



Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais



Impacto de múltiplos estressores em Nematoda de vida-livre: aspectos ecológicos, funcionais e moleculares

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Orientador: Prof. Dr. Juliano Zanette
Coorientadora: Profa. Dra. Fabiane Gallucci

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Tese apresentada ao Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais como requisito parcial para a obtenção do título de Doutora em Biologia de Ambientes Aquáticos Continentais.

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ATA DE DEFESA DE TESE DE DOUTORADO EM BIOLOGIA DE AMBIENTES AQUÁTICOS CONTINENTAIS – Nº 002/2025

Às 09h00 (nove horas) do dia 26 (vinte e seis) do mês de junho de 2025 (dois mil e vinte e cinco), via Webconferência no endereço eletrônico: (<https://conferenciaweb.rnp.br/sala/glauco-cesar-dias-delevedove>) reuniram-se docentes, discentes e comunidade em geral, para a Defesa Pública da Tese de Doutorado da acadêmica Nilvea Ramalho de Oliveira. A Tese intitulada “**Impacto de múltiplos estressores em Nematoda de vida-livre: aspectos ecológicos, funcionais e moleculares**” foi avaliada pela Banca Examinadora composta pelo Profº Dr. Juliano Zanette (Orientador); Profª. Dra. Fabiane Gallucci (Co-orientadora); Profª Dra. Cristina Stenert Maltchik Garcia (FURG); Profª Dra. Karina dos Santos Machado (FURG) e Profº Dr. Thiago Lopes Rocha (UFG). Após a defesa e arguição pública, a Banca Examinadora reuniu-se, para deliberação final, e considerou a acadêmica APROVADA. Desta forma, a acadêmica concluiu mais uma das etapas necessárias para a obtenção do grau de **DOUTOR EM BIOLOGIA DE AMBIENTES AQUÁTICOS CONTINENTAIS**. Nada mais havendo a tratar, às 12h35 (doze horas e trinta e cinco minutos) foi lavrada a presente ata, que lida e aprovada, foi assinada pelos membros da Banca Examinadora, pela Acadêmica e pela Coordenadora do Curso.

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“Ninguém ignora tudo. Ninguém sabe tudo. Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa. Por isso aprendemos sempre.”

Paulo Freire

“Nada na vida deve ser temido, apenas compreendido. Agora é a hora de compreender mais, para que possamos temer menos.”

Marie Curie

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RESUMO

As comunidades biológicas em ambientes costeiros estão cada vez mais expostas a múltiplos estressores associados às mudanças globais, como contaminação por metais, acidificação e aumento de temperatura. Esses fatores interagem de forma complexa, dificultando a previsão de seus impactos ecológicos. Essa tese investigou os efeitos de múltiplos estressores sobre Nematoda de vida livre, utilizando abordagens experimentais em microcosmo e simulações computacionais, com o objetivo de compreender as respostas ecológicas e moleculares em diferentes níveis de organização biológica. No primeiro capítulo, avaliamos os efeitos combinados de uma mistura metálica (Cu, Pb, Zn e Hg), acidificação por CO₂ e aumento de temperatura sobre assembleias naturais de Nematoda estuarinos. Observamos que a contaminação por metais foi o principal fator estruturante das assembleias, mas sua interação com os demais estressores revelou efeitos emergentes: enquanto gêneros dominantes declinaram, as oportunistas prosperaram sob contaminação, revelando respostas ecológicas complexas. Nesse contexto, a temperatura amplificou os efeitos da contaminação, enquanto a acidificação atuou como fator atenuante, enfatizando a necessidade de considerar interações entre múltiplos estressores na ecotoxicologia de ambientes costeiros. No segundo capítulo, analisamos como as relações taxonômicas e funcionais dessas assembleias respondem à contaminação por metais. Os resultados indicaram um aumento na distinção taxonômica sem grande perda de riqueza, sugerindo uma reorganização interna das assembleias. Além disso, houve convergência funcional em traços relacionados a estratégias de história de vida, alimentação e estrutura da cutícula nos tratamentos contaminados. No entanto, de forma inesperada, características geralmente associadas à sensibilidade tornaram-se mais frequentes nos tratamentos contaminados, evidenciando a complexidade das respostas funcionais ao estresse. No terceiro capítulo, caracterizamos estruturalmente a proteína metalotioneína MTL-1 do Nematoda *Caenorhabditis elegans* em ligações com diferentes íons metálicos. Por meio de modelagem tridimensional e simulações de dinâmica molecular, demonstramos que a ligação metálica aumenta a estabilidade estrutural da proteína desta espécie modelo, especialmente com Zn²⁺, Cd²⁺, Cu²⁺ e Hg²⁺, enquanto Pb²⁺ mostrou efeitos estabilizantes mais fracos. Os resultados evidenciaram a importância de modelos moleculares flexíveis para entender mecanismos de detoxificação em Nematoda. De forma integrada, esta tese contribui para o entendimento dos efeitos de estressores múltiplos sobre organismos bentônicos e destaca a necessidade de abordagem em diferentes níveis de organização biológica, para avaliar de forma adequada os impactos ambientais em sistemas costeiros.

Palavras-chave: metais, aumento de temperatura, acidificação, microcosmo, metalotioneína, dinâmica molecular

ABSTRACT

Biological communities in coastal environments are increasingly exposed to multiple stressors associated with global change, such as metal contamination, acidification and rising temperature. These factors interact in complex ways, making it difficult to predict their ecological impact. This thesis investigated the effects of multiple stressors on free-living nematodes, using experimental approaches in microcosm and computer simulations, with the aim of understanding the ecological and molecular responses at different levels of biological organization. In the first chapter, we evaluated the combined effects of a metal mixture (Cu, Pb, Zn e Hg), CO₂ acidification and temperature increase on natural assemblages of estuarine nematodes. We observed that metal contamination was the main factor structuring the assemblages, but its interaction with the other stressors revealed emergent effects: while dominant genera declined, opportunist genera thrived under contamination, revealing complex ecological responses. In this context, temperature amplified the effects of contamination, while acidification acted as a mitigating factor, emphasizing the need to consider interactions between multiple stressors in the ecotoxicology of coastal environments. In the second chapter, we analyzed how the taxonomic and functional relationships of these assemblages respond to metal contamination. The results indicated an increase in taxonomic distinction without a major loss of richness, suggesting an internal reorganization of the assemblages. In addition, there was functional convergence in traits related to life history strategies, feeding type and cuticle structure in the contaminated treatments. However, unexpectedly, traits usually associated with sensitivity become more frequent in the contaminated treatments, revealing the complexity of functional responses to stress. In the third chapter, we structurally characterize the MTL-1 metallothionein protein from *Caenorhabditis elegans* nematode in bonds with different metal ions. Using three-dimensional modelling and molecular dynamics simulations, we demonstrated that metal binding increases the protein structural stability of this model species, especially with Zn²⁺, Cd²⁺, Cu²⁺ e Hg²⁺, while Pb²⁺ showed weaker stabilizing effects. The results emphasize the importance of flexible molecular models to understand detoxification mechanisms in nematodes. In an integrated manner, this thesis contributes to the understanding of the effects of multiple stressors on benthic organisms and reinforces the need to approach in different levels of biological organization in order to adequately assess environmental impacts in coastal systems.

Key-words: metal, temperature rise, acidification, microcosm, metallothionein, molecular dynamics

APRESENTAÇÃO

Esta Tese de doutorado segue o modelo sugerido pelo Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais (PPGBAC) da Universidade do Rio Grande do Sul (FURG). A introdução geral apresenta o arcabouço teórico do tema, estruturado em três tópicos principais: 1) Estressores ambientais antrópicos em ecossistemas costeiros; 2) A importância de entender as respostas biológicas em múltiplas escalas de organização biológica e 3) Nematoda de vida-livre como modelo integrador de respostas a estressores ambientais. Também são apresentados os objetivos que nortearam o desenvolvimento da tese. Todas essas seções foram elaboradas conforme as normas da Associação Brasileira de Normas Técnicas (ABNT).

O capítulo 1 refere-se a um artigo que avaliou os efeitos combinados da contaminação por metais (Cu, Pb, Zn e Hg), acidificação por CO₂ e aumento de temperatura sobre assembleias naturais de Nematoda estuarinas, por meio de experimento de microcosmos. Este artigo intitulado “*Emergent properties of free-living nematode assemblages exposed to multiple stresses*” está publicado na revista *Science of the Total Environment*.

O capítulo 2 trata da resposta das assembleias de Nematoda estuarinas à contaminação por metais a partir de uma perspectiva das relações taxonômicas e funcionais. O manuscrito correspondente intitulado “*Taxonomic distinctness and functional traits convergence reveal complex nematode responses to metal stress*” está sendo preparado e será submetido na revista *Ecology Letters*.

O capítulo 3 refere-se à caracterização estrutural da proteína metalotioneína MTL-1 do Nematoda *Caenorhabditis elegans* em ligações com diferentes íons metálicos, utilizando modelagem tridimensional e simulações de dinâmica molecular. Este manuscrito intitulado “*Metal-binding specificity and structural dynamics of C. elegans metallothionein I: insights from 3D modeling and MD simulations*” está atualmente submetido na revista *PROTEINS: Structure, Function, and Bioinformatics*, e encontra-se em fase de revisão, na primeira rodada de correções.

A tese é finalizada com seção de Considerações finais e perspectivas, onde são discutidas as principais contribuições do trabalho para entendimento dos efeitos de múltiplos estressores sobre organismos bentônicos, bem como a importância de se considerar diferentes níveis de organização biológica na avaliação de impactos ambientais em sistema costeiros. As referências bibliográficas desta seção seguem as normas da ABNT.

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Capítulo 1

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Capítulo 2

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Capítulo 3

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Figure S5 Distance in Angstroms between residues and the Zn metal at each ion binding site (1-7). The graphs on the right correspond to replica 1 and the graphs on the left correspond to replica 2.

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Capítulo 1

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Table S4 Results of similarity percentage analysis (SIMPER) and the dissimilarity, from significative pairwise ($p < 0.05$) between temperature, acidification and metal mixture contamination levels. Nematode genera are ranked in order of importance of their contribution. More abundant (+); less abundant (-); similar abundance (=) and eliminated ($\emptyset$).

Capítulo 2

Table 1 Functional traits used for nematode assemblages.

Table 2 Outputs of the ANOVA and post-hoc analysis of Taxonomic distinctness indices (TD) and richness (S) parameters. TD values are presented as mean. Significant differences ($P < 0.05$) are indicated in bold.

Table 3 Functional traits analysis. A) PERMANOVA results and post hoc output, showing the effects of treatments on functional traits. B. Indicator trait analysis using r.g. statistic with permutation test (*indicspecies* package, in R). Significant values ($p < 0.05$) are highlighted in bold.

Capítulo 3

Table 1 Validation of the *C. elegans* metallothionein I model structure using AlphaFold.

Table S1 Percentage of secondary structure of MTL-1 3D model from AlphaFold and after molecular dynamics (200 ns) in AMBER, shown for duplicate simulations (Replica 1 and Replica 2). The secondary structure was determined quantitatively using the DSSP program (KABSCH; SANDER, 1983).

INTRODUÇÃO GERAL

1) Estressores ambientais antrópicos em ecossistemas costeiros

Regiões costeiras têm sofrido considerável impacto do aumento da poluição e das mudanças do clima nas últimas décadas, em grande parte devido às atividades antropogênicas (VIKAS; DWARAKISH, 2015). Dentre essas áreas, os estuários, em particular, estão sob forte pressão, já que tais impactos podem comprometer significativamente tanto a variabilidade natural quanto a capacidade de resiliência desses ambientes. Cerca de 60 % da população mundial vive em regiões costeiras e estuarinas e práticas como a agricultura, industrialização e o desenvolvimento urbano costeiro tem provocado alterações que extrapolam os limites naturais de variação desses ecossistemas (LINDENOOM, 2002). Esses impactos resultam em uma exposição contínua a uma variedade de fatores físicos, químicos e bióticos que podem se atuar como estressores ao deslocar organismos e comunidades para fora de suas faixas operacionais normais, comprometendo o equilíbrio ecológico.

Os estuários são corpos de água parcialmente fechados, permanentemente ou periodicamente conectados ao mar, e que recebem descarga de um ou mais rios (WOLANSKI; ELLIOT, 2016). Esse ambiente possui características únicas, com alta biodiversidade e produtividade biológica, funcionando como áreas críticas de alimentação, desova e berçário para muitos animais (RAKIB et al., 2022; SAVAGE et al., 2012). Além disso, oferecem uma ampla gama de serviços ecossistêmicos tais como a provisão de recursos alimentares, controle de inundações e proteção costeira, desempenhando um papel fundamental na manutenção e saúde dos ecossistemas costeiros (PÉREZ-RUZAFÁ et al., 2019). Por serem sistemas altamente dinâmicos, os estuários estão naturalmente adaptados a grandes variações em parâmetros ambientais como salinidade, disponibilidade de nutrientes, temperatura e pH. Estes dois últimos fatores, no entanto, fortemente associados às mudanças climáticas, têm apresentado – e também possuem previsão de – variações mais extremas do que as normalmente previstas para esse ambiente, desafiando a capacidade adaptativa desses ecossistemas.

Todos os compartimentos do ambiente estuarino têm sido afetados por atividades antrópicas, principalmente os sedimentos (HO; BURGESS, 2013), que atuam como importante sumidouros de poluentes. Esse compartimento tende a acumular contaminantes ao longo do tempo, muitas vezes em elevadas concentrações. Entre os diversos contaminantes, os metais são particularmente relevantes, por sua persistência, toxicidade e frequência de ocorrência (DE SOUZA MACHADO et al., 2016), o que contribui para que os estuários estejam entre os seis ambientes aquáticos mais contaminados do mundo por esses elementos (FÖRSTNER;

WITTMANN, 1979). Alguns dos metais mais recorrentes no estuário são zinco (Zn), cobre (Cu), chumbo (Pb) e mercúrio (Hg) comumente provindos de fontes industriais e do lançamento de esgoto urbano (BAISCH; NIENCHESKI; LACERDA, 1988; KIM et al., 2016; QIAN et al., 2015). O acúmulo desses elementos no sedimento representa uma ameaça significativa para a biota bentônica, comprometendo a saúde das comunidades que vivem nesse habitat e gerando efeitos deletérios e, em alguns casos, irreversíveis (JOHNSTON; ROBERTS, 2009).

No ambiente estuarino, os metais são transportados tanto em forma particulada quanto dissolvida. O sedimento e as partículas em suspensão desempenham papéis essenciais no processo de adsorção, complexação e sedimentação desses elementos (OYEWO et al., 2020). A permanência e/ou distribuição dos metais nos sedimentos dependem de processos como solubilização, especiação, precipitação (BENOIT et al., 1994), todos influenciados por condições ambientais (LV et al., 2021) que podem, por sua vez, intensificar seus efeitos sobre a biota.

Além dos contaminantes químicos, fatores associados às mudanças climáticas, como o aumento da temperatura e acidificação, também têm alterado significativamente os processos ecológicos e biogeoquímicos nesses ambientes (PRYA et al., 2023). O Painel intergovernamental sobre Mudanças Climáticas (IPCC) prevê que a absorção de CO₂ pelos oceanos continuará até 2100 em todos os cenários de emissão. Na atmosfera, estima-se que as concentrações dos gases de efeito estufa pode atingir entre 475–1313 ppm até o final do século (IPCC, 2023), o que resultará em uma queda de até 0,4 unidades de pH da superfície oceânica e aumento de 2.2 a 3.5 °C na temperatura média global da Terra (IPCC, 2023). Dessa forma, a integração dos efeitos abióticos decorrentes das mudanças climáticas aos impactos provocados por contaminantes químicos é essencial para uma avaliação mais acurada e realista das influências antrópicas sobre os sistemas estuarinos.

Embora alguns estudos tenham explorado os efeitos isolados de estressores tais como metais ou alterações climáticas sobre a comunidade biológica (GUTIÉRREZ et al., 2016; HAEGERBAEUMER et al., 2018; LEE; TORRES; MANRÍQUEZ, 2017; MAJDI et al., 2019; MEADOWS et al., 2015; PILÓ et al., 2016; VAFEIADOU et al., 2018), muitos aspectos ainda permanecem pouco compreendidos, especialmente quando se consideram diferentes níveis de organização biológica. Além disso, tais fatores raramente atuam de forma independente no ambiente. Prever os efeitos combinados de vários estressores é um desafio, visto que a sobreposição espacial e temporal de estressores pode intensificar ou modificar seus efeitos individuais, potencializando o surgimento de respostas ecológicas inesperadas – as chamadas propriedades emergentes (HALE; PIGGOTT; SWEARER, 2017). Estes efeitos são heterogêneos e imprevisíveis, resultantes da interação entre estressores e envolvem conexões e interações entre espécies ou conjuntos, que, quando observados em conjunto, representam mais do que a soma de

suas partes (MAYR, 1984). A detecção de tais respostas complexas tornam-se particularmente viáveis em experimentos de microcosmo - sistemas controlados em pequena escala com prazo relativamente longo para captar efeitos de múltiplos estresses (FERRAZ et al., 2022; HÖSS et al., 2015; JIANG; PATEL, 2008; MARTINEZ; QUIOBE; MOENS, 2019). Esse tipo de ensaio permite a observação de dinâmicas ecológicas em níveis mais altos de organização biológicas (ou seja, baseada na comunidade), oferecendo subsídios valiosos para a compreensão de padrões emergentes em sistemas complexos.

2) A importância de entender as respostas biológicas em múltiplas escalas de organização biológica

Compreender os impactos da contaminação por metais em ecossistemas estuarinos exige uma abordagem em várias escalas, capaz de integrar respostas observadas desde alterações na comunidade biológica até mecanismos moleculares que conferem sensibilidade ou tolerância aos organismos. As respostas em nível de comunidade são particularmente relevantes porque podem evidenciar mudanças estruturais e funcionais que afetam diretamente seu funcionamento (RAMSEY et al., 2005). Ao mesmo tempo, respostas podem ocorrer de forma sutil e precoce ao nível molecular, por exemplo, por meio de proteínas que interagem diretamente com os poluentes (KADIM et al., 2022).

Considerando o grau e a duração dos eventos de contaminação, mudanças na estrutura de comunidade são esperadas, resultando em: i) eliminação de espécies sensíveis devido ao efeito direto do contaminante; ii) substituição dessas espécies por outras menos sensíveis devido a liberação da competição; iii) mudanças nas interações da cadeia alimentar como consequência da diminuição da predação e herbivoria de espécies susceptíveis ao contaminante iv) aclimação fisiológica mediada por mecanismo bioquímicos de detoxificação e v) seleção de tolerância geneticamente herdada (i.e., microevolução devido a poluição). Essas mudanças decorrem da variabilidade na sensibilidade das espécies, ou seja, da diferente capacidade que cada uma possui de habitar e persistir em ambientes contaminados (MEDINA; CORREA; BARATA, 2007).

Respostas em nível de comunidade, podem ser geradas por quatro diferentes processos ecológicos: estocasticidade demográfica, dispersão, seleção de nicho e/ou por meio de filtros ambientais (VELLEND, 2010). Este último, é um dos potenciais fatores da diversidade beta (CAO et al., 2016). Nesse caso, uma maior variação ambiental dentro de um habitat produz uma maior diversidade beta. Isso ocorre devido a ampliação da disponibilidade de recursos e condições que podem suportar conjuntos de espécies com diferentes nichos (LEIBOLD et al., 2004). Por outro lado, a modificação do habitat causada pela contaminação de poluentes pode

promover a homogeneização da comunidade biológica (OLDEN; ROONEY, 2006), favorecendo espécies com maior tolerância e resultando na convergência taxonômica e/ou funcional das comunidades (CLAVEL; JULLIARD; DEVICTOR, 2011; YUAN, 2004).

Nesse contexto, respostas à contaminação podem seguir padrões associados com a relação filogenético, uma vez que a sensibilidade ou tolerância tende a ser conservada entre grupos taxonômicos relacionados (HAMMOND et al., 2012; MALAJ et al., 2016). Dependendo da escala filogenética, por exemplo dentro de família, até 83% da variação da tolerância (i.e., CL50;96 horas) das espécies pode ser explicada pela estrutura de parentesco entre as espécies (GUÉNARD et al., 2011). Por outro lado, o filtro ambiental também pode promover convergência funcional ao selecionar características adaptativas associadas à tolerância, independente da relação filogenética (HELMUS et al., 2010; MORENO et al., 2011; SEMPRUCCI; COLANTONI; BALSAMO, 2016). Considerando que os traços funcionais refletem características morfológicas, fisiológicas ou comportamentais ligadas à história de vida e ao funcionamento ecológico, esses atributos – como estratégias alimentares, modo de vida ou atributos morfofuncionais – podem ser favorecidos quando expostos à contaminação, moldando as respostas individuais e consequentemente influenciando processos ecossistêmicos (HOOPER et al., 2005). Além disso, a convergência funcional pode, entretanto, resultar em perda de diversidade funcional e, consequentemente, reduzir a resiliência ecológica frente a novas perturbações (MOUILLOT et al., 2013). Assim, embora a relação de parentesco entre as espécies possa influenciar a sensibilidade ou tolerância das espécies, a filtragem ambiental pode impulsionar uma convergência funcional entre organismos com características similares, e ambos os processos podem atuar de forma conjunta ou excludente na estruturação das comunidades em ambientes contaminados.

Considerando que a tolerância pode também estar associada a mecanismos bioquímicos, além dos traços funcionais clássicos, características associadas a maquinaria molecular dos organismos também podem responder à filtragem ambiental promovida pela contaminação. Particularmente na contaminação por metais, é bem estabelecido que as metalotioneínas, proteína altamente conservada ao longo do reino animal (ZILLER; FRAISSINET-TACHET, 2018), estão envolvidas no processo de homeostase de metais, no qual realizam o transporte, armazenamento e detoxificação (BLINDAUER; LESZCZYSZYN, 2010). Além disso, elas podem atuar na proteção contra radicais livres e danos fisiológicos, entre outras funções ainda não elucidadas (CAPDEVILA; ATRIAN, 2011). As metalotioneínas possuem baixo peso molecular (6 -7 kDa) e são ricas em cisteínas, sendo consideradas as primeiras linhas de defesa contra a contaminação. A capacidade de ligação aos metais se dá pela presença e distribuição conservada de resíduos de cisteína ao longo da cadeia polipeptídica. Porém, ainda não se

compreende completamente como as metalotioneínas reconhecem e interagem com diferentes tipos de metais. A especificidade por determinados íons metálicos, bem como os mecanismos envolvidos na seletividade e estabilidade dos complexos formados, permanece pouco elucidado. Além disso, diferentes isoformas de metalotioneínas, podem responder de maneiras diversas à contaminação, refletindo variações evolutivas, ecológicas ou funcionais (DALLINGER, 2024; TOMAS et al., 2013).

Características estruturais e funcionais das proteínas podem ser precisamente determinadas através de cristalografia de raios-X e espectroscopia de ressonância magnética nuclear (SCARFF et al., 2008). No entanto, estes métodos são caros e demandam muito esforço e tempo. Recentemente, estudos *in silico* acessando a estrutura tridimensional de proteínas têm promovido forte avanço na predição da sua funcionalidade (MADHUSUDHAN et al., 2005; WALKER; YALLAPRAGADA; TANGNEY, 2020). No caso das metalotioneínas, tem se destacado investigações sobre os sítios ligantes de metais dessas proteínas com objetivo de entender o seu papel no metabolismo bem como no entendimento sobre os mecanismos que envolvem toxicidade (SINGH et al., 2019a; ZHANG et al., 2012).

Portanto, as metalotioneínas por sua relevância funcional e ampla conservação evolutiva, surgem como elemento-chave para investigar como a pressão antrópica pela contaminação por metais afeta os mecanismos de resposta a adaptação dos organismos expostos. Nesse contexto, sua análise articulada as demais escalas biológicas superiores, permite uma abordagem integrada das respostas às perturbações ambientais. A integração entre níveis molecular, funcional e ecológico pode revelar padrões consistentes de adaptação e vulnerabilidade, ampliando a compreensão dos processos ecológicos e evolutivos em ambientes contaminados por metais.

3) Nematoda de vida livre como modelo integrador de respostas a estressores ambientais

Dentre os organismos que habitam os sedimentos e estão potencialmente expostos à contaminação, Nematoda de vida livre destacam-se por serem os metazoários mais abundantes e diversos nesse ambiente (HEIP; VINCX; VRANKEN, 1985). Suas densidades são da ordem de 1 milhão de indivíduos por metro quadrado de sedimento, e o número de espécies encontrado é geralmente uma ordem de grandeza superior ao de qualquer outro táxon (LAMBSHEAD; BOUCHER, 2003; MOKIEVSKY; AZOVSKY, 2002). Devido ao curto tempo de geração e taxas metabólicas relativamente altas, esses organismos desempenham um papel relevante na transferência de energia para níveis tróficos superiores (WOODWARD; PERKINS; BROWN, 2010), contribuindo significativamente para funções ecossistêmicas essenciais, tais como processos de decomposição e ciclagem de nutrientes (BONAGLIA et al., 2014; HUBAS et al., 2010; SCHRATZBERGER; INGELS, 2018). Por viverem entre os grãos de sedimento e

possuírem baixa mobilidade, permanecem nesse habitat ao longo de todo seu ciclo de vida (GIERE, 2009). Essa característica os torna sensíveis a alterações refletindo diretamente as condições ambientais locais, tanto em termos de composição quanto de abundância de espécies, permitindo a detecção de alterações associadas a estressores ambientais (BONGERS; FERRIS, 1999). Além disso podem contribuir de forma significativa para a avaliação de risco dos contaminantes ao gerar rápidas respostas em diversos níveis de organização biológica (FLEEGER; CARMAN; NISBET, 2003).

