood.

This knowledge gap, in addition to the lack of standardized protocols hinder the application of seaweed for biomonitoring. Some advances have already been made; *F. vesiculosus* has been recommended for the Atlantic region, but the protocol for using this species as a biomonitor is far from being optimized. The active biomonitoring technique, which has proved successful in other cryptogams such as terrestrial mosses [2] and freshwater mosses [10], has been recommended for use with seaweeds [15]. This approach involves culturing or obtaining the plant material from an unpolluted site and transferring it to the test area for exposure. The technique allows the use of pre-exposure treatments, including devitalization. Devitalization consists of killing an organism in a way that preserves the relevant properties for biomonitoring. This method has produced more stable results with other cryptogams [11,2,20], and it has simplified the logistics of the monitoring method, making it possible to preserve the material for longer times. Thus, the present study has two objectives: to compare the loss of material and the ability to accumulate PTEs of live and devitalized transplants of the seaweed *F. vesiculosus*; and to analyze their physicochemical properties and accumulation capabilities to examine the possible uptake mechanisms.

2. Material and methods

2.1. Sampling and pre-exposure treatments

Thalli of the brown algae *Fucus vesiculosus* L. were collected from an unpolluted coastal area (around SS1, Table 1) in NW Spain. Stones of approximate size 30 × 30×20 cm were also collected in the same site. The thalli were rinsed briefly in seawater and transported to the laboratory in polyethylene bags, along with the stones. Some of the thalli samples (25%) were preserved whole in seawater; the other thalli were processed by removing healthy apical segments using glass equipment. The three most apical dichotomies were selected from each thallus, to produce similarly-aged, homogeneous test material [16].

The selected apices were divided into three equal portions (each comprising 25% of the original material collected) and each was subjected to a different treatment: one portion was left untouched; another was oven-dried (at 50 °C for 8 h, 80 °C for 8 h, and 100 °C for 8 h), and the final portion was boiled for 5 min in a 2 L glass beaker heated on a hot plate.

2.2. Preparation and exposure of the transplants

The transplants were prepared by placing the fresh or boiled material (11 g) or the dried material (1.8 g) (the material lost approximately 5/6 of its weight during drying) in 9 × 9 cm flat fiberglass mesh bags (2 mm mesh size). The whole thalli were attached to the stones by gluing the basal part to the stone surface with waterproof silicone sealant. To prevent loss of material but without hampering water flow, the stones with attached seaweeds were covered with 2 cm mesh size polyethylene nets. These transplants imitated natural conditions.

A total of 5 stones each with 15 whole thalli attached were transported to each study site. A total of 45 bag transplants were also exposed in each site, 15 of each type, distributed in 15 strings that were attached to the meshes used to wrap the stones. In addition, 5 transplants of each type were transported to the study site but were not exposed in the water, as a time zero control (T0).

This was done in 6 study sites (SS) in the interior of rias on the NW coast of Spain (Table 1): 5 of the sites were potentially polluted due to their proximity to cities or to current or previous industrial areas, and the other was the same site where the seaweeds were collected.

2.3. Sample collection and processing

In each SS, samples were collected after exposure for 4, 8, and 20

Table 1
List of study sites. The coordinates are in WGS 84 with a web Mercator projection.

Study site	Start date	End date	Coordinates	Pollution source
SS1	31/10/2019	20/12/2019	42.22391, −8.75815	Next to an industrial city of 300,000 inhabitants
SS2	14/11/2019	04/12/2019	42.34626, −8.61331	Next to an old ceramics factory, known to discharge Pb-rich waste into the water
SS3	28/11/2019	18/12/2019	43.49986, −8.17095	Close to an industrial city of 65,000 inhabitants, in a ria with a low water renewal rate
SS4	06/02/2020	26/02/2020	42.94528, −9.17639	Next to an active metallurgic industry
SS5	20/02/2020	11/03/2020	42.40816, −8.6812	Next to a closed chlor-alkali plant, known to discharge Hg-rich waste into the water
SS6	05/03/2020	25/03/2020	42.79022, −8.91764	Not exposed to any known source of metal pollution

days. The length of the exposure period was selected to allow stabilization of element concentrations [41]. At each sampling time, transplants at the end of each string were collected, and the mesh envelopes were opened and 5 thalli removed. Thus, 5 samples of whole fresh seaweed attached to stones and 5 bag transplants subjected to each devitalization treatment were collected each time (i.e. 20 samples each time). The samples were briefly rinsed in the surrounding seawater, stored in polyethylene bags and transported to the laboratory. In addition, on day 20 of the exposure period, a sediment sample (upper 2 mm) was obtained in each SS. In the laboratory, the mesh bags were opened, and the three apical dichotomies were cut from the thalli attached to stones, so that these control samples were comparable to the bag transplants. The samples were then oven dried at 40 °C until constant weight, weighed, and homogenized in a tangential mill with zirconium oxide grinding vessels (Retsch ZM400). The material was stored in hermetically sealed vials in darkness until chemical analysis.

2.4. Chemical analysis

Prior to analysis, the samples were dried again at 40 °C in a forced air oven. The samples (1 g d.w.) were mineralized in Teflon vessels in a microwave oven (Milestone Ethos-1), in 3 successive steps (10 min at 100 °C, 7 min at 150 °C, 25 min at 190 °C), by adding 10 mL of HNO_3 (65%), 2 mL of H_2O_2 (30%) and 2 mL of MilliQ water. The concentrations of Al, V, Mn, Fe, Ni, Cu, Zn, Cd, Ba, and Pb were determined by inductively coupled plasma mass spectrometry (ICP–MS) (Agilent 7700x) by the University Research Support Services Unit (Universidade de Santiago de Compostela). The concentrations of Hg were determined in an elemental analyzer (DMA 80 Milestone) in the Ecology Unit of the same university.

The quality of the samples was ensured by analyzing certified reference materials (CRMs) and analytical replicates every 15 samples, in addition to blanks. The CRMs used were ERM-CD200 (*F. vesiculosus*), BCR-279 (*Ulva lactuca*) and BCR-277R for the sediments. All three materials are certified by the Joint Research Centre of the European Union. The limit of detection (LOD) was calculated as the mean blank value plus 3 times the standard deviation, and the limit of quantification (LOQ) as the mean value plus 10 times the standard deviation.

In order to investigate the causes of the differences in elemental accumulation, stored seaweed samples processed using the same protocol as describe above were analyzed in an FTIR-ATR spectrometer (Agilent Cary 630), in the 400 cm^{-1} to 4000 cm^{-1} region with a resolution of 4 cm^{-1}. A total of 14 samples were analyzed, 2 fresh and 2 dried unexposed (T0) seaweed samples, and 5 fresh and 5 dried samples exposed for 14 days.

2.5. Statistical analysis

All analyses were performed with R software [36]. The normality of the samples was checked using the Shapiro test. The data were not normally distributed either before or after logarithmic transformation. Due to the lack of normality, the differences in the elemental concentrations of different treatments were analyzed by the Kruskal-Wallis test and ad-hoc Dunn tests.

The trends in the elemental concentrations over time were tested by fitting linear models to the untransformed and transformed (logarithmic transformation) data. This was applied to the respective data for each SS and treatment for which material remained after 20 days. The quotient of the sigma values (the sum of the residuals of each model) of the linear and logarithmic model of each data set was calculated, producing a value that indicates whether the trend fitted best to a linear (values <1) or logarithmic (>1) model.

The fraction of the elemental concentrations of the transplants for which the sediment particles attached to the surface are responsible was calculated using Formula 1.

$$y = \frac{[Al]_{Sw}}{[X]_{Sw}} \Big/ \frac{[Al]_{Sed}}{[X]_{Sed}}$$

Formula 1. Calculation of the percentage of the elemental concentrations attributed to the sediment, where 'y' is the portion of the element X attributed to the sediment, $[Al]_{Sw}$ and $[Al]_{Sed}$ are the concentrations of aluminum in seaweed and sediment samples, and $[X]_{Sw}$ and $[X]_{Sed}$ are the concentrations of the studied element.

This was done under the assumption that all the Al in the seaweed samples was derived from the sediment, so that the concentration of any element present in the sediment particles associated with the seaweed samples should thus be proportional to the concentration of Al in the sediment samples. Thus, values < 1 imply that other factors are increasing the concentration of that element in the sample.

The FTIR results were analyzed and visualized using the ChemoSpec package for R [25], including the principal component analysis (PCA). The data were normalized with a probabilistic quotient normalization to remove any possible systematic deviation.

3. Results

The dry weights of the T0 transplants were similar (although weighing the material in different states such as dried or fresh to make the trasnplants led to differences). Thus, for T0, the average dry weight in grams of the apical segments of the whole algae attached to stones, expressed as mean ± sd, was 1.29 ± 0.34; for the fresh transplants, it was 1.53 ± 0.17, for the dried transplants, 1.65 ± 0.20, and for the boiled transplants, 1.05 ± 0.11. However, loss of material during the exposure period led to the final weights being very different (Fig. 1).

The weight of the whole algae attached to stones and the transplants of fresh material did not decrease throughout the experiment; all or most of the devitalized material was lost, in some SS within a week. The low remaining weight of some samples from devitalized transplants made it impossible to perform all chemical analyses, and the concentration of Hg was not determined in these cases.

Regarding the elemental determinations, the results of the quality control were generally satisfactory: the average percentage recovery of the algal CRMs for the certified elements (Cu, Zn, Cd, and Pb) was 84.6% for ERM-CD200 and 86.5% for BCR-279, and in all cases it was higher than 75%. The percentage recovery from sediments was below 40% for all elements except Ni: this was expected as the digestion method used is not designed to extract elements contained in silicates. The global error was below 10% for most elements, except Ni, Zn, Cd, and Pb, for which it was below 14%. For Mn, Fe, Ni, Cu, Zn, Ba, and Hg, the concentrations in all samples were above the LOQ. For some elements, the concentrations in all samples were above the LOD, but a few were below LOQ: 3% for Al, 5% for V, and 0.5% for Cd. Finally, the only element for which the results were not satisfactory was Pb. In this case, the concentrations in 34% of the samples were below LOQ, and in 6% they were under LOD, all non-exposed transplants.

The initial concentrations of elements in the transplants (Figure 1SM) varied slightly depending on the treatment applied; e.g., the boiled transplants consistently had the lowest concentrations of elements like V, Fe, and Ni. Some differences in the concentrations in the T0 transplants in different SS reflected temporal variations in the material collected. However, the differences were not large enough to have a notable impact on the enrichment results, except for Ba in SS4, as the initial high concentrations explain the loss of Ba during the exposure period in the SS.

The net elemental uptake of the transplants, defined as the concentration of each transplant minus the mean concentration of the T0 transplants of the same treatment in the same SS, is shown in Fig. 2 (and Figure 2SM for the remaining elements). Although the final

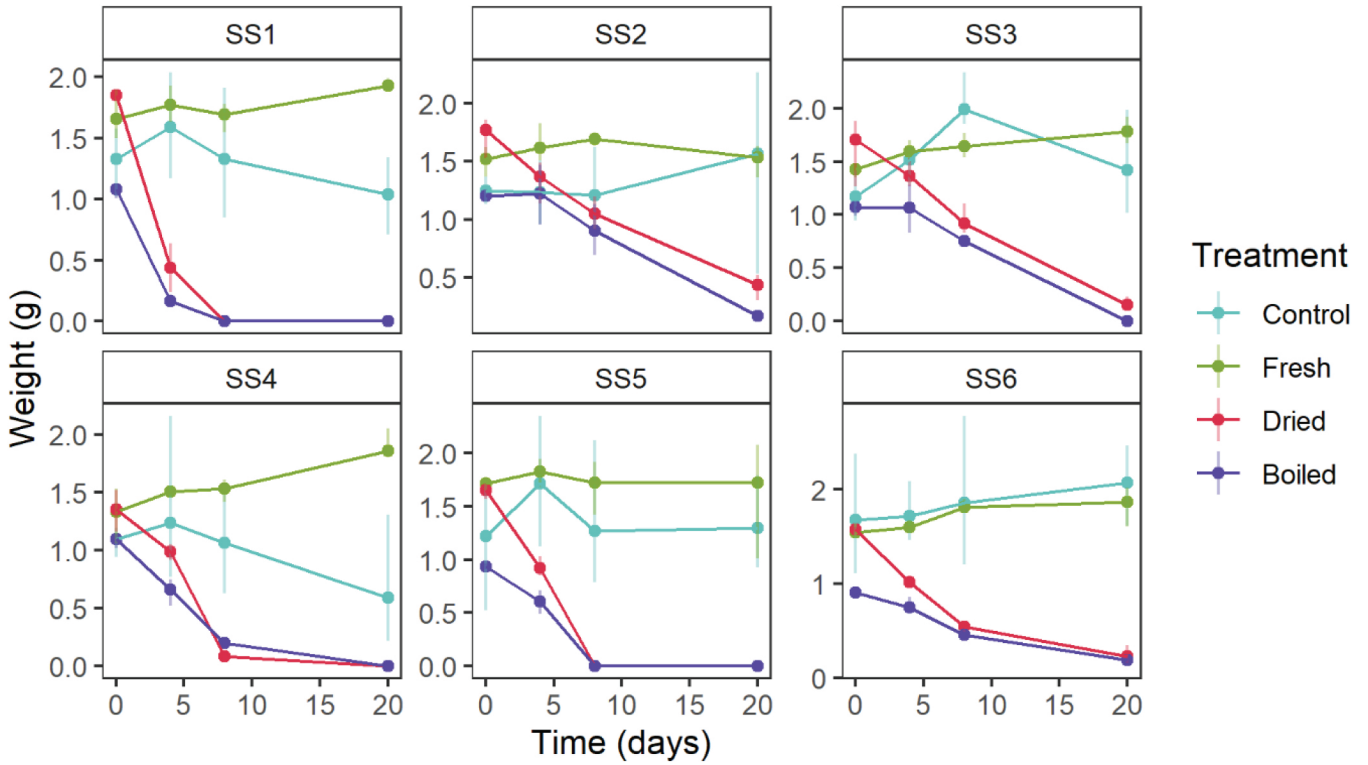


Fig. 1. Weight of the *F. vesiculosus* transplants collected during the exposure period. The points represent the average values, and the vertical bars the maximum and minimum values.

concentrations varied widely depending on the SS, some consistent patterns for each element and treatment were observed. In most cases, the treatments with live seaweed followed the same trend, whereas the devitalized transplants followed a different trend. For Al, V, Fe, Ni, Cu, Hg, and Pb, the concentrations in fresh transplants remained constant or increased slightly during the exposure period, whereas the concentrations in devitalized transplants increased sharply. For Mn, the concentrations in fresh algae remained constant, but the concentrations in devitalized transplants decreased during the 4 first days, before then stabilizing. For the other elements (Zn, Cd, and Ba), the patterns were less clear and varied across SSs.

For most elements and SSs, the concentrations differed significantly depending on the treatment considered. The cases for which the Kruskal-Wallis test revealed differences between treatments are shown in Fig. S3; Dunn test revealed between group differences (indicated by a compact letter display in the same Figure). In most cases, there were no significant differences between the treatments with fresh algae, nor between the devitalization treatments; significant differences were only observed between the live and devitalized transplants.

To evaluate whether the trends in the elemental concentrations during the study period stabilized at a specific value or increased linearly, both linear and logarithmic models were fitted for each element, SS, and treatment for which data for the 20-day exposure was available (Table 1SM). For around half of the treatments, the linear regression provided the best fit and for the other half the logarithmic regression model provided the best fit. The treatments for which the logarithmic model generally yielded the best fits were the devitalized transplants in SS2 and SS3. However, linear models provided the best fits for the devitalized transplants in SS6. Logarithmic models provided the best fits for the concentrations of Mn and Cd in devitalized transplants, owing to the rapid loss of these elements during the first 4 days and subsequent stabilization.

The proportion of the concentration of each element derived from the sediment particles on the surface of the seaweed, which was calculated by comparing the concentrations normalized with Al in the samples and the sediments, is shown in Fig. S4. Although it was assumed that all of the Al in the samples was derived from the sediment, some of the values were higher than 1, indicating that the concentration of those elements in the samples was lower than what the sediment particles would contribute in that case. For most elements, the proportion was similar among sampling points for each element, and higher in devitalized samples than in fresh samples. The element for which the concentrations were highest was Mn, which is consistent with the observed loss of the element during the exposure period. For V and Fe, the ratios were similar among treatments in all SSs except SS4, in which the concentrations of these elements were much higher in the devitalized samples than in the fresh samples (> than 1).

The FTIR spectra of fresh and dried transplants are shown in Fig. 3.

Differences between fresh samples, dried samples, and the respective controls were observed. The complete spectra from 400 to 4000 cm^{-1} and the grouping of the samples by PCA are represented in Figs. S5 and S6.

4. Discussion

Devitalization of *F. vesiculosus* samples led to rapid loss of material during the exposure period. This does not occur in moss, in which the devitalization by boiling and oven-drying with a temperature ramp causes a slight, stable loss of material, even when exposed in rivers [11]. In the present study, almost all the material of devitalized transplants collected after exposure for 20 days exposure was lost, and in some cases there was little to no material remaining after 8 days (Fig. 1). Thus, devitalizing the seaweed is not suitable for transplants intended to be exposed during long periods. For the 4-day exposure, only in a boiled transplant lost all the material, and all dried seaweed transplants had enough material for elemental analysis. Thus, dried transplants could be used for short exposure periods, and would be preferable to boiling as a devitalization treatment as slightly less material was lost and the dried

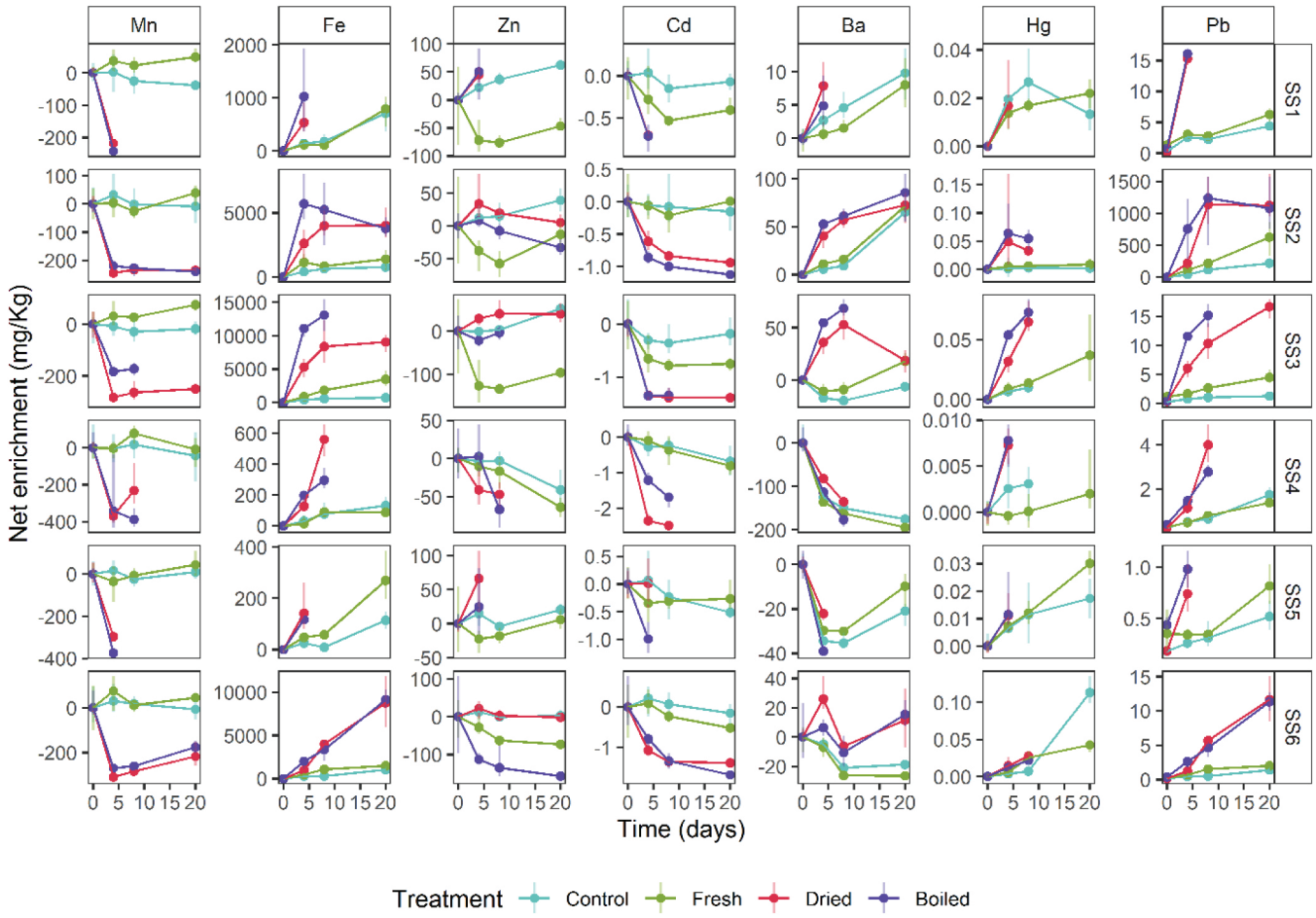


Fig. 2. Net enrichment of the transplants with different treatments throughout the exposure period. The points represent the average of the five experimental replicates, and the vertical bars represent the minimum and maximum concentrations. Al, V, Fe, and Ni followed the same pattern as Cu; the concentrations are shown in Fig. S2.

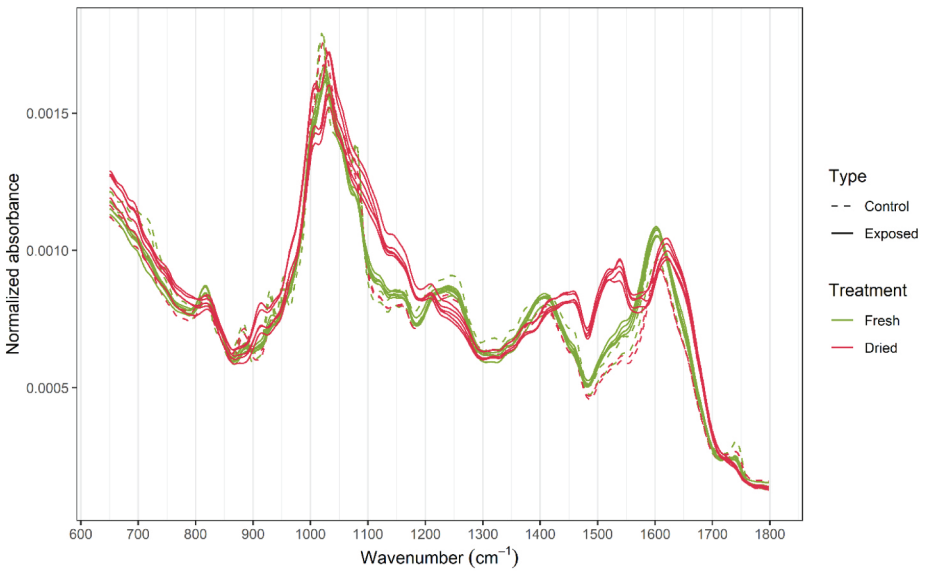


Fig. 3. Normalized absorption spectra of 14 samples in the region from 600 to 1800 cm^{-1}.

material can potentially be preserved for a long time under dry conditions. However, the devitalized seaweeds were much less durable than fresh transplants (either in bags or attached to rocks), the weight of which remained approximately constant. The differences in weight of the fresh seaweeds in bags and on rocks were negligible, and both preserved sufficient material, although the variation in the weight of the

transplants attached to rocks was much greater because they were not weighed before exposure, (the quantity was determined on the basis of the number of thalli). Thus, regarding weight loss, bags with fresh material are as good as the more natural approach of attaching the whole thalli to the rocks.

The other main consideration in selecting the best treatment is the differences in their metal-uptake capacity. The concentrations of most elements were much higher in the devitalized transplants (both methods) than in the live seaweed. Previous studies have shown different effects in metal uptake depending on the organism and element: in terrestrial mosses, *Sphagnum palustre* did not capture more Pb after devitalization [12]; in *Pseudoscleropodium purum* the concentrations of PTEs in live moss ranged from slightly lower to much higher than in oven-dried samples [3]; in *Hypnum cupressiforme* both similar [21] and higher uptake levels [20] have been reported; and the uptake of trace elements in devitalized *Platyhypnidium riparioides* was similar or slightly higher than in live transplants [7]. The only studies that have compared the differences in the accumulation of metals between devitalized and live seaweed are bioremediation studies, in which the biosorption of seaweed biomass (sometimes ground material) has been tested. In these studies, higher levels of uptake by devitalized seaweed have been suggested [32]; however, the only recent study comparing uptake levels showed that live *Ulva lactuca* can remove more Hg from spiked water than devitalized material [27].

The higher element uptake in devitalized transplants than in live transplants supports the hypothesis that the main uptake mechanism of metal accumulation in seaweed is chemical adsorption rather than active internalization [41], as devitalized seaweeds are not capable of the latter. However, the reason why the concentrations of elements in devitalized transplants range from similar to more than one order of magnitude higher than in live transplants requires further explanation. Numerous factors may contribute to these differences: 1) rupture of cell membranes during the devitalization process, which may expose binding sites in the interior of the membranes; 2) heat-induced chemical alteration of the cell walls and membranes, which can increase the affinity for certain elements, as occurs with other pre-exposure treatments [18]; 3) inactivation of the adaptations present in live seaweed to prevent the uptake of toxic elements; the mechanisms involved include the secretion of exudates to capture these elements [38], and possibly the excretion of chelated elements through active transport, both of which would contribute to lowering the concentrations of fresh seaweed; 4) differential loss of material in the devitalized transplants: if all of the components in seaweed do not have the same affinity for each element, the preferential loss of some components over others could cause differences in the accumulation capacity of the different transplants; 5) growth of fresh transplants; in the case of fresh moss transplants, growth has been found to lead to some variation in elemental concentrations due to the differential accumulation of differently aged plant parts [14], in which metal concentrations are different in *F. vesiculosus* [16]; and 6) physical modification of the seaweed surface, allowing pollutant-containing particles to adhere more easily to the surface of the devitalized transplants.

Two results that helped us test these hypotheses were the comparison of Al-normalized concentrations in samples and sediment, and the FTIR spectra of fresh and dried samples. For most elements, Al ratios were higher in devitalized samples, which indicates that a greater percentage of those elements can be accounted for by the contribution from sediments, suggesting that devitalized samples may have more adhered particles than fresh samples. This pattern was observed for almost all of the elements, except V, Fe, and Pb. One possible explanation may be that the sediment is the main contributor to the concentration of these elements in all samples. This is further supported by the fact that SS4 is the only site where there were differences between fresh and devitalized transplants in the Al ratios of Fe and Pb. The origin of these elements is usually geologic, and this SS is affected by a metallurgic industry that may be releasing them in soluble form, so the alteration of their Al ratios may indicate that this is the only case in which the soluble concentrations are high enough to make a significant difference. This result supports the hypothesis that the devitalization process alters the physical properties of the transplants, allowing more sediment particles to adhere to them.

The FTIR spectra of fresh and dried samples showed differences in the IR absorption profiles, and the PCA (Figure 6SM) clustered them in clearly defined groups. The group of samples with the most distinct profiles was the dried transplants after exposure, but differences were also observed in the spectra of exposed fresh samples and the controls, whereas the spectra of the two control groups were much more similar to each other. There are a few significant regions where the different treatments yielded different peaks: the peaks at around 750–950 cm^{-1} have been related to C-O and C-H bonds in uronic and mannuronic residues of alginate polysaccharides [22]; both the controls and exposed fresh samples generated peaks of different intensity at around 820, 880 and 930 cm^{-1}, while the exposed dried samples did not produce peaks at 880 and 930 cm^{-1}, having a peak at 910 cm^{-1} instead. The broad peak from 1220 to 1260 cm^{-1} was common to both controls and fresh samples, but it appeared shifted to 1210 cm^{-1} in exposed dried samples. This peak is related to sulphate ester groups found in fucoidan [8]. In the 1300–1800 cm^{-1} region all except the exposed dried samples also shared the same patterns, with two peaks around 1410 and 1600 cm^{-1}, which have been related to the carboxylate groups present in alginate [22,30]. Exposed dried samples, on the other hand, generated three peaks, with shifts towards higher wavenumbers. The different spectra in these regions imply that the quantities and/or composition of the biomolecules bearing the functional groups to which trace elements attach are also different, and therefore the alterations caused by the devitalization process (at least in the case of oven drying) are partly responsible for the differences in accumulation. However, fresh and dried controls have similar IR profiles, indicating that the heat exposure itself is not sufficient to alter the composition of the seaweed, and most of the changes occur during the exposure period. This suggests that the modifications occur either due to the differential loss of components, or that the devitalization allows chemical alteration to occur during the exposure period, possibly through decomposition.

The fact that these two accumulation mechanisms (adhesion of sediment particles and chemical binding to extracellular components) seem to be able to make a sizable contribution to the accumulation of PTEs suggests that, even in live seaweed, internalization is not a necessary uptake pathway for PTE accumulation. This is key to understand how seaweed interact with pollutants in their environment, for a few reasons: it suggests that most of the PTEs that seaweeds are exposed to are unable to affect the intracellular components, which helps to understand the high tolerance these organisms have to pollution [19]; it also implies that knowing the chemical species of PTEs and whether they are attached to particles is necessary to predict their impact on seaweeds, as the extracellular components are able to capture most species for many PTEs; finally, it also points to the effectiveness of the protection mechanisms seaweeds have against pollutants, since they can control their extracellular composition to avoid internalization [1]. Furthermore, these findings also have profound implications for the interpretation of biomonitoring studies, due to the implication that to extract precise information of the concentrations of seaweeds, it is necessary to know in which cellular compartments the PTEs are.

Another unexpected finding was the variation between elements. Whereas devitalized transplants were able to take up much higher amounts of most elements than live ones, the concentrations of Mn and Cd in them decreased during the exposure period. One possible explanation for the loss of Mn is that, as this element is essential for photosynthesis [24], seaweeds maintain high intracellular concentrations that could be lost due to membrane disruption caused by the devitalization processes. However, this does not explain the decrease in Cd, which is a toxic element with very little biological function, if any. Another possible explanation is the chemical modification of the functional

groups present on the surface of the cells to which PTEs can be bound, such as carboxyl, amino, sulphate and hydroxyl groups [9,26]. The alteration or creation of new functional groups during the devitalization process could affect the affinity of the material for specific elements. However, the uptake patterns of some elements are difficult to explain; for example, it has been suggested that the affinity of chemical elements for different ligands depends on their ionic and covalent index [33,35], but the accumulation of Cu and Cd, which are very similar in this regard, was completely different in the present study.

All of these patterns help us to understand the advantages and limitations of using live and devitalized seaweed transplants. In live transplants, the mechanisms for regulating PTE uptake are the same as in organisms occurring naturally in the ecosystem, and therefore they better reflect the elements affecting them. Live transplants are also more resilient and durable because they can maintain their structure which makes longer exposure periods possible; however, they are still capable of growth, which affects the reliability of the data. Devitalized transplants, on the other hand, have some advantages: 1) elemental concentrations are not affected by growth of the seaweed; 2) the concentrations of most elements are higher, which can be important for the detection of elements that display low concentrations such as Pb; some of the initial concentrations of this element in our experiment were below the LOD, but the post-exposure concentrations of devitalized transplants were much higher; and 3) they are also easier to use from a logistical point of view, as dried seaweed can be kept in the laboratory for longer. It would therefore be possible to collect large amounts of material, dry it, and used it for a long period, thus preventing differences in the initial concentrations such as those we found for Ba.

Based on these properties, both types of transplants may be useful in different scenarios. Fresh transplants would be better for producing results representative of longer periods, and when the objective is to obtain information on the impact of PTEs on the ecosystem. By contrast, devitalized transplants would probably be better for obtaining reliable data on the metal concentrations in seawater in a short time, although further information about the relationship between the concentrations in transplants and water must be obtained to confirm this possibility.

5. Conclusions

- For *F. vesiculosus*, transplants consisting of fresh apices in bags are easier to use and as effective for trace element biomonitoring as transplants of whole thalli (imitating natural conditions).
- Devitalizing the seaweeds affects the consistency and durability of the transplants, leading to complete disintegration of the material in as little as 8 days, making their use for periods longer than a week inviable.
- Uptake of trace elements was much higher in devitalized transplants than in fresh transplants, indicating the possibility of using the former for short exposure periods.
- The physicochemical modifications caused by the devitalization process affect the elemental uptake. After exposure, devitalized transplants contain different polysaccharides and functional groups, which affects their affinity for different elements.
- Devitalized transplants contain higher concentration of elements associated with the sediment, probably due to physical modification of the surface that enables binding of greater numbers of particles.
- PTEs physically or chemically bound to the surface of the seaweeds are very significant for metal accumulation, highlighting the importance of understanding the accumulation mechanisms to understand how they are affected by PTE pollution.

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Environmental Implication

Heavy metals such as the ones studied in this article are some of the pollutants with the greatest impact on coastal environments. This work improves the use of biomonitorization with seaweeds in several aspects: studying the use of devitalization and the application of devitalized transplants; helping to understand the results of biomonitorization studies; and advancing our understanding of the underlying uptake mechanisms. This way, it enables the use of a valuable tool to control pollution in seawater.

