PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

Avaliação do Impacto no Ambiente de Compostos Hidrossolúveis de *Pinus taeda* e *Araucaria angustifolia* (Coniferae) Utilizando Indicadores Biológicos

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TESE DE DOUTORADO

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FACULDADE DE BIOCIÊNCIAS

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Co-orientador: Dr. Leandro Vieira Astarita

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SUMÁRIO

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"Se seus sonhos estiverem nas nuvens, não se preocupe, pois eles estão no lugar certo; agora construa os alicerces" (Autor desconhecido)

"O Futuro pertence àqueles que acreditam na beleza de seus sonhos"
(Elleanor Roosevelt)

Dedicatória

A minha mãe Berenice

A meu marido Felipe

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Resumo

O impacto mais importante das coníferas no ambiente é atribuído à liberação de fitotoxinas/aleloquímicos (predominantemente compostos fenólicos) da biomassa no solo (Singh et al., 1999). Os polifenóis são considerados um dos grupos mais amplamente distribuídos entre as substâncias químicas produzidas pelas plantas e têm potencial aleloquímico devido à sua alta solubilidade em água e sua propriedade de inibir o crescimento de outras espécies de plantas (Inderjit 1996;. Graça et al 2002). A plantação de *Pinus* surgiu como uma solução para substituir a fonte de matéria-prima para produção de móveis, painéis, celulose, papel, compensado, entre outros, e é economicamente viável devido ao uso de espécies de crescimento rápido. A preocupação com o desenvolvimento desta atividade são as conseqüências do uso de espécies exóticas na prática da monocultura sobre o ecossistema local. Este estudo avaliou os efeitos do extrato aquoso de *Pinus taeda* (espécie exótica) e *Araucaria angustifolia* (espécie nativa): (1) sobre a composição bioquímica, estresse oxidativo e parâmetros reprodutivos de *Hyalella castroi*; (2) determinou as concentrações de compostos fenólicos hidrossolúveis em folhas de *P. taeda* e *A. angustifolia* coletados nos meses de inverno e verão de 2009 e 2010 no sul do Brasil, (3) quantificou a biomassa produzida por *P. taeda* e *A. angustifolia*; (4) determinou, em condições de laboratório, o tempo necessário para a lixiviação de compostos fenólicos hidrossolúveis das folhas; (5) quantificou a concentração de compostos fenólicos hidrossolúveis em corpo d'água perto das plantações; (6) avaliou o efeito de aleloquímicos extrato aquoso da *P. taeda* e *A. angustifolia* em sementes de *Lactuca sativa*, (7) determinou, por HPLC, o perfil dos compostos fenólicos no extrato hidrossolúvel de *P. taeda* e *A. angustifolia*; (8) avaliou o efeito de material vegetal seco de duas coníferas, *P. taeda* e *A. angustifolia* na atividade do sistema de transporte de elétrons (ETS) de *H. castroi* e (9) avaliou as alterações dos parâmetros físico-químicos e os níveis de compostos fenólicos hidrossolúveis em um corpo de água perto e outro distante das plantações de *P. taeda*. Os anfípodos foram coletados no verão e inverno, no Rio Grande do Sul, Brasil. Parte dos animais foi congelado no campo e o restante transportado para o laboratório. Os animais foram aclimatados por 7 dias e congelados, os outros animais foram expostos por mais 7 dias ao extrato aquoso de ambas as árvore, contendo diferentes concentrações de compostos fenólicos hidrossolúveis (0,10, 0,25, 0,5, 0,75 mg/L), e um grupo foi mantido até os experimentos terminarem apenas com a dieta (14 dias). Após o cultivo, os animais foram imediatamente congelados e dividido em cinco *pools* para determinar os níveis de arginina, arginina fosfato, glicogênio, proteínas, lipídeos, triglicerídeos, glicerol, o colesterol, a lipoperoxidação, e a atividade da catalase, SOD, GST,

Na^+/K^+ ATPase e ETS por técnicas espectrofotométricas. Parâmetros reprodutivos (número de casais reprodutivos, fêmeas ovígeras e ovos no marsúpio) foram analisados em animais expostos a ambos os extratos e nos grupos de controle. Folhas de *P. taeda* e *A. angustifolia* foram coletadas de árvores com mais de 20 anos de idade cultivadas em uma floresta comercial no município São Francisco de Paula. A atividade de radicais livres dos extratos aquosos de plantas também foi avaliado. Foram coletadas amostras em duas corpos d'águas: um no município de São José dos Ausentes (28°47'00"S - 49°50'53"W; 1200m de altitude) distante da plantação de *P. taeda*, e outro em São Francisco de Paula (29°23'36.2"S - 50°22'50.7"W; 900m de altitude), perto da plantação de *P. taeda*, no Rio Grande do Sul, Brasil durante o verão e inverno de 2009 e 2010. Os parâmetros medidos foram os níveis de compostos fenólicos totais, coliformes totais e fecais, dureza, nitrito, nitrato, sólidos totais, sulfato, demanda biológica de oxigênio (DBO), demanda química de oxigênio (DQO), oxigênio dissolvido, pH e temperatura da água. Nossos resultados revelaram que o extrato aquoso de *P. taeda* induz uma diminuição em todos os metabólitos e parâmetros reprodutivos estudados. Por outro lado, os níveis de lipoperoxidação e atividades de catalase, SOD e GST aumentaram durante a exposição. Já os animais expostos ao extrato de *A. angustifolia* não alterou a composição bioquímica e os parâmetros reprodutivos. Este extrato determinou uma diminuição nos níveis de lipoperoxidação, esta resposta sugere um efeito antioxidante do extrato da espécie nativa. As análises das folhas sugerem que os compostos produzidos por extrato hidrossolúvel de espécies Coniferae têm potenciais antioxidantes diferentes e afetam a anfípodos de forma divergente em termos da ETS. Dos parâmetros analisados no presente trabalho apenas DBO, oxigênio dissolvido e pH alteraram com a presença da plantação de *P. taeda* perto do corpo d'água e os resultados sugerem que a alteração está relacionada com a presença das acículas, bem como a alta concentração de compostos fenólicos verificada na água. Depois de analisar o perfil dos compostos fenólicos foi observada a presença de outros compostos fenólicos nos extratos de *P. taeda*, e esta combinação é provavelmente o fator que determinou o efeito deletério do extrato de *P. taeda*. Este padrão de resposta pode ajudar a explicar como as espécies exóticas de coníferas, como *P. taeda*, modificam o ambiente natural e podem causar alterações graves nos ecossistemas de água doce.

Abstract

The most important impact of conifers in the environment is attributed to the release of phytotoxins/allelochemicals (predominantly phenolic compounds) from the fallen litter layers (Singh et al., 1999). Polyphenols are considered to be one of the most widely distributed groups of the chemical substance produced to plants and had a potential allelochemicals due to its high water solubility and properties to inhibit growth of others species of the plants (Inderjit 1996; Graça et al. 2002). *Pinus* plantation has emerged as a solution to replace the source of feedstock for production of furniture, paneling, particle board, paper, cellulose, among others, and economically viable due to the use of fast-growing species. The concern for the development of this activity is the consequences of the use of exotic species and the practice of monoculture on the local ecosystem. This study assesses the effects the aqueous extract of *Pinus taeda* (exotic species) and *Araucaria angustifolia* (native species) has: (1) on the biochemical composition, oxidative stress, and reproductive parameters of *Hyalella castroi;* (2) determine the concentrations of hydrosoluble phenolics in leaves of *P. taeda* and *A. angustifolia* collected in months of winter and summer of 2009 and 2010 in the south of Brazil; (3) quantify the litter produced by *P. taeda* and *A. angustifolia*; (4) determine, in laboratory conditions, the time required for leaching of hydrosoluble phenolics from leaves; (5) quantify the concentration of hydrosoluble phenolics in body water near the plantations; (6) evaluate the allelochemicals effect of aqueous extract of the *P. taeda* and *A. angustifolia* in seeds of *Lactuca sativa*; (7) determine, by HPLC, the profile of phenolics in the hydrosoluble extract from *P. taeda* and *A. angustifolia*; (8) evaluate the effect of plant dry material of two conifers, *P. taeda* and *A. angustifolia* in the activity of the respiratory electron transport system (ETS) of *H. castroi* and (9) evaluated the changes physical-chemical parameters and hydrosoluble phenolics in one body water near and another distant from the plantations of *Pinus taeda*. Amphipods were collected in summer and winter, in Rio Grande do Sul, Brazil. Part of the animals was frozen in the field and the remainder transported to the laboratory. Animals were acclimated for 7 days and frozen, the other animals were exposed for a further 7 days to the aqueous extract of both tree, containing different concentrations of hydrosoluble phenolics (0.10, 0.25, 0.5, 0.75mg/L), and one group was kept until the experiments finish only with diet (14 days). After cultivation, the animals were immediately frozen and divided into five pools for determining the levels of arginine, arginine phosphate, glycogen, proteins, lipids,

triglycerides, glycerol, cholesterol, lipid peroxidation, and the activity of catalase, SOD, GST, Na^+/K^+ATPase and ETS by spectrophotometric technique. Reproductive parameters (number of breeding pairs, ovigerous females and eggs in the pouch) were analyzed in animals exposed to both extracts and the control groups. Leaves of *P. taeda* and *A. angustifolia* were collected from trees older than 20 years old cultivated in a commercial culture in São Francisco de Paula Municipality. The radical scavenging activity of the plant aqueous extracts was also evaluated. We collected samples in two body waters: one in São José dos Ausentes Municipality (28°47'00"S – 49°50'53"W; 1200 m a.s.l.) distant of the *Pinus taeda* plantation, and other in São Francisco de Paula Municipality (29°23'36.2"S – 50°22'50.7"W; 900 m a.s.l.) near the *P. taeda* plantation, both in Rio Grande do Sul, Brazil during summer and winter of 2009 and 2010. The parameters measured were total levels of phenolic compounds, total and fecal coliforms, hardness, nitrite, nitrate, total solids, sulphate, biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen, pH and water temperature. Our results revealed that the aqueous extract from *P. taeda* induces a decrease in all metabolites and reproductive parameters studied. On the other hand, the levels of lipoperoxidation and activities of catalase, SOD and GST increased during exposition. Already the animals exposed to the extract of *A. angustifolia* showed no alteration of the biochemical composition and reproductive parameters. This extract determined a decrease in the lipoperoxidation levels, this response suggests an antioxidant effect of the extract of native wood. The analyses of the leaves suggest that hydrosoluble compounds produced by extract of coniferae species have different antioxidant potentials and affect the amphipods in a divergent form in terms of the ETS. Out of the parameters analyzed in the present work only BOD, oxygen dissolved and pH seemed to change by the presence of the *Pinus taeda* plantation near of the body water and results suggest that the alteration are related with the presence of the needles as well as the high concentration of the phenolic compounds verified in the body water. After analyze of the profile of the phenolic compounds we observed that others phenolic compounds are present in the extracts of *P. taeda*, and this combination is probably the factor that determined the deleterious effect of the extract of *P. taeda.* This pattern of response can help to explain how exotic species of conifers such as *P. taeda*, modify the natural environment and can cause severe alterations in freshwater ecosystem.

Apresentação

A tese aqui apresentada é composta por quatro artigos científicos, os quais estão divididos em quatro capítulos:

O capítulo 1 é composto pelo artigo intitulado: "**Biochemical and reproductive changes of *Hyalella castroi* (Crustacea, Amphipoda) induced by hydrosoluble leaf extracts of exotic and native Coniferae species"** o qual está submetido ao periódico **Oecologia** que possui ISI 3,517 e qualis A1 segundo a área de Biodiversidade da CAPES. O presente artigo tem por objetivo avaliar o efeito do extrato aquoso de *Pinus taeda*, uma expécie exótica, e *Araucaria angustifolia*, uma espécie nativa, no metabolismo energético, lipoperoxidação e enzimas do estresse oxidativo, bem como, sob parâmetros reprodutivos de *Hyalella castroi*.