A diversidade de estratégias de história de vida entre os Nematoda resulta em distintos mecanismos de resposta frente à exposição a contaminantes. Em particular, há uma ampla variação na sensibilidade aos metais evidenciada tanto em estudos de campo quanto estudos laboratoriais (GYEDU-ABABIO et al., 1999; HAEGERBAEUMER et al., 2018; HAEGERBAEUMER; HÖSS; TRAUNSPURGER, 2019; HÖSS et al., 2017). Estudos com comunidades estuarinas demonstraram que alguns gêneros como *Sabatieria*, *Tershellinia* e *Ptycholaimellus* apresentam forte tolerância a uma grande variedade de metais (e. g. Zn, Cu)(GYEDU-ABABIO; BAIRD, 2006; SOMERFIELD; GEE; WARWICK, 1994). Em estudos controlados com cultivo de Nematoda estuarinos, *Diplolaimella diavengatensis* apresentou alta tolerância a Cd, Cu e Pb (VRANKEN; HEIP, 1986), enquanto *Litoditis marina* foi bastante sensível a Cd e Cu (MONTEIRO et al., 2018). Portanto, a presença de gêneros com diferentes níveis de sensibilidade, amplia e fortalece seu uso como ferramenta para ensaios de toxicidade.

Nesse contexto, as respostas de Nematoda à contaminação ambiental não se limitam apenas às mudanças na composição e abundância, mas também envolvem padrões funcionais que refletem adaptações morfofuncionais e ecológicas frequentemente observadas em espécies de Nematoda de vida-livre (Figura 1) (EGRES et al., 2019; KANDRATAVICIUS et al., 2018; LOSI et al., 2021; PORTUGAL et al., 2025). Variações na estrutura e permeabilidade da cutícula entre diferentes espécies, por exemplo, podem influenciar diretamente a tolerância à exposição a contaminantes (KAMMENG; VAN GESTEL; BAKKER, 1994). Nesse contexto, cutículas com menos ornamentações são geralmente associadas a uma maior permeabilidade a substâncias externas (FONSECA; FEHLAUER-ALE, 2012) embora exista evidências sugerindo que essas estruturas possam atuar como barreiras eficazes contra a entrada de contaminantes (EYUALEM-ABEBE; ANDRÁSSY; TRAUNSPURGER, 2006). Além disso, os Nematoda podem ser classificados quanto ao tipo de alimentação com base na morfologia bucal (Figura 1), sendo divididos em alimentadores de depósito seletivo (1A), não seletivo (1B), epístrato (2A) e onívoros/ predadores (2B) (WIESER, 1953). Essa classificação está relacionada com a forma como os organismos interagem com o sedimento e com a disponibilidade de recursos, o que também pode influenciar sua sensibilidade a contaminantes. Por exemplo, entre os organismos

do tipo 1, os 1B tendem a ser mais tolerantes por ingerirem partículas de forma menos seletiva, enquanto os do tipo 2, os 2B podem apresentar maior resiliência ao explorar presas oportunistas nos ambientes impactados (MOENS; VINCX, 1997; SEMPRUCCI; BALSAMO, 2014).

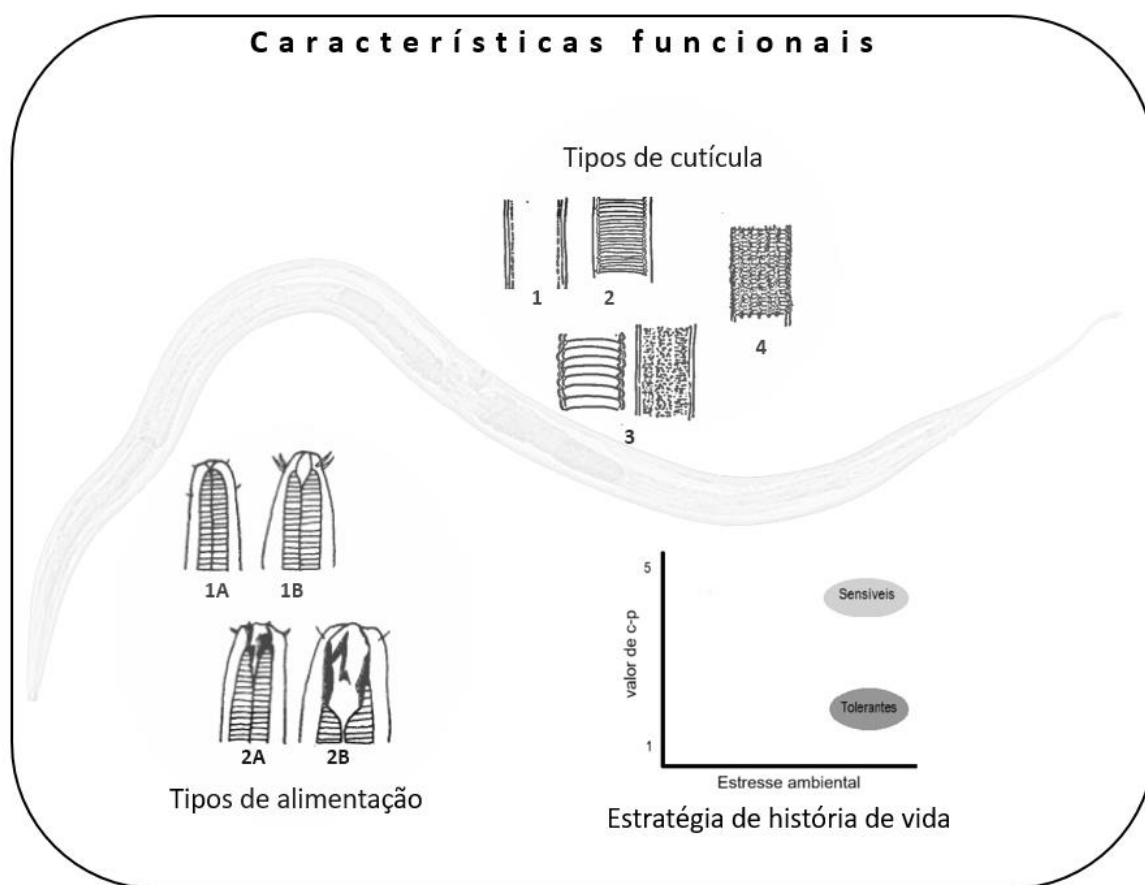


Figura 1. Características funcionais de Nematoda estuarinos. Tipos de alimentação: 1^a = alimentadores de depósito seletivo; 1B = alimentadores de depósito não seletivo; 2^a = alimentadores de epistrato e 2B = onívoro/predadores (Wieser, 1953, 1960). Tipos de cutícula: 1 = lisa; 2 = estriada; 3 = anelada ou coberta com pontos e 4 = ornamentada. Estratégia de história de vida, com base nos valores de c-p: 1= extremamente colonizadores; 2 = colonizadores; 3= intermediário; 4 = persistente e 5 = extremamente persistentes (Bongers et al 1990; Bongers and Bongers, 1989). Imagens adaptadas de Hyeong-Gi, 2017 e Matthew R. Lee, 2025 (<https://meiochile.matthewlee.org/>).

Um outro aspecto funcional amplamente utilizado é a classificação segundo a escala “colonizador-persistente” (cp) o qual compõe o Índice de Maturidade (IM) proposto por BONGERS, 1999. Essa escala ranqueia Nematoda em cinco classes (cp-1 a cp-5), onde as espécies mais próximas ao cp-1 são mais oportunistas (r-estrategistas), com rápido ciclo de vida, alta fecundidade e maior tolerância as perturbações, enquanto espécies cp-5 apresentam

crescimento mais lento, maior longevidade e são mais sensíveis a estressores ambientais. Deste modo, ambientes contaminados favorecem espécies com baixos valores de cp, enquanto ambientes mais preservados são dominados por espécies de cp mais elevados (BONGERS; BONGERS, 1998).

Apesar da relevância ecológica dos Nematoda estuarinos, ainda há escassez de informações sobre seus mecanismos moleculares de resposta à contaminação por metais. Grande parte do conhecimento disponível provém de estudos com o Nematoda terrestre *Caenorhabditis elegans*, bastante utilizado como organismo modelo em ensaios experimentais, devido a sua facilidade de cultivo, ciclo de vida curta e a existência de ferramentas genéticas avançadas. Para essa espécie, já foi demonstrado que a desintoxicação de metais absorvidos ou ingeridos envolve proteínas de ligação a metais (HÖCKNER; DALLINGER; STÜRZENBAUM, 2011; YE et al., 2010). Duas isoformas de metalotioneínas, *mtl-1* e *mtl2*, foram identificadas (Imagawa et al., 1990), apresentando diferentes afinidades por metais como zinco e cobre, devido à reatividade dos sítios de ligação contendo cisteína (BLINDAUER, 2008; BOFILL et al., 2009). No entanto, esses experimentos focaram apenas em zinco e cobre, ressaltando a necessidade de ampliar a investigação para outros metais com potencial tóxico. Até o momento, buscas realizadas no Genbank indicam sequências nucleotídicas de metalotioneínas descritas para quatro espécies de Nematoda: *C. elegans*, *C. remanei*, *C. briggsae* and *Trichinella spiralis* (GenBank), todas de ambiente terrestre ou parasita. Diante disso, *C. elegans* foi adotado como modelo para o estudo *in silico* da metalotioneína, visando elucidar os mecanismos moleculares associados à resposta a contaminantes metálicos, fornecendo uma base funcional que pode ser posteriormente comparada ou validada em espécies estuarinas. Essa abordagem, portanto, contribui com uma perspectiva complementar aos dados ecológicos obtidos com as comunidades naturais de Nematoda estuarinos expostas a contaminação por metais.

Objetivos

Objetivos gerais

Investigar os efeitos da contaminação por metais, isoladamente e com outros estressores associados às mudanças climáticas, sobre Nematoda de vida-livre, por meio de uma abordagem em diferentes níveis de organização: desde alterações emergentes sob cenário realista de múltiplos estressores, passando por padrões funcionais e taxonômicos das assembleias, até a estrutura molecular da proteína metalotioneína envolvida na tolerância aos metais.

Objetivos específicos

Avaliar os efeitos de múltiplos estressores (mistura de metais [Cu, Pb, Zn e Hg] acidificação induzida por CO₂ e aumento de temperatura) sobre uma assembleia natural de Nematoda estuarino, por meio de um experimento de microcosmo (Capítulo 1).

Analisar alterações na composição, abundância e riqueza de espécies, bem como possíveis efeitos emergentes resultantes da interação entre os estressores (Capítulo 1).

Investigar como diferentes níveis de contaminação por metais influenciam as assembleias de Nematoda estuarino, considerando relações taxonômicas entre os gêneros e traços funcionais dos organismos (*i.e* tipo de cutícula, tipo de alimentação e história de vida) (Capítulo 2).

Caracterizar as propriedades estruturais e a flexibilidade da proteína metalotioneína MTL-1 de *Caenorhabditis elegans* com diferentes íons metálicos por meio de modelagem tridimensional e simulações de dinâmica molecular (Capítulo 3).

Avaliar como distintos metais afetam a estabilidade estrutural da MTL-1 e compreender aspectos moleculares envolvidos na tolerância e detoxificação de metais, com potencial implicação para Nematoda estuarinos (Capítulo 3).

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CAPÍTULO 1

Emergent properties of free-living nematode assemblages exposed to multiple stresses

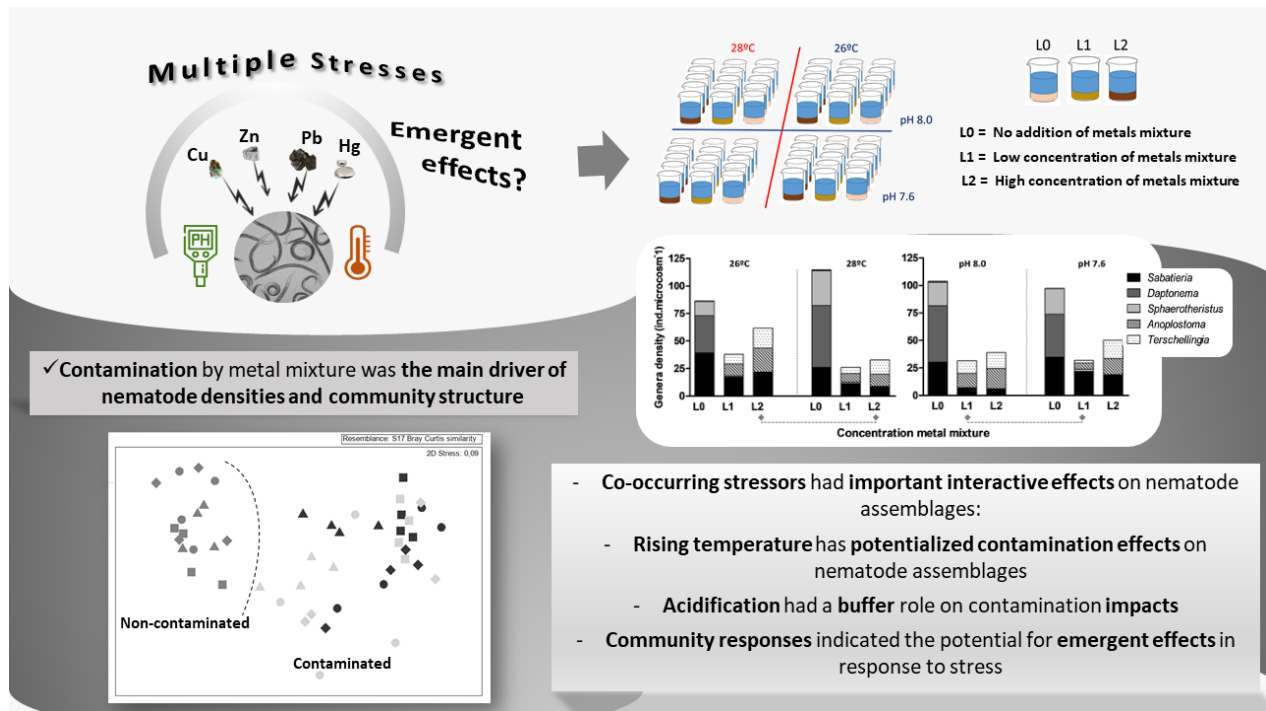
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Graphical abstract



Abstract

Biological communities are currently facing multi-stressor scenarios whose ecological impacts are challenging to estimate. In that respect, considering the complex nature of ecosystems and types and interaction among stressors is mandatory. Microcosm approaches using free-living nematode assemblages can effectively be used to assess complexity since they preserve the interactions inherent to complex systems when testing for multiple stress effects. In this study, we investigated the interaction effects of three stress factors, namely i- metallic mixture of Cu, Pb, Zn, and Hg (control [L0], low, [L1] and high [L2]), ii- CO₂ - driven acidification (pH 7.6 and 8.0), and iii- temperature rise (26 and 28 °C) on estuarine free-living nematode assemblages. Metal contamination had the greatest influence on free-living nematode assemblages, irrespective of pH and temperature scenarios. Interestingly, whilst the most abundant free-living nematode genera showed significant decreases in their densities when exposed to contamination, other, less abundant, genera were apparently favored and showed significantly higher densities in contaminated treatments. The augmented densities of tolerant genera may be attributed to indirect effects resulting from the impacts of toxicity on other components of the system, indicating the potential for emergent effects in response to stress. Temperature and pH interacted significantly with contamination. Whilst temperature rise had potentialized contamination effects, acidification showed the opposite trend, acting as a buffer to the effects of contamination. Such results show that temperature rise and CO₂-driven acidification interact with contamination on coastal waters, highlighting the importance of considering the intricate interplay of these co-occurring stressors when assessing the ecological impacts on coastal ecosystems.

Introduction

Anthropogenic activities are accelerating the degradation of coastal environments worldwide (Vikas and Dwarakish, 2015). Within this scenario, pollution and climate change are among the most serious environmental problems. The constant and accumulative input of contaminants to the environment has long been significantly impacting ecosystem functions and biodiversity (Johnston and Roberts, 2009). More recently, climate change has added to this scenario, aggravating diverse impacts to natural ecosystems, of which those caused by raises in temperature and acidification have been most often documented (Cabral et al., 2019; Hauser-Davis and Wosnick, 2022). As a result, biological communities are currently facing multi-stressor scenarios whose ecological impacts are challenging to estimate. In that respect, considering the complex nature of ecosystems and types and interaction among stressors is mandatory.

Aquatic sediments are sinks for multiple anthropogenic contaminants, particularly in estuarine systems, where complex interactions between salty water and freshwater favor the deposition of particulate matter (Simon et al., 2002). Trace metals, for example, are rapidly removed from the water column because of their sorption to suspended particles, which are predominantly fine and primarily composed of organic matter. These particles exhibit a preference for associating with metals. Subsequently, these metal-laden particles ultimately settle in sediments (Comber and Gunn, 1996; Honeyman and Santschi, 1988). Hence, many different types of metal elements are found in estuarine sediments, mainly in urban areas, often showing toxic levels for benthic organisms (De Souza Machado et al., 2016; Pan and Wang, 2012).

Despite the complex metal mixture present in estuarine sediments, the toxic effects of metallic elements on organisms are generally assessed individually (Brinke et al., 2010; Harada et al., 2007; Kang et al., 2018) and rarely combined (Hedfi et al., 2013). Nonetheless, metal mixtures can have antagonist effects (Mahmoudi et al., 2007; Fleeger et al., 2007), or manifest a competitive interaction dynamic between metals, reflecting the distinct toxicity to organisms (Das et al., 2020).

The ongoing changes in global climate patterns are adding further levels of complexity to already impacted ecosystems. The increase in greenhouse gas emissions has led to shifts in temperature and altered pH levels in aquatic environments (Fox-Kemper et al., 2021; Laffoley and Baxter, 2016). Rising temperatures have implications for species distribution (Barbarossa et al., 2021) and metabolic rates (Anestis et al., 2008), whereas acidification mainly impacts

calcifying organisms (Brennand et al., 2010) and food web dynamics (Ravaglioli et al., 2020). Since metals can undergo relevant alterations under different environmental conditions (Ann Hauser-Davis and Wosnick, 2022), which can potentially alter metal solubility and speciation, therefore changing their bioavailability, the ecological impacts caused by metal toxicity can clearly be influenced by global climate change (Stockdale et al., 2016; Zeng et al., 2015). Temperature, for instance, strongly influences the chemical transport and fate of metals (Hauser-Davis and Wosnick, 2022; Noyes et al., 2009) as well as their interplay with organisms (i.e., toxicokinetic and toxicodynamic processes) (Sokolova and Lannig, 2008). Temperature tends to increase metal uptake (Fritioff et al., 2005) and subsequent metal toxicity, particularly when the temperature approaches the upper tolerance limit of the organisms (Heugens et al., 2003).

Evidence of acidification, driven mainly by atmospheric CO₂ forces, has been reported for coastal waters (Gac et al., 2021). Even estuarine organisms adapted to wide fluctuations seem to be vulnerable to changes in ambient pH, such as bivalves and free-living nematode assemblages (Gazeau et al., 2010; Ferraz et al., 2022). Metal behavior is also sensitive to pH change. In the acidification scenario, the decreased concentration of OH⁻ and CO₃²⁻ ions can alter the solubility, adsorption, rates of redox processes, and, consequently, the toxicity of metals in estuarine environments (Millero et al., 2009). A speciation model for Cu²⁺, for example, has predicted a 115 % increase in the concentration of this metal in estuarine areas over the next 100 years as a result of the projected decrease in pH and increase in water temperature (Richards et al., 2011).

When multiple stressors act simultaneously, their combined effects can be additive, antagonistic, or synergistic. Moreover, interactions among elements of the system can exhibit complex and nonlinear patterns, wherein even minor changes in one element can have disproportionate or unpredictable effects on others (King et al., 2022). Understanding and predicting such complexity can pose challenges, particularly in highly dynamic systems. One approach to assess the results of such ecological interactions is by manipulating at the community level, not only when analyzing the composition and multivariate characteristics of communities but also by means of univariate ecological parameters. Diversity or numbers of individuals, for example, result from reproduction and social behavior, biomass, intraspecific and/or interspecific competition, and cooperation, among several other types of complex system interactions (Diamant et al., 2022; Libralato et al., 2019; Lidicker, 2008). Such community parameters can help clarify a rich system that can be foggy by the complex

interactions, which naturally are not predictable based on knowledge about individual organisms or populations alone.

To evaluate the effects of multiple stressors on a complex community, this study investigated the responses of a natural free-living nematode assemblage to simultaneous contamination by a metal mixture, CO₂-driven acidification, and temperature rise in a microcosm experiment. Microcosms are excellent options for longer-term, small-scale controlled manipulations to evaluate the impacts of multiples stressors on complex ecosystems (Benton et al., 2007). This method offers realistic responses at higher levels of biological organization (i.e., community-based) (Höss et al., 2014; Santos et al., 2018). Considering this approach, free-living nematodes are particularly advantageous because (i) species-rich assemblages are easy to maintain under laboratory conditions, (ii) they are relevant representatives of the organisms inhabiting aquatic sediments considering both abundance and diversity (Moens et al., 2013), and (iii) they play important ecological roles within benthic systems, such as nutrient cycling and serving as food resource for higher trophic levels (Schratzberger and Ingels, 2018).

Material and methods

This study is a complementary investigation to the research conducted by Altafim et al. (2023). The primary focus of their work was assessing the combined impacts of acidification, warming, and metal contamination on a meiofauna community in terms of higher taxon composition and abundance. Herein, the present study focuses on the combined impacts of the three stressors on free-living nematode assembles at the genus level. Whilst this summary provides an overview of the methodologies employed, readers can consult the original investigation by Altafim et al. (2023) for more details.

Experimental design

To evaluate the interaction effects of acidification, warming, and metal contamination on free-living nematode assemblages, a controlled microcosm experiment was set up in a fully crossed design with three fixed factors: “pH” (two levels: 8.0 and 7.6), “temperature” (two levels: 26 °C and 28 °C), and “contamination” (three levels of a mixture of Cu, Pb, Zn, and Hg) (Fig. 1). The temperature increase (+2 °C) falls within the projected range of the Representative Concentration Pathways (RCP) 4.5 by of the Intergovernmental Panel on

Climate Change - IPCC (IPCC, 2014), representing a moderate greenhouse gas trajectory leading to a global ocean surface temperature change at the end of the 21st century (2081–2100). The tested pH variation (0.4 pH unit) aligns with RCP 8.5 (Bindoff et al., 2022) and with Coupled Model Intercomparison Project Phase 6 (CMIP6) of the World Climate Research Program (Arias et al., 2021), which made predictions about potential future changes in ocean acidity. To evaluate the impact of contamination, sediment samples were spiked with a mixture of Cu, Pb, Zn, and Hg, commonly found in coastal areas worldwide (Kim et al., 2016; Qian et al., 2015). Therefore, three levels of exposure to these pollutants were established based on the sediment quality criteria (ISQG) outlined by the Canadian Council of Ministers of the Environment (CCME, 2002): control (no metals), low contamination level (L1, which corresponds to 37.5% of the threshold effect levels – TEL, ISQG), and high contamination level (L2, which corresponds to 75% of the threshold effect levels – TEL, ISQG). Each level of each factor was replicated five times; one experimental unit was kept only for chemical analysis and four for the analysis of free-living nematode assemblages. The exposure period lasted for 20 days. This duration corresponds to the period during which experimental microcosm pilot remained in a ‘healthy’ state, thereby preventing potential collapse, at the same time it allows direct and indirect community responses to be observed (Gallucci et al., 2015; Santos et al., 2018).

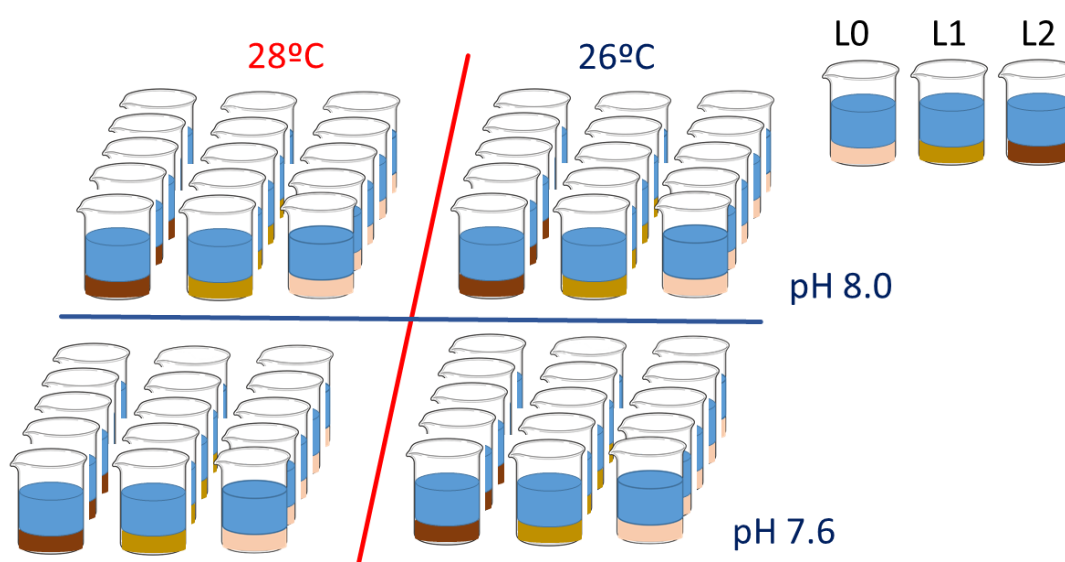


Fig. 1. Schematic representation of the experimental design. L0 = non-spiked sediments; L1 = low level of contamination tested; L2 = high level of contamination tested.

Sediment preparation

The sediment for the microcosm assay was sampled in intertidal sand and mud banks at Barra do Rio Itaguapé (Bertioga-SP) (23.763 45.773 °W), a non-urbanized and pristine area far from pollution sources, in December 2017. To ensure a better adsorption of metals to the sediment, the sediment was collected with a sand: mud ratio of 4:1 (kg/kg). The sediment was stored at 4 °C in the dark prior to use. A sample of the homogenized sediment was desiccated at 60 °C for particle size characterization (Wentworth, 1922) and the analysis of organic matter (with incineration at 500 °C) and carbonate (digestion with 0.1 M HCl) contents (Luczak et al., 1997).

