Thaís Fávero Massocato

SUBSÍDIOS PARA BIORREMEDIAÇÃO DE ÁGUAS CONTAMINADAS POR DRENAGEM ÁCIDA DE MINAS UTILIZANDO *ULOTHRIX* SP. (CHLOROPHYTA) PROVENIENTE DA REGIÃO CARBONÍFERA DE SANTA CATARINA.

Dissertação submetida ao programa de Pós-Graduação em Biologia de Fungos, Algas e Plantas da Universidade Federal de Santa Catarina para a obtenção do Grau de Mestre em Biologia de Fungos, Algas e Plantas.

Orientador: Prof. Dr. José Bonomi Barufi
Coorientador: Prof. Dr. Leonardo Rubi Rörig

Florianópolis, 2017

Ficha de identificação da obra elaborada pelo autor,
através do Programa de Geração Automática da Biblioteca Universitária da UFSC.

Fávero Massocato, Thaís
Subsídios para biorremediação de águas contaminadas por drenagem ácida de minas utilizando Ulothrix sp. (chlorophyta) proveniente da região carbonífera de Santa Catarina. / Thaís Fávero Massocato ; orientador, José Bonomi Barufi; coorientador, Leonardo Rubi Rörig - SC, 2017.
72 p.

Dissertação (mestrado) - Universidade Federal de Santa Catarina, , Programa de Pós-Graduação em , Florianópolis, 2017.

Inclui referências.

1. . 2. Biologia de Fungos, Algas e Plantas. 3. Drenagem Ácida de Minas. 4. Algas Filamentosas. 5. Metais. I. Bonomi Barufi, José. II. Rubi Rörig , Leonardo. III. Universidade Federal de Santa Catarina. Programa de Pós Graduação em . IV. Título.

“Subsídios para biorremediação de águas contaminadas por drenagem ácida de minas utilizando *Ulothrix* sp. (*Chlorophyta*) proveniente da região carbonífera de Santa Catarina”

por

Thaís Fávero Massocato

Dissertação julgada e aprovada em sua forma final pelos membros titulares da Banca Examinadora (Port. **01**/PPGFAP/2017) do Programa de Pós-Graduação em Biologia de Fungos, Algas e Plantas - UFSC, composta pelos Professores Doutores:

Banca Examinadora:

Prof. Dr. José Bonomi Barufi

Profa. Dra. Ana Teresa Lombardi

Prof. Dr. Admir José Giachini

Prof. Dr. Paulo Antunes Horta Júnior

Prof. Dr. Elisandro Ricardo Drechsler dos Santos
Coordenador do Programa de Pós-Graduação
em Biologia de Fungos, Algas e Plantas

Florianópolis, **14 de março de 2017**

Aos meus pais que sempre me apoiaram, aos meus irmãos que continuam ao meu lado mesmo longe e aos meus amigos que fazem com que eu me sinta sempre em casa.

AGRADECIMENTOS

Este trabalho não poderia ter sido realizado sem o apoio da minha família. Aos meus pais, Laércio e Cleusa, que sempre me incentivaram nas minhas escolhas e me ensinaram que um profissional não é feito só de títulos, mas de valores. Obrigada por serem meus grandes mestres! Aos meus irmãos, Fábio e Gabriel, que sendo grandes homens me ensinam a ser uma grande mulher.

Às minhas amigas Ellie, Meli e Mari que sempre estiveram ao meu lado desde o início da minha caminhada aqui em Floripa e que se tornaram amigas pra vida toda.

Ao meu grande amor, Matheus, que surgiu no meio desse trabalho e que me deu ainda mais energia pra seguir em frente.

Agradeço infinitamente aos meus amigos da república da agronômica que sempre me ouviam, me ajudavam e faziam comidas deliciosas. E às amigas, Lis e Sara, que foram grandes companheiras nos momentos difíceis. Também agradeço a família do Pólo Áquatico pelos treinos e momentos de descontração os quais me faziam lembrar que meus dias não se resumiam a ficar só no Laboratório. Também agradeço aos amigos de mestrado e da Botânica por todas as horas de aulas, trabalho e bar.

Aos meus amigos e colegas do Laboratório de Ficologia! Pelos momentos de trabalho, pelas risadas na hora do café e por estarem sempre unidos, até nas horas dos perrengues.

Ao meu orientador, José Bonomi Barufi, por sempre me ajudar independente do momento que eu batia à sua porta. Obrigada por sempre me ouvir e levar em consideração minhas ideias, mesmo quando não concordávamos. Aprendemos muito nesses 3 anos.

Ao meu coorientador Leonardo Rörig que nos momentos de desespero me mostrava uma luz no fim do túnel.

Aos Professores Paulo Horta e Nadine Schubert pela ajuda e dedicação profissional, e pelas importantes participações ao longo desse projeto.

Esse trabalho não poderia ter sido realizado sem a participação das seguintes pessoas e laboratórios. Aqui vão os meus sinceros agradecimentos à Eliana e ao Laboratório Central de Microscopia Eletrônica (LCME) pelas análises de Microscopia Eletrônica de Transmissão e Varredura. Agradeço também a Carmen que teve uma participação fundamental neste trabalho me ensinando as técnicas de Microscopia Eletrônica, bem como, a interpretação destes dados. Ao

Laboratório Multiusuário de Estudos de Biologia (LAMEB) onde realizei as leituras no equipamento TECAN. Ao Juliano que me auxiliou com as tão sonhadas análises químicas e ao Laboratório de Espectrometria de Massa e Atômica (LEMA), onde estas foram realizadas. Todos da Universidade Federal de Santa Catarina (UFSC).

Por fim, à Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) pelo apoio financeiro.

RESUMO

Dentre os principais processos oriundos da atividade extrativista de minério de carvão, a produção de Drenagem Ácida de Minas (DAM) é um dos principais fenômenos responsáveis por grande parte da degradação dos recursos hídricos e do solo. Além da elevada concentração de metais e pH entre 2 e 4, os ambientes contaminados por DAM são caracterizados como oligotróficos, tornando-se favoráveis para o crescimento de um pequeno grupo de organismos adaptados a locais extremos. Organismos presentes em locais contaminados por DAM podem ser denominados acidófilos ou ácido-tolerantes, por exemplo, comunidades de algas. O desenvolvimento dessas comunidades em ambientes contaminados por DAM e seu potencial de bioacumulação de metais pesados levam ao interesse no estudo da aplicação de algas no processo de biorremediação. Dentre as espécies de algas isoladas de águas contaminadas por DAM na região de Sideropólis (SC), foi identificada a microalga filamentosa do gênero *Ulothrix*. O presente trabalho avaliou a tolerância e o potencial de bioacumulação relacionados a três metais pesados por parte de *Ulothrix* sp LAFIC 010. Foram realizados experimentos utilizando diferentes concentrações de Zn, Mn e Ni (individualmente e em combinações) no desempenho fisiológico da alga. Também foi testado o potencial de *Ulothrix* sp. LAFIC 010 para bioacumulação desses metais quando mantida em efluente proveniente de local contaminado por DAM. Os resultados mostraram que somente os cultivos submetidos às concentrações acima de 0,27 mM de Zn apresentaram queda na taxa de crescimento e danos aos processos fisiológicos. Para Mn e Ni não houve diferenças significativas entre os tratamentos, mesmo com o aumento de 8 vezes nas concentrações destes metais no meio. Nos cultivos em que os metais foram combinados, somente os tratamentos submetidos às concentrações mais altas de Zn apresentaram danos de crescimento, independente da presença dos demais metais. Nos ensaios de combinações dos metais realizados neste trabalho, foi observado que Mn e Ni, mesmo em altas concentrações, não diminuíram o efeito tóxico de Zn. Isso porque possivelmente os íons de Zn podem apresentar maior afinidade pelos sítios de ligação da alga do que os íons de Mn e Ni. Nos experimentos dos metais separados e em combinações foi observado desarranjo dos cloroplastos nos tratamentos com altas concentrações de Zn. Também foi indicado acúmulo de Mn na parede celular e Ni no vacúolo. Foi constatado crescimento de *Ulothrix* sp. LAFIC 010 em percentual de 5.89 por dia durante 14 dias de cultivo em DAM. A baixa

remoção de metais Zn, Mn e Ni atrelada à biomassa de *Ulothrix* sp. LAFIC 010 cultivada em DAM pode ter sido em virtude da alta concentração de prótons no meio, os quais supostamente competem pelos mesmos sítios de ligação que os metais estudados. Sugere-se que a distribuição desta alga em meio contaminado não é afetada pela concentração de Ni e Mn, ao menos no pH avaliado. Vale ressaltar que as concentrações dos metais usadas neste trabalho foram maiores do que as encontradas em ambientes contaminados por DAM e que, mesmo em concentrações elevadas, a alga *Ulothrix* sp. LAFIC 010 exibiu crescimento. Conclui-se que *Ulothrix* sp. LAFIC 010 tolera e cresce em condições com concentrações de metais maiores do que as reportadas até então para ambientes contaminados por DAM. Entretanto, não foi possível associar a remoção de metais pesados do ambiente à presença das algas, de modo que testes adicionais são necessários para comprovar o potencial de *Ulothrix* sp. LAFIC 010 para biorremediação de efluente contaminado por DAM.
Palavras-chave: *Ulothrix* sp., *biorremediação, zinco, manganês, níquel.*

ABSTRACT

Among the main processes arising from the extractive activity of coal ore, the production of Acid Mine Drainage (AMD) is one of the main phenomena responsible for much of the degradation of water and soil resources. In addition to the high concentration of metals and a pH between 2 and 4, environments contaminated by AMD are characterized as oligotrophic, becoming favorable for the growth of a small group of organisms adapted to extreme conditions. Organisms present in sites contaminated by AMD may be referred to as acidophilic or acid tolerant, where algae communities are a common example. The development of these communities in environments contaminated with AMD and their potential for bioaccumulation of heavy metals leads to interest in the study of algae as an application in the bioremediation process. Among the species of algae isolated with water contaminated by AMD in the region of Sideropólis (SC), a filamentous microalga from the genus *Ulothrix* was identified. The present study evaluated the tolerance and bioaccumulation potential related to three heavy metals by *Ulothrix* sp. LAFIC 010. Experiments were performed using different concentrations of Zn, Mn and Ni (individually and in combination) on the physiological performance of the alga. The potential of *Ulothrix* sp. LAFIC 010 to bioaccumulate these metals when maintained in effluent from a contaminated site by AMD was also evaluated. The results indicated that only the cultures subjected to concentrations above 0.27 mM Zn showed a decrease in growth rate and damage to physiological processes. For Mn and Ni, there were no significant differences between the treatments, even with the 8-fold increase in the concentrations of these metals in the medium. In the cultures with the three metals combination, only the treatments with the highest concentrations of Zn presented reduced growth, regardless of the presence of the other metals. In the tests of combinations of metals performed in this study, it was observed that Mn and Ni, even in high concentrations, did not decrease the toxic effect of Zn. This is because Zn ions may have a higher affinity for algal binding sites than Mn and Ni ions. Chloroplast damaged was observed in treatments with high concentrations of Zn. Mn accumulation was also observed in the cell wall and Ni in the vacuole. The growth rate of Ulothrix sp. LAFIC 010 was verified to be 5.89% (of total biomass) per day during a period of 14 days of being cultured in AMD. The low removal of the metals Zn, Mn and Ni coupled with the low biomass of *Ulothrix* sp. LAFIC 010 cultivated in AMD may have

been due to the high concentration of protons in the medium, which may compete for the same binding site as the metals studied. It is suggested that the distribution of this alga in contaminated medium is not affected by the concentration of Ni and Mn, at least in the evaluated pH. It is worth mentioning that the concentrations of the metals used in this study were higher than those found in environments contaminated with AMD, however even in such high concentrations, the alga Ulothrix sp. LAFIC 010 achieved growth. We conclude that *Ulothrix* sp. LAFIC 010 tolerates and grows under conditions with higher metal concentrations than previously reported for AMD contaminated environments. However, it was not possible to associate the presence of *Ulothrix* sp. LAFIC 010 to the heavy metal removal, and additional experiments are required to ensure the potential for bioremediation of effluents contaminated by AMD.
Key words: *Ulothrix* sp.*, bioremediation, zinc, manganese, nickel.*

LISTA DE FIGURAS

INTRODUÇÃO GERAL e MATERIAIS E MÉTODOS

Figura 1.1 Locais de ligação de metal em uma acélula típica de alga. A letra M do alfabeto representa a espécie de metal (independente do seu estado de oxidação). Notar que os metais se ligam a estruturas tanto interna quanto externamente. Figura obtida em Mehta e Gaur, (2005).....22

Figura 1.2 Microscopia de *Ulothrix* sp. Foto tirada pela autora.....24

Figura 3.1 Indicação do Município de Siderópolis no sul do estado de Santa Catarina; (B) Fotografia de satélite da Lagoa Língua do Dragão, local de coleta da água; (C) e (D) Imagens da margem da Lagoa Língua do Dragão. Notar aspecto amarelado da água decorrente da atuação da DAM; (E) Crescimento de biofilme algal em água contaminada por DAM, apresentando predominância de *Ulothrix* sp.....26

Figura 3.2 Delineamento experimental para o teste de tolerância da alga *Ulothrix* sp. LAFIC 010 cultivada com a presença de diferentes concentrações dos metais zinco, manganês e níquel, durante 10 dias.....28

Figura 3.3 Delineamento experimental para o teste de tolerância de *Ulothrix* sp. LAFIC 010 submetida aos metais zinco, manganês e níquel em 27 combinações, durante 8 dias.....30

Figura 3.4 Processo de montagem para terceiro experimento; (A) Escalonamento do cultivo de *Ulothrix* sp. LAFIC 010; (B) Pré-filtração da água contaminada por DAM; (C) tratamentos: DAM sem alga e DAM com alga.....33

CAPÍTULO 1

Figure 5.1 Physiological responses of *Ulothrix* sp. LAFIC 010 to the metals Zn (A, B, C), Mn (D, E, F) and Ni (G, H, I).....49

Figure 5.2 Filaments of *Ulothrix* sp. LAFIC 010 using SEM.....51

Figure 5.3 Transmission electron microscopy of *Ulothrix* sp. LAFIC 010 specimens cultivated in different heavy metal concentrations.....52

Figure 5.4 Physiological responses of *Ulothrix* sp. LAFIC 010 to varying concentrations and combinations of Zinc, Manganese and Nickel, totaling 27 treatments.....55

Figure 5.5 Transmission electron microscopy of *Ulothrix* sp. LAFIC 010 specimens cultivated under different concentrations and combinations of heavy metals 58

Figure 5.6 Chemical analysis of the concentrations of Zinc (A), Manganese (B) and Nickel (C) in treatments with and without *Ulothrix* sp. LAFIC 010 biomass over the course of 14 days.....60

Figure 5.7 Percentage of removal of Zn, Mn, and Ni by *Ulothrix* sp. LAFIC 010 over a a period of 14 days in Acid Mine Drainage.....61

Figure 5.8 Energy-dispersive X-ray spectroscopy of cell wall components from *Ulothrix* sp. LAFIC 010 cultivated for 14 days in water contaminated with Acid Mine Drainage.....62

Figure 5.9 Transmission electron microscopy of *Ulothrix* sp. LAFIC 010 cultivated in AMD.....63

LISTA DE TABELAS

MATERIAIS E MÉTODOS

Tabela 3.1 Concentração total de metais pesados ($mg.L^{-1}$) em amostras de água de Siderópolis e Florianópolis, SC. *Valores máximos ($mg.L^{-1}$) de metais pesados permitidos pela Resolução Conama 357/2005 - Água doce. Tabela apresentada em trabalho de.....28

CAPÍTULO 1

Table 5.1 Summary of one-way repeated measures analysis of variance (ANOVA) of physiological responses of *Ulothrix* sp. to different concentrations of Zinc, Manganese and Nickel during toxicity experiment. $\alpha < 0.05$. N=5. Bold indicates significance when $P < 0.05$.....48

Table 5.2 Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDS) data resulting from the exposure of *Ulothrix* sp. LAFIC 010 to different concentrations of Zinc, Manganese and Nickel during toxicity experiment. The values correspond to the mean (±SD) of compound %. (*): samples where elements were recorded to only one replicate avoiding calculation of SD.....50

Table 5.3 Summary of three-way repeated measures analysis of variance (ANOVA) of physiological responses of *Ulothrix* sp. LAFIC 010 to treatments with combinations of the heavy metals Zinc, Manganese and Nickel during toxicity experiment. $\alpha < 0.05$. N=5. Bold indicates significance when $P < 0.05$.....54

Table 5.4 Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDS) data resulting from the exposure of *Ulothrix* sp. LAFIC 010 to different concentrations and combinations of Zinc, Manganese and Nickel. The values correspond to the mean (±SD) of compound %. (*): samples where elements were recorded to only one replicate avoiding calculation of SD.....56

Table 5.5 Summary of results of one-way repeated measures analysis of variance (ANOVA) of chemical water analysis comparing the concentration of the metals Zinc, Manganese and Nickel in AMD water

with and without *Ulothrix* sp. LAFIC 010 biomass. N=3. $\alpha < 0.05$. Bold indicates significance when $P < 0.05$.....60

SUMÁRIO

1. INTRODUÇÃO GERAL.....18
2. OBJETIVOS.....25
2.1 Objetivo Geral.....25
2.2 Objetivos Específicos.....25
3. MATERIAL E MÉTODOS.....25
3.1 Área de coleta de água e biofilme algal.....25
3.2 Coleta, identificação e cultivo de biofilme algal.....27
3.3 Testes de tolerância.....27
3.4 Avaliação do Potencial de Remoção dos metais zinco, manganês e níquel.....30
3.5 Alterações ultraestruturais em *Ulothrix* sp. LAFIC 010.....33
3.7 Análises estatísticas.....35
4. REFERÊNCIAS.....35
5. CAPÍTULO 1 39
5.1 Introduction.....40
5.2 Material and Methods.....44
5.3 Results.....48
5.4 Discussion.....63
5.5 References.....70
6. CONSIDERAÇÕES FINAIS.....78

1. INTRODUÇÃO GERAL

Diante do acelerado crescimento populacional e consequente exploração dos recursos naturais, grande atenção tem sido dada aos problemas ambientais causados por ações antrópicas nas últimas décadas. Diversos setores industriais são responsáveis pelos fenômenos de degradação e comprometimento do solo, contaminação das águas superficiais e poluição da atmosfera. Dentre as principais atividades poluidoras estão a indústria petroquímica, agricultura intensiva, indústria têxtil e mineração (Freitas *et al*., 2011; Moura, 2014).

Responsável por 99,98 % das atividades extrativistas de carvão mineral do Brasil, os estados do sul do Brasil exercem importante papel no crescimento econômico do país. Juntamente com o estado do Rio Grande do Sul, a região carbonífera de Santa Catarina deu suporte para a intensa extração de minério de carvão iniciada em meados do século 19 (Barbosa, 2001). Desde então, a atividade tem gerado profundas modificações no cenário ambiental destes locais provocando impactos no solo, poluição do ar e contaminação dos sistemas hídricos (Martins Pompêo *et al*., 2004). Tendo em vista que a região carbonífera é portadora de três importantes bacias hidrográficas (bacia do Rio Araranguá, bacia do Rio Tubarão e bacia do Rio Urussanga), as quais garantem recursos hídricos para uma série de cidades e atingem aproximadamente 413 mil habitantes (Castilhos e Fernandes, 2011), maior atenção deve ser dada aos problemas de poluição ambiental dos recursos hídricos decorrentes das práticas extrativistas, de modo a minimizar os impactos negativos e garantir a qualidade dos mesmos.