O capítulo 2 é composto pelo artigo intitulado: **"Biological effects of hydrosoluble compounds from exotic and native Coniferae species"** o qual está submetido ao periódico **Ecological Indicators** que possui ISI 2,967 e qualis A2 segundo a área de Ciências Biológicas I da CAPES. O presente artigo tem por objetivo: (1) determinar as concentrações de fenólicos hidrossolúveis nas acículas de *Pinus taeda* e folhas de *Araucaria angustifolia* coletadas nos meses de inverno e verão de 2009 e 2010; (2) quantificar a biomassa liberada para o ambiente por *P. taeda* e *A. angustifolia*; (3) verificar em condições de laboratório quanto tempo às folhas liberariam todo o conteúdo de fenólicos hidrossolúveis por lixiviação; (4) verificar em corpos de água com diferentes distâncias de plantações de *Pinus* a concentração de fenólicos hidrossolúveis; (5) avaliar o efeito do extrato aquoso de *P. taeda* e *A. angustifolia* em sementes de *Lectuca sativa*; (6) verificar a composição de fenólicos de *P. taeda* e *A. angustifolia* por HPLC.

O capítulo 3 é composto pelo artigo intitulado: "**Hydrosoluble compounds of exotic and native Coniferae species interfere in the activity of the respiratory electron transport system of *Hyalella castroi*"** o qual está submetido ao periódico **Environmental Pollution** que possui ISI 3,395 e qualis A1 segundo a área de Biodiversidade da CAPES. O

presente artigo tem por objetivo verificar o potencial antioxidante dos extratos aquosos de *Pinus taeda* e *Araucaria angustifólia* e seu efeito no sistema de transporte de elétrons de *Hyalella castroi*.

O capítulo 4 é composto pelo artigo intitulado: "**Evaluation of the effects of *Pinus taeda* in two bodies water in Brazilian highlands"** o qual está submetido ao periódico **Journal of Hydrology** que possui ISI 2,514 e qualis A2 segundo a área de Biodiversidade da CAPES. O presente artigo tem por objetivo avaliar parâmetros físico-químicos e verificar se estes estão alterados em presença de plantações de *Pinus taeda* no sul do Brasil.

Final dos quatro capítulos está apresentada às conclusões gerais, as quais visam dar uma visão de todos os resultados obtidos durante a tese.

Por últimos encontram-se os apêndices que seguem a seguinte ordem:

Apêndice 1: é composto pelos comprovantes de submissão dos artigos

Apêndice 2: é composto pelas normas de publicação de cada um dos periódicos a qual os artigos que formam esta tese foram submetidos.

Capítulo 1

Biochemical and reproductive changes of *Hyalella castroi* (Crustacea, Amphipoda) induced by hydrosoluble leaf extracts of exotic and native Coniferae species

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1. Bolsista de Produtividade do CNPq

Running title: Biochemical changes of *H. castroi* induced by exotic and native Coniferae species

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Declaration of authorship: We declare that all author participated equally in this work.

ABSTRACT

This study assesses the effects the aqueous extract of *Pinus taeda* (exotic species) and *Araucaria angustifolia* (native species) has on the biochemical composition, oxidative stress, and reproductive parameters of *Hyalella castroi*. Amphipods were collected in summer and winter, in Rio Grande do Sul, Brazil. Part of the animals was frozen in the field and the remainder transported to the laboratory. Animals were acclimated for 7 days and frozen, the other animals were exposed for a further 7 days to the aqueous extract of both tree, containing different concentrations of hydrosoluble phenolics (0.10, 0.25, 0.5, 0.75mg/L), and one group was kept until the experiments finish (14 days). After cultivation, the animals were immediately frozen and divided into five pools for determining the levels of arginine, arginine phosphate, glycogen, proteins, lipids, triglycerides, glycerol, cholesterol, lipid peroxidation, and the activity of catalase, SOD, GST and Na^+/K^+ATPase by spectrophotometric technique. Reproductive parameters (number of breeding pairs, ovigerous females and eggs in the pouch) were analyzed in animals exposed to both extracts and the control groups. Our results revealed that the aqueous extract from *P. taeda* induces a decrease in all metabolites and reproductive parameters studied. On the other hand, the levels of lipoperoxidation and activities of catalase, SOD and GST increased during exposition. The animals already exposed to the extract of *A. angustifolia* showed no alteration of the biochemical composition and reproductive parameters. This extract determined a decrease in the lipoperoxidation levels, this response suggests an antioxidant effect of the extract of native wood.

Keyword: *Pinus taeda*; *Araucaria angustifolia;* Crustacea; Oxidative stress; Biochemical composition

1. INTRODUCTION

The use of exotic species for culture represents an important impact on the natural environment, causing changes in species, communities and ecosystems. Various species of pine were introduced in the 1960s to the southern and southeastern regions of Brazil, replacing the disturbed native *Araucaria angustifolia* forest (Montagna and Yamazoc 1978). As a result, there are more than two million hectares planted with *Pinus* spp. in this country (IBGE 2003). *Araucaria angustifolia* is a native coniferous that grows wild in Brazil and it is a species of great ecologic and economic importance. Extensive *Pinus* spp. plantation is associated with environmental impact due to their ability to invade native areas and their production of allelochemical compounds (Richardson and Higgins 1998). The success of this genus as invasive is related to the capacity they have to colonize marginal, nutrient-poor habitats, and producing a massive reservoir of seeds from plantings of the same species (Moran et al. 2000).

The major consequences of invasive plants are the loss of biodiversity and the modification of the natural cycles, as well as the change in appearance of the natural landscape. This process is called biological contamination and refers to damage caused by species that are not part, of course, of a given ecosystem, but they are naturalized and start to disperse and cause changes in their activity hampering natural recovery (Aubert and Oliveira Filho 1994).

Allelochemicals produced by exotic species play an important role in natural ecosystems. These effects are seen in terrestrial and phytoplankton succession, inhibition of nitrogen fixation, nitrification and others (Kohli et al. 1997). The most important impact of conifers in the environment is attributed to the release of phytotoxins/allelochemicals (predominantly phenolic compounds) from the fallen litter layers (Singh et al. 1999).

However, polyphenols are involved in plant defenses against herbivorous and microorganisms and these compounds are considered to be one of the most widely distributed groups in plants (Graça et al. 2002).

The genus *Pinus* contains high levels of phenolic compounds, with known toxic activity in biological systems (Arise et al. 2009) , and only a small group of animals can feed and detoxify these compounds in the liver (Whitman and Ghazizadeh 1994), whilst for the most part animals do not possess these attributes. Davi and Gnudi (1999) reported that phenolic compounds, especially chlorinated, may be life-threatening to humans even at low concentrations. The World Health Organization (WHO) recognises that there are associated heath risks to humans as a result of noxious substances found in some phenolic compounds with a maximum admissible concentration (MAC) in drinking water ranging from 60 to 400 mg/l in relation to their toxicity degree (EPA 1984).

The mode of action of allelochemicals produced by gymnosperms has remained unexplored. Since most of the studies with allelochemicals focus on the germination and seedling growth of the tested plants (Singh et al. 1999), there is a lack of information related to the effect of those compounds on the native fauna.

Regarding native fauna, hyalellids offer an excellent model for toxicity tests and bioassays for evaluation of the quality of water or sediment of an aquatic ecosystem (Gerhardt et al. 2005; Dutra et al. 2008, 2009, 2011). Dutra et al. (2007) showed that the energy reserves of these hyalellids seem to be used in two different ways: (a) the adults use them for their own metabolic needs in response to simultaneously acting environmental factors such as temperature, food availability and its composition, feeding rhythms amongst others; or (b) the reserves are transferred to reproductive traits and to the offspring through eggs and are used by the young animals in their development. Reproductive events are

important in the life cycles of these animals, leading to high energy expenditures and a close correlation with lipoperoxidation levels. Environmental conditions (e.g., trophic conditions and photoperiod) and reproduction are supposed to be the main processes influencing the seasonal patterns of variation in biochemical composition in these animals.

The aim of the present study was to evaluate the effect of the aqueous extract of the *Pinus taeda*, an exotic species, and *Araucaria angustifolia*, a native species, on the energy reserves, lipid peroxidation, and enzymes of oxidative stress as well as reproductive traits in *Hyalella castroi*.

2. MATERIAL AND METHODS

Animals were collected and maintained in accordance with Brazilian laws (No. 23378-1- SISBIO/IBAMA) and were used with approval from the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) (License 002/09).

2.1. Plant material

Green leaves of *Pinus taeda* and *Araucaria angustifolia* were collected from trees older than 20 years cultivated in a commercial culture in São Francisco de Paula Municipality (29°23'36.2"S – 50°22'50.7"W; 900 m a.s.l.), Rio Grande do Sul, Brazil. The leaves were stored in a paper bag and dried in oven at 40ºC for 72h. The dry material was processed in a knife grinder and stored at -20ºC until used. The levels of phenolic compounds were used as parameter for preparing four different concentrations of aqueous extracts. The phenolic compounds were analyzed in the ground samples (0.2 g) homogenized in water or 80% methanol (1:20 w/v). The extracts were centrifuged at 2.500*g* for 30 min at 4ºC, and the supernatants were used for quantification using the Folin-

120 Ciocaulteau method (Poiatti et al. 2009). The methanolic extracts were used as the
121 reference to determine the total phenolic compounds present in the leaves.

122 Considering that the dry material of *P. taeda* and *A. angustifolia* have different
123 concentrations of primary and secondary metabolites, the level of hydrosoluble phenolic
124 compounds was used as a reference to calculate the adequate amount of dry mass added in
125 each aquarium. Levels of hydrosoluble phenolic were measured two hours, three days and
126 seven days after the supplementation of plant material in the aquarium, no differences were
127 verified, in order to verify the final concentration in the water (Table 1).

128

129 *2.2. Hyallela castroi*

130 The collection was made in the summer of 2009/2010 (December, January and
131 February) and winter 2009/2010 (June, July and August); and animals were collected
132 together with macrophytes *Callitriche rimosa* from their habitat with fish traps. *Hyallela*
133 *castroi*, 1,500 males and 1,500 females each year, were collected in São José dos Ausentes
134 Municipality (28°47'00"S – 49°50'53"W; 1200 m a.s.l.), Rio Grande do Sul, Brazil.
135 Animals were transported in cooled water in insulated containers to the Laboratory of
136 Conservation Physiology of PUCRS. Twenty animals of each sex were immediately
137 cryoanesthetized, in order to assess whether there were any differences between the animals
138 collected in the wild (control group) and the animals that received diets *ad libitum* (ration
139 and macrophyte) for 7 days (Diet 7) or 14 days (Diet 14) in cultivation aquariums and
140 others that received this diet for 7 days and after were exposed to the extracts of plants for 7
141 days.

142 The animals were fed a combination of commercial feed for fish and the
143 macrophyte (*Callitriche rimosa*), presented 351.99 Kcal/100g to total caloric value, as
144 standardize by Gering et al. (2009).

145 In order to establish the profile of variation in the biochemical composition, lipid
146 peroxidation and oxidative stress levels in the amphipods, individuals of *Hyalella castroi*
147 were submitted to an aqueous extract that contained four different concentrations of
148 phenolics (0.10, 0.25, 0.50 and 0.75 mg/L). The concentrations were standardized
149 according to the amount of hydrosoluble phenolic compounds in the plant material. These
150 concentrations were chosen based on previous bioassays made in our laboratory with
151 *Hyalella castroi* exposed to aqueous extracts containing hydrosoluble phenolics in
152 concentrations equal or higher than 1.0 mg/L (for *Pinus taeda*) which showed mortality
153 rates of 70%.

154

155 *2.3. Experimental procedure*

156 Adult animals were kept submerged in aerated aquariums with declorated water (20
157 L), divided with netting in order to maintain chemical contact but to prevent any physical
158 interaction between males and females (the water passed through both sides of the
159 aquarium). Previous studies in our laboratory demonstrated that this arrangement is
160 important to keep the animals alive (Gering et al. 2009). The mean temperature was 23 ±
161 1ºC and the photoperiod was 12 hours of light. The animals were acclimated in the
162 aquariums for seven days, during which they received food (macrophytes and artificial diet)
163 *ad libitum*, daily only during periods when most of the animals were active (Dutra 2007).
164 After this acclimation period, 20 animals of each sex were cryoanesthetized (Diet 7) for
165 determination of all biochemical parameters.