Prior to spiking, all sediment samples used in the assay were defaunated as described by Schratzberger et al. (2002). Defaunated sediments were homogenized and divided into fractions for spiking with different dilutions of stock solution (or distilled water only for the contamination control). The metal stock solution was prepared by dissolving inorganic copper (CuSO_4), lead (PbCl_2), zinc (ZnSO_4) and mercury (HgCl_2) salts in distilled water. A fraction of the sediment was mixed with the stock solutions to achieve nominal concentrations representing the highest exposure level (L2): 18.7 mg kg⁻¹ for Cu, 30.2 mg kg⁻¹ for Pb, 124 mg kg⁻¹ for Zn, and 0.13 mg kg⁻¹ for Hg. The remaining stock solution was diluted to 50% and added to another portion of sediment, representing the lower exposure concentration (L1), with nominal concentrations of 9.3 mg kg⁻¹ for Cu, 15.1 mg kg⁻¹ for Pb, 62 mg kg⁻¹ for Zn, and 0.06 mg kg⁻¹ for Hg. This way, the addition of the same volume of stock solution was ensured in both contamination treatments. A separate fraction of the sediment was designated as the sediment control group. This group underwent the same treatment as the spiked sediments, with the exception that distilled water was added instead of the stock solution, using the same volume. Spiked sediment samples were manually homogenized for 15 min and stored at 4 °C in the dark for 7 days to allow the sorption of the contaminants into the sediment (ASTM, 2010). Finally, the sediments were once again thoroughly mixed and divided among the replicate samples. This means that each portion of sediment used in the experimental units was obtained by replicating a specific quantity of spiked sediment corresponding to the designated level of metal contamination.

Experimental setup

Sediments containing the free-living nematode assemblages to be used in the microcosm assays were collected in the field in December 2017. The material was gently homogenized, and a 100-g aliquot was carefully placed in the test chambers (500-mL glass beakers). Then, 250 mL of natural estuarine water (salinity 20, filtered through a 63 μm mesh) sampled in the same place where the nematode samples were obtained, was added to each test chamber. The free-living nematode assemblages were left in the microcosms for 3 days to acclimate to the tested temperature and pH levels before the addition of the contaminated sediment. This duration ensures the stabilization of the community in the microcosm, serving as a baseline for comparison in subsequent analyses (Gallucci et al., 2015). In treatments where $p\text{CO}_2$ was manipulated, the pH was gradually reduced until reaching the desired level by the third day of acclimation. Subsequently, the over laying water was drained, and 100 g of either defaunated control sediment or metal-spiked sediment was added. The sediments within the microcosms were carefully mixed to ensure homogeneity, and the test chambers were refilled with natural estuarine water, maintaining the described characteristics and adjusting the pH to the targeted level in $p\text{CO}_2$ -manipulated treatments. Throughout the experiment, the microcosms were continuously aerated and kept in the dark to prevent the growth of microalgae, as outlined by Schratzberger et al. (2002). Various physical-chemical parameters were monitored, including salinity (using an induction refractometer Shibuya, model I45), pH (seawater scale), measured by a portable pH/ORP meter (Hanna HI 9126), and temperature, using analog glass thermometers. The dissolved oxygen levels were measured at the end of the experiment using a portable oximeter (Hanna HI9146–04), whereas total alkalinity was determined using an automatic titrator (Hanna HI901C).

Since the pH was adjusted for each tested level at the beginning of the experiments, the study was conducted under static conditions without exchanging water or sediment. Real-time pH measurements showed no significant variation throughout the exposure period, indicating that there was no expected significant change in the carbonate system parameters between the beginning and end of the experiment.

CO₂-driven acidification and warming

The $p\text{CO}_2$ manipulation was achieved using a CO₂ injection system connected to the Apex Fusion® software, which allowed for the automated control of the desired pH within the microcosms with a resolution of ± 0.01 pH unit. Indirect CO₂ injection was employed by manipulating the $p\text{CO}_2$ in airtight boxes containing the experimental units, all of which were

under the same pH treatment (following Gattuso et al., 2011; Lee et al., 2017, with adaptations). The CO₂ was bubbled directly into a beaker containing distilled water, and the diffused CO₂ then permeated the atmosphere inside the box. A separate beaker, identical to the experimental units, was used for continuous pH monitoring, with an electrode connected to the Apex Fusion® system. This setup ensured greater stability in the *p*CO₂ manipulation of the test systems. At the end of the experiment, the total alkalinity of each replicate was measured to assess pH-related variations in the samples.

A room temperature of 26 °C was maintained using air conditioning, whereas the respective experimental units were kept at 28 °C by placing them in a water bath inside the boxes, with the temperature being regulated automatically by aquarium thermostats. Throughout the exposure period, the temperature was constantly monitored using analog glass thermometers.

Microcosm sampling and sample processing

After 20 days of exposure, the overlaying water in the microcosms was drained, and one replicate was stored in a plastic container for chemical analysis. The remaining four replicates were fixed with formalin (4%) for the analysis of the free-living nematode assemblages.

Carbonate chemistry analysis

At the end of the experiment, seawater samples were collected from each treatment for the quantification of carbonate chemistry parameters. Total alkalinity (TA) was quantified with the aid of an automatic titrator (Hanna HI901C) combined with a pH meter and an electrode (Hanna Instruments, ref. HI1131). Other parameters of carbonate speciation, such as the total partial pressure of CO₂ (*p*CO₂), inorganic carbon (TIC), HCO³⁻, CO₃⁻², and saturation states for calcite and aragonite (Ω_{cal} , Ω_{arag}), were estimated according to Pierrot et al. (2006) (CO2SYS program for MS Excel), using the dissociation constants of carbonic acid in seawater described elsewhere (Mehrbach et al., 1973), refitted by Dickson and Millero (1987), and KSO₄, as described in Dickson (1990).

Free-living nematode assemblage processing

Fixed sediments were washed in a 45- μ m mesh sieve, and nematodes were separated from the sediment by flotation with colloidal silica solution (Ludox®) (Heip et al., 1985). After that, all organisms were stored in formalin (4%) and stained with Rose Bengal. The samples were screened under a stereomicroscope to determine the total density of nematodes (results reported in Altafim et al., 2023). For each sample, 10% of the total nematodes was randomly picked and mounted on permanent slides for identification to genus level. Free-living nematodes were identified to the genus level using the Nemys database (Bezerra et al., 2020) and pertinent taxonomic literature (Leduc and Zhao, 2023; Brito de Jesus et al., 2023; Gagarin, 2019; Schmidt-Rhaesa, 2014; Gagarin and Thanh, 2008). If the densities were lower than 100 individuals, all nematodes were identified.

Metal analysis

Sediment samples were collected at the beginning and the end of the experiment to determine the trace metal concentrations. Sediment samples were stored in plastic containers at 4 °C and lyophilized. All glassware was acid-cleaned using 5% HNO₃ prior to the analyses. The acid digestion method was used for the analysis of Cu, Pb, and Zn, adapted from the method USEPA 3050B (USEPA, 1996). Briefly, 1 g of each lyophilized sediment sample was weighed into a 50 mL beaker, and 5 mL of HNO₃ (1:1) was added. The vessels were then covered with a glass lid and heated for 15 min (95 °C) in a laminar flow hood. Then, 2.5 mL of concentrated HNO₃ was added at 30 min intervals until the sample was completely oxidized, as indicated by the disappearance of the brown foam in the sample. The samples remained in the heating changer for 90 min. Subsequently, 1 mL of deionized water and 1.5 mL of H₂O₂ were added for the oxidation of organic matter over a period of 120 min, with regular addition of 0.5 mL of H₂O₂, without exceeding the limit of 5 mL of the solution. The extract resulting from the digestion was quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Varian equipment, model 710ES) in the Marine Inorganic Chemistry Laboratory (Oceanographic Institute of the University of São Paulo), following the USEPA 6010c methodology (USEPA, 2007).

Mercury analysis was adapted from the USEPA 7471B method for solid and semisolid waste analysis (USEPA, 1994). Triplicates of 0.6 g of sediment were weighed and stored in Falcon tubes. Subsequently, 1.7 mL of Milli-Q water and 1.7 mL of aqua regia (1 HNO₃: 3 HCl) were added to Falcon tubes and heated in a water bath at 95 °C for 2 min. Samples were cooled, and 16.4 mL of Milli-Q water and 4.9 mL of potassium permanganate (5%) were

added. Samples were heated again in a water bath for 30 min. After cooling, 2 mL of sodium chloride- hydroxylamine sulphate (12%) was added. The mercury extracted from the samples was analyzed in an ICP/OES with a coupled vapor generated accessory (VGA).

Data analysis

Initially, the effects of sediment metal contamination, temperature, pH, and their interactions on free-living nematode assemblages were evaluated using multivariate PERMANOVA (Anderson, 2001). Three fixed factors were established in a full-factorial design: (i) sediment metal contamination (three levels: Control, L1, and L2), (ii) pH (two levels: 8.0 and 7.6), and (iii) temperature (two levels: 26 °C and 28 °C). The PERMANOVA tests were conducted on Bray–Curtis similarity matrices. The residuals were permuted using unrestricted permutation of raw data. The effects of the three factors and their interactions on genus richness were also assessed by means of PERMANOVA, with the same design as for the multivariate data, but performed on a Euclidian-distance matrix. In this case, PERMANOVA was used instead of the traditional ANOVA after testing for the homogeneity of variances (Cochran's test) and obtaining significant p -values ($p < 0.05$) even after transformation. Although PERMANOVA was designed for multivariate analysis on distance matrices, it can be used for univariate ANOVA, and because it calculates p -values using permutations rather than relying on tabulated p -values, it does not have the traditional ANOVA assumptions (Anderson et al., 2008). Results were considered significant at $p < 0.05$. Post-hoc analyses were performed for the terms (isolated factors or interactions) that had a significant effect on the main test. In cases where the interaction of factors was significant, if the factors involved also presented isolated effects, only the interaction was considered for further analyses (since the effects of these factors are dependent on each other). The multivariate structure of free-living nematode assemblages was further visualized by performing non-metric multi-dimensional scaling ordination (nMDS) with the Bray–Curtis similarity measure (Clarke and Ainsworth, 1993). A SIMPER (similarity percentage analysis) (Clarke and Warwick, 1994) was performed to determine the contribution of individual genus towards dissimilarity among treatments.

Results

Metal and physical-chemical characterization

Metal analysis indicated that the sediment spiking procedure was effective, corresponding to the pre-established concentrations for exposure, in the initial times (Fig. 2; Table S1). After 20 days of exposure, a 50 % reduction in the concentration of metals was observed for all exposure conditions (Fig. 2; Table S1).

The sediment consisted of sand (1.71 % fine and 57.44% very fine), silt (40.16%), and clay (0.06%). Only a small fraction of the sediment matrix contained medium and large sand grains (0.62%). The total carbon content of the sediment was 1.64%, with 1.6% being organic matter and 0.04% being inorganic matter.

The physical-chemical factors of the water remained within the pre- established standard for the experiment, with only slight variations for DO and salinity (Table S2). The measured pH and temperature values were close to the adjusted values and showed variations among the pH factor levels. Analysis of carbonate speciation confirmed that treatments subjected to CO₂-driven acidification exhibited higher $p\text{CO}_2$ (average = 559.84 uatm) and increased concentrations of CO₂ values total inorganic carbon, and bicarbonate. Conversely, these treatments showed lower concentrations of carbonates (average = 138.10. $\mu\text{mol/Kg}$) and reduced saturations of calcite (average = 3.36) and aragonite (average = 2.08). Notable changes were not observed regarding temperature increase (Table S3). In microcosms where both temperature and pH were simultaneously altered, the average partial CO₂ pressure was lower compared to those in the treatments involving pH change alone. Consequently, the concentrations of carbonate species in these samples were higher than their respective counterparts in the pH 7.6 and temperature 26 °C treatment.

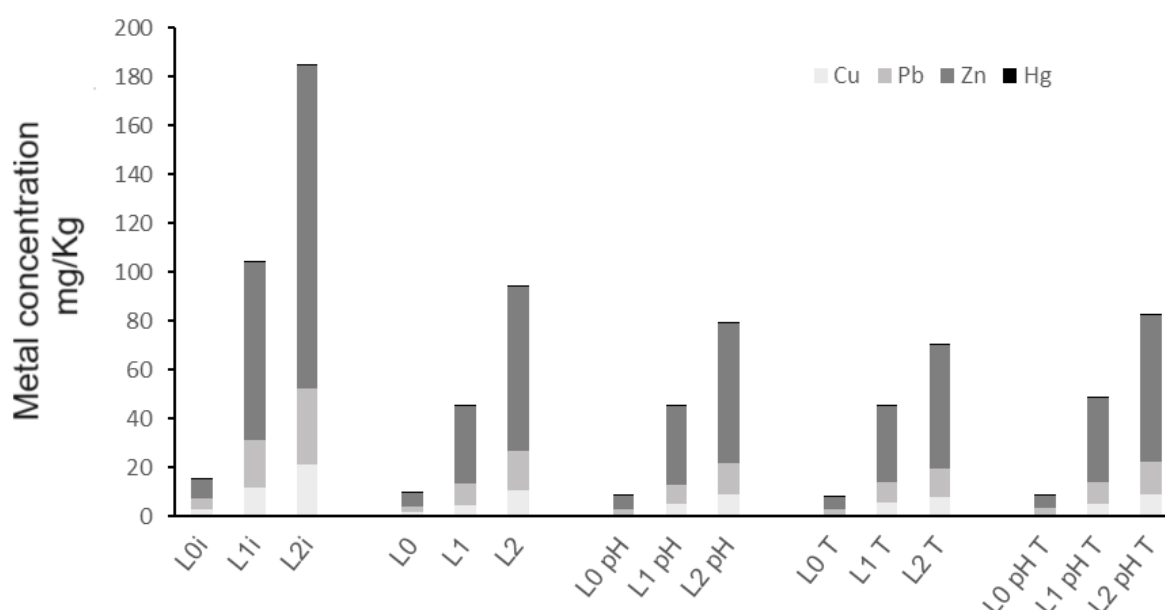


Fig. 2. Total metal concentrations in spiked sediments. L0_i, L1_i, and L2_i = initial treatments; L0, L1, and L2 = pH 8 and 26°C; L0 pH, L1 pH, and L2 pH = pH 7.6 and 26°C; L0 T, L1 T, and L2 T = pH 8 and 28°C; L0 pH T, L1 pH T, and L2 pH T = pH 7.6 and 28°C.

Free-living nematode assemblage

A total of 3684 nematode specimens were identified, which accounted for 46 genera, belonging to 24 families. Ten genera (belonging to seven families) accounted for approximately 90 % of the total abundance: *Sabatieria* (26%), *Daptonema* (19.8 %), *Anoplostoma* (11.5%), *Sphaerotheristus* (10.09%), *Terschellingia* (9.8%), *Pseudochromadora* (5.5%), *Cobbia* (2.2%), *Halalaimus* (1.8%), *Oxystomina* (1.7%), and *Cyarttonema* (1.6%). Other genera contributed each with <1 % of the total abundance.

The multivariate structure of free-living nematode assemblages showed a significant interaction between all factor pairs but not for their trifactorial combination (Table 1). Despite the observed interactions, Metal contamination clearly was the dominant driver of free-living nematode assemblage composition and multivariate structure, followed by pH and temperature (Fig. 3). Independent from the temperature and pH scenario, the contaminated treatments L1 and L2 were significantly different from the control L0 (Table 1, post-hoc).

Table 1

Results of three-way PERMANOVA and post hoc analyses for the multivariate structure of nematode assemblages from the different experimental treatments. Bold values indicate $p < 0.05$. L0: without contamination, L1: 37. 5% TEL and L2: 75% TEL.

Source	df	MS	Pseud-F	<i>P(perm)</i>
Temperature	1	4021.8	3.5834	0.003
pH	1	3343.2	2.9788	0.006
Contamination	2	28069	25.009	0.001
Temperature x pH	1	2612.4	2.3276	0.039
Temperature x Contamination	2	2224.9	1.9823	0.037
pH x Contamination	2	2362.6	2.1051	0.023
Temperature x pH x Contamination	2	1375.1	1.2252	0.239
Residue	36	1122		

Interactions	Level	Group	<i>P</i>
Temperature x pH	pH 7.6	26°C - 28°C	0.004
	pH 8.0	26°C - 28°C	0.183
	28°C	pH 7.6 - pH 8.0	0.001
	26°C	pH 7.6 - pH 8.0	0.069

Temperature x Contamination	L2	26°C - 28°C	0.004
	L1	26°C - 28°C	0.183
	L0	26°C - 28°C	0.069
	26°C	L2 - L1	0.001
		L2 - L0	0.002
		L1 - L0	0.002
	28°C	L2 - L0	0.002
		L1 - L0	0.001
		L2 - L1	0.171
pH x Contamination	L1	pH 7.6 - pH 8.0	0.021
	L0	pH 7.6 - pH 8.0	0.425
	L2	pH 7.6 - pH 8.0	0.106
	pH 7.6	L2 - L1	0.006
		L2 - L0	0.001
		L1 - L0	0.001
	pH 8.0	L2 - L0	0.001
		L1 - L0	0.001
		L2 - L1	0.485

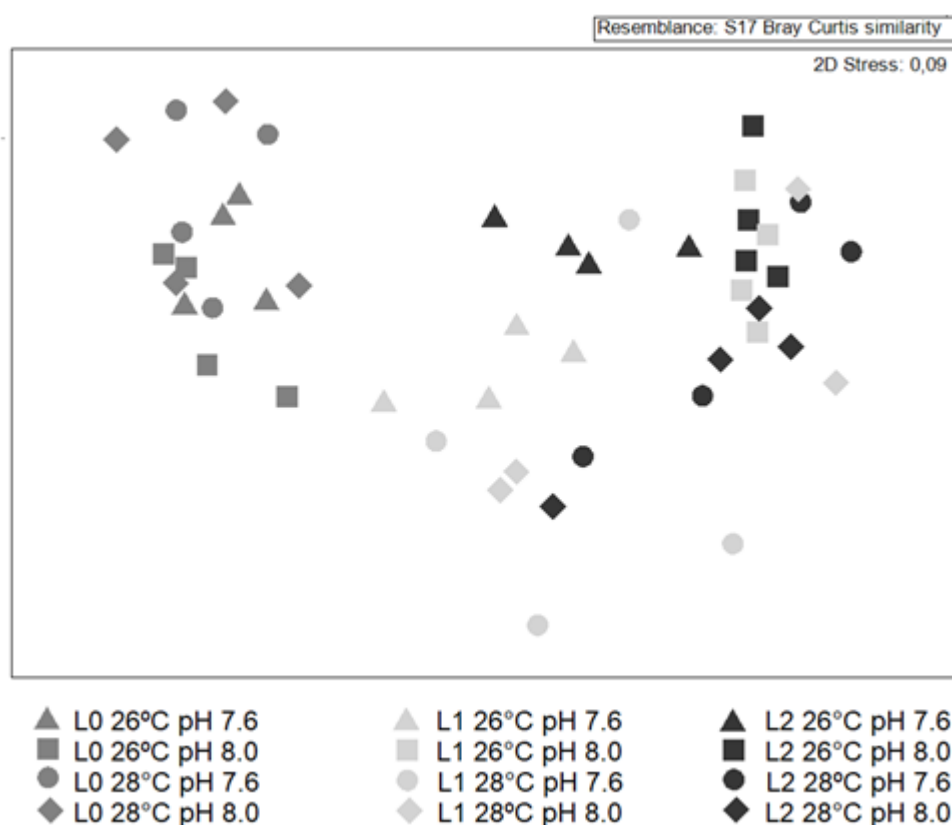


Fig. 3. Multidimensional scaling (nMDS) from free-living nematode genus data. Black-grey scale indicates different contamination treatments, and different geometric symbols indicate the temperature and pH treatments.

Simper analysis (Table S4) indicated that differences among contamination levels were mainly due to lower abundances of *Sabatieria*, *Daptonema* and *Sphaerotheristus*, followed by higher abundances of *Anoplostoma* and *Terschellingia* in contaminated treatments (Fig. 4). Variations were particularly conspicuous at L2, where *Daptonema* and *Sphaerotheristus* (dominant genera at L0) were absent and *Anoplostoma* and *Terschellingia* showed their highest densities (Fig. 4).

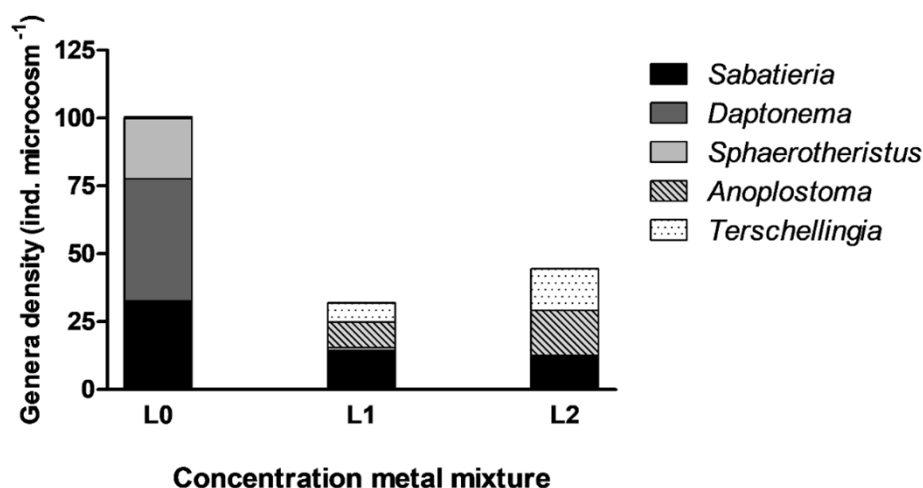


Fig. 4. Mean densities of the free-living nematode genera which were most responsible to observed differences between contamination treatments according to SIMPER analysis. L0: without contamination, L1: 37.5 % TEL, and L2: 75 % TEL.

This interplay among the densities in different free-living nematode genera resulted in differences in nematode genus richness (Table 2; Fig. 5). Significant effects were observed only for the ‘Contamination’ factor in isolation, with no significant interaction with the other two stress factors (Table 2). In this case, richness was significantly higher in the lower concentration (average = 13 genera/microcosm) of the contaminated treatment (L1) when compared to the control (L0) (average = 10 genera/microcosm) and the highest concentration (L2) (average = 10 genera/microcosm), regardless of temperature and pH (Table 1, Fig. 5). At this treatment (L1), genera that were absent at L2 were present (*Daptonema* and *Sphaerolaimus*; Fig. 5), whereas those that were absent from the controls were already present (*Anoplostoma* and *Terschellingia*; Fig. 5), resulting in a higher genus richness at this particular treatment (Table S4, Fig. 5).

Table 2

PERMANOVA and post hoc analysis for genus richness. Values in bold represent $P < 0.05$

	df	MS	Pseudo-F	P (perm)
Temperature	1	28.5	3.0	0.092
pH	1	6.0	0.65	0.441
Contamination	2	52.2	5.6	0.006
Temperature x pH	1	17.5	1.9	0.196
Temperature x Contamination	2	4.0	0.44	0.702
pH x Contamination	2	8.4	0.9	0.442
Temperature x pH x Contamination	2	19.1	2.0	0.141
Residue	36	9.2		

<i>Post hoc</i> “Contamination”	
<i>group</i>	P
L2 – L1	0.031
L1 – L0	0.010
L2 – L0	0.275

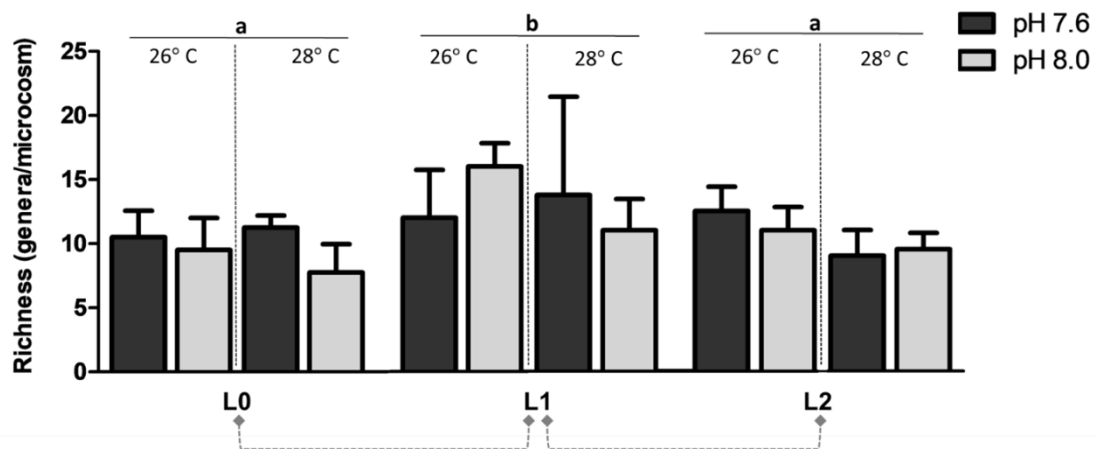


Fig. 5. Mean values ($\pm$ standard deviation) of free-living nematode genus richness for all experimental treatments. Significant differences among L0, L1, and L2 are indicated with different lowercase letters.

For the multivariate data, ‘Temperature’ and ‘pH’ showed both significant interactions with ‘Contamination’ (Table 1). For temperature and contamination interactions, significant effects of temperature were observed only for the treatments with the highest metal mixture concentrations (L2) (Table 1). In this case, SIMPER analysis revealed that the differences between 26 °C and 28 °C at L2 were due to reduced abundances (ca 50 %) of *Sabatiera*,

Anoplostoma, and *Therschelingia* at the increased temperature treatment (28 °C) (Fig. 6A). On the other side, considering the pH effects in the contaminated treatments, significant differences were observed only in L1, mainly due to higher abundances of the genus *Sabatieria*, nearly tripling in the acidified treatment (pH 7.6) in comparison to pH 8.0 (Fig. 6B, Table S4). Finally, the post-hoc test for the interaction between temperature and pH showed that significant differences occurred only at the altered treatments, i.e., differences among temperatures were significant only at pH 7.6, and differences among pH values were significant only at 28 °C. This can be mainly attributed to higher abundances of the genus *Sabatieria* at the acidified treatment, but only at 26 °C, and to higher abundances of *Daptonema* at 28 °C, but only at pH 8.0 (Table 1).