Dentre os principais processos oriundos da atividade extrativista de minério de carvão, a Drenagem Ácida de Minas (DAM) é um dos fenômenos responsáveis por grande parte da degradação dos recursos hídricos e do solo (Gazea *et al*., 1996). Este fenômeno é descrito para diversas regiões do mundo em áreas de extração de ouro, cobre, níquel e ferro (Akcil e Koldas, 2006; Novis e Harding, 2007). Os efeitos da DAM nos sistemas lóticos implicam em alterações multifatoriais na estrutura do ambiente contaminado (Gray, 1997). Alterações nos aspectos químicos, físicos, biológicos e ecológicos do ecossistema são citados por modificações, por exemplo, como aumento da acidez, diminuição do pH, aumento na solubilização e concentração de metais, aumento da turbidez da água, perda de espécies sensíveis, modificação do habitat natural (Gray, 1997). A contaminações de ecossistemas

lóticos por DAM tem sido citada em países como Irlanda, Estados Unidos, Nova Zelândia, África do Sul (Gray, 1997; Smucker *et al*., 2014; Novis and Harding, 2007; Oberholster *et al*., 2014), dentre outros lugares, o que torna a contaminação por DAM um problema ambiental em escala mundial. Os processos descritos para controle e tratamentos de ambientes contaminados por DAM envolvem atividades de remedição por métodos químicos, físicos e biológicos (Mello *et al*., 2014). No âmbito químico, por exemplo, busca-se neutralizar a acidez da água através de procedimentos de alcalinização (Mello *et al*., 2014). Uma das estratégias físicas de controle da DAM é a tentativa de isolar a área impactada para que não ocorra a contaminação em outros locais não impactados (Mello *et al*., 2014). A remediação por seres vivos é chamada de biorremediação e consiste na aplicação de organismos resistentes à acidez e alta concentração de metais e que sejam capazes diminuir e/ou reverter o processo de contaminação (Malik, 2004).

A produção de DAM é decorrente da oxidação de sulfetos presentes nas rochas quando expostos à água e ao oxigênio. A pirita (FeS_2) é o principal mineral sulfetado responsável pela produção de DAM. A formação de DAM é decorrente de um série de reações de oxidação de sulfetos, que em parte, podem ser catalisadas por microrganismos que apresentam elevada atividade, principalmente em pH inferior a 3,5 (Moura, 2014). As sequências de reações que formam a DAM estão representadas a seguir (Mello *et al*., 2014):

$$FeS_{2(s)} + H_2O_{(l)} + 7/2\ O_{2(g)} \leftrightarrow Fe^{2+}{}_{(aq)} + 2SO_4{}^{2-}{}_{(aq)} + 2H^+{}_{(aq)} \quad [1.1]$$

$$Fe^{2+}{}_{(aq)} + 1/4O_{2(g)} + H^+{}_{(aq)} \leftrightarrow Fe^{3+}{}_{(aq)} + 1/2H_2O \quad [1.2]$$

$$2\ Fe^{3+}{}_{(aq)} + FeS_{2(s)} \leftrightarrow 3\ Fe^{2+}{}_{(aq)} + 2\ S^O{}_{(s)} \quad [1.3]$$

$$2\ S^O{}_{(s)} + 12\ Fe^{3+}{}_{(aq)} + 8\ H_2O_{(l)} \leftrightarrow 12\ Fe^{2+}{}_{(aq)} + 2\ SO_4{}^{2-}{}_{(aq)} + 16\ H^+ \quad [1.4]$$

Etapa em pH maior que 3, pois é quando ocorre a oxidação do hidróxido férrico ($Fe(OH)_3$).

$$2\ SO(s) + O_2(g) + 2H_2O \leftrightarrow SO_4{}^{2-}(aq) + 4\ H^+(aq) \quad [1.5]$$

$$FeS_{2(s)} + 14\ Fe^{3+}{}_{(aq)} + 8\ H_2O_{(l)} \leftrightarrow 15\ Fe^{2+}{}_{(aq)} + 2\ SO_4{}^{2-}{}_{(aq)} + 16\ H^+{}_{(aq)} \quad [1.6]$$

As equações [5] e [6] são catalisadas principalmente pela bactéria *Acidithiobacillus ferrooxidans*.

Durante a oxidação de sulfetos, há liberação de alta concentração de íons H^+, resultando em baixo pH, e alta concentração de metais dissolvidos, dentre eles os elementos Fe, As, Cu, Zn, Al e Mn (Novis e Harding, 2007). A elevada concentração de íons H^+ que torna o ambiente fortemente ácido e o aumento na concentração de metais comprometem o equilíbrio físico-químico natural do meio aquático (Novis e Harding, 2007). Dessa forma, a poluição causada por DAM é provavelmente o impacto mais significativo decorrente da ação extrativista de minério na Região Carbonífera de Santa Catarina (Moura, 2014).

Em ambientes contaminados por DAM, além da elevada concentração de metais e pH tipicamente entre 2 e 4, a disponibilidade de nutrientes é considerada baixa, sendo estes locais caracterizados como oligotróficos (Johnson e Hallberg, 2005). Nesse sentido, ambientes impactados por DAM tornam-se favoráveis para o crescimento de um pequeno grupo de organismos adaptados a situações extremas, denominados acidófilos ou ácido-tolerantes. Dentre os grupos de organismos capazes de crescer neste tipo de ambiente estão bactérias, fungos e comunidades de algas (Novis e Harding, 2007). Em trabalho de Druschel *et al.* (2004), a arquea *Ferroplasma acidarmano* foi isolada de áreas impactadas por DAM na Califórnia. Algas do gênero *Chlamydomonas* foram identificadas em ambientes fortemente ácidos como lagos ácidos de mineração e lagoas vulcânicas (Silva, 2011). Stevens *et al*. (2001) descreveram a presença de algas dos gêneros *Euglena*, *Chlamydomonas*, *Microspora* e predominância de *Klebsormidium* em águas contaminadas por DAM em Ohio, nos Estados Unidos. Em trabalho realizado na Região Carbonífera de Santa Catarina, Freitas et. al (2011) fizeram o levantamento de espécies de algas presentes em ambiente contaminado por DAM. Dentre os gêneros encontrados foram identificados *Microspora*, *Eunotia*, *Euglena*, *Mougeotia* e *Frustulia*.

De acordo com Gross (2000), para crescer em ambientes ácidos, com elevada concentração de H^+, algas acidófilas exibem mecanismos de redução do influxo de prótons nas células, aumento na eficiência das bombas de prótons e mecanismo para concentração de CO_2 para fotossíntese. Estes mecanismos permitem que o citoplasma permaneça com pH em nível alcalino, apropriado para o funcionamento de enzimas dependentes de pH, as quais podem ser desnaturadas quando os valores desse parâmetro são reduzidos. No entanto, segundo Spijkerman *et al.*

(2007), para superar o gradiente de prótons e uso de mecanismos que permitem manter o pH dentro do limite normal de funcionamento celular, há maior demanda energética e metabólica, o que implica em decréscimo na taxa fotossintética de algas que se desenvolvem em meio ácido.

Além do baixo pH, a alta concentração de metais pesados também é fator limitante para o desenvolvimento de organismos em ambientes hostis, como os contaminados por DAM (Novis e Harding, 2007). Os mecanismos de ação e as respostas fisiológicas podem ocorrer de forma isolada, causados por um agente único principal, como o pH, ou pela interação entre diferentes fatores impactantes, como por exemplo a variação de pH e da composição dos metais presentes no meio (Oberholster *et al*., 2014). Estudos tem sido feitos para se entender se a distribuição das espécies de algas presentes em águas contaminadas por DAM são relacionadas ao gradiente de pH e/ou às concentrações dos metais. Rousch e Sommerfeld (1999) avaliaram se o pH ou as concentrações de Mn e Ni, separadas ou em combinações, foram responsáveis pela distribuição de espécies de algas. Os autores concluíram que as elevadas concentrações de metais podem ser mais importantes na determinação da distribuição de algas do que o pH em sistemas aquáticos afetados por DAM. Entretanto, a toxicidade dos metais é pH-dependente. Segundo Oberholster *et al.,* (2014), algas da espécie *Klebsormidium klebsii*, *Microspora tumidula* e *Oedogonium crassum* isoladas de locais impactados por DAM apresentaram bioacumulações dos metais Al, Fe, Mn e Zn variáveis entre os pHs 3, 5 e 7, indicando que a bioacumulação dos metais varia com o gradiente de pH.

Além pH, a presença de outros metais no meio influenciam na captação dos metais pela alga. De acordo com Mehta e Gaur (2005), a presença de um metal pode alterar a distribuição de outros metais entre os componentes celulares, interferindo na captação desses elementos pela alga. Chong *et al*. (2000) mostraram que no caso de 10 cepas de algas cultivadas em solução contendo Zn e Ni, o primeiro metal provocou queda na bioacumulação de Ni quando comparada à mesma em meio que não continha Zn. No entanto, ainda grande parte dos trabalhos analisa a eficiência na captação dos metais isoladamente, o que diverge de situações reais de poluição, as quais normalmente envolvem a presença de múltiplos metais sob influências das interações de aspectos físicos da água, como o pH (Zeraatkar *et al*., 2016).

Sabe-se que são dois os principais mecanismos fisiológicos utilizados por algas extremófilas para resistir a tais condições com elevada concentração de metais: a adsorção dos metais por ligantes presentes na superfície celular e a captação ativa de metais pela célula (Fig. 1.1).

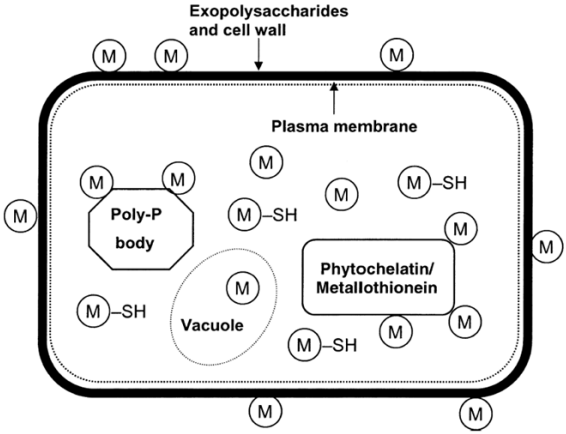

Figura.1.1 Locais de ligação de metal em uma célula típica de alga. A letra M representa a espécie de metal (independente do seu estado de oxidação). Notar que os metais se ligam a estruturas tanto interna quanto externamente. Figura obtida em Mehta e Gaur (2005).

A adsorção é o processo em que a célula é capaz de complexar metais na superfície externa por meio da ligação dos íons metálicos à parede celular, à membrana e polissacarídeos, impedindo a entrada dos metais no interior do citoplasma (Mehta e Gaur, 2005). Por outro lado, a captação ativa de metais pela célula é decorrente de mecanismos de seu sequestro para interior da célula. Os íons metálicos são associados a moléculas intracelulares, como fitoquelatinas e metalotioneínas, que atuam como ligantes citoplasmáticos na complexação dos metais. Os metais também podem ser incorporados para o interior de organelas, como tilacóides e vacúolos (Mehta e Gaur, 2005; Soldo *et al*., 2005). No entanto, segundo Mehta e Gaur (2005), a presença de um metal pode mudar e interferir na distribuição de outros metais dentre os componentes celulares. A adsorção e a captação ativa de metais pelas células são mecanismos que podem operar em conjunto ou isoladamente para proteger as células contra os efeitos nocivos dos íons metálicos. O principal objetivo de ambas as estratégias acima citadas é evitar a

exposição e dano de estruturas celulares sensíveis, como DNA e proteínas, aos metais reativos (Soldo *et al.*, 2005). Dessa forma, a capacidade de crescimento e desenvolvimento de comunidades algais em ambientes contaminados, bem como a captação de metais, leva ao estudo da aplicação destes organismos na prática de biorremediação.

Atualmente, o processo de biorremediação é uma alternativa para a descontaminação de um ambiente, propondo o cultivo de organismos resistentes em locais poluídos visando a remoção completa ou parcial dos contaminantes (Vidali, 2001). O uso de organismos na biorremediação tem sido aplicado ao tratamento de poluentes tais como águas residuais, derramamento de petróleo e efluentes tóxicos nos diversos setores da indústria (Macek *et al*., 2000). Dentre eles, o uso para descontaminação de águas contaminadas por DAM tem sido foco de vários trabalhos (Das *et al*., 2009; Orandi *et al*., 2012; Stevens *et al*., 2001). Em pesquisa desenvolvida por Rose *et al.*, (1998), o uso de *Spirulina* sp. como parte do processo de tratamento de água contaminada por DAM mostrou resultados otimistas para o uso da microalga a fim de concentrar e captar os metais dissolvidos. Em trabalho de Oberholster *et al*. (2014) observou-se que três algas filamentosas (*Oedegonium crassum*, *Klebsormidium klebsii* e *Microspora tumidula*) isoladas de ambientes contaminados por DAM, e posteriormente mantidas em laboratório, exibiram capacidade de bioacumulação dos metais alumínio, ferro, manganês e zinco quando cultivadas em água contaminada por DAM.

Juntamente à desposluição e restauração de um ambiente contaminado por metais, a prática de biorremediação pode ser atrelada à recuperação dos metais bioacumulados na biomassa algal por processo denominado dessorção (Volesky, 2007). De acordo com Volesky (2007) a recuperação dos metais pela biomassa de um organismo seria o último passo de todo o processo de descontaminação de um efluente. No entanto, a purificação e obtenção do metal ainda agregam dificuldade e elevado custo.

Para a elaboração de um projeto de biorremediação, é importante que seja feita a identificação da comunidade microbiana que ocorra naturalmente no ambiente em que se visa o tratamento (Uhlik *et al*., 2013). Dessa forma, organismos ocorrentes no local podem ser usados para o tratamento. Ademais, alguns aspectos devem ser analisados para a escolha do organismo e o método de biorremediação. Tais características são: capacidade de crescimento e assimilação de metais, facilidade de manipulação e manutenção da cultura, tempo de

crescimento do organismo, capacidade competitiva com outros organismos, além da possível obtenção da biomassa para fins biotecnológicos (Lourenço, 2006).

Segundo Novis e Harding (2007), *Ulothrix* está entre os principais gêneros de algas filamentosas que ocorrem naturalmente em ambientes fortemente ácidos contaminados por DAM. Algas desse gênero pertencem ao filo Chlorophyta, e possuem talo filamentoso unisseriado com um cloroplasto parietal (Bicudo e Menezes, 2006). De acordo com Bicudo e Menezes (2006), elas podem também apresentar talo fixo ao substrato quando jovens, e livres e flutuantes quando adultas, sendo considerado um gênero cosmopolita (Fig. 1.2).

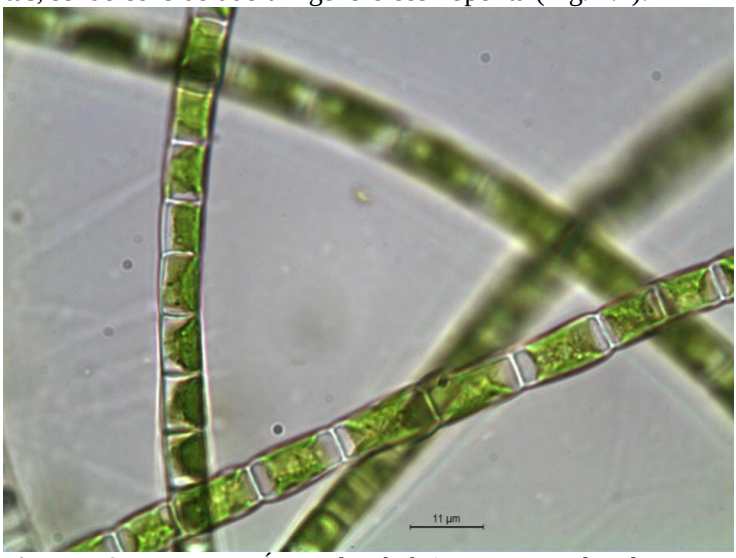

Figura 1. 2 Microscopia Óptica de *Ulothrix* sp. Foto tirada pela autora.

Estudos têm apontado o potencial de *Ulothrix* sp. como organismo biorremediador na captação de metais pesados (Orandi e Lewis, 2013; Lawrence *et al*., 1998). Em trabalho levantado por Paknikar *et al*. (2003), foi proposto o uso de biomassa liofilizada de *Ulothrix* para bioadsorção e remoção de metais em soluções. Orandi *et al*. (2012) observaram que no biofilme de microrganismos isolados de local contaminado por DAM de minas de cobre predominava *Ulothrix* sp. Esses autores observaram também que a capacidade de remoção de metais como Cu, Ni, Mn, Zn, Sb, Se, Co, Al, variou entre 20-50% indicando que esta microalga apresenta características de bioacumulação

de metais pesados. No entanto, poucos estudos foram realizados analisando as respostas fisiológicas dessa alga frente aos metais zinco, manganês e níquel cujas concentrações são elevadas em ambientes contaminados por DAM. Ademais, trabalhos de interações entre os metais e seus efeitos nos organismos são escassos, porém de forte importância uma vez que simulam um real efluente contaminado. Outro aspecto relevante para o entendimento e seleção de uma alga como organismo biorremediador é a forma e localização celular na qual o organismo acumula os metais superficialmente ou em seu interior.

2. OBJETIVOS

2.1 Objetivo Geral

A presente dissertação propõe incrementar o conhecimento a respeito do potencial de *Ulothrix* sp. LAFIC 010 para o tratamento de águas contaminadas por Drenagem Ácida de Minas, avaliando a tolerância da espécie e a sua capacidade de biorremoção dos metais zinco, manganês e níquel.

2.2 Objetivos Específicos

- Avaliar os efeitos de Zn, Mn e Ni na fisiologia de *Ulothrix* sp. LAFIC 010, quando cultivada em meio com metais diluídos individualmente e em combinações;
- Avaliar alterações ultraestruturais das células de *Ulothrix* sp. LAFIC 010, em meio com adição de Zn, Mn e Ni;
- Inferir se *Ulothrix* sp. LAFIC 010 bioacumula os metais Zn, Mn e Ni, e sua eventual localização intracelular;
- Avaliar o crescimento, concentração de pigmentos (clorofila *a*, *b* e carotenóides) e alterações ultraestruturais de *Ulothrix* sp. LAFIC 010 cultivada em meio contaminado por DAM.

3. MATERIAL E MÉTODOS

3.1 Área de coleta de água e biofilme algal

O local de coleta da água e da alga chama-se Lagoa Língua do Dragão e situa-se no município de Siderópolis na região banhada pela bacia do Rio Araranguá, localizado a sudeste do estado de Santa Catarina na Região Carbonífera (Fig. 3.1). Segundo Freitas (2011), o rio

Araranguá apresenta elevado comprometimento qualitativo da água resultante de atividades antrópicas como derramamento de esgoto doméstico, efluentes industriais, contaminação por agrotóxicos e, principalmente, por resíduos da extração de carvão. O principal resíduo decorrente da atividade de mineração é denominado Drenagem Ácida de Minas (DAM).

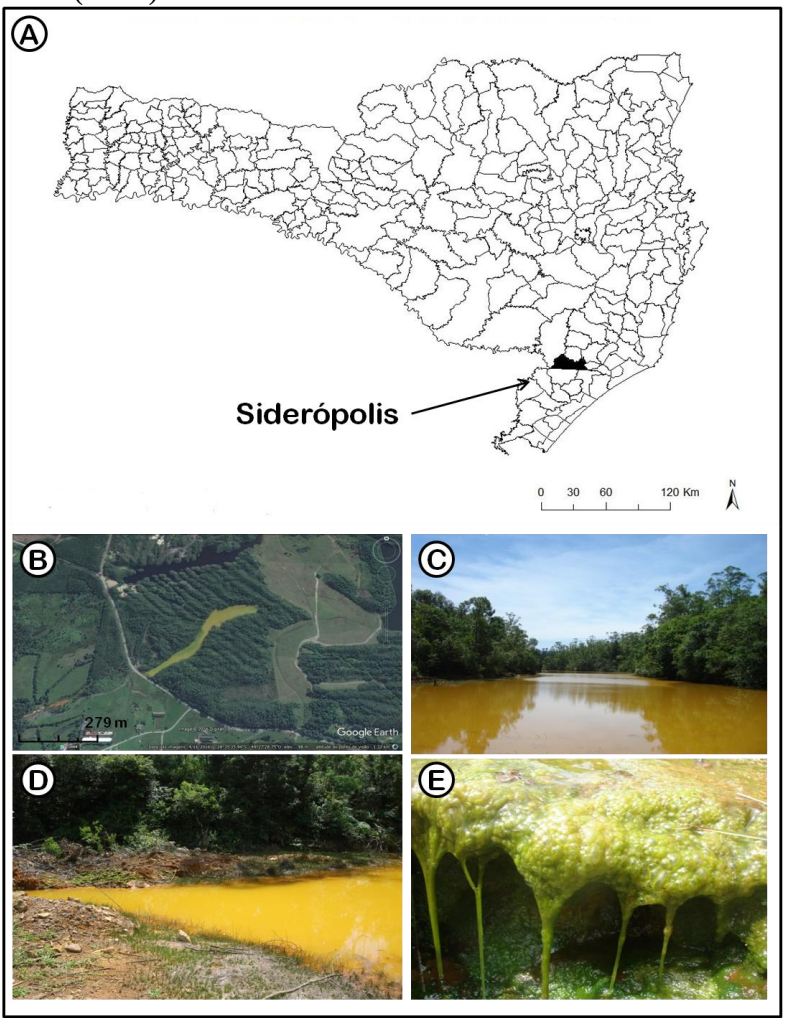

Figura 3.1 Indicação do Município de Siderópolis no sul do estado de Santa Catarina; (B) Fotografia de satélite da Lagoa Língua do Dragão, local de coleta da água; (C) e (D) Imagens da margem da Lagoa Língua do

Dragão. Notar aspecto amarelado da água decorrente da atuação da DAM; (E) Crescimento de biofilme algal em água contaminada por DAM, apresentando predominância de *Ulothrix* sp.