CRediT authorship contribution statement

Antón Vázquez-Arias: Investigation, Formal analysis, Writing – original draft. **Jesús R. Aboal**: Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **J. Ángel Fernández**: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132216.

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6.2. Capítulo 2: Assessing the effectiveness of seaweed transplants in reflecting seawater pollution levels

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Assessing the effectiveness of seaweed transplants in reflecting seawater pollution levels

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Abstract

Seaweed transplants have been widely used to monitor coastal marine pollution, yet their effectiveness in reflecting seawater elemental concentrations remains uncertain. This study investigated the relationship between elemental concentrations in *Fucus vesiculosus* transplants (both fresh and devitalized) and seawater samples representative of the transplants' exposure period collected using autosamplers. The transplants were deployed across 22 coastal sites in NW Spain over 14 days. No significant correlations were found between seawater elemental concentrations and those in transplants for any element. Similarly, seawater physicochemical properties (pH, dissolved oxygen, conductivity, and temperature) and the pre-exposure concentrations in transplants had minimal influence on post-exposure levels. Elemental concentrations in native seaweeds at the exposure sites were not correlated with those in transplants (except for Zn in the fresh transplants) and did not reflect seawater concentrations either. These findings highlight that element concentrations in seaweed do not follow a straightforward linear relationship between exposure and tissue concentrations. Instead, they result from a complex interplay of various, yet unknown environmental factors that influence element bioavailability in the water and the physicochemical properties of the seaweed. This complexity calls into question the suitability of seaweed transplants as effective biomonitors for marine pollution.

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Keywords: PTE, heavy metal, macroalgae, biomonitoring, Cd, Pb

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30

31 1. Introduction

32 Chemical pollution is a major driver of global change, posing a threat to most ecosystems
33 worldwide. To control its impact, institutions and regulatory agencies have implemented
34 measures to monitor and limit pollutant concentrations (European Parliament and of the Council,
35 2010; People's Republic of China, 1982; US EPA, 1972). However, direct measurements of
36 pollutant levels in environmental matrices present several challenges: 1) concentrations are
37 often low, necessitating high sensitivity for accurate quantification (Bulska and Ruszczyńska,
38 2017); 2) concentrations have high spatiotemporal variability, requiring extensive sampling
39 efforts for accurate representation (Elbaz-Poulichet et al., 2006); and 3) similar concentrations
40 can pose different risk levels depending on the pollutants' bioavailability (de Paiva Magalhães
41 et al., 2015).

42 To mitigate these drawbacks, biomonitoring has been proposed as an alternative technique.
43 Biomonitoring involves analyzing the organisms present in an environment to infer its ecological
44 characteristics. For pollutants, this often entails measuring their concentrations in resident
45 organisms (Zhou et al., 2008), as they uptake pollutants over time, reaching higher
46 concentrations and potentially providing information about bioavailable pollutants over longer
47 exposure periods. This approach has been used with several organisms across different
48 environments. For example, mosses have been used to monitor environmental concentrations
49 of some potentially toxic elements (PTEs) and polycyclic aromatic hydrocarbons (PAHs) (Aboal
50 et al., 2010; Boquete et al., 2015; Foan et al., 2015; Schröder and Pesch, 2010), with varying
51 success depending on the specific pollutant, species, and environmental matrix.

52 In coastal environments, seaweeds have been regarded as promising biomonitors due to their
53 abundance, year-round availability, sessile nature, and capacity to accumulate high levels of
54 pollutants (Pasumpon et al., 2023). Consequently, they have been used in hundreds of
55 biomonitoring studies using both passive (i.e. using native seaweed, García-Seoane et al.
56 (2018b)), and active (i.e. using transplants, García-Seoane et al. (2018a)) techniques. Among
57 them, brown algae in the genus *Fucus* have been some of the most common species in coastal
58 biomonitoring studies (García-Seoane et al., 2018b).

59 For biomonitoring to be an effective tool for quantifying environmental pollution, it is necessary
60 to understand what environmental parameters are reflected in the biomonitor organism
61 concentrations. Ideally, pollutant concentrations in biomonitors should show a strong positive

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linear correlation with their environmental levels (Wolterbeek, 2002). For native seaweeds, this relationship has been studied to some extent in the 70s (Foster, 1976; Melhuus et al., 1978), but findings have been inconsistent. More recent studies have also yielded mixed results. While some authors have reported positive correlations between seaweed and seawater concentrations, especially in heavily polluted regions (Bonanno et al., 2020; Chakraborty et al., 2014), most studies have failed to find significant correlations (Akcali and Kucuksezgin, 2011; Chernova and Shulkin, 2019). This lack of correlation may be attributed, to some extent, to a lack of comparability between the sample types, as seaweed concentrations may represent cumulative exposure over a period, while seawater concentrations are snapshots of specific time points. Recent studies with *F. vesiculosus* transplants have demonstrated that their concentrations can take up to several weeks to stabilize for some elements (García-Seoane et al., 2020; Vázquez-Arias et al., 2023b, 2023a). This implies that concentrations in seaweeds take some time to respond to changes in environmental concentrations, so if water concentrations change over time, seaweed and seawater samples collected simultaneously may not be comparable.

Unlike for native seaweeds, the relationship between elemental concentrations in seaweed transplants and their environment has never been addressed, despite the advantage of known exposure times that allow for better comparability. Recent studies supported the use of transplants for biomonitoring (García-Seoane et al., 2018a), and proposed using devitalized material—seaweeds killed without losing biomonitoring properties—as an alternative to fresh material (Vázquez-Arias et al., 2023a). Comparing the elemental concentrations of fresh and devitalized seaweed transplants with those of seawater and native seaweeds could reveal their responsiveness to environmental concentrations and offer insights into the largely unknown accumulation mechanisms and dynamics. For instance, many active biomonitoring studies use pollution indices such as enrichment ratios (ERs)—the difference between pre- and post-exposure concentrations normalized by exposure time—to account for initial variability (Sandu et al., 2012; Sergeeva et al., 2021; Vázquez-Arias et al., 2024), assuming that transplants integrate pollutants over time rather than reaching a dynamic equilibrium with the medium — an assumption that has yet to be tested.

This study aims to examine the relationship between elemental concentrations in native and transplanted *Fucus vesiculosus* samples and those in seawater collected continuously over the same exposure period. This will enable testing the following hypotheses: 1) post-exposure PTE concentrations in transplants reflect the total concentration of PTEs in seawater; 2) ERs show stronger correlations with seawater than post-exposure concentrations, assuming that

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transplants integrate PTEs over time; 3) transplants reflect seawater concentrations better than native samples due to their matching sampling periods; 4) seaweed physiology influences the relationship between seawater and seaweed concentrations; and 5) physicochemical characteristics of the water also influence that relationship.

2. Material and methods

2.1. Transplant preparation

To compare the concentrations of PTEs in the brown algae *Fucus vesiculosus* L. and in the surrounding water, transplantation experiments were conducted in 22 exposure sites (ES) along the coast of Galicia, NW Spain (Figure 1), over a ten-month period. Transplant preparation followed the methodology described in Vázquez-Arias et al. (2023a). Thalli of *F. vesiculosus* were collected from a reportedly unpolluted site in NW Spain (García-Seoane et al., 2019), briefly rinsed in seawater, and transported to the laboratory in polyethylene bags. There, the material was separated to apply two treatments: one half was preserved in seawater to create fresh transplants, while the other half was processed to produce devitalized transplants. For the devitalized transplants, the three most apical dichotomies were selected from healthy thalli without reproductive structures to ensure homogeneity, as elemental concentrations vary throughout the thallus (García-Seoane et al., 2021). These apices were then oven-dried at 50 ◦C for 8 h, 80 ◦C for 8 h, and 100 ◦C for 8 h. Once the dry material was ready, fresh transplants were prepared by selecting and cutting the apices of the preserved material in the same manner.

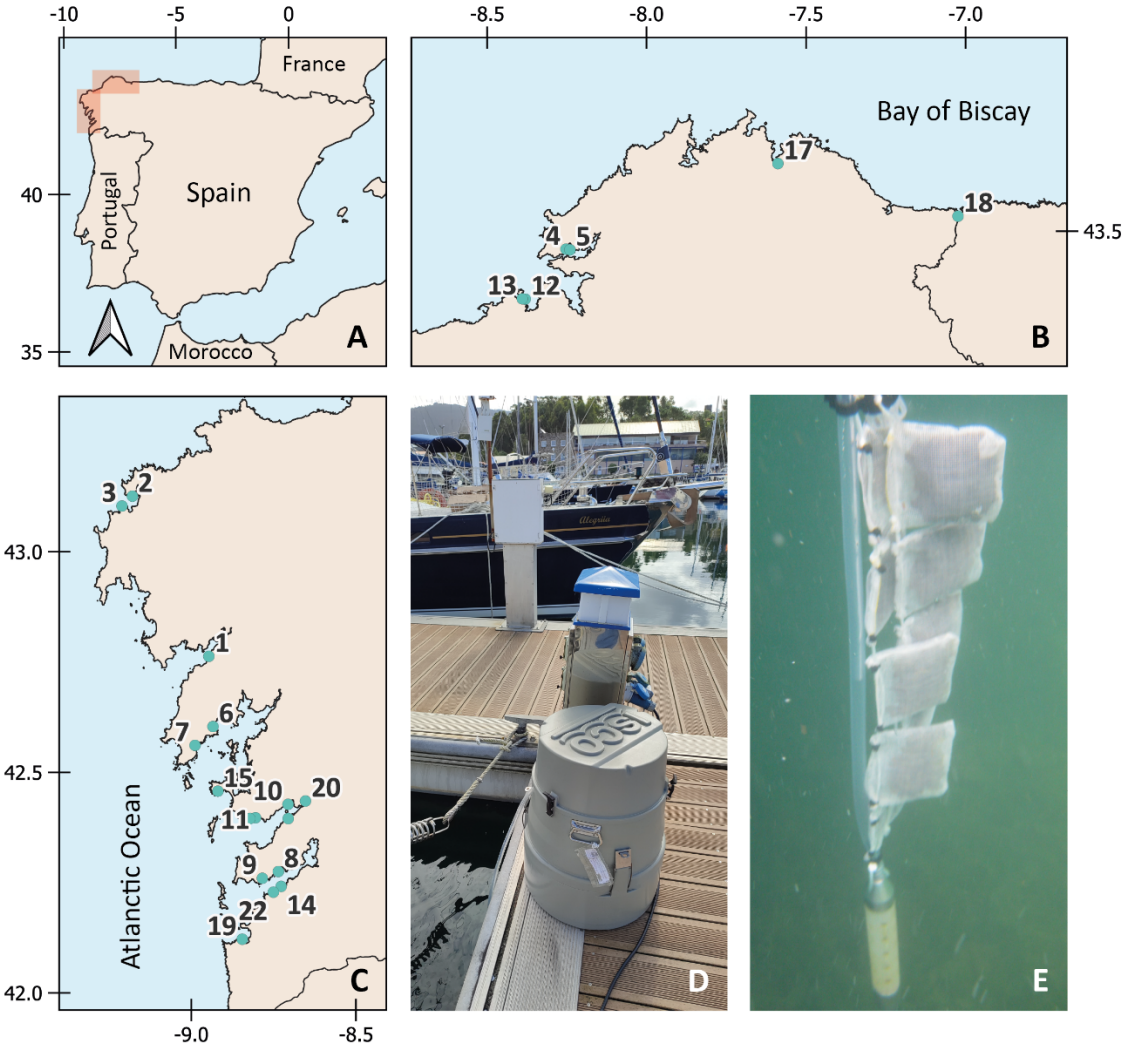
Both fresh and devitalized transplants were prepared by placing the apices in 9 × 9 cm flat fiberglass mesh bags (2 mm mesh size). Due to the significant weight loss during the drying process (approximately 5/6 of the initial weight), 1.8 g of dry material was used per transplant, compared to 11 g of fresh material.

2.2. Exposure and water collection

To ensure comparability between seaweed and seawater samples, transplants were exposed for two weeks, and composite seawater samples were collected continuously over the same period. Water samples were obtained using autosamplers (3700 Teledyne ISCO, Figure 1D) comprised of a body containing 24 one-liter bottles, and a hose that is submerged for water suction. The autosamplers were placed on floating docks for two reasons: 1) they require a continuous

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127 energy supply, which was available at power pedestals on the docks; and 2) the floating nature
128 of the docks ensures that the hose tip remains at constant depth despite tidal height fluctuations.
129 The autosamplers were programmed to collect 50 mL of water every hour, totaling
130 approximately 16.8 L of water over the 14-day exposure period. One autosampler was deployed
131 at each of the 22 ES (Figure 1), with the hose positioned at a depth of around 4 meters. In
132 exceptionally shallow harbors, the hose was coiled around the autosampler to reduce the depth
133 in one or two meters.



134

135 **Figure 1**. **A**. Location of the study region in the Iberian Peninsula. **B**. and **C**. Location of the
136 exposure sites. **D**. Body of the autosampler on a floating dock. **E**. Seaweed transplants exposed
137 underwater attached to the hose of the autosampler. Coordinates are in ETRS89.

138

139 Five transplants of each treatment were attached to the hose tip (Figure 1E). Additionally, two
140 extra transplants of each treatment were transported to the ESs and brought back to the

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laboratory to obtain the pre-exposure (initial) concentrations of the transplants. A temperature data logger was also attached to the hose tip.

After 14 days, the autosamplers were collected, the transplants were put in plastic bags, and everything was taken to the laboratory. Additionally, three samples of native *F. vesiculosus* were collected in 15 out of the 22 harbors where native populations were growing in or around them. For each water sample, 50 mL were taken from every bottle and combined to make a composite sample of ~1.2 L. The pH, conductivity, and dissolved oxygen content (DO) were measured, after which the samples were transferred to a polyethylene bottle and frozen until analysis.

The seaweed transplants were removed from the mesh bags, oven-dried at 40°C until reaching constant weight, weighed, and ground in a tangential mill with zirconium oxide grinding vessels (Retsch ZM400). The homogenized material was stored in hermetically sealed vials in darkness until chemical analysis. Native samples were processed in the same way by selecting the three most apical dichotomies and following the same drying and homogenization procedures.

2.3. Chemical analysis

The seaweed samples were oven-dried again at 40°C before analysis. One gram of each sample was mineralized in a Teflon vessel in a microwave oven (Milestone Ethos-1) in three steps (10 min at 100°C, 7 min at 150°C, 25 min at 190°C) by adding 10 mL of 65% HNO_3, 2 mL of 30% H_2O_2 and 2 mL of MilliQ water. The concentrations of Al, V, Cr, Mn, Fe, Ni, Cu, Zn, Cd, and Pb were determined by inductively coupled plasma mass spectrometry (ICP–MS) (Agilent 7700x) by the University Research Support Services Unit (RIAIDT, Universidade de Santiago de Compostela). Seawater samples were analyzed in the same way, excluding the digestion steps.

The analytical quality of the seaweed concentrations was validated using analytical blanks, certified reference materials (CRM: ERM-CD200, *F. vesiculosus*, Joint Research Centre of the European Union), and analytical replicates. For seawater samples analytical replicates were used. The average concentrations of the blanks were subtracted from the sample concentrations. Nearly all concentration values were above the limit of quantification, except for Al in four samples and Cd in one sample. The average percentage recovery of the CRMs for the certified elements (Cu, Zn, Cd and Pb) were between 90% and 110%. The global errors for the seaweed samples, estimated from the analytical replicates, were below 10% for all elements except Pb, for which it was 15%. The global errors were much higher in the case of seawater samples, ranging from 8.7% in Ni to 51% for Cd. The concentrations of Zn, Cd and Pb in some seawater

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samples were below the limit of detection. Despite the high analytical variability in seawater, the differences observed between sites were greater than these errors, and thus the results were used for comparison with seaweed concentrations.

2.4. Statistical analysis

All analyses were carried out using R 4.4.1 (R Core Team, 2024). To assess the relationship between elemental concentrations in post-exposure transplants and seawater, ERs and seawater, native seaweeds and seawater, and between the different seaweed sample types (the post-exposure concentrations of fresh and dried transplants and native seaweeds) we performed correlation analyses using the cor_test() function in the rstatix package (Kassambara, 2019). Data was right-skewed, so Spearman correlations were applied.

To evaluate the influence of the physicochemical parameters of seawater on the concentrations of post-exposure transplants and native seaweeds, and the impact of pre-exposure concentrations on the post-exposure concentrations of the transplants, we constructed generalized linear models (GLMs) using the glm() function in the lme4 package (Bates et al., 2015). For transplanted seaweed GLMs, the post-exposure concentrations of each element were used as the response variable, and water concentrations and physicochemical variables (pH, dissolved oxygen, conductivity and average water temperature) as predictor variables. For native samples, the predictor variables were the same, excluding pre-exposure concentrations. As concentration distributions in seaweed were right-skewed, a Gamma distribution with a logarithmic link function was used. Model evaluation was conducted visually using Q-Q residual plots and residuals vs leverage plots. The results were satisfactory for all models. Data visualization was done using the package ggplot2 (Wickham, 2016).

Bioconcentration factors (BCFs) were calculated as the ratio between elemental concentrations in seaweed and in seawater.

To control the false discovery rate due to the large number of tests performed, p-values were adjusted using the Benjamini and Hochberg (1995) method.

3. Results

The median concentrations and interquartile range of the concentrations of each element in post-exposure seaweed transplants, native seaweeds and seawater can be seen in Table 1SM of

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the supplementary material. We observed large differences in elemental concentrations across different sample types (i.e. fresh transplants, dried transplants, native seaweeds and seawater), with seaweed samples displaying much higher concentrations than seawater. The summary of the BCF in seaweeds can be found in Table 2SM. The BCFs were highly variable, with the IQR being higher than the median for most elements in all three seaweed sample types.

The order of elements from highest to lowest median concentrations in seawater was: Al > Fe > Ni > Mn > V > Cu > Zn > Cr > Pb > Cd. For fresh seaweed transplants the order was: Al > Fe > Mn > Zn > Cu > Pb > Ni > V > Cr > Cd; for dried transplants: Al > Fe > Zn > Mn > Cu > V > Pb > Cr > Ni > Cd; and for native samples: Al > Fe > Zn > Mn > Cu > Ni > Pb > V > Cr > Cd. Some elements were in the same or similar position across sample types, such as Cd in the lowest position, and Al and Fe in the highest ones. However, there were notable exceptions: Ni was one of the most concentrated elements in seawater but one of the lowest in seaweed, whereas Zn ranked much higher in seaweed compared to seawater. Among seaweed sample types, the order was relatively consistent, especially for fresh and native seaweeds.

Figures 2 and 3 illustrate the elemental concentrations in seaweed samples compared to seawater. As can be observed in these figures, no clear relationships were found between elemental concentrations in the seawater and in seaweeds. This was confirmed by the Spearman correlation coefficients, which were not significant regardless of the treatment (adjusted p-value > 0.05).

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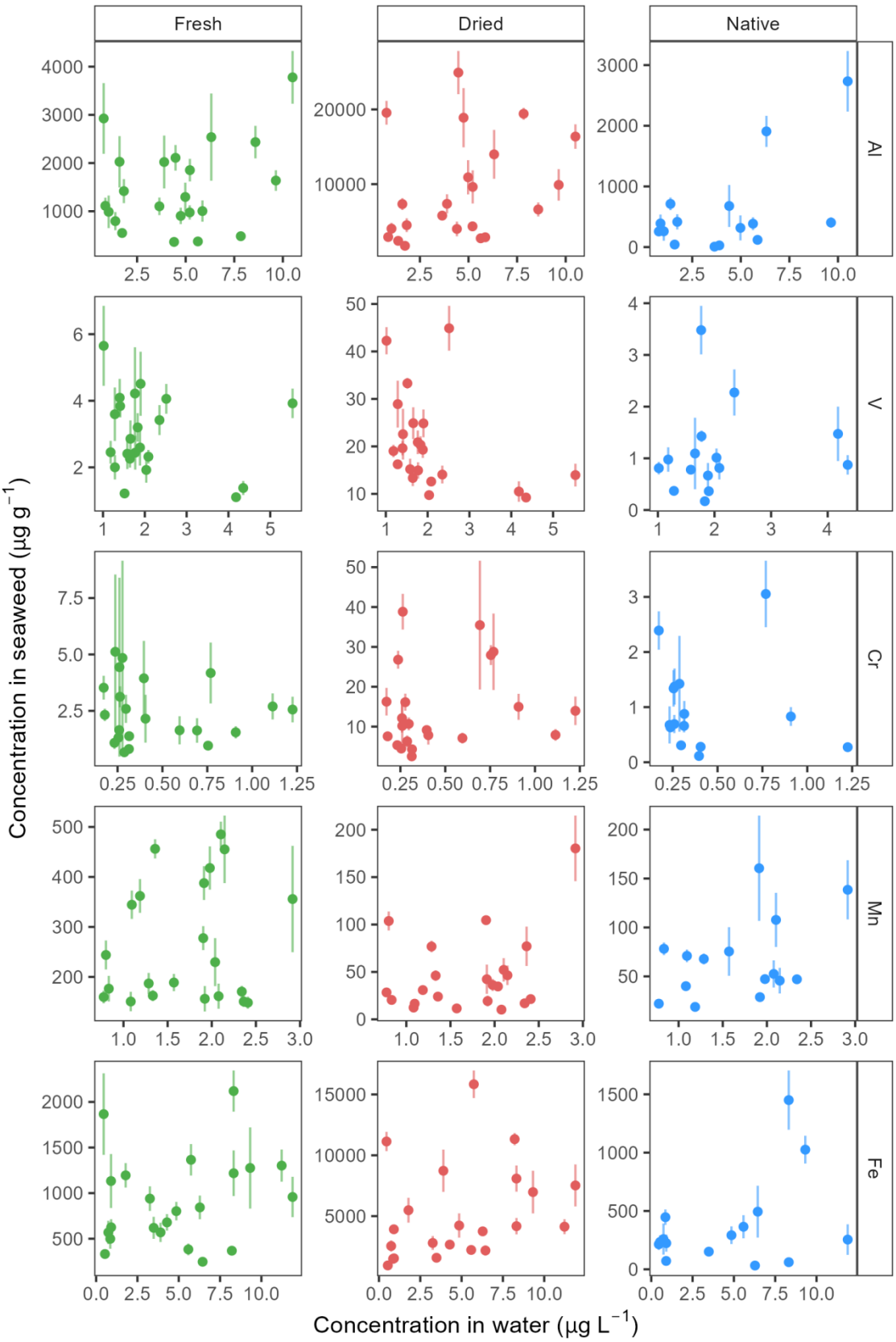


Figure 2. Scatterplot of the concentrations of Al, V, Cr, Mn and Fe in seaweed and seawater. "Fresh" represents the final concentrations in fresh seaweed transplants, "Dried" in dried

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226 transplants, and “Native” in native seaweeds. Points represent the mean concentration of
227 samples from each site (5 replicates for transplants and 3 for native samples), and error bars
228 indicate standard deviations. The x-axis range in the native samples is shorter in some cases due
229 to the absence of native seaweeds at certain sites.

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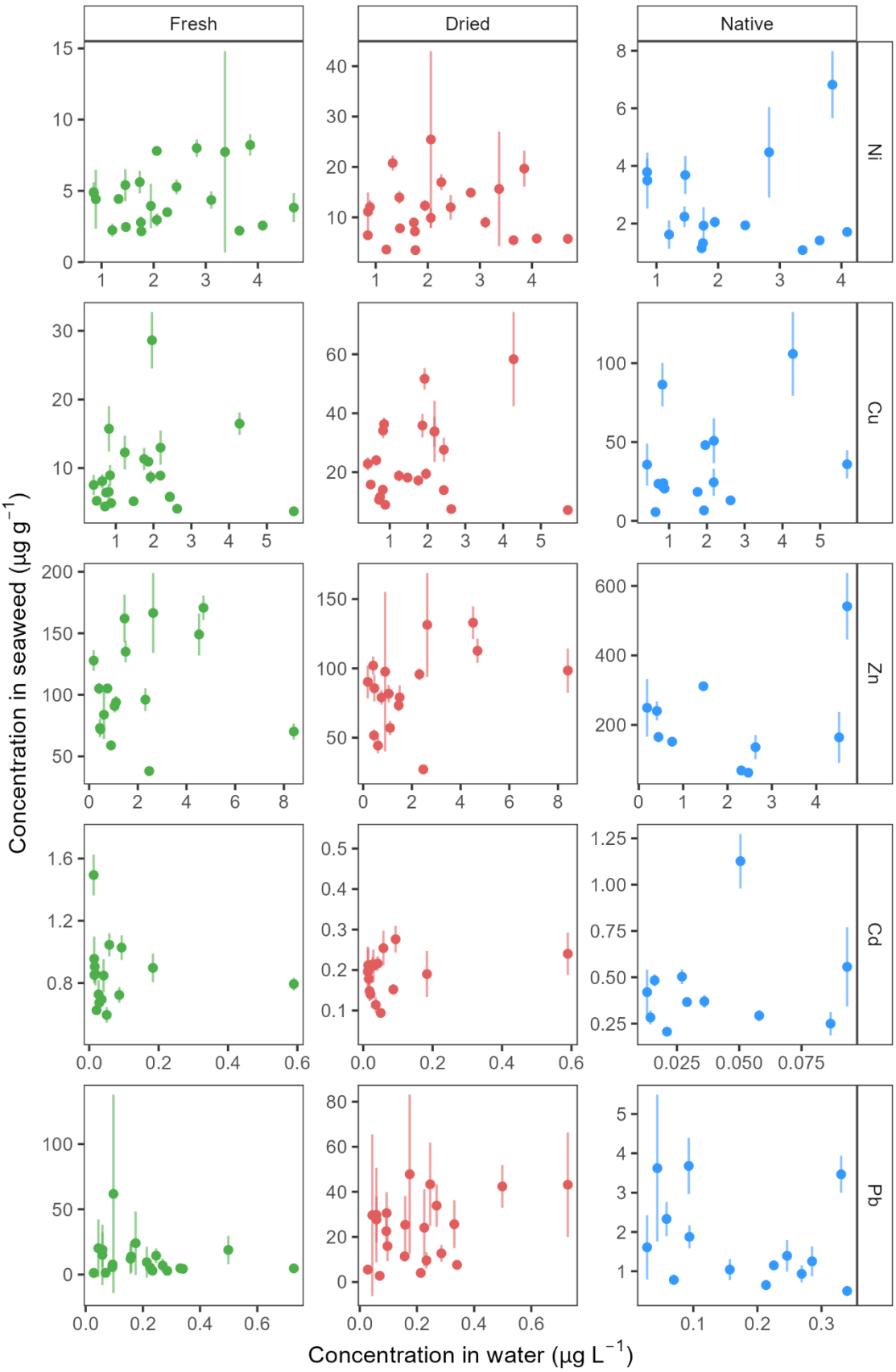


Figure 3. Scatterplot of the concentrations of Ni, Cu, Zn, Cd, and Pb in seaweed and seawater. "Fresh" represents the final concentrations in fresh seaweed transplants, "Dried" in dried

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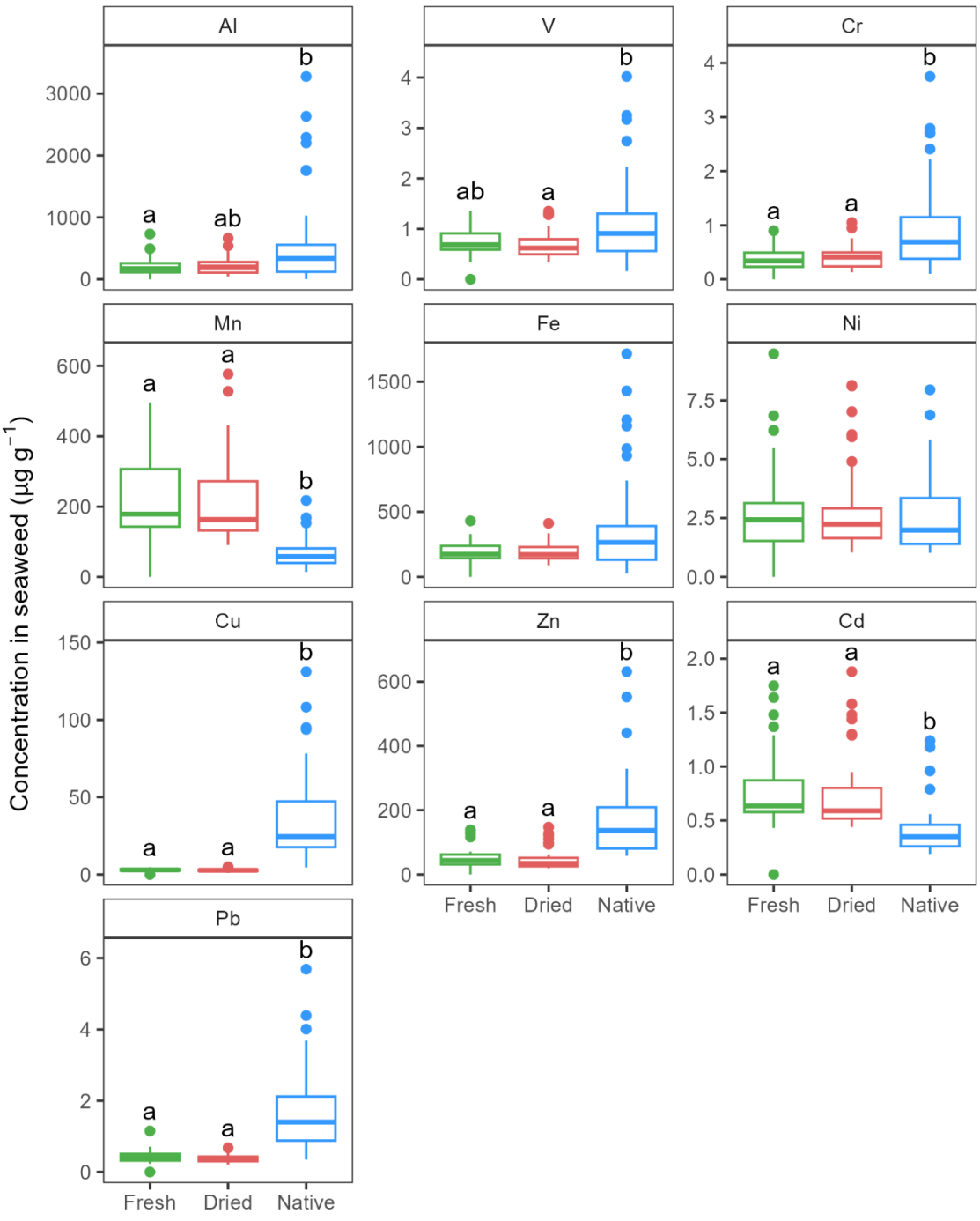
transplants, and “Native” in native seaweeds. Points represent the mean concentration of samples from each site (5 replicates for transplants and 3 for native samples), and error bars indicate standard deviations. The x-axis range in the native samples is shorter in some cases due to the absence of native seaweeds at certain sites.

We also tested the correlation across different seaweed sample types (Table 3SM). Correlation between fresh and dried transplants were significant for all elements but V, Mn, and Pb. In contrast, native seaweeds showed no significant correlations with dried transplants and only for Zn with fresh transplants.

Pre-exposure concentrations in transplants exhibited seasonal variation, with higher concentrations in winter for half of the elements (Cd, Cu, Mn, Ni, and Zn), and in summer for the other half (Al, Fe, Cr, Pb, and V) (Figure 1SM). Yet, for most elements, these concentrations were lower than those found in native seaweeds from the ESs except for Ni and higher for Mn and Cd (Figure 4).

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247

248 **Figure 4.** Boxplots of the pre-exposure elemental concentrations in fresh and dried seaweed
249 transplants made with thalli from a reportedly unpolluted site, and in native seaweeds across
250 the exposure sites. The letters show the groups that were significantly different for the elements
251 in which there were significant differences.

252

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To study if ERs reflect concentrations in seawater better than post-exposure concentrations, we performed correlation tests between the ERs of each element and seawater, but none of them were significant (adjusted p-value > 0.05).

The results of the GLMs used to identify the variables explaining the variability in the concentrations of each element in seaweed samples are shown in Table 1. Seawater concentrations did not have a significant effect on the post-exposure concentrations in transplants for any element, and neither did the pre-exposure concentrations. The environmental variables only had a significant effect on the concentrations of Cd in the dried transplants, which was positively affected by pH, and negatively by DO and temperature. For fresh transplants, the initial concentration of Mn had a marginally significant effect (adjusted p-value = 0.096) on its post-exposure concentration. Visually, pre-exposure concentrations of Cd in fresh transplants also appeared to influence post-exposure ones (Figure 2SM), but the adjusted p-value was not significant. Similarly, none of the explanatory variables had a significant effect on the elemental concentrations in native seaweeds.

Table 1. Parameter estimates for the GLMs modeling elemental concentrations in seaweeds. The response variable for fresh and dried transplants was the post-exposure concentration. Statistically significant values (adjusted p-value < 0.05) are in bold and underlined, and marginally significant values (adjusted p-value < 0.1) are in bold. Abbreviations: Water conc. = concentration of the element in seawater (μg L^{-1}); Initial conc. = concentration of the element in pre-exposure transplants (μg g^{-1}); DO = dissolved oxygen in seawater (mg L^{-1}); Cond = conductivity in water (mS cm^{-1}); Temp = average water temperature during the exposure period (°C).