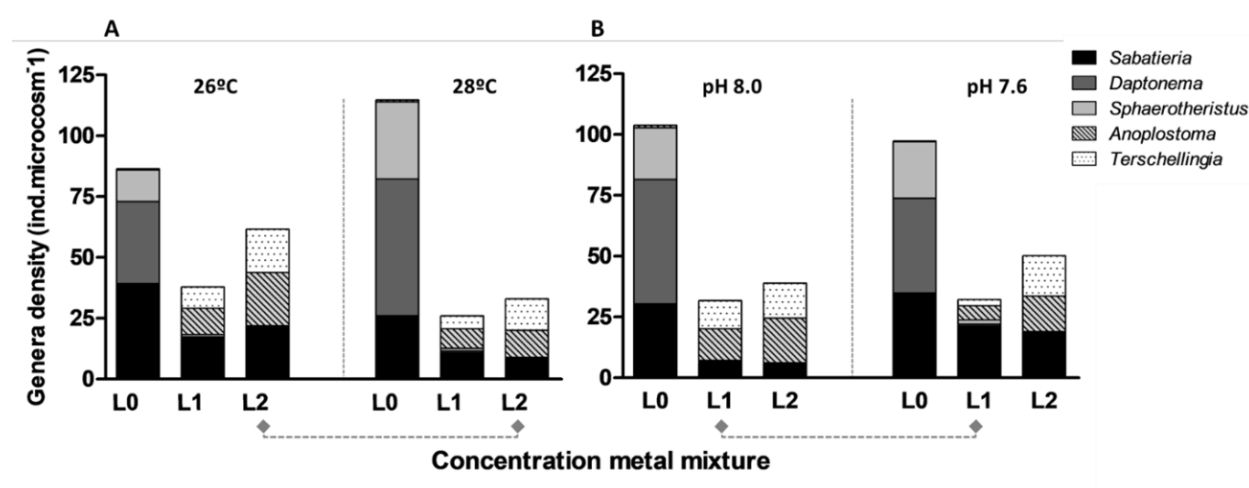


Fig. 6. Distribution of the five most dense (mean) free-living nematode genera from SIMPER analyses correspondent to (A) temperature and (B) pH interaction in different metal mixture treatments. L0: without contamination, L1: 37.5% TEL, and L2: 75% TEL. Significant differences among temperature and pH treatments are indicated by the dashed line with diamonds at the tip.

Discussion

In coastal habitats, both environmental and anthropogenic stressors often occur simultaneously. Through an analysis of the effects of the factorial combinations of three stressors (temperature, CO₂-induced acidification, and a mixture of trace metals) on a diverse assemblage of estuarine free-living nematodes, we found significant interactions among most pairs of stressors, demonstrating that co-occurring anthropogenic stressors can have important

interactive effects on natural communities. These findings emphasize the critical importance of considering the intricate interplay of multiple co-occurring stressors when assessing their impacts on coastal ecosystems. Despite the remarkable decrease in overall free-living nematode densities (Altafim et al., 2023), analysis of community composition and multivariate structure revealed diverse patterns. Whilst certain genera, which were dominant in the control assemblages, exhibited reduced densities or complete disappearance in the contaminated sediments, other less prevalent genera displayed higher densities in the contaminated setting (Tables S4). Such variable effects in response to metal contamination have previously been documented in the scientific literature. For example, in a study conducted by Hermi et al. (2009), the free-living nematode species *Araeolaimus bioculatus* became extinct in the presence of mercury, whereas the abundances of *Marylynnia stekhoveniata* and *Prochromadorella neapolitana* increased. Similarly, Brinke et al. (2011) observed a higher abundance of the genus *Daptonema* in the control treatment, but this genus disappeared entirely at the highest cadmium (Cd) treatment level. In contrast, other studies have reported an increase in the abundances of two *Daptonema* species when exposed to a combination of cobalt and zinc (Beyrem et al., 2011) as well as chromium (Boufahja et al., 2011). Similar diversified patterns have also been reported as a community response to other classes of contaminants, such as antifouling biocides (Ferraz et al., 2020) and domestic sewage (Santos et al., 2018).

Different biota can often exhibit a wide range of responses to specific toxicants as far as tolerance aspects are concerned (Guénard et al., 2011; Romero-Blanco and Alonso, 2022). In this case, sensitive species can experience negative impairment due to sublethal effects or face decimation as a result of lethality (Baird and Van den Brink, 2007). Changes in such parameter in the community may trigger ecological processes involving trophic cascades or a release from competition, which, as a consequence, may lead to positive responses in tolerant species (Carman et al., 1997). As such, whereas the decrease in the densities of certain free-living nematode genera in response to contamination observed here is likely a direct outcome of metal toxicity, the increased densities of other genera more likely stem from indirect effects arising from the impact of toxicity on other components of the system (Fleeger et al., 2003). For instance, the significant decline in the population sizes of the dominant genera reduces grazing pressure, potentially leading to an increase in the abundance and/or biomass of their food sources, mostly microalgae and bacteria. This, in turn, could have favored less abundant tolerant species that feed upon similar resources. The free-living nematode genera that showed lower densities when exposed to the metal mixture (*Daptonema*, *Sabatieria*, and

Sphaerotheristus) are classified as non-selective deposit feeders (sensu Wieser, 1953), primarily consuming bacteria and small protozoa. Likewise, those genera that showed increased abundances when exposed to contamination are also classified as selective (*Terschellingia*) and non-selective (*Anoplostoma*) deposit feeders, therefore feeding on the same food items. Apart from a potential rise in food resources, release from competition per se may also have contributed to increases in the abundances of tolerant genera, as previously suggested in other microcosm exposures with nematode assemblages (Ferraz et al., 2020; Santos et al., 2018).

Several free-living nematode genera have a high tolerance to various stressors (Ridall and Ingels, 2021). These taxa are generally characterized as colonizers or r-strategists, exhibiting rapid growth rates and reproductive capabilities (Bongers et al., 1991). This is the case of *Terschellingia*, which is characterized as “colonizer” on a scale which classifies free-living nematodes from high colonizers (= tolerant) to high persisters (= sensitive). *Terschellingia* (cp = 2, sensu Bongers et al., 1991) and, to a lesser extent, *Anoplostoma*, less opportunistic on the c-p scale (i. e., cp = 3. Sensu Bongers et al., 1991), thrive in environments with organic enrichment (Kapusta et al., 2006). This opportunistic life- history strategy may contribute to their enhanced resilience to contamination at the population level. Additionally, at the individual level, certain mechanisms associated with physiological aspects have been linked to metal tolerance in free-living nematodes. These mechanisms encompass cellular detoxification systems, such as glutathione, heat shock proteins, phytochelatin, and metallothioneins, which play a role in regulating intracellular metal levels (Hughes et al., 2009; Martinez-Finley and Aschner, 2011) protecting free-living nematodes from metal toxicity.

Although the responses of free-living nematode assemblages to contamination were rather similar at the different exposure temperatures, the contamination effects were more intense at higher temperatures, particularly for the most contaminated treatment. The decrease in nematode densities at L2 was more conspicuous at 28 °C than the decrease observed in L2 at 26 °C. This greater metal toxicity at a higher temperature is not unexpected since there is extensive evidence of increased metal toxicity with increasing temperatures (Hauser-Davis and Wosnick, 2022; Heugens et al., 2001; Laskowski et al., 2010; Sokolova and Lannig, 2008). Higher environmental temperatures can induce metal compounds to solubilize, increasing the concentration of free metal ions (Sokolova and Lannig, 2008). At the same time, higher temperatures might increase metal uptake and accumulation in aquatic organisms due to increased metabolic rates in ectotherms (Portner, 2002; Veltman et al., 2010, 2008).

For free-living nematodes, for which diet-borne exposure to contamination is particularly relevant (Ferraz et al., 2020; Offermann et al., 2009), it is likely that the increase in feeding rates may have led to an increased exposure to metal- contaminated food. Additionally, metals could be more available to nematodes due to a possible increase in metal accumulation in the microbiota as a result of the temperature rise (Laderriere et al., 2022). Together with a higher intake of these food items due to increased metabolism, this may have caused the interactive effects between trace metals and temperature rise observed in the current study.

Interestingly, the opportunistic response exhibited by the genera *Anoplostoma* and *Terschellingia* to contamination was significantly less pronounced at the higher temperature, although other nematode densities decreased the most at this scenario. A higher sensitivity of the microbiota to contamination at the higher temperature (Sun et al., 2013) might have partially counterbalanced the effects of competition release and/or rise in food resources due to increased toxic effects of metals to such living resources. Whilst *Terschellingia* primarily consumes bacteria (Vafeiadou et al., 2014), *Anoplostoma* primarily feeds on ciliates (Moenz and Vincx, 1997). Both components of the microbiota show sensitivity to metal contamination, particularly at higher temperatures (Gomiero et al., 2012; Li et al., 2020). As such, metal impacts to this biota may have contributed to a decrease in the fitness performance of both free- living nematode genera under the highest temperature condition.

Contrary to temperature rise, which has potentialized contamination effects on free-living nematode assemblages, acidification apparently buffers contamination impacts. Such an interactive effect was observed for the multivariate data, where the reduction in densities of the genera *Sabatieria* and, to a lesser extent, *Sphaerotheristus* were less pronounced at the acidified treatment (pH 7.6). This is the opposite effect to that observed for a few other coastal invertebrate species, where a relative increase in metal toxicity under acidification has been reported (Lewis et al., 2016; Roberts et al., 2013). Whilst a higher toxicity with decreasing pH values is often expected due to the predominance of free metal ions at low pH values and, therefore, higher bioavailability (Millero et al., 2009), the opposite trend (i.e., a decrease in metal toxicity with decreasing pH) might result from increased competition between the metal ions and H^+ at the organism cell surface (Franklin et al., 2000; Macfie et al., 1994). In free-living nematodes, the external cortical layer of the cuticle is predominantly composed of nematode collagen. This layer contains disulfide and sulfhydryl groups (Bird, 1971; Lee, 1966), which serve as binding sites for heavy metals. Finally, in addition to the biological interactions between contaminants and the biological cell membrane, a low pH can also weaken the strength of the metal association and prevent the retention of metals by

sediments (Belzile et al., 2004; Guven and Akinci, 2013). Consequently, this reduction in metal retention can lead to a decreased intake of organic- contaminated matter through diet-borne exposure, a significant route of contamination for free-living nematodes (Ferraz et al., 2020).

Conclusion

The results of the present study demonstrate that low to moderate levels of metal contamination in the sediment have a strong effect on free-living nematode densities and assemblage structure. Despite the overall decrease in the densities of the most abundant genera, opportunistic genera also showed a positive response to contamination, indicating that ecological impacts are rather complex. Temperature and pH interacted significantly with contamination. Whilst temperature rise had potentialized contamination effects, acidification showed the opposite trend, acting as a buffer to the effects of contamination. These results show that in coastal water, temperature rise and CO₂-driven acidification interact with contamination, highlighting the importance of considering the combined effects of these co-occurring stressors on coastal ecosystems. Additionally, community responses indicated the potential for emergent effects in response to stress, underscoring the complex interactions among the components of ecological systems. This highlights the importance of more complex approaches in ecotoxicological studies for a thorough understanding of the ecological impacts on coastal ecosystems.

Credit authorship contribution statement

Nilvea Ramalho Oliveira: Formal analysis, Investigation, Method ology, Writing – original draft. Giam Luca Altafim: Conceptualization, Investigation, Methodology, Writing – review & editing. Aline Vecchio Alves: Conceptualization, Investigation, Methodology, Writing – review & editing. Rodrigo Brasil Choueri: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, writing – original draft, Writing – review & editing, Formal analysis, Resources. Juliano Zanette: Investigation, Writing – review & editing. Rubens Cesar Lopes Figueira: Investigation, Resources, Supervision. Fabiane Gallucci: Conceptualization, Formal analysis, Methodology, Resources, Supervision, Visualization, writing – original draft, Writing – review & editing.

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

No data was used for the research described in the article.

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Supplementary data

Table S1. Metal concentrations in the microcosm (mg/Kg) for initial treatments (L0_i, L1_i and L2_i) and finals (L0, L1 and L2), with pH (8 and 7.6) and temperature (26 and 28° C) ranges.

	Conc. level	Cu	Pb	Zn	Hg
		mg/Kg	mg/Kg	mg/Kg	mg/Kg
Initial	L0	2.73	4.28	7.98	0.03
	L1	11.57	19.47	73.08	0.13
	L2	20.99	31.30	132.34	0.23
pH 8 and 26° C	L0	1.76	2.32	5.55	0.06
	L1	4.69	8.38	31.89	0.06
	L2	10.66	15.73	67.44	0.13
pH 7.6 and 26° C	L0	0.64	2.27	5.54	0.22
	L1	4.72	8.29	32.05	0.05
	L2	8.64	13.07	57.07	0.08
pH 8 and 28° C	L0	0.60	2.12	4.91	0.02
	L1	5.27	8.37	31.42	0.06
	L2	7.58	11.87	50.47	0.10
pH 7.6 and 28° C	L0	0.63	2.59	4.93	0.02
	L1	5.13	8.81	34.33	0.06
	L2	8.65	13.50	60.31	0.08

Table S2 Mean and standard deviation of the physical-chemical parameters in the different treatments.

	Concent. level	Dissolved oxygen	stand. dev.	Salinity	stand. dev.	pH	stand. dev.
pH 8 and 26° C	L0	5,70	0,14	20,20	0,45	8,31	0,05
	L1	5,89	0,32	20,20	0,45	8,18	0,01
	L2	5,85	0,12	20,20	0,84	8,25	0,03
pH 7,6 and 26° C	L0	5,35	0,08	20,20	0,00	7,52	0,05
	L1	5,31	0,45	20,00	0,00	7,50	0,05
	L2	4,99	0,18	20,00	1,22	7,55	0,03
pH 8 and 28° C	L0	4,91	0,08	18,00	0,74	8,21	0,06
	L1	5,10	0,52	19,00	3,49	8,14	0,10
	L2	4,99	0,55	18,20	1,10	8,04	0,11
pH 7,6 and 28° C	L0	5,21	0,09	17,80	1,10	7,64	0,04
	L1	5,14	0,27	17,80	0,45	7,60	0,03
	L2	4,90	0,34	20,20	0,84	7,57	0,06

Table S3. Total alkalinity and composition of carbonate species in the water samples of the tests (standard deviation) for the different treatments. TA = Total alkalinity, CiT = Total inorganic carbonic, $p\text{CO}_2$ = partial pression of CO_2 , bicarbonate (HCO_3^-) levels, carbonate levels (CO_3^{2-}), carbon dioxide concentrations (CO_2), and the saturation states of calcite (Ω_{Ca}) and aragonite (Ω_{Ar}).

	pH8 + T26			pH8 + T28			pH7,6 + T26			pH7,6 + T28		
	L0	L1	L2	L0	L1	L2	L0	L1	L2	L0	L1	L2
TA ($\mu\text{mol/kg}$)	1823 (163,44)	1455 (22,12)	1601 (108,03)	1772 (188,52)	1482 (120,13)	1324 (50,05)	2034 (216,39)	1721 (120,13)	1861 (99,54)	1831 (92,69)	1513 (39,76)	1417 (54,49)
CiT ($\mu\text{mol/kg}$)	1613 (123,14)	1330 (18,38)	1448 (77,01)	1467 (224,10)	1341 (128,97)	1249 (46,97)	1998 (217,73)	1703 (128,97)	1840 (109,67)	1743 (76,79)	1482 (31,34)	1393 (60,56)
$p\text{CO}_2$ (μatm)	257,60 (41,33)	346,91 (37,83)	340,17 (46,22)	181,38 (108,71)	292,26 (269,18)	521,79 (85,26)	1257,33 (222,97)	1278,53 (269,69)	1261,13 (248,91)	568,42 (115,84)	712,82 (130,40)	971,82 (165,49)
HCO_3^- ($\mu\text{mol/kg}$)	1446 (93,62)	1227 (17,27)	1324 (55,90)	1237 (327,48)	1230 (123,69)	1175 (44,71)	1903 (207,12)	1622 (123,69)	1754 (104,38)	1644 (71,05)	1413 (30,78)	1326 (58,58)
CO_3^{2-} ($\mu\text{mol/kg}$)	158,50 (37,45)	92,04 (5,40)	113,79 (23,75)	223,73 (144,46)	102,46 (2,38)	59,54 (8,07)	56,48 (5,91)	41,52 (2,38)	44,92 (4,10)	79,58 (19,55)	41,49 (12,43)	36,36 (6,19)
CO_2 ($\mu\text{mol/kg}$)	8,23 (1,46)	10,35 (0,82)	10,04 (1,37)	5,98 (3,56)	9,15 (7,39)	15,09 (2,27)	38,85 (6,73)	39,09 (7,40)	41,20 (7,63)	19,67 (3,37)	27,49 (5,88)	30,33 (5,78)
Ω_{Ca}	4,26 (1,02)	2,49 (0,13)	3,08 (0,64)	5,97 (3,84)	2,76 (0,06)	1,63 (0,22)	1,55 (0,17)	1,14 (0,06)	1,21 (0,11)	2,15 (0,52)	1,13 (0,36)	0,98 (0,16)
Ω_{Ar}	2,64 (0,63)	1,55 (0,08)	1,93 (0,40)	3,68 (2,36)	1,71 (0,03)	1,02 (0,14)	0,95 (0,10)	0,70 (0,03)	0,74 (0,07)	1,31 (0,33)	0,68 (0,23)	0,61 (0,10)

Table S4. Results of similarity percentage analysis (SIMPER) and the dissimilarity, from significative pairwise ($p < 0.05$) between temperature, acidification and metal mixture contamination levels. Nematode genera are ranked in order of importance of their contribution. More abundant (+); less abundant (-); similar abundance (=) and eliminated ($\emptyset$).

Temperature x pH			
Level	Group		Accumulated contribution (%)
pH 7.6	28° C	26° C	67
	Sabatieria (-)	Sabatieria (+)	28,70
	Daptonema (+)	Daptonema (-)	47,64
	SpHaerotheristus (+)	SpHaerotheristus (-)	60,36
	Therschellingia (-)	Therschellingia (+)	68,67
	Anoplostoma (-)	Anoplostoma (+)	76,58
pH 8.0	28° C	26° C	69
	Daptonema (-)	Daptonema (+)	24,74
	Sabatieria (=)	Sabatieria (=)	42,63
	Anoplosotma (+)	Anoplosotma (-)	54,28
	Terschellingia (+)	Terschellingia (-)	65,13
	SpHaerotheristus (+)	SpHaerotheristus (-)	75,96
26° C	pH 7.6	pH 8.0	64
	Sabatieria (+)	Sabatieria (-)	31,91
	Daptonema (=)	Daptonema (=)	48,51
	Anoplostoma (+)*	Anoplostoma (-)	59,21
	Therschellingia (+)	Therschellingia (-)	68,39
	SpHaerotheristus (-)	SpHaerotheristus (+)	76,45

Temperature x Contamination			
Level	Group		
L2	28° C	26° C	52
	Sabatieria (-)	Sabatieria (+)	25,65
	Anoplostoma (-)	Anoplostoma (+)	51,0
	Therschellingia (-)	Therschellingia (+)	71,34
	Pseudochromadora (-)	Pseudochromadora (+)	77,39
	Tylechida (+)	Tylenchida (-)	80,48
28	L2	L0	92
	Daptonema ($\emptyset$)	Daptonema (+)	39,55
	SpHaerotheristus ($\emptyset$)	SpHaerotheristus (+)	61,05
	Sabatieria (-)	Sabatieria (+)	79,01
	Therschellingia (+)	Therschellingia ($\emptyset$)	83,03
	Cobbia ($\emptyset$)	Cobbia (-)	86,03
28	L1	L0	86
	Daptonema (-)	Daptonema (+)	41,49

	SpHaerotheristus (-)	SpHaerotheristus (+)	63,89
	Sabatieria (-)	Sabatieria (+)	81,10
	Cobbia (-)	Cobbia (+)	84,45
	Anaplostoma (-)	Anaplostoma (+)	87,50
26	L1	L2	50
	Sabatieria (-)	Sabatieria (+)	28,55
	Anoplostoma (+)	Anoplostoma (-)	48,93
	Therschellingia (+)	Therschellingia (-)	66,4
	Pseudochromadora (-)	Pseudochromadora (+)	72,48
	Viscosia (-)	Viscosia (+)	75,22
	Halailamus (=)	Halailamus (=)	
26	L2	L0	83
	Daptonema (elim)	Daptonema (+)	25,81
	Sabatieria (-)	Sabatieria (+)	50,19
	Anoplostoma (+)	Anoplostoma (-)	61,91
	SpHaerotheristus (Ø)	SpHaerotheristus (+)	72,60
	Therschellingia (+)	Therschellingia (Ø)	84,42
26	L1	L0	79
	Daptonema (Ø)	Daptonema (+)	29,49
	Sabatieria (-)	Sabatieria (+)	56,38
	SpHaerotheristus (-)	SpHaerotheristus (+)	68,18
	Anoplostoma (+)	Anoplostoma (-)	74,41
	Therschellingia (+)	Therschellingia (Ø)	78,78
Contamination x pH			
Level	Group		
L1	pH 7.6	pH8.0	63
	Sabatieria (+)	Sabatieria (-)	27,32
	Therschellingia (-)	Therschellingia (+)	42,71
	Anoplostoma (-)	Anoplostoma (+)	56,59
	Pseudochromadora (-)	Pseudochromadora (+)	63,68
	Halailamus (=)	Halailamus (=)	66,54
pH 7.6	L1	L2	61
	Sabatieria (=)	Sabatieria (=)	28,75
	Therschellingia (+)	Therschellingia (-)	50,23
	Anoplostoma (+)	Anoplostoma (-)	65,01
	Pseudochromadora (+)	Pseudochromadora (-)	71,45
	Viscosia (elim)	Viscosia (+)	74,26
pH 7.6	L2	L0	82
	Daptonema (elim)	Daptonema (+)	29,75
	Sabatieria (-)	Sabatieria (+)	52,03
	SpHaerotheristus (Ø)	SpHaerotheristus (+)	69,98
	Therschellingia (+)	Therschellingia (Ø)	76,97

	Anoplostoma (+)	Anoplostoma (Ø)	83,30
pH 7.6	L1	L0	77
	Daptonema (-)	Daptonema (+)	33,6
	Sabatieria (-)	Sabatieria (+)	57,14
	SpHaerotheristus (-)	SpHaerotheristus (+)	77,06
	Cobbia (+)	Cobbia (-)	81,89
	Anoplostoma (-)	Anoplostoma (+)	84,75
pH 8.0	L2	L0	92
	Daptonema (Ø)	Daptonema (+)	36,1
	Sabatieria (-)	Sabatieria (+)	56,8
	SpHaerotheristus (Ø)	SpHaerotheristus (-)	71,21
	Anoplostoma (+)	Anoplostoma (-)	78,88
	Therschellingia (+)	Therschellingia (Ø)	85,03
pH 8.0	L1	L0	90
	Daptonema (-)	Daptonema (+)	36,99
	Sabatieria (-)	Sabatieria (+)	57,94
	SpHaerotheristus (Ø)	SpHaerotheristus (+)	72,66
	Anoplostoma (+)	Anoplostoma (-)	78,36
	Therschellingia (+)	Therschellingia (Ø)	83,84

CAPÍTULO 2

Taxonomic distinctness and functional traits convergence reveal complex nematode responses to metal stress

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Abstract

Metal contamination acts as a powerful ecological filter in estuarine ecosystems, yet the relative contributions of taxonomic relatedness and functional convergence to benthic community responses remain poorly understood. In this study, we investigate how metal contamination influences the structure of estuarine nematode assemblages by integrating taxonomic distinctness metrics and functional trait analysis, testing whether changes in assemblage structure were primarily driven by lineage-level sensitivities and/or by functional convergence. We conducted a microcosm experiment with two metal mixture concentrations (low level [L1] and high level [L2]). Our results showed that metal exposure led to increased taxonomic distinctness without substantial major genus loss, suggesting reorganization along taxonomic hierarchies rather than full turnover. This pattern was most pronounced at L1 treatment, where richness and dispersion increased, consistent with the Intermediate Disturbance Hypothesis. Conversely, diversity was reduced at L2 possibly due to more intense metal filtering. Functional composition also shifted markedly under contamination with convergence in life-history strategies, feeding types, and cuticle features of nematodes. Unexpectedly, certain traits typically associated with sensitivity, such as high c-p value and smooth cuticle, became more prevalent under contaminated treatments, highlighting the complexity of trait-environment relationships. These findings suggest that both taxonomic filtering and functional convergence jointly shape nematode responses to metal stress, although not always in predictable ways. Overall, this study underscores the importance of integrating taxonomic structure with functional traits to better understand the ecological consequences of pollution in benthic systems.

Key-words: estuarine free-living nematode, metal mixture, microcosm, taxonomic hierarchies, functional convergence

Introduction

Pollution is one of the major drivers of current biodiversity loss (Sigmund *et al.* 2023). Among the ecosystems affected, estuarine regions are particularly vulnerable due to their role as interfaces between terrestrial and marine environments (Chapman & Wang 2001). These regions often receive large quantities of contaminants, which are deposited in the sediments and can accumulate to high concentrations over time (Ho & Burgess 2013). Metals, for example, are commonly found in estuarine sediments (Wolanski & Elliot 2016), posing significant risks to local biodiversity. Their effects on biological benthic community are therefore very concerning in terms of ecotoxicological risk (Luoma & Rainbow 2005).

The environmental filters imposed by metal contamination on biological communities can alter their composition and ecological dynamics. Because sensitivity or tolerance to contaminants is often phylogenetically conserved across taxonomic groups, responses to this exposure may follow a pattern of taxonomic relatedness (Hammond *et al.* 2012). In this case, closely related species tend to exhibit similar sensitivities (Malaj *et al.* 2016). For instance, diatoms exhibit varying sensitivities to herbicides depending on their taxonomic classification: higher sensitivity has been observed in species from the orders Thalassiosirales and Fragilariales, while more resistant species are typically found in Cymbellales, Naviculales, and Bacillariales (Larras *et al.* 2014). Likewise, in groups such as chironomids and mayflies, a strong phylogenetic has been detected in their responses to pollution (Carew *et al.* 2011). This pattern has been also observed in other taxa, including fungal (Mikryukov *et al.* 2020) and amphibian (Chiari *et al.* 2015) communities, indicating that sensitivity to contamination tend to be phylogenetically conserved across a wide range of taxonomic groups.