3.2 Coleta, identificação e cultivo de biofilme algal

A microalga coletada em águas contaminadas por DAM na região de Sideropólis (28°37'S, 49°24'O), foi identificada como sendo *Ulothrix* sp., isolada em 2012, e tem sido cultivada no Laboratório de Ficologia do Departamento de Botânica da Universidade Federal de Santa Catarina como *Ulothrix* sp. LAFIC 010. As culturas isoladas foram mantidas em Meio Ácido Modificado (MAM) (Olaveson and Stokes, 1989) previamente autoclavado. A identificação das amostras coletadas foi feita ao microscópio óptico, por meio de fotomicrografias e por consulta de obras taxonômicas (Bicudo e Menezes, 2006) e especialistas da área.

3.3 Testes de tolerância

A fim de se conhecer efeitos fisiológicos e morfológicos dos metais zinco (Zn), manganês (Mn) e níquel (Ni) em *Ulothrix* sp. LAFIC 010, foram realizados ensaios de tolerância com os metais individualmente e em seguida em diferentes combinações. Os metais citados acima foram escolhidos uma vez que apresentaram altas concentrações em água contaminada por DAM, segundo o trabalho de Moura (2004) (Tabela 3.1), que coletou amostras no mesmo local do presente estudo.

Tabela 3.1 Concentração total de metais ($mg.L^{-1}$) em amostras de água de Siderópolis e Florianópolis, SC. *Valores máximos ($mg.L^{-1}$) de metais permitidos pela Resolução Conama 357/2005 - Água doce. Tabela apresentada em trabalho de Moura (2014).

Metais (mg.kg ¹)	Siderópolis	Florianópolis	VMP*
Cd	<0.001	<0.001	0.001
Cu	<0.005	<0.005	_
Fe	140.8	<0.120	_
Mn	20.1	<0.01	0.1
Ni	0.371	<0.02	0.025
Pb	<0.008	<0.008	0.01
Zn	36.02	<0.116	0.18

Primeiramente foram realizados testes com cada um dos metais em seis concentrações diferentes (Fig. 3.2).

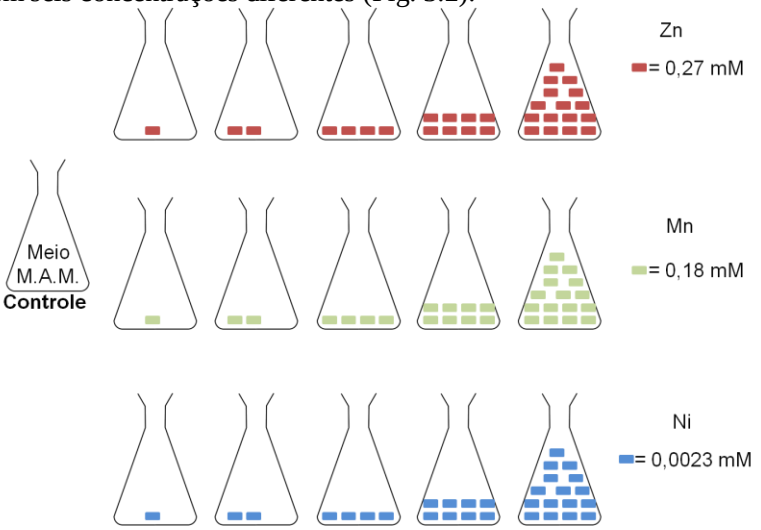

Figura 3.2 Delineamento experimental para o teste de tolerância da alga *Ulothrix* sp. LAFIC 010 cultivada com a presença de diferentes concentrações de Zn, Mn e Ni durante 10 dias.

As concentrações de Zn, Mn e Ni foram respectivamente: 0 (controle); 0,27; 0,55; 1,1; 2,2; 4,4 mM; 0 (controle), 0,18; 0,37; 0,73;

1,46; 2,9 mM; e 0 (controle); 0,0023; 0,0046; 0,0092; 0,0185; 0,0369 mM. As concentrações foram determinadas a partir do trabalho de Moura (2004). As soluções foram preparadas a partir de solução estoque de sulfato de zinco, sulfato de manganês e sulfato de níquel, sendo as concentrações ajustadas em frascos de 250 mL contendo meio de cultura MAM. Os testes de tolerância foram realizados em microplacas de poliestireno de 12 cavidades com 3 mL de solução em cada poço. Quantidades apropriadas de filamentos concentrados de *Ulothrix* sp. LAFIC 010 foram inoculados para se obter uma concentração inicial em cada cultura correspondente de 0,1540 de Densidade Óptica de Clorofila a 680 nm.

No presente estudo, foram avaliadas as taxas de crescimento e fisiologia da microalga *Ulothrix* sp. LAFIC 010 após 10 dias de exposição aos metais individualmente. Para cálculo da taxa de crescimento, acompanhou-se a Densidade Óptica (λ= 680 nm) e Fluorescência da clorofila *a*, medidas diariamente por meio do equipamento TECAN (RCHISTO infinite M200).

Ao início e término do experimento foram realizadas medidas de análise da fotossíntese a partir do parâmetro de rendimento quântico efetivo do fotossistema II [Y(II)] com o uso de um fluorômetro de pulso de amplitude modulada (Junior PAM, Walz, Germany). A obtenção do Y(II) foi gerada pelo equipamento por meio da seguinte equação: $Y(II) = (F_m'-F)/F_m'$, em que F = Fluorescência em estado estacionário, e F_m' = Fluorescência máxima após pulso de saturação. Também foram realizadas análises das algas em Microscopia Eletrônica de Varredura juntamente à Espectroscopia de Energia Dispersiva e Microscopia Eletrônica de Transmissão (mais detalhes abaixo).

Após os ensaios com os metais ajustados separadamente foram realizados experimentos com os três metais em diferentes combinações. As concentrações 0; 0,55; e 4,4 mM, para zinco; 0; 0,37; e 2,9 mM, para manganês; e 0; 0,0046; e 0,0369 mM, para níquel foram definidas totalizando 27 combinações (Fig. 3.3) As concentrações acima citadas foram selecionadas em virtude do valor encontrado desses metais em água contaminada por DAM, segundo o trabalho de Moura (2014): 0,55 mM para Zn, 0,37 mM para Mn e 0,0046 mM para Ni, e as maiores concentrações abordadas no primeiro experimento: 4,4 mM para Zn, 2,9 mM para Mn e 0,0369 mM para Ni.

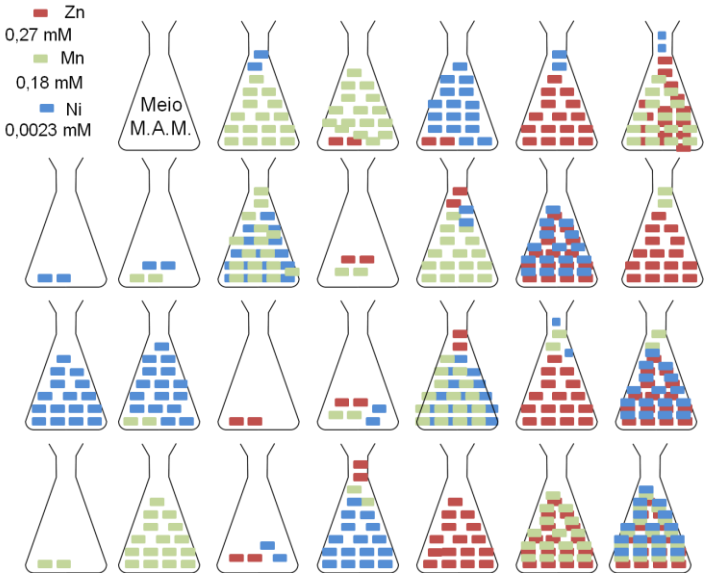

Figura 3.3 Delineamento experimental para o teste de tolerância de *Ulothrix* sp. LAFIC 010 submetida aos metais zinco, manganês e níquel em 27 combinações, durante 8 dias.

Pretendeu-se com este experimento analisar os efeitos dos metais na alga e suas possíveis interações (sinergias e/ou antagonismos). Este ensaio foi conduzido de maneira similar ao experimento anterior, com 5 réplicas para cada tratamento durante 8 dias.

3.4 Avaliação do Potencial de Remoção dos metais zinco, manganês e níquel

Posteriormente à avaliação do crescimento de *Ulothrix* sp. LAFIC 010 cultivada em diferentes concentrações de metais, foi realizado um terceiro experimento para analisar o potencial da bioacumulação de Zn, Mn e Ni pela alga. O experimento consistiu em dois tratamentos: **1-** água contaminada com DAM inoculada com *Ulothrix* sp. LAFIC 010, **2-** apenas água contaminada com DAM (Fig. 3.4). Para tal finalidade, foi coletada água poluída por DAM em Siderópolis e transportada em bombonas de plástico de volume de 50 L

para o Laboratório de Ficologia, onde foram mantidas em temperatura ambiente por duas semanas. Em laboratório, a água coletada foi filtrada (0,45 □m) antes do início dos experimentos. As culturas em crescimento previamente à inoculação foram mantidas sob parâmetros controlados de luz e temperatura como descrito acima. A cultura preliminar foi mantida durante 20 dias para se obter uma biomassa fresca concentrada de 2,8 $g.L^{-1}$ (i.e. totalizando 22,4 g de biomassa algal). Os filamentos foram coletados, pesados em balança analítica, e 4,2 g de biomassa fresca foram obtidos e transferidos para cada réplica de água contaminada por DAM. O experimento foi realizado em triplicata e os frascos foram mantidos em agitador orbital de mesa (modelo Tecnal te 1400) a 110 rpm sob iluminação de 45 $\mu mol.m^{-2}.s^{-1}$ e temperatura controladas em 25 ± 2ºC, fotoperíodo 12 horas claro/escuro durante 14 dias.

As avaliações do estado fisiológico da alga foram realizadas ao início e término do experimento. Para verificar mudanças morfofisiológicas foram realizadas Microscopia Eletrônica de Varredura (MEV), acoplada com Espectroscopia de Energia Dispersiva (EDS), e Microscopia Eletrônica de Transmissão. Medidas de crescimento por biomassa seca, concentração de clorofilas *a* e *b* e carotenóides totais também foram realizadas. O acompanhamento do crescimento da microalga foi realizado por meio do cálculo da porcentagem de crescimento (Lignell e Pedersén, 1989) usando o peso de biomassa seca inicial e final, a partir da seguinte equação: % de crescimento = 100 $[(B_F/B_I)^{1/t} -1]$ em que B_I corresponde à biomassa seca inicial, B_F é a biomassa seca final e t é o tempo de medida final após o começo do teste.

A análise de pigmentos foi realizada por meio da concentração de clorofila *a*, *b* e carotenóides extraídos em metanol seguindo o protocolo de Wellburn (1994). Nas análises, foram utilizadas 0,01 ± 0,005 g de biomassa (n=3) oriunda do tratamento contendo alga e biomassa previamente inoculada na água contaminada por DAM, para comparação das concentrações de pigmentos pré e pós-cultivo em DAM. Foi adicionado 1,0 mL de metanol com pureza analítica (P.A). em cada amostra e mantida por 1h:30min no escuro a 4 °C. Posteriormente, as amostras foram centrifugadas a 4000 rpm por 20 minutos e o sobrenadante recolhido para leitura em espectrofotômetro TECAN (RCHISTO infinite M200), nos comprimentos de onda de 470, 653, 666 e 750 nm. As leituras nos comprimentos de onda 470, 653 e 666 foram subtraídas pelas leituras nos comprimentos de 750 a fim de

evitar ruídos. As concentrações das clorofilas foram calculadas, utilizando-se as equações [3.1] e [3.2], e de carotenóides por meio da equação [3.3], de acordo com Wellburn (1994).

Clorofila *a* = (15,65 x A666) – (7,34 x A653)
[3.1]
Clorofila *b* = (1,67 x A664) – (7,60 x A647) – (24,52 x A630)
[3.2]
Carotenóides Totais = [(1000 x A470) - (2,86 x Ch *a*) - (12,2 x Ch *b*)] / 221 [3.3]

Onde:
A666: absorbância da amostra no comprimento de onda de 666 nm;
A653: absorbância da amostra no comprimento de onda de 653 nm;
A470: absorbância da amostra no comprimento de onda de 470 nm

Para avaliar a bioacumulação de Zn, Mn e Ni por *Ulothrix* sp. LAFIC 010, amostras de água obtidas durante a condução do experimento foram analisadas por espectrometria induzida de emissão atômica com plasma acoplado (ICP-MS - Perkin Elmer, modelo NexIon 300D, Shelton, USA). As análises de água foram feitas imediatamente após a inoculação da biomassa (tempo 0) e após 1, 6, 24, 168 e 336 horas. Estes intervalos de tempo foram escolhidos para avaliar os dois principais passos da biossorção: adsorção e captação ativa dos metais pela biomassa de *Ulothrix* sp. LAFIC 010. Para tal finalidade, 15 mL de água de cada réplica foram filtrados (0,45 □m). Todas as amostras foram mantidas a 4°C até o momento da análise. As concentrações foram mensuradas mediante soluções padrão com as seguintes concentrações: 1008 ± 5 $mg.L^{-1}$ para Zn, 1000 ± 4 $mg.L^{-1}$ para Mn e 1000 ± 3 $mg.L^{-1}$ para Ni. As análises foram conduzidas na Central de Análises Químicas no Departamento de Química (CFM, UFSC). A amostragem, preservação e métodos analíticos foram realizados de acordo com métodos padrão (APHA / AWWA, 1998). Todas as análises de cada amostra foram feitas em triplicata. Foram obtidas as concentrações dos metais presentes na água e a partir desses valores foram calculadas as taxas de remoção dos metais Zn, Mn e Ni pela biomassa algal. A taxa de remoção de cada metal foi calculada a partir

da concentração máxima dos metais na água (100%) utilizando-se da média das concentrações no "tempo 0" e nos tratamentos em que não havia alga.

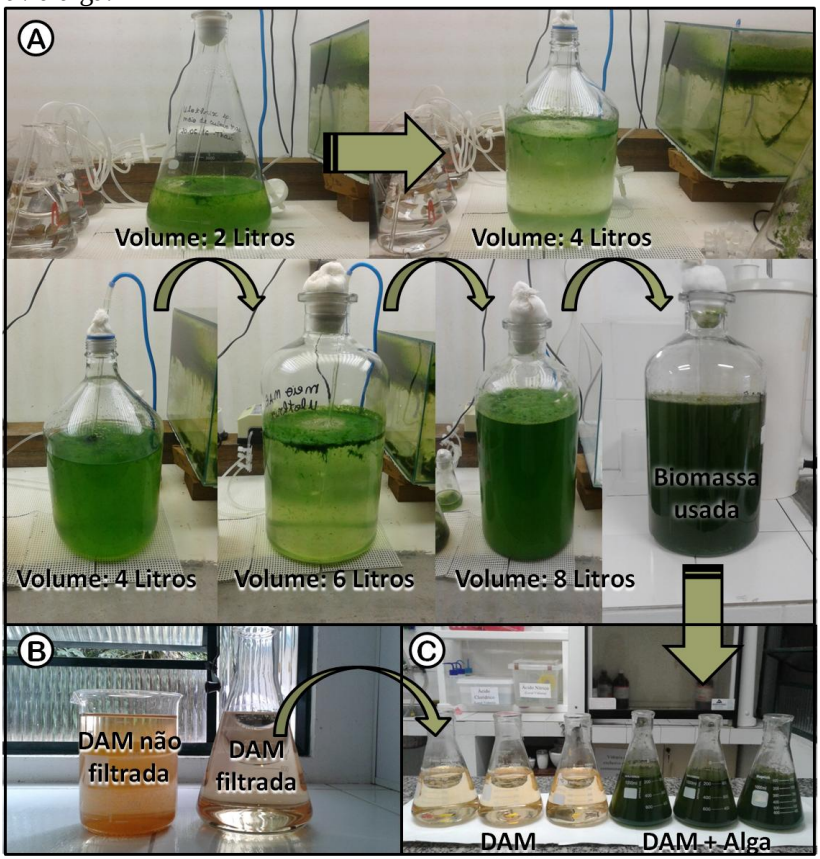

Figura 3.4 Processo de montagem para terceiro experimento; (A) Escalonamento do cultivo de *Ulothrix* sp. LAFIC 010; (B) Pré-filtração da água contaminada por DAM; (C) tratamentos: DAM sem alga e DAM com alga.

3.5 Alterações ultraestruturais em *Ulothrix* sp. LAFIC 010

Para investigar se houve adsorção e/ou captação ativa de metais em *Ulothrix* sp. LAFIC 010, nos três experimentos, foram realizadas análises ultraestruturais de Microscopia Eletrônica de Varredura (MEV),

acoplada com Espectroscopia de Energia Dispersiva (EDS), para a caracterização de materiais metálicos presentes nas amostras, e Microscopia Eletrônica de Transmissão para avaliar mudanças nas células. Para o preparo do material para MEV-EDS foi utilizado o protocolo modificado de Ouriques e Bouzon (2003). As amostras foram fixadas em solução de glutaraldeído (2,5%), sacarose (0,2 M) e cacodilato de sódio (0,1 M) tamponado a (pH 7,2). As amostras fixadas foram lavadas em solução tampão cacodilato (0,1 M) três vezes de 15 minutos cada e desidratadas em série crescente de concentrações de etanol (30, 50, 70, 90 e 100%), com duração de 15 minutos para cada solução. A secagem foi feita com hexadimetildisilazane (HMDS). Após a secagem, as secções foram colocadas em suportes metálicos, ou stubs, e foram recobertas com carbono. As amostras foram observadas e documentadas em Microscópio Eletrônico de Varredura (MEV) Jeol JSM-6390LV.

As amostras para análise de Microscopia Eletrônica de Transmissão (TEM) foram realizadas de acordo com o método de Simioni *et al.*, (2014). Os filamentosos de biomassa foram fixados em solução de glutaraldeído (2,5 %), sacarose (0,2 M) e cacodilato de sódio (0,1 M) tamponado a (pH 7,2). As amostras fixadas foram lavadas em solução tampão cacodilato (0,1 M) três vezes de 15 minutos cada. Foi realizada pós-fixação com tetróxido de ósmio (2%) em solução tampão cacodilato 0,2 M por 2 h. Em seguida, realizaram-se três lavagens de 15 min cada com tampão de cacodilato de sódio (0,1 M). O material foi desidratado num gradiente crescente de séries de acetona (30, 50, 70, 90 e 100%), permanecendo 15 min em cada solução. A série final de 100% de acetona foi realizada duas vezes. O material foi então infiltrado em resina Spurr em séries graduadas de resina Spurr e acetona durante 3 dias, seguido por duas infiltrações com resina pura durante 12h e polimerizado em forno a 70 °C durante 24h. Após a polimerização, as amostras foram seccionadas em micrótomo manual, com navalha de tungstênio, em espessura de secção de 5,0 µm. As secções foram dispostas em lâminas para microscopia, e posteriormente coradas em 1% de acetato de uranilo durante 20 minutos e 1% de citrato de chumbo durante 10 minutos. As amostras foram então examinadas sob TEM JEM 1011 (JEOL Ltd., Tóquio, Japão, a 80 kV). Todas as análises de microscopia foram realizadas no Laboratório Central de Microscopia Eletrônica de Universidade Federal de Santa Catarina.