166 After the first seven days, the remaining amphipods were divided into four groups
167 and dispersed to other aquariums, and fed *ad libitum* with the same diet for a further seven
168 days. The experimental groups consisted of: animals that received only the diet for another
169 7 days (diet 14) (Group 1); Amphipods exposed to compounds released from the *Pinus*
170 *taeda* material with the final hydrosoluble phenolic concentration of 0.10 mg/L (Group 2),
171 0.25 mg/L (Group 3), 0.50 mg/L (Group 4) and 0.75 mg/L (Group 5). Likewise amphipods
172 exposed to compounds released from the *Araucaria angustifolia* material with the final
173 hydrosoluble phenolic concentration of 0.10 mg/L (Group 6), 0.25 mg/L (Group 7), 0.50
174 mg/L (Group 8) and 0.75 mg/L (Group 9). All amphipods from groups 2 to 9 were exposed
175 to the plant material for a period of 7 days.
176 When the bioassay period ended, all amphipods were cryoanesthetized, weighed on
177 an electronic balance (± 0.001), and stored frozen at -80°C until the determination of the
178 biochemical parameters. Each of the experiments was repeated three times in the different
179 months (December, January and February or June, July and August) of each year.
180
181 *2.4. Reproductive traits*
182 After a period of 24 hours in the laboratory, 10 couples were placed in each 20-liter
183 aquarium, for a total of eleven aquariums each month and 110 animals used for
184 experiments monthly; this experiment was repeated six times. The animals were observed
185 daily: for 7 days, during which they were only fed the diet; and for an additional 7 days
186 with diet and phenolic treatments. The number of reproductive pairs, ovigerous females and
187 eggs in the marsupium (brood pouch) was counted in each day. According to Castiglioni
188 and Bond-Buckup (2008) the reproductive pairing is defined as the period when the male
189 guards a potential mate by carrying her beneath his ventral surface for several days before

she becomes available for mating. Pairs remain attached in this way (male dorsal to female) through the female's molt, and the new clutch of eggs is fertilized by the guarding male as the eggs pass into the brood pouch; the same author defined ovigerous females as those carrying their eggs in her marsupium (brood pouch).

2.5. Survival and Mortality

The survival and mortality of the animals were recorded during the course of the experiments. The animals were considered dead in the absence of movement of the abdominal pereiopods after a period of observation (10 minutes).

2.6. Biochemical Analyses

The metabolic determinations for *H. castroi* were done in total homogenates of five pools of five males and five females for each point (per experimental group). One pool of *H. castroi* was used for determination of glycogen and proteins; the second pool for quantification of lipids, triglycerides, glycerol and cholesterol; the third pool for determination of lipoperoxidation levels and antioxidant enzymes; the fourth pool for quantification of arginine and arginine phosphate levels, and the fifth pool for determination of Na^+/K^+ATPase. Metabolic parameters and enzymatic activity were determined in quadruplicate by spectrophotometric methods.

a. Glycogen was extracted from tissue following the method described by Van Handel (1965). Glycogen levels in the animals were determined as glucose equivalent, after acid hydrolysis (HCl) and neutralization (Na_2CO_3), following the method of Geary et al. (1981). Glucose was quantified using a Biodiagnostic kit (glucose-oxidase). The results are presented as mg/g of animal weight.

214 b. Proteins were measured by the reactions of the proteins with the copper ions (Labtest
215 Kit). The results are expressed in mg/g of animal weight.
216 c. Lipids were extracted from tissue homogenized with an Omni Mixer Homogenizer in a
217 2:1 (v/v) chloroform-methanol solution, according to Folch et al. (1957). Total lipids in this
218 homogenate were determined by the sulfophosphovanillin method (Meyer and Walter
219 1981). Triglycerides were measured by the reactions of lipase, glycerokinase, 1-P-glycerol
220 oxidase, and peroxidase enzymes (Biodiagnostic Kit / GPO Trinder). The levels of total
221 cholesterol were measured by the reactions of the enzymes cholesterol esterase, cholesterol
222 oxidase, and peroxidase (Labtest Kit/Liquiform).The levels of glycerol were measured by
223 the reactions of the enzymes glycerokinase, ADP dependent hexokinase, and glucose-6-
224 phosphate-dehydrogenase (Enzytec Kit). The results are expressed as mg/g of animal
225 weight.
226 d. Arginine and arginine phosphate were determined using the method of Bergmeyer
227 (1985). The arginine was determined by the change in absorbance at 339 nm in the reaction
228 catalyzed by octopine dehydrogenase: arginine + pyruvate + NADH + $H^+ \leftrightarrow$ octopine +
229 $NAD^+ + H_2O$. To hydrolyze arginine and arginine phosphate to phosphate, 100 µl of 1 mol l
230 $^{-1}$ HCl was added to 100 µl of tissue (homogenate) and incubated in tightly capped tubes
231 for 90 mins in boiling water. The hydrolysates were then cooled and neutralized with 100
232 µl of 1 mol l -1 NaOH. The arginine (assay) was repeated, and the previous concentration
233 of arginine subtracted to obtain the level of arginine phosphate. The results were expressed
234 in mmol/g.
235 e. The membrane was extracted from five animals, according to Barnes et al. (1993). The
236 pool was homogenized (10% W/V) in cold Tris buffer (40 mM) and phenylmethylsulfonyl

fluoride (1 mM; Sigma, St. Louis, MO, USA), pH adjusted to 7.40. The homogenate was centrifuged at 10,000 g at 4 °C, and the supernatant was collected and centrifuged at 40,000 g (4 °C). The pellet was resuspended in the same buffer and centrifuged again at 40,000 g (4 °C). This last supernatant was then used as the source of Na^+/K^+ATPase. Na^+/K^+ATPase activity was measured according to the method described by Esmann (1988), and standardized for this genus according to Dutra et al. (2008). Incubation medium A contained ATP (5 mM; from Sigma), NaCl (60 mM), KCl (10 mM), and MgCl (40 mM), with the pH adjusted to 7.40. In incubation medium B, KCl was replaced by ouabain (1 mM; Sigma). Aliquots of homogenate were incubated at 30 °C in media A and B, for 30 min with the equivalent of 10 mg of the proteins. The enzyme reaction was stopped by addition of 10% trichloroacetic acid. The inorganic phosphorus released was determined using the method of Chan and Swaminathan (1986), in a spectrophotometer at 630 nm. Any difference in phosphorus concentration between medium A and B was attributed to Na^+/K^+ATPase activity. All determinations were done at least in quadruplicate. Results are expressed in μmol of the Pi/mg protein/min.

f. Lipoperoxidation levels were quantified by the method of Buege and Aust (1978), by measuring reactive substances to Thiobarbituric Acid (TBA-RS), using the extraction method of Llesuy et al. (1985). The results are expressed in nmol of TBARS/mg of protein.

g. Catalase activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm, and was expressed as μmoles H_2O_2 per milligram of protein per minute in accordance with Boveris and Chance (1973).

h. Superoxide dismutase activity was determined by measuring the auto oxidation of adrenalin as described by Misra and Fridovich (1972) and was expressed as units per milligram of protein.

i. The glutathione S-transferase activity was measured according to Boyland and Chasseaud (1969) by measuring the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB) with reduced glutathione (GSH) activity as a function of increasing absorbance values at 340 nm. The activity was expressed as units per milligram of protein.

2.7. Statistical Analysis

The results are expressed as mean ± standard error, and all the metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). A three-way ANOVA test was used for statistical analysis followed by a Bonferroni test. The significance level adopted was 5%. Tests were performed with the program Statistical Package for the Social Sciences (SPSS 11.5) for Windows.

3. RESULTS

No significant difference was observed between the experiments carried out with plant material collected in summer and winter; therefore all data were divided by sex and treatment and results were analyzed together in the same group, independent of the season.

Numbers of mating pairs and ovigerous females of *H. castroi* submitted to the different treatments are listed in Table 2. The females maintained on the diet for 7 days or 14 days showed no differences in the number of eggs. In the same groups (diet 7 and 14 days) the values verified for mating pairs and ovigerous females showed similarity.

In the groups treated with *Pinus* material in all concentrations of hydrosoluble phenolics, we found a small number of mating pairs and no ovigerous females or eggs in the marsupium. When we compared with the control group (Diet 7 and 14) similar numbers of eggs, mating pairs and ovigerous females were observed in the treatments where *A. angustifolia* material was used.

Independent of the sex, animals exposed to soluble compounds released from *Pinus taeda* showed lower survival rates (0.10mg/L – 83.72; 0.25mg/L –75.19%; 0.50mg/L – 69.36%; 0.75mg/L – 52.27%) in relation to the control groups (Diet 7 – 99. 96%; Diet 14 – 99.51%). Previously, treatment with *A. angustifolia* material had not affected this parameter (0.10mg/L – 98.97; 0.25mg/L – 98.91%; 0.50mg/L –99.69%; 0.75mg/L – 99.52) (Table 3).

In females and males for all metabolic parameters analysis no difference was observed between wild or maintained animals on the diet for 7 or 14 days.

The levels of arginine phosphate decreased approximately 31 fold in females exposed to *P. taeda* material. The same pattern was observed in males where the level of this parameter was 42 times higher ($p<0.05$) in animals collected in the wild or maintained on the diet (7 or 14 days) when compared with animals treated with *P. taeda* material. There was no significant difference ($p>0.05$) in the behavior of arginine phosphate levels in females and males of *H. castroi* subjected to the different treatments ($p>0.05$) (Table 4). The arginine phosphate content of the control group and the group treated with *A. angustifolia* material showed no significant difference (Table 5), and there was no significant difference between females and males of *H. castroi*. When the levels of arginine phosphate of the animals treated with *P. taeda* and *A. angustifolia* were compared we verified a significant difference ($p<0.05$).

304 Variation in arginine content in females and males of *H. castroi* of the control and
305 treated group with soluble compounds released from *P. taeda* was shown in Table 4. When
306 the females were exposed to different concentrations of soluble compounds released from
307 *Pinus taeda*, arginine levels decreased up to 11 times. The same pattern of response was
308 observed for males when treated with an aqueous extract that contained the four
309 concentrations of soluble compounds, decreasing the level approximately 3 fold. There was
310 no significant difference in the curve obtained to arginine levels between males and females
311 of *H. castroi* exposed to the different treatments. Table 5 shows the variation in arginine
312 content in females and males of *H. castroi* treated with different concentrations of soluble
313 compounds released from *Araucaria angustifolia*. We did not verify any significant
314 difference between the control and groups treated with soluble compounds released from
315 *Araucaria angustifolia*. When the levels of arginine in animals treated with *P. taeda* and *A.*
316 *angustifolia* materials were compared, we identified a significant difference ($p<0.05$).
317 Table 4 shows the variation in the levels of metabolites in females and males of *H.*
318 *castroi* treated with different concentrations of soluble compounds released from *P. taeda*.
319 The levels of glycogen were significantly reduced when females were exposed to soluble
320 compounds from *P. taeda*. The same pattern of response was observed in males in the same
321 treatment. These levels decreased approximately 1.8, 2.3, 4.7 and 7.6 fold, respectively
322 with the concentrations of soluble compounds (0.1, 0.25, 0.5 and 0.75 mg/L). There was a
323 significant difference in the curve of the glycogen levels between males and females of *H.*
324 *castroi* exposed to the different treatments ($p<0.001$) (Table 4). Table 5 shows the variation
325 in glycogen content in females and males of *H. castroi* treated with an aqueous extract that
326 contained different concentrations of hydrosoluble phenolics extracted from *A. angustifolia*;
327 we did not verify any significant difference between the control and treated groups. When

328 the glycogen levels of the animals treated with *P. taeda* and *A. angustifolia* were compared,
329 we verified a significant difference ($p<0.05$).

330 In females exposed to an aqueous extract that contained all concentrations of
331 hydrosoluble phenolics of *Pinus taeda* the protein levels decreased approximately 3.7, 4.5,
332 6.4 and 13.1 fold with the increase of the phenolic concentration. In control groups the
333 levels of total protein found in males were 2 and 14 times higher ($p<0.05$) than in the other
334 groups treated with an aqueous extract that contained phenolics and this response was dose
335 dependent (Table 4). There was no significant difference in the behavior of total-protein
336 levels in females and males of *H. castroi* subjected to the different treatments ($p>0.05$).
337 Table 5 shows the variation in total protein content in females and males of *Hyalella*
338 *castroi* treated with an aqueous extract that contained different concentrations of
339 hydrosoluble phenolics extract from *Araucaria angustifolia*. The levels of total proteins
340 between the control and treated groups showed no significant difference. When the levels
341 of total proteins of the animals treated with an aqueous extract from *Pinus taeda* and
342 *Araucaria angustifolia* were compared a significant difference was detected ($p<0.05$).