On the other hand, environmental filtering can also promote a functional convergence, by decreasing or eliminating species with sensitive traits to contamination (Justino *et al.* 2023; Kuzmanovic *et al.* 2017; Moreno *et al.* 2011; Semprucci & Balsamo 2014). Under these conditions, unrelated species may develop or retain similar functional traits as a result of selective pressures imposed by pollution, favoring organisms with adaptive characteristics that confers tolerance to environmental contamination. Such characteristics can be associated with different biology of the taxa, for instance, feeding strategy, life strategies or morpho-functional traits (Alves *et al.* 2014; Armenteros *et al.* 2009; Schratzberger *et al.* 2007). Ecological studies based on functional traits suggest that species sharing such characteristics tend to perform similar ecological functions, supporting the idea of functional convergence under environmental pressures (Li & Shipley 2018; Nie *et al.* 2016). Thus, while phylogenetic constraints may shape species-specific sensitivities to contaminants, environmental filtering can drive functional

convergence by favoring traits associated with tolerance. These processes can interact to shape the community responses to contamination and the extent to which mechanisms predominate may vary among taxonomic groups.

Investigating these patterns in diverse and ecologically relevant organisms is crucial for understanding the mechanisms through which metal pollution affects benthic communities. In this context, nematodes – as free-living meiobenthic organisms – are especially valuable for studying responses to metal contamination due to their high diversity and variation in sensitivity to pollutants (Gyedu-Ababio *et al.* 1999; Höss *et al.* 2011). As the most abundant metazoans in sediments ecosystems (Heip *et al.* 1985), nematodes exhibit a wide array of life-history strategies. Studies conducted both in laboratory settings (e.g., microcosm (Boufahja *et al.* 2011; Haegerbaeumer *et al.* 2018), single species assays (Monteiro *et al.* 2018) and in the field (Danovaro *et al.* 1995) have revealed differential sensitivities to metal exposure among estuarine nematodes.

In this study, we examine the taxonomic relatedness and functional traits of nematode assemblages exposed to metal contamination. Given the challenges of constructing robust phylogenies for this taxonomically diverse group, we employed Taxonomic Distinctness (TD) as a proxy for phylogenetic proximity. Our main objective is to assess whether nematode responses to metal stress are influenced by shared taxonomic lineage and/or driven by functional trait convergence. We hypothesize that TD decreases with increasing metal contamination, reflecting the dominance of more closely related taxonomic groups and suggesting a homogenization of assemblage taxonomic structure. Conversely, assemblages exposed to lower levels or no metal contamination are expected to show higher TD, indicating the presence of species from more distantly related taxonomic lineages and a more taxonomically diverse and distinct assemblage structure. Simultaneously, we expect that functional trait convergence may arise under selective pressure from metal contamination, favoring traits that confer tolerance.

Material and Methods

The data used to test the hypotheses from this study was retrieved from an experiment in which a free-living nematode assemblage was exposed to two concentrations of a metal mixture (L1= low and L2= high concentration) (Oliveira *et al.* 2024). The experiment was conducted using controlled microcosms with a mixture of Cu, Pb, Zn and Hg. The nominal concentration representing the L1 were: 9.3 mg kg⁻¹ for Cu, 15.1 mg kg⁻¹ for Pb, 62 mg kg⁻¹ for Zn and 0.06 mg kg⁻¹ for Hg. For L2, the concentrations were 18.7 mg kg⁻¹ for Cu, 30.2 mg kg⁻¹ for Pb, 124 mg kg⁻¹ for Zn and 0.13 mg kg⁻¹ for Hg. The experiment ran for 20 days under varying

temperature and pH conditions. For the present study, we analysed data from treatments conducted at 26°C and pH 8.0, focusing solely on the factor “contamination,” which included three levels: control, L1, and L2. For methodological details, refer to Oliveira et al. (2024).

To assess phylogenetic relationships, we used taxonomic distinctness (TD) following Warwick & Clarke (1998). This approach was chosen because nematode phylogenetic relationships remain complex and still under development (Ahmed & Holovachov 2021) complicating the construction of robust phylogenetic trees for all lineages. Moreover, the identification of nematodes was conducted up to genus level. TD, based on hierarchical Linnaean taxonomic, offers insights into how environmental stressors selectively affect lineages and alter taxonomic composition (Fu *et al.* 2012; Liu *et al.* 2016). We evaluated four TD metrics: 1) quantitative taxonomic distinctness (Δ) and 2) presence/absence taxonomic distinctness (Δ^+), which reflects shifts in average taxonomic distances and indicate if related taxa respond similarly to contamination; 3) total taxonomic distinctness ($S\Delta^+$), measuring overall taxonomic breadth and potential loss of deeper lineages, and 4) variation in taxonomic distinctness (Δ^+), assessing structural variability and indicating whether assemblage responses are uniform or skewed toward certain taxa. We also tested for differences in genus richness (S) across treatments. All statistical analyses, including ANOVA and Tukey's HSD post hoc test were performed using R software (R Development Core Team 2009).

For functional investigation, we selected three morpho-functional traits which have previously shown evidence of response to stress (Mitwally & Fleeger 2016; Semprucci *et al.* 2022): cuticle morphology, feeding mode and life history, encompassing 13 traits categories (see Table 1). For the analysis, we followed the procedure describe by Bremner *et al.* 2003, 2006. Two matrices were constructed: one with taxon abundance per replica and another detailing each taxon's traits. Traits were coded using the fuzzy coding method (Chevent *et al.* 1994) where each category received a score of 0 (no affinity) and 3 (full affinity) based on literature (Bongers 1990; Bongers *et al.* 1991; Soetaert *et al.* 1995; Wieser and Kanwisher 1960, Wieser, 1957). These matrix were combined by multiplying each taxon's trait scores by its abundance, generating a final matrix of trait-weighted abundance. The resulting data were log (x+1) transformed and analyzed using PERMANOVA with the vegan package (Oksanen *et al.* 2012). Where significant effects were found, pairwise comparisons were performed using the *adonis2* function with 999 permutations.

Additionally, to identify traits significantly associated with each contamination treatment, we adapted the Indicator Species Analysis using *indicspecies* package (De Cáceres & Legendre, 2009), treating each trait as an indicator variable. Significance was tested via the r.g. statistic and permutation test. Finally, a two-dimensional nMDS plot based on Euclidean distances was

created using *ggplot2* (Wickham 2016) to visualize the functional traits structure across treatments.

Table 1. Functional traits used for nematode assemblages.

Traits (abbreviation)	Categories
Cuticle morphology (C)	1. Smoot cuticle (SC) 2. Striated (S) 3. Covered with dots or annulated (CDA) 4. Ornamented (O)
Feeding type (FT)	1. Selective deposit feeders (1A) 2. Non-selective deposited feeders (1B) 3. Epistrate feeders (2A) 4. Predator and omnivore (2B)
Life-history strategies (c-p value)	1. Extreme colonizers 2. Colonizers 3. Intermediary 4. Persisters 5. Extreme persisters

Results and Discussion

Taxonomic distinctness

Impacts of metal contamination was evident for both quantitative taxonomic distinctness (Δ) and total taxonomic distinctness ($S\Delta^+$) (Table 2). Quantitative taxonomic distinctness was significantly higher in contaminated treatments (both L1 and L2) when compared to the control, indicating that nematode genera in contaminated treatments were taxonomically more distant than in the control. In contrast, the other two parameters, Δ^+ (presence/absence-based distinctness) and Λ^+ (variation in distinctness), did not differ significantly among treatments (Table 2). This pattern suggests that changes in taxonomic structure were not primally driven by complete turnover in genera composition but rather shifts in the relative abundance of taxa. The stability of Δ^+ and Λ^+ implies that most genera remained present across treatments, although their relative contributions to the assemblage structure changed substantially.

These results reflect two distinct processes: I) selective loss of closely related genera, and/or increase in densities of genera belonging to more distant lineages. Particularly at the low concentration treatment (L1), higher Δ , $S\Delta^+$, and taxonomic richness (S), suggests the

recruitment of taxa from broader taxonomic groups, contributing to greater taxonomic dispersion (Table 2, Fig. 1). In contrast, the L2 treatment, which had the highest metal concentration, did not exhibit the same level of richness and taxonomic spread as L1. The significant difference between L1 and L2 ($p = 0.027$; Table 1) suggest that the increase in taxonomic dispersion observed at lower contamination levels did not persist at higher concentrations, likely due to the stronger pressure imposed by higher metal toxicity, which prevented recruitment. This phenomenon aligns with the “Intermediate Disturbance Hypothesis” (IDH) (Connell 1978; Grime 1973; Kautsky & Kautsky 1989), where intermediate levels of disturbance (such L1) can promote higher diversity by controlling the abundances of dominant taxa, whereas stronger disturbances (such L2) may lead to decline in diversity due to the inability of certain genera to persist. In our dataset, the increase of diversity in L1 can be attributed to the emergence of four nematode families which were absent in the control treatment (Selachinematidae, Microlaimidae, Aegialoalaimidae and Haliplectidae; Fig. 1B). Particularly, for nematodes, evidence supporting the consistency of IDH has been reported in various studies addressing sediment disturbance (Armonies & Reise 2000; Gheskiere *et al.* 2004; Mundo-ocampo & Rocha-olivares 2010; Schratzberger *et al.* 2009).

Although we did not use explicit phylogeny, the hierarchical structure of taxonomic classification used in TD metrics serves as a meaningful proxy for evolutionary divergence (Bevilacqua *et al.* 2021; Ellingsen *et al.* 2005; Roque *et al.* 2014). As such, these observed patterns do not corroborate our hypothesis that taxonomically similar genera would respond similarly to metal exposure. The significant changes in quantitative taxonomic distinctness (Δ) between control and contaminated treatments indicate that metal stress reorganized the abundance of taxa along the taxonomic hierarchy without eliminating many genera. Indeed, only few genera present in L0, such as *Chromadora*, *Chromadorita*, and *Throcamus* (all Chromadoridae) (see Fig. 1), were not detected in L2, suggesting that some lineages were particularly sensitive. Conversely, some genera like *Campylaimus* and *Monhystera* were observed exclusively in L2, possibly reflecting recruitment of taxa potentially better adapted to metal-contaminated conditions. This pattern supports the idea that while overall genus richness was maintained, shifts in dominance and presence at different taxonomic levels occurred in response to metal stress.

Table 2. Outputs of the ANOVA and post-hoc analysis of Taxonomic distinctness indices (TD) and richness (S) parameters. TD values are presented as mean. Significant differences ($P < 0.05$) are indicated in bold.

Treatment	Δ	$S\Delta+$	S	$\Lambda+$	$\Delta+$
L0	50.55	747.91	9.50	279.99	79.05
L1	71.22	1175.97	14.25	343.42	81.04
L2	67.14	867.68	10.50	412.1	82.71

Taxonomic distinctness parameters	df	MS	F value	<i>p valor</i>
Quantitative taxonomic distinctness (Δ)	2	479.4	28.11	0.0001
Total taxonomic distinctness ($S\Delta+$)	2	195080	10.32	0.004
Richness (S)	2	50.17	8.439	0.008
Variation in taxonomic distinctness ($\Lambda+$)	2	17460	3.161	0.091
Presence/absence taxonomic distinctness ($\Delta+$)	2	13.474	2.94	0.104

<i>Pos hoc</i>	Group	<i>p valor</i>
Quantitative taxonomic distinctness (Δ)	L1 - L0	0.0001
	L2 - L0	0.0007
	L2 - L1	0.104
Total taxonomic distinctness ($S\Delta+$)	L1 - L0	0.004
	L2 - L0	0.465
	L2 - L1	0.027
Richness (S)	L1 - L0	0.009
	L2 - L0	0.700
	L2 - L1	0.032

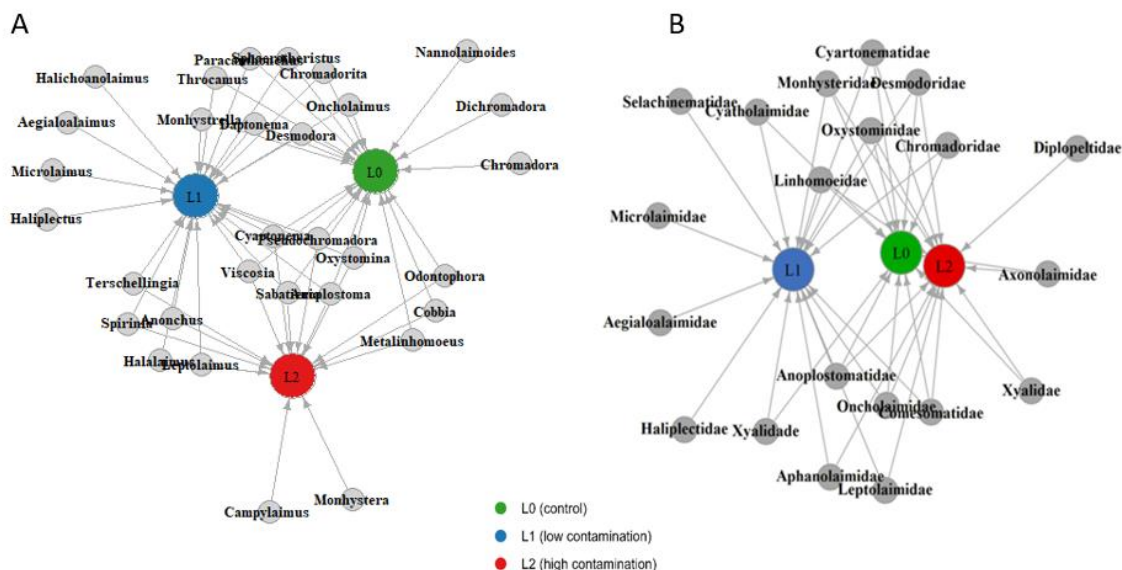


Figure 1. Taxonomic network of nematode assemblages exposed to metal contamination. A) Nematodes organized at genus level and B) Nematodes organized at family level.

Functional trait analysis

Concerning the functional composition of nematode assemblages, the analysis revealed a significant effect of contamination (Table 3A; Fig.2). The partial overlap between L1 and L2 in the nMDS plot indicates that both treatments under metal stress shared a distinct set of similar functional traits, which was clearly distinct from the control (L0) (Fig. 2). These results indicate functional convergence under contaminated conditions, corroborating our second hypothesis that functional trait convergence could occur as result of selective pressures imposed by metal contamination.

Table 3. Functional traits analysis. A) PER-MANOVA results and post hoc output, showing the effects of treatments on functional traits. B. Indicator trait analysis using r.g. statistic with permutation test (*indicspecies* package, in R). Significant values ($p < 0.05$) are highlighted in bold.

A. PER-MANOVA summary				
Treatment	df	MS	F value	<i>p valor</i>
Metal contamination	2	56.021	6.719	0.001
Residual	9	37.25		
Total	11	93.541		
<i>Pos hoc</i>				
Group		<i>p valor</i>		
L1 - L0		0.021		
L2 - L0		0.023		
L2 - L1		0.182		

B. Indicator traits per treatment			
Treatment	Functional traits	IndVal (stat)	<i>p valor</i>
L0	cp2 life-history	0.979	0.01
	striated cuticle	0.932	0.01
	1B feeding type	0.928	0.01
L1 + L2	cp4 life-history	0.930	0.01
	cp3 life-history	0.920	0.01
	smooth cuticle	0.892	0.01
	Dotted/annulated cuticle	0.855	0.01
	1A feeding type	0.855	0.01

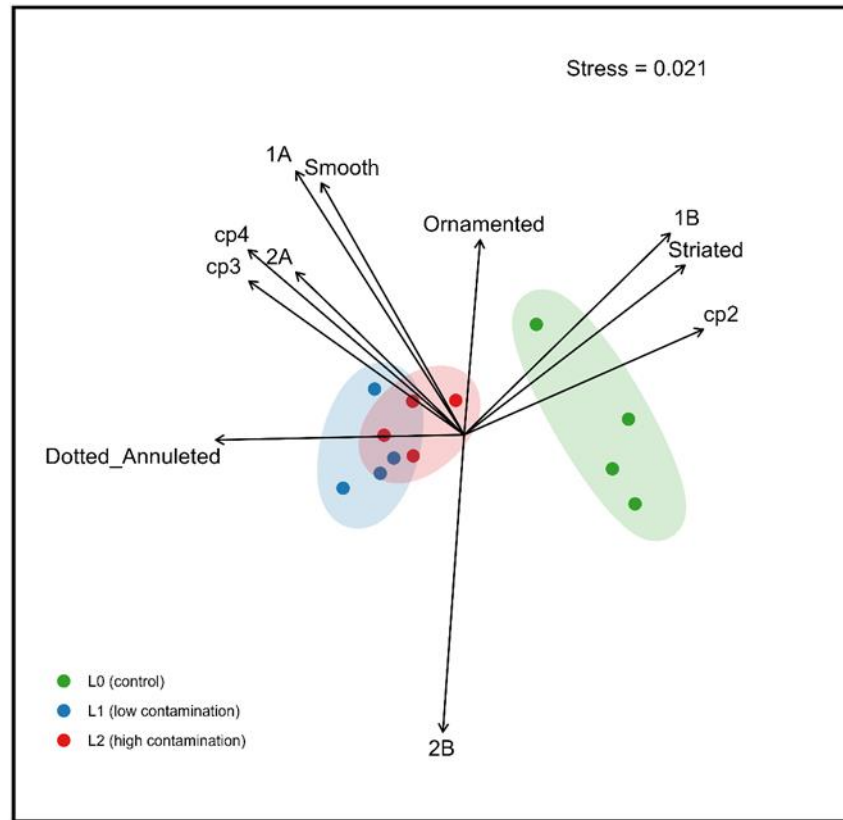


Figure 2. Multidimensional scaling (nMDS) of categories from functional traits of nematodes exposed to metal mixture (Cu, Pb, Zn, and Hg). Feeding type: selective deposit feeders (1A); non-selective deposited feeders (1B); epistrate feeders (2A) and predator and omnivore (2B). Life- history strategies: colonizers (cp2); intermediary (cp3) and persisters (cp4). Cuticle morphology: smooth cuticle; striated; covered with dots or annulated and ornamented.

Among the functional traits that potentially involved in trait convergence, cuticle characteristics have garnered particular attention due to their ecological significance in mediating stress tolerance (Raes *et al.* 2007; Semprucci *et al.* 2018). The relationship between cuticle and functional role it has been highlighted by studies showing that variations in the cuticle permeability and morphology are linked to differences in tolerance to contamination among nematodes species (Kammenga *et al.* 1994). In general, less ornamented cuticles are considered more permeable (Fonseca & Fehlaue-Ale 2012) whereas highly ornamented cuticles may act as a barrier against contaminants (Eyualet-Abebe *et al.* 2006). Contrary to these expectations, our study found that smooth cuticle was associated with the highest metal contamination (L2 treatment) (Table 3 B; Fig. 2). Although no definitive explanation to this pattern is currently available, it challenges the assumptions that external ornamentation always confers greater protection.

The functional role of ornamentation under condition environmental stress remains a subject of debate (Justino *et al.* 2023; Semprucci *et al.* 2022), and most existing research has focused more on the biochemical composition and sensory functions of the cuticle rather than on its external morphology (Basyoni & Rizk 2016; Davies & Curtis 2011; Song *et al.* 2014). The nematode cuticle, primarily composed of structural proteins, serves a dual role: it acts both as physical barrier and as a sensory interface with the environment, capable of triggering stress and immune responses when disrupted (Dodd *et al.* 2018). Although studies directly linking nematode cuticle ornamentation to environmental conditions are still scarce, recent findings point to potentially meaningful ecological associations. For instance, habitat with elevated organic matter – often associated with organic pollution – have shown positive correlation between organic matter content and certain cuticle traits, such as rows of dots, suggesting potential trait-response to pollution (Justino *et al.* 2023). Additionally, ornamentation patterns like punctured or annulated ornamentation, particularly when occurring alongside other morphological traits, have been associated to human-disturbed environments (Semprucci *et al.* 2022). However, these features also appear to be more strongly influenced by phylogenetic lineage, potentially limiting their utility as purely environmental indicator (Armenteros *et al.* 2019). Overall, these observations underscore the need for further research to clarify the extent to which cuticle traits are shaped by environmental stressor, especially by metal contamination, and how these traits contribute to functional response and ecological resilience of nematode assemblages.

Mouth morphology, on the other hand, is closely related to the feeding behavior of nematodes and therefore can provide insights into their functional responses to environmental contamination (Chauvin *et al.* 2020; Ekschmitt & Korthals 2006; Heininger *et al.* 2007; Schratzberger *et al.* 2009; Yeates 2003). In our study, the 1B feeding type, which feed mainly on bacteria, organic particles and diatom cells (Moens & Vincx, 1997) was predominant in the control treatment, whereas 1A feeders, which feed mainly bacteria and small detritus (Moens & Vincx, 1997) were more frequent under contaminated treatments (Table 3B, Fig. 2). A similar pattern was reported in previous study that found a higher prevalence of 1A feeders in sites contaminated with PCB and PBDEs, often associated with cp-3 life history strategies (Franzo & Del Negro 2019). This trend was also present in our data, where higher c-p values were recorded under contamination (Table 3B, Fig.2).

As for the cuticle, this finding contrast with the expected pattern, where selective (1A) and non-selective (1B) deposited feeders are typically associated with disturbed or organically enriched environments (Asif & Fazul Rahaman 2024; Bohlen & Edwards 2015; Quist *et al.* 2019). Both 1A and 1 B feeding type lack buccal armature and consume similar food items. However,

the distinction between them lies in the size of the buccal cavity. Type 1A feeders possess a very narrow buccal opening, allowing the ingestion of diminutive bacteria-sized particles. In contrast, type 1B have wider buccal cavity capable of handling broader range of food items, including small metazoans (Moens & Vincx 1997). The predominance of 1A feeders under contamination may reflect ecological dynamics not fully captured by traditional classifications. One possibility is that metal contamination stimulated microbial growth, increasing high-quality food resources that benefited selective feeders. Alternatively, 1A nematodes present might be tolerant specialists capable of persisting under stress when their preferred food remains available. Certain contaminants can stimulate microbial growth, increasing the availability of high-quality food for selective feeders (Schratzberger *et al.* 2009; Yeates *et al.* 1993).

Lastly, considering the functional trait about life history, our results suggest an unexpected shift in strategies composition under metal contamination. In our study, nematode genera with higher c-p values were associated with the metal-contaminated treatment, whereas those with lower c-p values were more prevalent in the treatment without metal (Fig. 2; Table 3). This pattern deviates from classical expectations according to the coloniser-persistent model (c-p scale) (Bongers 1990; Bongers & Bongers 1998), where nematodes are ranked from cp-1 (opportunistic (r-strategist), disturbance-tolerant species) to cp-5 (slow-growing, k-strategist, and more sensitive to disturbance (*sensu* Bongers, 1990)). Typically, contaminated environments are expected to favor colonizers (cp-1, cp-2), while reducing persisters (cp-3 to cp-5).

Our findings, however, contradict this assumption by showing the persistence of disturbance-sensitive taxa under contaminated treatment (e.g., *Halalaimus* cp = 4). One possible explanation for this pattern lies in the trade-off between resistance and resilience. Species responses to stress are strongly influenced by life-history strategies. Genera with low c-p values commonly invest less energy in somatic maintenance, making them less resistant to stress at individual level (Hoffmann & Parsons 1989). However, their high reproductive output and rapid turnover rates confer greater resilience at population level (Begon *et al.* 2005). In contrast, species with higher c-p values show slow life histories and are generally more resistant to stress due to efficient energy allocation mechanisms (Ponge 2013), but their low reproductive rates result in limited resilience and slow recovery of populations after disturbance (Aktipis *et al.* 2013; Palacios *et al.* 2012). Given the short duration of our experiment (20 days), it is plausible that the observed patterns reflect short-term resistance rather than long term resilience. This highlights a key limitation of the c-p scale: while it is a useful conceptual tool, it may oversimplify the diversity of ecological strategies and stress responses among nematode taxa. Similar inconsistencies have been noted in other studies (Brinke *et al.* 2010; Heininger *et al.* 2007; Höss

et al. 2011; Ristau *et al.* 2015; Semprucci *et al.* 2016), emphasizing the need for caution when interpreting c-p value as direct indicators of tolerance to pollution.

Overall, the differences observed in the functional composition among treatments (Fig. 2) suggest a reorganization of life-history strategies in response to metal contamination. However, considering all context, it is important to note that such shifts in trait composition may not strictly reflect functional adaptation, but rather taxonomic turnover, where genera with particular traits increase or decrease in dominance depending on metal contamination levels irrespective of a real functional response. In such cases, the observed changes could result either from selective pressures favoring specific functional traits or simply from the replacement of taxa with preexisting trait combinations. Still, the restructuring of trait composition indicates that, even if mediated by taxonomic shift, the functional roles within the assemblage likely experienced meaningful changes in response to metal stress.

In fact, the general response of functional traits to contamination may appear more nuanced and context-dependent than previously thinking, varying according to the type of disturbance, community composition and the selected traits for analysis (Egres *et al.* 2019; Grassi *et al.* 2023; Justino *et al.* 2023; Losi *et al.* 2021). The functional convergence observed, despite deviation from classical tolerance patterns, may reflect adaptive response shaped by environment filtering. Although the direction of these responses was unexpected, we observed distinct and consistent changes in functional composition between control and contaminated treatments. Therefore, our findings highlight the value of integrating taxonomic and functional traits approaches to better understand the process shaping the assembly under metal-contaminated condition. This integrative perspective reveals that trait patterns may emerge not only adaptation, but also from taxonomic reorganization under selective pressures. Despite the complexity of interpreting trait responses, the consistent functional shifts observed here suggest that contamination can reorganize ecological roles within benthic assemblages. Finally, this underscores the need for more refined frameworks that account for both functional and taxonomic dimensions when assessing ecological resilience and vulnerability in polluted ecosystems.