3.7 Análises estatísticas

Para a análise estatística das respostas fisiológicas de *Ulothrix* sp. LAFIC 010 no Experimento 1 foi feita uma análise de variância (ANOVA) unifatorial na qual o fator avaliado foi a concentração dos metais Zn, Mn e Ni, isoladamente. Para o Experimento 2, foi feita uma ANOVA bifatorial para constatar o efeito da concentração de cada metal pesado e a interação entre eles. A ANOVA unifatorial foi feita no Experimento 3 para avaliar o potencial de *Ulothrix* sp. LAFIC 010 na remoção de Zn, Mn e Ni na água contaminada por DAM. Para isso, a comparação entre a concentração dos metais na água com e sem a alga foi realizada. As diferenças significativas foram determinadas pelo teste de Student-Newman-Keuls ao nível de 5% de significância. Previamente às ANOVAs foram realizados testes de normalidade e homogeneidade de variâncias de Cochran. Todas as análises estatísticas foram executadas no software Statistica v.10 para Windows (Statsoft Inc., 2010).

4. REFERÊNCIAS

Akcil, A., Koldas, S., 2006. Acid Mine Drainage (AMD): causes, treatment and case studies. J. Clean. Prod. 14, 1139–1145.

Barbosa, J. P., 2001. Projeto conceitual para recuperação ambiental da bacia carbonífera sul catarinense. *Rio de Janeiro: CETEM* .

Bicudo, C. E. M.; Menezes, M. Gêneros de algas de águas continentais do Brasil. 2 ed. São Carlos: RiMa, 2006. 502p.

Castilhos, C.., Fernandes, F.R.., 2011. A bacia carbonífera sul catarinense e os impactos e passivos da atividade da indústria extrativa mineral de carvão na territorialidade. Recur. minerais e sustentabilidade terrritorial Gd. minas I, 343.

Chong, A.M.Y., Wong, Y.S., Tam, N.F.Y., 2000. Performance of different microalgal species in removing nickel and zinc from industrial wastewater. Chemosphere 41, 251–257.

Das, B.K., Roy, A., Koschorreck, M., Mandal, S.M., Wendt-Potthoff, K., Bhattacharya, J., 2009. Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. Water Res. 43, 883–894.

Druschel, G.K., Baker, B.J., Gihring, T.M., Banfield, J.F., 2004. Acid mine drainage biogeochemistry at Iron Mountain, California.

Geochem. Trans. 5, 13–32.
Freitas, A.P.P., Schneider, I.A.H., Schwartzbold, A., 2011. Biosorption of heavy metals by algal communities in water streams affected by the acid mine drainage in the coal-mining region of Santa Catarina state, Brazil. Miner. Eng. 24, 1215–1218.
Gazea, B., Adam, K., Kontopoulos, A., 1996. A review of passive systems for the treatment of acid mine drainage. Miner. Eng. 9, 23–42.
Gray, N. F., 1997. Environmental impact and remediation of acid mine drainage: a management problem. Environmental Geology 30.1: 62-71.
Gross, W., 2000. Ecophysiology of algae living in highly acidic environments. Hydrobiologia 433: 31–37.
Johnson, D.B., Hallberg, K.B., 2005. Acid mine drainage remediation options: A review. Sci. Total Environ. 338, 3–14.
Lawrence, J.R., Swerhone, G.D.W., Kwong, Y.T.J., 1998. Natural attenuation of aqueous metal contamination by an algal mat. Can. J. Microbiol. 44, 825–832.
Lignell, A., Pedersén, M., 1989. Agar composition as a function of morphology and growth rate. Studies on some morphological strains of Graciliaria secundata and Graciliaria verrucosa (Rhodophyta). Bot. Mar. 32, 219–227.
Lourenço S. O. Cultivo de Microalgas Marinhas: Princípios e Aplicações. São Carlos: RiMa, 2006. 606 p.
Macek, T., Macková, M., Káš, J., 2000. Exploitation of plants for the removal of organics in environmental remediation. Biotechnol. Adv. 18, 23–34.
Malik, A., 2004. Metal bioremediation through growing cells. Environ. Int. 30, 261–278.

Martins Pompêo, M.L., Moschini-Carlos, V., Alexandre, N.Z., Santo, E., 2004. Qualidade da água em região alterada pela mineração de carvão na microbacia do rio Fiorita (Siderópolis, Estado de Santa Catarina, Brasil). Acta Sci. - Biol. Sci. 26, 125–136.
Mehta, S.K., Gaur, J.P., 2005. Use of algae for removing heavy metal ions from wastewater: progress and prospects. Crit. Rev. Biotechnol. 25, 113–152.
Mello, J.W. V, Duarte, H.A., Ladeira, A.C.Q., 2014. Origem e Controle do Fenômeno Drenagem Ácida de Mina. Cad. Temáticos Quim. Nov. na Esc. 8, 24–29.

Moura, A.J. Microrganismos Endofíticos Associados À Planta De Ambientes Impactados E Não Impactados Pela Drenagem Ácida De Mina De Carvão (Dam) 128. (Mestrado em Biotecnologia e Biociências) – Univ. Federal de Santa Catarina, Florianópolis, 2014.

Novis, P.M., Harding, J.S., 2007. Extreme acidophiles – freshwater algae associated with acid mine drainage. In: Seckbach, J. (Ed.), Algae and Cyanobacteria in Extreme Environments. Springer, Dordrecht, pp. 443–463.

Oberholster, P.J., Cheng, P.H., Botha, A.M., Genthe, B., 2014. The potential of selected macroalgal species for treatment of AMD at different pH rangesin temperate regions. Water Res. 60, 82–92.

Olaveson, M.M., Stokes, P.M., 1989. Responses of the acidophilic alga Euglena mutabilis (EUGLENOPHYCEAE) to carbon enrichment at pH 3. J. Phycol. 25, 529–539.

Orandi, S., Lewis, D.M., 2013. Synthesising acid mine drainage to maintain and exploit indigenous mining micro-algae and microbial assemblies for biotreatment investigations. Environ. Sci. Pollut. Res. 20, 950–956.

Orandi, S., Lewis, D.M., Moheimani, N.R., 2012. Biofilm establishment and heavy metal removal capacity of an indigenous mining algal-microbial consortium in a photo-rotating biological contactor. J. Ind. Microbiol. Biotechnol. 39, 1321–1331.

Ouriques, L., Bouzon, Z., 2003. Ultrastructure of germinating tetraspores of *Hypnea musciformis* (Gigartinales, Rhodophyta). Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 137, 193–201.

Paknikar, K.M., Pethkar, A. V., Puranik, P.R., 2003. Bioremediation of Metalliferous Wastes and Products using Inactivated Microbial Biomass. Indian J. Biotechnol. 2, 426–443.

Rose, P.D., Boshoff, G. a, van Hille, R.P., Wallace, L.C., Dunn, K.M., Duncan, J.R., 1998. An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters. Biodegradation 9, 247–257.

Rousch, J.M., Sommerfeld, M.R., 1999. Effect of manganese and nickel on growth of selected algae in pH buffered medium. Water Res. 33, 2448–2454.

Silva, M. V., 2011. Avaliação do potencial de uma microalga acidofílica quanto a biorremoção de metais traço em diferentes

compartimentos ambientais. Diss. (Mestrado em Ciência e Tecnol. Ambient.) – Univ. do Val. do Itajaí, Itajaí 93.

Simioni, C., Rover, T., Schmidt, É.C., de L. Felix, M.R., Polo, L.K., Santos, R. dos, Costa, G.B., Kreusch, M., Pereira, D.T., Ouriques, L.C., Bouzon, Z.L., 2014. Effects of brefeldin A on the endomembrane system and germ tube formation of the tetraspore of *Gelidium floridanum* (Rhodophyta, Florideophyceae). J. Phycol. 50, 577–586.

Soldo, D., Hari, R., Sigg, L., Behra, R., 2005. Tolerance of Oocystis nephrocytioides to copper: Intracellular distribution and extracellular complexation of copper. Aquat. Toxicol. 71, 307–317.

Smucker, N. J., Morgan L. V., 2014. Acid mine drainage affects the development and function of epilithic biofilms in streams. Journal of the North American Benthological Society 30.3: 728-738.

Spijkerman, E., Barua, D., Gerloff-Elias, A., Kern, J., Gaedke, U., Heckathorn, S.A., 2007. Stress responses and metal tolerance of Chlamydomonas acidophila in metal-enriched lake water and artificial medium. Extremophiles 11, 551–562.

Stevens, A.E., Mccarthy, B.C., Vis, M.L., 2001. Metal content of Klebsormidium-dominated (Chlorophyta) algal mats from acid mine drainage waters in southeastern Ohio ’. J. Torrey Bot. Soc. 128, 226–233.

Uhlik, O., Leewis, M.C., Strejcek, M., Musilova, L., Mackova, M., Leigh, M.B., Macek, T., 2013. Stable isotope probing in the metagenomics era: A bridge towards improved bioremediation. Biotechnol. Adv. 31, 154–165.

Vidali, M., 2001. Bioremediation - An overview. J. Ind. Pollut. Control 27, 161–168.

Volesky, B., 2007. Biosorption and me. Water Res. 41, 4017–4029.

Wellburn, A.R., 1994. The Spectral Determination of Chlorophylls a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. J. Plant Physiol. 144, 307–313.

Zeraatkar, A.K., Ahmadzadeh, H., Talebi, A.F., Moheimani, N.R., McHenry, M.P., 2016. Potential use of algae for heavy metal bioremediation, a critical review. J. Environ. Manage. 181, 817–831.

5. CAPÍTULO 1

("Artigo a ser submetido no periódico indexado Water Research")

Title

Tolerance to high concentrations of zinc, manganese and nickel and biosorption capacity of *Ulothrix* sp. LAFIC 010 (Chlorophyta)

Authors

Massocato, T. F.[1] - Ramos, J. C.[2], - Bascuñan, V. L. F.[2] - Simioni, C.[3] - Rörig, L. R.[1] Bonomi Barufi, J.[1]

[1] Laboratory of Phycology, Department of Botany, Federal University of Santa Catarina, 88049-900, Florianópolis, SC, Brazil

[2] Laboratory of Mass and Atomic Spectrometry. Chemistry Department. Federal University of Santa Catarina, 88049-900, Florianópolis, SC, Brazil.

[3] Postdoctoral Research of Post-Graduate Program in Cell Biology and Development, Department of Cell Biology, Embryology and Genetics, Federal University of Santa Catarina, 88049-900, Florianópolis, SC, Brazil

Abstract

The production of Acid Mine Drainage of (AMD) is one of the main phenomena responsible for much of the degradation of water and soil resources. Organisms present in sites contaminated by AMD can have the potential to bioaccumulate heavy metals, stimulating their application in bioremediation processes. *Ulothrix* sp. LAFIC 010 was identified among the species of algae isolated from water contaminated by AMD in the region of Sideropólis (Brazil). The present study evaluated its tolerance and bioaccumulation potential related to zinc, manganese and nickel. Experiments were performed to see the effects of different concentrations of Zn, Mn and Ni (individually and in combination) on the physiological performance of the alga. Also, the potential of *Ulothrix* sp. LAFIC 010 to bioaccumulate these metals was

evaluated when maintained in effluent from a contaminated site by AMD. The results showed that only the cultures submitted to concentrations above 0.27 mM Zn showed a decrease in growth rate and damage to physiological processes. There was no observed effect of Mn and Ni on Ulothrix physiology, even with an 8-fold increase in concentrations of these metals in the medium. In cultures with combined metals, only the treatments with the highest concentrations of Zn presented reduced growth, regardless of the presence of other metals. Additionally, we observed that Mn and Ni did not decrease the toxic effect of Zn. Mn accumulation was indicated in the cell wall and Ni in the vacuole. Our results suggest that the distribution of this alga in contaminated medium is not affected by the concentration of Ni and Mn, at least under the pH that was evaluated. We conclude that *Ulothrix* sp. LAFIC 010 tolerates and grows under conditions with higher metal concentrations than previously reported for AMD.

Key words: *Ulothrix* sp.*, bioremediation, zinc, manganese, nickel.*

5.1 Introduction

Industrial activities are responsible for the degradation and impairment of soils, contamination of surface waters and pollution of the atmosphere (Alloway and Ayres, 1997). Mining is among the main polluting activities in the world (Wong, 2003). Pollution by mine residues is a common and large worldwide problem that can cause great changes in the structure and functioning of many ecosystems (Wong, 2003). Coal mining generates Acidic Mine Drainage (AMD), which is responsible for the acidification and metal contamination of extensive areas around de world (Freitas, 2011; Smucker, 2014). AMD is produced when sulfides present in coal ores are oxidized after exposure to oxygen and water. As a result, H^+ is released, the pH decreases, and metal dissolution occurs in the soil and rocky matrix. The strong acidity compromises the natural physico-chemical balance of the aquatic environments, leading to the collapse of benthic communities, toxicity of heavy metals, disruption of buffer systems, conversion of carbonate and bicarbonate to carbonic acid (problems for photosynthesis), and increase in oligotrophy due to the oxidation of organic matter and unavailability of N and P (Gray, 1997; Novis and Harding, 2007).

Bioremediation processes have been considered as alternatives for environment decontamination through the use of resistant organisms to achieve the complete or partial removal of the contaminants (Vidali, 2001). Bioremediation has been used for treatment of effluents such as sewage, oil spills and toxic waste from several types of industry (Macek *et al*., 2000). The use of algae for decontamination of water impaired by AMD has been the focus of several studies (Stevens *et al*., 2001; Malik, 2004; Das *et al*., 2009). Orandi *et al*. (2012) showed the ability of some microalgal assemblages to grow in synthetic AMD even in limiting nutrient concentrations and toxic levels of metals. Fyson *et al*. (2006) performed a microcosm experiment that revealed that primary production of *Eunotia exigua* and *Chlamydomonas* sp. allowed the development of sulfate reducing bacteria, which lead to a reduction of dissolved iron from 14 mg/L to 0.2 mg/L. In addition, the use of *Spirulina* sp. as part of the AMD contaminated water treatment process showed optimistic results, suggesting the use of microalgae as a biological tool for concentration and uptake of dissolved metals (Rose *et al*. 1998). Additionally, Oberholster *et al*. (2014) verified growth and high bioaccumulation of metals by three filamentous algae isolated from environments contaminated by AMD (*Oedegonium crassum*, *Klebsormidium klebsii* and *Microspora tumidula*).

Nevertheless, for the success of bioremediation, some aspects must be considered regarding the algal species and the bioremediation method. Among them, some features need to be highlighted: capacity of heavy metal uptake, ease of handling and maintenance of the cultures, growth rate of the algae, as well as the possibility of obtaining useful biomass, for example for biofuel production (Sturm and Lamer, 2011; Park *et al*., 2011). The difference in metal uptake capacity can be observed even intraspecifically, and thus an extensive bioprospection study is sometimes necessary (Monteiro *et al*., 2011). The growth time of the organism is an important parameter since the increased biomass concentrations positively increase final metal bioremoval (Zeraatkar *et al*., 2016). According to Terry and Stone (2002), higher concentrations of *Scenedesmus abundans* biomass diminished the competition between copper and cadmium for the binding algae sites.

Essentially, heavy metals can be processed by algae following two strategies: adsorption and/or active uptake by cellular tissues (Mehta and Gaur, 2005). The adsorption is a fast process that does not consumes energy. It occurs on the cellular surface via the interaction

between the metallic ions and metal-functional groups, such as carboxyl, phosphate, hydroxyl, amino, sulphur, sulphide, and thiol present in the cell wall (Zeraatkar *et al*., 2016). The active uptake process requires metabolic energy, taking more time and involving several possible mechanisms before achieving metal penetration into the cell and its compartmentalization in some internal cellular structures (Talebi *et al*. 2013). The intracellular accumulation of heavy metals is due to the binding of the metal ions to thylakoids, vacuoles, and cytoplasmic ligands, such as phytochelatins and metallothioneins, and other intracellular molecules (Mehta and Gaur, 2005; Soldo *et al*., 2005). Despite the fact that many parameters play roles in the process of heavy metal accumulation (Bajguz, 2000), it is clear that different species of algae can accumulate heavy metal ions to various degrees (Jordanova *et al*., 1999).

Algae species isolated from polluted environments are frequently more resistant to metals, and additionally, the tolerant species had a greater capability of accumulating heavy metals (Wong 2000). Novis and Harding (2007) have shown that the green algae genus, *Ulothrix*, is amongst the main filamentous genera naturally occurring adhered to the substrate in strongly acidic environments contaminated by AMD. In addition, these algae have great potential to capture heavy metals (Orandi and Lewis, 2013; Lawrence et al., 1998). Paknikar (2003) proposed the use of *Ulothrix*-lyophilized biomass for the adsorption and removal of metals in solution. The potential to use the biomass of *Ulothrix zonata* was also reported by Nuhoglu (2002) and Nuhoglu (2003) for the removal of copper and chromium, respectively. *Ulothrix gigas* showed pronounced efficiency in sequestering Cu (up to 3500 mg/L) and As (up to 500 mg/L) from AMD (Orandi *et al*., 2007). In another study, Orandi *et al*. (2012) have found that *Ulothrix* sp. was the predominant species in the biofilm of microorganisms found in an area contaminated by copper in AMD. Those authors have also observed that the species was able to remove between 20-50% of metals such as Cu, Ni, Mn, Zn, Sb, Se, Co, and Al from contaminated water, indicating good potential for metal biosorption.

Our research aimed to expand the knowledge of the potential application of *Ulothrix* spp. for the bioremediation of acidic and metallic residues, mainly AMD. In this context, the strain *Ulothrix* sp. LAFIC 010 was evaluated in terms of its tolerance to the main heavy metals present in AMD from a coal mining region in Southern Brazil. Solutions with Zn, Mn and Ni were tested in two different experimental

approaches, providing each metal separately and in combined treatments. Additionally, the alga was inoculated in AMD samples and the uptake of Zn, Mn and Ni were evaluated in different exposure times. Moreover, algal filaments from the different treatments were analyzed by scanning and transmission electron microscopy to evaluate internal cellular location of heavy metals as well as cellular ultrastructural modifications.

5.2 Material and Methods

Algae strain and culture conditions

Filaments of *Ulothrix* sp. LAFIC 010 were isolated from water contaminated with AMD in Siderópolis (28°37'S, 49°24'W), Brazil. Cultures were maintained in the Laboratory of Phycology (LAFIC), at the Federal University of Santa Catarina (UFSC), in Florianópolis. Filaments were cultivated in sterile Modified Acid Medium (MAM) (Olaveson and Stokes, 1989) at 24 ± 2°C in a 12:12 h light-dark cycle with an irradiance of 60±5 µmol photons.m^{-2}.s^{-1} obtained with cool white fluorescent bulbs. MAM was (in g.L^{-1}) $(NH_4)_2SO_4$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.01, $MgSO_4 \cdot 7H_2O$ 0.5, KH $_2PO_4$ 0.3, NaCl 0.03, (in mg.L^{-1}) $FeSO_4 \cdot 7H_2O$ 4.98, H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 5H_2O$ 2.22, $NaMoO_4 \cdot 2H_2O$ 0.39, $CuSO_4 \cdot 5H_2O$ 0.079, $Co(NO_3)_2 \cdot 6H_2O$ 0.0494, biotin 1.0, B12 10.0, and thiamine 20.0. The medium was adjusted to pH 3.6 with sulfuric acid to be in accordance with the average pH of the sampling site. To prepare stock cultures for tolerance tests, algal filaments from culture collection were inoculated in 250 mL Erlenmeyer flasks containing 150 mL of sterile modified MAM medium. Na_2-EDTA was not added to avoid metal chelation. 3 experiments were performed: metal tolerance (experiments 1 and 2) and capacity of heavy metal bioaccumulation in AMD (experiment 3). In all procedures, *Ulothrix* filaments were evaluated by using electronic microscopy assays, detailed as follows.