343 When these animals were treated with an aqueous extract that contained different
344 concentrations of hydrosoluble phenolic of *Pinus taeda* their lipid levels decreased between
345 2 and 5 times fold approximately and this decrease was dose dependent. There was no
346 significant difference in the behavior of total lipid levels between females and males of *H.*
347 *castroi* submitted to an aqueous extract that contained the different concentrations of
348 hydrosoluble phenolic of *Pinus taeda* ($p>0.05$) (Table 4). The total lipid content of the
349 control group and those treated with an aqueous extract that contained different
350 concentrations of hydrosoluble phenolics extract from *Araucaria angustifolia* showed no
351 significant difference (Table 5). There was no significant difference in the behavior of total

lipid levels between females and males of *H. castroi* submitted to an aqueous extract that contained the different concentrations of hydrosoluble phenolic of *Araucaria angustifolia* ($p>0.05$). When the total lipid levels of of the animals treated with an aqueous extract from *Pinus taeda* and *Araucaria angustifolia* were compared we verified a significant difference ($p<0.05$).

Table 4 shows the variation in triglycerides content in females and males of *H. castroi* control group and those treated with an aqueous extract that contained hydrosoluble phenolics extracted from *Pinus taeda*. When amphipods were treated with an aqueous extract that contained different doses of hydrosoluble phenolics of *Pinus taeda* the levels decrease 3 times in all concentrations. In males collected in the wild or maintained on the diet for 7 or 14 days triglycerides levels were higher than in the animals treated with an aqueous extract that contained hydrosoluble phenolics. There was no significant difference in the behavior of triglycerides levels in females and males of *H. castroi* treated with an aqueous extract that contained the different concentrations of hydrosoluble phenolics of *Pinus taeda* ($p>0.05$). Table 5 shows the variation in triglycerides content in females and males of *Hyalella castroi* treated with an aqueous extract that contained different concentrations of hydrosoluble phenolics extracted from *Araucaria angustifolia*. We did not verify any significant difference between the control and groups treated with an aqueous extract that contained hydrosoluble phenolics extracted from *Araucaria angustifolia*. When the levels of triglycerides of the animals treated with the aqueous extracted from *Pinus taeda* and *Araucaria angustifolia* were compared we verified a significant difference ($p<0.05$).

In animals that were treated with an aqueous extract that contained the different concentrations of hydrosoluble phenolics of *Pinus taeda* their glycerol levels decreased

376 until approximately 2.7 times in males and 7 times in females. There was no significant
377 difference in the behavior of glycerol levels in females and males of *H. castroi* submitted to
378 the different treatments ($p<0.05$) (table 4). Table 5 shows the variation in glycerol content
379 in females and males of *Hyalella castroi* treated with an aqueous extract that contained
380 different concentrations of hydrosoluble phenolics extracted from *Araucaria angustifolia*.
381 We did not verify any significant difference between the control and groups treated with an
382 aqueous extract that contained hydrosoluble phenolics extracted from *Araucaria*
383 *angustifolia*. When the glycerol levels of the animals treated with an aqueous extracted
384 from *Pinus taeda* and *Araucaria angustifolia* were compared we verified a significant
385 difference ($p<0.05$).

386 In animals exposed to an aqueous extract that contained the different concentrations
387 of hydrosoluble phenolics of *Pinus taeda*, we observed that levels of cholesterol decreased
388 approximately 1.5, 2.5, 2.8 and 2.95 times and this response was dose dependent. In males
389 we observed the same pattern of reduction as the females ($p>0.05$) (Table 4). The
390 cholesterol content of the control and groups treated with an aqueous extract that contained
391 different concentrations of hydrosoluble phenolics extracted from *Araucaria angustifolia*
392 showed no significant difference (Table 5). There was no significant difference in the
393 behavior of cholesterol levels between females and males of *H. castroi* submitted to an
394 aqueous extract that contained different concentration of hydrosoluble phenolic of
395 *Araucaria angustifolia* ($p>0.05$).When the levels of cholesterol of the animals treated with
396 an aqueous extracted from *Pinus taeda* and *Araucaria angustifolia* were compared we
397 verified a significant difference ($p<0.05$).

398 In both sexes, the group collected in the wild or maintained on the diet for 7 or 14
399 days showed similar levels of Na^+/K^+ATPase activity. When these crustaceans were

exposed to an aqueous extract that contained hydrosoluble phenolics of *Pinus taeda* the levels of activity decreased approximately 44 times in all concentrations used. There was no significant difference in the behavior of levels of Na^+/K^+ATPase activity in both sexes during the time of experimentation (Figure 1). The Na^+/K^+ATPase activity of the control and groups treated with an aqueous extract that contained different concentrations of hydrosoluble phenolics extracted from *Araucaria angustifolia* showed no significant difference (Figure 1). There was no significant difference in the behavior of Na^+/K^+ATPase activity between females and males of *H. castroi* treated with an aqueous extract that contained different concentrations of hydrosoluble phenolic of *Araucaria angustifolia* ($p>0.05$).When the levels of Na^+/K^+ATPase activity of the animals treated with *Pinus taeda* and *Araucaria angustifolia* were compared we verified a significant difference ($p<0.05$).

The variation in lipoperoxidation levels in females and males of *H. castroi* was shown in figure 2A, the animals that were exposed to an aqueous extract with hydrosoluble phenolics of *Pinus taeda*, the lipoperoxidation levels increased between 3 and 4 times, respectively. There was no significant difference in the behavior of lipoperoxidation levels in females and males submitted to the different treatments ($p<0.05$). Figure 2B shows the variation of lipoperoxidation levels in females and males of *Hyalella castroi* treated with an aqueous extract that contained different concentrations of hydrosoluble phenolics extracted from *Araucaria angustifolia*. We verified a significant difference between the control group and the group treated with an aqueous extract that contained hydrosoluble phenolics extracted from *Araucaria angustifolia*, because the animal treated with an aqueous extract that contained phenolics from *Araucaria angustifolia* the levels of lipoperoxidation decreased (Figure 2). When the levels of lipoperoxidation of the animals treated with an

aqueous extract of *Pinus taeda* and *Araucaria angustifolia* were compared we verified a significant difference ($p<0.05$).

The activity levels of catalase, superoxide dismutase and glutathione S-transferase (GST) in both sexes of control and treated groups with an aqueous extract that contained hydrosoluble phenolics of *Pinus taeda* are shown in Figure 3. The groups collected in the wild or maintained on the diet for 7 or 14 days showed similar levels of catalase, SOD and GST activity. When these animals were exposed to an aqueous extract that contained hydrosoluble phenolics of *Pinus taeda* the levels of activity increased by approximately 11 fold. There was no significant difference in the behavior of the levels of catalase, SOD and GST activity in either sex during the time of experimentation. There was no significant difference in the behavior of catalase, SOD and GST activity between females and males of *H. castroi* submitted to an aqueous extract that contained different treatments of hydrosoluble phenolic of *Araucaria angustifolia* ($p>0.05$) (Figure 3B).When the levels of catalase, SOD and GST activity of the animals treated with an aqueous extracted from *Pinus taeda* and *Araucaria angustifolia* were compared we verified a significant difference ($p<0.05$).

4. DISCUSSION

In this work the amphipods treated with soluble compounds of *P. taeda* showed a lower survival rate compared with both control animals and animals treated with *A. angustifolia* and this response is dose-dependent. According to Guerra (2001) the toxicity of phenolic compounds in aquatic environments has been mainly investigated through acute toxicity tests on freshwater organisms (Buikma et al. 1979; De Grave et al. 1980). Buttino and Filippi (1991) reported that phenolic compounds are extremely toxic for aquatic

447 organisms at concentrations of mg/L, as chlorophenols influences the organoleptic
448 properties for fish and shellfish at concentrations of μg/L.

449 Our results revealed that an aqueous extract containing hydrosoluble phenolics from
450 *Pinus taeda* induces significant depletion of arginine phosphate, arginine, glycogen,
451 proteins, lipids, triglycerides, glycerol, cholesterol, and Na^+/K^+ATPase activity as well as
452 the reproductive traits analyzed (the numbers of reproductive pairs, ovigerous females and
453 eggs in the marsupium). Hooftman and Vink (1980) reported that pentachlorophenol
454 inhibited the reproduction of *O. diadema* at concentrations of 0.75mg/L, whereas
455 *Crassostea gigas* and Mytillus *edulis* (Dimik and Breese 1965) showed an increase in
456 abnormal embryos at exposures of 0.069 and 0.2mg/L, respectively.

457 Studies in phenolics extracted from pine have reported that these compounds have a
458 negative impact on insect development and shows deterrent properties against moose and
459 hares feeding on willows and birch and voles feeding on pine (Beninger and Abou-Zaid
460 1997) Additionally, phenolics are associated with terpenoids in coniferous trees, which can
461 have different deterrent properties as well (Elliot and Loudon 1987; Epple et al. 1996). The
462 mixture of different, overlapping defenses might be useful to withstand multiple attackers.
463 According to Stolter et al. (2010) the different reports of deterrent effects of specific
464 phenolics and terpenoids on different herbivores underline the necessity of detailed
465 chemical analyses, as shown in the present study, to unravel the effects of various
466 antifeedants.

467 According to Naylor et al. (1989) and Roddie et al. (1996) the decrease in feeding
468 rate cause by a toxicant can be related to reductions in an aquatic organism's energy
469 assimilation, which, in turn, could lead to a reduction in resource allocation to growth,

reproduction, and survival, and finally translate into effects on the population level (Maltby and Naylor 1990; Maltby 1994; Maltby et al. 2001; Irving et al. 2003; Dutra et al. 2011). Calow and Sibly (1990) suggested that the feeding rate could lead to differences in the intrinsic rate of population growth, depending on whether the reduction in reproduction was due to reduced food intake or to increased metabolic cost. In the present work we suggest that the hydrosoluble phenolics contained in an aqueous extracted from *Pinus taeda* are unpalatable and this characteristic led to a decrease in the feeding rate and an increase in the allocation of the energy for functions other than reproduction. These points must be investigated.

On the other hand, the levels of lipoperoxidation, catalase, SOD and GST activity increased during the period of exposition to an aqueous extract that contained phenolics of *Pinus taeda*. On the contrary, the animals exposed to an aqueous extract that contained different concentrations of hydrosoluble phenolic of *Araucaria angustifolia* did not show alterations in the metabolic parameters or reproductive patterns, and the lipoperoxidation levels showed a significant decrease. Phenolic compounds are known for their defensive properties as antioxidants (Osawa et al. 1991), although allelopathic effects are also recognized (Swain 1977; Anderson and Velimirov 1982; Steinberg 1984, 1988; Johnson and Mann 1986; Dumay et al. 2004).

Studies on the variations of phosphoarginine in crustaceans are needed to better understand the role of this compound. The major aspect of previous studies which have evaluated phosphoarginine were conducted using species subjected to starvation, anoxia or hypoxia and recovery. Few studies have treated the variations of arginine phosphate in crustaceans submitted to toxicants (Dutra et al. 2011; Fernandes et al. 2011, Oliveira et al. 2011). According to Uda et al. (2006) the invertebrate phosphagen (arginine phosphate) is a

494 well-established marker of cellular energy status, donating a phosphate to ADP when the
495 ATP pool is depleted. We verified in the present work that when the animals were exposed
496 to an aqueous extract that contained different concentrations of phenolics extracted from
497 *Pinus taeda* the levels of phosphagen decrease significantly. Levels of phosphoarginine
498 decreased were verified by Taylor et al. (2010) to other toxicants; this response suggests a
499 role in energetic balance of these animals when exposed to toxicants.

500 In crustaceans the metabolism of arginine is unknown. Li et al. (2008) suggest that
501 fish have particularly high requirements for dietary arginine because this amino acid is
502 abundant in protein and tissue fluid, and its *de novo* synthesis is limited or even completely
503 absent. In fish and other aquatic animals, arginine is an essential amino acid that plays a
504 crucial role in regulating endocrine and reproductive function, cell signaling,
505 osmorregulation, growth, development, immunity and survival (Li et al. 2008). The
506 significant decrease of the levels of arginine observed in animals treated with an aqueous
507 extract that contained phenolics of *Pinus taeda* reinforced its play mainly in reproductive
508 capacity and survival of amphipods. Aragão et al. (2005) working with fish submitted to
509 acute stress observed a substantial decrease in plasma concentration of arginine and
510 ornithine.