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CAPÍTULO 3

Metal-binding specificity and structural dynamics of *C. elegans* metallothionein I: insights from 3D modeling and MD simulations

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Abstract

Metallothioneins (MTLs) are small, cysteine-rich proteins known for their ability to bind metal ions and exhibit flexible, disordered structures. The structural and functional characteristics of metallothionein I (MTL-1) from *Caenorhabditis elegans* were investigated, focusing on its behavior in both metal free (MTL-1 Apo) and metal-bound states with Zn^{2+} , Cd^{2+} , Cu^{2+} , Hg^{2+} , and Pb^{2+} divalent metal ions. Using molecular dynamics simulations and 3D modelling via AlphaFold, we characterised the flexibility and stability of MTL. The MTL-1 Apo form displayed high flexibility, aligning with its intrinsically disordered protein (IDP) nature, with 89.3 % of its structure composed of coils, bends, and turns. Metal binding significantly enhanced the protein's stability, particularly with Zn^{2+} , Cd^{2+} , Cu^{2+} , and Hg^{2+} , reducing root mean square deviation (RMSD), root mean square fluctuation (RMSF), accessible surface area (SASA) and radius of gyration (R_g) values, indicating structural compaction. Conversely, Pb^{2+} showed a weaker stabilising effect, with more dynamic and less stable structure. Structural analysis revealed that conserved cysteine residues form strong metal-thiolate bonds, critical for coordination, with additional contributions from non-cysteine residues, such as Glu and Lys. The study underscores the importance of incorporating intrinsically disordered protein models in MD simulations to provide deeper insights into how metallothionein's flexibility and stability vary in response to different metal ions, providing insights into their biological interactions and behaviour under diverse environmental conditions.

Keywords: metalloprotein, tridimensional protein, molecular dynamic simulation, AMBER, structural biology

Introduction

Metallothioneins (MTL) are ubiquitous and highly conserved proteins throughout biological systems of animals, higher plants, eukaryotic microorganisms and many prokaryotes.¹⁻³ MTLs have been considered the first lines of defence against metal contamination among proteins related to detoxification. It is known that these proteins are involved in metal homeostasis, performing transport, storage, and detoxification.⁴ They have a low molecular weight (6 -7 kDA) and are rich in cysteines (>25%) of comparatively higher sulfur content.⁵ The binding to metals has been attributed to the presence and conserved distribution of these cysteine residues along the polypeptide chain.

In mammals, for example, four isoforms of metallothionein are present, MTL-1 to MTL-4.³ In the invertebrate model organism *Caenorhabditis elegans* (Nematoda, Rhabditidae), two forms can be found, MTL-1 and MTL-2, which contain 75 and 63 amino acid residues, respectively. *C. elegans* serves as a useful model for studying MTL function in response to metal exposure, with both isoforms playing critical roles in protection against metal toxicity.⁶ Studies have indicated that *mtl-1* gene expression is rapidly activated in response to metal exposure, showing a marked preference for Zn over Cd (CD spectra methods^{7,8}). In contrast, *mtl-2* gene expression appears to be primarily constitutive and localized in gut cells.⁹ Consistent with these findings, only MTL-1 appears to play a crucial role in protecting against depleted uranium (238U) contamination.¹⁰ Therefore, MTL-1 seems more importance in physiological responses to metal exposure.

A wide range of metals can adversely affect organisms, particularly non-essential metals, which have no biological function and are toxic to the organism in minimal amounts. However, even essential metals can become toxic if concentrations exceed safe limits.¹¹ Both kinds of metal ions can bind to MTL and affect their conformation, contributing to their biochemical activity in the biological system and consequently to its stability. Despite that, research has mainly focused on the effects of Zn²⁺ and Cd²⁺ on MTL isoforms in different species.¹²⁻¹⁷ From the structural perspective, albeit a small protein, metallothionein constitutes a highly complex system, with the same peptide with the ability to display different final 3D folds, which confers high flexibility.⁴ In addition, they lack a well and stable defined tertiary and secondary structure in their apo forms (demetalated condition) aligned with characteristics of intrinsically disordered proteins (IDPs).¹⁸ This protein exhibits a flexible and highly dynamic conformation.¹⁹⁻²² The possible intrinsic disorder of MTs increases the complexity of evaluation and understanding their interactions with different metals.

Structural and functional characteristics of more stable proteins can be precisely determined using X-ray crystallography and nuclear magnetic resonance spectroscopy.²³ However, these techniques have some limitations when applied to IDPs.^{9,24} In addition, these methods are expensive and require extensive effort and time. Recently, *in silico* studies accessing the three-dimensional structure of proteins and molecular dynamics have generated significant progress in predicting their functionality.^{25–27} In the case of metallothionein, investigations into the metal-binding sites have been carried out in order to understand their role in metabolism, as well as mechanisms involving toxicity.^{28–30} Using computational models, we can gain deeper insights into the dynamic behaviour of MTLs and their roles in metal exposure, advancing our understanding of their biochemical functions and potential applications in managing metal toxicity.

Thus, this study was aimed to understand the effects of the divalent metals Zn^{2+} , Cd^{2+} , Cu^{2+} , Pb^{2+} , and Hg^{2+} on the isoform I of metallothionein from *C. elegans* (MTL-1), considering its molecular behaviour and protein conformation. To achieve this goal, we carried out a theoretical simulation of the complexation of MTL-1 modelled protein, with each set of metal ions, using all-atom molecular dynamics (MD). This approach allowed us to simulate and analyse how different metals influence the conformation and dynamics of MTL-1, revealing critical aspects that may not be easily observed by experimental techniques alone. The results obtained from these simulations were compared with experimental data to support and complement our findings.

Material and Methods

Molecular modelling of MTL-1 from *C. elegans*

The three-dimensional structure was obtained from AlphaFold, which predicted the model for metallothionein 1 (P17511, MTL-1). After that, the modelling structures were evaluated using Ramachandran plot, QMEAN³¹ and Molprobit score.³² To visually analyse the model, PyMoL software (The PyMOL Molecular Graphics System, Version 2.5.2 Schrödinger, LLC) was utilised. The secondary structure was determined quantitatively using the DSSP program.³³

System preparations for molecular dynamic simulations

To investigate the conformation and structural stability of MTL-1 bound to different metals, we assembled six systems: one MTL-1 Apo system without metal ions, and five Holo MTL-1 systems, each loaded with seven ions of Zn^{2+} , Cd^{2+} , Cu^{2+} , Pb^{2+} , and Hg^{2+} , respectively.

The systems were neutralised by Na⁺ or Cl⁻ ions. The all-atom molecular dynamic simulations (MD) were conducted in duplicate for each system using the Amber 23 software package.³⁴ Averages and standard deviations were calculated for each system, and a coefficient of variation analysis was performed on the duplicates to assess the relative variability of the results. The simulations were run over 200 ns, which provides a sufficient timescale to reach a plateau in the conformational changes and to capture alterations in their structural stability and flexibility. The results were analysed in terms of 1) root mean square deviation (RMSD) of the backbone, which measures the overall changes in conformation from the reference structure; 2) root mean square fluctuation (RMSF), assessing the flexibility of individual residues over time to evaluate structural stability and dynamics of system, 3) accessible surface area (SASA) determined by changes in the accessibility of protein to solvent using ‘surf’ command in the cpptraj module; and finally 4) radius of gyration (R_g), used as a measure of the compactness of a protein conformational. All cysteines and histidines in the systems were deprotonated; that is, they lost a proton being treated as a thiolate and imidazolate group, respectively. It is expected that cysteines need to exist in the form of deprotonated thiolates for stable metal ion complex formation because it seems that coordination of divalent metal ions requires the deprotonation of thiolate groups, at least prior to binding formation, making interaction stronger.^{7,35,36} Additionally, the histidine can facilitate protein stabilisation by maintaining a high affinity for metal ions, through the imidazole side chain which, serves as a potential metal-binding site.³⁷

For MD simulations, the MTL-1 full metalated approach was adopted, where it can bind up to seven divalent metals distributed in two metal–cysteine clusters: four ions in the α -protein domain and three ions in the β -protein domain.^{8,38,39} The tridimensional structure was compared with the partial structure deposited in the PDB (PDB ID: 8AQ9) derived from NMR experiments, which was loaded with zinc. Visual analysis of MTI-Apo was also conducted to identify putative metal binding sites with a focus on the cysteines. General amber force field (GAFF)⁴⁰ and FF19SB⁴¹ force fields were used. The systems were solvated in an octagonal box with 10 Å in each direction of the protein by optimal point charge (OPC) water molecules.⁴² Energy minimisation was performed in five steps, using cycles of steepest descent and conjugate gradients. The heavy atoms were restrained by a harmonic potential of 1,000 kcal mol⁻¹ x Å² initially. In the last step, 5,000 cycles of steepest descent and 30,000 cycles of conjugate gradients and no restraints were used. The heating and equilibration stage was divided into 14 steps. The temperature was gradually increased until it reached 300 K. Langevin dynamics (thermostat) was employed with a collision frequency of 3.0 ps⁻¹. A harmonic potential of 25 kcal mol⁻¹ x Å² was employed in the initial steps and was turned off during step 14. The heating procedure lasted 650 ps until step 14 and was performed using an NVT ensemble. After that, a 2-

ns equilibration phase was employed in an NPT ensemble. The SHAKE algorithm was applied to restrict the vibration of the ligations of all hydrogen atoms. The particle mesh Ewald method was used for calculating electrostatic interactions using a cutoff value of 10.0 Å. The integration time step in the MD simulations was 2 fs. The simulation frames were saved every 10 ns for analysis.

Results and Discussion

Moderate-quality metallothionein model from AlphaFold, offering interesting insights through its flexibility

Modelling small and flexible proteins is a challenge, and recently some attempts have been made to understand their molecular mechanisms.^{43,44} Three-dimensional modelling can also aid in this understanding. The model for MTI of *C. elegans* was generated using structural information from AlphaFold predictions (Fig.1B).

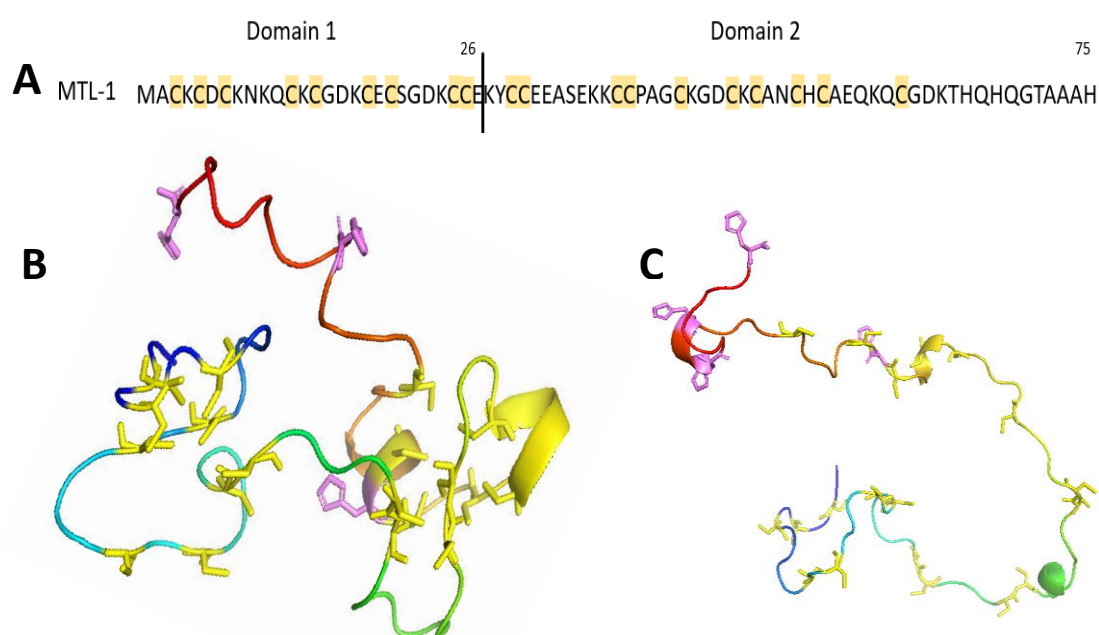


Figure 1. Sequence and 3D structural models of metallothionein I isoform (MTL-1) of *C. elegans*. A) Amino acid sequence, with cysteine residues highlighted in yellow. B) 3D model of MTL-1 generated by AlphaFold, showing deprotonated Cys and His residues as yellow and violet sticks, respectively. C) Metallothionein without metal (MTL-1 Apo) after 200 ns of MD simulation, in centre specified coordinates onto reference structure, with Cys and His residues highlighted in yellow and violet, respectively.

The parameters evaluating the geometry and target-template alignment of the protein model indicated moderate quality, with MolProbity score of 2.35 and a GMQE score of 0.60,

respectively. The Ramachandran analysis indicated about 98.5% in allowed regions with just 1.5% residues in disallowed regions for MTI of *C. elegans* (Table 1).

Table 1. Validation of the *C. elegans* metallothionein I model structure using AlphaFold.

Ramachandran analysis	%	Molprobit	GMQE	QMEAN
Residues in most favoured	65.2	2.35	0.60	-3.43
Residues in additional allowed regions	30.3			
Residues in generously allowed regions	3.0			
Residues in disallowed regions	1.5			

The high negative value of QMEAN (-3.43) of MTL-1 model indicated structural patterns deviation, pointing to low-confidence regions identified by AlphaFold. Despite that, it has been recognised that proteins with segments of low confidence may contain important information about conditional order, i.e., the final protein conformation is not extremely stable but rather is on the edge of stability.⁴⁵ This means that the folded state can be easily altered by small variations in environmental conditions (e.g., pH⁴⁶ or modifications to the amino acid sequence). However, such changes may not be captured by AlphaFold predictions due to its limitations, such as those arising from partially documented structures or methodological constraints.⁴⁷ This behaviour is consistent with the properties of intrinsically disordered proteins (IDPs), which is quite common; for example, over 30% of regions within the humane proteome cataloged in AlphaFold are disordered.⁴⁸ Therefore, in this context, AlphaFold predictions, which include structural annotations for disordered proteins, can be a valuable resource when experimental protein resolutions (e.g., X-ray crystallography and nuclear magnetic resonance (NMR)) are absent or difficult to obtain, such as metallothionein.

Metal binding enhances conformational stability in flexible MTL-1

In this study, metallothionein without metal (MTL-1 Apo) was used as reference structure. The coefficient of variation (CV) for the simulation duplicates indicated high confidence in their reliability. The SASA and R_g showed low variability, while the RMSD and RMSF showed moderate variability, although still within an acceptable range (see Figures S1-S4). As expected, the MTL systems with and without metal exhibited relatively high flexibility (high values of RMSD, Fig. 2A) if compared to conventional proteins, a characteristic trait of intrinsically disordered proteins.⁴⁹ MTL-1-Apo exhibited an extended structure (Fig. 1C), consisting of approximately 86% turn, bend, and coil conformations (Table S1), which predictably resulted in the highest of molecular dynamic parameters results (Fig. 2A-D).

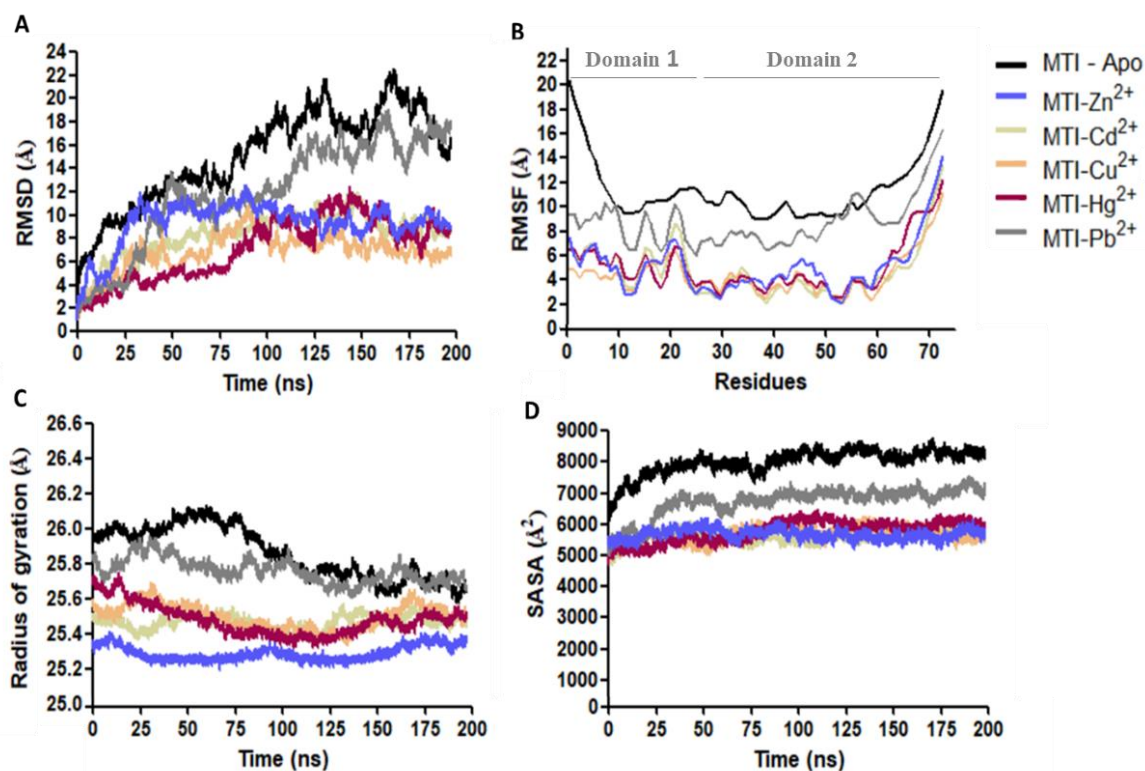


Figure 2. All-atom molecular dynamics simulations of *C. elegans* metallothionein I isoform in the absence (MTL-1-Apo) and binding of different divalent metal ions. Except for MTL-1-Apo, each system is formed by MTI protein and 7 homometallic ions. A) root mean square deviation (RMSD), B) root mean square fluctuation (RMSF), C) radius of gyrations (R_g), and D) solvent accessible surface area (SASA). Each parameter represents the average of the duplicate simulations.

This outcome corroborates experimental studies by circular dichroism spectroscopy^{50–52} and MD studies⁵³ that indicate that metallothionein is relatively unstructured in the absence of metal ions, content minimal traditional secondary structural (see Table S1) elements such as α -helices or β -pleated sheets prevailing random coil structure.⁵² This structural condition of MT-Apo, observed in different species, appears necessary to precede the cooperative unfolding transition, characterized by a coordinated all-or-nothing change in the protein's structure, indicating that this native conformation is crucial for the unfolding process.^{54,55} This observation has broader implications since some studies suggest that MTL-1 Apo is not simply an unstructured protein. Instead, it is proposed that in physiological conditions, the native structure may adopt folded conformation, contrary to the extended structure.^{56,57} Others suggest that MT-Apo (2A human MT) samples several conformational states ranging from globular-like compact to extended conformations.⁵⁸ Although these observations provide valuable insights, ongoing studies are needed to fully understand these structural dynamics. Indeed, the biological function of the ‘denatured-like structure’ of MTL Apo is particularly remarkable. Beyond its crucial role

in metal sequestration, it significantly contributes to the protein's interaction with cellular oxidants, underscoring its importance in cellular redox regulation and stress response.⁵⁹

Conversely, analysis of the metal-bound systems revealed that Cd^{2+} , Zn^{2+} , Cu^{2+} , and Hg^{2+} binding stabilised MTL-1, as indicated by lower RMSD values compared to MTL-1 Apo (Fig. 2A). The trajectory for these metal set reached equilibrium and converged after 80 ns of the simulation, except for Pb^{2+} . This same pattern was observed for the other parameters analysed (see Fig. 2 B-D). Experimental data have revealed that the presence of metals induces the formation of a more ordered and stable structure and stabilizes MTL protein structure upon binding. This stabilisation has been observed in various organisms, including mammals⁶⁰, plants^{28,61}, earthworms¹⁵, fungi⁶² and bacteria.⁶³ Although different isoforms exhibit varying affinities, laboratory data for *C. elegans* MTL-1 specifically demonstrated that this isoform folds more effectively in the presence of zinc than cadmium.⁹ Our simulation data aligns with this observation, as the Zn^{2+} system showed slightly lower values for the radius of gyration (Fig. 2C). This parameter represents changes in protein compaction reflecting the distribution of mass around the centre of mass. Despite the small variation, this may indicate a tendency toward compaction induced by Zn^{2+} metal, suggesting a more compact and stable structure, even if not causing a drastic structural change. On the other hand, Pb^{2+} , in particular, did not confer the same structural stability as the other metals tested, as evidenced by higher RMSD, SASA and radius of gyration values, which indicated a more flexible and dynamic conformation. This suggests that Pb^{2+} leads to a less compact structure, jeopardising the overall stability of the protein. Although there is experimental evidence that Cys residues from MTL can bind to Pb^{2+} ,⁶⁴ this process is quite sensitive to other factors, which will be further discussed in the next section. Experimental studies exploring the interaction of MTL-1 with Cu^{2+} and Hg^{2+} are limited. Therefore, our simulation study sought to address this gap by investigating the binding and structural stability of MTL-1 in the presence of these metals.

The RMSF data revealed that metal binding of Cd^{2+} , Zn^{2+} , Cu^{2+} , and Hg^{2+} induced similar patterns in the variation of residue flexibility, with only a slight distinction in fluctuation intensity, except for Pb^{2+} (Fig. 2B). This means that the most flexible and most rigid regions of the protein remain consistent, regardless of the metal bound, which may indicate a structured global function of this protein when exposed to metal. In this context, the selectivity of MTL for a specific metal may not strongly depend on the conformation of the protein but on other local factors, such as the binding affinity of the metals and the chemistry of the metal involved. The most significant changes were observed in the region near the N-terminal, particularly at residues Asp16, Lys17, Gli21, and Asp22 (Fig. 2B). Regions closest to the C-terminus of a protein which can also exhibit higher RMSF values, especially if the C-terminus is not involved in stable

secondary structures or without interactions. The residues that are more flexible are often located in the loop regions of proteins.⁶⁵ In addition, glycine, due to its small size and flexibility promotes mobility. Meanwhile lysine, with its long positively charged side chain, and aspartic acid, with its negatively charged carboxyl group, further enhance dynamics and flexibility by interacting with the solvent. Furthermore, considering the MTL-1 fold in *C. elegans*, biophysical studies have proposed a two-domain model for MTL-1,³⁸ with domain 1 comprising residues 1-26 and domain 2 comprising 27-75. Similarly, mammalian MTL-3 has a two-domain structure and experimental studies indicated that the β -domain (residues 1-31) is structurally more flexible, while the α -domain (residues 32-68) is more stable.⁶⁶ Although the primary protein sequences of mammalian MTL-3 and *C. elegans* MTL-1 are dissimilar, MTL-1 showed similar behaviour, exhibiting higher flexibility residues in domain I, with peaks and higher absolute value of RMSF if compared to domain 2 (Fig. 2B). Therefore, our results align with these findings, indicating that the flexibility patterns of MT in *C. elegans* mirror the established structural characteristics of mammalian metallothionein isoform. This similarity may suggest that the three-dimensional behaviour of the protein structure may be conserved across species.

The consistence interdependence among these metrics— RMSD, RMSF, radius of gyration, and SASA— demonstrates the overall stability effects that the metal binding causes to structure MTL-1. The SASA variation, ranging from 5000 to 6000 Å² in the Cd²⁺, Zn²⁺, Cu²⁺, and Hg²⁺ systems, does not indicate big changes in the exposure of the protein's surface to the water solvent. Similarly, the minimal variation of the radius of gyration (approximately 0.3 Å) did not lead to drastic shifts in the compaction or extension of MTL-1. The results for both parameters suggest that the overall conformation of metallothionein is maintained similarly among the different metals, and small difference are likely related to specific interactions with the binding site residues, or the specific characteristics of each metal.

Variability and influence of distinct metal ions in MTL-1 coordination

The putative binding sites were coordinated through direct bonds between the metal ions and amino acids. The distances among them were < 2.0 Å for Cu²⁺, < 2.5 Å for Zn²⁺, Cd²⁺, and Hg²⁺, and < 3.0 Å for Pb²⁺ (see Figures S5-S10). Distances below 3.0 Å indicate strong coordination bonds. As predicted, cysteine was the most frequently involved amino acid in these interactions, forming stable complexes with the metal ions. The binding site structure remained largely unchanged when substituting Zn²⁺, Cd²⁺, Co²⁺, or Hg²⁺, except for Pb²⁺ (Fig. 3). These metal-thiolate bonds (S-donor) coordinated primarily covalently, indicating a high binding affinity. Cysteine is essential for the function of metallothionein as sequester metals, with certain

cysteine positions playing a more important role in the binding site and structuration of MTL.⁵³ Here, residues such as Cys12, Cys26, Cys31, Cys40 and Cys55 were often involved in coordination with tested metals, which suggests that they are fundamental to the structure of MTL-1, being essential for effective metal sequestration (Fig. 3).