	Al	V	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
	Fresh transplants									
Intercept	-1.8	-4.5	-2.6	4.8	1.5	1.6	-0.37	0.69	-0.9	0.15
Water conc.	0.13	0.049	-0.19	0.068	0.042	0.034	0.17	-0.045	-0.28	-2.8
Initial conc.	0.0023	-0.048	-0.66	**0.003**	0.0018	0.099	0.091	0.0089	0.84	0.0089
pH	0.31	0.17	-0.1	-0.013	0.18	-0.013	-0.097	0.076	0.09	-0.46
DO	0.092	0.06	0.1	0.038	0.06	0.059	0.045	0.064	0.054	0.44
Cond	0.11	0.07	0.044	0.013	0.055	0.002	0.031	0.041	0.0015	0.012
Temp	0.0047	0.044	0.11	-0.051	0.032	-0.052	0.061	0.041	-0.054	0.17
	Dried transplants									
Intercept	5.2	1.3	0.3	4	6.8	4.2	-0.078	2.9	-1.2	4.5
Water conc.	0.088	-0.079	0.12	0.18	0.044	0.035	0.15	0.048	0.22	1.4
Initial conc.	-0.0011	-1.4	-2.5	-0.0027	-0.0046	-0.091	0.011	0.0036	0.46	-1.5
pH	0.49	0.11	0.33	0.44	0.36	0.14	0.25	0.38	**0.64**	-0.11
DO	-0.15	-0.082	-0.15	-0.03	-0.14	0.091	-0.036	-0.2	**-0.2**	-0.085
Cond	0.081	0.051	0.064	0.05	0.056	0.0011	0.054	0.037	-0.017	0.025

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Temp	-0.16	0.011	-0.064	-0.33	-0.12	-0.2	-0.068	-0.11	**-0.2**	-0.064
	Native seaweeds									
Intercept	4.1	1.3	1.3	6.5	5.5	3.9	2.7	4.3	0.35	2.4
Water conc.	0.1	-0.068	0.13	0.13	0.071	0.035	0.3	-0.029	-0.48	-0.46
pH	0.54	0.22	0.36	0.2	0.56	0.1	-0.11	0.052	-0.0068	-0.15
DO	-0.061	-0.0076	0.034	0.025	-0.1	0.1	-0.055	0.064	0.1	0.21
Cond	0.059	0.02	0.0062	-0.013	0.023	-0.027	0.018	0.029	-0.03	-0.012
Temp	-0.15	-0.079	-0.16	-0.17	-0.15	-0.13	0.01	-0.11	-0.0082	0.037

4. Discussion

4.1. Effect of seawater concentrations on transplant elemental levels

This study is the first to compare elemental concentrations in seaweed transplants, with a known exposure period to seawater samples representative of the same period. We detected a wide range of elemental concentrations in seawater (one order of magnitude of variation among sites, which were homogeneously distributed across the range, Table 1SM) which should allow us to detect significant correlations with seaweeds concentrations, if any. However, within our range of concentrations and environmental variables, elemental concentrations in seawater did not explain the concentrations of *F. vesiculosus* transplants, either fresh or dried.

Only a few previous studies have examined the correlation between elemental concentrations in seaweed and seawater, and they have been limited to native seaweeds and discrete seawater samples collected simultaneously. While some of these studies reported finding consistent correlations (Bonanno et al., 2020; Chakraborty et al., 2014; Reis et al., 2016, 2014), others found no relation for most elements and seaweed species (Akcali and Kucuksezgin, 2011; Billah et al., 2017; Boubonari et al., 2008b, 2008a; Chernova and Shulkin, 2019; Ribeiro et al., 2018). Notably, studies with positive results were often constrained to a rather limited number of sampling sites (Bonanno et al., 2020; Reis et al., 2016), or applied arguable statistical methods (Chakraborty et al., 2014; Reis et al., 2014). Other than the previous studies, positive correlations between seaweed and seawater concentrations are generally rare, and some of the works with the necessary data to study this correlation omit the comparison (for example Gubelit et al., 2023; Ryabushko et al., 2024), potentially due to, in our opinion, the lack of positive correlations. These findings suggest that, in most cases, seawater elemental concentrations do not explain the ones in seaweeds.

The aforementioned studies are not directly comparable to ours due to a major limitation: the mismatch between the seaweed exposure period and the point measurements of seawater.

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Elemental concentrations in seaweed require time to respond to changes in environmental concentrations as shown in the supplementary material 4 of Vázquez-Arias et al. (2023b). In that study, Hg, Cr, and Cu reached levels similar to those in native seaweeds within 5 days, whereas Zn and Cd required up to 60 days. Consequently, if concentrations in seawater change over time, discrete water samples may not accurately represent the concentrations in seaweeds exposed over a longer period. The variation in stabilization times across elements suggests that our 14-day exposure period might not be ideal for Mn and Cd, but still offers a more representative sampling compared to single time-point measurements. Yet, despite this improved comparability, we found no significant effect of the seawater concentrations on those in seaweed transplants, indicating that *F. vesiculosus* transplants do not accurately reflect elemental concentrations in seawater. Below, we discuss some of the factors that could contribute to the lack of correlation between element concentrations in seawater and seaweeds.

4.2. Effect of seawater physicochemical properties

Variation in seawater physicochemical variables (pH, DO, conductivity and temperature) had minimal impact on transplant concentrations, despite the known influence of these factors on metal bioavailability in marine environments (Hong et al., 2011). Except for temperature, which was recorded every 10 minutes throughout the exposure period, other variables were measured on the composite sample after collection. Therefore, they may not reflect the exact properties of seawater at the site during exposure, but they are still valuable for cross-site comparisons, as they are influenced by the chemical characteristics of the water. Despite these biases, the lack of explaining power of the physicochemical variables is likely due to the complexity of their interaction with the uptake process. On one hand, their influence on elemental availability is complex and depends on the studied element (Wei et al., 2024). On the other hand, these variables can also impact on the properties of the surface polysaccharides of the seaweeds through processes like the neutralization of acidic groups (Kuzuhara et al., 2018), further complicating their interaction with dissolved elements.

The only exception to this pattern was Cd in dried transplants, where physicochemical variables did have a significant effect. As dried seaweeds have no metabolism, their uptake is likely to be more influenced by the properties of the environment, but why Cd was the only element in which this effect was noticeable, is unclear.

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4.3. Impact of initial concentrations

The GLM results indicated that pre-exposure concentrations had no influence on post-exposure concentrations in transplants, except for Cd and Mn. For these two elements, a notable alignment between pre- and post-exposure concentrations in fresh transplants was observed (Figure 1SM), although this effect was not statistically significant after correcting for multiple comparisons for Cd, and only marginally significant for Mn. Cadmium is one of the elements with longer stabilization times (Vázquez-Arias et al., 2023b), which is consistent with our results. Interestingly, Cd and Mn were the only elements with higher concentrations in pre-exposure transplants than in native seaweeds at most ESs. This suggests that, when exposed in the environment, the discharge of these elements may be slower than their uptake, requiring more time to reach equilibrium. This could explain why pre-exposure concentrations still influenced post-exposure levels after 14 days. Additionally, Cd and Mn concentrations were higher in fresh than in dried transplants. The drying process likely ruptures cell membranes, allowing the release of those elements in dried transplants more rapidly than in fresh ones. This may account for the lack of a significant effect of pre-exposure levels on post-exposure concentrations in dried samples.

Other biomonitors, such as mosses, have been proposed to uptake pollutants by integrating them over time in a cumulative process (Harmens et al., 2008). Under that scenario, pre-exposure concentrations would affect post-exposure ones. Although later studies have contradicted this hypothesis (Aboal et al., 2010), many biomonitoring studies still use pollution indices such as ERs to remove the effect of pre-exposure concentrations (e.g. Sergeeva et al., 2021). Contrarily, the independence of pre- and post-exposure concentrations found in this study for most elements, supports the hypothesis that the uptake process in seaweeds is not cumulative. Instead, elemental concentrations in seaweeds tend to stabilize over time, reaching a dynamic equilibrium with the environment. In this scenario, including pre-exposure concentrations by using ERs would not remove the effect of initial concentrations, introducing noise instead. The use of ERs in transplants is further complicated by the high variability of the natural concentrations, which follow an intrinsic seasonal pattern already described in the literature (García-Seoane et al., 2021), and which cannot be prevented without the use of cultured material.

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4.4. Comparisons among native and transplanted seaweeds

Similar to transplanted seaweeds, the GLMs failed to explain the variability in elemental concentrations in native seaweeds. This is consistent with most previous studies comparing elemental concentrations in seawater and native seaweed (see previous section 4.1). Furthermore, concentrations in native seaweeds were not significantly correlated to those of the transplants for most elements. The only exception was Zn, which showed a significant correlation with fresh transplants. The lack of correlation between dried transplants and native seaweeds is expected, as the devitalization process disrupts cell membranes, halts metabolism, and alters uptake properties (Vázquez-Arias et al., 2023a). However, the divergence between native seaweeds and fresh transplants is more surprising, as these materials are supposed to be more similar. Potential factors contributing to this discrepancy include differences in exposure periods, phenotypic variation between native and exposed seaweeds, and the exposure methodologies (e.g., transplants being permanently submerged in harbors). Regardless of the factors, provided that the use of transplants did not improve the correlations between the concentrations of PTEs in seawater and seaweed, our results did not favor the use of transplants over the collection of native seaweeds.

Post-exposure concentrations of most elements (Al, V, Cr, Fe, Ni, and Cu) were much higher in dried transplants than in fresh ones, while Mn, Zn, Cd and Pb were higher in fresh transplants, which is mostly consistent with Vázquez-Arias et al. (2023a). Except for Mn and Pb, all elements were significantly correlated between fresh and dried transplants (V marginally significant), largely overlapping with the elements present at higher levels in dried transplants. These findings suggest that, for the elements with higher concentration in dried transplants and significant correlations with fresh ones, uptake is mostly passive, and the devitalization process either: 1) exposes new binding sites for those elements, 2) chemically alters some of the already exposed binding sites, or 3) inactivates metabolic mechanisms that regulate elemental uptake. A recent study on subcellular elemental distribution in *F. vesiculosus* (unpublished results, Vázquez-Arias et al.) showed that some of these elements (Ni, Cu, and Zn) are primarily localized in physodes (intracellular membrane-bounded organelles which accumulate phenolic compounds). This, along with the fact that Ni and Cu reached higher concentrations in dried transplants, supports the hypothesis that devitalization increases the availability of metal binding sites.

For Zn, whose concentrations in dry transplants were lower than those of fresh ones, yet still significantly correlated, the second and third hypotheses may also apply, but acting in the opposite direction. For Pb, the lack of correlation and lower concentrations in dried transplants

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may be explained by the disruption of metabolism-dependent uptake mechanisms. Finally, Mn and Cd in fresh transplants may not have yet reached equilibrium, limiting the interpretation of comparing the values across samples types.

4.5. Implications for biomonitoring

The use of seaweed transplants for monitoring coastal marine pollution has been recommended as a way to improve the temporal interpretation of biomonitoring studies, reduce data variability caused by phenotypic differences, and monitor areas without native seaweeds (García-Seoane et al., 2018a). However, our findings do not support their effectiveness. In this study, elemental concentrations in transplants showed no correlation with those in seawater, even when accounting for the physicochemical properties of seawater. This was also true for native seaweeds, but unlike for transplants, which are exposed under artificial conditions, knowing the natural concentrations in ecologically significant organisms is far more valuable given the potential negative impacts on the ecosystems they inhabit.

The lack of the correlation between seaweed and seawater concentrations - a key requirement for an effective biomonitor organism - is likely due to the complex interactions between them. Chemically, physicochemical variables in seawater can have complex, synergistic effects on element bioavailability and on the properties of surface polysaccharides, making these interactions challenging to interpret. Biologically, seaweeds possess distinct uptake and regulatory mechanisms for each element, which significantly affect their concentrations in an element-specific manner. Hence, while seaweed concentrations tend to stabilize over time, eventually reaching equilibrium with their surroundings, the factors driving this equilibrium remain largely unknown. This uncertainty limits the effectiveness of seaweed transplants for biomonitoring purposes, calling into question the validity of numerous past studies that have applied this technique (García-Seoane et al., 2018a).

5. Conclusions

- Under the conditions of our study, *F. vesiculosus* transplants did not reflect the concentrations of chemical elements in seawater.
- Native *F. vesiculosus* performs equally poorly for PTE biomonitoring.
- Seaweeds do not integrate elemental concentrations over time. Rather, they reach a dynamic equilibrium with the environment.

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- This equilibrium cannot be reliable predicted based on the elemental concentrations and physicochemical variables of the seawater, likely due to the complex interactions between these variables and the elements both matrices.
- Seaweed physiology affects metal accumulation and discharge.
- Uptake depends mostly on passive mechanisms.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT for assistance with refining language and improving the clarity of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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6.3. Capítulo 3: Do we know the cellular location of heavy metals in seaweed? An up-to-date review of the techniques

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Discussion

Do we know the cellular location of heavy metals in seaweed? An up-to-date review of the techniques

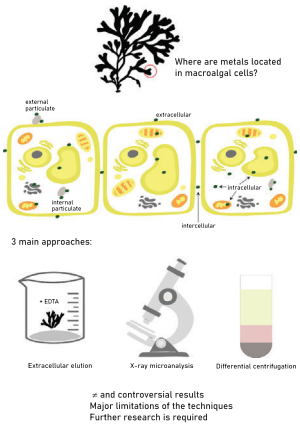
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HIGHLIGHTS

- The subcellular location of metals in seaweed has been seldom studied.
- The available bibliography shows contradicting results.
- The techniques applied to this point have conceptual and methodological problems.
- The partitioning of metals inside and outside the cells is unknown.
- Further studies should apply modern techniques to elucidate this question.

GRAPHICAL ABSTRACT



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ABSTRACT

Seaweeds are dominant organisms in coastal environments. However, in the context of global change, the integrity of these organisms is threatened by metal pollution. It is therefore important to understand how seaweeds are affected by metal concentrations in the water. Measuring the concentrations of metals in seaweed provides information about the effects of metal pollution on the seaweeds themselves and their ecosystems. Nonetheless, correct interpretation of this type of analysis requires knowledge of the cellular location of the pollutants, as the effects will differ depending on whether the metals are present in particles adhered to the surface, attached to external polysaccharides or dissolved in the cytoplasm. Thus, the objectives of this study were to compile the available information on the subcellular distribution of metals in seaweeds and to conduct a critical review of the information. We found that the existing studies provide contrasting, sometimes contradictory, results. Thus, metals have been detected entirely intracellularly and also mainly outside of the cells. In all of the studies reviewed, which used different techniques (mainly extracellular elution, X-ray microanalysis and centrifugation), methodological and/or conceptual problems were identified that raise questions about the effectiveness of each approach. To obtain reliable information about the distribution of metals in algal cells, further studies must be conducted that take into consideration the differences between elements and algal species and the limits of the methods used to measure the elements.

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1. Introduction

Throughout history, particularly in the last two centuries, human activities such as industry, mining and agriculture have discharged large amounts of metals and metalloids into the marine environment, with severe impacts on its ecosystems (Ansari et al., 2003; Islam and Tanaka, 2004; Pan and Wang, 2012). Seaweeds are dominant organisms in coastal waters, the part of the ocean most impacted by anthropogenic activities. Seaweeds play crucial roles in these ecosystems, as they are the main primary producers, providing food for many other species (Mann, 1973; Smale et al., 2013); and they also are ecosystem engineers, providing shelter, increasing the biodiversity and affecting the structure of intertidal communities (Umanzor et al., 2019; Watt and Scrosati, 2013). Seaweeds are also important for humans in more direct ways: they are the source of many relevant sea products such as food, medicines, and feed for fish farms (Beaumont et al., 2008; Paz et al., 2019), and they also provide other invaluable ecosystem services, such as coastal protection (Smale et al., 2013) and carbon sequestration (Krause-Jensen and Duarte, 2016), making them a key tool against global warming. They are therefore valuable participants in the fight against global change, and they can also be used to understand this phenomenon. Thus, seaweeds have been used as bioindicators (Alquezar et al., 2013), biomonitors (García-Seoane et al., 2018a, 2018b) and model organisms (Contreras-Porcia et al., 2017) to study marine ecosystems and the changes these are undergoing. However, global change is also a threat to seaweeds. For example, metal and metalloid pollution has been shown to be toxic to seaweeds, causing oxidative stress and DNA damage, limiting their growth and ultimately reducing their abundance and diversity in polluted areas (Babu et al., 2014; Contreras-Porcia et al., 2017; Munda and Hudnik, 1986).

Despite the strong impact that metal pollution can have on seaweeds, the fate of the toxic elements that reach seaweeds has been largely overlooked. Toxic elements are known to be adsorbed onto the surface of algal cells (Mehta and Gaur, 2005), but also to penetrate the cells (Espinoza et al., 2021). The importance of determining the location of metals accumulated by seaweeds is twofold. First, it will allow us to better understand and predict the impact that metal pollution will have on these organisms. Secondly, it will enable better interpretation of the results of studies in which seaweeds are used to investigate coastal pollution, as the adsorption of pollutants on the surface of algae and the intracellular uptake of the pollutants by the cells have very different implications. For this reason, several studies have attempted to elucidate the subcellular location of metals in seaweed by using different approaches; however, the results are often contradictory, and the validity of some of the methods is questionable. Thus, the purpose of the present study was to conduct a critical review of studies tackling this topic, by analyzing the methods and the results obtained, along with other indirect evidence.

2. Heavy metals in seaweed

2.1. Cellular compartments

Previous studies with other cryptogams have identified the different metal fractions that accumulate in different cellular compartments (Pérez-Llamazares et al., 2011a): (I) the external particulate fraction, composed of extracellular particles adhered to the surface of the cells; (II) the intercellular fraction, composed of elements in the solution that bathes the exterior of the cell wall matrix and the cell membrane, but that are not bound to the cells; (III) the extracellular fraction, composed of elements bound to the cell wall and outer layer of the plasma membrane; (IV) the intracellular fraction, composed of elements present within the cytoplasm or within cell organelles, as well as those bound to the internal layer of the plasma membrane; and (V) the internal particulate fraction, composed of insoluble particles comprising crystalline deposits from inside the cells. This classification can also be applied to seaweed.

In each compartment, metals can be free or bound to different molecules, and the concentrations will therefore depend on different processes. Metal cations outside the cells that are not bound to biomolecules can be either dissolved in the intercellular solution or form part of mineral particles adhered to the surface of the seaweed, i.e. the external particulate fraction. Particles adhered to the surface of seaweed have been found to make an important contribution to the metal content, especially due to sediment particles adhered to the surface of the shoot, which may remain even after scrupulous washing (Luoma et al., 1982; Villares et al., 2005, 2001). For some heavy metals the contribution of the external particle fraction is considered negligible, as it usually only reaches 5 % (i.e. Cd, Co, Mn, V and Zn) or slightly higher (up to ca. 15 % for Ag, Cu and Ni, with some exceptions); however, for other elements abundant in the sediments (i.e. Al, Cr, Fe and Pb) the proportion can reach high values ranging from ca. 30 to 100 % (Supplementary Material 1).

The extracellular fraction is composed of elements bound to negatively charged functional groups in the cell wall and/or on the external surface of the plasma membrane, especially carboxyl groups (Davis et al., 2003) and others such as amino, sulphate and hydroxyl groups (He and Chen, 2014; Murphy et al., 2008). These groups can be found in polysaccharides and proteins that predominate on the surface of algal cells, such as alginates and fucoidans (He and Chen, 2014; Salgado et al., 2005).

The intracellular metal can be attached to the interior of the plasma membrane, dissolved in the cytoplasm or present in organelles. The exact location and the molecules to which the metals are bound to may vary depending on the group of algae. For example, in brown algae, metals have often been detected in physodes (Lignell et al., 1982; Salgado et al., 2005; Smith et al., 1986), a type of vacuole that accumulates phenolic compounds (Schoenwaelder, 2002), whereas in green algae, metal-rich granules have been detected in the chloroplasts (Espinoza et al., 2021).

Finally, inside cells, metals can also be found in organic crystallizations of molecules, such as oxalate, that form the internal particulate matter. Although these crystallizations have been found to combat heavy metal toxicity in other organisms like plants and fungi (Franceschi and Nakata, 2005; Shao and Sun, 2007), there is no evidence supporting this function in seaweed (Leliaert and Coppejans, 2004; Pueschel, 1995).

In the extracellular compartments, heavy metals are passively bound by physico-chemical processes; however, in order to reach the intracellular compartment, metals must pass through the plasma membrane. As metal ions are positively charged, they cannot pass through the hydrophobic membrane and would have to enter via membrane transporters. While essential metals (e.g. Zn, Cu) use high affinity metal-specific transporters to get imported into the cytoplasm, non-essential metals (e.g. Cd, Pb) can enter through various channels, including those for essential metals. For instance, the Zrt/Irt-like proteins (ZIPs) mediate the uptake of mainly Fe and Zn but they can also transport Cd into the cells. This has been already proved for microalgae (Blaby-Haas and Merchant, 2012) and hypothesized for seaweed, where the same ZIP transporters are present (Clemens, 2006). Other main membrane transporter families involved in both the specific and unspecific metal transport includes the cation diffusion facilitators (CFC), metal tolerance proteins transporters (MTPs), ATP-binding cassette (ABC) transporters, or the natural resistance-associated macrophage proteins (NRAMPs) among many others. These orchestrate metal trafficking to ensure the cell homeostasis, and their study helps to better understand metal compartmentalization within the cells. However, while the role of these transporter families is deeply studied in other group of organisms like plants (Hall and Williams, 2003; Thomine et al., 2000) or prokaryotes (Chandrangsu et al., 2017; Ma et al., 2009; Nies, 2003) the way these operate on seaweed is still far from being understood.

2.2. Relevance of the cellular location of metals

Metals can be lethal to seaweeds, but their toxicity will depend on their cellular location. Most toxic effects occur when the metals are inside the algal cells. In this location, metals can lead to the formation of reactive oxygen species (ROS), which can cause oxidative damage to cellular constituents such as DNA, RNA, proteins and lipids (Contreras-Porcia et al., 2017). Thus, heavy metals have been shown to affect many functions and

components, such as ultrastructure, membrane integrity, photosynthetic rate and growth (Andrade et al., 2004; dos Santos et al., 2014; Huang et al., 2013).

Seaweeds are expected to have mechanisms to prevent toxic elements from entering the cells, which would explain their tolerance to metals. The first barrier to the entry of toxic elements is the cell wall, whose negative charges allow it to adsorb metal cations dissolved in the water. It has been shown that seaweeds with more negatively charged groups in the cell wall can accumulate more metals in it (Fourest and Volesky, 1997), and that these seaweeds can increase the amount of biomolecules with negative charges in response to heavy metal toxicity (Andrade et al., 2010). This indicates that not only is the cell wall acts as a binding site for cations, but also that seaweeds can actively use the cell wall to prevent the toxic effects of the cations.

However, this is not the only mechanism that seaweeds possess to prevent metal toxicity. Once the metals have entered the cells, seaweeds generate antioxidants to prevent the oxidation of cellular constituents by ROS, thereby preventing toxic damage, and they can also produce metal chelators to inactivate the cations (Moenne et al., 2016; Pinto et al., 2003). The chelates are then accumulated in organelles such as vacuoles and chloroplasts (Andrade et al., 2004), or physodes in brown algae (Lignell et al., 1982). Thus, not all of the intracellular metals will be available, and the availability will depend on the organelles where they are located and the molecules with which the metals form complexes.

Determining the cellular location is also important for correct interpretation of the results of experiments in which seaweeds are used to study global change. For example, in biomonitoring, in which seaweeds are used to obtain information about pollutants in the environment, knowing whether the metals are extracellularly (reversibly) bound or are immobilized inside cells is key for interpretation of the findings.

3. Previous studies

3.1. Extracellular elution

Extracellular elution has been used to determine where heavy metals are located in seaweeds. We have identified two different methodological approaches in the existing literature. In both cases, the technique comprises one or more washing steps with extractants (e.g. chelating agents, heavy metals of higher covalent index and ionic ratios than the studied metal, leading to displacement by cationic competition, or acids). In the first approach, i.e. the Sequential Elution Technique (SET), 3–4 washing steps are carried out to separate the metal into the cellular locations described above (Pérez-Llamazares et al., 2011a); however, this method has scarcely been used in seaweed (Costas and López, 2001). In the second approach (i.e. in Table 1 all the references included as "extracellular elution", except for Costas and López, 2001) it is assumed that the total concentration of any metal can be described as follows: Total concentration$_{metal}$ = Intracellular concentration$_{metal}$ + Extracelullar concentration$_{metal}$. The intracellular concentration is determined after washing the extracellular fraction with EDTA. From a conceptual point of view, this method is an oversimplification, because the intracellular fraction also includes the internal particulate fraction, and the extracellular fraction also includes the external particulate and the intercellular fractions. Furthermore, as in some studies the extracellular extract (obtained by washing with EDTA) is not chemically determined (e.g. García-Ríos et al., 2007; Ryan et al., 2012; Turner et al., 2009), and is just calculated by subtracting the intracellular fraction from the total concentration, this sometimes leads to incoherent results (e.g. intracellular concentrations higher than the total concentration: see Table 1).

On the other hand, according to Pérez-Llamazares et al. (2011a), there are three problems associated with using a single EDTA washing step. First, the extracellular extractant may cause changes in membrane permeability. The integrity of the plasma membrane can be ensured by considering how some nutrients (i.e. K, Ca, and Mg) are distributed between the extra- and intracellular fractions when the membrane is not disrupted. It has been reported that EDTA produces alterations in the permeability of the plasma membrane in green algae (Henriques et al., 2019) and that the magnitude of the changes depend on the concentration used. The change in permeability produced by different concentrations of EDTA (5 and 10 mM) has also been demonstrated in brown algae (see Supplementary Material 2). This brings into question the results of previous studies using these concentrations of EDTA, as both fractions considered (intra and extracellular) may be mixed together. Secondly, the extractant used may partially extract the metal bound to the external particles, which comprises an important fraction of the total concentration in seaweed as it is shown in Supplementary Material 1 for brown algae. Pérez-Llamazares et al. (2011b) showed that, with other extracellular extractants (i.e. $NiCl_2$) the extracellular fraction obtained includes the metal (i.e. Zn) both bound to cation exchange sites and to particles on the plant surface; however, this was not tested in any of the other studies reviewed. Thirdly, to prevent overestimation of the intracellular fraction, it is essential to use a chelating agent or metal that effectively extracts the extracellularly bound elements. In other studies with cryptogams, the plant is incubated in a metal solution for a short time (ca. 30 min) (after verifying that the solution does not cause alterations to membrane permeability) to check if the extractant effectively removes the extracellular metal. Studies analyzing the kinetics of metal uptake show that during the first minutes or hours there is a rapid phase of uptake consistent with an extracellular adsorption (Wang and Dei, 1999), and therefore after a short incubation period an effective extracellular extractant should remove most of the metal. The concentration of the extractant, the duration of the extraction and the number of extracellular extractions must be considered. However, until now, no researchers have tested the efficiency of EDTA as a heavy metal extractant in this way, and the only evidence for the effectiveness of EDTA is the results of speciation calculations reported by Vasconcelos and Leal (2001). For this reason, we conducted a simple experiment to test the effectiveness of EDTA for extracting metals from seaweed (Supplementary Material 3). The results show that despite the short incubation with Cd, only between 4 % and 9 % of the total metal in the seaweed was detected in the EDTA extraction solution, demonstrating that EDTA does not works as a chelating agent in the brown algae studied. Thus, it appears impossible to find a concentration of EDTA that is high enough to extract all of the extracellular metal, but low enough not to damage the membrane. The lack of an effective extractant could explain most of the findings summarised in Table 1 (and others such as Kearns and Turner (2016) for Bi; and Turner et al. (2007) for Pt, Pd and Rh), leading to detection of high intracellular concentrations, in direct contrast to the results of numerous studies using other cryptogams and included in a review paper by Pérez-Llamazares et al. (2011a). Furthermore, the concentrations of organelles obtained by differential centrifugation techniques are lower of those obtained by EDTA chelation (Huang et al., 2013), further indicating the inefficient extraction by EDTA (see Table 1).

3.2. X-ray microanalysis

X-ray microanalysis has also been used to localize metals at subcellular level. This technique is commonly applied in combination with Transmission Electron Microscopy (TEM) to create images of the cells and visualize their condition, as well as potential areas for metal enrichment. Then, X-ray microanalysis is applied to specific sub-cellular structures to obtain an emission spectrum based on the electromagnetic radiation of the area analyzed. The results obtained are qualitative, but the method has been used to compare the concentration of metals in different compartments of the cells, especially between the outer layers and the intracellular compartment (see Table 1). Studies using this technique are, surprisingly, not as common as those using extracellular elution, and they only appeared between the 1980s and early 2000s. There are no replicate studies analyzing the concentration of the same metal in the same species, which makes it impossible to make direct comparisons. The two elements most commonly analyzed are Cu and Zn, which show large differences between different species (see Table 1).

As with the previous technique, differences in the results could be due to the natural variability in metals and species, but also to methodological

Table 1
Percentages of total metal concentrations (extracellular [ext] + intracellular [int]) detected intracellularly in macroalgae reported in the literature. Data are classified by the technique used to estimate the cellular location (Tech.; E, extracellular elution; M, X-Ray microanalysis; C, centrifugation). Brackets after the species name include the phylum: (*R*), Rhodophyta; (*C*), Chlorophyta; and (*P*), Ochrophyta (class Phaeophyceae). Exposure time is expressed in days (Exp.). When qualitative data are available, the sign "=" represents a similar distribution of the concentration between both locations [int] ≈ [ext]; "++" [int]>> [ext, undetectable]; "+" [int] > [ext]; "-" [int] < [ext]; and "--" [int, undetectable] >> [ext]. Note that [int] higher than 100 % indicates anomalous results corresponding to negative [ext]. Genus and species names are currently accepted taxonomic names (if the papers used synonyms or outdated names they were updated in accordance with algaebase.org). For papers that did not report the numerical values of the data, the values were extracted with the WebPlotDigitizer software, available at https://automeris.io/WebPlotDigitizer/.

Tech.	Reference	Genus/species	Exp.	Al	Cr	Mn	Co	Ni	Cu	Zn	As	Sr	Cd	Hg	Pb
E	(Ryan et al., 2012)	*Ascophyllum nodosum (P)*	–		130	91	76	136	91	71	91		100		212
E	(Roncarati et al., 2015)	*Ectocarpus siliculosus (P)*	10						47[1]						
E	(Ryan et al., 2012)	*Fucus vesiculosus (P)*	–		150	98	104	114	115	109	112		130		113
E	(Andrade et al., 2006)	*Lessonia nigrescens (P)*	4.17						46[2]						
E	(Vasconcelos and Leal, 2001)	*Ulva* sp. *(C)*	1						55[3]				72[4]		53[5]
E	(Costas and López, 2001)	*Ulva lactuca (C)*	0.25						62				22		
E	(Ryan et al., 2012)	*Ulva lactuca (C)*	–		103	71	133	103	95	88	93		167		75
E	(Henriques et al., 2019)	*Ulva lactuca (C)*	3		100	80		50	70		<40		<40	100	60
E	(Turner et al., 2009)	*Ulva lactuca (C)*	–						39[6]	86[7]					
E	(Masakorala et al., 2008)	*Ulva lactuca (C)*	–						31[8]						46[9]
E	(Valdés et al., 2018)	*Ulva lactuca (C)*	1						78[10]						
E	(García-Ríos et al., 2007)	*Chondrophycus poiteaui (R)*	3, 7										114[11]		
E	(García-Ríos et al., 2007)	*Gracilaria cornea (R)*	3, 7										44[12]		
E	(Huang et al., 2013)	*Gracilaria edulis (R)*	20						18[13]						
E	(Huang et al., 2013)	*Gracilariopsis lemaneiformis (R)*	20						22[14]						
E	(Vasconcelos and Leal, 2001)	*Porphyra* sp. *(R)*	1						61[15]				86[16]		53[17]
E	(Ryan et al., 2012)	*Vertebrata lanosa (R)*	–		113	21	78	84	81	94	86		89		58
M	(Skipnes et al., 1975)	*Ascophyllum nodosum (P)*	2–6							+		–			
M	(Pedersen, 1981)	*Fucus vesiculosus (P)*	n.a.						=						
M	(Lignell et al., 1982)	*Fucus vesiculosus (P)*	5	=									=		
M	(Smith et al., 1986)	*Fucus vesiculosus (P)*	–						+						
M	(Amado Filho et al., 1996)	*Padina gymnospora (P)*	4							- -					
M	(Amado Filho et al., 1999)	*Padina gymnospora (P)*	–							–					
M	(Salgado et al., 2005)	*Padina gymnospora (P)*	–						+ +						
M	(Talarico, 2002)	*Colaconema savianum (R)*	10–15										=		
M	(Andrade et al., 2004)	*Ulva flexuosa (C)*	5							=					
C	(Zhao et al., 2015)	*Neopyropia yezoensis (R)*	4										42[18]		
C	(Zhao et al., 2019)	*Neopyropia yezoensis (R)*	6												55[19]

Values included in the table represent the mean value of all data in the paper, and:
[1] Median values of concentrations used were 53 % and 49 %, with ranges of 39–67 % and 30–49 % for each locality respectively.
[2] Median of registered times, 47 %; range 36–59 %.
[3] Temporal mean, 86 %; range 74–97 %. Mean value of concentrations used, 87 %; range 71–95 %.
[4] Temporal mean, 67 %; range 41–81 %. Mean value of concentrations used, 43 %; range 41–46 %.
[5] Temporal mean, 61 %; range 51–70 %. Mean value of concentrations used, 46 %; range 45–47 %.
[6] Median concentration of the antifouling paint tested, 16 %; range 5–100 %.
[7] Median concentration of the antifouling paint tested, 72 %; range 48–145 %.
[8] Median concentration of the surfactant tested, 32 %; range 25–64 %.
[9] Median concentration of the surfactant tested 47 %; range 16–43 %.
[10] Median values of concentrations tested 71 and 85 %, with ranges of 75–89 % and 67–81 % for each locality respectively.
[11] Median value of concentrations/days tested, 106 %; range 6–183 %.
[12] Median value concentrations/days tested, 28 %; range 20–125 %.
[13] Median value of concentrations tested, 15 %; range 13–33 %.
[14] Median value of concentrations tested, 19 %; range 18–33 %.
[15] Temporal mean, 64 %; range, 59–77 %. Mean value of concentrations tested, 58 %; range, 50–63 %.
[16] Temporal mean, 61 %; range, 50–72 %. Mean value of concentrations tested, 45 %; range, 43–49 %.
[17] Temporal mean, 69 %; range, 57–92 %. Mean value of concentrations tested, 76 %; range, 54–87 %.
[18] Median value of concentrations/time tested, 46 %; range 21–59 %.
[19] Median value of concentrations/time tested, 55 %; range 36–73 %.

problems. The sensitivity of the technique at the time these papers were published was very low, and the very high limit of detection made the determination of the target elements impossible in some cases. In addition, some authors reported that the extracellularly bound metals could be lost during the fixation of the samples for microscopic observation due to the washing steps and use of strong chemicals (Lignell et al., 1982; Pedersen, 1981; Smith et al., 1986). Nevertheless, in the last two decades great advances have been made in this technique, leading to the development of the Energy-dispersive X-ray spectroscopy (EDX) technique, which is currently the most common version of x-ray microanalysis. However, the only recent study that applied this technique to seaweed did so after washing the material with EDTA (Espinoza et al., 2021), enabling detection of the presence of Cu inside the cells, but not enabling comparison with the extracellular concentrations.