511 The animals treated with hydrosoluble phenolics presented in an aqueous extract of
512 *Pinus taeda* lower levels of glycogen than animals collected in the wild or maintained only
513 on the diet, independent of the length of time that they remained on the diet (7 or 14 days).
514 The levels of glycogen can be decreased to maintain the levels of ATP and are associated
515 with the hyperglycemic response determined by the toxicant. Kumar and Rajini (2009)
516 observed a hyperglycemic potential with another facet of a toxicant, for example
517 organophosphorus insecticides. Koundinya and Ramamurthi (1979) studying *S.*

518 *mossambicus* exposed to fenitrothion verified an increase in blood glucose in association
519 with decreased hepatic glycogen. Deotare and Chakrabarti (1981) exposed rats to a sub
520 chronic dose of pesticide and verified a slight increase in blood glucose and a depletion of
521 liver glycogen.

522 A similar response was observed in lipids in both males and females when exposed
523 to the amphipods in aqueous extract that contained different concentrations of phenolic
524 compounds from *Pinus taeda*. In other works the lipid content decreased during exposure to
525 different pesticides because of its use as an energy reserve, parallel to glycogen (Sancho et
526 al. 1998; Rambabu and Rao 1994; Dutra et al. 2008, 2009, 2011).

527 Guerra (2001) working with *Daphnia magna, Artemia salina, Brachionus plicatilis*
528 and *Vibrio fisheri* verified damages in all models after exposure to sublethal doses of
529 phenols, affecting the nervous and circulatory systems. Karnovic-Ozretic and Orzetic
530 (1988) observed a significant decrease in erythrocytes, total proteins and cholesterol in the
531 blood plasma of *Mugilus auratus* following 8-day exposure to 7.5mg/L of phenol. The
532 same pattern of response was observed in cholesterol and total proteins when animals were
533 exposed to an aqueous extract that contained phenolics of *Pinus taeda* in concentrations
534 lower than 1mg/L.

535 Decreases in protein content observed in crustaceans treated with an aqueous extract
536 that contained phenolics of *Pinus taeda* might also be due to the formation of lipoproteins,
537 used to repair damaged cells raised to increase the lipoperoxidation or direct utilization by
538 cells for energy requirements resulted in stress as observed by Sancho et al. (1998) and
539 Rambabu and Rao (1994) for other crustaceans and other toxicants. Bagchi et al. (1995) has
540 observed an increase in liver lipid peroxidation levels after organophosphate exposure,

where a high peroxidation status is observed in the plasma and erythrocyte membrane of the rats gavaged with pesticides.

In the present work the aqueous extracts containing phenolic compounds of *P. taeda* or *A. angustifolia* played in different ways, as where the compounds extracted from *P. taeda* induced an increase in the lipoperoxidation levels, already the compounds extracted from the native tree (*A. angustifolia*) decreased the level of lipoperoxidation likely exhibiting an antioxidant effect. This result can be related to the composition of these phenolics or to the different proportions between them. Further studies are required for characterizing these compounds.

The increase in the lipoperoxidation identified in the animals exposed to an aqueous extract that contained different concentrations of hydrosoluble phenolics of *P. taeda* can lead to a decrease in Na^+/K^+ATPase activity, because according to Rodrigo et al. (2007) the interaction of reactive oxygen species with biological membranes produces a variety of functional modifications due to either direct interaction with the molecular cell machinery and/or oxidative modification of the environment of biological macromolecules. Lipid peroxidation contributes to the loss of cellular functions through the inactivation of membrane enzymes and cytoplasmic proteins. The same response was verified by Dutra et al. (2009, 2010, 2011) by exposing *H. pleoacuta* and *H. castroi* to different toxicants. An important factor to be noted is that although in water in environment we verified an acidification which could alter the Na^+/K^+ATPase activity, in experimental conditions this has not been checked, since the pH showed no variation.

In the present work the activity of antioxidant enzymes in *H. castroi* submitted to aqueous extracts of *A. angustifolia* showed no significant differences. On the other hand, when exposed to aqueous extracts that contained hydrosoluble phenolics of *Pinus taeda* the

565 levels of activity of the antioxidant enzymes increased. The same was found when Arise et
566 al. (2009) studied the effect of the aqueous extract of *Eucalyptus globulus* in the rat livers
567 and identified that these animals showed a significant increase in SOD activity. This
568 increase may indicate a free radical generating potential of the extract, which consequently
569 triggered increased synthesis of SOD to scavenge the free radicals produced. These authors
570 identified a significant increase of the level of malondialdehyde in a dose dependent
571 manner, and suggest stimulation of the peroxidation of membrane lipids by the extract. The
572 increase in the level of the antioxidant enzyme (superoxide dismutase) may also not be
573 sufficient to cope with the level of oxidant influx caused by the *E. globulus*. It has been
574 reported that membrane lipid peroxidation results in the loss of polyunsaturated fatty acids,
575 decreased membrane fluidity and severe structural changes (Van Ginkel and Sevanian
576 1994).

577 In this work we suggest that the effects found are mainly caused by the
578 hydrosoluble phenolics present in *P. taeda* an exotic species that were considered the main
579 allelopathic compound. According to Canhoto and Laranjeira (2007) although phenols and
580 oils are obvious candidates to explain the toxicological effect of eucalypt leachates there is
581 a possibility that other compounds may also be involved, because an aqueous extraction can
582 dissolve other compounds as saponosides and sugars.

583 Our results revealed that an aqueous extract that contained phenolic compounds of
584 *Pinus taeda* induces significant reduction in arginine, arginine phosphate, glycogen,
585 proteins, lipids, triglycerides, glycerol, cholesterol, and Na^+/K^+ATPase activity, as well as a
586 significant increase in lipoperoxidation levels. The results demonstrate the significant
587 reduction in reproductive traits and survival of the amphipods. In natural environments it

588 can lead to changes in the trophic structure of future limnic environments because these
589 amphipods are important links in the food chain.
590
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Legends of figures

Figure 1: Levels of Na^+/K^+ATPase in *Hyalella castroi* submitted to aqueous extract that contained different concentrations of hydrosolubles phenolics of the *P. taeda* (A) and *A. angustifolia* (B). The results show the mean and standard error. The same letter represents a significant difference. All significances present are in relation to the environmental.

Figure 2: Levels of Lipoperoxidation in *Hyalella castroi* submitted to aqueous extract that contained different concentrations of hydrosolubles phenolics of the *P. taeda* (A) and *A. angustifolia* (B). The results show the mean and standard error. The same letter represents a significant difference. All significances present are in relation to the environmental.

Figure 3: Levels of Catalase, Superoxide Dismutase and Glutathione S-transferase in *Hyalella castroi* submitted to aqueous extract that contained different concentrations of hydrosolubles phenolics of the *P. taeda* (A) and *A. angustifolia* (B). The results show the mean and standard error. The same letter represents a significant difference. All significances present are in relation to the environmental.

Table 1: Nominal test concentration and Effective concentration determined in water of the hydrosolubles phenolics of the *P. taeda* or *A. angustifolia*.

	***P. taeda* 0.1mg/L**	***P. taeda* 0.25mg/L**	***P. taeda* 0.5 mg/L**	***P. taeda* 0.75 mg/L**	***A. angustifolia* 0.1mg/L**	***A. angustifolia* 0.25mg/L**	***A. angustifolia* 0.5mg/L**	***A. angustifolia* 0.75 mg/L**
Nominal test concentration	0.10	0.25	0.5mg/L	0.75	0.1	0.25	0.5	0.75
Effective concentration determined in water (mg/L)	0.096 ± 0.005	0.23 ± 0.06	0.48 ± 0.05	0.73 ± 0.09	0.094 ± 0.003	0.26 ± 0.03	0.49 ± 0.02	0.76± 0.03

Table 2: Number of mating pairs and ovigerous females of *Hyalella castroi* observed during 7 days, 14 days and exposed during 7 days to different concentrations of soluble compounds released from *P. taeda* or *A. angustifolia* dry mass. The N in each point is 120 couples.

	***Diet* 7 days**	***Diet* 14 days**	***P. taeda* 0.1mg/L**	***P. taeda* 0.25mg/L**	***P. taeda* 0.5mg/L**	***P. taeda* 0.75 mg/L**	***A. angustifolia* 0.1mg/L**	***A. angustifolia* 0.25mg/L**	***A. angustifolia* 0.5mg/L**	***A. angustifolia* 0.75 mg/L**
Number of Mating Pairs	63	75	45	18	15	12	68	65	59	64
Ovigerous Females	52	67	25	**-**	**-**	-	55	57	53	57
Mean Number of Eggs	31 ± 4	30 ± 2	18 ± 3	-	-	-	28 ± 3	30 ± 5	31± 4	31 ± 5

Table 3: Survival rates (%) of males and females of *Hyalella castroi* exposed to different concentrations of soluble compounds released from *P. taeda* or *A. angustifolia* dry mass.

	***Diet* 7 days**	***Diet* 14 days**	***P. taeda* 0.1mg/L**	***P. taeda* 0.25mg/L**	***P. taeda* 0.5mg/L**	***P. taeda* 0.75 mg/L**	***A. angustifolia* 0.1mg/L**	***A. angustifolia* 0.25mg/L**	***A. angustifolia* 0.5mg/L**	***A. angustifolia* 0.75 mg/L**
Survival	99.96%	99.51%	83.72%	75.19%	69.36%	52.27%	98.97%	99.91%	99.69%	99.52%

Table 4: Levels of metabolites in *Hyalella castroi* submitted to different concentrations of soluble compounds released from *P. taeda* dry mass. The results show the mean and standard error. The N in each point varied from 48 to 60 animals. The same letter represents a significantive difference. All significances present are in relation to the environmental.

	Arginine phosphate	**Arginine**	**Glycogen**	**Proteins**	**Lipids**	**Cholesterol**	**Triglycerides**	**Glycerol**
Environmental	6.94±0.28^{abcd}	2.91±0.26^{abcd}	1.64±0.16^{abcd}	1.60±0.06^{abcd}	9.88±0.39^{abcd}	1.28±0.08^{abcd}	2.72±0.23^{abcd}	0.68±0.02^{abcd}
	8.60±0.19^{abcd}	1.60±0.04^{abcd}	1.49±0.11^{abcd}	1.16±0.02^{abcd}	11.43±0.10^{abcd}	1.58±0.02^{abcd}	3.32±0.04^{abcd}	0.70±0.02^{abcd}
Control 7	7.85±0.32	2.17±0.30	2.07±0.12	1.38±0.05	10.32±0.25	1.41±0.08	2.47±0.11	0.63±0.02
	8.82±0.41	1.57±0.04	1.30±0.13	1.18±0.02	11.30±0.10	1.64±0.03	3.46±0.09	0.63±0.03
Control 14	6.83±0.28	3.02±0.26	1.76±0.12	1.60±0.04	9.74±0.40	1.40±0.10	2.40±0.29	0.67±0.02
	9.67±0.47	1.53±0.04	1.58±0.11	1.19±0.03	11.49±0.07	1.56±0.11	3.38±0.07	0.61±0.02
0.1mg/L	0.41±0.06^{a}	0.31±0.03^{a}	0.05±0.007^{a}	0.42±0.03^{a}	3.41±0.07^{a}	0.87±0.05^{a}	0.79±0.13^{a}	0.27±0.01^{a}
	0.61±0.03^{a}	0.66±0.01^{a}	0.84±0.01^{a}	0.52±0.002^{a}	2.78±0.17^{a}	0.55±0.06^{a}	1.50±0.07^{a}	0.46±0.04^{a}
0.25mg/L	0.38±0.02^{b}	0.30±0.01^{b}	0.29±0.01^{b}	0.35±0.03^{b}	3.21±0.10^{b}	0.55±0.04^{b}	0.62±0.12^{b}	0.19±0.06^{b}
	0.36±0.02^{b}	0.56±0.04^{b}	0.66±0.07^{b}	0.35±0.008^{b}	2.37±0.07^{b}	0.24±0.02^{b}	0.58±0.08^{b}	0.26±0.01^{b}
0.5mg/L	0.22±0.02^{c}	0.27±0.005^{c}	0.16±0.01^{c}	0.24±0.01^{c}	2.19±0.03^{c}	0.49±0.04^{c}	0.63±0.14^{c}	0.12±0.005^{c}
	0.32±0.01^{c}	0.50±0.03^{c}	0.33±0.01^{c}	0.09±0.003^{c}	2.051±0.06^{c}	0.18±0.01^{c}	0.52±0.07^{c}	0.26±0.007^{c}
0.75mg/L	0.22±0.02^{d}	0.27±0.006^{d}	0.11±0.02^{d}	0.12±0.003^{d}	1.91±0.12^{d}	0.47±0.04^{d}	0.61±0.11^{d}	0.09±0.003^{d}
	0.23±0.02^{d}	0.49±0.05^{d}	0.20±0.02^{d}	0.08±0.004^{d}	2.00±0.04^{d}	0.01±0.0008^{d}	0.07±0.007^{d}	0.22±0.01^{d}

Table 5: Levels of metabolites in *Hyalella castroi* submitted to aqueous extract that contained different concentrations of soluble compounds released from *A. angustifolia* dry mass. The results show the mean and standard error. The N in each point varied from 48 to 60 animals.