Tolerance test conditions

Tolerance tests consisted of two different experimental designs: Experiment 1, to determine the isolated effects of Zn, Mn and Ni, and Experiment 2, to determine the combined effects of the three heavy metals on the physiology of *Ulothrix* sp. LAFIC 010. Additional concentrations of Zn, Mn and Ni to modified MAM medium were, respectively: 0 (control), 0.275, 0.55, 1.1, 2.2, and 4.4 mM; 0 (control), 0.182, 0.37, 0.73, 1.46, and 2.9 mM; and 0 (control), 0.0023, 0.0046, 0.0092, 0.0185, and 0.0369 mM. These concentrations were obtained by the addition of $ZnSO_4$, $MnSO_4$ and $NiSO_4$ stock solutions to growth media. In the second experiment, the Zn, Mn and Ni metals were combined in different concentrations (0, 0.55, and 4.4 mM Zn; 0, 0.37, and 2.9 mM Mn; 0, 0.0046, and 0.0369 mM Ni), totaling 27 metal combination treatments. The metal concentrations used were based on values previously found at the AMD sample site (Moura, 2014), but

concentrations up to 8 times higher were also tested to verify the tolerance limits of the strain. MAM media was autoclaved after the addition of the metals. Tolerance tests were run in 12-well polystyrene microplates with 3 mL solution in each well. Appropriate quantities of concentrated filaments of *Ulothrix* sp. LAFIC 010 were inoculated to obtain an initial concentration in each culture corresponding to 0.154 Chlorophyll *a* Optical Density at 680 nm The first experiment lasted 10 days, while the second one 8 days. Both experiments 1 and 2 were performed with 5 replicates.

Chlorophyll *a* optical density and fluorescence were determined along the experiments by using an Infinite M200 multimode microplate reader (Tecan - RCHISTO). Absorbance was obtained at 680 nm. Fluorescence settings were: excitation filter at 472nm; emission filter at 685nm; gain of 160; and area definition with 100 measuring points of the well. Photosynthetic performance was estimated as *in vivo* fluorescence of chlorophyll *a* of photosystem II (PSII) using a portable pulse modulation fluorometer (Junior PAM, Walz, Germany). Effective quantum yield (Y[II]) was measured in the beginning and in the end of the experiments, according to Genty *et al*. (1989). Growth rates were estimated according to equation 4.1, where V_i is the Chl *a* Optical Density or Chl *a* Fluorescence at the initial time; V_f is the Chl a Optical Density or Chl *a* Fluorescence at the end of the experiment, and *t* is elapsed time (days) between V_i and V_f.

$$GR = \frac{(Vf - Vi)}{t} \qquad [4.1]$$

Metal bioaccumulation in AMD

The potential of Zn, Mn and Ni bioaccumulation by the algae was evaluated in the experiment by comparing the concentrations of metals in AMD samples with inoculation of *Ulothrix* sp. LAFIC 010 living biomass (contact treatment) and without any inoculation (control treatment). AMD sample was collected from the same site where the algal filaments were isolated. Culture conditions were the same as described previously. The biomass for this experiment was obtained by growing the algae in eight liters of culture during 20 days to obtain a concentrated fresh biomass of 2.8 $g.L^{-1}$, achieving 22.4 g of total algal biomass. For contact treatment, 4.2 g of fresh biomass were transferred to each of 3 replicates consisting of 1,000 mL Erlenmeyer flasks with 800 mL of AMD sample. The same sample volume and replicate number was used for the control treatment. All flasks were kept on a

shaker at 110 rpm under 45 µmol.m^{-2}.s^{-1} of photosynthetically active radiation in a 12:12 light:dark cycle at 24 ±2°C for 14 days.

During the experiment, the dissolved concentration of zinc, manganese, and nickel were analyzed using inductively coupled plasma-mass spectroscopy (ICP-MS - Perkin Elmer, model NexIon 300D, Shelton, USA). Analyses were done immediately after biomass inoculation (t_0) and after 1, 6, 24, 168 and 336 hours. These intervals of time were chosen to evaluate the two main steps of possible heavy metal incorporation by the cells: adsorption, which is normaly a quick process, and active transport (absorption), which is a slower process. Water sample, preservation and analytical procedures were carried out according to the Standard Methods of Water and Wastewater Analysis (APHA/AWWA,1998). Metal removal rates by the algal biomass were calculated considering that the maximum concentration of metals in the water (100%) was the mean concentration control replicates at t_0.

Algal biomass from the beginning (before inoculation) and the end of the experiment was filtered and stored at -80°C for further pigment analysis. The algal pellets were extracted with methanol for one hour at 4°C. The obtained suspensions were centrifuged and the supernatant used for the determination of Chl *a*, Chl *b* and carotenoids fluorescence using an Infinite M200 multimode microplate reader (Tecan - RCHISTO). Pigment fluorescence data were converted to concentrations using the equations described by Wellburn (1994).

Relative growth rates were calculated by the percentage increase of dry biomass along the incubation time. The dry biomass was obtained by maintaining the fresh biomass in an oven at 100 °C for 48 hours and weighing it in an analytical balance to obtain the dried biomass. The initial and final values were applied to the formula of Lignell and Pedersén (1989).

Electron Microscopy Analyses

To investigate the metal incorporation in *Ulothrix* sp. LAFIC 010, algal filaments were submitted to Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) with simultaneous application of Energy-dispersive X-ray spectroscopy (EDS). These analyses were performed at the end of the experiments.

In Experiment 1, all samples were processed for TEM. However, in Experiment 2, to avoid repetition of conditions, samples containing only one type of metal were not analyzed, as well as those showing deleterious effects resulting in unavailability of biomass. Considering

these aspects and to ensure the detection of intracellular effects, the following treatments of heavy metal combinations were evaluated: control; 0 (Zn), 2.9 mM (Mn), 0.0369 mM (Ni); 0.55 mM (Zn), 0 (Mn), 0.0369 mM (Ni); 0.55 mM (Zn), 0.37 mM (Mn), 0.0046 mM (Ni); 0.55 mM (Zn), 2.9 mM (Mn), 0 (Ni); 0.55 mM (Zn), 2.9 mM (Mn), 0.0046 mM (Ni); 0.55 mM (Zn), 2.9 mM (Mn), 0.0369 mM (Ni); and 4.4 mM (Zn), 2.9 mM (Mn), 0.0369 mM (Ni).

TEM was performed according to the method of Simioni *et al*. (2014). The biomass was fixed with 2.5% glutaraldehyde and sucrose (0,2 M) in sodium cacodylate buffer 0.1 M (pH 7.2). Thereafter, fixed samples were washed three times with sodium cacodylate buffer 0.1 M (pH 7.2) for 15 min. The material was post-fixed with 2% osmium tetroxide (OsO_4) and 0.2 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature. Then, three washes, 15 min each, were performed with 0.1 M sodium cacodylate buffer. The material was dehydrated in a series of increasing concentrations of acetone (30, 50, 70, 90, and 100%), 15 min each step. The final series of 100% acetone was performed twice. Treated samples were then infiltrated in graded series of acetone-Spurr's resin for 3 days, followed by two infiltrations with pure resin for 12h, and finally polymerized in an oven at 70 °C for 24 h. The ultrathin sections (60 nm) were obtained with a diamond knife on an ultramicrotome and subsequently stained with 1 % uranyl acetate for 20 min and 1 % lead citrate for 10 min. Samples were then analyzed under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan) using an accelerating voltage of 80 kV. Cell sections were selected randomly and photographed. After image analyses, the effects of the metals were compared in the following cellular features: whole cell, cytoplasm, chloroplast, vacuole and vacuolar deposits, starch grains, pyrenoid, and cell walls.

For SEM, the protocol was modified from Ouriques and Bouzon (2003). The biomass was fixed using the same procedure as for TEM. Fixed samples were washed in cacodylate buffer solution 0.1 M and then dehydrated in an ethanol series of 30, 50, 70, 90, and 100%, v/v, and dried using Hexamethyldisilazane (HMDS). For the SEM-EDS analysis, the samples were sputter-coated with carbon and then examined under SEM JSM 6390 LV (JEOL Ltd., Tokyo, Japan) using an accelerating voltage of 20 kV. These procedures were performed for all samples from Experiment 1.

For the same reasons as for TEM, it was not possible to evaluate the procedure for all samples in SEM analyses. In Experiment 2, the

treatments analyzed were: control; 0 mM (Zn), 0.37 mM (Mn), 0.0046 mM (Ni); 0 mM (Zn), 0.37 mM (Mn), 0.0369 mM (Ni); 0 mM (Zn), 2.9 mM (Mn), 0.0046 mM (Ni);0 mM (Zn), 2.9 mM (Mn), 0.0369 mM (Ni); 0.55 mM (Zn), 0 mM (Mn), 0.0046 mM (Ni); 0.55 mM (Zn), 0 mM (Mn), 0.0369 mM (Ni); 0.55 mM (Zn), 0.37 mM (Mn), 0 mM (Ni); 0.55 mM (Zn), 0.37 mM (Mn), 0.0046 mM (Ni); 0.55 mM (Zn), 0.37 mM (Mn), 0.0369 mM (Ni); 0.55 mM (Zn), 2.9 mM (Mn), 0 mM (Ni); 0.55 mM (Zn), 2.9 mM (Mn), 0.0046 mM (Ni); and 0.55 mM (Zn), 2.9 mM (Mn), 0.0369 mM (Ni).

Statistics

Statistical evaluation of physiological responses was performed using a one-way analysis of variance (ANOVA) in Experiment 1, where metals were the factors assessed to obtain the isolated heavy metal effects. For Experiment 2, a two-way ANOVA was performed to access the effect of each metal and the interaction among them. A one-way (ANOVA) was performed on the results of Experiment 3 to evaluate the potential of *Ulothrix* sp. LAFIC 010 to remove Zn, Mn and Ni from AMD. Previous assumptions to ANOVA were verified performing tests of normality and homogeneity of variances (Cochran test). When significant effects were detected ($p<0.05$), a post-hoc test of Student-Newman Keuls was applied. All statistical analyses were performed in the software Statistica v.10 for Windows (Statsoft Inc., 2010).

5.3 Results

Experiment 1 -Tolerance of individual metals

Ulothrix sp. LAFIC 010 showed tolerance to high concentrations of zinc, manganese and nickel. Physiological responses of *Ulothrix* sp. LAFIC 010 were significantly influenced by the presence of Zn, while the addition of Mn or Ni was effective to cause variations only for Chlorophyll *a* fluorescence (Table 5.1).

Table 5.1. Summary of one-way repeated measures analysis of variance (ANOVA) of physiological responses of *Ulothrix* sp. LAFIC 010 at different concentrations of Zinc, Manganese and Nickel during tolerance experiment. $\alpha < 0.05$. N=5. Bold indicates significant values at $P < 0.05$.

Source of variation	Degrees of freedom	Zn (mM)		Mn (mM)		Ni (mM)	
		F value	*p*	*F* value	*p*	*F* value	*p*
O.D. Chl *a*	5	**22.210**	**0.000**	1.264	0.311	1.047	0.413
Fluor. Chl *a*	5	**102.211**	**0.000**	**5.260**	**0.002**	**3.331**	**0.020**
Yield (II)	5	**20.099**	**0.000**	2.264	0.080	0.496	0.776
Error	24						

Under concentrations of 2.2 and 4.4 mM of Zn, the Optical Density and Fluorescence of Chl *a* exhibited a sharp reduction (Fig. 5.1a, d), but no effect was observed in Y(II) (Fig. 5.1g), although a reduction in the photosynthetic response was detected at 4.4 mM Zn. No significant variation was observed in the optical density and Y(II) of *Ulothrix* sp. LAFIC 010 in any concentration of Mn or Ni tested (Figs. 5.1b, 5.1c, 5.1h, 5.1i) . The only parameter sensitive for both metals, Mn and Ni, was the Chlorophyll *a* fluorescence for 2.9 mM Mn (Fig. 5.1e), and 0.0185 and 0,0369 mM Ni (Fig. 5.1f).

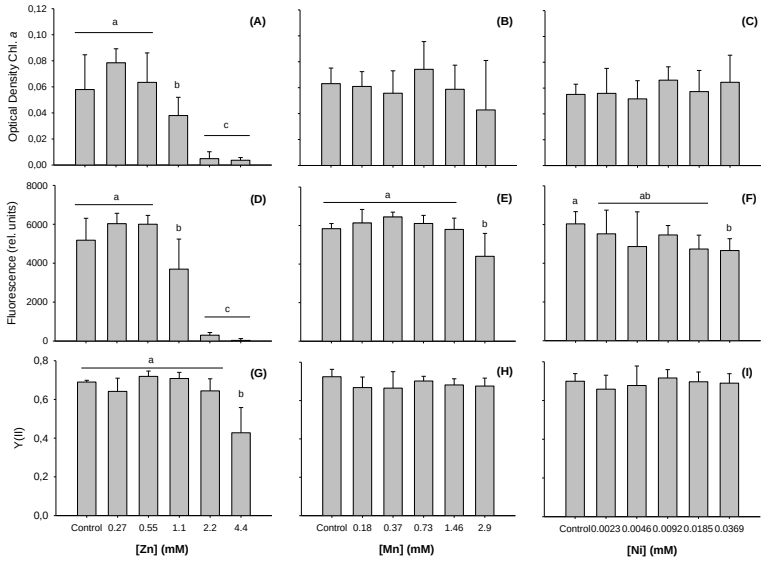

Figure 5.1 Physiological responses of *Ulothrix* sp. LAFIC 010 in the experiment for metal tolerance with Zn (A, B, C), Mn (D, E, F) and Ni (G, H, I). The parameters optical density (λ = 680 nm), cellular fluorescence, and effective quantum yield (Y(II)) were evaluated. The metals Zinc, Manganese and Nickel were provided separately. Letters indicate

significantly different treatments according to post-hoc test of Student-Newman Keuls (mean ±SD. n = 5).

SEM-EDS spectra showed the presence of the elements O, Na, Si, P, S, and Ca on the cellular surfaces of *Ulothrix* sp. LAFIC 010 (Tab. 5.2). These compounds remained in similar contents after metal treatments, for any type or concentration of metal tested. Data from SEM-EDS spectra of this experiment revealed that Zn, Mn, and Ni, were not present on the surface of *Ulothrix* sp. LAFIC 010 filaments (Figure 5.2).

Table 5.2 Scanning Electron Microscope (SEM) and Energy-dispersive X-ray spectroscopy (EDS) data of *Ulothrix* sp. LAFIC 010 exposed to different concentrations of Zinc, Manganese and Nickel during tolerance experiment. The values correspond to the mean (±SD) of the compound %. (*): samples where elements were recorded for only one replicate, thus avoiding calculation of SD.

Treatments (mM)		O	Na	Si	P	S	Ca	Mg	Cl	Mo	Br	K	Al
	Control	74.28 ± 2.07	4.93 ± 0.95	1.72 ± 0.42	12.41 ± 2.67	3.71 *	3.06 ± 0.85	0.41 *	1.41 *	5.2 *			
Zn	**0.55**	73.33 ± 1.03	4.47 ± 1.37	2.15 ± 0.15	13.35 ± 0.48	3.28 ± 0.36	2.88 ± 0.87	0.8 ± 0.12					
	1.1	76.88 ± 0.56	5.45 ± 0.18	1.48 ± 0.13	9.08 ± 0.68	4.73 ± 0.38	2.36 ± 0.18						
Mn	**0.37**	76.65 ± 3.79	3.69 ± 0.08	1.81 ± 0.7	9.61 ± 2.32	3.25 ± 0.17	2.55 ± 0.61		1.07 *	6.62 *	2.83 *		
	2.9	76.1 ± 0.4	4.59 ± 0.35	0.58 *	7.08 ± 0.34	3.34 ± 0.24	4.5 ± 0.56		3.94 ± 0.36			0.71 *	
Ni	**0.0046**	76.23 ± 0.48	4.25 ± 0.62	1.06 ± 0.48	9.74 ± 0.17	3.29 ± 0	2.87 ± 0.32		1.85 ± 0.63	5.19 *		0.82 *	0.43 *
	0.0369	74.78 ± 2.46	5.33 ± 0.57	1.58 ± 0.6	14.53 ± 1.87	2.9 *	2.4 ± 0.51	0.95 ± 0.7	0.92 *				

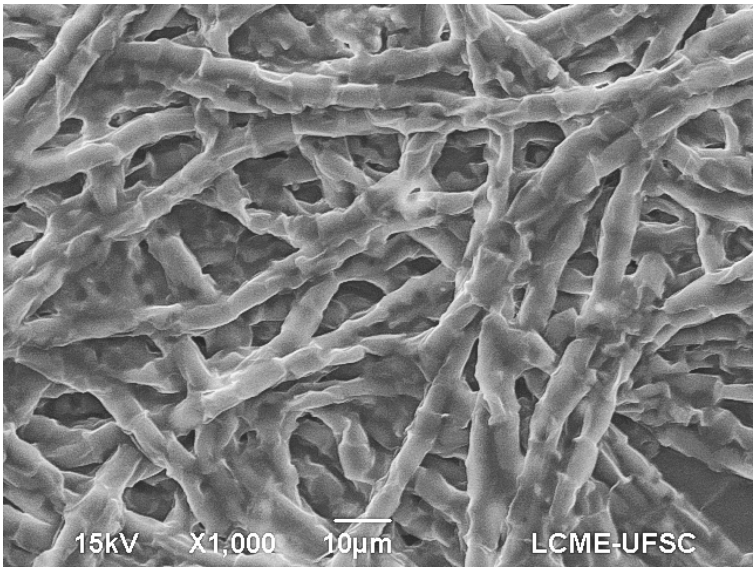

Figure 5.2. SEM of the filaments of *Ulothrix* sp. LAFIC 010 from the control treatment.

The presence of the metals in the medium affected the algal cellular ultrastructure, as can be seen in the TEM images (Fig. 5.3). *Ulothrix* sp. LAFIC 010 cells from the control treatment showed chloroplasts occupying most of the cellular surface, and inside the chloroplast it was possible to observe large pyrenoids surrounded by starch grains (Fig. 5.3a). The cell wall appeared translucent (Fig 5.3b). Chloroplasts presented elongated thylakoids forming grana (Figs. 5.3a-d). Additionally, cells showed a vacuole occupying almost half the size of the cell (Fig 5.3d). Comparing the images from the control with treatments submitted to each metal separately, it was possible to infer that the structural changes were different for each metal. In samples under the lowest Zn concentration (Figs. 5.3e-h), it was possible to observe cell wall thickening. Under higher Zn concentration (Figs. 5.3i-l), the cells presented changes in the chloroplasts, which exhibited disorganized thylakoids and higher quantity of plastoglobuli. Moreover, vacuoles in these samples were also disarranged (Fig. 5.3l). In the treatments with Mn (Figs. 5.3m-t), especially under the higher concentration tested (2.9 mM, Fig. 5.3q-t), cells presented electron-dense areas in the cell wall, and inside the cell the chloroplasts also

presented higher concentration of plastoglobuli (Fig. 5.3s). These treatments also presented electron-transparent regions without any type of storage products. For the Ni treatments (Figs. 5.3u-b'), electron-dense materials were observed inside of the vacuole (Figs. 5.3z, b'). In addition, an increase of plastoglobuli, mainly in the higher concentration (Fig. 5.3a'), was observed in these treatments.