3.3. Differential centrifugation techniques

Another way of fractionating the metal content of seaweed is to physically separate the cell compartments. For this purpose, differential centrifugation techniques can be used to isolate the cell walls, soluble fraction and organelle-containing fraction, in which the metal concentration is determined (Zhao et al., 2019, 2015). The results of these studies are shown in the Table 1, considering as extracellular the metals they detect in the cell wall, and the remainder (referred in the articles as organelles and soluble fraction) as intracellular fraction. In another study this technique was even used to determine the metal content of chloroplasts, nuclei, mitochondria and ribosomes (Huang et al., 2013). However, there were some problems associated with this method: the freezing and homogenization steps can disturb membrane integrity, homogenization can mix the different components, affecting

the metal distribution, and the buffer can alter the cation equilibrium. Furthermore, the partitioning does not correspond to that established in Section 2.1., because the extracellular and external particulate fractions attached to the external side of the membrane are not explicitly included in the extracellular fraction, and the internal side of the membrane is not included in the intracellular fraction. It is also not clear to which compartment the soluble fraction belongs, as it could be either intracellular or intercellular.

3.4. Indirect sources

In addition to the aforementioned techniques that directly investigate the partitioning of metals in seaweed, other studies have provided evidence supporting the presence of metals in one or other of the compartments. For instance, seaweeds have been used extensively in bioremediation strategies to treat wastewater (Znad et al., 2022). In this discipline, seaweeds can be used as a biotechnological tool to remove toxic elements from water, often as treated or inactivated biomass (Mehta and Gaur, 2005). In these studies most of the metal is assumed to be adsorbed on the exterior of the cells (Davis et al., 2003), as different results support this idea: I) dried seaweed can accumulate more metals than live seaweed (Mehta and Gaur, 2005), indicating that active transport and accumulation in organelles are not the main mechanisms of metal accumulation; II) ion exchange occurs, i.e. the biomass releases previously adsorbed cations when exposed to new ones (Davis et al., 2003; do Nascimento Júnior et al., 2019; Figueira et al., 2000); and III) when isolated, the cell wall polysaccharides are also capable of capturing metals (Zhao et al., 2018).

Another group of studies providing information about the location of metal in seaweed include those focusing on uptake and release kinetics. For high concentrations of metal, the uptake kinetics can usually be divided in two phases: during the first minutes or hours there is a period of rapid accumulation that has been attributed to extracellular adsorption, and is then followed by a linear, slower uptake that could correspond to intracellular uptake (Wang and Dei, 1999), supporting the presence of metal in both compartments. For the release kinetics, experiments carried out with brown algae transplants have shown that seaweed grown in polluted sites exposed to unpolluted water can release previously accumulated metals within 5 days (Supplementary Material 4), which is consistent with rapid, reversible binding in the extracellular compartment. A decrease in the concentration of metals accumulated by seaweed was also detected throughout a year, and robust seasonal variation observed (García-Seoane et al., 2021). The authors point out that this variation is consistent with the seasonal cycles in the concentration of alginates of the seaweed, suggesting that adsorption to these molecules is one of the major mechanisms for accumulation.

4. Current state of knowledge and final remarks

The evidence obtained supports the presence of metals both inside and outside the cells of seaweed; however, the findings regarding the cellular partitioning of the metals are very variable depending on the technique, the metal and the algal species considered in each study (Table 1). A high level of variation between studies considering the same species and metal has also been observed, as in the case of Cu concentrations in *Ulva lactuca*, with intracellular concentrations ranging from 38 to 95 %. Part of the differences can be attributed to individual variability as well as to the bioavailability of metals, which will differ according to the source of contamination and the environmental compartment (i.e. sediments may presents high metal contents complexed with organic matter whereas in sea water free bioavailable ions predominate) (Gaulier et al., 2019; Klink et al., 2019). Therefore, it is to be expected that in areas with higher sedimentary inputs, a large fraction of the metals will be found extracellularly, due to the difficulty of complexed ions to cross membranes.

However, the methodological problems here identified may also be a major source of variation. Thus, the elution technique has been applied without testing the efficiency of the extractants; past studies with X-ray microanalysis lacked the sensitivity and resolution to detect natural concentrations in seaweed, and some of the extracellular metal fraction may have been lost during fixation of the samples; finally, centrifugation does not account for the mixing of the different compartments during the process.

Although solving these problems could improve the reliability of the findings, there remain some unavoidable issues: in both the elution and centrifugation techniques the fate of metals in the particles attached to the surface is difficult to determine and isolate; and centrifugation cannot distinguish between the metals attached to the outer and inner faces of the plasma membrane, which has different biological implications. Thus, regardless of the improvements in these techniques, some limits to the accuracy that can be achieved will remain.

The X-ray microanalysis technique has improved greatly since it was last used in studies to determine the cellular location of metal in seaweeds. Both the accuracy and sensitivity have increased, and now it allows to map the distribution of elements in the cells, providing semi-quantitative results. Hopefully, these advances will enable use of this technique to accurately determine the subcellular location of metals, including their presence in small structures like particles and organelles, and even in compartments that were not considered by previous models, such as the bacteria on the surface of seaweed. However, methodological problems with the fixation of the samples must first be solved, and methods that prevent the loss of extracellular metals must be developed.

Thus, to date, the cellular location of metals in seaweed remains unknown and represents an important knowledge gap in relation to understanding the impact of metal pollution on these organisms. Such knowledge is essential to enable prediction of the effects of global change on seaweeds and to understand the information that seaweeds can provide about global change. More research using up-to-date techniques is needed to close this gap, as this would give us a deeper understanding of coastal pollution, the effect it can have on the organisms leaving in these ecosystems, and how it can affect the services they provide us. This would allow us to control and regulate metal pollution in a proper, comprehensive way.

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CRediT authorship contribution statement

Antón Vázquez-Arias: Investigation, Writing – original draft. **Carme Pacín:** Investigation, Writing – original draft. **Ángela Ares:** Writing – review & editing. **José Ángel Fernández:** Conceptualization, Funding acquisition, Writing – review & editing. **Jesús Ramón Aboal:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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6.4. Capítulo 4: Nanoscale distribution of potentially toxic elements in seaweeds revealed by synchrotron X-ray fluorescence

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Nanoscale distribution of potentially toxic elements in seaweeds revealed by synchrotron X-ray fluorescence

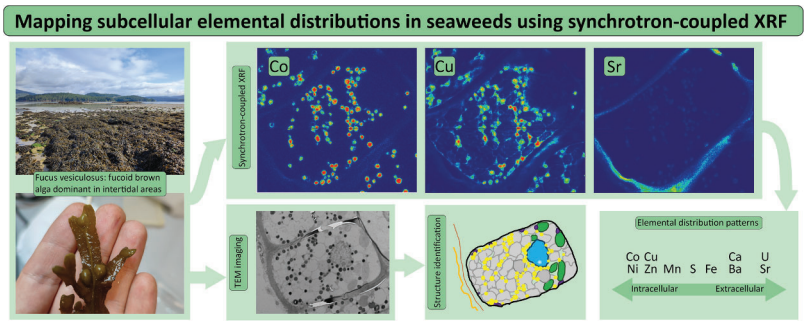
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HIGHLIGHTS

- PTE distribution in *Fucus vesiculosus* mapped at the nanoscale using Synchrotron XRF.
- Co, Ni, Cu, and Zn are found primarily intracellularly, accumulated in physodes.
- The highest concentrations of Ba and U are in the cell wall.
- Physodes play a key role in regulating intracellular PTE concentrations.

GRAPHICAL ABSTRACT



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XRF

ABSTRACT

Assessing the impact of potentially toxic elements (PTEs) on coastal waters requires understanding their interaction with seaweeds, as they are foundational organisms in nearshore ecosystems. While seaweeds are known to accumulate PTEs, information on the mechanisms and locations of this accumulation is very limited. We employed synchrotron-based X-ray fluorescence (S-XRF) to map the distribution of various chemical elements at nanometric resolution in *Fucus vesiculosus*, a brown alga dominant in intertidal zones. Our results revealed that different PTEs have distinct subcellular accumulation patterns, which are largely consistent across different samples and cell layers. The distribution of Ba and U was predominantly located in the cell walls, while Co, Ni, Cu, and Zn were concentrated within physodes, specialized organelles containing phlorotannins. These findings suggest that *F. vesiculosus* regulates PTE uptake either by preventing their accumulation, or by sequestering them into specialized organelles. Physodes seem to play a key role in this regulation, concentrating divalent elements to minimize their toxicity and potentially storing them for intracellular functions. The use of S-XRF allowed us to map elemental distributions on seaweed cells with unprecedented detail, furthering our understanding of the subcellular structures responsible for PTE accumulation, and setting the foundation for studying their uptake mechanisms.

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1. Introduction

Seaweeds, especially brown algae, form the foundation of coastal marine ecosystems in cold and temperate regions, acting as primary producers and ecosystem engineers, and fostering highly diverse and productive ecosystems [1,2]. These organisms inhabit coastal waters, which are particularly vulnerable to environmental degradation due to the wide range of productive, recreational, and industrial activities they sustain. Furthermore, they receive discharge from rivers, which are often used for waste disposal [3]. Consequently, pollution is one of the primary stressors in coastal waters, and seaweeds, as sessile species, are continuously exposed to it.

Potentially toxic elements (PTEs) can damage seaweed health [4] and, while PTE concentrations in seaweed have generally been decreasing for decades [5], ongoing ocean acidification may increase PTE bioavailability, exacerbating their toxicity [6]. Yet, even for pollutants as chemically simple as PTEs, little is known about their interaction with seaweeds and its potential biological and ecological consequences. Seaweeds accumulate PTEs in high concentrations, but the location and mechanisms of this accumulation remain largely unknown. Bridging these knowledge gaps is critical for understanding how PTEs impact seaweeds, as PTE toxicity varies depending on whether they are bound to the cell walls or internalized, and on which intracellular structures they can reach. A recent review of the available information on the fate of elements accumulated by seaweeds found inconsistent and conflicting results [7]. Different studies reported PTE accumulation in different compartments, with some authors finding most of the concentrations inside the cells, while others detected them bound extracellularly, even for the same element and algal species. As Vázquez-Arias et al. [7] noted, these contradictions may stem from shortcomings of the methodologies applied. For instance, the sequential elution technique (SET) uses extractants to isolate the element fractions bound to different structures, but it has often been applied without testing the effectiveness of the extractants (e.g. [8]). Centrifugation has been used to separate cellular compartments mechanically and then analyze them separately, but this can lead to the release and mobilization of PTEs during the process (e.g. [9]). Finally, earlier microanalysis studies lacked the resolution or sensitivity to distinguish subcellular structures, and most protocols involved chemical sample fixation, which can lead to elemental mobilization (e.g. [10]).

Fortunately, the limitations of micro-/nano-analysis have been tackled in recent years. To prevent the mobilization of chemical elements during chemical fixation, the use of cryofixation has been proposed, as it immobilizes elements in place by freezing the samples [11,12]. In terms of resolution, two new technologies have been developed recently that provide enough resolution and sensitivity to map the distribution of PTEs inside cells: nanoscale secondary ion mass spectrometry (NanoSIMS) and synchrotron-based X-ray fluorescence (S-XRF). Both of these have similar resolution (down to 20 to 50 nm) and sensitivity (mg kg^{-1} or even μg kg^{-1}, depending on the element), yet each has distinct advantages and limitations [13,14]: S-XRF allows quantitative analysis, is more sensitive to metals, and provides simultaneous information on all detectable elements, but it cannot detect light elements or differentiate isotopes. Conversely, NanoSIMS can detect light elements and specific isotopes, but cannot provide quantitative data and is limited to detecting up to 7 elements simultaneously. These techniques have been successfully used to study elemental distributions in different organisms [15-17], but few studies have applied them to seaweeds. NanoSIMS has only been used to study the distribution of As [18], Br and I [19] in the kelp *Laminaria digitata*. Synchrotron-based XRF has been mostly applied to analyze elemental concentrations in the surface of seaweeds [20-22], but a recent study mapped the distribution of Fe, As, Sr and Zn in cells of *L. digitata* [23]. These results, however, barely scratch the surface of the fate and mechanisms of accumulation of PTEs in seaweeds. The data gathered so far are limited to very few species and elements, excluding important PTEs such as Cu and Pb, and the elemental maps obtained did not find the subcellular structures involved in accumulation.

The seaweed investigated in these studies is a kelp species, which plays an essential role in coastal waters as an ecosystem engineer [24]. However, no research has yet explored the distribution of PTEs in fucoids, the other major group of habitat-forming seaweeds, despite being especially exposed to land runoff in the intertidal environments in which they dominate. This gap could be effectively addressed using S-XRF, as its multi-elemental simultaneous analysis can provide foundational information on the distribution of a wide range of elements. Resolving the fate of PTEs within fucoids would offer valuable insights into their potential impact on fucoid health, and help us predict how pollution might affect the ecosystems they sustain. Moreover, addressing this knowledge gap would benefit the applications of these species. Fucoid extracts are frequently used in the food and pharmaceutical industries [25,26], so the PTE content of the extracts could depend on their bindings sites, influencing product safety. Furthermore, fucoids are widely used in biomonitoring studies [27,28], whose interpretation depends on the accumulation dynamics of PTEs, which are in turn affected by the uptake mechanisms. Lastly, fucoids are rich in physodes, a cellular structure exclusive to brown algae which has been proposed, but not confirmed, to protect against PTE toxicity [25].

Therefore, the aim of this study is to analyze the subcellular distribution of chemical elements, particularly PTEs, using S-XRF on the fucoid alga *Fucus vesiculosus*. This approach allows us to test the hypothesis that elemental concentrations in fucoids vary across subcellular structures (nuclei, plastids, mitochondria, physodes, vacuoles and cell walls) depending on the cell layers of the seaweed (meristoderm, cortex and medulla) and the pollution sources they were exposed to during growth.

Table 1
Sampling sites, and their a priori pollution sources. Coordinates are in WGS84.

Code	Coordinates	Pollution sources
S1	43.46106, −8.26274	Small city with heavy industry
S2	43.36667, −8.38843	Medium-size city
S3	43.35128, −8.21464	None
S4	43.32796, −8.37884	Medium-size city
S5	42.48176, −8.89400	None
S6	42.40825, −8.68063	Former chlor-alkali industry
S7	42.34627, −8.61331	Former ceramics industry. Known Pb pollution
S8	42.22358, −8.75932	Medium-sized city with heavy industry

2. Material and methods

2.1. Sample collection

Samples of *Fucus vesiculosus* L. were collected from eight sites along the coast of NW Spain (Table 1) on July 4th, 2023. The sites were selected to represent a diverse range of pollution sources, including two mostly unpolluted locations. At each site, several thalli were picked up, briefly rinsed in seawater, placed in a cooler (ca. 5 °C), and transported to the laboratory. In the lab, the three most apical dichotomies of healthy thalli free of reproductive structures were cut. Two apices from each site were placed in polypropylene tubes with a small amount of seawater from the same sampling site and stored in a cold storage room (5 °C). The remaining material was dried and homogenized for analytical determination.

2.2. Analytical determinations with ICP-MS

The samples were further dried before analysis at 40 ºC in an oven to remove any residual moisture before analysis. From each sample, 1 g (dry weight) was digested in Teflon vessels using a microwave oven (Milestone Ethos-1) with a temperature ramp (100 ºC for 10 min, 150 ºC for 7 min, 190 ºC for 25 min), and adding 10 mL of HNO_3 (65 %), 2 mL of

H_2O_2 (30 %) and 2 mL of MilliQ water. Total concentrations of Mn, Fe, Co, Ni, Cu, Zn, Sr, Ba and U were determined by inductively coupled plasma mass spectrometry (ICP–MS, Agilent 7700x) at the University Research Support Services Unit (RIAIDT, Universidade de Santiago de Compostela). Quality control was ensured through replicates and the use of reference material (ERM-CD200, *Fucus vesiculosus*). For all elements, the variation among the replicates was less than 5 %, and the percentage recovery of the reference material for the only two elements certified (Cu and Zn) was 105 %.

2.3. Sample preparation for transmission electronic microscopy (TEM) and S-XRF

Within 24 h of sampling, the eight polypropylene tubes containing the selected apices were transported refrigerated to the Centro Nacional de Biotecnología (CNB-CSIC), where they were fixed for synchrotron-based XRF (S-XRF) analysis. The samples were fixed by cryofixation rather than standard chemical fixation to prevent the mobilization of trace elements [12], following a protocol adapted from Ender et al. [18]. Transverse sections, 150 μm in thickness, were cut from the most apical centimeter of the thalli using a vibratome and placed on gold-plated copper planchettes coated with 1- hexadecene. The sections were then subjected to high-pressure freezing (HPF) using a Leica EM ICE (Leica Microsystems). Cryosubstitution of vitrified fragments was carried out using a Leica EM AFS2 (Leica Microsystems) with a 1 % solution of osmium tetraoxide (OsO_4) in anhydrous acetone at −90 °C, followed by dehydration with 100 % anhydrous acetone at room temperature. The samples were then embedded in epoxy resin TAAB 812 (TAAB Laboratories) with increasing concentrations. Polymerization was conducted in 100 % epoxy resin for 2 days at 60ºC. A few fragments of each sample processed, and at least one from each sample, had well preserved regions, except for sample S5, which was excluded from the analyses.

Once fixed, the thallus anatomy was examined using optical microscopy on 500 nm sections stained with toluidine blue 1 %. For TEM, ultrathin sections (70 nm) were obtained, transferred to 200 mesh formvar carbon coated nickel grids, and stained with saturated aqueous uranyl acetate (25 min) and lead citrate 0,2 % (1 min) at room temperature. Finally, for S-XRF, 200 nm thick sections were cut and mounted onto silicon membranes (Silson, 0.5 μm membrane thickness) without staining. All section were obtained using an ultramicrotome Leica EM UC6 (Leica Microsystems). All electron and optical microscopy work was performed at CNB-CSIC.

2.4. TEM imaging

Ultrathin sections were visualized on a JEOL JEM 1400 Flash electron microscope (operating at 100 kV). Micrographs were taken with a Gatan One view digital camera at various magnifications.

2.5. S-XRF analysis

To study the subcellular elemental distributions, 200 nm sections were analyzed using S-XRF at the ID16B beamline of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The mapping was performed in air at room temperature by using a focused beam of 60 × 60 nm^2 at 29.6 keV with a flux of 10^{12} ph/s. The exposure time per pixel was 200 ms. We obtained an overview image of each sample to cover the three distinct cell layers of the thallus (from the outer surface to the inner part: meristoderm, cortex and medulla) at low spatial resolution (300 ×300 nm pixel size) and scanning an area of approximately 200 × 300 μm. Then, we obtained high-resolution images of specific cells (60 ×60 nm pixel size). For one sample, we acquired high-resolution images of cells from each cell layer, but as most elements were concentrated in the surface cells, the close-up images for the other samples were focused on the meristoderm. Elements with completely uniform XRF distribution patterns were discarded, as well as those exactly matching the distribution pattern of Os, as high concentrations of OsO_4 added during fixation could be interfering with the detection of those elements. Therefore, the following chemical elements were identified within the samples: Ag, Ba, Ca, Cl, Co, Cr, Cu, Fe, Mn, Ni, Os, S, Sr, U and Zn. Since Cl can be found in the embedding resin, its results, as for Os, do not reflect natural distributions. The elements analyzed varied in their potential for environmental toxicity and nutritional importance, but we focused on those with a higher potential to impact seaweed health due to environmental pollution (Ag, Ba, Co, Cr, Cu, Mn, Ni, U, and Zn); these are the elements we refer to as potentially toxic elements (PTEs).

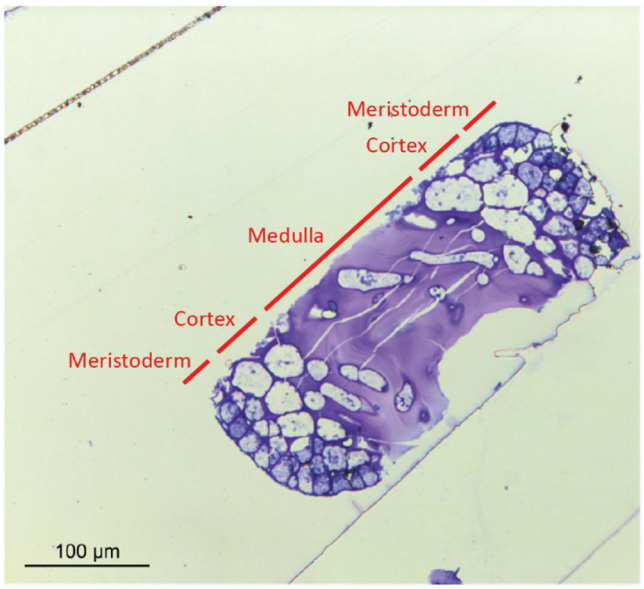


Fig. 1. Transverse section of *Fucus vesiculosus*. The names in the image highlight the three different cell layers. Section at 500 nm thickness, stained with toluidine blue.

The analysis of the XRF spectra recorded for each pixel allowed the obtention of relative abundance values for each element. Then, a reference sample (AXO DRESDEN GmbH) was analyzed to calibrate these results and transform them into concentration values.

2.6. S-XRF data processing

The data were fitted using PyMca 5.9.2 [29], which was also employed to export TIFF images for each analysis and element, as well as uniband images. The uniband images were further analyzed using Fiji [30] to identify cellular structures and select them as regions of interest (ROIs). Only visually identifiable structures were selected, discarding ambiguous, ruptured and overlapping regions. Using a custom script, the concentration values for each element in the pixels of each cellular structure, cell layer, and sample were exported, creating a dataset of 314 957 entries.

Data analysis was performed using R software version 4.4.1 (R [31]). Comparison between analytical techniques was carried out using Spearman correlations. Differences among groups were tested using the Scheirer-Ray-Hare test, and pairwise comparisons were conducted using Mann-Whitney U tests. The large n of the dataset greatly increased the power of the tests, leading to extremely low p-values in every comparison, even between populations with small differences without biological significance. Consequently, the tests were not included in the article, and instead we visualized the differences between cell layers and between subcellular structures using smoothed density estimates with gaussian kernels. Data visualization was performed using the ggplot2 package [32].

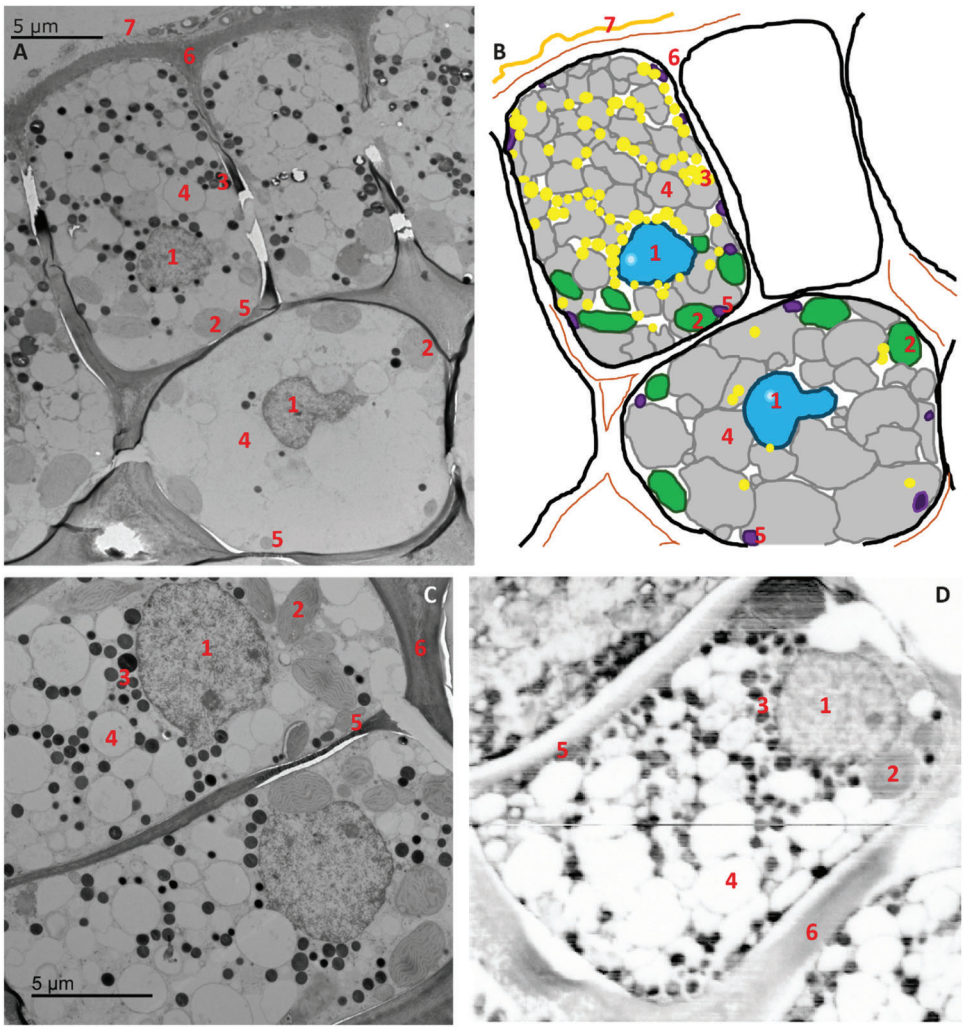


Fig. 2. Images of the cells of *Fucus vesiculosus* with numbered subcellular structures. **A**. TEM image of cells from the meristoderm (upper cells) and cortex (lower cells). Images A and C were obtained from 70 nm ultrathin sections (see Material and methods section). **B**. Diagram highlighting the structures identified in image A with color. **C**. TEM image of two meristoderm cells. **D**. Image obtained with S-XRF on 200 nm unstained sections. The color of each pixel represents the concentration of Os, added during the fixation of the samples. The numbers represent the following structures: 1: nucleus; 2: plastids; 3: physodes; 4: vacuoles; 5: mitochondria, 6: cell wall, 7: polysaccharides.

3. Results

3.1. Visualization of cell layers and ultrastructure

The anatomy of the thallus (Fig. 1) was observed under optical microscope, and consists of the following regions: the meristoderm, a single layer of palisade cells on the surface; the cortex, consisting of one to four layers of less densely packed cells located just below the meristoderm; and the medulla, located in the center of the thallus, composed of a polysaccharide matrix interspersed with sparse, elongated cells.

Sample preparation for TEM and S-XRF required different cut thickness, sample holders, and staining technique, which prevented the visualization of the exact same cells with both methods. Nonetheless, TEM images were invaluable for assessing sample preservation after fixation, and for identifying the subcellular structures to be recognized in S-XRF images. Fig. 2 illustrates the ultrastructural elements identified in the TEM and S-XRF images.

3.2. Comparison between S-XRF and ICP-MS results

The elemental concentrations of the samples as analyzed using ICP-MS and the average concentrations detected with S-XRF in the ROIs of the sections, as well as the ratios and correlations between them, are presented in Table 2. The absolute values determined by both techniques were markedly different, sometimes by more than one order of magnitude. The correlations between the results of both techniques showed low coefficients for most elements, with none being statistically significant.

3.3. General distribution patterns

The elemental distributions across different regions of *F. vesiculosus* thalli were visualized using a color scale to represent concentrations. Figs. 3 and 4 display the concentrations of representative elements for each pixel in the 300 nm-resolution images. The remaining elements can be seen in the annex SM1.

Distinct accumulation patterns were observed for each element. For example, the highest concentrations of Ni were found intracellularly, while other elements such as Sr were accumulated almost exclusively extracellularly. Despite this variation, the accumulation patterns were largely consistent across samples, with the same structures exhibiting the highest concentrations. A notable exception was seen with Ni, Cu and Zn, where intracellular concentrations were significantly higher than extracellular ones in all samples except S2, in which the

Table 2
Elemental concentrations (μg g^{-1}) in samples of *Fucus vesiculosus* collected in seven sites (S1-S8) along the coast of NW Spain. The first half of the table shows ICP-MS results, while the second half presents the average concentrations of the pixels inside ROIs in 300 nm-resolution S-XRF images. Only elements detected with both techniques are shown. Correlation: Spearman correlation coefficients between the values for both techniques. Cor. p-value: p-value for these correlations. Average ratio: cross-site average concentration of each element measured by ICP-MS divided by the average measured by S-XRF.

ICP-MS results								
	Mn	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**Sr**	**Ba**
S1	78.4	348	1.02	1.65	10.0	47.2	885	10.7
S2	23.0	64.2	0.17	0.99	7.66	51.8	660	5.87
S3	184	749	1.26	2.33	2.97	21.8	688	14.0
S4	145	1941	1.95	2.39	15.7	127	651	17.8
S6	108	643	0.56	3.66	8.77	55.6	728	12.9
S7	160	803	2.04	1.64	4.12	50.8	730	13.2
S8	84.6	104	0.96	1.95	5.39	90.7	809	7.71
Average S-XRF results								
S1	97.5	102	79.5	77.8	194	131	61.9	867
S2	89.5	101	76.7	133	228	172	49.8	835
S3	155	121	87.9	101	304	495	61.5	959
S4	98.4	102	81.7	125	207	249	51.8	814
S6	92.8	116	74.5	91.5	170	154	62.5	841
S7	104	129	81.3	80.5	165	204	61.5	918
S8	113	135	103	154	246	290	59.7	984
Correlation	0.71	0.11	0.46	0.00	−0.32	0.07	0.54	−0.14
Cor. p-value	0.09	0.84	0.30	1.00	0.50	0.91	0.24	0.78
Average ratio	1.04	5.77	0.01	0.02	0.04	0.26	12.60	0.01

extracellular concentrations reached similar levels.

To better analyze and visualize subtle differences in concentration, we sorted pixel values by cell layer and subcellular structures. Most structures could be reliably identified by their accumulation patterns, with the exception of mitochondria. In *F. vesiculosus*, mitochondria are small organelles located near the cell membrane that get strongly stained by OsO_4. However, other structures also fit that description, so they were grouped as peri-membrane organelles.

This data was used to study concentration differences across cell layers, by visualizing the density distributions of the concentrations for each subcellular structure across cell layers (Fig. 5 for representative elements, additional elements in the annex SM2).

For the same subcellular structures, elemental concentrations were generally similar across cell layers, although small differences were observed in some cases. For example, Sr concentrations showed almost complete overlap across layers for most structures, except for the cell wall. The cell walls of layers closer to the surface have higher concentrations of Sr, but their overall accumulation pattern remained distinct from that of other structures. This trend was observed for most elements: the highest concentrations were usually found in layers closer to the surface. However, since the variation across subcellular structures was greater than across cell layers, we pooled the data of all layers for each structure in further analyses.

3.4. Ultrastructural distribution patterns

To study subcellular elemental accumulation patterns, we visualized the density estimates of the element concentrations in each subcellular structure (Figs. 6 and 7 for representative elements, rest in annex SM3).