	Arginine phosphate	Arginine	Glycogen	Proteins	Lipids	Cholesterol	Triglycerides	Glycerol
Environmental	6.94±0.28	2.91±0.26	1.64±0.16	1.60±0.06	9.88±0.39	1.28±0.08	2.72±0.23	0.68±0.02
	8.60±0.19	1.60±0.04	1.49±0.11	1.16±0.02	11.43±0.10	1.58±0.02	3.32±0.04	0.70±0.02
Control 7	7.85±0.32	2.17±0.30	2.07±0.12	1.38±0.05	10.32±0.25	1.41±0.08	2.47±0.11	0.63±0.02
	8.82±0.41	1.57±0.04	1.30±0.13	1.18±0.02	11.30±0.10	1.64±0.03	3.46±0.09	0.63±0.03
Control 14	6.83±0.28	3.02±0.26	1.76±0.12	1.60±0.04	9.74±0.40	1.40±0.10	2.40±0.29	0.67±0.02
	9.67±0.47	1.53±0.04	1.58±0.11	1.19±0.03	11.49±0.07	1.56±0.11	3.38±0.07	0.61±0.02
0.1mg/L	7.75±0.27	2.29±0.11	1.79±0.14	1.46±0.09	9.95±0.21	1.12±0.07	2.54±0.24	0.59±0.03
	8.26±0.75	1.52±0.04	1.34±0.06	1.13±0.007	11.51±0.09	1.62±0.04	3.56±0.10	0.67±0.03
0.25mg/L	6.90±0.29	2.40±0.25	1.90±0.14	1.56±0.07	10.20±0.36	1.23±0.10	2.76±0.18	0.66±0.01
	9.19±0.30	1.59±0.02	1.25±0.02	1.12±0.01	11.11±0.03	1.66±0.04	3.59±0.12	0.66±0.006
0.5mg/L	7.70±0.33	2.16±0.27	2.12±0.11	1.41±0.06	10.28±0.26	1.41±0.08	2.56±0.13	0.64±0.02
	8.93±0.14	1.59±0.04	1.25±0.08	1.15±0.02	11.09±0.12	1.57±0.02	3.62±0.07	0.66±0.007
0.75mg/L	6.84±0.28	2.79±0.25	1.97±0.09	1.59±0.05	9.42±0.25	1.58±0.11	2.24±0.25	0.67±0.02
	8.57±0.31	1.62±0.03	1.31±0.04	1.14±0.01	11.30±0.10	1.62±0.02	3.58±0.09	0.71±0.01

Figure 1

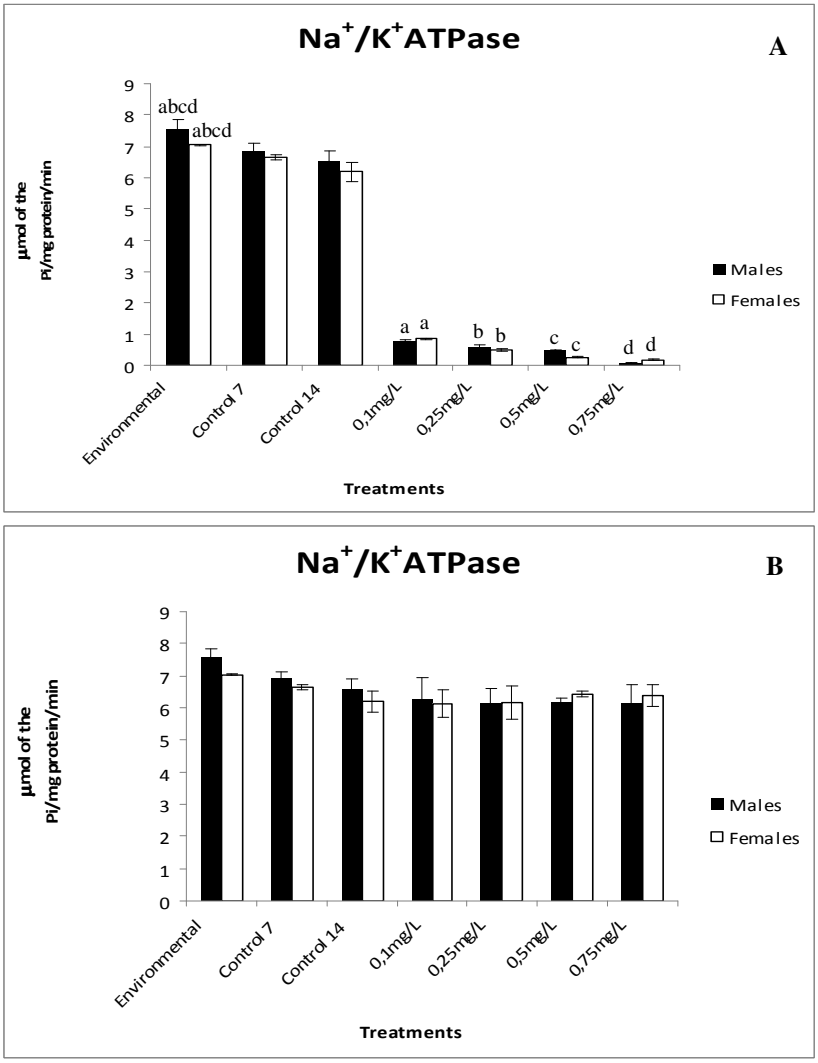

Na+/K+ATPase
A
µmol of the Pi/mg protein/min
abcd
abcd
a a
b b
c c
d d
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatments
Na+/K+ATPase
B
µmol of the Pi/mg protein/min
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatments

Figure 2

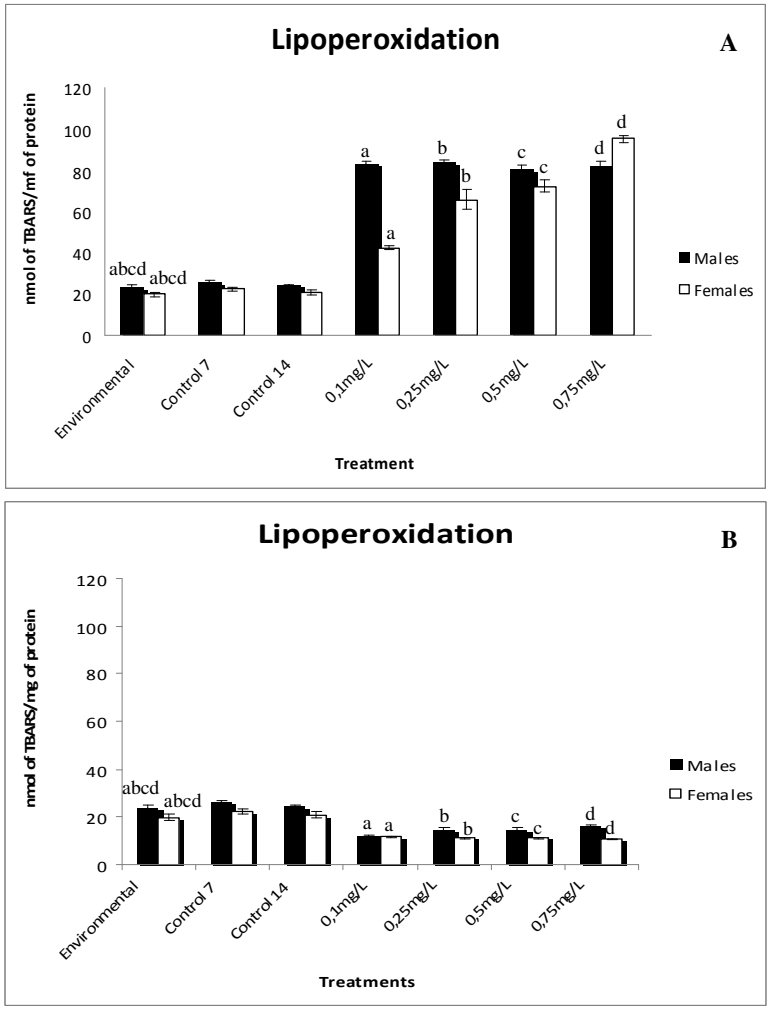
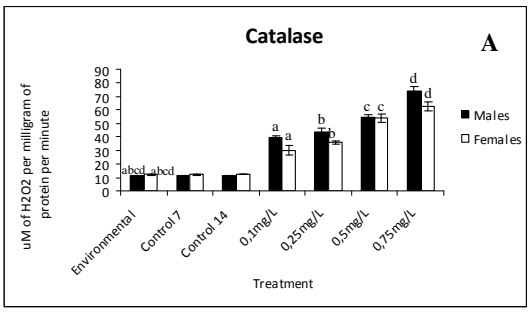
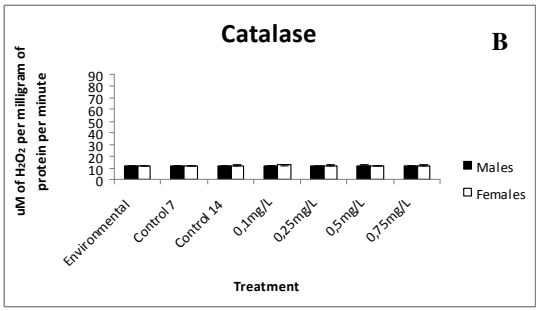
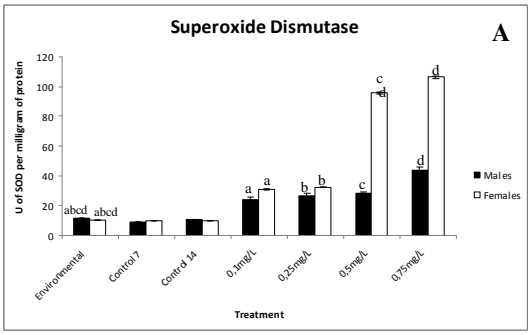
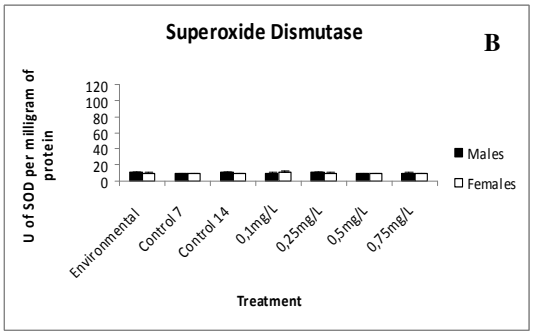

Lipoperoxidation
A
nmol of TBARS/mf of protein
120
100
80
60
40
20
0
abcd
abcd
a
a
b
b
c
c
d
d
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment
Lipoperoxidation
B
nmol of TBARS/mg of protein
120
100
80
60
40
20
0
abcd
abcd
a
a
b
b
c
c
d
d
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatments