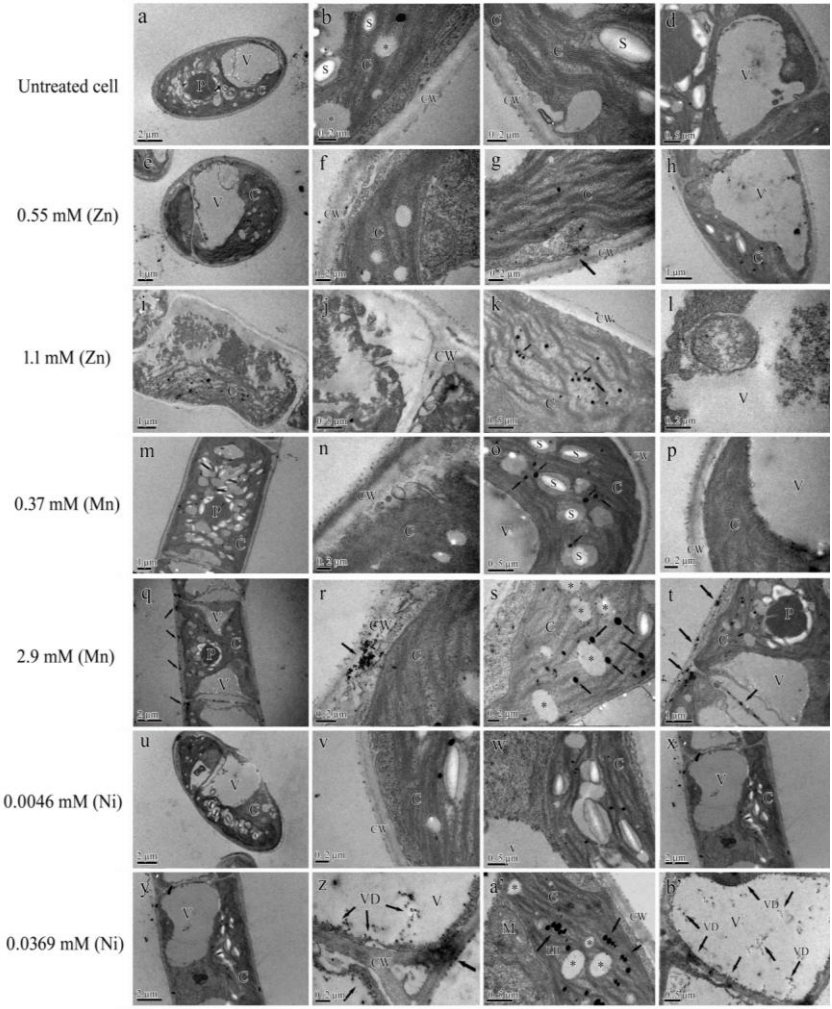

Figure 5.3. Transmission electron microscopy of *Ulothrix* sp. LAFIC 010 cultivated with different metal concentrations provided separately. a-d: Untreated cells. a, Overview of the cellular structure; b, Detail of the structure of the cell

wall; c, Chloroplasts with thylakoid forming grana; d, Detail of the vacuole. e-h: Cells treated with 0.55 mM Zn. e, Cell overview; f, Thickening of the cell wall; g, Detail of the electron-dense material in the cell wall (arrow); h, Detail of the vacuole. i-l: Cells treated with 1.1 mM Zn. i, Overview of the altered cellular structure; j, Detail of the extensive layer of cell wall; k, Cells with altered structure of the chloroplast with details of thylakoids disarrangements and numerous plastoglobuli (arrows); l, Presence of vacuole with electron-dense structures inside. m-p: Cells treated with 0.37 mM Mn. m, Cell overview; n, Detail of the cell wall organization with the presence of vesicles; o, Chloroplasts intact, but with the presence of plastoglobuli (arrows) and starch grains; p, Detail of the vacuole. q-t: Cells treated with 2.9 mM Mn. q, Cell overview; r, Electron-dense structures present in the cell wall; s, Chloroplasts with numerous plastoglobuli (arrows) and absence of starch grains (asterisk); t, Detail of the vacuole and of the electron-dense punctuations present in the cell wall (arrows). u-x: Cells treated with 0.0046 mM Ni. u, Cell overview; v, Cell wall detail; w, Detail of the chloroplast with presence of a smaller number of thylakoids per granum; x, Detail of the vacuole. y-b': Cells treated with 0.0369 mM Ni. y, Cell overview; z, Detail of the cell wall with presence of electron-dense material and vacuoles with a deposit of electron-dense material; a', Chloroplasts with the presence of several plastoglobuli and absence of starch grain (asterisk); b', Details of the vacuoles with electron-dense material. Abbreviations: Chloroplast (C); Cell Wall (CW). Mitochondria (M); Pyrenoid (P); Starch Granule (S); Vacuoles (V); Vacuolar Deposit (VD).

Experiment 2 - Tolerance of combined metals

No interactive effects were observed in the experiment with the combinations of metals (Zn, Mn, and Ni). Zn was the unique factor causing variation in the physiological responses evaluated (Tab. 5.3).

Table 5.3 Summary of the three-way repeated measure analysis of variance (ANOVA) for the physiological responses of *Ulothrix* sp. LAFIC 010 to metal combination treatments of Zinc, Manganese and Nickel during a tolerance experiment. $\alpha < 0.05$. N=5. Bold indicates significant values at $P < 0.05$.

Source of variation	Degrees of freedom	O.D. Chl *a*		Fluor. Chl *a*		Yield (II)	
		F value	*p*	*F* value	*p*	*F* value	*p*
Zn	2	**681.539**	**0.000**	**2975.455**	**0.000**	**782.333**	**0.000**
Mn	2	2.627	0.077	1.814	0.168	0.893	0.412
Ni	2	0.081	0.922	2.044	0.135	3.006	0.054
Zn*Mn	4	0.844	0.501	0.747	0.562	0.852	0.495
Zn*Ni	4	0.120	0.975	0.513	0.726	0.959	0.433
Mn*Ni	4	1.440	0.226	0.998	0.412	0.878	0.480
Zn*Mn*Ni	8	1.141	0.342	1.284	0.259	0.771	0.629
Error	108						

The three parameters showed a marked decrease in values when the Zn concentration was 4.4 mM, independent of the concentration of Mn and Ni (Fig. 5.4a,b,c). Additionally, there was a slight, but significant reduction in the fluorescence of Chl *a* when 0.55 mM Zn was applied in comparison to conditions where Zn was absent (Fig. 5.4b).

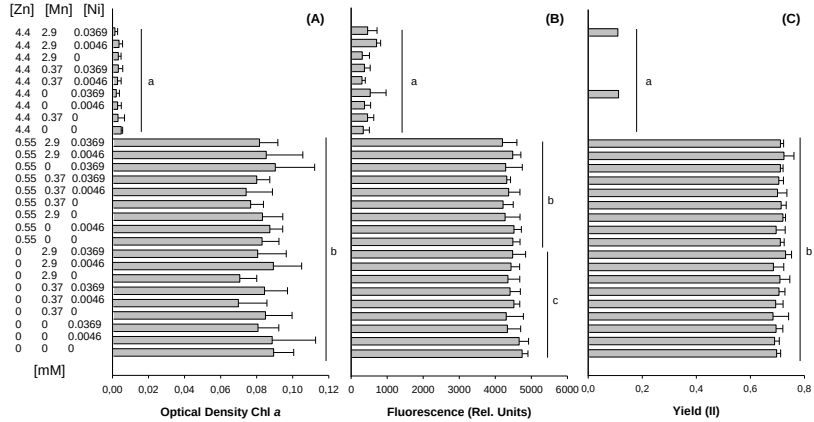

Figure 5.4 Physiological responses of *Ulothrix* sp. LAFIC 010 in an experiment of metal tolerance tests with zinc, manganese and nickel combined, totaling 27 combinations. The parameters optical density (λ = 680 nm), cellular fluorescence and effective quantum yield (Y(II)) were evaluated. Letters indicate significantly different treatments according to the Student-Newman Keuls post-hoc test (mean ±SD. n = 5). Results without standard deviation had no live replicates at the end of the experiment.

The results of SEM-EDS (Tab. 5.4) were similar to the first experiment, with spectra showing the elements O, Na, Si, P, S, and Ca on the cellular surfaces with few variations of these compounds among treatments. However, the presence of Mn on the cellular surfaces was detected in treatments with the highest concentration of Mn (2.9 mM) and Zn (0.55 mM), regardless of the presence of Ni.

Table 5.4 Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDS) data of *Ulothrix* sp. LAFIC 010 expososed to different concentrations and combinations of Zinc, Manganese and Nickel. The values correspond to the mean (±SD) of compound %. (*): samples where elements were recorded for only one replicate, avoiding the calculation of SD.

Treatments (mM)			O	Na	Si	P	S	Ca	Mg	Cl	Mo	K	Al	Zn	Mn
Zn	**Mn**	**Ni**													
0	**0**	**0**	74.28 ± 2.07	4.93 ± 0.95	1.72 ± 0.42	12.41 ± 2.67	3.71 *	3.06 ± 0.85	0.41 *	1.41 *	5.2 *				
0	**0.37**	**0.0046**	76.93 ± 0.79	3.03 ± 0.42		12.41 ± 1.51	3.83 ± 0.15	2.33 ± 0.22	1.98 ± 0.21	1.33 *			0.92 *		
0	**0.37**	**0.0369**	74.09 ± 3.38	2.49 ± 0.91	0.98 ± 0.02	11.88 ± 0.91	3.88 ± 0.14	3.23 ± 0.82	1.74 ± 0.58					3.89 ± 0.2	
0	**2.9**	**0.0046**	73.1 *	3.46 *	3.67 *	11.6 *	4.73 *	1.24 *	1.42 *				0.78 *		
0	**2.9**	**0.0369**	75.27 ± 0.19	3.46 ± 0.24		13.73 ± 0.18	3.85 ± 0.19	2.22 ± 0.6	1.46 ± 0.15						
0.55	**0**	**0.0046**	74.81 ± 1.08	3.5 ± 0.62	3.72 ± 0.58	13.1 ± 1.18	4.19 ± 0.65	1.34 *	1.61 ± 0.12						
0.55	**2.9**	**0**	62.2 ± 0.57	4.52 ± 0.39	13.03 ± 1.51	13.62 ± 0.6	3.45 ± 0.2	1.21 *	0.96 ± 0.44						1.59 ± 0.37
0.55	**0.37**	**0**	76.46 ± 2.33	3.42 ± 0.4	1.99 ± 1.21	12.22 ± 2.26	5.64 *	1.02 ± 0.11	1.4 ± 0.21		5.78 *				
0.55	**0.37**	**0.0046**	70.58 ± 2.22	5.16 ± 0.77	3.84 ± 2.77	15.12 ± 0.7	3.5 ± 0.21	1.08 *	1.41 ± 0.33						
0.55	**0.37**	**0.0369**	75.11 ± 0.57	4.65 ± 0.79	3.75 ± 0.67	12.44 ± 1.44	3.32 *	1.59 ± 0.07	1.34 ± 0.3						
0.55	**0**	**0.0369**	74.06 ± 1.85	4.75 ± 0.11	0.92 *	13.52 ± 0.2	4.16 ± 0.17	1.42 *	1.94 ± 0.15			0.77 *			
0.55	**2.9**	**0.0046**	67.68 ± 4.24	3.36 ± 0.02	15.55 ± 1.13	9.81 ± 0.7	3.58 *	0.93 *	0.94 *						1.74 *
0.55	**2.9**	**0.0369**	64.83 ± 2.47	3.86 ± 0.07	11.13 ± 1.09	13 ± 0.46	4.18 ± 1.2	1.19 ± 0.47	0.94 ± 0.16						1.7 *

When compared to the control (Fig. 5.5a-d), the samples cultivated in a combination of Zn 0, Mn 2.9 mM, and Ni 0,0369 mM (Figs. 5.5e-h) did not show internal cellular alterations under TEM analysis. The only exception was the presence of electron-dense structures in the cell wall (Fig. 5.5f). In the combination of Zn 0.55 mM, Mn 0, and Ni 0.0369 mM (Figs. 5.5i-l), samples also exhibited electron-dense materials in the cell wall (Fig. 5.5j), aside from chloroplasts with disorganized thylakoids and hyaline regions without starch grains. Electron-dense materials in the cell wall were also observed in the combinations Zn 0.55 mM, Mn 0.37 mM, and Ni 0.0046 mM (Figs. 5.5m-p) and Zn 0.55 mM, Mn 2.9 mM, and Ni 0.0369 mM (Figs. 5.5y-b'). In the combinations Zn 0.55 mM, Mn 2.9 mM, and Ni 0 mM (Figs. 5.5q-t) and Zn 0.55 mM, Mn 2.9 mM, and Ni 0.0046 mM (Figs. 5.5u-x), the cellular ultrastructure features of samples were similar to the control. The treatment under the highest concentrations of the three metals (Zn 4.4 mM, Mn 2.9 mM, Ni 0.0369 mM, Fig. 5.5c'-f') exhibited cells with several alterations, mainly in the cytoplasm, where chloroplasts presented disorganization with rupture of thylakoids and presence of plastoglobuli. The cell wall of these cells also presented electron-dense materials.

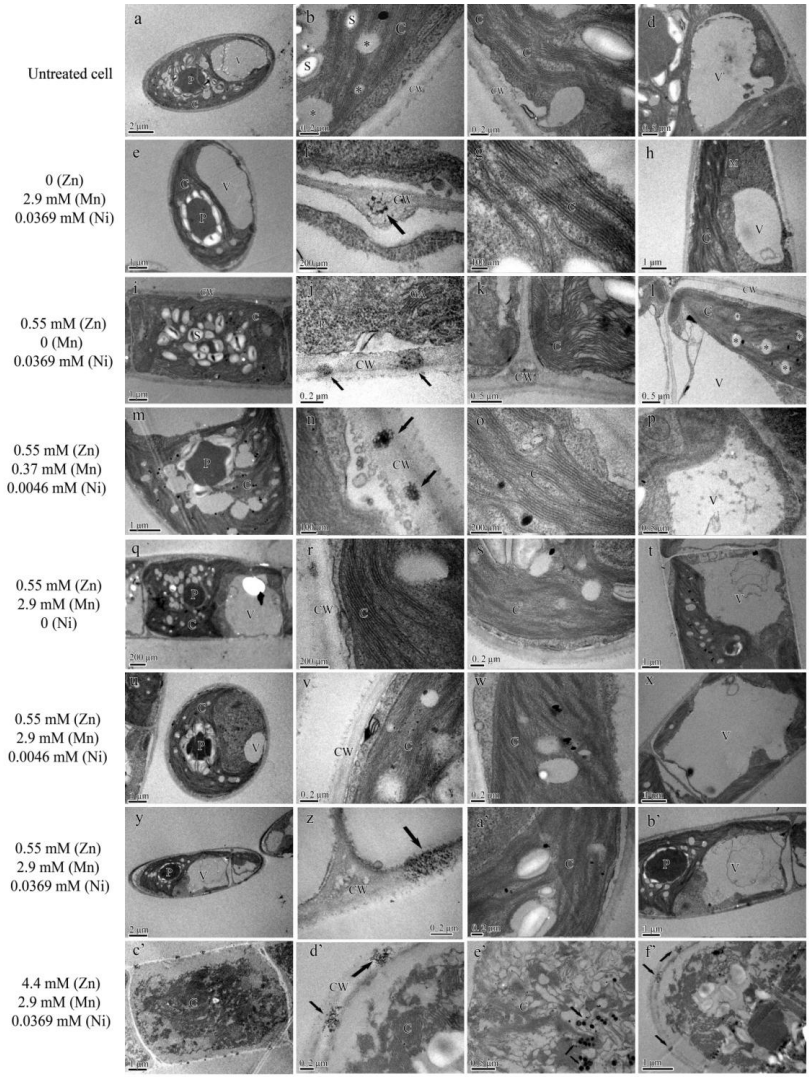

Figure 5.5 Transmission electron microscopy of *Ulothrix* sp. LAFIC 010 cultivated in different combinations of metal concentrations. a-d: Untreated cell. a, Overview of the cellular structure; b, Detail of the structure of the cell wall; c, Chloroplasts with thylakoid-forming grana; e-h: Cells treated with 0 (Zn); 2.9 mM (Mn); 0.0369 mM (Ni). e, Cell Overview; f, electron-dense structures (arrow) appeared in the cell wall; g, Chloroplasts and thylakoids; h, Detail of the vacuole. i-l: Cells treated with 0.55 mM (Zn); 0 (Mn); 0.0369 mM (Ni). i, Cell

overview, detail of numerous starch granules; j, Detail of the cell wall with the presence of electron-dense material (arrows); k, Detail of the chloroplast; l, Detail of the vacuole and the chloroplast without presence of starch grains (asterisk); m-p: Cells treated with 0.55 mM (Zn); 0.37 mM (Mn); 0.0046 mM (Ni). m, Cell overview; n, Detail of the cell wall with the presence of electron-dense material (arrows); o, Detail of the chloroplast; p, Detail of the vacuole. q-t: Cells treated with 0.55 mM (Zn); 2.9 mM (Mn); 0 (Ni). q, Cell overview; r, Cell wall detail; s, Detail of the chloroplast; t, Detail of the vacuole. u-x: Cells treated with 0.55 mM (Zn); 2.9 mM (Mn); 0.0046 mM (Ni). u, Cell overview; v, Detail of the cell wall; w, Detail of the chloroplast; x, Detail of the vacuole. y-b': Cells treated with 0.55 mM (Zn); 2.9 mM (Mn); 0.0369 mM (Ni). y, Cell overview; z, Detail of the cell wall with presence of eletrondense material material (arrow); a', Detail of the chloroplast; b', Cell overview, detail of vacuole. c'-f': Cells treated with 4.4 mM (Zn); 2.9 mM (Mn); 0.0369 mM (Ni). c', Overview of the altered cellular structure; d', Cells with altered structure of the chloroplast and eletrondense material present in the cell wall (arrows); e', Cells with altered structure of the chloroplast with detail of disorganized thylakoids and numerous plastoglobuli (arrows); f', Cells with altered structure of the chloroplast with detail of the electron-dense material in the cell wall (arrows). Abbreviations: Cell wall (CW); Chloroplast (C); Golgi Apparatus (GA); Mitochondria (M); Pyrenoid (P); Vacuoles (V); Vacuolar Deposit (VD). These pictures were obtained for samples after 8 days of treatment.

Experiment 3 - bioaccumulation of metal from AMD by Ulothrix *sp.* LAFIC 010

The presence and variation of the three metals (Zn, Mn, and Ni) in AMD with and without *Ulothrix* sp. LAFIC 010 filaments are showed in Figure 5.6. The metal concentrations in the AMD sample were 0.0303 mM Zn, 3.313 mM Mn, and 0.0041 Ni. The contact of the biomass with AMD for 14 days promoted the decay in concentrations of the three metals, reaching 0.0114 mM Zn, 1.291 mM Mn, and 0.0014 mM Ni. However, concentrations of all three metals also decreased in the treatments without algal biomass, reaching 0.0119 mM Zn, 1.295 mM Mn, and 0.0022 mM Ni.

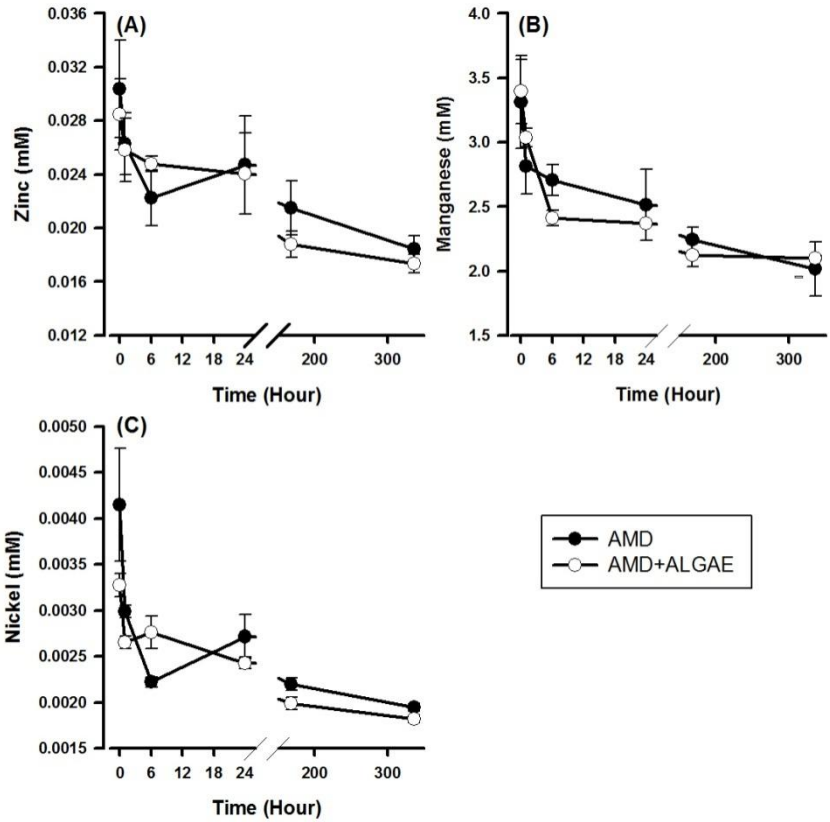

Figure 5.6 Chemical analysis of the concentrations of Zinc (A), Manganese (B) and Nickel (C) in the treatments with and without *Ulothrix* sp. (biomass during 14 days).