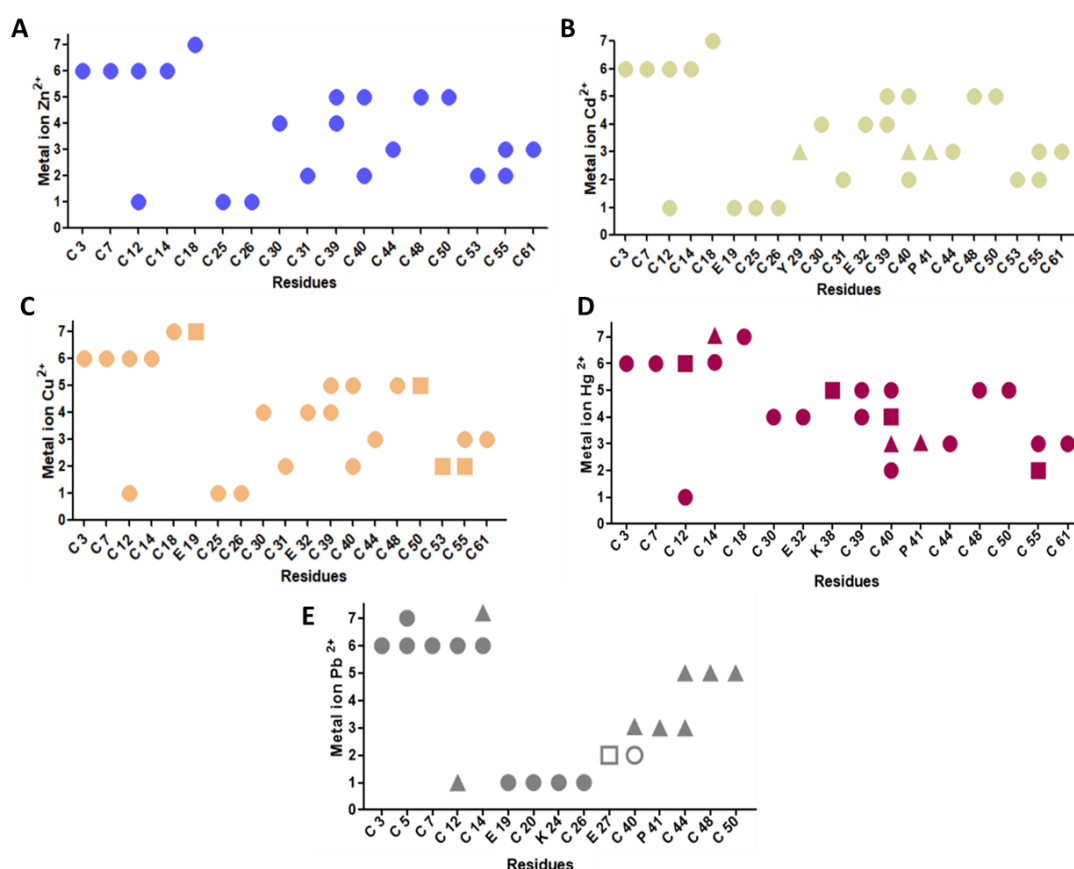


Figure 3. Residues involved in the binding site of each MTL-1 system with seven ions from the divalent metals A) Zn, B) Cd, C) Cu, D) Hg, and E) Pb. Residues on the x-axis are represented by single-letter amino acid codes of MTL-1 sequence: C = cysteine, D = aspartic acid, E = glutamic acid, K = lysine, P = proline, and Y = tyrosine. Triangle symbols denote residues that were part of the binding site exclusively in Replica 1, squares denote residues unique to Replica 2, and circles represent residues observed in both replicas. Closed symbols indicate a binding distance of less than 3.0 Å between residue and metal ion, while open symbols signify a distance of less than 5.0 Å.

In our study, all metal complexes showed some level of clustering or compaction throughout the simulation that is characteristic of cluster structures. At the same time, Pb²⁺ presented a more intermediate distribution, aligning more with the beaded-like structure (Fig. 4). The compact arrangement involves bridging thiolates, i.e., the sulphur in cysteine forms a ‘bridge’ between two metals ions, coordinating with both and thereby linking them together, which herein engaged Cys12, Cys39, Cys40 and Cys55 residues participation especially on the binding Zn²⁺, Cd²⁺, Cu²⁺, and Hg²⁺ metal. The clustering organization is a key feature of the cooperative metalation pathway proposed for human MTLs,^{50,67} in which metalation is facilitated

to promote protein structuring. On the other hand, non-cooperativity occurs when one metalation event does not facilitate the subsequent metalation events. Although we just tested the full metalation, without evaluating the effects of ion addition, we observed that Pb^{2+} metal set did not fit well into the binding site coordination of metallothionein and did not follow the typical co-operative pattern of other metals used. The non-cooperative behaviour of Pb^{2+} may have led to a more disordered or flexible MTL structure, with significant variations in ion positions and protein conformation, which can be observed in the simulation as a greater variation in RMSD and radius of gyration, indicating a less stable structure (Fig. 2).

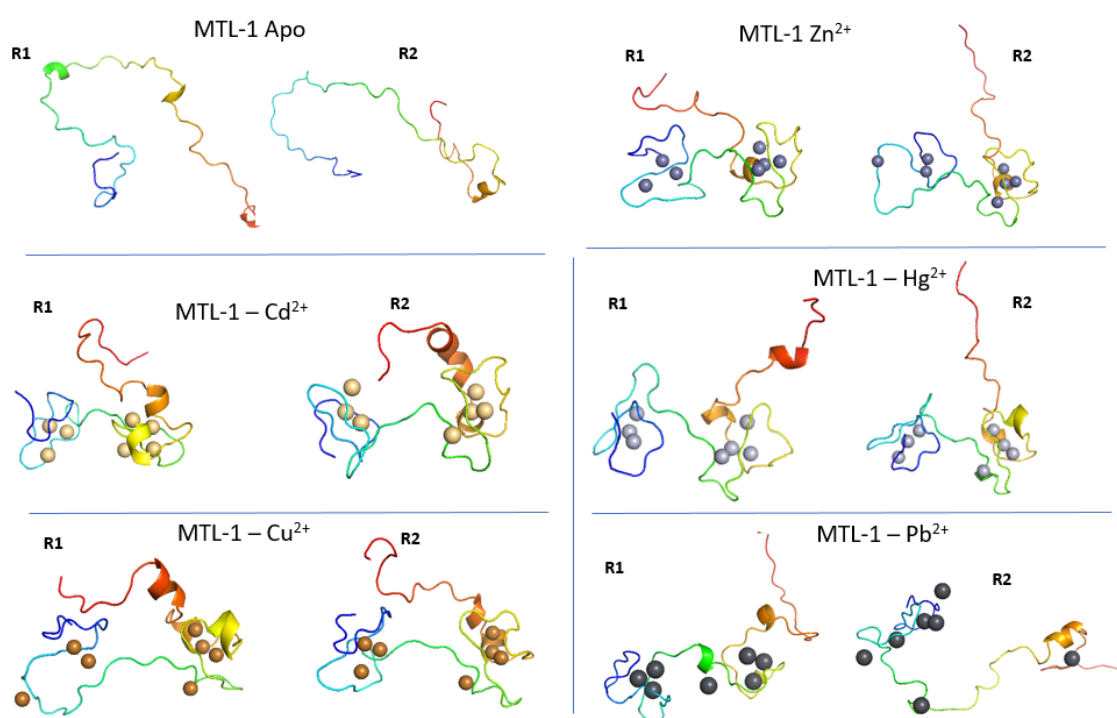


Figure 4. Three-dimensional structure of MTL-1 duplicates in the Apo form (without metal) and complexes with five different divalent metals (Zn^{2+} , Cd^{2+} , Hg^{2+} , Cu^{2+} and Pb^{2+}) obtained from molecular dynamics simulation (200 ns) using the AMBER software. The structures were visualised using PyMOL. Simulations were performed in two independent replicas (R1 and R2).

Global stability in metallothionein proteins is influenced by various factors, including physicochemical conditions,⁶⁸ but metal characteristics are particularly important. Generally, the affinity of a divalent metal ion for a specific ligand (or set of ligands) is correlated with the metal's second ionisation enthalpy.⁶⁹ This correlation follows the Irving-Williams series⁷⁰, which ranks the relative stability of complexes formed by divalent metal ions in the following order: $\text{Mg}^{2+} < \text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$. In our study, after the general convergence of systems (Fig. 2), Cu^{2+} had the highest MTL-1 stability considering the RMSD value parameter, followed by Zn^{2+} , Hg^{2+} and Cd^{2+} with slight differences. The same scenario was observed in another study which proposed the order of metal ion affinity to Cys residue, $\text{Cu} > \text{Zn} > \text{Hg} >$

Cd, using density functional theory calculations.⁷¹ On the other hand, Pb^{2+} ions caused the lowest MTL stability, leading to higher RMSD values (Fig. 2A).

In experimental conditions, the binding of Pb^{2+} to MTL is characterised by a high degree of variability and complexity, with some complexes becoming more stable while others may dissociate and become highly sensitive to pH changes if compared to other metals.^{72,73} This could partially explain the diversity of conformer variations in the simulations for this metal. Deviations were observed in the binding sites for some Pb^{2+} metal ions across duplicate runs and in additional simulations not shown here. In this case, ions 3 (for Replica 2), 4 and 5 had a lack of strong and constant bonds with residues, a phenomenon not observed with any other metal tested. The affinity of Pb^{2+} for MTL can be relatively weak compared to other metals like zinc or cadmium. This is primarily due to the nature of the interactions involved. Studies have shown that Pb^{2+} binding to MTL is less stable, leading to a higher likelihood of dissociation under physiological conditions.⁷⁴ In addition, Cys residues remained the main binding sites for Pb with MTL-1, where an absence of bridging cysteine sulphur was observed, a feature that has previously been associated with increased flexibility in $\text{Pb}_7\text{-MT2(II)}$ from rabbit liver.⁷⁵

Furthermore, while in our simulation, the deprotonation of all cysteines in the MTL system with Pb^{2+} may have favour the affinity of Pb^{2+} ions for the deprotonated thiolate ligands, potentially increasing the stability of the complex, it could also promote changes in electrostatic interactions and coordination geometry of protein. Finally, the binding of Pb^{2+} with MT can have a time-dependent behaviour, which may vary over time due to the unique coordination properties of metal ⁷⁶. This could influence the stability and conformation of the metallothionein complex, leading to potential variations in binding site occupancy and overall protein structure during molecular dynamics simulations. In this context, further deeper studies are required to understand the exact mechanisms governing the unique behaviour of Pb^{2+} in metallothionein complexes.

Metal interactions in MTL-1: cysteines and beyond

Cys residues allow the formation of high-affinity complexes with metals, forming ‘anchor points’, which can be crucial for protein function, at first. However, versatility in coordination with metals may be a crucial aspect of the function of IDPs. The binding sites on metallothionein can be highly dynamic and capable of adapting rapidly to different metals, which can be essential for the regulation and function of this protein in response to variable conditions. The increased presence of cysteines in MTL sequences also correlates with increased disorder propensity within the sequences that flank the cysteines, which potentially increases

conformational plasticity under reducing conditions, such as our simulation condition.⁷⁷ In this context, besides cysteine, other residues may play a crucial role in metal coordination. In our study, Glu residues were particularly important for coordinating Cd and Hg ions along with Cys (see Figures S6 and S8). Additionally, Glu was involved in binding one of the Cu ions. Lys, Tyr, and Pro also emerged as bond-stabilising residues, with Lys participating in the binding of Pb. These non-Cys residues maintained a distance of less than 2.5 Å throughout the entire simulation, indicating strong coordination interaction between metals and amino acid residues in MTL-1.

Experimental and modelling studies have been providing evidence that other residues such as Glu, Lys, Ser, and Asp can participate in metal coordination together with cysteines^{53,78,79} affecting the modulation of the complex's structure and stability. The specific binding of non-Cys residues with metals can be attributed to a combination of factors, such as specific electrostatic interactions or the local conformation. Glu residue, for example, has a carboxylate group (-COO^-), which can interact with metals to make the bond more stable, even if it's not the first choice in terms of chemical affinity. This residue at positions 19 and 32, flanked by nearby two Cys residues each, may have altered the binding affinity of the metal ions,⁷⁸ especially for Hg^{2+} which has a high polarizability, that mean is easily its electron cloud can be distorted by an external electric field. The presence of non-Cys residues in MTL-1 is as crucial as the presence of Cys, as these non-Cys residues can influence which metals are preferentially bound and how effectively they are chelated.⁸⁰ Furthermore, other explanatory factors, such as the size and geometry of the binding site cavity, are also important aspects that can help in understanding the selectivity of the metal ion.⁸¹

As mentioned, Pb^{2+} was observed to induce the least stability among the metals tested, showing varied binding patterns across different residues. Experimental studies appoint indirect evidence that the coordination of Pb^{2+} in thiol-rich environments, such as glutathione and zinc fingers, showed consistent with the preference of Pb^{2+} for strong coordination with sulfur atoms, which are functional analogues to the interactions observed in your simulated metallothionein.⁸² Confirming, Pb^{2+} bound strongly to some cysteine residues (Figure S9 and S10). On the other side, the coordination chemistry of Pb^{2+} , together with the expanded conformation and dynamics of the protein, seems to favour a condition for a transient interaction with non-Cys residues. Indeed, studies on lead binding to metallothionein indicate that the metalation process varies with different experimental conditions.⁷³ Furthermore, although MTL plays a role in the binding and detoxification of Pb^{2+} , its capacity and efficiency in this function are less responsive to lead than to other metals such as Zn and Cd.⁸³ These characteristics, such as a high rate of associated changes or moderate affinities at specific binding sites, are common in IDP interactions. These

interactions are likely a combination of conformational selection and induced fit mechanisms, relying on existing structural elements and structural changes after binding.⁸⁴

Conclusions

This study provides valuable insights into the structural flexibility and metal-binding properties of *C. elegans* metallothionein I (MTL-1) using molecular dynamics simulations and AlphaFold-based modelling. Despite moderate confidence in some structural predictions due to the intrinsically disordered nature of MTL-1, the simulations reveal that metal binding significantly stabilises the protein structure, with Zn^{2+} and Cu^{2+} inducing the highest levels of compaction and stability. The exception was Pb^{2+} , which showed weaker stabilisation, suggesting non-cooperative binding and greater flexibility in the protein structure. These findings not only confirm previous experimental observations about MTLs dependence on metal coordination but also provide new insights into the structural dynamics of metallothioneins in response to different metals.

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Supplemental material

Metal-Binding specificity and structural dynamics of *C. elegans* metallothionein I: insights from 3D modeling and MD simulations

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Table S1. Percentage of secondary structure of MTL-1 3D model from AlphaFold and after molecular dynamics (200 ns) in AMBER, shown for duplicate simulations (Replica 1 and Replica 2). The secondary structure was determined quantitatively using the DSSP program (KABSCH; SANDER, 1983).

Secondary Structure (%)	MTL-1 3D model AlphaFold	MTL-1 Apo (Replica 1)	MTL-1 Apo (Replica2)
Bend	34.7	21.3	16.0
Turn	13.3	8.0	12.0
Alpha Helix (4-12)	6.7	0.0	10.7
3-10 Helix	4.0	13.3	4.0
Coil	41.3	57.3	57.3
Total Bend/Turn/Coil	89.3	86.6	85.3

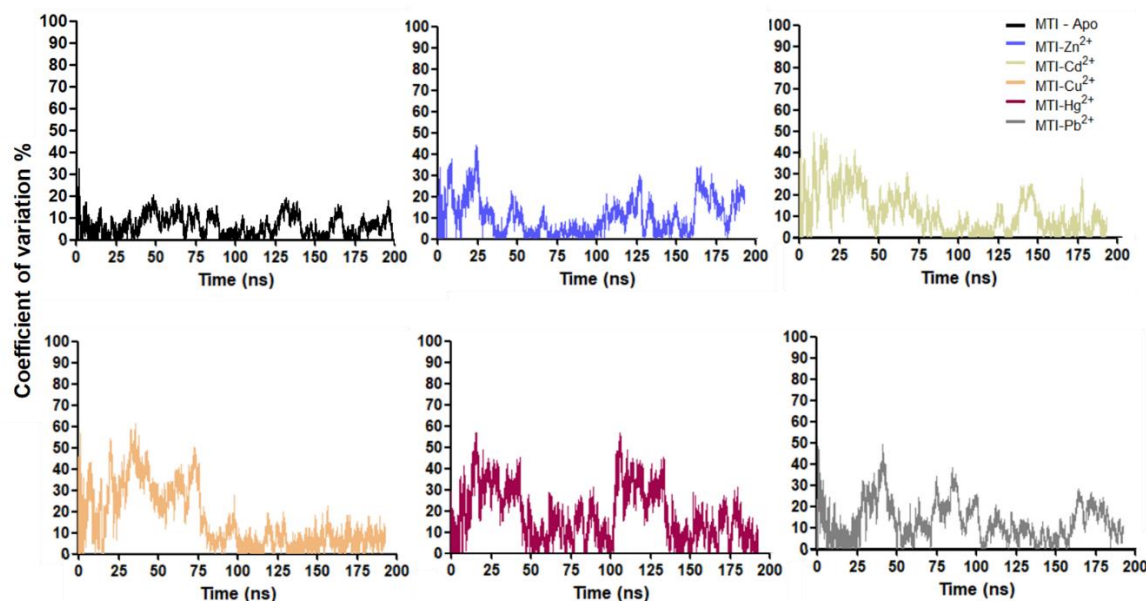


Figure S1. Coefficient of variation (%) of the RMSD (root mean square deviation) parameter for duplicate molecular dynamics simulations of all atoms of isoform I of metallothionein from *Caenorhabditis elegans*, in the absence of metal ions (MTL-1 Apo) and with various divalent metal ion bindings.

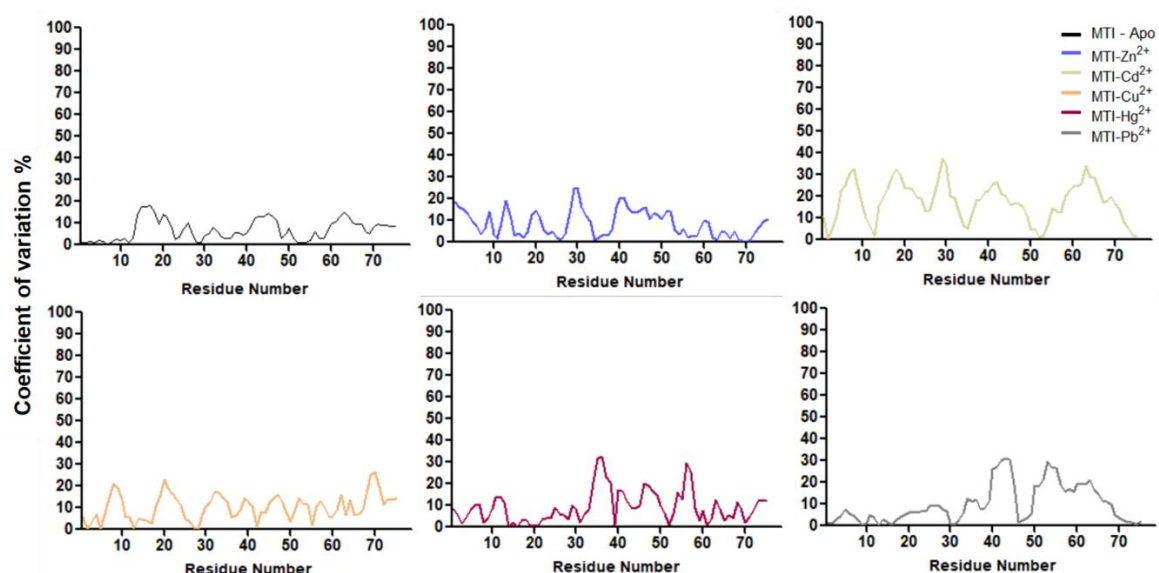


Figure S2. Coefficient of variation (%) of the RMSF (root mean square fluctuation) parameter for duplicate molecular dynamics simulations of all atoms of isoform I of metallothionein from *Caenorhabditis elegans*, in the absence of metal ions (MTL-1 Apo) and with various divalent metal ion bindings.

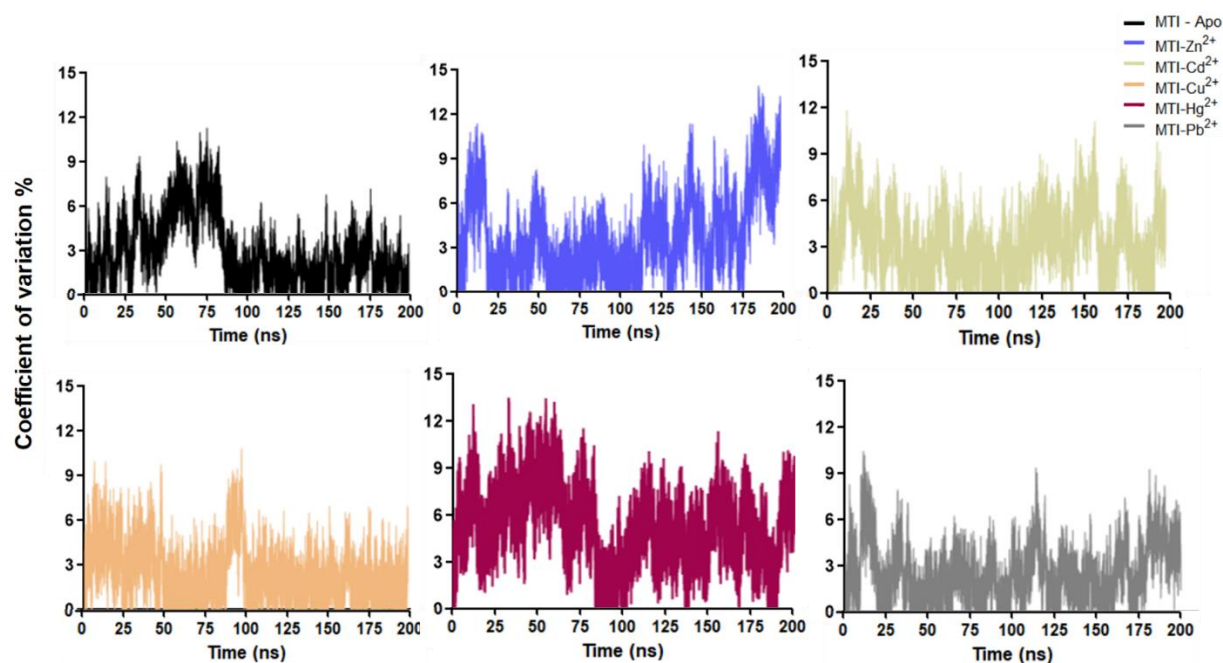


Figure S3. Coefficient of variation (%) of the SASA (accessible surface area) parameter for duplicate molecular dynamics simulations of all atoms of isoform I of metallothionein from *Caenorhabditis elegans*, in the absence of metal ions (MTL-1 Apo) and with various divalent metal ion bindings.

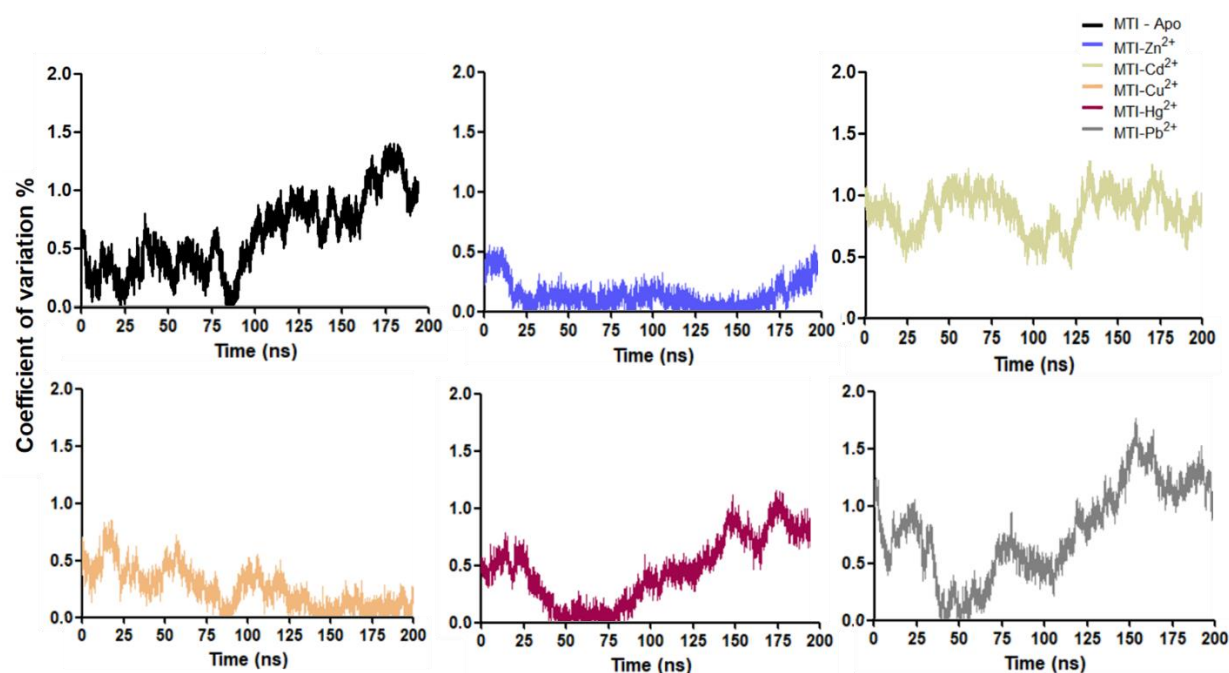


Figure S4. Coefficient of variation (%) of radius of gyration (R_g) parameter for duplicate molecular dynamics simulations of all atoms of isoform I of metallothionein from *Caenorhabditis elegans*, in the absence of metal ions (MTI-1 Apo) and with various divalent metal ion bindings.