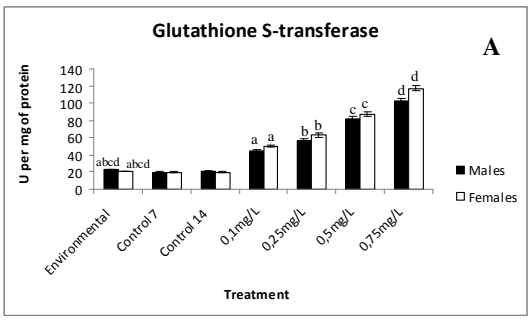
Figure 3

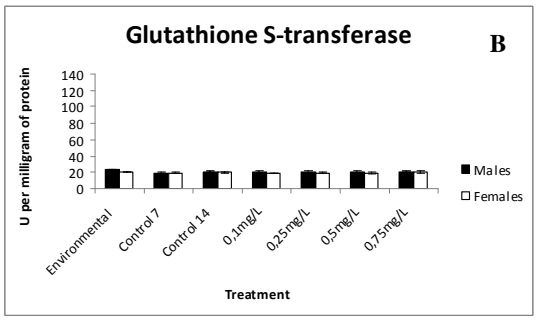

Catalase
A
uM of H2O2 per milligram of protein per minute
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment


Catalase
B
uM of H2O2 per milligram of protein per minute
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment


Superoxide Dismutase
A
U of SOD per milligram of protein
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment


Superoxide Dismutase
B
U of SOD per milligram of protein
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment


Glutathione S-transferase
A
U per mg of protein
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment


Glutathione S-transferase
B
U per milligram of protein
Males
Females
Environmental
Control 7
Control 14
0,1mg/L
0,25mg/L
0,5mg/L
0,75mg/L
Treatment

Capítulo 2

Biological effects of hydrosoluble compounds from exotic and native Coniferae species

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Abstract

Polyphenols are considered to be one of the most widely distributed groups of the chemical substance produced to plants and had a potential allelochemicals due to its high water solubility and properties to inhibit growth of others species of the plants. Therefore the aims of the present study were: (1) determine the concentrations of hydrosoluble phenolics in leaves of *Pinus taeda*, an exotic species, and *Araucaria angustifolia*, a native species, collected in months of winter and summer of 2009 and 2010 in the south of Brazil; (2) quantify the litter produced by *P. taeda* and *A. angustifolia*; (3) determine, in laboratory conditions, the time required for leaching of hydrosoluble phenolics from leaves; (4) quantify the concentration of hydrosoluble phenolics in body water near the plantations; (5) evaluate the allelochemicals effect of aqueous extract of the *P. taeda* and *A. angustifolia* in seeds of *Lactuca sativa*; (6) determine, by HPLC, the profile of phenolics in the hydrosoluble extract from *P. taeda* and *A. angustifolia.* Leaves of *P. taeda* and *A. angustifolia* were collected from trees older than 20 years old cultivated in a commercial culture in São Francisco de Paula Municipality. After analyze of the profile of the phenolic compounds we observed that others phenolic compounds are present in the extracts of *P. taeda*, and this combination is probably the factor that determined the deleterious effect of the extract of *P. taeda.* The results of this study allow us to suggest that *P. taeda* and *A. angustifolia* showed important differences related to environmental interaction. These differences may explain why the exotic species of the Coniferae, with *P. taeda,* can interfere in the natural ecosystem.

Keyword: *Pinus taeda*, *Araucaria angustifolia*, Phenolic, Deleterious effects

Introduction

Allelochemicals produced by exotic species play an important role in natural ecosystems. These effects are seen in terrestrial succession, phytoplankton succession, inhibition of nitrogen fixation, nitrification and others (Kohli et al. 1997). The most important impact of conifers in the environment is attributed to the release of phytotoxins/allelochemicals (predominantly phenolic compounds) from the fallen litter layers (Singh et al. 1999).

Polyphenols are considered to be one of the most widely distributed groups in plants (Graça et al. 2002). Phenolic acids are potential allelochemicals due to its high water solubility and properties to inhibit plant growth (Inderjit 1996). These compounds have high solubility in water (Inderjit 1996) and are known to affect photosynthesis, protein synthesis, mineral absorption, synthesis of chlorophyll, as well as alter membrane permeability and water balance in plants (Rice 1984). Phenolic compounds can be released into the environment by rainwater, oozing or by the degradation of plant parts. However, polyphenols are also involved in plant defenses against herbivorous and microorganisms (Manninem et al. 2002).

Many Asteraceae, Myrtaceae, Rutaceae and Rosaceae that occur in arid environments release terpenoids and phenolic substances soluble in water, as allelopathic compounds. In these regions, such as *Eucalyptus* cultures, the area around the older plants is populated only when the plant material and allelopathic substances are decomposed by soil microorganisms or are destroyed by fire (Larcher 2000).

Among coniferous, the genus *Pinus* contains high levels of phenolics compounds, with known toxic activity in biological systems (Arise et al. 2009). Although this genus is exotic in Brazil, the cultures with *Pinus* have been increased since 1954, replacing the south native forests characterized by *Araucaria angustifolia*, another coniferous species. Furthermore, *Pinus taeda* is one of the most cultivated species in Brazil for wood and paper production, reaching approximately two million hectares. Extensive *Pinus* spp. plantation is associated with environmental impact due to the ability to invade native areas and produce allelochemical compounds (Richardson and Higgins 1998). The allelopathy is known to be exhibited by plants of almost every group. In gymnosperms, conifers represent the great group exhibiting allelopathy. Out of the seven taxonomic families of conifers six *viz.* Araucariaceae, Cupressaceae,

Pinaceae, Podocarpaceae, Taxaceae, and Taxodiaceae are reported to show this phenomenon (Singh et al. 1999).

Besides the production of polyphenols acting as allelochemicals, cultures of *Pinus* show a high density of individuals/area, resulting in shading and deposition of large amounts of biomass in the soil. This material deposited in the soil can act as a source of release of phenolic compounds, impregnating both the soil and the drainage water, which may contaminate natural areas and alter the physical and chemical properties of the environment.

Therefore the aims of the present study were: (1) to determine the concentrations of hydrosoluble phenolics in leaves of *Pinus taeda*, an exotic species, and *Araucaria angustifolia*, a native species, collected in months of winter and summer of 2009 and 2010; (2) to quantify the litter produced by *P. taeda* and *A. angustifolia*; (3) to determine, in laboratory conditions, the time required for leaching of hydrosoluble phenolics from leaves ; (4) to quantify the concentration of hydrosoluble phenolics in body water near the plantations; (5) to evaluate the allelochemicals effect of aqueous extract of the *P. taeda* and *A. angustifolia* in seeds of *Lactuca sativa*; (6) to determine, by HPLC, the profile of phenolics in the hydrosoluble extract from *P. taeda* and *A. angustifolia.*

Material and Methods

Plant material

Leaves of *P. taeda* and *A. angustifolia* were collected from trees older than 20 years old cultivated in a commercial culture in São Francisco de Paula Municipality (29°23'36.2"S - 50°22'50.7"W; 900 m a.s.l.), Rio Grande do Sul, Brazil.

Collected leaves were categorized according to their color: Green (fresh mature leaves from trees), yellow (fallen litter layer from the floor of coniferous culture) and black (partially decomposed leaves from the floor). Leaves from each category were separated in paper bags and were further dried in oven at 40ºC for 72h. The dry material was processed in a knife grinder and stored at -20ºC until use.

Phenolic concentrations

Phenolic compounds were analyzed in the ground samples (0.2 g) and homogenized in water or 80% methanol (1:20 w/v). Each category of leaves was sampled. Extracts were centrifuged at 2.500*g* for 30 min at 4ºC, and the supernatants

were used for quantification using the Folin-Ciocaulteau method (Poiatti et al. 2009) during summer and winter of 2009 and 2010. Gallic acid was used as reference for establishing the calibration curve. Methanolic extracts were used as the reference for determination of the total phenolic compounds in the leaves. Phenolic compounds in the water samples collected in different sites were also analyzed using the method indicated above. The results are presented as mg/g DW.

Leaching from leaves

The time necessary for leaves of *P. taeda* and *A. angustifolia* to release hydrosoluble compounds in water was evaluated. Fresh Green needles were collected in summer and winter (2009 and 2010). Samples of 0.5 g were immersed in 500 ml of sterile distilled water in Erlenmeyers. All flasks were kept at 25±2°C with light intensity of 31 µmol m^{-2} s^{-1} in a 16-h photoperiod. The level of phenolics in the water was used as indicator of time for leaching and concentration decay. These levels were evaluated at 0, 7, 15, 30, 60, 90, 120, 150 and 180 days, using the Folin-Ciocaulteau method (Poiatti et al. 2009). The results are presented as mg/g DW.

Determination of phenolics by HPLC

Green leaves of *P. taeda* or *A. angustifolia* (1 g dry matter) were extracted with sterile distillated water in vortex for 10 minutes. Extracts were centrifuged at 2.500*g* for 30 min at 4ºC ans were filtered through 0.45 µm filter. Samples were maintained at –20 ºC until chromatographic analyses were carried out. All solvents were purchased from Mallinkrodt (USA). The HPLC analyses were carried out in a Agilent Technologies, 1200 Series chromatograph, operated at 45 ºC. Separations were performed on a MetaSil ODS column (5 µm; 150 x 4.6 mm) and detection was achieved with a UV/V detector set at 280 nm. Compounds were separated by a linear gradient program with the following solvents: A, water: phosphoric acid (98:2 v/v) and B, acetonitrile (100%). The gradient of the mobile phase was from 20 to 30% of B from 0 to 30 min and 100% of B from 30 to 35 min. The flow rate was kept constant at 1 ml/min and injection volume was 10 µL. Standard curves of peak area *versus* concentration were plotted and the equation of the regression line was determined. Compounds were identified based on the retention time of pure standards and quantified by reference to peak areas of the standard curves.

Litter fall estimation

The litter fall of *P. taeda* and *A. angustifolia* trees was seasonally estimated using 6 collectors with 1 m^2 of area installed at the perimeter of each plantation. Accumulated litter fall was collected during summer and winter in 2009 and 2010. Samples were sorted and only leaves were collected. Other components (bark, twigs, debris) were discarded. Leaves were oven-dried at 40°C for 72h and dry mass was determined. The results are presented as g/m^2.

Natural occurrence of phenolics in water

Water samples were collected in two body waters localized near (inside a range of 500 m) and far from commercial culture with *P. taeda* or *A. angustifolia* (more than 5000 m away). Body waters were localized in São José dos Ausentes Municipality (28°47'00"S - 49°50'53"W; 1200 m a.s.l.) – far from a commercial culture, and São Francisco de Paula Municipality (29°23'36.2"S - 50°22'50.7"W; 900 m a.s.l.) – near a commercial culture, both in Rio Grande do Sul, Brazil. All samples were collected during summer and winter of 2009 and 2010. The levels of phenolic compounds in the water were quantified using the Folin-Ciocaulteau method (Poiatti et al. 2009).

Seed germination

Seeds of *L. sativa* were used to evaluate the presence of allelochemicals in the aqueous extracts of *P. taeda* and *A. angustifolia.* Extracts were prepared from green leaves collected in the summer and winter of 2009 - 2010. The level of phenolics in the extracts was used as parameter for the establishment of different concentrations. Treatments consisted in four concentrations of extracts (0.1, 0.25, 0.5 and 0.75 mg phenolics/L) and the control (water). For each coniferous species, three Petry dishes (replicate) with thirty seeds each and five treatments, including control, were tested. Two sterile papers were placed at the bottom of a Petry dish and impregnated with 10 mL of extract or water. All treatments were maintained at 25±2°C with light intensity of 31 µmol m^{-2} s^{-1} in a 16-h photoperiod. The germination rates were determined daily in a cumulative way for eight days. Percentage of germination and speed of germination index (SGI) were calculated. SGI has advantage over percent germination, because it is usually more sensitive indicator of allelopathic (Wardle et al. 1991). The parameters shoot and root length, as well as, changes in the morphology were also evaluated for seedlings development.

Statistical Analysis

The results are expressed as mean ± standard error. A two-way ANOVA test was used for statistical analysis followed by a Bonferroni test for data obtained with different concentration of phenolic. When no significant differences were observed between samples collected in 2009 and 2010, results were analyzed independently of the year. The difference between concentrations of phenolics within each species evaluated was determined with T test to independent data. The significance level adopted was 5%. Tests were performed with the program Statistical Package for the Social Sciences (SPSS 11.5) for Windows.