In the 300 nm-resolution images vacuoles and cytoplasm could not be distinguished, so they were analyzed together. These structures had the lowest concentrations for most elements, although other structures had similarly low concentrations for certain elements. The highest concentrations of Co, Cu, Ni, and Zn were found intracellularly, specifically in the physodes. In all but one sample (S2), the concentrations of these elements in physodes were much higher than in any other structure, while in S2 cell wall concentrations were comparable or even higher. Physodes also showed above-average concentrations of Ca, Mn and Sr. Other intracellular organelles had much lower concentrations for most elements, with some exceptions. Plastids, for instance, had higher concentrations of Fe, and Mn compared to other intracellular compartments.

Conversely, the highest concentrations of S, Ca, Sr, Ba and U were found extracellularly, in the cell wall and other extracellular polysaccharides. While concentrations in these two structures were generally similar, there were some minor differences. For Ca, Co, Cu, Ni and Zn, the cell wall had slightly higher concentrations, whereas other polysaccharides had higher concentrations of S in most samples.

Some elements were also found in mineral particles. Most particles were located on the surface of the alga, but some were inside the cells. For instance, Fe concentrations in mineral particles were on average two orders of magnitude higher than in organic material. The same pattern was found for Cr, Mn, Co, Ni and Cu, although with smaller differences. For Ag, concentrations in seaweed material were indistinguishable from the background, with the notable exception of a single pixel outside the cells of S8 (annex SM1).

The 60 nm-resolution images enabled better visualization of the cellular ultrastructure. Most patterns observed at this resolution were already observable in 300 nm-resolution images, but the finer detail allowed for the differentiation between vacuoles and cytoplasm, as it can be seen in Fig. 1D, and in the annex SM4. Generally, concentrations in the cytoplasm were similar or higher than those in vacuoles without exceeding those of other intracellular organelles.

4. Discussion

Using S-XRF, we successfully resolved the subcellular location of a wide range of chemical elements in *F. vesiculosus*. This study is the first to examine the distribution of elements in fucoids at this scale and provides the most comprehensive and detailed information available on seaweed accumulation patterns to date. Surprisingly, previous information for seaweeds was limited to a few elements in the kelp-forming *L. digitata*, and even less in the filamentous brown alga *Ectocarpus siliculosus*. Verhaeghe et al. [12] used NanoSIMS to determine that Br and I were predominantly located outside the cells of *L. digitata*, and Ender et al. [18] reported similar results for As. The most detailed study until now [23] used S-XRF to show that Sr was primarily found in cell walls, while Fe and Zn were colocalized inside the cells, but with considerable variation across thallus regions and between the analyzed species *L. digitata* and *E. siliculosus*.

In our samples of *F. vesiculosus*, the distribution of Sr aligns with these findings, as we observed it primarily attached to external

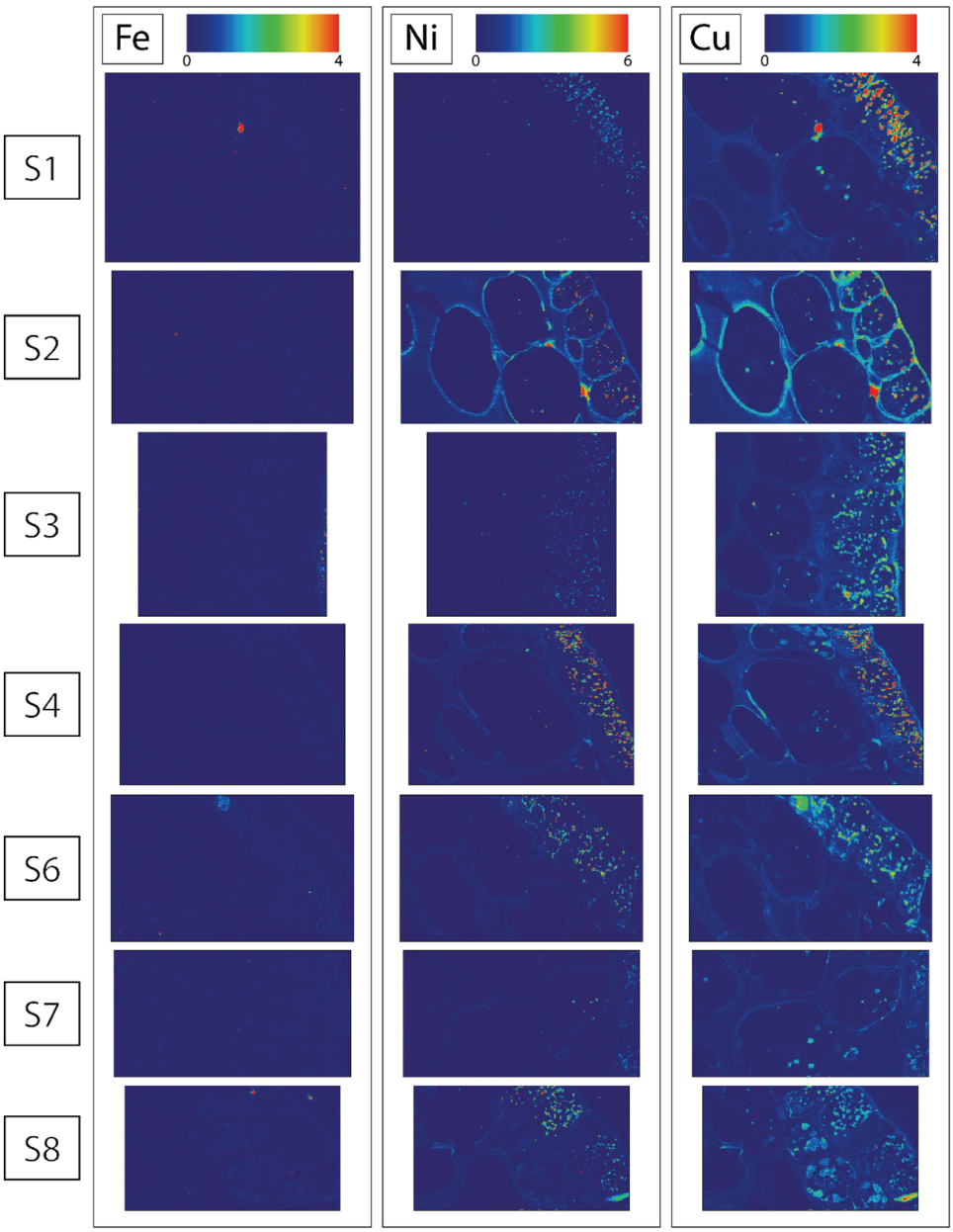


Fig. 3. Uniband images of Fe, Ni and Cu concentrations in the 300 nm-resolution S-XRF images of *Fucus vesiculosus* samples (S1-S8). The color represents the concentration of each pixel in mg g^{-1}. Concentration ranges vary among elements (see color scale values).

polysaccharides, either in the cell wall or otherwise. This distribution resulted in higher concentrations in the medulla, which is largely composed of extracellular polysaccharides. For Fe, in addition to mineral particles on the seaweed's surface, we identified structures with high concentrations in some cortical cells which may be akin to the cisternae-like metal-accumulating structures found in *L. digitata* [23]. The next highest Fe concentrations were found in the cell wall, particularly close to the surface, resembling findings in *E. siliculosus* [23]. Unlike in that study, we did not find Zn to be colocalized with Fe. This may be due to the abundance of physodes in *F. vesiculosus*, which contain high concentrations of Zn but almost no Fe.

Out of the PTEs detected, Co, Ni, Cu and Zn were predominantly located inside physodes. These elements share similar atomic weights, are commonly found as divalent cations, and are essential for metabolism [33], mostly due to their role as cofactors for hundreds of enzymes. The only other PTE found intracellularly was Mn, also a divalent cation of similar atomic weight, which was primarily located inside plastids, likely due to its function as part of photosystem II in photosynthesis [34]. In contrast, Ba and U, which lack known physiological functions, were mainly located outside the cells, similarly to Sr in our experiment and As in Ender et al. [18], other non-essential elements. These findings suggest that seaweeds can effectively regulate the accumulation of PTEs by restricting the internalization of non-essential elements and chelating essential elements intracellularly inside specialized organelles. This is further corroborated by the fact that in polluted sites, where PTEs were higher overall, structures like the vacuoles and cytoplasm did not have increased levels. These findings support the hypothesis that physodes found in fucoids play an essential role in protecting the cells against PTE toxicity. Physodes are small vesicles containing phlorotannins, compounds recognized by their metal-binding capabilities, leading some authors to suggest they may have a role chelating PTEs as a protection mechanism [35,36]. However, other studies have indicated that phlorotannin content is not directly correlated with metal-binding capacity [37,38], and in the fucoid

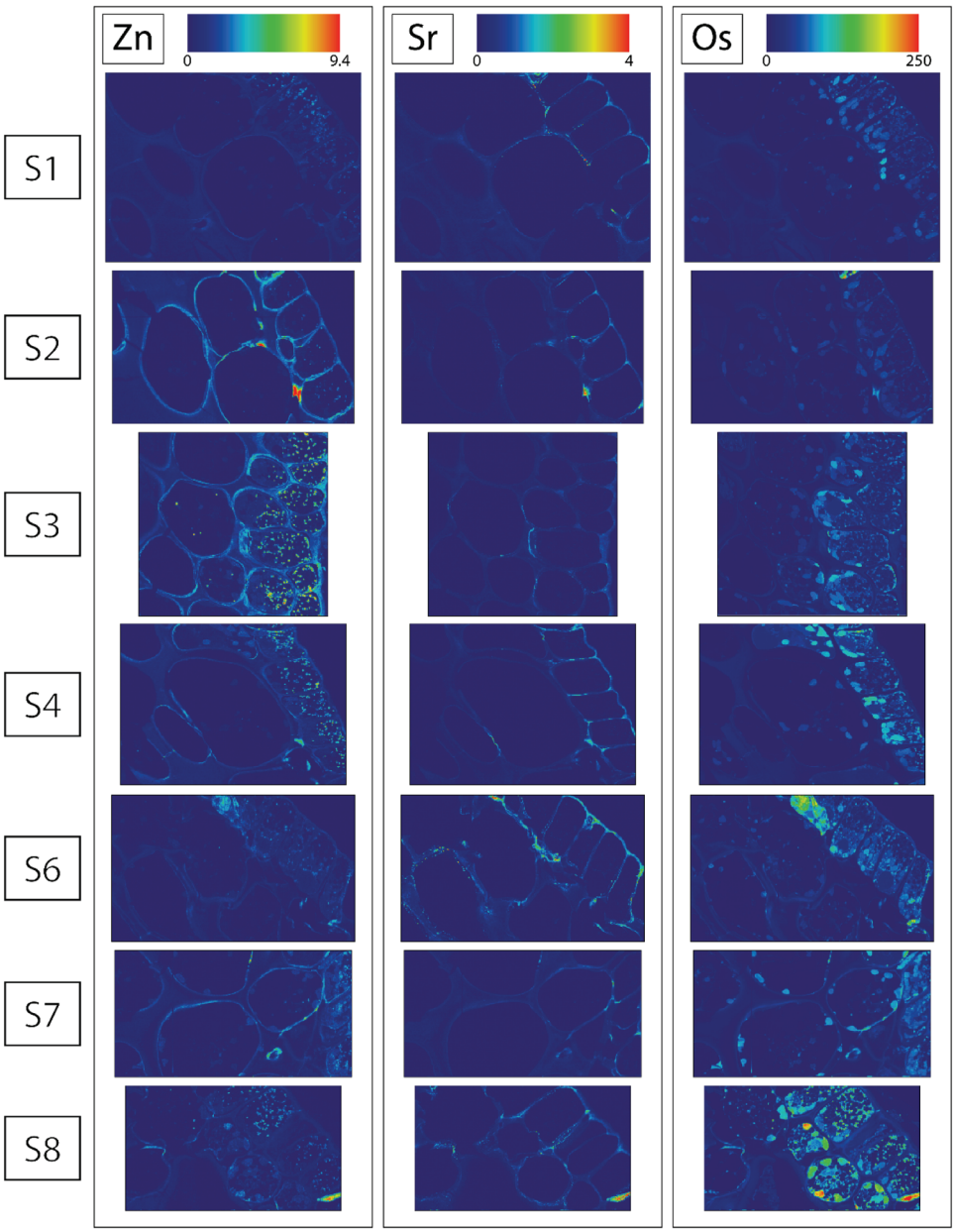


Fig. 4. Uniband images of Zn, Sr and Os concentrations in the 300 nm-resolution S-XRF images of *Fucus vesiculosus* samples (S1-S8). The color represents the concentration of each pixel in mg g^{-1}. Concentration ranges vary among elements (see color scale values).

Ascophyllum nodosum, physodes do not appear to be a protection mechanism against Cu toxicity [39]. In contrast, our results demonstrate that in *F. vesiculosus,* physodes sequester most of the intracellular concentrations of some PTEs.

Still, there are open questions regarding metal accumulation in seaweed. The cause of intracellular accumulation of divalent ions could be explained by various hypotheses: 1) they are internalized through transporters that handle less toxic elements, as observed in other organisms [40], and then sequestered into physodes to mitigate toxicity; 2) they are internalized for their importance in cellular metabolism and stored in physodes; and 3) they play a role in UV protection. Regarding the last hypothesis, physodes in fucoids are positioned above chloroplasts to shield them from UV radiation [41], and some compounds of the elements accumulated in physodes, such as ZnO_2, are known to absorb UV wavelengths [42]. Thus, seaweeds might be taking up divalent ions to protect themselves from radiation. Another unresolved question is the nature of the uptake mechanisms; while extracellular accumulation is understood as chemical binding to extracellular polysaccharides [43], the internalization mechanisms and their dynamics remain unclear. To address these questions, it would be valuable to analyze the distribution of additional PTEs that could not be detected in his study such as Pb and Cd, which are not important for metabolism. Besides, understanding the distribution of PTEs with different chemical properties could elucidate whether internalization is restricted to elements with specific intracellular functions.

The concentration values detected by S-XRF differed notably from those obtained by ICP-MS, a discrepancy expected due to several factors affecting elemental concentrations in fixed samples: 1) the presence of certain elements in the fixatives, such as Cl and Os; 2) biomass changes due to the resin embedding process; 3) potential element wash-off due to mobilization during fixation; and 4) the removal of surface mineral particles during fixation and sectioning, which can represent an important fraction of total concentrations in fresh samples. These limitations are inherent to the protocol used, meaning that concentration

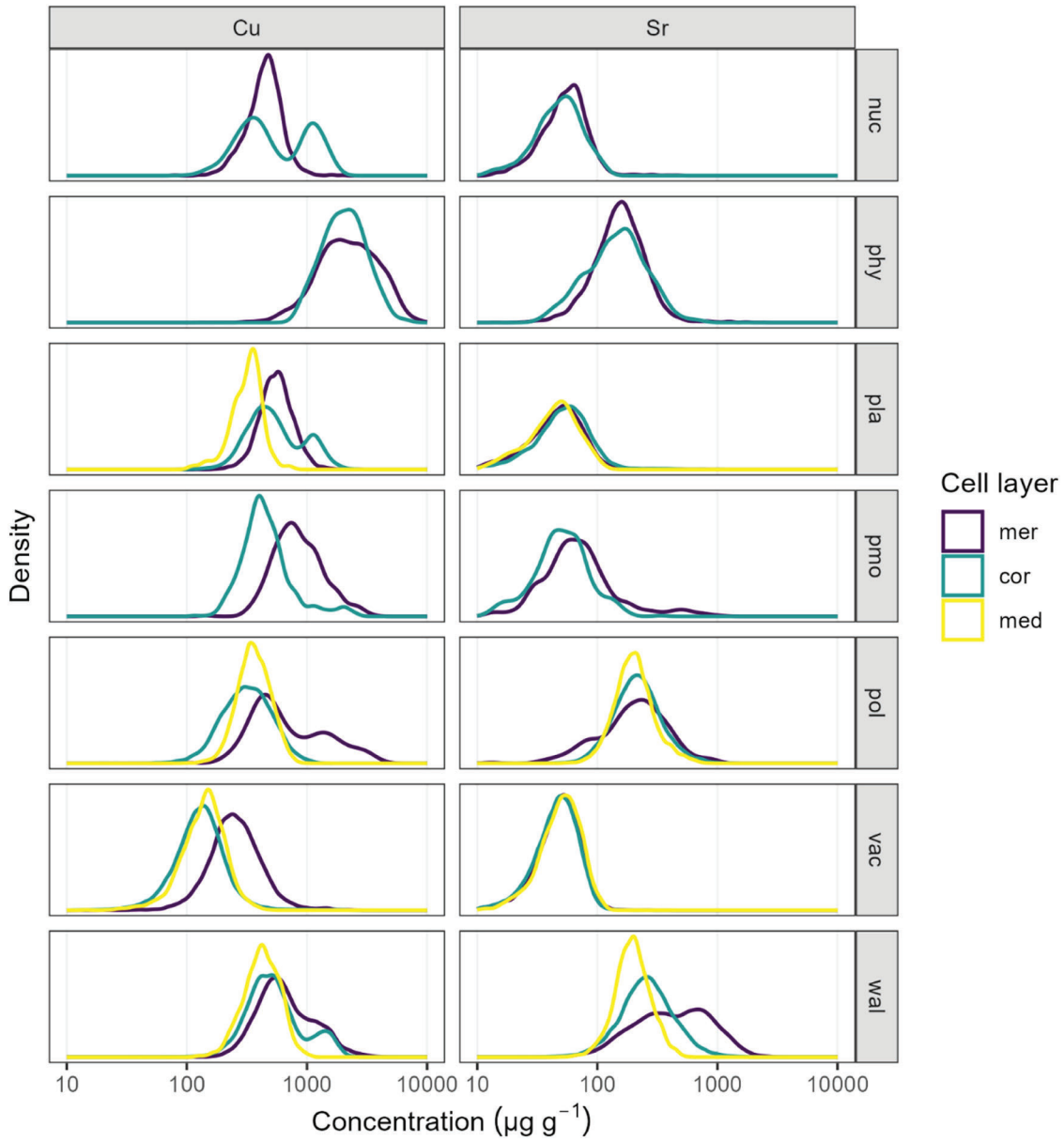


Fig. 5. Distribution estimates of Cu and Sr pixel concentrations in the 300 nm-resolution S-XRF images of *Fucus vesiculosus* samples (S1-S8). The x-axis is on a logarithmic scale. Cell layers: mer: meristoderm; cor: cortex; med: medulla. Subcellular structures: nuc: nuclei; phy: physodes; pla: plastids; pmo: peri-membrane organelles; pol: extracellular polysaccharides; vac: vacuoles and cytoplasm; wal: cell wall.

values are not directly comparable to those from fresh or dried samples. One option to mitigate these biases would be to apply S-XRF to frozen samples, for example using other Synchrotron beamlines such as ID16A at ESRF. However, this was not necessary for our objective, as accumulation patterns were clearly detectable. These factors, along with our focus on specific regions which may not be representative of the entire thallus [44], also explain the weak correlation between elemental concentrations measured by both techniques. The sections scanned with S-XRF varied among samples and excluded particles present in the material analyzed by ICP-MS. Therefore, while our measurements for each subcellular structure were consistent, they did not necessarily correlate with the concentrations of the complete thallus.

5. Conclusion

We used Synchrotron-based XRF to elucidate the subcellular distribution of several chemical elements in the brown algae *Fucus vesiculosus*. Most PTEs were localized extracellularly (Ba and U) or inside physodes (Co, Ni, Cu and Zn), but not in other intracellular compartments. These findings suggest that *F. vesiculosus* tightly regulates the uptake of PTEs, either by preventing their internalization or by chelating them using specialized organelles. Notably, physodes seem to play an important role protecting against PTE toxicity in *F. vesiculosus*, in contrast with observation made in other brown algae species.

S-XRF has proven to be an invaluable tool for revealing elemental distributions at the nanometric scale in seaweed, as it has in other organisms. Nonetheless, there are still unanswered questions regarding elemental accumulation in seaweeds. For instance, uptake mechanisms are still largely unknown; while extracellular adhesion must be mediated by chemical binding to extracellular macromolecules, internalization mechanisms are yet to be understood. Additionally, the fate of several elements significant for their biological roles or potential toxicity, such as Mg, Pb, and Cd, remains unknown. Current research is also limited to brown seaweeds, while red and green seaweeds, which

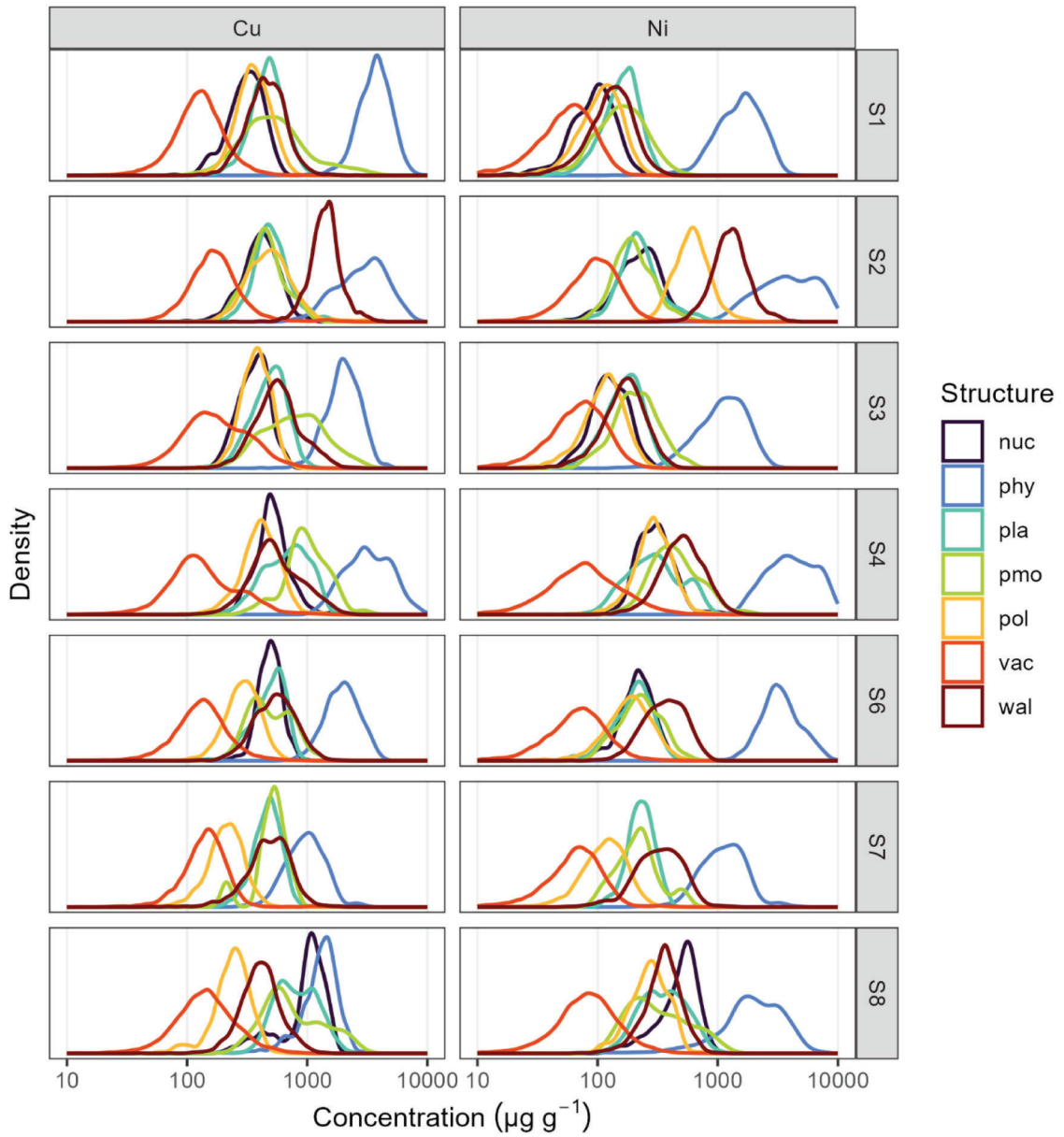


Fig. 6. Distribution estimates of Cu and Ni pixel concentrations in the 300 nm-resolution S-XRF images of *Fucus vesiculosus* samples (S1-S8). The x-axis is on a logarithmic scale. Subcellular structures: nuc: nuclei; phy: physodes; pla: plastids; pmo: peri-membrane organelles; pol: extracellular polysaccharides; vac: vacuoles and cytoplasm; wal: cell wall.

are less dominant in coastal ecosystem but also have high ecological importance, remain unaddressed. This is particularly interesting due to their metabolic and chemical differences; for example, their lack of physodes implies the presence of different regulatory mechanisms for PTE toxicity.

Future research should expand the range of elements and species analyzed to gain a comprehensive understanding of uptake mechanisms and influencing factors. The application of other techniques such as NanoSIMS could enable the analysis of elements inaccessible for S-XRF, as has been done for As, Br and I. Furthermore, studies with more complex experimental designs and applying molecular techniques would be valuable for obtaining direct confirmation of the uptake pathways.

Environmental Implication

This study reveals the nanoscale distribution of several elements in the brown alga *Fucus vesiculosus*, many of which had unknown accumulation patterns in seaweeds until now. Since the fate of PTEs in seaweed cells determines their toxicity, this knowledge is crucial for understanding the impact of pollution by different PTEs on seaweed health. *F. vesiculosus* is a fucoid seaweed, which are foundational organisms in coastal marine ecosystems, making it essential to understand how pollution affects them in order to assess its impact on the ecosystem as a whole.

CRediT authorship contribution statement

Antón Vázquez-Arias: Writing – original draft, Visualization, Software, Investigation, Formal analysis, Conceptualization. **Rémi Tucoulou:** Writing – review & editing, Methodology, Investigation. **M. Teresa Boquete:** Writing – review & editing, Investigation. **Beatriz Martín-Jouve:** Writing – review & editing, Methodology, Investigation. **Jesús R. Aboal:** Writing – review & editing, Investigation, Funding

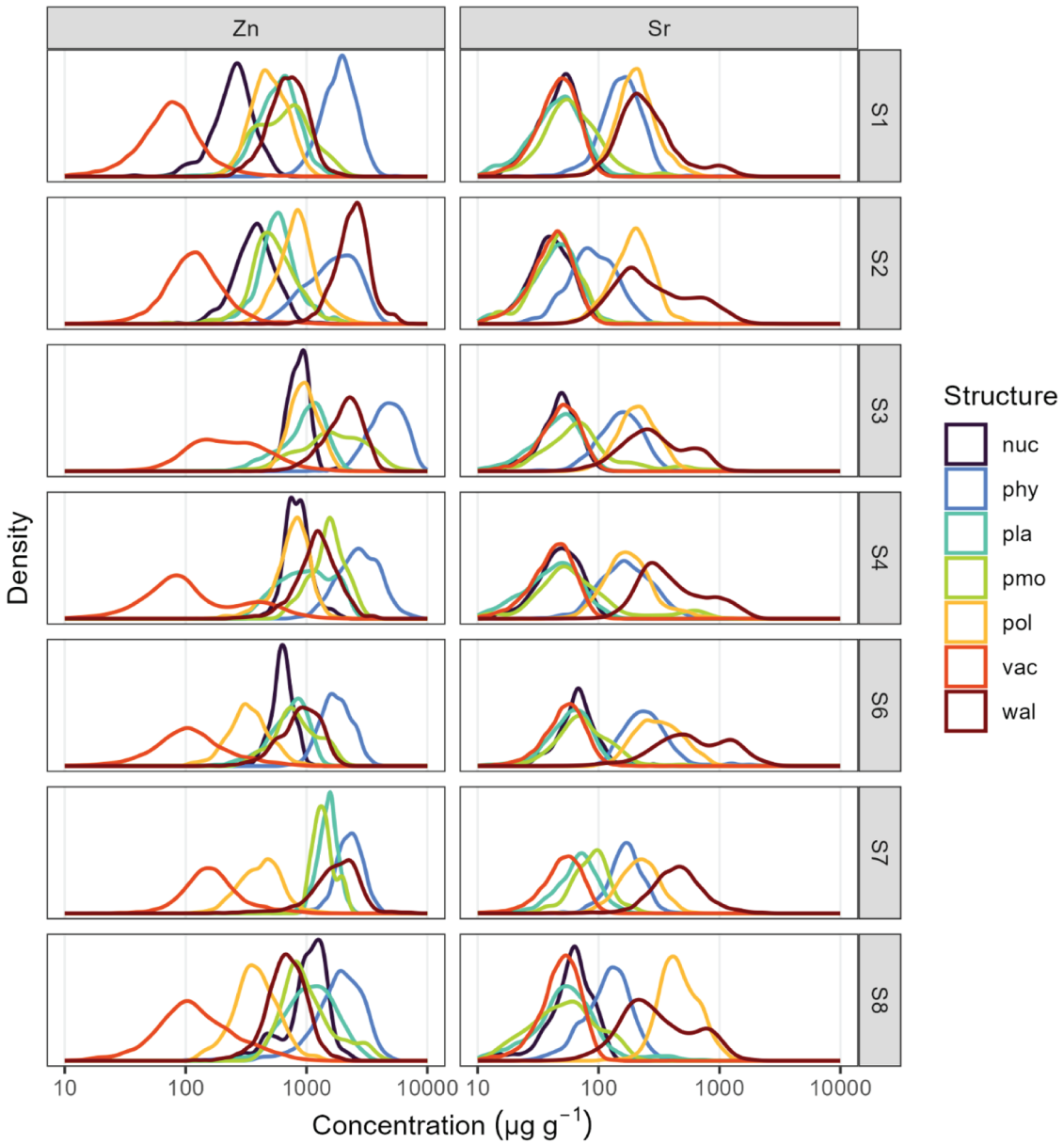


Fig. 7. Distribution estimates of Zn and Sr pixel concentrations in the 300 nm-resolution S-XRF images of *Fucus vesiculosus* samples (S1-S8). The x-axis is on a logarithmic scale. Subcellular structures: nuc: nuclei; phy: physodes; pla: plastids; pmo: peri-membrane organelles; pol: extracellular polysaccharides; vac: vacuoles and cytoplasm; wal: cell wall.

acquisition, Conceptualization. **Conxi Rodríguez-Prieto:** Writing – review & editing, Visualization. **J. Ángel Fernández:** Writing – review & editing, Investigation, Funding acquisition.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT for assistance with refining language and improving the clarity of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Anton Vazquez-Arias reports financial support was provided by Spain Ministry of Science and Innovation. M. Teresa Boquete reports financial support was provided by Spain Ministry of Science and Innovation. Anton Vazquez-Arias reports equipment, drugs, or supplies was provided by ESRF. Jesus R. Aboal reports financial support was provided by Spain Ministry of Science and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136454.

Data Availability

Data will be made available on request.

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7. Capítulo sen publicar

7.1. Capítulo 5: Deciphering uptake mechanisms of potentially toxic elements in seaweeds using high resolution imaging analysis

7.1.1. Abstract

Pollution by potentially toxic elements (PTEs) poses a significant threat to coastal marine environments, yet their interaction with seaweeds, foundational organisms in these ecosystems, remain poorly understood. The key to this interaction lies in the fate and uptake mechanisms of PTEs by seaweeds, which despite determining their toxicity are largely unexplored. To investigate these mechanisms, we analyzed the subcellular location of PTEs in transplanted thalli of the brown seaweed *Fucus vesiculosus* using nanoscale secondary ion mass spectrometry (NanoSIMS).

We found that Pb was predominantly accumulated in the cell wall, with minimal intracellular localization. Its concentrations increased slightly upon exposure at a polluted site and decreased at an unpolluted site, reflecting an equilibrium between environmental bioavailability and cell wall binding sites. In contrast, metabolically important PTEs such as Mn and Zn exhibited higher intracellular concentrations, which responded similarly when exposed at a site different from their collection site, likely as a stress response to changes in environmental conditions. These findings suggest that the intracellular concentrations of these elements are metabolically regulated and responsive to environmental conditions, enabling uptake and discharge. Conversely, as Pb internalization is restricted, its concentrations are not metabolically controlled.

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7.1.2. Introduction

Coastal ecosystems are essential for marine life and human communities, as they sustain rich biodiversity, support key activities, and supply vital resources[1,2]. Yet, these ecosystems are among the most vulnerable to anthropogenic impact, as they host all sorts of industrial, transportation and recreational activities, as well as being at the receiving end of rivers, which are often used for waste disposal[3]. This has led to the accumulation of pollutants in coastal areas, causing significant environmental damage and disrupting marine ecosystems.

Among the most harmful pollutants in coastal waters are potentially toxic elements (PTEs). For instance, the use of Cu and Zn in antifouling paints has resulted in severe contamination of harbor waters[4,5]; discharges from rivers affected by acid mine drainage containing Cu, As, and other PTEs, has damaged ecosystems around river mouths[6–8]; and concentrations of Pb and Hg in marine environments have increased tenfold compared to preindustrial levels[9,10].

In these environments, seaweeds are foundational organisms, providing food and acting as ecosystem engineers[11,12]. Understanding their interactions with PTEs is therefore crucial for assessing and predicting the impact of pollution on the ecosystems they support. Additionally, seaweeds are commonly used in biomonitoring studies to assess pollution in coastal ecosystems[13,14], with the interpretation of these studies relying on our understanding of seaweed-PTE interactions. Still, until recently, little was known about the pathways and fate of the PTEs taken up by seaweeds[15].