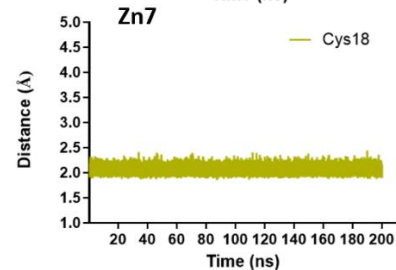
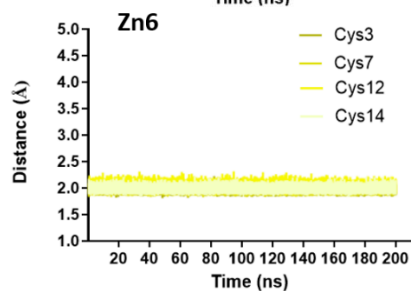
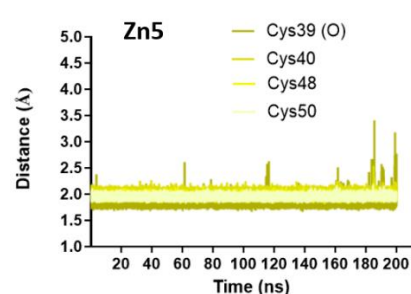
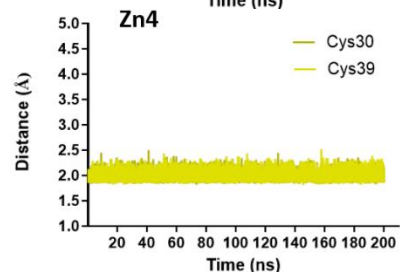
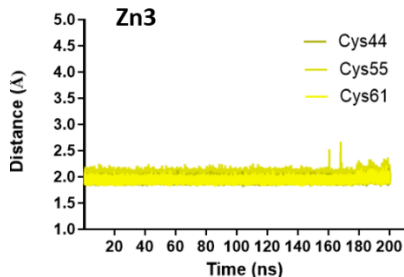
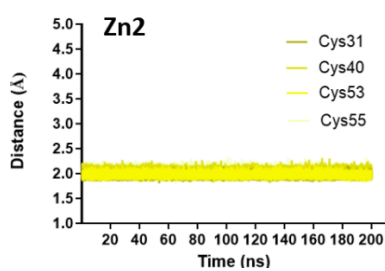
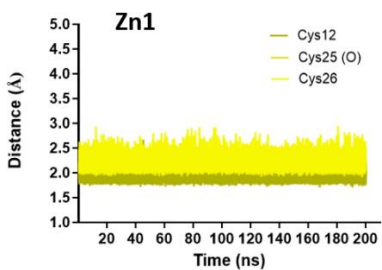


Figure S5. Distance in Angstroms between residues and the Zn metal at each ion binding site (1-7). The graphs on the right correspond to replica 1 and the graphs on the left correspond to replica 2.

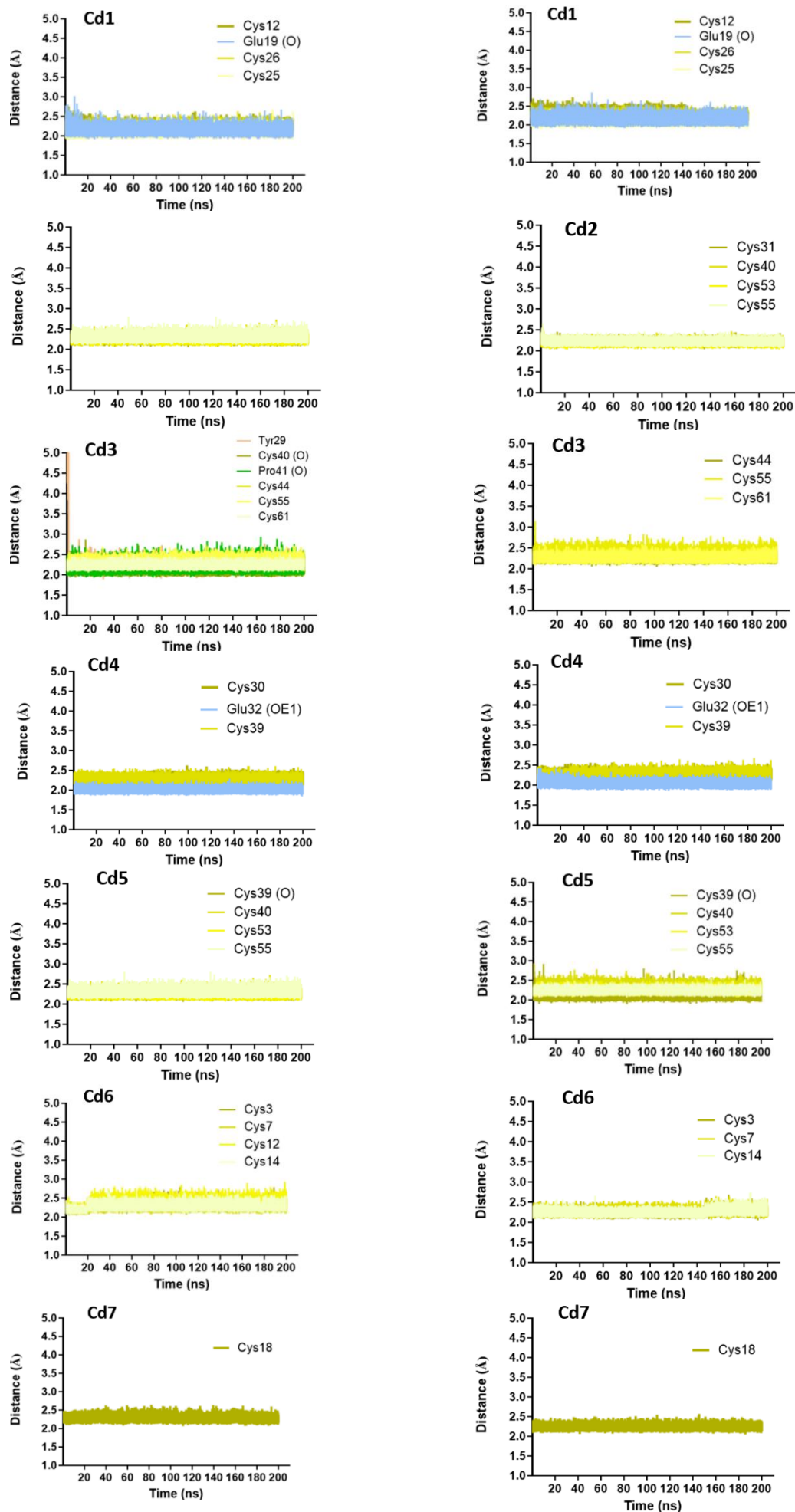


Figure S6. Distance in Angstroms between residues and the Cd metal at each ion binding site (1-7). The graphs on the right correspond to replica 1 and the graphs on the left correspond to replica 2.

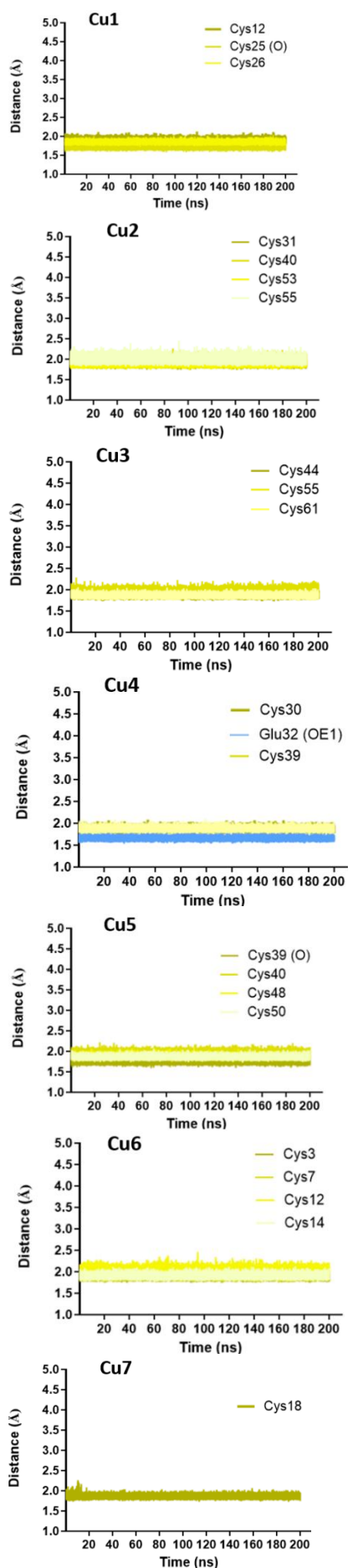
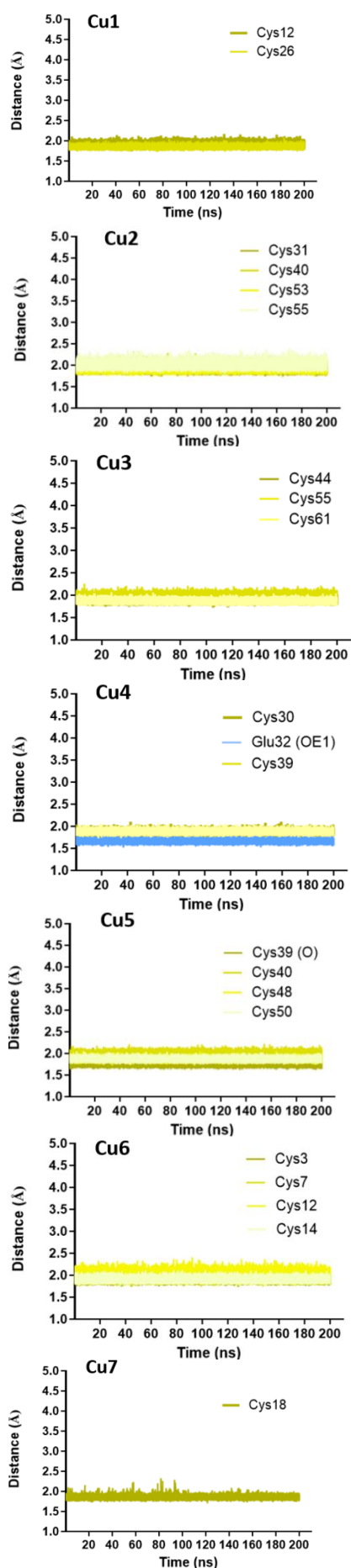


Figure S7. Distance in Angstroms between residues and the Cu metal at each ion binding site (1-7). The graphs on the right correspond to replica 1 and the graphs on the left correspond to replica 2.

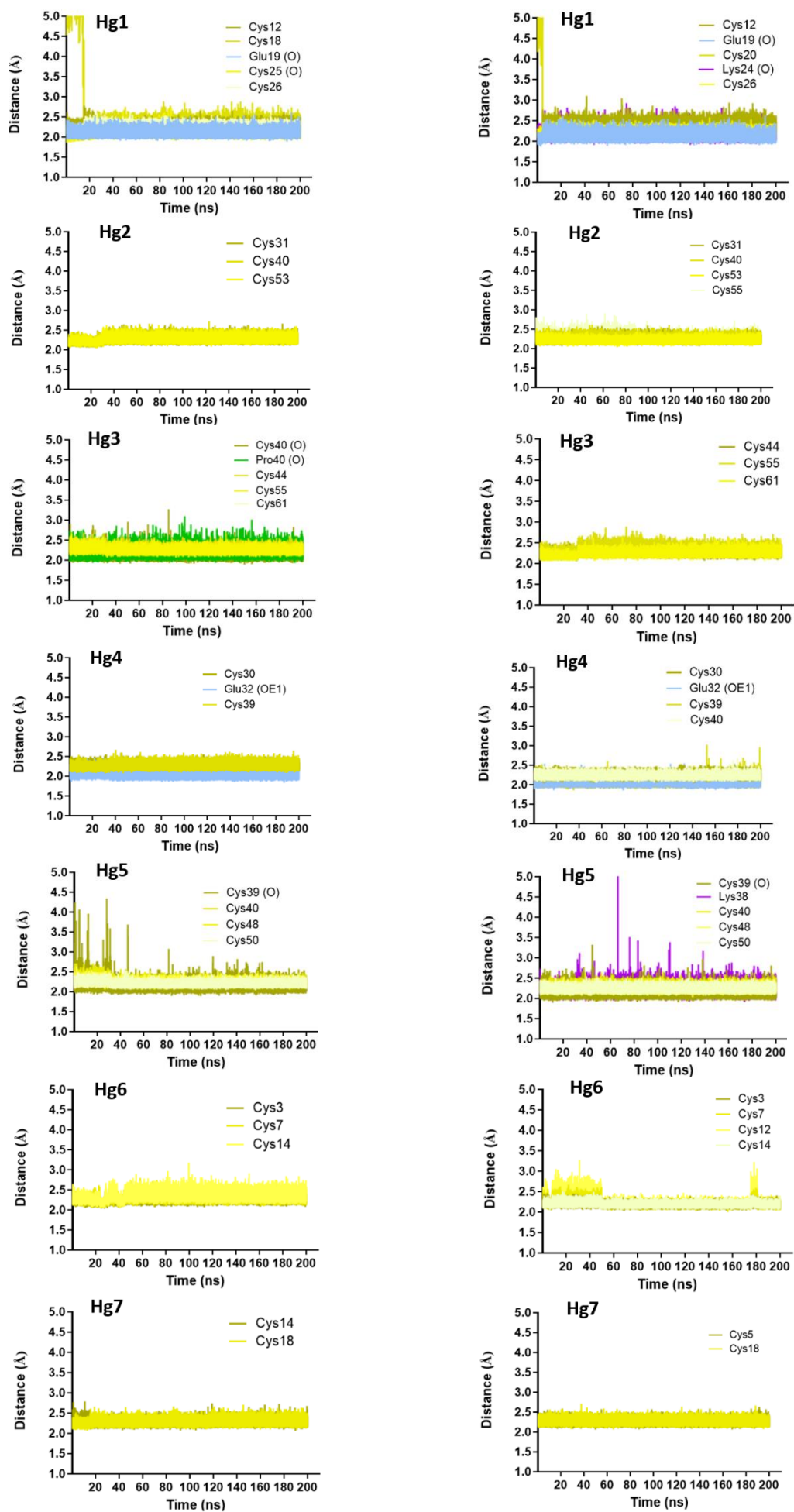


Figure S8. Distance in Angstroms between residues and the Hg metal at each ion binding site (1-7). The graphs on the right correspond to replica 1 and the graphs on the left correspond to replica 2.

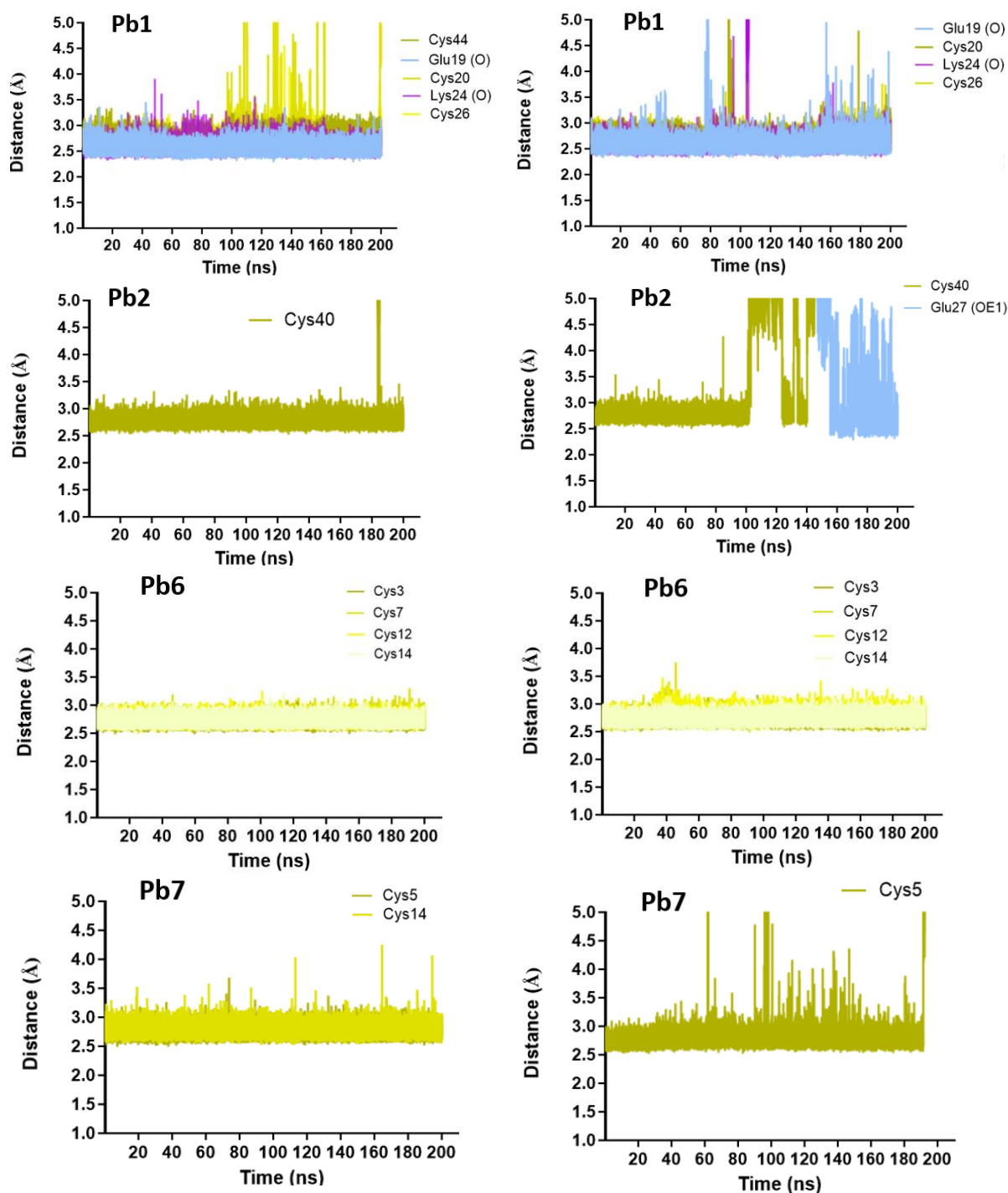


Figure S9. Distance in Angstroms between residues and the Pb metal at each ion binding site (1,2,6 and 7). The graphs on the right correspond to replica 1 and the graphs on the left correspond to replica 2.

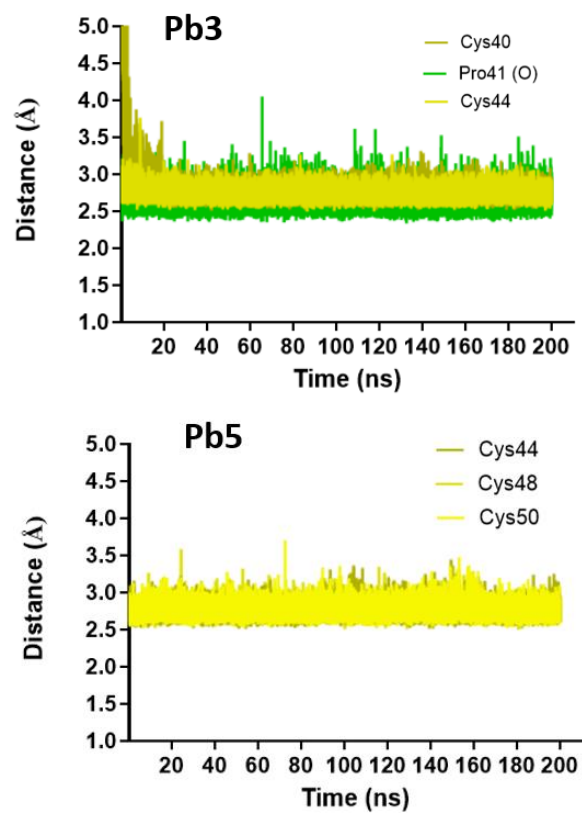


Figure S10. Distances $< 3 \text{ \AA}$ between residues and the Pb3 and Pb5 ion binding sites, observed only in replica 1, with no corresponding interactions in replica 2.

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Esta tese investigou os efeitos da contaminação metálica, isoladamente e em interação com outros estressores (acidificação por CO₂ e o aumento da temperatura), sobre Nematoda de vida livre. A abordagem adotada analisou as respostas desses organismos em diferentes níveis biológicos – desde alteração em cenários realistas de múltiplos estressores, passando por padrões funcionais e taxonômicos das assembleias até o estudo da estrutura molecular da metalotioneína, proteína chave na tolerância aos metais.

No capítulo 1, foi demonstrado que os efeitos da contaminação por metais sobre assembleias de Nematoda estuarinos resultaram em respostas ecológicas complexas, especialmente quando interagiram com variáveis abióticas relacionadas às mudanças climáticas. Embora a contaminação metálica tenha se destacado como o principal fator estruturante da assembleia, a presença simultânea de acidificação e o aumento de temperatura modulou essas respostas, revelando efeitos emergentes não previsíveis a partir da ação isolada dos estressores. Esses achados reforçam a interdependência das respostas biológicas diante da exposição a múltiplos fatores (SULMON et al., 2015) e apontam para a relevância de se considerar misturas de metais, assim como ocorre na natureza, ao invés de investigar seu efeito isolado (ANYANWU et al., 2018). Nesse sentido, estratégias baseadas em modelos ecológicos integrativos devem ser cada vez mais incorporadas aos estudos de impacto ambientais, reunindo várias áreas do conhecimento e ampliando a compreensão desses múltiplos efeitos (ORR et al., 2020).

No capítulo 2, com base no mesmo experimento de microcosmo do capítulo 1, aprofundou-se a análise para investigar os efeitos da mistura de metais em aspectos taxonômicos e funcionais da assembleia, oferecendo uma perspectiva complementar e permitindo entender não apenas quais grupos respondem à contaminação, mas como essas respostas se organizaram em termos de afinidade taxonômica e estratégias funcionais. Nossos resultados mostraram que os metais promoveram uma reorganização taxonômica hierárquica, mas sem implicar em mudanças drásticas na assembleia. Ao contrário, os tratamentos com menores concentrações de metais promoveram um aumento da riqueza e na dispersão dos taxa. No entanto, na concentração mais alta, a filtragem foi intensificada reduzindo a diversidade, mas mantendo a dispersão dos taxa. A contaminação por metais levou à convergência funcional, porém com padrão inesperado, como a prevalência de traços funcionais tradicionalmente considerados sensíveis sob condições de contaminação. Esse resultado desafia pressupostos generalistas sobre a sensibilidade e tolerância associadas à traços funcionais e destaca a necessidade de contextualizar ecologicamente os padrões observados em comunidades impactadas.

O capítulo 3 adicionou uma nova camada de entendimento ao explorar, por meio da modelagem *in silico*, as propriedades estruturais da proteína metalotioneína (MTL-1) do Nematoda modelo *Caenorhabditis elegans* com diferentes ligantes metálicos. Os resultados mostraram que a ligação aos diferentes íons metálicos Zn^{2+} , Cd^{2+} , Cu^{2+} e Hg^{2+} aumentaram a estabilidade da MTL-1, enquanto Pb^{2+} teve um efeito estabilizador mais fraco. A elevada flexibilidade da estrutura também foi compatível com achados experimentais prévios em metalotioneínas humanas (Yuan et al., 2021). Essa abordagem, embora limitada à uma investigação preliminar com parametrização simplificada baseada em íons solvatados, reforça o grande potencial dos estudos moleculares na compreensão dos mecanismos de detoxificação.

Entretanto, é importante destacar que a validação experimental da funcionalidade biológica dessa proteína ainda é necessária, especialmente em Nematoda estuarinos. Técnicas como a expressão recombinante da proteína permitem a produção de MTL-1 em sistema heterólogos (bactérias ou leveduras), viabilizando sua purificação e testes diretos de ligação aos metais. Abordagens mecanistas como silenciamento gênico por RNAi ou *knockout*, podem revelar a importância funcional da MTL-1, ao demonstrar como sua redução ou ausência afeta a tolerância ao estresse metálico. No entanto, nesta Tese, não foi possível realizar ensaios laboratoriais com Nematoda estuarinos devido à baixa biomassa disponível, que comprometeu procedimentos como extrações de RNA e análises moleculares. O estabelecimento de cultivos em laboratório desses organismos, especialmente dos gêneros *Anoplostoma* e *Therschellingia* que apresentaram alta tolerância aos metais, pode, no futuro, viabilizar esses estudos em condições controladas. Além disso, a identificação de MTL-1 e possíveis outras isoformas em espécies estuarinas ainda é limitada pela falta de genomas sequenciado, dificultando abordagens moleculares mais precisas e comparativas. Ainda assim, embora a ausência de validação experimental represente uma limitação, os resultados *in silico* oferecem uma base para hipóteses que poderão ser testadas em pesquisas futuras com Nematoda estuarino.

Considerando os resultados obtidos, sugere-se algumas outras perspectivas que seriam promissoras para o avanço na pesquisa na área:

- O uso de ensaios de microcosmos, pode ser ampliado para incluir camadas adicionais de análise biológicas, por exemplo, filogenéticas, genômicas e bioquímicas que permitirão uma compreensão mais aprofundada dos mecanismos de resposta de comunidades bentônicas sob estresse ambiental.
- A incorporação de um número maior de traços funcionais nas análises pode aumentar a sensibilidade e especificidade na detecção de impactos ambientais, que muitas vezes não são detectados por métricas ecológicas tradicionais.

- A interação de outros diferentes íons metálicos divalentes com metalotioneínas pode ser aprofundada em simulações futuras, com o uso de abordagens complementares, como a parametrização específica de centros metálicos por meio do MCPB.py (*Metal parameter builder*) do pacote Amber.

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ANEXO

Artigo publicado que compõe a Tese

Oliveira, Nilvea Ramalho; Altafim, Giam Luca; Alves, Aline Vecchio; Choueri, Rodrigo Brasil; Zanette, Juliano; Figueira, Rubens Cesar Lopes; Gallucci, Fabiane. Emergent properties of free-living nematode assemblages exposed to multiple stresses. *Science of the total environment*, v. 912, p. 168790, 2024. <https://doi.org/10.1016/j.scitotenv.2023.168790>

Artigos publicados durante o período de doutorado que não compõem a Tese (julho/2020 – dezembro/2024)

Oliveira, Nilvea Ramalho; Cunha, Beatriz Pereira; Vilas-Boas, Ana Carolina; Gallucci, Fabiane; Monteiro, Luana; Custódio, Márcio Reis; Fonseca, Gustavo. Establishing the baselines of *Diplolaimella dievengatensis* (Nematoda: Monhysteridae) for life-history experiments. *Zoologischer Anzeiger*, v. 293, p. 303-313, 2021. <https://doi.org/10.1016/j.jcz.2021.07.002>

Oliveira, Nilvea Ramalho; Moens, Tom; Fonseca, Gustavo; Nagata, Renato Mitsuo; Custódio, Márcio Reis; Gallucci, Fabiane. Response of life-history traits of estuarine nematodes to the surfactant sodium dodecyl sulfate. *Aquatic Toxicology*, v. 227, p. 105609, 2020. <https://doi.org/10.1016/j.aquatox.2020.105609>