Comparing the results between treatments with and without biomass, significant statistical differences were shown for Mn after 6 hours and for Ni after 1 and 6 hours, and 7 and 14 days (Table 5.5).

Table 5.5 Summary of the results of one-way repeated measures analysis of variance (ANOVA) of chemical water analysis comparing the concentration of the metals Zinc, Manganese and Nickel in AMD water with and without *Ulothrix* sp. LAFIC 010 biomass. N=3. $\alpha < 0.05$. Bold indicates significant

values at P < 0.05.

	[Zn] mM		[Mn] mM		[Ni] mM	
Time (hour)	*F* value	*p*	*F* value	*p*	*F* value	*p*
0	0.533	0.506	0.103	0.764	5.879	0.072
1	0.054	0.828	2.960	0.160	**38.633**	**0.003**
6	4.128	0.112	**14.317**	**0.019**	**25.572**	**0.007**
24	0.055	0.826	0.697	0.451	3.920	0.119
168	4.414	0.104	2.263	0.207	**15.886**	**0.016**
336	2.648	0.179	0.481	0.526	**14.866**	**0.018**

The results in Fig. 5.7 showed an increasing removal rate during the first hour after inoculation for Ni and Zn, and after 6 hours for Mn. After 6 hours, the removal rates were 33.5% for Ni, 27.2% for Mn, and 18.3% for Zn. Considering the period from 6 to 336 hours (14 days) the removal slightly increased. The maximum removal was recorded after 14 days at rates of 41.4% for Ni, 28.5% for Mn, and 20.7% for Zn.

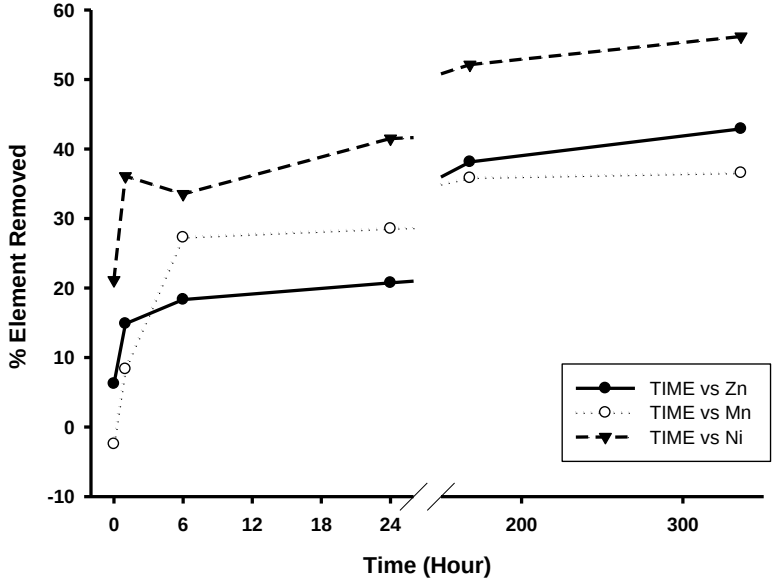

Figure 5.7. Removal percentages of Zn, Mn, and Ni by the biomass of *Ulothrix* sp. LAFIC 010 over 14 days of growth in Acid Mine Drainage.

The algal growth reached 5.89% per day, totaling approximately 0.4 $g.L^{-1}$ of dried biomass in each replicate. Additionally, Chl *a*, Chl b, and carotenoid concentrations in the dry biomass were not significantly different between algae exposed and not exposed to AMD.

According to the SEM-EDS spectra (Fig. 5.8), the main elements present on the *Ulothrix* sp. LAFIC 010 cell surface were O (71.56%), P (13.70%), Na (3.77%), Ca (2.62%), Si (1.97%), Mg (1.96%) and Fe (1.40%). The representative metal adsorption on the biofilm was Fe. Similarly to previous experiments, Zn, Mn and Ni were not detected on the surface of *Ulothrix* sp. LAFIC 010 filaments.

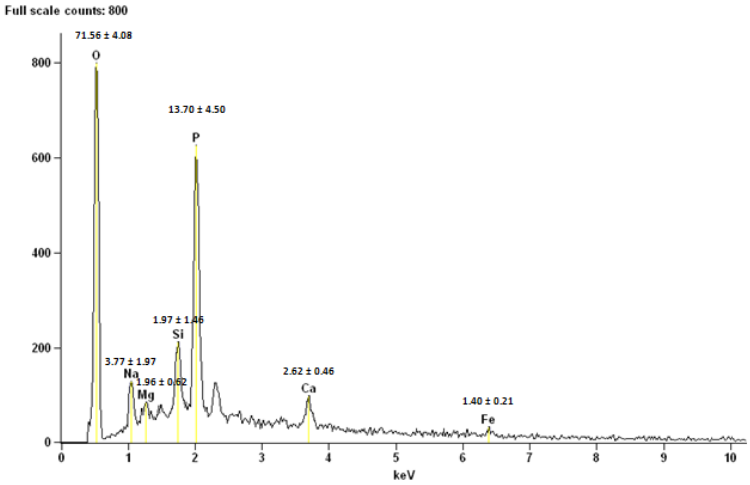

Figure 5.8 Energy-dispersive X-ray spectroscopy of the cell wall components from *Ulothrix* sp. LAFIC 010 cultivated for 14 days in water contaminated with Acid Mine Drainage.

Filaments of *Ulothrix* sp. LAFIC 010 (Control of Experiment 1 and 2) without the addition of AMD or metals (Fig. 5.9a-d) were compared with those cultivated in AMD treatments (Figs. 5.9e-l). The cells cultivated in AMD presented electron-dense material in all cell walls, chloroplasts with higher concentration of plastoglobuli, and organized thylakoids. It was possible to observe electron-dense material dispersed throughout the cell wall across the entire cell (Figs. 5.9e-f), and this material is evidenced in Figures 5.9g-h. In these cells, the chloroplasts showed integrity, but with higher concentration of plastoglobolis and electron-transparent regions without starch grains

(Figs. 5.9 i-j). Moreover, electron-dense materials were observed inside vacuoles in the cells of *Ulothrix* sp. LAFIC 010 AMD-cultivated samples (Figs. 5.9k-l).

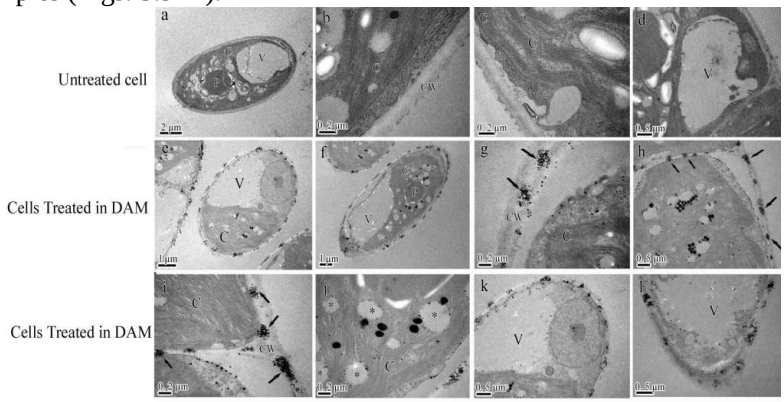

Figure 5.9 Transmission electron microscopy of *Ulothrix* sp. LAFIC 010 cultivated in AMD. a, b, c, d: Untreated cell. a, Overview of the cellular structure; b, Detail of the structure of the cell wall; c, Chloroplasts with thylakoid forming grana; d, Detail of the vacuole. e, f, g, h, i, j, k, l: Cell cultivated in AMD treatment for 14 days. e and f, Cell overview, cells presenting chloroplast occupying lower cell volume, but with characteristic thylakoids and pyrenoid with few starch grains. g and h, Electron-dense material (*arrows*) in the cell wall. i and j, chloroplasts with presence of several plastoglobuli and without presence of starch grain (asterisk). k and l, Details of the vacuoles. Abbreviations: Chloroplast (C); Cell Wall (CW); Pyrenoid (P); Vacuoles (V); Vacuolar Deposit (VD).

5.4 Discussion

The results presented in this study provide additional evidence for the capacity of *Ulothrix* sp. LAFIC 010 to survive in environments with high concentrations of Zn, Mn and Ni, reinforcing its potential for bioremediation. The main damages in *Ulothrix* sp. LAFIC 010 could be observed just in higher concentrations of zinc. It is known that Zn is an essential micronutrient for cellular metabolism, such as for chlorophyll formation (Mane *et al*., 2014), and it acts as an important cofactor for many biochemical processes (Mikulic and Beardall, 2014). Despite this, our study demonstrated that at high concentrations, Zn can be toxic even in an extremophilic algae. Negative effects of Zn on the physiological responses and ultrastructural features (TEM) of *Ulothrix* sp. LAFIC 010

could already be observed at the lowest Zn concentration tested (1.1 mM). The greatest ultrastructural changes were observed at 4.4 mM Zn, when chloroplast thylakoids were degraded and effective quantum yield diminished. The inhibition of cell division and destruction of chlorophyll were mentioned as examples of the toxic effects of Zn by Pawlik-Skowron´ska (2001). According to Mikulic and Beardall (2014), exposure to high levels of zinc can cause damages to photosystem II (PSII) and decrease the efficiency of the Calvin cycle in microalgae. The negative effects of Zn in six freshwater algal species were detected by Mane *et al*. (2014). In that study, Zn affected chlorophyll, protein, carbohydrate, starch, and amino acids starting at 1 mg Zn/L, i.e., the equivalent of 0.015 mM Zn, much lower than the levels tested in our study.

Although Zn-coupled toxic effects were detected in *Ulothrix* sp. LAFIC 010, the filaments analyzed under SEM or TEM did not present direct evidence of external or intracellular Zn localization. This metal can be detected inside algal cells, such as the case of *Chlorella salina*, which accumulated Zn in the cell wall and in the vacuoles (Garnham *et al*. 1992). *Chlamydomonas acidophila* isolated from an acidic environment and then cultivated in 1.5 mM of Zn presented unaffected chloroplasts (Nishikawa *et al*. 2003). On the other hand, those authors observed 85% decrease in cell size in comparison to control cells. In addition, Zn-treated samples showed a slight increase in number of starch grains and the presence of membrane whorls. It is noteworthy to reinforce that in the case of *Ulothrix* sp. LAFIC 010, threefold Zn was utilized in the highest and most deleterious concentration tested.

Ulothrix sp. LAFIC 010 showed high tolerance to Mn and Ni. The physiological responses were not affected by these metals, even considering the highest concentrations and combinations among them. In addition, cells did not present apparent damages, according to the TEM results. Mn and Ni are micronutrients for the development and metabolism of all aquatic microalgae (Andersen, 2005). For example, even low concentrations of Mn were able to stimulate chlorophyll formation (Mane *et al*., 2014). Lustigman *et al*. (1995) described that when *Chlorella* was pre-exposed to low concentrations of Ni, the total chlorophyll concentration was found to be greater than that of the control (without any Ni contact). However, at higher concentrations Mn and Ni metals could inhibit chlorophyll physiological activities (Malcolm, *et al*., 2004; Tam *et al*., 2001).

Algae present several mechanisms for metal detoxification. Intracellular metals can be immobilized by metal binding peptides, compartmentalized in vacuoles by binding in peptides forming organometalic complexes, and/or complexed in the cytoplasm by synthesis of phytochelatins and polyphosphate bodies **(**Pawlik-Skowron´ska, 2001; Priya *et al*., 2014). According to Nishikawa *et al*. (2003), many researchers have reported that electron-dense bodies are composed by polyphosphates, which have the ability to accumulate metals and also protect algal cells from metal toxicity. According to the TEM results from the individual metal experiment, *Ulothrix* sp. LAFIC 010 showed electron-dense material in different parts of the cells in the treatments with Mn and Ni, indicating a possible role in bioaccumulation of these metals. In the case of cells exposed to Mn, electron-dense bodies were evident in cell walls (clearly seen at the highest Mn concentration 2.9 mM). In addition, the samples treated strongly with Ni (0.0369 mM) presented signs of electron-dense areas inside cellular vacuoles. As reported by Chong *et al*. (2000), the cell wall is the main site for metal binding in microalgae and variations in metal removal efficiency could be explained by differences in thickness and composition of algal cell wall. Other studies with bacterial and fungal strains showed that Ni is mainly limited to the cell surface or to periplasm and the cell membrane (Malik, 2004). Despite this, we suggest that, for *Ulothrix* sp. LAFIC 010, Ni was allocated to the vacuoles. The exact role of vacuoles in metal detoxification is not yet clear, but vacuolation could contribute to compartmentalization of toxic metals, as suggested by Davies *et al* (1992).

Considering the fact that most industrial and mining effluents contain a mixture of metals, the combination of metals may act in antagonistic, synergistic, or non-interactive ways (Harris and Ramelow, 1990). In the tests of metal combinations carried out with *Ulothrix* sp. LAFIC 010, Zn toxic effects were also detected regardless of the presence of Ni and Mn, even if they were in high concentrations. A possible reason could be a higher affinity of algae binding sites for Zn when compared to Mn and Ni, causing the Zn to first occupy the sites, blocking the sorption of the other metals. This was suggested by Garnham *et al*. (1992), which verified Zn and Mn competing for the same cell binding sites. These authors referred this process as an inhibition of Mn uptake by Zn, since the same uptake system could be responsible for the transport of both metals. Chong *et al*. (2000) showed that *Chlorella vulgaris*, C. *sorokiniana*, and eight other different

microalgal species had higher affinity for binding Zn than Ni. When cultured in a solution containing both Zn and Ni, Chong *et al*. (2000) observed that Zn caused a decrease in Ni bioaccumulation of these species when compared to their Ni-bioaccumulation cultured in medium without Zn, indicating that Zn could suppress the Ni-uptake by algal cells. Additionally, in the case of *Ulothrix* sp. LAFIC 010, Mn alone and in combination with Ni did not change the toxic effect of Zn. However, for other species, like *Chlamydomonas* sp., Mn decreased Zn toxicity (Sunda and Huntsman, 1998). These authors reported cellular Zn increasing while external Mn concentrations decreased. Futhermore, Mn and Ni toxicity were non-interactive since the combinations of these metals caused the same physiological response in *Ulothrix* sp. LAFIC 010 when compared to the effects of metals alone. The same was reported by Rousch and Sommerfeld (1999), where Mn toxicity was not diminished by Ni, probably due to the existence of a different uptake mechanism for each metal.

Despite the fact that Zn could be competing for the same cellular uptake system as described previously, SEM results suggests that the adsorption of Mn occurred due to a synergistic effect of the combination of Mn and Zn. Results from SEM analyses of the first experiment showed that none of the 3 metals appeared to be associated with cell surfaces. On the other hand, in the second experiment, the presence of Mn was identified in the treatments with Zn. Similar results were obtained by Harris and Ramelow (1990) in a study with the algae *Scenedesmus quadricauda*. The authors described that the alga showed increased Cu binding in the presence of Ag-Zn combination. The explanation of this behavior was that copper binding could be enhanced by the presence of other metals. Such a variety of results suggest that there is no standard behavior for the fate of different metals, alone or combined with others, in the presence of microalgae. Each species can show different reactions, which points to the need for specific studies.

In the TEM analysis, cells exposed to Zn (4.4 mM) showed similar features to those from the combination of the three metals (Zn 4.4 mM, Mn 2.9 mM, Ni 0.0369 mM), with a deterioration of the thylakoids over almost the entire cell. This process was also observed in the first experiment for treatments with Zn alone in concentrations higher than 4.4 mM. Alteration of chloroplasts due to Zn was observed in leaf cells from maize (Jiang *et al*., 2007). When maize was grown in solution with 10 mM Zn, chloroplast stroma was not uniform, exhibiting alterations compared with the control. Also under 4.4 mM Zn, 2.9 mM

Mn, and 0.0369 mM Ni, electron-dense structures were detected in the cell wall. Algae cell walls have many metal binding sites due to the presence of polysaccharides and proteins in their composition (Schiewer and Wong, 2000). However, the composition and distribution of the cell wall differs among algal species, influencing the capacity of metal ion biosorption (Zeraatkar *et al*., 2016). According to Domozych *et al*. (1980), *Ulothrix* spp. cell walls have a high degree of cellulose and water soluble polysaccharides, which may thus be responsible for the electron dense material in cell the wall of *Ulothrix* sp. LAFIC 010. This organic material could be related to the incorporation of Mn and Ni.

Metal concentrations in the AMD utilized in our experiments were different from previous samples collected at the same site (Moura, 2014). While Zn concentrations (0.028 mM) were lower than the previous study (0.55 mM), the opposite was observed for Mn, with 2.888 mM in our sample and 0.365 mM in samples from Moura (2014). For Ni concentrations, both studies showed similar values (0.0033 mM – this study, and 0.0063 mM - Moura, 2014). These variations suggest that metal concentrations in AMD are influenced by several factors, like seasonality, rainfalls and type and intensity of mining operations.

The metal removal rate by *Ulothrix* sp. LAFIC 010 was highest for Ni (41.4%), followed by Mn (28.5%) and then Zn (20.7%). Orandi *et al*. (2012) observed that a biofilm produced by *Ulothrix* presented similar removal rate of Ni and Mn (45–50 %) when cultivated in AMD from a copper mine, while the removal of Zn was much lower in comparison to the others. Similarly, in a study with Al, Fe, Mg, Mn, and Zn bioaccumulation in *Oedogonium crassum*, *Klebsormidium klebsii* and *Microspora tumidula*, the three species bioaccumulated less Zn than the other metals (Oberholster *et al*., 2014). The experiment was conducted at three different pH levels (3, 5 and 7) and under all of them, the selected species expressed a protective mechanism that prevented large quantities of Zn from entering the cells compared to the uptake of other metal ions.

Ulothrix sp. LAFIC 010 showed two phases for metal uptake during the 14 days of the experiment. The first phase occurred during the first six hours, possibly associated to the process of adsorption (Zeraatkar *et al*. 2016). This phenomenon may be rapid, with passive physical (electrostatic) or chemical (surface complexation) interactions, resulting in metal complexation on the extracellular surface of algal cells. The adsorption process occurs in both living and dead cells, and is a metabolism-independent binding process. After six hours, the

concentrations of the three metals slightly decreased, probably associated with the second phase of biosorption. The active uptake process was described as a slow and metabolism-dependent process, involving active transport of metals across the cell membrane into the intracellular organelles (Mehta and Gaur, 2005).

Removal rates of metals by algae like *Ulothrix* sp. LAFIC 010 may depend not only on the specific physiology of the species, but also on the presence of other microorganisms (fungi, bacteria) and environmental conditions. When metal biosorption potential tests are applied to real industrial effluents, multiple aspects should be taken into account such as metal concentration, hardness, dissolved organic carbon, and pH (Orandi *et al*., 2012). These physicochemical variables affect both metal speciation and metal binding at the organism-water interface, which influence the removal of Zn, Mn and Ni from AMD. Most studies with microalgae (*Chlorella*, *Chlamydomonas*, *Scenedesmus* and *Pseudokirchneriella* sp.) have shown an increase in metal toxicity with the increase of pH, as a result of decreased competition between the metal ion and H^+ at the cell surface (Arunakumara and Zhang, 2008). According to Oberholster *et al*. (2014), bioaccumulation of Al, Fe, Mg, Mn, and Zn decreased in *Oedogonium crassum, Klebsormidium klebsii* and *Microspora tumidula* according to pH reduction. The competition between protons (H^+) and metals for binding sites at the algal cell surface could suggest that the biomass of *Ulothrix* sp. LAFIC 010 did not assimilate more metals since the low pH could be affecting the uptake of metals by the biomass. As *Ulothrix* sp. can be cultivated in a range of pH (Rousch and Sommerfeld, 1999), it is important to investigate the bioaccumulation potential of metals in a range of pH to be sure if the process is pH-dependent for this alga. Besides pH, the presence of a high concentration of multi-metal ions could be causing competition for the same algal binding sites, influencing the uptake of metal ions by algal cells. Corder and Reeves (1994) found improvement in Ni biosorption by autoclaving cyanobacteria biomass. However, none of the species studied could bind Ni efficiently in actual effluent sample due to the presence of high concentration of Na ions. Similarly, a decline in Cu concentration in the medium induced decrease of competition between Cu and Zn ions thus allowing increased removal of Zn by biofilm dominated by *Ulothrix* sp. (Orandi *et al*. 2012). The authors concluded that Zn removal could be adversely affected by the presence of competing ions.