Over the last two decades, two techniques have emerged that enable the imaging of elemental concentrations with enough sensitivity to map natural distributions of PTEs at the nanoscale, leading to important advances in this field[16]. These techniques are synchrotron-based X-Ray diffraction (S-XRF) and nanoscale secondary ion mass spectrometry (NanoSIMS), both of which can map elemental concentrations in a sample at resolutions as low as tens of nanometers[17]. These technologies have different operating principles: S-XRF uses an X-ray beam to excite the sample and analyzes the resulting fluorescence emission spectrum, while NanoSIMS employs an ion beam to scatter secondary ions from the sample, which are then analyzed by mass spectrometry. These different principles lead to distinct

USC DE COMPOSTELA

advantages and limitations. S-XRF provides data on a wide range of elements within a single spectrum, but is primarily limited to heavy elements and overlap between fluorescence peaks can cause interferences in the analysis of fixed samples. With NanoSIMS, the number of elements analyzed simultaneously is lower, but it can also be used to detect light elements that are beyond the reach of S-XRF[17] and is able to provide higher resolution[18].

For now, very few studies have applied these techniques to seaweed. NanoSIMS studies have focused exclusively on *Laminaria digitata*, a kelp species, examining the distribution of As, Br, and I—all of which were found to accumulate extracellularly[19–21]. Similarly, S-XRF analysis conducted on *Laminaria digitata* detected Sr outside the cells, while Fe and Zn were colocalized intracellularly. Lastly, most of the information available on PTEs distributions at nanoscale comes from a study on the fucoid *Fucus vesiculosus*, which revealed two distinct accumulation patterns for PTEs[22]. Elements without significant metabolic roles (e.g., As, Ba, and U) were found to accumulate extracellularly, whereas metabolically essential PTEs (e.g., Co, Ni, Cu, and Zn) were internalized and sequestered into physodes—specialized organelles containing phenolic compounds known as phlorotannins. Although these studies provide a strong foundation for understanding PTE uptake in seaweeds, several questions remain unanswered. First, even though the metabolic role of elements has been proposed to be relevant for their accumulation patterns, key PTEs without metabolic functions (i.e. Pb, Cd, and Hg) could not be detected in previous S-XRF studies. Second, while some inferences about accumulation mechanisms and dynamics can be drawn from existing data—for example, elements attached to the cell wall are likely bound by ionic interactions—the dynamics and mechanisms controlling intracellular concentrations remain poorly understood. Key uncertainties include the rate of the internalization process, its reversibility, and the molecular mechanisms governing membrane transport. Addressing these gaps would improve our understanding of the PTE toxicity under different conditions and the potential to mitigate it by decreasing environmental pollutant levels.

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In this study, we aim to address both limitations. We conducted a transplantation experiment between two sites with differing pollution levels to investigate the cellular structures responsible for capturing and discharging PTEs in response to a change in environmental concentrations. The samples were analyzed using NanoSIMS, which enabled us to resolve the distribution of Pb, which was undetectable in previous S-XRF studies, among other PTEs.

7.1.3. Material and methods

7.1.3.1. Transplant preparation and exposure

A transplantation experiment was conducted between two exposure sites (ES) in NW Spain with different pollution levels but otherwise similar environmental conditions. The first site, located at 42°47'25.3"N, 8°55'04.1"W (WGS84), is characterized by low PTE concentrations in seaweed, included Pb. The second site, located at 42°20'46.7"N, 8°36'47.9"W near an inactive porcelain factory, exhibits residual Pb pollution, resulting in elevated Pb concentrations in sediments and local seaweed populations[15].

Thalli of *Fucus vesiculosus* L. and two stones roughly 20 x 20 x 10 cm in size were collected from each site. Transplants mimicking natural conditions were prepared by attaching four medium-sized thalli to each stone: thalli from the polluted site were attached to two stones, while thalli from the unpolluted site were attached to the other two. The thalli were secured to the upper surface of the stones using waterproof silicone sealant. To prevent material loss without hindering water flow, the stones were covered with 2 cm mesh size polyethylene nets. The prepared transplants were left at 4°C overnight. The next day, one transplant from each site was placed at each ES, ensuring that each site contained one transplant with local seaweed (autotransplant) and one with seaweed from the opposite site (cross-transplant). To avoid confusion, the following codes will be used in the text and figures: autotransplants in the unpolluted site (U-U), autotransplants in the polluted site (P-P), cross-transplants from unpolluted to polluted (U-P), and cross-transplants from polluted to unpolluted (P-U).

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The transplants were collected after 12 days, a period sufficient for changes in PTE concentrations in seaweeds[15]. The transplants were dismantled by removing the mesh nets and scraping off the silicone sealant. The thalli were collected and placed in polyethylene bags, which were transported to the laboratory. To ensure homogeneity, the three apical dichotomies of healthy thalli without reproductive structures were selected. Two apices from each of the four samples were further selected for SEM and NanoSIMS analysis. These were stored in polypropylene tubes with a few mL of water to prevent dehydration, and kept at 4°C. The remaining material was oven-dried at 40°C until constant weight, homogenized with a tangential mill with zirconium oxide grinding vessels (Retsch ZM400), and stored in darkness until chemical analysis.

7.1.3.2. ICP-MS analysis

The samples were dried at 40 ºC in an oven to remove residual moisture before analysis. From each sample, 1 g (dry weight) was digested in Teflon vessels using a microwave oven (Milestone Ethos-1) with the following temperature ramp: 100 ºC for 10 minutes, 150 ºC for 7 minutes, and 190 ºC for 25 minutes. The digestion was performed with 10 mL of HNO3 (65%), 2 mL of H2O2 (30%), and 2 mL of Milli-Q water. Total concentrations of Na, Ca, Mn, Zn, and Pb were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x) at the University Research Support Services Unit (RIAIDT, Universidade de Santiago de Compostela). Quality control was ensured through replicate analyses and the use of reference material (ERM-CD200, Fucus vesiculosus). For all elements, variation among replicates ranged from 9 to 11% and the percentage recoveries for the two certified elements—Zn and Pb—were 113% and 96% respectively.

7.1.3.3. Sample preparation for Scanning Electron Microscope (SEM) and NanoSIMS

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Within 24 h of collection, the samples in polypropylene tubes were transported under refrigeration to the Centro Nacional de Biotecnología (CNB-CSIC) in Madrid,

Spain, for preparation for SEM and NanoSIMS analysis. The samples were prepared by cryofixation to prevent the mobilization of trace elements[20]. The fixation protocol is described in Vázquez-Arias et al.[22]. Briefly, 150 µm-thick transverse sections were subjected to high-pressure freezing, vitrified in a 1% osmium tetroxide solution in acetone, and dehydrated with anhydrous acetone. Finally, the samples were embedded in epoxy resin. All 4 samples had well-preserved regions suitable for analyses.

From each resin block, 200 nm-thick sections were obtained, transferred onto 5 x 5 mm silicon wafers, and stained with saturated aqueous uranyl acetate for 25 min at room temperature. The sections were then mounted on SEM aluminum holders using conductive carbon tape.

7.1.3.4. SEM imaging

The mounted samples were taken to the Research Support Services Unit of the University of Santiago de Compostela (RIAIDT-USC) for SEM analysis. Images were captured to visualize the samples and identify the regions best preserved for NanoSIMS analysis. Low-resolution exploratory images were obtained using a Zeiss EVO 15, while close-up images were captured with a Zeiss Gemini 500. The cellular ultrastructure was visualized using backscatter detectors.

7.1.3.5. NanoSIMS analysis

The mounted sections were analyzed using nanoscale secondary ion mass spectrometry (NanoSIMS) analysis at the Kochi Institute for Core Sample Research (KOCHI-JAMSTEC) in Japan. Analyses were conducted with a CAMECA NanoSIMS 50L using a primary O^- beam (Hyperion RF source apparatus). Areas measuring 48 x 48 µm were scanned with an approximate spatial resolution of 200 nm and an acquisition time of 10 ms per pixel, obtaining 256 x 256-pixel images. Positive secondary ion images of ^{23}Na, ^{40}Ca, ^{55}Mn, ^{64}Zn, ^{208}Pb, and $^{238}U^{16}O$ were obtained to map the spatial distributions of these elements. These ions were acquired

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simultaneously by multidetection with six electron multipliers at a mass resolving power of approximately ~3,000 (CAMECA NanoSIMS definition). Multiple regions of each sample were analyzed, obtaining five images of each region.

As uranyl acetate was used during the staining process, UO maps were generated to assist in visualizing ultrastructural elements and aligning with SEM images; however, they do not reflect natural U distributions. Pb was selected for analysis among the key PTEs without metabolic functions (Pb, Cd, and Hg) due to its extreme toxicity and comparatively high environmental concentrations.

7.1.3.6. Data processing

Image processing was performed using ImageJ[23]. The .im NanoSIMS data files were opened using the OpenMIMS plugin[24], which was used to export them as PNG files for visualization and as TIFFs for statistical analysis. To overlay SEM and NanoSIMS images, an affine transformation was applied to the NanoSIMS images using the Landmark Correspondence tool. For regions were SEM images with at least 2000x magnification and corresponding NanoSIMS data available, the intensity values of the different elements in the ultrastructural components of the samples were extracted. This level of magnification was necessary to accurately identify small organelles. For each of the four samples, this analysis was done in two regions, except for the worst preserved sample, where only one region could be analyzed. Ultrastructural elements were selected as regions of interest (ROIs) over the SEM images, which were then applied to the TIFF files to retrieve element intensity values across pixels within the ROIs. Cellular structures were identified following previous studies[22]. The main structures identified were the cell wall, nuclei, plastids, vacuoles, mitochondria, and physodes. Extracellular polysaccharides were also identified but were grouped with the cell wall due to their similar composition and indistinct boundaries. Cytoplasmic regions were excluded from analysis as they could not be consistently identified across all samples due to tightly packed vacuoles and limited contrast. Most analyses focused on regions close to the

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surface (meristoderm and cortex), as these cellular layers are primarily responsible for PTE accumulation[22].

7.1.3.7. Data analysis

The extracted data were analyzed and using R software version 4.4.1[25], with data visualization performed using the ggplot2[26] package. The median concentration for each subcellular structure was calculated by aggregating data from all pixels within its ROI across all five layers. The median was used instead of the mean to ensure robustness against pixels from surrounding structures that may have been incorrectly included due to minor errors in image alignment or ROI selection.

The use of statistical test to compare concentration values was hindered by two factors: slight differences in intensity values introduced by NanoSIMS analysis, and the large number of pixel values. As previously reported[22], applying statistical tests to pixel values can inflate statistical power, resulting in low p-values for differences that lack biological significance. A possible alternative could be comparing the medians values of subcellular structures, but this is complicated by the differences in quantity. While the large number of physodes allowed for meaningful comparisons, the continuity of the cell wall and the limited number of nuclei restricted such comparisons. Therefore, sample comparisons were performed visually. For these comparisons, structures from different cellular layers (meristoderm and cortex), as prior studies[22] reported only minor differences in elemental concentrations between these layers.

7.1.4. Results

The NanoSIMS concentration maps, SEM images, and their overlay can be seen in figures 1 and 2 for selected elements and samples, and figures 1 through 4 of the supplementary material for the rest. Each element exhibited distinct distribution patterns that were generally consistent across different samples. Na showed some variability, with high concentrations in the cell walls of most samples but higher

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intracellular concentrations in the vacuoles of certain samples. For Ca, the highest concentrations were found in the cell wall, followed by the physodes and plastids. Mn was predominantly detected intracellularly, particularly within the plastids. Zn was primarily concentrated in the physodes, although in some samples significant concentrations were also observed in the cell wall. Pb was mostly concentrated in the cell wall, with minimal intracellular presence; intracellularly, it was restricted to the physodes.

Figure 3 shows the median concentrations of each element by sample and ultracellular structure, enabling the study of the effect of the exposure site. For all elements except Pb, the cross-transplants (U-P and P-U) experienced similar effects regardless of the exposure site. Therefore, the concentrations of Ca, Mn and Zn in U-P were higher than in U-U, and in P-U than in P-P, while Na concentrations in the cross-transplants experienced a decrease compared to the autotransplants of the same origin. These trends were consistent across subcellular structures. For Pb, however, the effect of being moved to a different exposure site followed the expected pattern. Samples collected from the unpolluted site had higher Pb concentrations after being exposed at the polluted site (U-P) than after being re-exposed at the unpolluted one (U-U). Conversely, Pb concentrations decreased in samples collected from the polluted site (P) and exposed at the unpolluted site (P-U) compared to those re-exposed at the polluted site (P-P).

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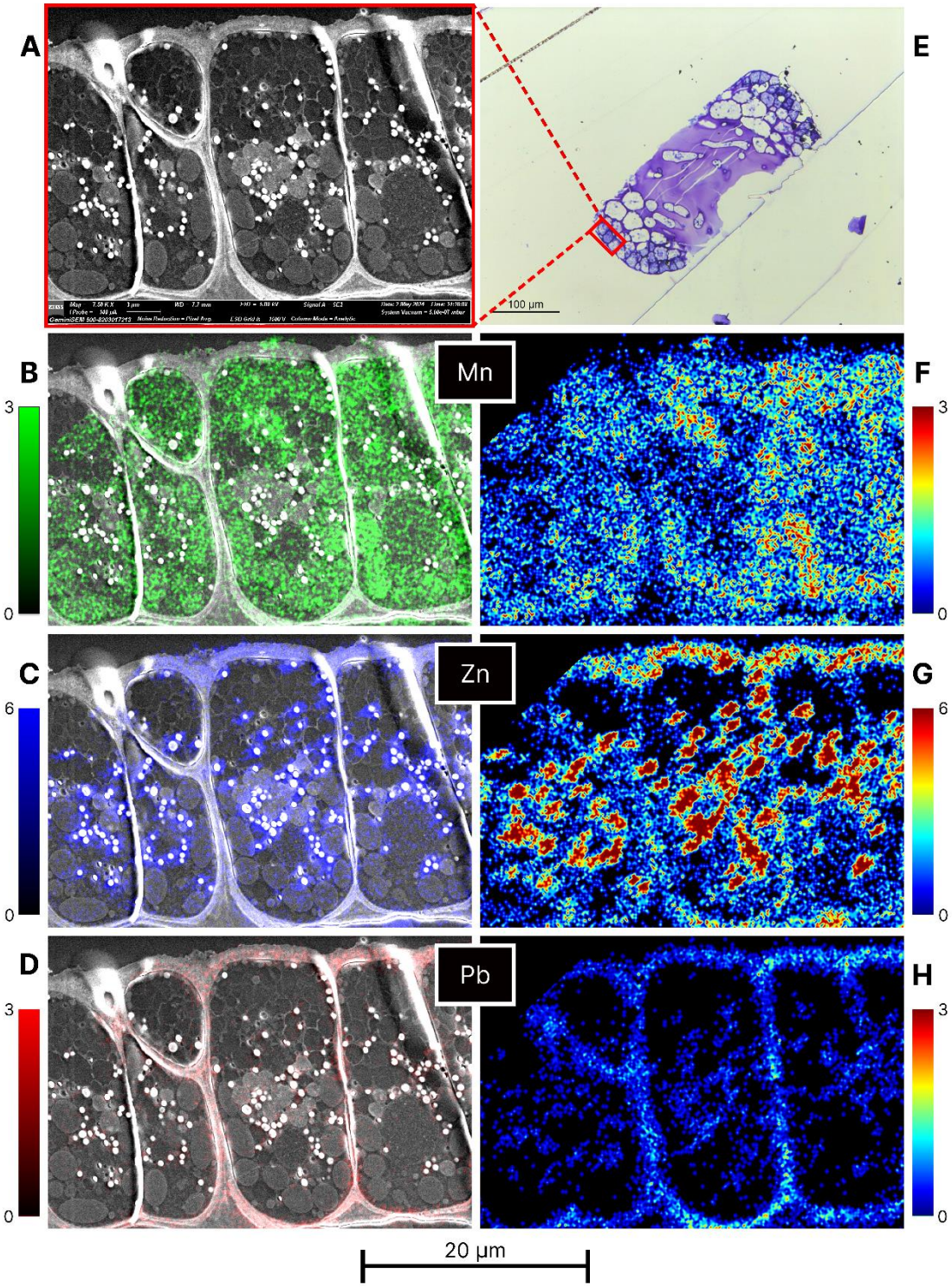


Figure 1. SEM and NanoSIMS images of the P-U sample. A: SEM image. B-D: Elemental distribution maps of Mn, Zn, and Pb overlayed over the SEM image. E: Optical image of a section from a different *F. vesiculosus* sample stained with toluidine blue. F-H: Distribution maps with a color scale over for enhanced contrast. Color scale units are NanoSIMS read counts. Bottom scale applies to SEM and NanoSIMS images.

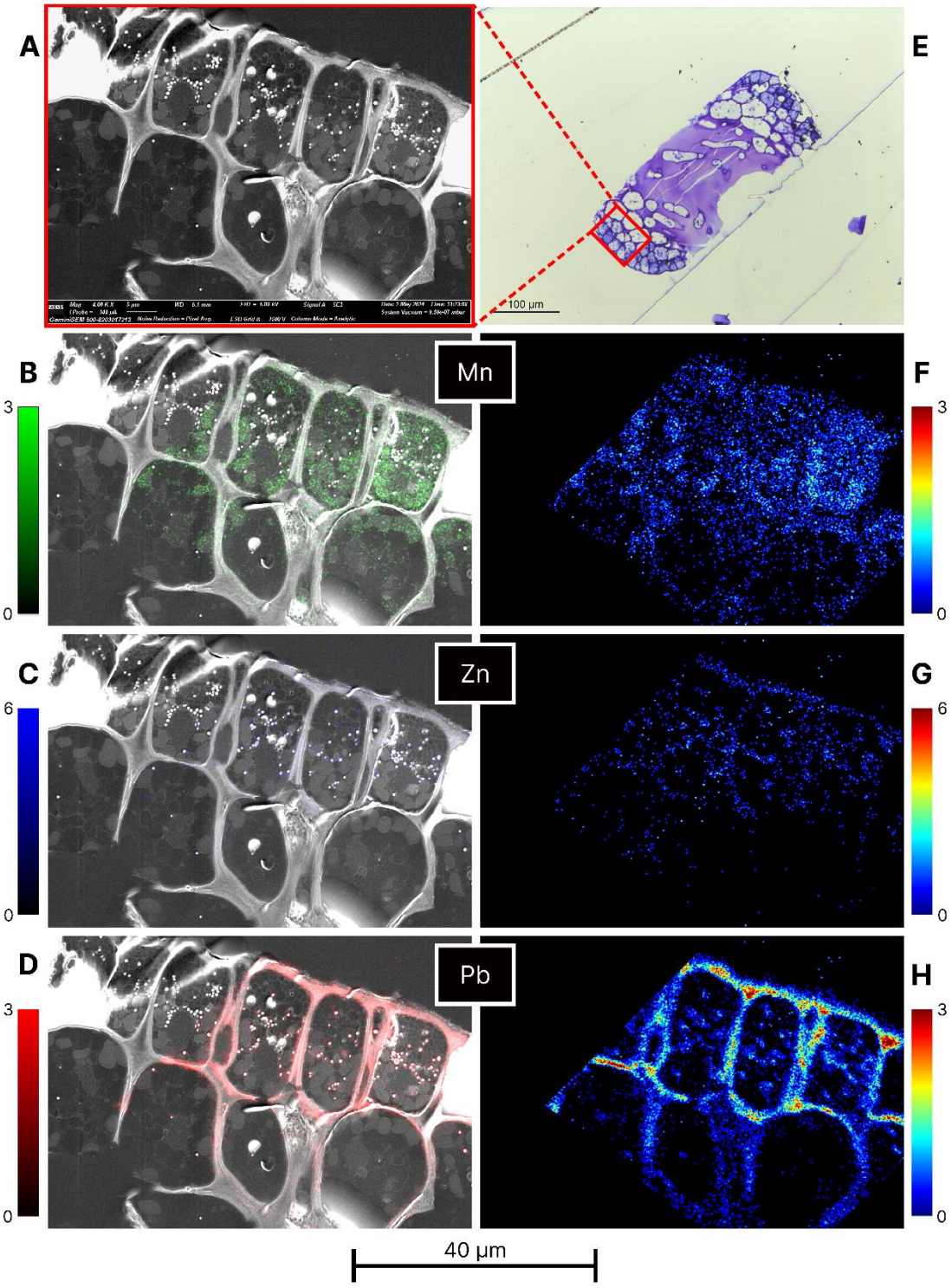


Figure 2. SEM and NanoSIMS images of the P-P sample. A: SEM image. B-D: Elemental distribution maps of Mn, Zn, and Pb overlayed over the SEM image. E: Optical image of a section from a different *F. vesiculosus* sample stained with toluidine blue. F-H: Distribution maps with a color scale over for enhanced contrast. Color scale units are NanoSIMS read counts. Bottom scale applies to SEM and NanoSIMS images.

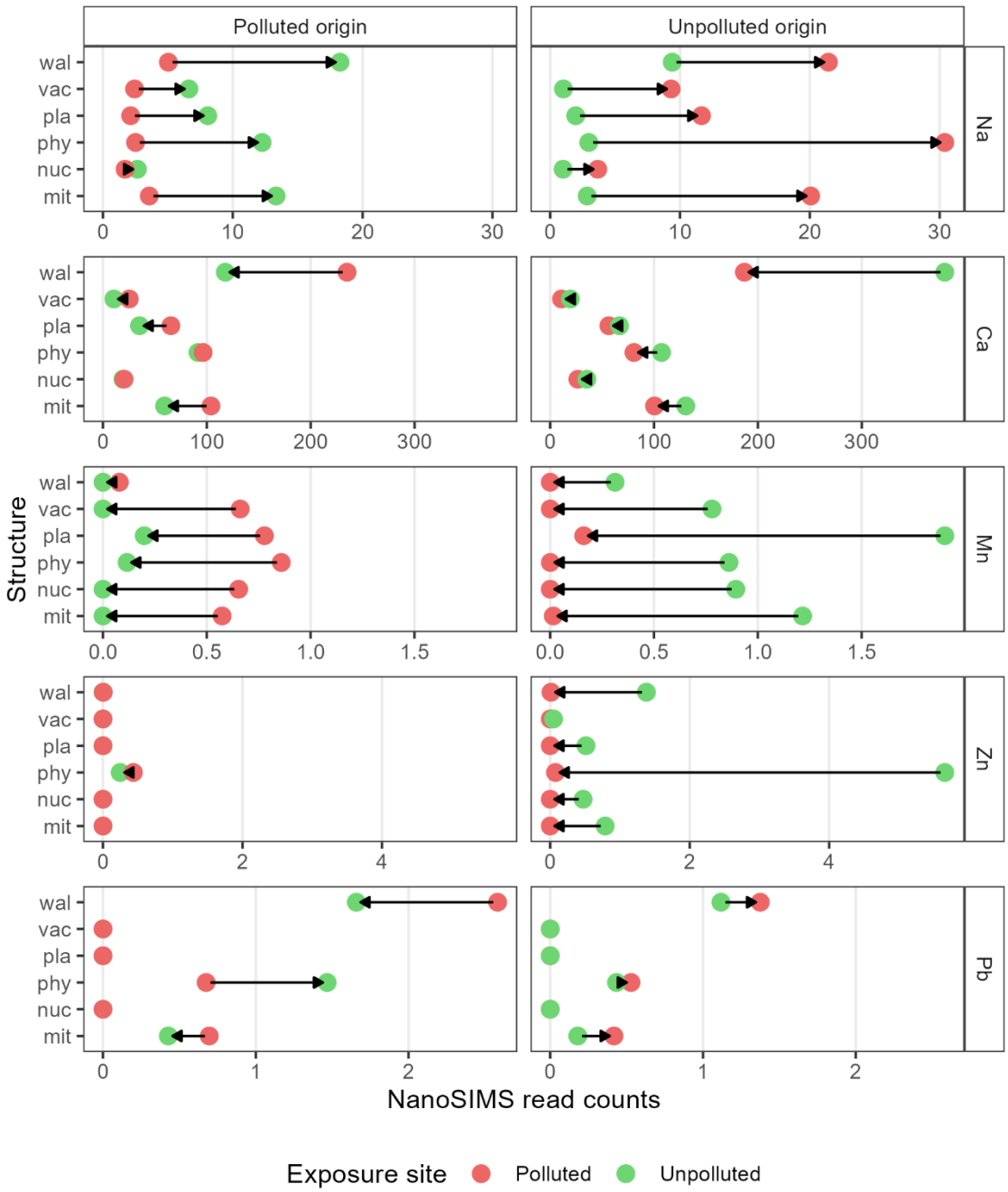


Figure 3. Median intensity values obtained with NanoSIMS for the different subcellular structures in each sample. Each column represents the site where they were collected. The direction of the arrows represents the effect of exposure at a different site. Subcellular structures: nuc: nuclei; phy: physodes; pla: plastids; mit: mitochondria; vac: vacuoles; wal: cell wall and extracellular polysaccharides.

To ensure this effect was not caused by differences during NanoSIMS analysis, we compared the variance between different analysis of the same sample and analyses of different samples (figure 5 of the supplementary material). The variance across samples was considerably higher.

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We also compared the total concentrations of each element and sample determined with ICP-MS, as shown in Figure 4. In this case, concentrations did not depend on whether the samples were autotransplants or cross-transplants, but were instead influenced by the exposure site. Mn concentrations increased when transplants were exposed at the polluted site rather than the unpolluted site, regardless of their collection site. The concentrations of the other elements were higher in transplants exposed in the polluted site compared to transplants of the same origin exposed at the unpolluted site.

7.1.5. Discussion

7.1.5.1. Elemental distributions

Despite its importance as an environmental pollutant, the localization of Pb within seaweeds was previously unknown. This study demonstrates that Pb is predominantly accumulated outside the cells, and only a minimal fraction is internalized. These findings align with previous results suggesting seaweeds prevent the internalization pf PTEs with limited metabolic functions[22]. The binding of Pb to the cell wall is also consistent with studies showing that brown seaweeds use cell wall polysaccharides as protection mechanisms against Pb and Cd pollution[27]. The ability of seaweeds to prevent the internalization of these elements might explain why PTEs with lower toxicity to humans such as Cu are more toxic to algae than Cd and Pb[28,29].

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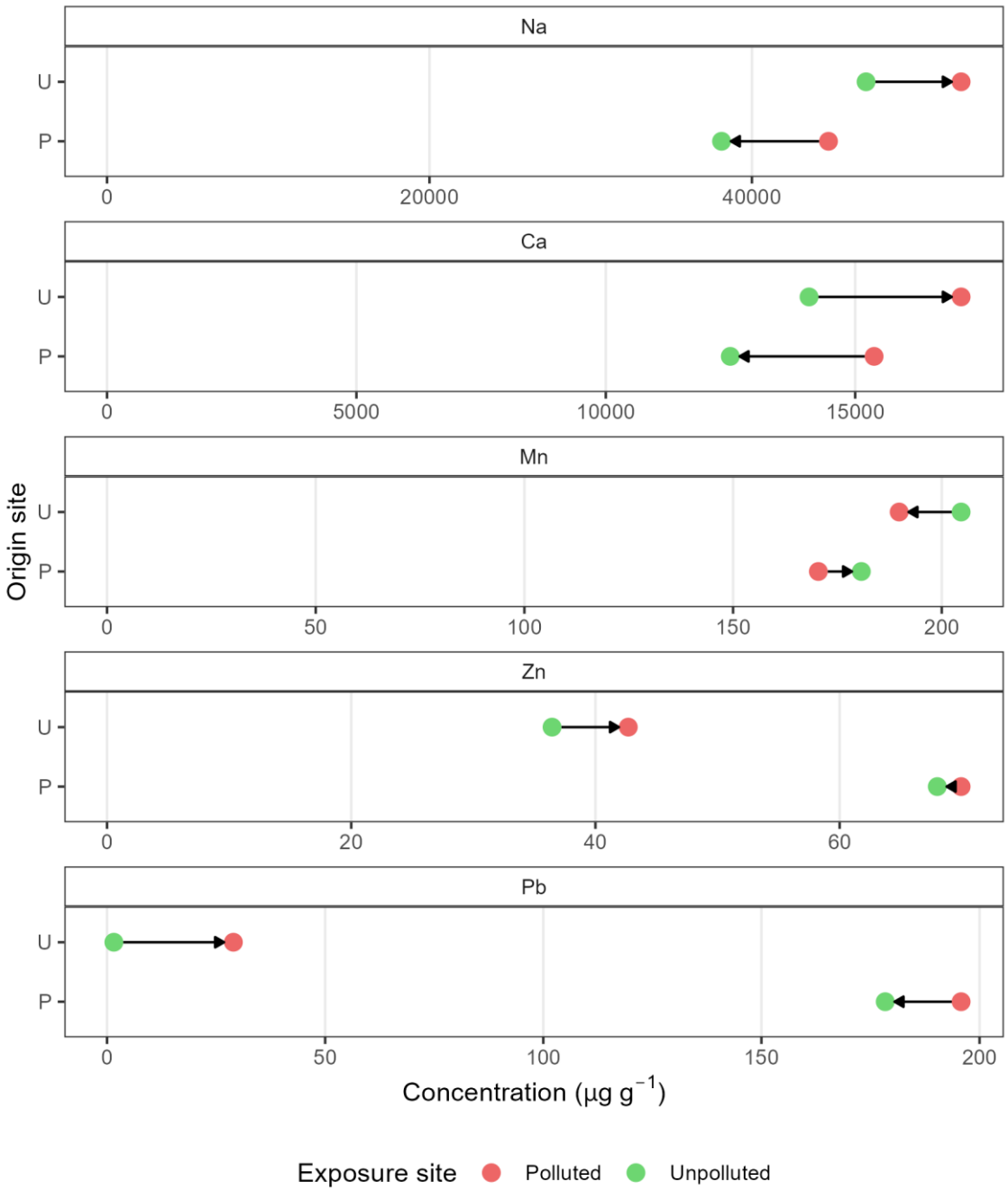


Figure 4. Total concentration values for each sample detected by ICP-MS. U and P indicate the origin site, with U for sample collected from the unpolluted site and P for samples from the polluted site. The direction of the arrows illustrates the effect of exposure at a different site.

The distribution of other elements (Ca, Mn, and Zn) matched those found through S-XRF analysis[22]. The highest concentrations of Ca were found in the cell walls, where it plays an important structural role[30]. However, as an essential nutrient, its concentrations in mitochondria, plastids, and physodes were also high as

compared to vacuoles. Mn, while an important nutrient for seaweeds due to its role in photosystem II, can also exert toxic effects on brown seaweeds[31]. Its biological function explains its elevated concentrations inside plastids. High concentrations were also found in physodes, possibly due to the affinity of physodes for metallic cations. Since physodes act as storage sites for elements that have both toxic effects and metabolic functions such as Co, Ni, Cu and Zn[22], this may also be the case for Mn. Zn, another essential PTE regulated through confinement in physodes, was highly associated with these organelles, although some samples also displayed high concentrations in the cell walls, a pattern consistent with previous observations[22].

Aside from Pb, Na was the only other element in our study whose distribution in seaweeds had not yet been analyzed. Due to living in euhaline environments, seaweeds regulate Na primarily by preventing its internalization to maintain normal metabolic activity[32]. This is consistent with our results, which found Na to be predominantly associated with the cell wall and physodes, the cellular structures where elements are stored to mitigate toxicity. High Na concentrations were also detected in mitochondria, but this may be an artifact of the analysis. Structures with high concentrations of certain elements, such as Na in the cell wall and Zn in physodes, exhibit a halo of high concentrations around them. Because mitochondria are usually associated to the cell wall, their apparent Na concentrations may be inflated by the halo effect. Larger and more widely distributed structures are less affected by this bias, but mitochondria concentrations for elements with high cell wall concentrations should be interpreted with caution.

7.1.5.2. Comparison across transplants

The transplantation experiment was designed to study the effect of exposure to different PTE concentrations, with the aim of gaining insights into the uptake mechanisms and dynamics. However, this analysis presented two major challenges. The first was ensuring data comparability. We tried to minimize the differences among analyses with identical sample preparation and analysis procedures, but matrix effects—such as sample charge and topology—can influence the ion yield,

USC DE COMPOSTELA

potentially causing systematic differences[33]. To address this, we compared the variation across analyses of different regions of the same sample, and across different samples. The variation was found to be considerably higher across different samples. Therefore, we are reasonably confident in the comparability of the data.

The second challenge was addressing the effect of the transplantation process itself. Although autotransplants were treated in the same way as cross-transplants to account for the effects of transportation and transplant preparation, autotransplants were then exposed to environmental conditions they were already acclimated to, whereas cross-transplants were exposed to new conditions. Changes in environmental physicochemical conditions can cause stress in seaweeds[34], potentially influencing membrane permeability, as has been shown in plants[35]. This might explain why, for most elements, moving seaweeds from the polluted site to the unpolluted site and vice versa had the same effect when compared to autotransplants of the same origin. This contrasts with the absolute concentrations values, which in our study (Figure 3) and in previous studies[15] exhibit a more predictable pattern: transplants moved to more polluted sites increase their PTE concentrations, while those moved to less polluted sites decrease them. The only element with lower total concentrations in the polluted site was Mn, but this is expected, as the unpolluted site has been reported to have high Mn concentrations[(under review)]. The inconsistency between the effect of exposure at a different site on total and subcellular concentrations is not surprising, as S-XRF results[22] also showed no correlation with total concentrations. This discrepancy is likely due to the presence of mineral particles on the seaweeds' surface. These particles often contain elemental concentrations significantly higher than those within seaweeds, and are included in the total concentration analyses. However, sample preparation for NanoSIMS, particularly sectioning, may remove these particles, especially those larger than the section thickness. As a result, these particles could account for a substantial portion of the concentrations detected in ICP-MS analyses, so that the patterns observed in total concentrations may reflect changes in surface-deposited mineral particles, whereas changes in the

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concentrations of subcellular structures are more representative of the bioavailable fraction.

The disconnection between total and ultrastructural PTE concentrations also explains another unexpected pattern in our data. The polluted site was selected due to its extremely high Pb concentrations, previously detected in the sediment and in seaweeds[36]. The ICP-MS analysis of our samples confirmed this pollution, with samples originating from the polluted site showing much higher Pb concentrations than those from the unpolluted site, and an increase in Pb in samples transplanted from the unpolluted to the polluted site. However, while the total Pb concentrations in samples exposed at the polluted site were orders of magnitude higher than the U-U sample, the differences detected with NanoSIMS were far smaller. This supports the hypothesis that the increase in total Pb concentrations is primarily due to mineral particles attached to the surface. This is also consistent with the source of Pb at the polluted site. As the contamination originates from a porcelain factory that closed more than 20 years ago, the Pb currently present at the site is likely mostly associated with mineral particles.

However, Pb was the only element for which the direction of the effect of the cross-transplantation on the ultrastructural concentrations aligned with pattern observed for total concentrations. The reason for this might lie in the biological function of the elements and their regulatory mechanisms. Pb is almost exclusively found extracellularly, whereas the rest of the elements have metabolic functions that require internalization and, potentially, excretion mechanisms. Metabolic stress caused by changes in environmental conditions is therefore unlikely to affect Pb capture, which is mediated by chemical binding and independent of metabolic regulation. In contrast, the intracellular concentrations of the other elements might be influenced by changes in metabolism.

Therefore, the primary pattern observed in response to exposure at a site with different pollution levels does not necessarily reflect what happens to naturally occurring seaweeds when PTEs are introduced into their environment, being instead a response to metabolic stress. Nonetheless, these findings provide useful information on the accumulation mechanisms. For Pb, the results confirm that the

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binding to the cell wall is partially reversible, as concentrations decrease substantially after 12 days of exposure at the unpolluted site. The accumulation of the other elements is also reversible, with seaweeds able to discharge them in response to metabolic stress. This reversibility is evident across different intracellular organelles, suggesting a continuous flux of these elements between them. In the case of physodes, this discharge may involve exchange with the cytoplasm, but physodes have also been reported to fuse with the cell membrane releasing their contents into the apoplast[37], which could serve as an alternative pathway for PTE expulsion.

7.1.6 Conclusion

Our results indicate that Pb, due to its toxicity and lack of biological function, is excluded from brown seaweed cells and is therefore retained in the cell wall. In contrast, PTEs with intracellular functions such as Mn and Zn are internalized and sequestered into physodes. Consequently, Pb concentrations in seaweed cells are not metabolically controlled but instead depend on the equilibrium between environmental bioavailable concentrations and cell wall binding sites. Conversely, intracellular concentrations of biologically important PTEs can be metabolically regulated and can vary in response to factors such as stress. These findings help explain the reduced toxicity of Pb for seaweed compared to metabolically active PTEs such as Cu,

7.1.7. Acknowledgements

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8. Discusión xeral

As macroalgas mariñas lévanse empregando para a biomonitorización de contaminación en augas costeiras desde os anos 50 ata a actualidade (Black e Mitchell, 1952; Sidondi *et al.*, 2024), con máis de 400 artigos empregando esta técnica durante ese período (García-Seoane *et al.*, 2018b). Esta popularidade é debida ás súas vantaxes como biomonitores: son abundantes, facilmente accesibles, están dispoñibles todo o ano, e son resistentes á contaminación e outras perturbacións ambientais (García-Seoane *et al.*, 2018b). Ademais, acumulan concentracións moi altas de PTEs, algo no que algúns autores se basean para destacar o seu potencial para a biomonitorización (Sidondi *et al.*, 2024). Non obstante, isto non é suficiente para que un organismo sexa un bo biomonitor, xa que para iso deberían dar información que permita chegar a conclusións que van máis alá do biomonitor en si (Markert *et al.*, 1999). Con este obxectivo os parámetros medidos no biomonitor (neste caso as concentracións de PTEs) deben estar correlacionados coa contaminación ambiental. Ademais disto, ten que haber un protocolo establecido para que haxa comparabilidade entre estudos, e débense ter en conta os factores externos que poidan afectar ás concentracións do biomonitor para poder interpretar os datos correctamente (Markert *et al.*, 1999). Así a todo, a pesar dos centos de traballos de biomonitorización con algas publicados na literatura científica, ata agora non había un protocolo estandarizado, a información sobre a correlación entre as concentracións de contaminantes en algas e no medio era escasa e contraditoria, e os mecanismos de acumulación e os factores que lles afectan eran descoñecidos. Nesta tese abórdanse todos estes problemas da biomonitorización de PTEs en augas costeiras usando algas coa intención de avaliar o seu potencial e mellorar a súa utilidade.

8.1. Emprego das algas como biomonitores

Para axudar á estandarización, seguíronse as recomendacións de dúas revisións sobre o uso desta técnica (García-Seoane *et al.*, 2018b, 2018a), nas que se

recomenda o uso de biomonitorización activa coa alga *F. vesiculosus*. Para isto, no primeiro capítulo próbase o uso de transplantes tratados de catro maneira distintas en sitios con distintos perfís de contaminación. Un dos tratamentos imita condicións naturais expoñendo algas adheridas a rochas, mentres que os outros tres tipos consisten en ápices de algas dentro de bolsas de malla, ben en fresco, secas, ou cocidas, os dous últimos sendo tratamentos de desvitalización. En termos de estandarización, este traballo revela que o uso de bolsas de algas frescas é equivalente ós transplantes que imitan condicións naturais, o que favorece o uso dos transplantes en bolsas debido a que a súa aplicación é loxisticamente máis simple. En canto á desvitalización, estes transplantes teñen un comportamento moi distinto do dos frescos, o que fai que cada tipo teña un conxunto distinto de vantaxes. Os transplantes frescos non se degradan durante o período de exposición, o que fai que se poidan expoñer durante tempos máis longos, e ó ser equiparables a algas no seu estado natural son máis representativas da exposición dos organismos que están de maneira nativa no ecosistema. Os transplantes desvitalizados, en cambio, teñen concentracións máis altas da maior parte de elementos, o que fai que sexa máis fácil analiticamente medir elementos con concentracións baixas; ó estar mortos non medran, algo que podería afectar ás concentracións; e pódense preparar e gardar en seco no laboratorio, o que facilita a loxística da súa aplicación. Dentro dos transplantes desvitalizados, tanto os frescos coma os secos teñen un comportamento moi similar, pero a consistencia dos secos é algo superior, polo que o seu uso é máis recomendable.

Os resultados deste traballo non só aportaron información metodolóxica, senón que tamén puxeron de manifesto as limitacións do noso coñecemento sobre os mecanismos de bioconcentración de PTEs en algas. As vías de captación elemental en animais e en plantas son coñecidas: en moitos animais os PTEs absórbense por vía respiratoria ou dixestiva, pasan ó sistema circulatorio e acumúlanse en distintos tecidos (Salthammer *et al.*, 2018). En plantas os contaminantes absórbense polas raíces ou polas follas, e chegan a outros tecidos a través dos tecidos condutores (Song *et al.*, 2017). Polo tanto, atopar concentracións de PTEs elevadas en tecidos distintos dos de absorción implica que estes contaminantes están dispoñibles para

os organismos e que foron internalizados. Nas algas pardas non hai tecidos condutores, e a absorción elemental ocorre por todo o talo da alga. Aínda así, a utilidade da biomonitorización con algas parte da asunción de que a detección de concentracións elevadas de PTEs no seu talo ten as mesmas implicacións que detectalas en organismos máis complexos, algo que non é necesariamente certo. Se as algas internalizasen os PTEs e os acumulasen dentro das células, esperaríase que ó romper as membranas celulares (algo que ocorre ó desvitalizar as algas) a carga de PTEs fose menor. Non obstante, os nosos resultados demostran o contrario: para a maior parte dos PTEs as concentracións son máis altas nas algas desvitalizadas. No capítulo un propoñemos varias hipóteses que poden explicar este fenómeno: 1) ó romperse as membranas expóñense sitios de unión no interior das células; 2) o tratamento por calor altera a estrutura química das macromoléculas de membranas e paredes celulares, incrementando a súa afinidade por certos PTEs; 3) inactívanse os mecanismos de regulación de PTEs presentes en algas vivas; 4) durante o período de exposición as algas desvitalizadas, que teñen menor consistencia, perden compoñentes celulares con menor capacidade para a acumulación, levando a concentracións medias máis altas; 5) o crecemento dos transplantes frescos incrementa a masa sen aumentar a captación, levando a concentracións medias menores; e 6) modifícase a superficie da alga, o que permite a acumulación de máis partículas de sedimento.

Para probar estas hipóteses, aplicáronselles dúas técnicas máis ás mostras: analizáronse con espectroscopía infravermella con transformada de Fourier e reflectancia total atenuada (FTIR-ATR), que permite analizar a composición molecular das algas, dando información dos polisacáridos da superficie (Gómez-Ordóñez e Rupérez, 2011); e calculáronse as ratios da concentración de cada elemento coa de aluminio tanto para as algas coma para o sedimento, o que informa sobre a porcentaxe da concentración de cada elemento que vén do sedimento. Os resultados de FTIR-ATR suxeriron que a desvitalización e posterior exposición das mostras alteran a composición dos seus polisacáridos extracelulares, algo que necesariamente afecta á fracción dos PTEs asociados a esas moléculas por unións electrostáticas. A comparación cos sedimentos indica

que a adhesión de partículas de sedimento á superficie tamén debe ser unha fonte importante de PTEs en algas, e que parte das diferenzas nas concentracións elementais entre algas desvitalizadas e frescas pode deberse a un incremento na adhesión de partículas causada por unha modificación das propiedades físicas da superficie.

Estes resultados indican que a captación de PTEs non ocorre principlamente por internalización, e que é un proceso complexo que depende de diversos factores. Ademais, se a unión a macromoléculas e a adhesión de partículas minerais son responsables dunha boa parte da captación, pode que as concentracións en macroalgas non reflictan a biodispoñibilidade ambiental dos PTEs.

Non obstante, para saber se as algas son útiles como biomonitores, é preciso coñecer se as súas concentracións de PTEs teñen unha correlación forte coas ambientais (Markert *et al.*, 1999). Isto xa se medira en diversas algas nativas con resultados variados. A maior parte deses estudos non detectaron correlacións fortes (Akcali e Kucuksezgin, 2011; Billah *et al.*, 2017; Boubonari *et al.*, 2008b, 2008a; Chernova e Shulkin, 2019; Ribeiro *et al.*, 2018), e os que si, en xeral teñen limitacións metodolóxicas que poñen en cuestión a súa fiabilidade (Bonanno *et al.*, 2020; Chakraborty *et al.*, 2014; Reis *et al.*, 2016, 2014). Ademais, todos estes artigos tiñan unha limitación maior: a recolección da auga facíase unha única vez no momento no que se recollían as algas. Isto é un problema porque as concentracións elementais nas algas tardan en estabilizarse en resposta ás condicións ambientais, como se ve no material suplementario do capítulo tres. Se os niveis de PTEs na auga varían ó longo do tempo, medilos nun momento concreto pode non ser representativo das concentracións ás que estiveron expostas as algas. No capítulo dous da tese afróntase este problema: expóñense transplantes de algas durante 12 días, e durante ese período cóllese auga cada hora, obtendo unha mostra composta representativa da auga á que estiveron expostos os transplantes durante o seu período de exposición. A pesar da comparabilidade absoluta entre as mostras de auga e dos transplantes, non se observa unha correlación entre elas, nin nos transplantes frescos nin nos secos. Ademais, a ausencia dunha correlación non se debe ó efecto doutros factores como as concentracións iniciais dos

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

transplantes ou os parámetros fisicoquímicos do medio, cuxo efecto se incluíu na comparación. Neste traballo tamén se recolleron algas nativas nos puntos de exposición o mesmo día que se recolleron os transplantes, pero as súas concentracións tampouco estiveron correlacionadas coas da auga.

O feito de que as concentracións finais non dependan das iniciais e que as réplicas teñan concentracións moi similares ó final do período de exposición, implica que as concentracións nas algas tenden a equilibrarse nun valor ó longo do tempo. Isto é algo que tamén se viu noutros traballos, e que encaixa cos resultados do capítulo un. Os valores de concentración de PTEs nos que se estabilizan deben depender de factores fisiolóxicos ou ambientais, pero sendo unha interacción complexa, onde o efecto das concentracións ambientais se ve enmascarado por outros factores. Polo tanto, a pesar de que as algas son capaces de captar concentracións altas de PTEs e chegar a un equilibrio co medio, a complexidade da interacción dificulta a interpretación dos resultados e limita a súa aplicación para a biomonitorización. Isto é certo incluso nos transplantes de algas secas, nos que a pesar de que nin o metabolismo nin o crecemento das algas teñen un impacto sobre as súas concentracións, estas non se poden predicir en base ás concentracións ambientais e ós parámetros fisicoquímicos medidos (pH, condutividade, oxíxeno disolto e temperatura). Igual que no primeiro traballo, as concentracións de PTEs nestes transplantes son máis altos que nos de algas frescas, confirmando que a carga destes elementos depende máis de procesos pasivos como a adhesión de partículas e a unión por enlaces electrostáticos que á internalización activa nas células.

Os resultados destes dous capítulos remarcan que a interacción entre as algas e os PTEs non é ben coñecida: coñécense distintas vías que poden estar contribuíndo ó incremento das concentracións como son a adhesión de partículas, a unión electrostática, e a internalización, pero non se saben os mecanismos polos que ocorre cada unha delas nin a súa contribución relativa. O primeiro paso para entender o proceso de captación é saber onde ocorre, xa que sen saber se os PTEs quedan na parede, atravesan as membranas, ou se adhiren a macromoléculas específicas, non se pode coñecer por que métodos chegan a estes compoñentes.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Responder a esta pregunta é de vital importancia, non só por entender as limitacións e axudar á interpretación dos resultados de estudos de biomonitorización, senón tamén porque a localización dos PTEs ten un impacto importante sobre a súa toxicidade e pode axudar a explicar e predicir o impactos destes contaminantes sobre as algas e os ecosistemas que sustentan.

8.2. Localización celular dos PTEs

Este é o obxectivo do capítulo tres, onde se fai unha revisión crítica da información dispoñible sobre a localización dos PTEs nas algas, centrándose en se son capaces de atravesar as membranas celulares. Ata hai pouco, a información sobre a localización dos PTEs obtivérase usando unha destas tres fontes: microanálise, técnicas de elución secuencial, e centrifugación. Non obstante, estes estudos chegaron a resultados contraditorios, incluso para a mesma especie e elemento. Nese capítulo conclúese que a principal causa desta contradición non é a variabilidade natural das localizacións dos PTEs nas algas, senón que vén dada polos problemas metodolóxicos das técnicas empregadas. A microanálise consiste na análise química de rexións da mostra de escala micrométrica (Rinaldi e Llovet, 2015). A maior parte dos artigos de microanálise fixéronse antes do ano 2000, e as técnicas dispoñibles non tiñan a resolución necesaria para diferenciar os distintos elementos ultraestruturais das células das algas con suficiente sensibilidade para detectar elementos traza. Ademais, para poder aplicar esta técnica, a preparación das mostras con frecuencia implicaba fixación química, un proceso que consiste en somerxer cortes da alga en distintos axentes fixadores, que poden lavar os elementos asociados a algúns compartimentos celulares (Moore *et al.*, 2012). Outra técnica é a de elución secuencial, que consiste en usar distintos axentes químicos que extraian os PTEs asociados a compoñentes celulares concretos, e despois medir as concentracións neses extractos (Pérez-Llamazares *et al.*, 2011). O maior problema deste método é que para que funcione correctamente é preciso ter axentes que extraian a totalidade da concentración dun elemento asociado a un certo compoñente sen afectar en absoluto as

concentracións do mesmo elemento asociado a outros compoñentes, algo que en moitas ocasións non se comprobaba para os extractantes empregados. Finalmente, a técnica de centrifugación consiste en separar os distintos compoñentes celulares por centrifugación, e logo medir as concentracións celulares en cada un deles (Zhao *et al.*, 2015). Isto podería permitir detectar as concentracións de PTEs firmemente asociadas a macromoléculas, pero aqueles con enlaces electrostáticos débiles ou almacenados dentro de orgánulos con membrana poderíanse mover debido á rotura das membranas.

Aínda que se solucionasen os problemas na aplicación das técnicas de elución e centrifugación, o seu potencial para aportar información precisa sobre a localización dos PTEs é limitado. Isto débese a que, como se viu nos capítulos anteriores, a distribución dos PTEs nas algas segue patróns complexos, nos que poden estar involucradas as partículas adheridas ó exterior, as paredes celulares, as membranas celulares tanto pola cara interna coma pola externa, e diversos compartimentos intracelulares, que poden incluír cristais orgánicos nos que algunhas especies acumulan PTEs. Utilizando elución secuencial, a selección de extractantes para saber exactamente que fracción da concentración de cada elemento está extraendo de cada compartimento é difícil. No caso da centrifugación, á parte de non poderse garantir que non hai mestura dos PTEs de distintos compartimentos, non necesariamente todos eles se poden separar por densidade.

A técnica co maior potencial para diferenciar todos os compartimentos celulares garantindo que non hai mobilización de PTEs é a microanálise, ou máis ben o nanoanálise, xa que se necesita resolución sub-micrométrica para poder mapear os elementos a escala subcelular. O problema metodolóxico de evitar a mobilización de PTEs durante a fixación das mostras pódese mitigar grazas á criofixación (Moore *et al.*, 2012), unha técnica que baixa a temperatura das mostras nun tempo tan curto que as vitrifica, impedindo a formación de cristais de xeo que poidan romper as membranas. Este proceso fixa a localización dos elementos minimizando a súa mobilización nos pasos posteriores, que permiten substituír o xeo por axentes fixadores e, finalmente, por resina, deixando a mostras fixada nun

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

bloque sólido de resina apto para estudos de nanoanálise. A outra limitación dos primeiros estudos de microanálise foi a resolución e sensibilidade das técnicas dispoñibles no momento, que non permitían distinguir os compartimentos subcelulares con precisión. Isto solucionouse grazas á aparición das técnicas de NanoSIMS e S-XRF, que permiten a obtención de mapas de distribución de elementos cunha resolución que pode baixar ata os 50 nm (Witt *et al.*, 2020). Estas técnicas permitiron descubrir a localización de múltiples elementos en distintos tipos de células animais e vexetais (Colvin *et al.*, 2016; Moore *et al.*, 2014; Penen *et al.*, 2016), pero a súa aplicación a algas foi moi limitada. Só catro estudos ata agora as empregaran, e todos sobre a mesma especie, a alga parda *Laminaria digitata*. A aplicación de NanoSIMS sobre esta permitiu analizar a distribución de As , Br, e I, que se detectaron principalmente nas paredes celulares (Ender *et al.*, 2019; Lebeau *et al.*, 2021; Verhaeghe *et al.*, 2008), e o emprego de S-XRF revelou a localización de Sr, que tamén está principalmente asociado ás paredes, e de Zn e Fe, que se colocalizan dentro das células (Mijovilovich *et al.*, 2023). Non obstante, estes estudos aportaron información de moi poucos elementos, dos cales só dous son PTEs (As e Zn), estiveron limitados a unha soa especie, e non aclararon os mecanismos de carga destes elementos.

Estes son os ocos de coñecemento que enchen os capítulos catro e cinco desta tese. O obxectivo do capítulo catro céntrase en describir a distribución do maior número de elementos nas células de *F. vesiculosus*. Para isto recolléronse mostras desta especie de puntos da costa galega con distintos niveis de contaminación, e analizáronse empregando S-XRF. Isto aportou información das distribucións de Ag, Ba, Ca, Co, Cr, Cu, Fe, Mn, Ni, S, Sr, U, e Zn nas células de algas cunha precisión sen precedentes. Esta análise revelou un patrón claro na captación de PTEs: aqueles sen funcións metabólicas intracelulares como o Ba e o U acumúlanse na parede celular, mentres que aqueles que ademais de ser potencialmente tóxicos tamén son nutrientes importantes como o Co, Ni, Cu, e Zn acumúlanse en fisoides, pequenos orgánulos cheos de florotaninos, que son compostos fenólicos propios de algas pardas (Gómez e Huovinen, 2020).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Respecto ó rol dos fisoides na bioloxía das algas pardas, propuxéranse diversas posibles funcións: protección contra a herbivoría, protección contra a radiación ultravioleta, formación da parede celular, e protección contra a toxicidade por PTEs, entre outras (Gómez e Huovinen, 2020; Meichssner *et al.*, 2023; Schoenwaelder, 2002). Os resultados deste traballo apoian esta última teoría. Non só iso, senón que a clara división baseada na súa función intracelular indica que as algas pardas son capaces de evitar a internalización de PTEs sen utilidade biolóxica, mentres que aqueles que si que a teñen son internalizados, e acumulados en orgánulos especializados para evitar a súa toxicidade.

Ademais dos elementos asociados a fisoides e á parede, outros elementos tiveron patróns distintos: o Mn apareceu nos plastos, o que é esperable debido á súa función como parte do fotosistema II, empregado na fotosíntese (Costa *et al.*, 2017); nalgunhas mostras, o Fe e o Cr, e en menor medida Mn, Co, Ni, e Cu atopáronse en concentracións moi altas en partículas minerais na superficie das algas, e en acumulacións no interior dalgunhas células; o Ca, a pesar de non ser un PTE, tamén tivo concentracións moi altas nas paredes celulares, probablemente debido á súa función en manter a súa estrutura (Terauchi *et al.*, 2016); e a Ag foi indetectable en todas as mostras menos nunha, na que se detectaron altas concentracións nun píxel situado na superficie da alga, probablemente debido á presenza dunha nanopartícula deste elemento.

A calibración das análises co S-XRF empregando unha mostra de referencia permitiu cuantificar as concentracións de elementos. Isto permitiu comparar as concentración medias detectadas nos tecidos das algas con medicións feitas empregando ICP-MS sobre as mostras de alga a partir das cales se fixeron as fixacións para S-XRF. Estes dous valores mostraron grandes diferenzas, ás veces tanto como dúas ordes de magnitude, e non se detectou ningunha correlación entre ambas. Hai distintas hipóteses que poden explicar estas diferenzas: as determinacións con ICP-MS foron feitas sobre as mostras secas, que teñen un peso moi distinto das mostras fixadas para S-XRF; puido haber algunha pequena perda ou adición de elementos durante o proceso de fixación; as células analizadas con S-XRF son aquelas da superficie, que poden ter concentracións distintas da media

USC UNIVERSIDADE DE SANTIAGO

do talo; e o proceso de fixación e corte das algas para S-XRF puido quitar as partículas da superficie, que en cambio si que se terían medido nas análises con ICP-MS. Este último punto é especialmente importante, xa que a diferenza dos demais, que deberían ter un efecto máis ou menos constante entre mostras, se as partículas da superficie son unha fonte importante de elementos que varía entre mostras, isto podería explicar a ausencia dunha correlación entre as concentracións medidas con ambas as técnicas.

Este estudo aportou a fonte máis extensa e detallada de información sobre as distribucións elementais en algas, pero tivo tamén certas limitacións. Por unha banda, non foi capaz de detectar algúns dos PTEs máis perigosos, como son o Pb, o Cd e o Hg. Por outra, o seu enfoque descritivo non achegou demasiada información sobre os mecanismos de captación dos elementos.

Estes dous puntos son os que se investigaron no traballo do capítulo cinco. Neste tamén se analizaron os patróns de distribución de elementos en células de *F. vesiculosus* a escala nanométrica, pero cunha selección distinta de mostras. Neste caso fíxose un experimento de transplantes, onde se colleron algas dun sitio contaminado e dun non contaminado, e algunhas de cada sitio expuxéronse nos dous, obtendo catro mostras: un autotransplante de cada sitio, e dous transplantes cruzados, un dunha alga do punto non contaminado exposto no contaminado, e un do contaminado exposto no non contaminado. A vantaxe deste enfoque respecto ó do capítulo catro é que permite ver os cambios nos patróns de acumulación causados pola exposición a concentracións distintas. Isto serve para estudar os compartimentos celulares encargados da carga e descarga, permitindo inferir información sobre os mecanismos involucrados. Ademais, mentres que no capítulo catro as mostras se analizaron con S-XRF para detectar o maior número de elementos posible, neste experimento empregouse NanoSIMS, permitíndonos analizar elementos que non se puideron detectar con S-XRF, especialmente o Pb. Para os elementos que se analizaron con ambas as técnicas (Ca, Mn, e Zn), os resultados foron moi similares, confirmando a súa fiabilidade. No caso do Pb, as súas distribucións apoian a hipótese proposta no anterior capítulo: o feito de que case todo o Pb se detectase nas paredes confirma que as algas son capaces de

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

evitar a internalización de PTEs sen funcións metabólicas. Tamén se detectaron concentracións baixas de Pb intracelularmente, pero sempre asociadas a fisoides, reforzando a hipótese de que teñen unha función de protección contra a toxicidade de PTEs. Ademais do Pb, outro elemento analizado neste traballo para o cal non había datos previos de localización celular é o Na. A súa distribución tamén estivo principalmente asociada á parede, inda que con concentracións moito máis altas que as de Pb, tanto intracelulares como extracelulares. No ambiente euhalino no que viven as algas, a maior parte de organismos teñen mecanismos para regular as concentracións de Na intracelular e así evitar que interfira co metabolismo (van Ginneken, 2018), polo que atopar as concentracións máis altas na parede é o esperable.

Ademais de revelar os patróns de distribución de novos elementos en algas, a comparación entre as distintas mostras tamén revelou patróns interesantes. O resultado esperable *a priori* era que os autotransplantes do sitio contaminado tivesen as concentracións máis altas da maior parte de elementos, que os autotransplantes do sitio non contaminado tivesen as máis baixas, e que os transplantes cruzados estivesen nun punto intermedio. Así, levar os transplantes do sitio non contaminado ó contaminado incrementaría as súas concentracións, e o contrario baixaríaas. No caso das concentracións totais das mostras medidas por ICP-MS, todos os elementos seguiron este patrón, detectando as concentracións máis altas de Na, Ca, Pb, e Zn no sitio contaminado e as máis baixas no non contaminado, e á inversa para o Mn. Isto encaixa co esperado, xa que se viu no capítulo un que as concentracións de Pb son moi altas no sitio contaminado, e sábese que o sitio non contaminado ten concentracións de Mn máis altas ca a media. Con todo, para a maior parte dos elementos, as concentracións medidas con NanoSIMS non seguiron este patrón en absoluto. En vez de isto, ambos transplantes cruzados experimentaron o mesmo efecto respecto ó autotransplante collido do mesmo sitio. No caso de Ca, Mn, e Zn, o cambio de sitio produciu un descenso nas concentracións, mentres que no caso do Na produciu un incremento. Este efecto foi consistente para as distintas estruturas subcelulares. O único elemento que non seguiu este patrón foi o Pb. Para este elemento, as

concentracións medidas por NanoSIMS comportáronse igual que as medidas por ICP-MS, cos autotransplantes tendo as concentracións máis extremas. Unha posible explicación para os cambios nas concentracións de Na, Ca, Mn, e Zn nos transplantes cruzados podería ser que a carga ou descarga experimentadas non se deban a que as súas concentracións se están equilibrando coas do medio, senón a un proceso activo inducido polo estrés causado polos cambios nas concentracións ambientais. Como só os transplantes cruzados experimentaron estes cambios ambientais, serían os únicos nos que o estrés metabólico afectaría ó metabolismo, modificando a carga e descarga destes elementos. O feito de que todos os elementos nos que se detecta este efecto teñan funcións intracelulares, e polo tanto o seu metabolismo estea regulado metabolicamente, apoia esta hipótese. O Pb, en cambio, é o único elemento medido neste traballo que non ten función metabólica, polo que se as algas regulan a súa concentración evitando a súa internalización independentemente das condicións ambientais, é esperable que o estrés metabólico non teña un efecto nas súas concentracións.

Os resultados dos capítulos catro e cinco teñen implicacións importantes no campo da biomonitorización. As concentracións elementais detectadas nas determinacións analíticas de concentracións absolutas de PTEs en algas reflicten os elementos asociados a distintos compoñentes (partículas minerais, parede celular, e orgánulos intracelulares), cada un dos cales ten uns mecanismos e dinámicas de carga diferentes. A maior parte das concentracións totais de PTEs en algas parece ser causada pola presenza de partículas minerais na superficie, co cal a carga ocorre progresivamente por adhesión ou desprendemento destas partículas, sen posibilidade de que estes PTEs teñan un efecto tóxico sobre as algas. Que a aportación de PTEs desta fonte sexa maior ou menor dependerá das concentracións de PTEs no sedimento, e de cantas partículas de sedimento haxa en suspensión no punto de exposición. Elementos pouco solubles en auga que habitualmente se asocian con fontes xeolóxicas como Al e Fe teñen concentracións altas nas partículas minerais, polo que as súas concentracións en algas dependerán principalmente desta fonte.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

En contraste, os PTEs disoltos na auga si que poden interaccionar co talo das algas. Unha fracción destes queda asociada ós polisacáridos da parede celular, que teñen cargas negativas ás que se poden unir catións por unións electrostáticas. Estes son enlaces polo menos parcialmente reversible, como ilustra a descarga de moitos elementos que se ve no capítulo cinco. Xa que os catións asociados á parede non poden interaccionar co metabolismo intracelular, a toxicidade destes elementos é nula ou reducida, o que explica por que as algas producen polisacáridos extracelulares en exceso en resposta á contaminación por PTEs. Nos mapas de distribución obtidos por S-XRF e NanoSIMS PTEs sen funcións metabólicas como o Ba, Pb e U son detectados principalmente neste compartimento, xa que as algas evitan a súa internalización nas células.

Por último, outra fracción dos elementos disoltos na auga é internalizada polas células das algas, especialmente naqueles elementos precisos para o metabolismo. Non obstante, debido ó potencial tóxico que teñen no compartimento intracelular, estes elementos son os máis sometidos a regulación metabólica. En *F. vesiculosus*, os fisoides xogan un rol fundamental nesta función, xa que serven para acumular concentracións excesivas de elementos como Mn, Co, Ni, Cu, e Zn. A acumulación nos fisoides, así como noutros compartimentos celulares, tamén e reversible, e pode depender non só das concentracións ambientais destes elementos, senón tamén en resposta a outros factores metabólicos como o estrés.

Esta complexidade dificulta a interpretación das concentracións absolutas das algas en estudos de biomonitorización, xa que varían de elemento en elemento, e non reflicten necesariamente os PTEs biodispoñibles, nin os disoltos na auga.

Ademais, incluso se fose posible separar as concentración asociadas a partículas das que forman parte da biomasa da alga, estas últimas tampouco terían por que ser útiles para a biomonitorización. Nos elementos con funcións intracelulares, o control metabólico podería estar tendo un efecto maior que as concentracións ambientais, co que estas concentracións da alga non estarían dando información directa da contaminación do medio.

USC DE COMPOSTELA

Estes descubrimentos teñen diversas implicacións que deberían guiar a investigación futura nesta área. En canto ós estudos de biomonitorización, tanto pasiva como activa, os autores destes traballos deberían ser cautos á hora de interpretar os seus resultados: tendo en conta a complexidade do proceso de carga e a variabilidade das concentracións, mapear a contaminación dunha rexión xeográfica baseándose só en datos de algas pode levar a resultados erróneos. Por outra banda, tamén sería importante replicar estes resultados con outros grupos de algas. Na revisión de García-Seoane et al. (2018b) sinálanse as algas do xénero *Ulva* como outro bo candidato para a biomonitorización, pero non se comprobou se transplantes desta especie teñen correlacións cos valores de concentración da auga. Con todo, a pesar de que a ultraestrutura das células de algas verdes e vermellas é distinta da de algas pardas, a presenza doutros polisacáridos externos nestes grupos como os carraxenatos suxire que é probable que se atopen resultados similares.