Additionally, another factor that could influence metal biosorption is the biomass concentration (Mehta and Gaur, 2005; Zeraatkar *et al*., 2016). A higher concentration of biomass increases metal removal due to greater availability of metal binding sites. This phenomenon was shown by Mehta and Gaur (2001) where increased biomass of *Chlorella vulgaris* by 100-fold intensified Ni and Cu removal from 13 to 65% and 10 to 85%, respectively, from solutions containing 5 $mg.L^{-1}$ Ni or Cu. In a study with *Scenedesmus abundans*, Terry and Stone (2002) observed binding site competition between Cu (II) and Cd (II) which was attenuated with increasing biomass concentration. Since increasing biomass concentrations could enhance final metal bioremoval, the results obtained in the present study could be greater with an increase in the biomass of *Ulothrix* sp. LAFIC 010 per unit of volume, which could result in increased metal removal capacity.

The AMD sample used in our study originated from coal mining residues, mostly pyrite ($FeS_{2(s)}$). The oxidation of pyrite and formation of AMD releases high amounts of Fe^{2+} as part of reaction products. In the SEM-EDS results, the main metal associated to *Ulothrix* sp. LAFIC 010 biomass was iron, which could be explained by the high concentration of this metal in the contaminated water. Efficient bioaccumulation of Fe was observed by Oberholster *et al*. (2014) by *O. crassum, K. klebsii and M. tumidula.* These species were cultivated in AMD with three different pHs, and Fe bioaccumulation increased as the pH decreased. The authors proposed that Fe precipitation at pH values $\geq$4.0 made the metal inaccessible to the algae, whereas at a lower pH dissolved Fe speciation changed due to microbial oxidation/reduction processes, causing Fe to be more readily available for bioaccumulation by microalgae.

Via TEM analysis, it is possible to observe that after 14 days in AMD, *Ulothrix* sp. LAFIC 010 cells exhibit increasing numbers of electron-dense material in the cell wall. As described previously, this phenomenon also occurred in both Experiments 1 and 2 under treatments of 2.9 mM of Mn. Althought the chemical analysis did not show any difference between the treatments in Experiment 3 it is possible to infer by TEM analisys that *Ulothrix* sp. LAFIC 010 could be accumulating the metals by the formation of the electron dense material in the cell wall.

Pigment analyses showed that most cells exposed to AMD survived for the 14 days. Decline in chlorophyll content after 15 days of exposition to 1.0 mg. mL^{-1} Zn and Mn was described for six microalgae

(*Anabaena ambigua*, *Anabaena subcylindrica*, *Nostoc commune*, *Nostoc muscorum*, *Spirogyra* sp., and *Spirulina* sp.) (Mane *et al*., 2014). Since Zn and Mn concentrations used in the study described above were equivalent to 0.01 mM Mn and the concentration of this metal in the AMD used in the present study was 2.888 mM, it is possible to infer that *Ulothrix* sp. LAFIC 010 can tolerate extremely high concentrations of Mn, considering that filaments grew 5.9% per day.

In conclusion, the results obtained in the present study suggest that the growth of *Ulothrix* sp. LAFIC 010 in medium enriched with metals is not affected by the concentration of Ni and Mn, at least in the pH tested in the present study. Zn most likely has a higher affinity to *Ulothrix* sp. LAFIC 010 biomass than Mn and Ni, resulting in toxic effects. It is noteworthy that the concentrations of metals used in this study were higher than those found in environments contaminated by AMD and that even at high concentrations, *Ulothrix* sp. LAFIC 010 survived and even grew. Future studies should focus on understanding the relationship among the factors that compete to influence metal bioaccumulation by the algae. The water quality (pH, temperature, metal concentration) and the features of the algae e.g. cell wall composition, are parameters that need to be considered for the investigation of bioremediation potential. *Ulothrix* sp. LAFIC 010 is an extremophilic microalga that thrives in conditions where only few species could survive. This confers capacity to exclude eventual competitors, reinforcing its potential for metal bioremediation of effluents. The possibility of using the produced biomass for obtainment of high value compounds could be an additional advantage.

Acknowledgments The authors would like to acknowledge the staff of the Central Laboratory of Electron Microscopy (LCME), Multiuser Laboratory of Biology Studies (LAMEB), and Laboratory of Mass and Atomic Spectrometry (LEMA), all from the Federal University of Santa Catarina, Brazil (UFSC). This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

5.5 References

Alloway, B.J., Ayres, D.C., 1997. Chemical Principles of Environmental Pollution. CRC Press, 2nd edn, Blackie Academic and Professional, London.

Anderson, R.A., 2005. Algal Culturing Techniques, 1st Edition, Journal of Chemical Information and Modeling.

Arunakumara, K.K.I.U., Zhang, X., 2008. Heavy metal bioaccumulation and toxicity with special reference to microalgae. J. Ocean Univ. China 7, 60–64.

Bajguz, A., 2000. Blockade of heavy metals accumulation in Chlorella vulgaris cells by 24-epibrassinolide. Plant Physiol. Biochem. 38, 797–801.

Chong, A.M.Y., Wong, Y.S., Tam, N.F.Y., 2000. Performance of different microalgal species in removing nickel and zinc from industrial wastewater. Chemosphere 41, 251–257.

Corder, S.L., Reeves, M., 1994. Biosorption of nickel in complex aqueous waste streams by cyanobacteria. Appl. Biochem. Biotechnol. 45–46, 847–859.

Das, B.K., Roy, A., Koschorreck, M., Mandal, S.M., Wendt-Potthoff, K., Bhattacharya, J., 2009. Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. Water Res. 43, 883–894.

Domozych, D.S., Stewart, K.D., Mattox, K.R., 1980. The comparative aspects of cell wall chemistry in the green algae (chlorophyta). J. Mol. Evol. 15, 1–12.

Davies, K. L., Davies, M. S., Francis, D., 1992. Zinc-induced vacuolation in root meristematic cells of cereals. Annals of botany 69.1: 21-24.

Freitas, A.P.P., Schneider, I.A.H., Schwartzbold, A., 2011. Biosorption of heavy metals by algal communities in water streams affected by the acid mine drainage in the coal-mining region of Santa Catarina state, Brazil. Miner. Eng. 24, 1215–1218.

Fyson, A., Nixdorf, B., Kalin, M., 2006. The acidic lignite pit lakes of Germany-Microcosm experiments on acidity removal through

controlled eutrophication. Ecol. Eng. 28, 288–295.

Garnham, G.W., Codd, G.A., Gadd, G.M., 1992. Kinetics of uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *ChloreUa salina* robiology Biotechnology Kinetics of uptake. Appl. Microbiol. Biotechnol. 37, 270–276.

Genty, B., Briantais, J. M., and Baker, N. R. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990:87–92.

Gray, N. F. Environmental impact and remediation of acid mine drainage: a management problem. Environmental Geology 30.1 (1997): 62-71.

Harris, P., Ramelow, G., 1990. Binding of metal ions by particulate biomass derived from Chlorella vulgaris and Scenedesmus quadricauda. Environ. Sci. Technol. 24, 220–228.

Jiang, H.M., Yang, J.C., Zhang, J.F., 2007. Effects of external phosphorus on the cell ultrastructure and the chlorophyll content of maize under cadmium and zinc stress. Environ. Pollut. 147, 750–756.

Jordanova, A., Strezov, A., Ayranov, M., Petkov, N., Stoilova, T., 1999. Heavy metal assessment in algae, sediments and water from the Bulgarian Black Sea coast. Water Sci. Technol. 39, 207–212.

Lawrence, J.R., Swerhone, G.D.W., Kwong, Y.T.J., 1998. Natural attenuation of aqueous metal contamination by an algal mat. Can. J. Microbiol. 44, 825–832.

Lignell, A., Pedersén, M., 1989. Agar composition as a function of morphology and growth rate. Studies on some morphological strains of Graciliaria secundata and Graciliaria verrucosa (Rhodophyta). Bot. Mar. 32, 219–227.

Lustigman, B., Lee, L.H., Khalil, A., 1995. Effects of nickel and pH on the growth of Chlorella vulgaris. Bull. Environ. Contam. Toxicol. 55, 73–80.

Macek, T., Macková, M., Káš, J., 2000. Exploitation of plants for the removal of organics in environmental remediation. Biotechnol. Adv. 18, 23–34.

Malcolm, H.M., Wood, M., Kingdom, U., 2004. Concise International Chemical Assessment Document 63 MANGANESE AND ITS COMPOUNDS.

Malik, A., 2004. Metal bioremediation through growing cells. Environ. Int. 30, 261–278.

Mane, P., Kadam, D., Bhosle, A., 2014. Influence of Manganese and Zinc on Biochemical Profiles of Selected Algal Species: A Laboratory Study. Bioremediat. J. 18, 111–127.

Mehta, S.K., Gaur, J.P., 2005. Use of algae for removing heavy metal ions from wastewater: progress and prospects. Crit. Rev. Biotechnol. 25, 113–152.

Mehta, S.K., Gaur, J.P., 2001. Removal of Ni and Cu from single and binary metal solutions by free and immobilized Chlorella vulgaris. Eur. J. Protistol. 271, 261–271.

Mikulic, P., Beardall, J., 2014. Contrasting ecotoxicity effects of zinc on growth and photosynthesis in a neutrophilic alga (Chlamydomonas reinhardtii) and an extremophilic alga (Cyanidium caldarium). Chemosphere 112, 402–411.

Monteiro, C.M., Castro, P.M.L., Malcata, F.X., 2011. Capacity of simultaneous removal of zinc and cadmium from contaminated media, by two microalgae isolated from a polluted site. Environ. Chem. Lett. 9, 511–517.

Moura, A.J. Microrganismos Endofíticos Associados À Planta De Ambientes Impactados E Não Impactados Pela Drenagem Ácida De Mina De Carvão (Dam) 128. (Mestrado em Biotecnologia e Biociências) – Univ. Federal de Santa Catarina, Florianópolis, 2014.

Nishikawa, K., Yamakoshi, Y., Uemura, I., Tominaga, N., 2003. Ultrastructural changes in Chlamydomonas acidophila (Chlorophyta) induced by heavy metals and polyphosphate metabolism. FEMS Microbiol. Ecol. 44, 253–259.

Novis, P.M., Harding, J.S., 2007. Extreme acidophiles – freshwater algae associated

with acid mine drainage. In: Seckbach, J. (Ed.), Algae and Cyanobacteria in
Extreme Environments. Springer, Dordrecht, pp. 443–463.

Nuhoglu, E.M.Y., 2003. The removal of chromium (VI) from synthetic wastewater by Ulothrix zonata 12, 376–381.

Nuhoglu, Y., Malkoc, E., Gürses, A., Canpolat, N., 2002. The removal of Cu (II) from aqueous solutions by Ulothrix zonata. Bioresour. Technol. 85, 331–333.

Oberholster, P.J., Cheng, P.H., Botha, A.M., Genthe, B., 2014. The potential of selected macroalgal species for treatment of AMD at different pH rangesin temperate regions. Water Res. 60, 82–92.

Olaveson, M.M., Stokes, P.M., 1989. Responses of the acidophilic alga Euglena mutabilis (EUGLENOPHYCEAE) to carbon enrichment at pH 3. J. Phycol. 25, 529–539.

Orandi, S. A., Yaghubpur, A. Sahraei, H. Behrouz, M., 2007. Influence of acid mine drainage on aquatic life at Sar Cheshmeh copper mine Nanometric jarosite / alunite in carbonaceous matter rich cherts : Marble bar drill core (# 1 ABDP): Indications for acid-sulfate conditions in a hydrothermal system. J. Geochemical Explor. 2007–2007.

Orandi, S., Lewis, D.M., 2013. Synthesising acid mine drainage to maintain and exploit indigenous mining micro-algae and microbial assemblies for biotreatment investigations. Environ. Sci. Pollut. Res. 20, 950–956.

Orandi, S., Lewis, D.M., Moheimani, N.R., 2012. Biofilm establishment and heavy metal removal capacity of an indigenous mining algal-microbial consortium in a photo-rotating biological contactor. J. Ind. Microbiol. Biotechnol. 39, 1321–1331.

Ouriques, L., Bouzon, Z., 2003. Ultrastructure of germinating tetraspores of Hypnea musciformis (Gigartinales, Rhodophyta). Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 137, 193–201.

Paknikar, K.M., Pethkar, A. V., Puranik, P.R., 2003. Bioremediation of Metalliferous Wastes and Products using Inactivated Microbial

Biomass. Indian J. Biotechnol. 2, 426–443.

Park, J.B.K., Craggs, R.J., Shilton, A.N., 2011. Wastewater treatment high rate algal ponds for biofuel production. Bioresour. Technol. 102, 35–42.

Pawlik-Skowrońska, B., 2001. Phytochelatin production in freshwater algae Stigeoclonium in response to heavy metals contained in mining water; effects of some environmental factors. Aquat. Toxicol. 52, 241–249.

Priya, Madhu, et al., 2014. "23 Microalgae in Removal of Heavy Metal and Organic Pollutants from Soil." *Microbial biodegradation and bioremediation*. 521-539.

Rose, P.D., Boshoff, G. a, van Hille, R.P., Wallace, L.C., Dunn, K.M., Duncan, J.R., 1998. An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters. Biodegradation 9, 247–257.

Rousch, J.M., Sommerfeld, M.R., 1999. Effect of manganese and nickel on growth of selected algae in pH buffered medium. Water Res. 33, 2448–2454.

Schiewer, S., Wong, M.H., 2000. Ionic strength effects in biosorption of metals by marine algae 41, 271–282.

Simioni, C., Rover, T., Schmidt, É.C., de L. Felix, M.R., Polo, L.K., Santos, R. dos, Costa, G.B., Kreusch, M., Pereira, D.T., Ouriques, L.C., Bouzon, Z.L., 2014. Effects of brefeldin A on the endomembrane system and germ tube formation of the tetraspore of *Gelidium floridanum* (Rhodophyta, Florideophyceae). J. Phycol. 50, 577–586.

Smucker, N.J., Drerup, S.A., Vis, M.L., 2014. Roles of benthic algae in the structure, function, and assessment of stream ecosystems affected by acid mine drainage. J. Phycol. 50, 425–436.

Soldo, D., Hari, R., Sigg, L., Behra, R., 2005. Tolerance of Oocystis nephrocytioides to copper: Intracellular distribution and extracellular complexation of copper. Aquat. Toxicol. 71, 307–317.

Stevens, A.E., Mccarthy, B.C., Vis, M.L., 2001. Metal content of Klebsormidium-dominated (Chlorophyta) algal mats from acid mine drainage waters in southeastern Ohio ’. J. Torrey Bot. Soc. 128, 226–233.

Sturm, B.S.M., Lamer, S.L., 2011. An energy evaluation of coupling nutrient removal from wastewater with algal biomass production. Appl. Energy 88, 3499–3506.

Sunda, W.G., Huntsman, S. a., 1998. Interactions among Cu2+, Zn2+, and Mn2+ in controlling cellular Mn, Zn, and growth rate in the coastal alga Chlamydomonas. Limnol. Oceanogr. 43, 1055–1064.

Talebi, A.F., Tabatabaei, M., Mohtashami, S.K., Thidfar, M., Moradi, F., 2013. Comparative Salt Stress Study on Intracellular Ion Concentration in Marine and Salt-adapted Freshwater Strains of Microalgae. Not. Sci. Biol. 5, 309–315.

Tam, N.F.Y., Wong, J.P.K., Wong, Y.S., 2001. Repeated use of two Chlorella species, C. vulgaris and WW1 for cyclic nickel biosorption. Environ. Pollut. 114, 85–92.

Terry, P.A., Stone, W., 2002. Biosorption of cadmium and copper contaminated water by Scenedesmus abundans. Chemosphere 47, 249–255.

Vidali, M., 2001. Bioremediation - An overview. J. Ind. Pollut. Control 27, 161–168.

Wellburn, A.R., 1994. The Spectral Determination of Chlorophylls a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. J. Plant Physiol. 144, 307–313.

Wong, J.P.K., Wong, Y.S., Tam, N.F.Y., 2000. Nickel biosorption by two chlorella species, C. Vulgaris (a commercial species) and C. Miniata (a local isolate). Bioresour. Technol. 73, 133–137.

Wong, M.H., 2003. Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. Chemosphere 50, 775–780.

Zeraatkar, A.K., Ahmadzadeh, H., Talebi, A.F., Moheimani, N.R.,

McHenry, M.P., 2016. Potential use of algae for heavy metal bioremediation, a critical review. J. Environ. Manage. 181, 817–831.

5. CONSIDERAÇÕES FINAIS

O presente estudo apontou que a microalga *Ulothrix* sp. LAFIC 010 apresentou respostas fisiológicas e ultraestruturais comprometidas quando cultivada em meio com elevadas concentrações de zinco (>2.2 mM). No entanto, foi observada alta tolerância e crescimento da microalga frente às elevadas concentrações de manganês (até 2.9 mM) e níquel (até 0.0369 mM). Os efeitos deletérios de zinco foram observados tanto nos experimentos dos metais isolados quanto em combinações, o que indica que a presença de manganês e níquel não diminui a toxicidade de zinco, ao menos nas concentrações apresentadas neste estudo. Também foi observado que as toxicidades de manganês e níquel não são sinérgicas uma vez que as combinações destes metais provocaram a mesma resposta fisiológica em *Ulothrix* sp. LAFIC 010 quando comparadas aos efeitos do metais sozinhos.

As análises de Microscopia Eletrônica de Transmissão indicaram que, tanto nos experimentos dos metais isolados quanto em combinações, cada metal alterou a célula de *Ulothrix* sp. LAFIC 010 em determinados locais. Nos tratamentos sob maiores concentrações de zinco, as células apresentaram alterações nos cloroplastos, sendo visualizado desarranjo nos tilacóides. Nos tratamentos de manganês as células apresentaram pontuações eletro-densas na parede celular. Este mesmo fenômeno foi observado nas células submetidas às altas concentrações de níquel, no entanto, tal aspecto foi visualizado nos vacúolos.

No experimento em que a biomassa de *Ulothrix* sp. LAFIC 010 foi mantida em água proveniente de local contaminado por Drenagem Ácida de Minas, foi observada remoção dos metais zinco, manganês e níquel do meio em porcentagem de 41.4% para Ni, 28.5% para Mn, e 20.7% para Zn. No entanto, as concentrações dos metais presentes no meio não diferiram significativamente com as concentrações do controle (ausência de alga). Sugere-se que em virtude do baixo pH pode ter havido competição entre prótons (H^+) e os metais pelos mesmos sítios de ligação da alga, inviabilizando a maior bioacumulação de metais pela espécie. Outro aspecto importante que pode ter contribuído para a baixa remoção de metais foi a biomassa inoculada no meio, a qual teria sido insuficiente para causar maior remoção dos metais estudados. Além disso, é possível que os metais presentes na água tenham sido adsorvidos ao material da parede dos frascos de cultivo em vez de serem

retidos pela biomassa algal, o que explicaria a similaridade de remoção entre os tratamentos e o controle.

Concluímos que *Ulothrix* sp. LAFIC 010 é uma alga tolerante às altas concentrações de metais que poderão eventualmente ser observadas em resíduos contaminados com DAM. Isso a torna uma espécie candidata ao uso para aplicação na área de biorremediação para efluentes contaminados por metais. Sugerimos que estudos futuros devam focar na compreensão da relação entre aspectos que influenciam na bioacumulação de metais pelas algas como, por exemplo, condições físico-químicas da água (pH, temperatura, concentração de metal) e características da alga (e.g. composição da parede celular), os quais são parâmetros que precisam ser considerados para investigação do potencial de um organismo para biorremediação.