En canto á localización e ós mecanismos de carga de PTEs en algas, estes estudos mostraron o potencial das técnicas de nanoanálise para comprendelos, pero aínda se precisa moita máis investigación. En primeiro lugar, os resultados actuais limítanse ás algas pardas, mentres que para algas vermellas e verdes pluricelulares aínda non hai ningunha información. As algas pardas destacan polo seu rol como enxeñeiras de ecosistemas (Umanzor *et al.*, 2019), pero algunhas das especies de algas de maior importancia comercial pertencen ós outros grupos, como é o caso do nori, unha alga vermella. En segundo lugar, hai moitos elementos de interese para os cales aínda non hai información dispoñible. Entre estes destacan o Cd e o Hg, pero tamén hai outros elementos contaminantes emerxentes ou menos estudados como o litio (Li) e o talio (Tl) que se deberían estudar para avaliar o potencial que teñen novas fontes de contaminación para afectar ás comunidades de algas costeiras. Finalmente, para entender os mecanismos polos que ocorre a internalización de PTEs sería preciso a aplicación doutras técnicas xenéticas e moleculares que permitisen identificar as proteínas responsables do seu transporte. Esta información permitiría entender o impacto da contaminación por PTEs sobre distintos grupos de algas, o que axudaría a predicir como se verían

afectados os ecosistemas costeiros en resposta a descensos ou incrementos das concentracións mariñas destes elementos baixo un escenario de cambio global.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

9. Conclusións

- There are significant differences in accumulation of PTEs between live and devitalized *Fucus* vesiculosus transplants, indicating that seaweed physiology and metabolism influence elemental uptake.
- Passive uptake mechanisms such as mineral particle adhesion to the seaweed surface and electrostatic interactions with external polysaccharides account for the majority of total PTE concentrations found in seaweeds.
- Total PTE concentrations in seaweeds, whether native of transplanted, do not reflect environmental PTE concentrations.
- Total PTE concentrations in seaweeds tend towards an equilibrium with the environment, but equilibrium levels cannot be reliably predicted based on environmental concentrations and water physicochemical characteristics.
- Before the application of nanoscale imaging techniques in recent years, information on the fate of PTEs accumulated by seaweeds was unreliable.
- In *F.* vesiculosus, different PTEs have distinct distribution patterns: non-metabolic PTEs such as Ba, Pb, and U are primarily associated with cell walls, while metabolically important PTEs such as Mn, Co, Ni, Cu, and Zn are concentrated intracellularly.
- *F. vesiculosus* regulates PTE toxicity by limiting the internalization of non-metabolic PTEs and sequestering metabolically relevant ones in physodes.
- Metabolic responses to environmental stressors can result in altered uptake or release of metabolically important PTEs.

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11. Anexos

11.1. Anexo I: Material suplementario do capítulo 2

Table 1SM. Median and interquartile range (IQR) of the elemental concentrations in post-exposure fresh transplants (Fresh), post-exposure dried transplants (Dried), native seaweed samples growing in the exposure sites (Native), and seawater samples (Water). Concentrations are in μg g^{-1}.

		Al	Cd	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Fresh	Median	1200	0.842	2.24	7.81	823	209	4.38	8.58	2.73	87.4
	IQR	1100	0.243	2.01	5.87	643	199	2.54	10.7	1.63	63.1
Dried	Median	6970	0.193	10.4	19.1	4030	32.8	10.5	24.7	19.2	80.4
	IQR	9210	0.0685	9	19.9	5080	31.2	8.02	20.4	10.3	45.6
Native	Median	385	0.36	0.693	23.9	255	52.5	1.94	1.39	0.873	136
	IQR	359	0.163	0.887	22.5	223	34	2.07	1.11	0.537	123
Water	Median	4.61	0.0325	0.305	1.61	4.57	1.91	2	0.175	1.77	1.1
	IQR	4.05	0.0477	0.409	1.37	6.64	0.885	1.58	0.176	0.637	1.86

Table 2SM. Median and interquartile range (IQR) of the bioconcentration factors in post-exposure fresh transplants (Fresh), post-exposure dried transplants (Dried) and native seaweed samples growing in the exposure sites (Native).

		Al	Cd	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Fresh	Median	336	21.8	5.22	6.04	172	146	2.08	44.3	1.54	86.7
	IQR	374	38.3	8.92	6.46	477	97.5	1.85	68.7	1.12	98.4
Dried	Median	1870	5.94	29.5	15.3	922	21.9	5.14	126	11.5	52.8
	IQR	2130	6.26	28.9	14.5	1520	21.8	4.31	183	9.21	67.0
Native	Median	154	12.6	2.68	23.3	79.6	37.0	1.09	8.56	0.493	159
	IQR	247	13.1	3.49	18.0	247	29.4	0.970	30.1	0.481	292

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Table 3SM. Spearman correlation coefficient (Coef.) and p-value of the Spearman correlation test for correlations between post-exposure fresh transplants (Fresh), post-exposure dried transplants (Dried), native seaweed samples growing in the exposure sites (Native), and seawater samples (Water). P-values have been adjusted with the BH method. Underlined and bolded p-values are significant ($p < 0.05$), and bolded ones are marginally significant ($p < 0.1$).

		Al	V	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
Fresh ~ Dried	Coef.	0.58	0.51	0.52	0.37	0.6	0.57	0.74	0.61	0.65	0.44
	p-value	**0.017**	**0.050**	**0.044**	0.29	**0.012**	**0.022**	**0.0004**	**0.0075**	**0.0037**	0.13
Fresh ~ Native	Coef.	-0.057	-0.25	-0.086	0.42	-0.14	0.29	0.51	0.8	0.21	0.34
	p-value	1	0.53	0.95	0.3	0.9	0.58	0.13	**0.002**	0.67	0.37
Fresh ~ Water	Coef.	0.14	-0.18	-0.12	0.047	0.28	0.027	0.055	0.23	-0.19	-0.22
	p-value	0.91	0.53	0.93	1	0.41	1	1	0.52	0.67	0.42
Dried ~ Native	Coef.	-0.029	-0.29	0.13	0.28	0.075	0.34	0.3	0.53	0.21	0.39
	p-value	1	0.53	0.93	0.57	0.99	0.54	0.45	0.088	0.67	0.3
Dried ~ Water	Coef.	0.3	-0.5	0.17	0.19	0.41	0.037	0.077	0.36	0.25	0.24
	p-value	0.4	0.05	0.91	0.58	0.15	1	1	0.26	0.67	0.42
Native ~ Water	Coef.	0.35	0.23	-0.31	0.26	0.24	-0.15	0.32	-0.16	0.15	-0.42
	p-value	0.4	0.53	0.66	0.57	0.65	0.99	0.45	0.82	0.84	0.3

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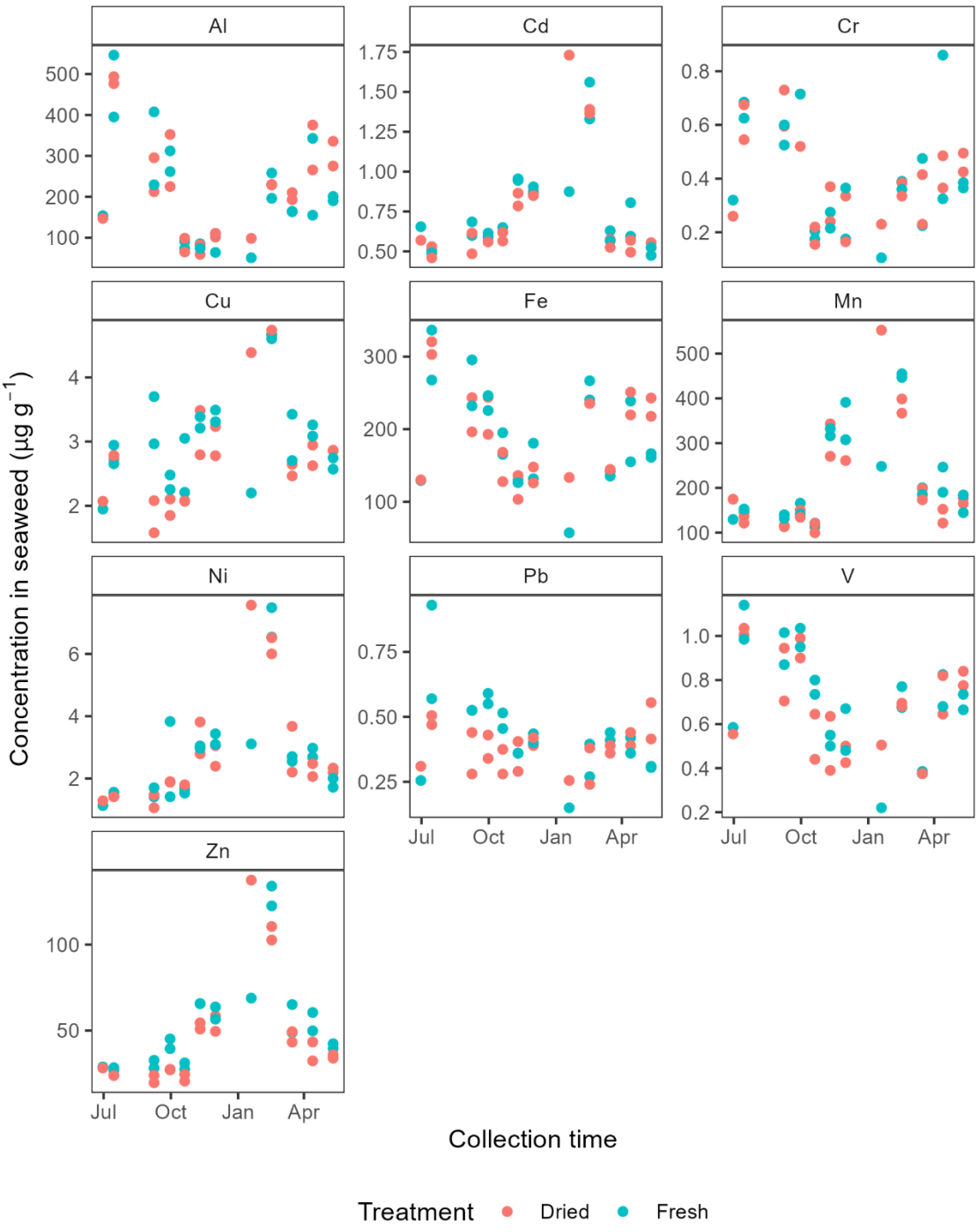


Figure 1SM. Elemental concentration in pre-exposure fresh and dried transplants over the time of the experiment in µg g^{-1}.

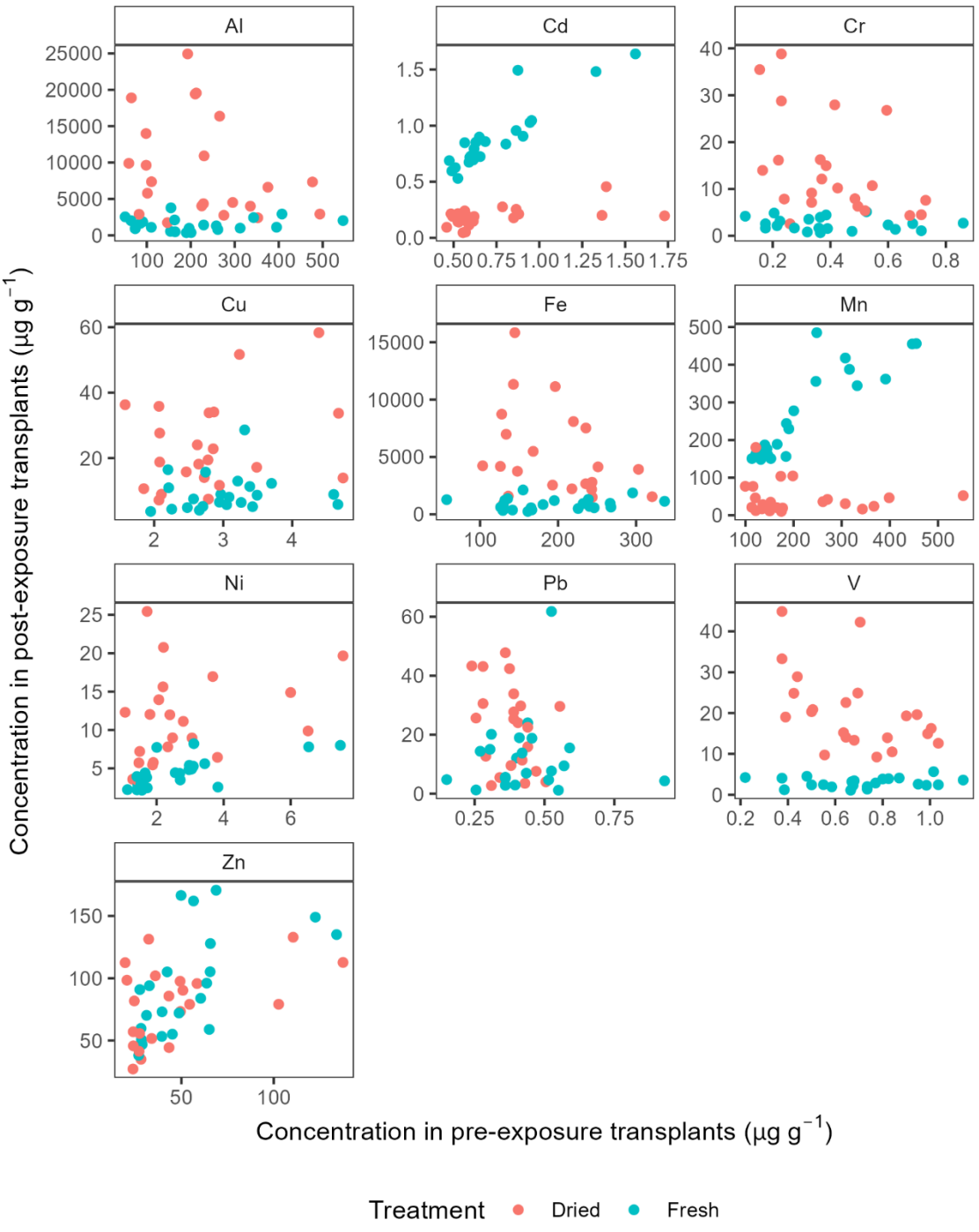


Figure 2SM. Comparison of pre- and post-exposure elemental concentrations in fresh and dried transplants.

11.2. Anexo II: Material suplementario do capítulo 5

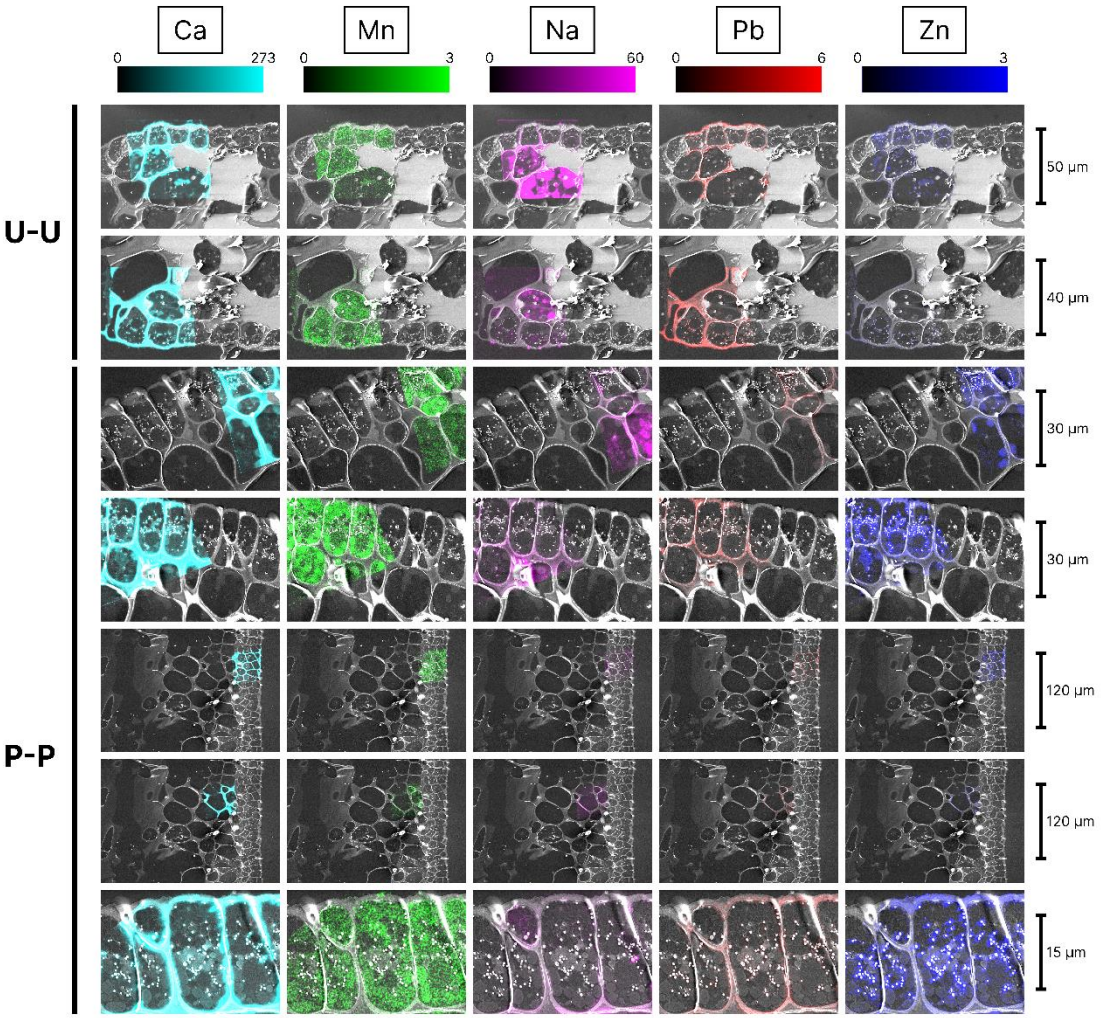


Figure 1. Elemental distribution maps of different regions of the samples U-U and P-P obtained with NanoSIMS, overlayed over SEM images of the same region. Color scale units are NanoSIMS read counts.

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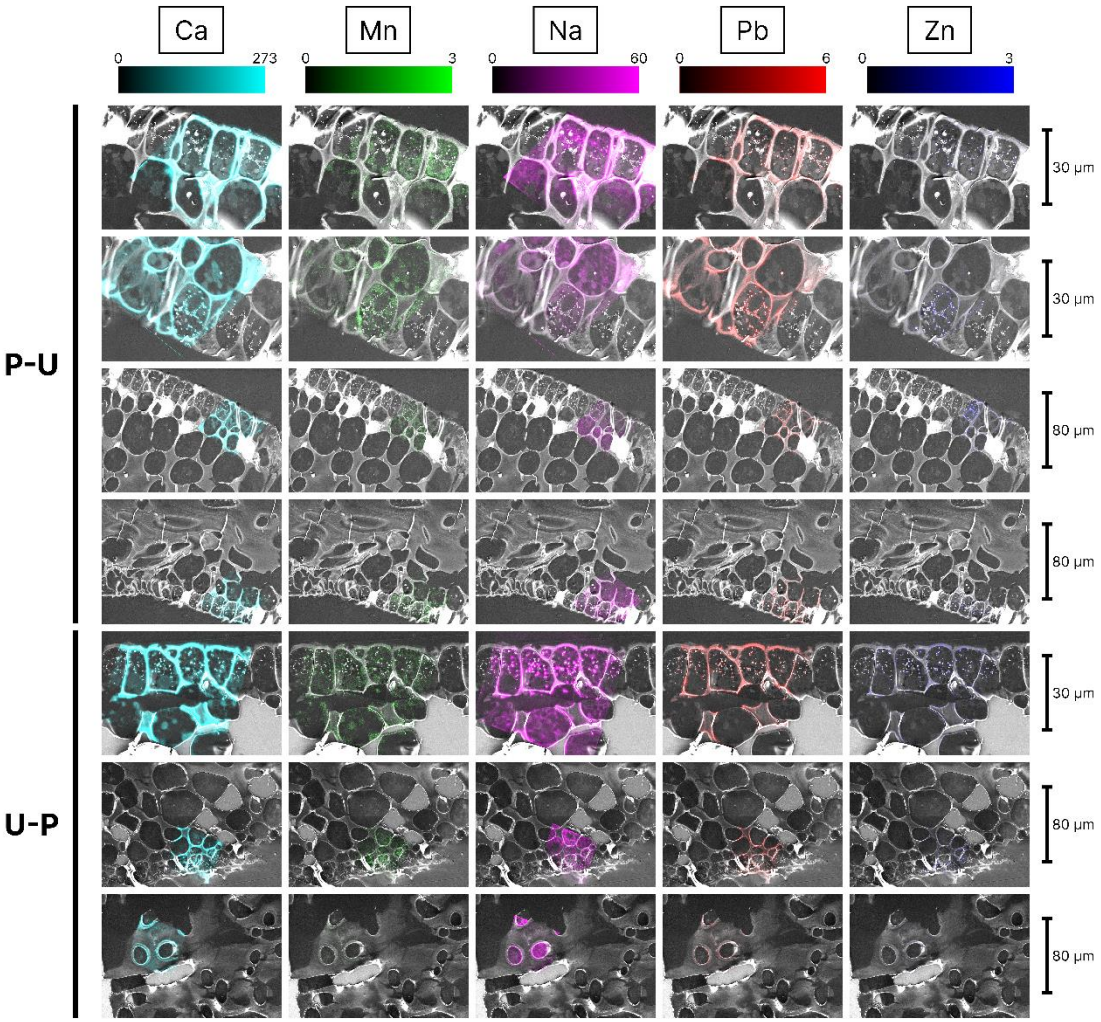


Figure 2. Elemental distribution maps of different regions of the samples P-U and U-P obtained with NanoSIMS, overlayed over SEM images of the same region. Color scale units are NanoSIMS read counts.

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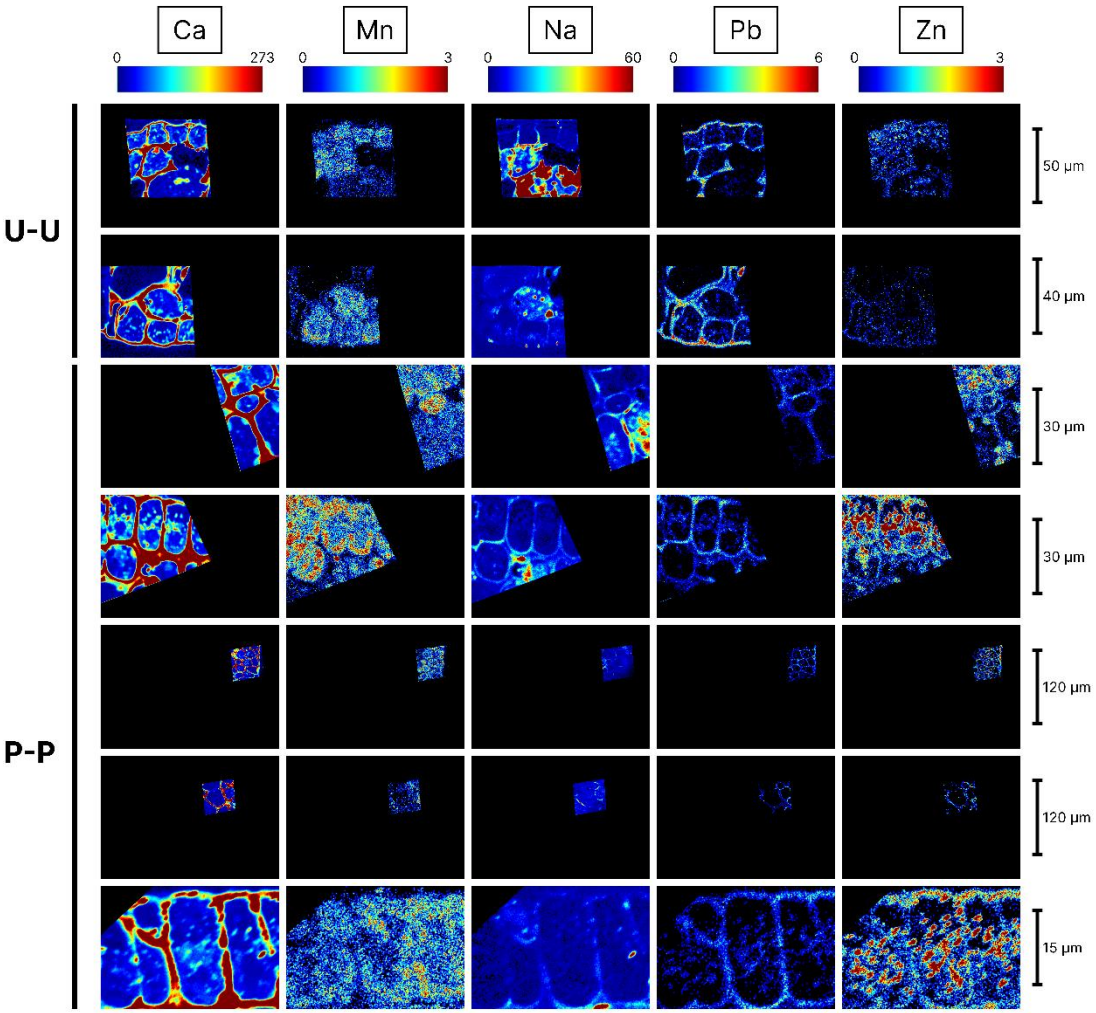


Figure 3. Elemental distribution maps of different regions of the samples U-U and P-P obtained with NanoSIMS. Color scale units are NanoSIMS read counts.

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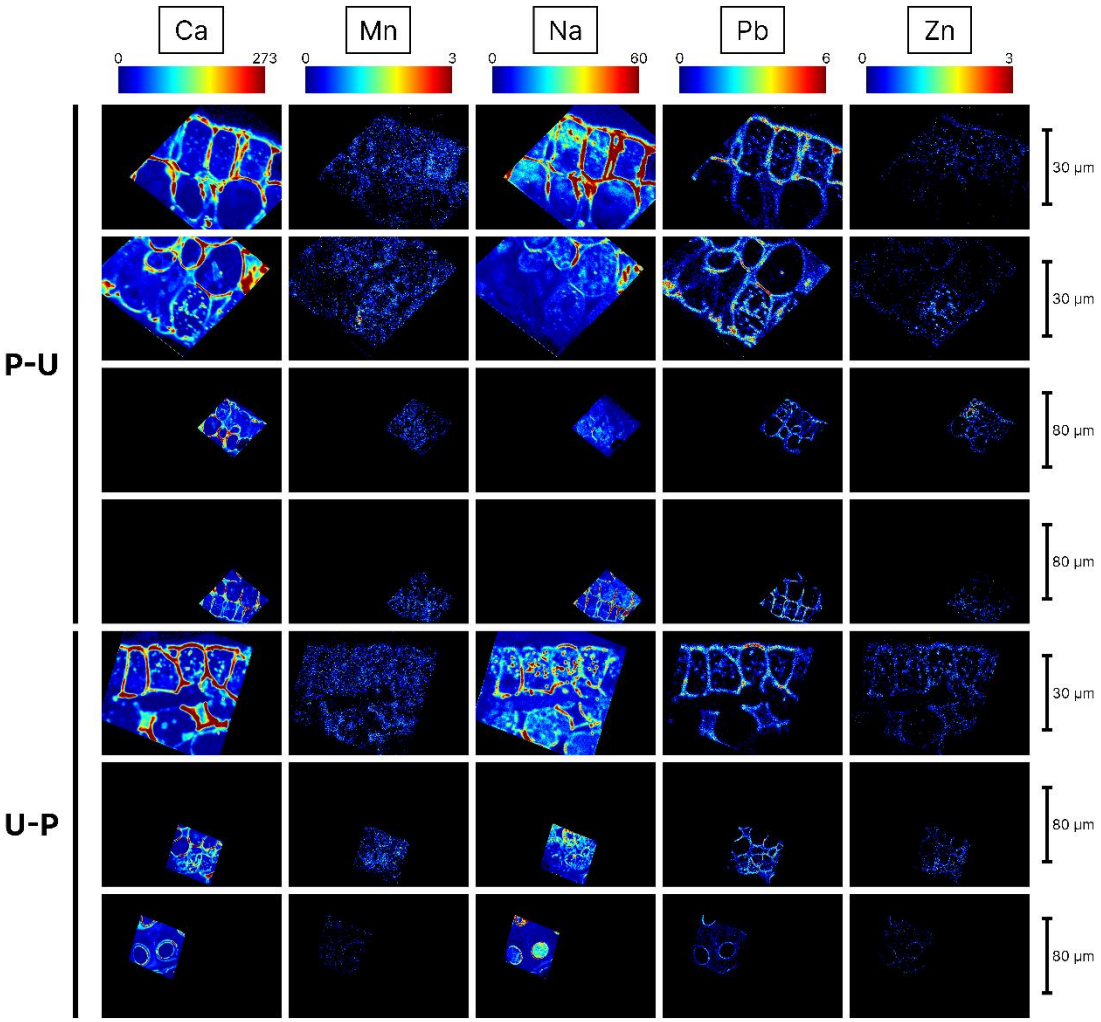


Figure 4. Elemental distribution maps of different regions of the samples P-U and U-P obtained with NanoSIMS. Color scale units are NanoSIMS read counts.

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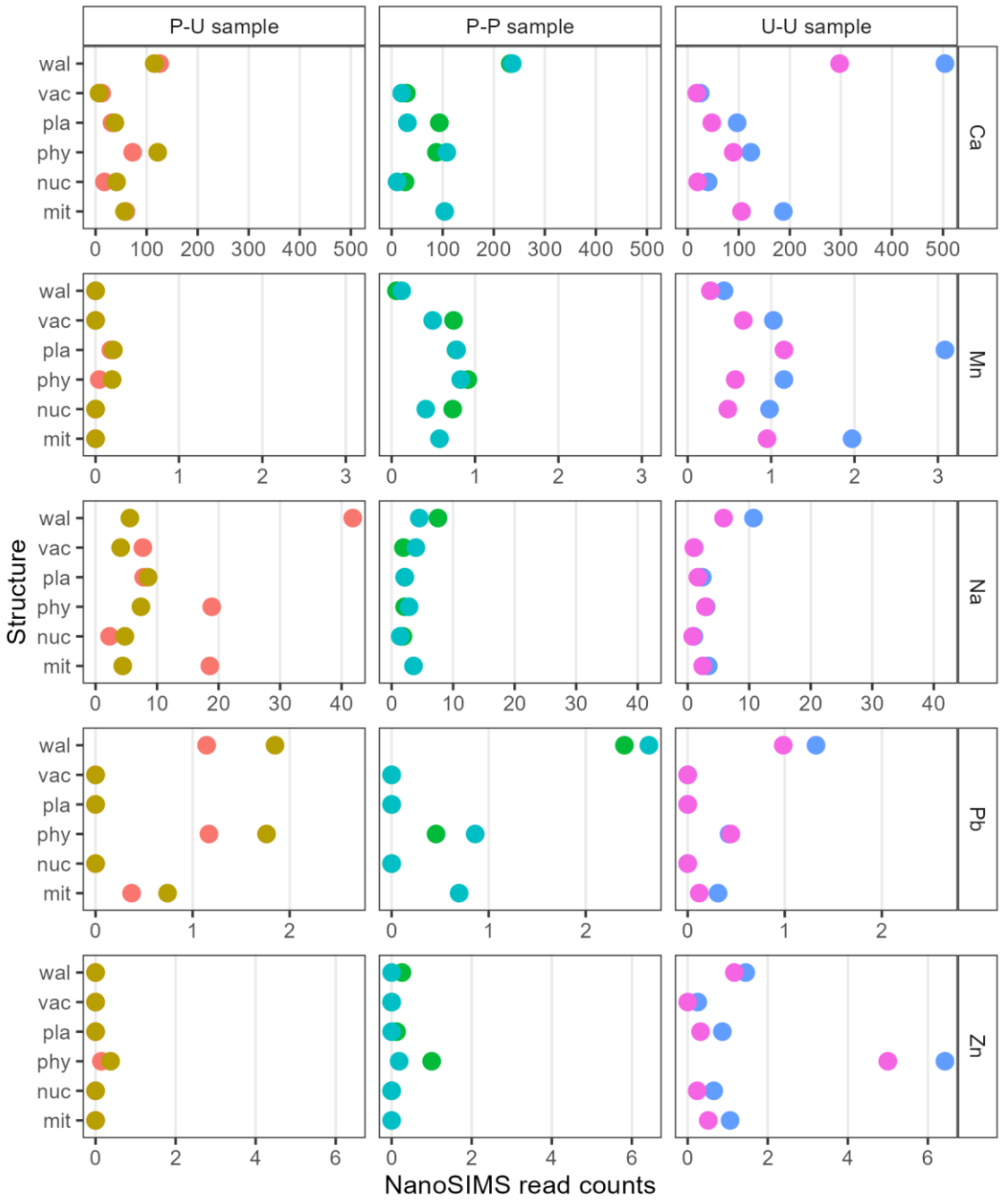


Figure 5. Median intensity values obtained with NanoSIMS for the different subcellular structures in each sample and subregion. Each column represents a different sample, and each color in each column corresponds to a different region within that sample. Subcellular structures: nuc: nuclei; phy: physodes; pla: plastids; mit: mitochondria; vac: vacuoles; wal: cell wall and extracellular polysaccharides.

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11.3. Anexo III: Permiso das revistas

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As algas pardas lévanse empregando máis de medio século para monitorizar a contaminación por elementos potencialmente tóxicos (PTEs) nos ecosistemas costeiros. Aínda así, o seu uso como biomonitores estivo limitado pola falta dun protocolo estandarizado e pola escasa comprensión dos mecanismos implicados na captación de contaminantes. Esta tese aborda ambos problemas, revelando unha complexidade previamente descoñecida nos mecanismos de captación. Dita complexidade reduce a eficacia das algas pardas como biomonitores, pero contribúe a comprender mellor o impacto dos PTEs tanto na súa saúde como na dos ecosistemas que sustentan.