Results

The green needles of *Pinus taeda* showed the highest level of hydrosoluble phenolic compounds (32.18 mg/g DM) in the summer and the lowest level in the winter, when they did not exceed 8.17 mg/g DM. Yellow and black needles showed concentrations of hydrosoluble phenolics lower than 20% and 10%, respectively compared with the green needles, independently of the season (Table 1). Whereas, the highest amount of litter of *P. taeda* was deposited in the winter (121.27 g/m^2), compared with the summer (65.55 g/m^2) (Figure 1).

The green leaves of *A. angustifolia* showed the double level of hydrosoluble phenolic compounds during summer when compared with winter (Figure 1). In general, when both species are compared, the phenolic level in *A. angustifolia* was 2.64 times lower in summer and 4.25 times lower in winter ompared with *P. taeda*.

Regardless the type of extraction or the season, the lowest level of phenolics in *A. angustifolia* was observed in leaves partially decomposed (black leaves) collected on the floor of the culture. There are no differences in these levels between green and yellow leaves (Table 1).

There is no difference in litter production by *A. angustifolia* in winter and summer. In general, the biomass produced by *A. angustifolia* was three times lower than *P. taeda* (Figure 1).

The leaching experiments indicated that the maximum level of hydrosoluble compounds in both P. *taeda* and *A. angustifolia* was released in seven days (Figure 2). However, needles from *P. taeda* remain releasing high amount of phenolics for two months. In contrast, these levels in A. *angustifolia* were lower than in *P. taeda* and remain liberating phenolics for four months.

The natural level of hydrosoluble phenolics in water was dependent on the presence of *Pinus* spp. plantation near the body water. Water samples collected in summer and winter presented the average concentration of 20.56mg/L and 12.82mg/L, respectively for sites near *Pinus* plantation. However, water samples collected in summer and winter on sites far from those plantations, presented 0.46 mg/L and 0.23mg/L of phenolics, respectively (Figure 3).

The percentage of germination and the speed of *L. sativa* were reduced by the extracts of *P. taeda* (Table 2). Moreover, the germination speed index and the root length was significant reduced with the increment of extract concentration. However, the seedlings shoot were no significant affected by these extracts. Extracts of *A. angustifolia* did not affect any parameter evaluated for *L. sativa* seeds (Table 3).

Phenolic compounds varied qualitatively and quantitatively according the season and species evaluated (Table 4). Aqueous extracts of *P. taeda* presented high concentration of catechin, but low concentration of cumarine, independently of the season (Table 4). Neither *p*-coumaric acid nor piceatanol were identified in samples collected in summer nor caffeic acid in winter. Aqueous extracts of *A. angustifolia* presented high concentrations of catechin, independently of the season (Figure 4). The highest level of cumarine was identified in samples collected in summer (0.22 ± 0.06 mg/g DM), but this level was reduced in the winter (0.12 ± 0.03 mg/g DM). Caffeic acid, *p*-coumaric acid and piacetanol were not detected in summer (Table 2).

Discussion

Wojdyło et al. (2007) reported that polyphenolic compounds are present in all plants, and they have been reported to have multiple biological effects, including antioxidant activity (Kahkonen et al. 1999).

Sergul et al. (2009) measured the total phenolic content of many species, such as *Inula aucherana, Fumaria officinalis, Crocus ativus, Vicum album, Tribulus terestris Polygonatum multiflorum, Alkanna tinctoria* and *Taraxacum officinale* and reported that levels ranged from 4.04 mg/g to 42.29 mg/g dry weight. Moreover, Bajpai et al. (2005) reported concentrations ranged from 6.80 to 32.10 mg gallic acid equivalents per g dry weight. Concentrations of phenolic compounds observed in the present work corresponded to the values described in the literature for different species.

Likely the present work Cosmulescu and Trandafir (2011) showed a seasonal variation in the total phenols content in *Juglans regia l*eaves. According to Cosmulescu

and Trandafir (2011) these differences in terms of total phenols is related to change in ecological parameters like soil composition, maturation level, cultivar and harvest year.

There is a lack of information considering not only the levels of phenolic compounds in *P. taeda* and *A. angustifolia*, but also their relation with leaching (Figure 1) and the amount of phenolics in water collected in the environment (Figure 3).

Leaching experiments are even after death, the allelopathic substances are still in their tissues, where they are released by evaporation, if volatile products, or by leaching through dew and rain, if they are soluble in water, being dragged the ground, where, upon reaching the necessary concentration, may influence the development of microorganisms and plants found therein (Neves 2005).

The decrease of seed germination and the germination speed index (GSI) of *L. sativa* indicate the alellopathic effect of *Pinus (*Ferreira & Borghetti 2004). Moreover, the root of seedlings was also inhibited by soluble compounds released by *P. taeda*. However, Cuchiara et al. (2007) reported no effect of allelochemicals from *Ricinus communis* in seedlings development of *Lectuca sativa*. On the other hand, no alellopathic effect was observed in extracts of *A. angustifolia.*

The results of this study allow us to suggest that hydrosoluble compounds extracted from *P. taeda* have allelopathic effects on seed *L. sativa*, as well as prevent the growth of the seedling through the interference with metabolic pathways in the roots.

According to Souto et al. (1994), the allelophatic effect of phenolic compounds produced by *P. radiata* and *E. globulus* leaded to inhibition of growth and development of lettuce. The same authors reported that the toxicity of the extracts was higher in the early stages of decomposition, according to the phytotoxic compounds released. However, there was no more inhibition six months after the decomposition. These results are similar to and corroborate those observed in this study, in which greater allelopathic activity was found in green needles. Fernandez et al. (1996) reported that extracts of *Pinus halepensis* needles, with different ages, influenced the germination of *Lactuca sativa* and *Linum strictum*. This effect was attributed to phenolic compounds. On the other hand, Ferreira et al. (2007) described that ethanolic extracts of *Pinus eliott* effect for the variables germination and early growth of *B. pilosa* and lettuce.

Sartor et al. (2009) observed the allelopathic effect of aqueous extract of *Pinus taeda* needles on the germination and development of black oat (*Avena strigosa*) seedlings. These extracts were composed of needles in vegetative stage (green needles),

moderately decomposed (dry needles) and in advanced decomposition (decomposed needles). The stage of green affected the germination and the velocity of the process and showed dose-dependent and negative effect with the increasing concentration of the crude extract.

According Inderjit and Duke (2003) and Ashraf et al. (2008) allelopathic interference thresholds also vary with plant processes involved and the sensitivity of the recipient species as well as generally, allelopathic inhibitors interfere with key physiological processes in receptor plants, resulting in reduction of plant growth and development.

Rashid et al. (2010) explored the allelopathic potential of *Pueraria montana* (Kudzu) as a function of its phenolics. Aqueous and methanol extracts of different kudzu inhibited all germination indices. In the present work we verified a reduction in percentage of the seeds that germinated when treated with aqueous extracts from *P. taeda*, only 58% of the seeds germinated when in the control group 97% of seeds germinated; this response was ~~is~~ not observed for *A. angustifolia.*

According to Blume and Saunders (1981) the synthesis and accumulation of phenolic compounds are important aspects of secondary plant metabolism and many of the biosynthetic reactions leading to the major classes of plant phenolics are known (Hanson and Harvin 1979) like simple phenols, phenolic acids (benzoic acid derivatives and cinnamic acid), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignins and lignans (Naczk and Shahidi 2004).

In the present work we found some differences between phenolics of coniferae and between seasons. The concentration of hydrosoluble phenolics in summer and winter was 4.64 and 6.92 mg/g for *P. taeda*, respectively and 11.16 and 4.93 mg/g for *A. angustifolia* in summer and winter, respectively. However, seasonal variation has been observed only in *A. angustifolia* (Table 4). Phenolic profile of *O. glandulosum* showed gallic acid, vanillic acid, coumaric acid, rutin, ferrulic acid and naringenin in the extracts (Naima et al. 2011). Proestos et al. (2005) showed that the most abundant phenolic acids were ferulic acid and caffeic acid in aromatic plants.

Cannac et al. (2007) analyzed the phenolic compounds in the needles of *Pinus laricio* and showed that 3-vanillyl propanol is the major compound. The presence of flavonoids and simple phenolics like phenolic acid in different pine species has been reported by many reportes (Ye-sil-Celiktas et al. 2009; Senthilmohan et al. 2003;

Rohdewald et al. 2002), but plant phenolics are quite variable, including stilbenes, coumarins, tannins, lignans and xanthones.

The analyze and the profile of some phenolic compounds, indicate that there are others toxic compounds present in the extracts of *P. taeda*. Probably, this combination is the factor that determined the deleterious effect of the extract of *P. taeda.* The results of this study allow us to suggest that *P. taeda* and *A. angustifolia* showed important differences related to environmental toxicity. These differences may explain why the exotic species *P. taeda* is interfering in the natural ecosystem formerly occupied by the native *A. angustifolia*.

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Table 1: Levels of phenolic compounds in leaves of *P. taeda* and *A. angustifolia* extracted with methanol or water. The levels are present as mean ± standard error. Results are expressed in mg/g Dry Matter. Green - fresh mature leaves, Yellow – fallen leaves, Black - partially decomposed leaves.

Types of leaves	Extract	*Pinus taeda*		*Araucaria angustifólia*	
		Summer	**Winter**	**Summer**	**Winter**
Green	Methanolic	39.25 ± 2.75	28.86 ± 2.35	14.86 ± 1.15	6.79 ± 0.62
	Hydrosoluble	32.18 ± 5.23	8.17 ± 0.43	12.32 ± 1.23	5.29 ± 0.41
Yellow	Methanolic	9.34 ± 0.62	10.63 ± 0.12	14.31 ± 1.33	6.42 ± 0.32
	Hydrosoluble	5.81 ± 0.17	3.63 ± 0.11	12.29 ± 1.12	5.26± 0.21
Black	Methanolic	4.56 ± 0.34	1.45 ± 0.07	4.19 ± 0.32	3.17± 0.31
	Hydrosoluble	3.24 ± 0.25	0.51 ± 0.04	2.02 ± 0.21	1.03 ± 0.11

Table 2: Effects of aqueous extracts of *P. taeda* in the germination speed index (GSI), the germination speed (GS), the percentage of germination and the shoot and root length of *Lectuca sativa* seedlings. The level of total phenolics in the extracts was used as parameter for the establishment of different concentrations (Treatments), and are expressed as mg phenolics/L.

Treatments	***GSI***	***GS***	***Germination (%)***	***Shoot (cm)***	***Root (cm)***
Control	92.03	1.36	96.92	1.94	0.88
0.10mg/L	78.54	2.59	64.29	1.86	0.27
0.25mg/L	69.09	2.27	59.37	1.83	0.30
0.50mg/L	66.94	2.31	63.98	1.81	0.29
0.75mg/L	58.42	2.28	57.97	1.84	0.24

Table 3: Effects of aqueous extracts of *A. angustifolia* in the germination speed index (GSI), the germination speed (GS), the percentage of germination and the shoot and root length of *Lectuca sativa* seedlings. The level of total phenolics in the extracts was used as parameter for the establishment of different concentrations (Treatments), and are expressed as mg phenolics/L.

Treatments	***GSI***	***GS***	***Germination (%)***	***Shoot (cm)***	***Root (cm)***
Control	94.21	1.41	97.64	1.92	0.88
0.10mg/L	93.67	1.38	94.87	1.89	0.83
0.25mg/L	93.14	1.39	94.49	1.90	0.80
0.50mg/L	94.19	1.39	92.12	1.91	0.84
0.75mg/L	93.78	1.38	93.53	1.90	0.82

Table 4: Evaluation of phenolic compounds presented in aqueous extracts of *P. taeda* and *A. angustifolia*. The levels are present as mean ± standard error. Results are expressed in mg/g dry mass.

Phenolics	***Pinus taeda***		***Araucaria angustifólia***	
	Summer	**Winter**	**Summer**	**Winter**
Catechol	0.26 ± 0.06	0.54 ± 0.11	1.54 ± 0.27	0.66 ± 0.10
Catechin	2.38 ± 0.56	3.65 ± 0.86	4.90 ± 0.13	2.70 ± 0.25
4-hydroxybenzoic acid	0.27 ± 0.07	0.44 ± 0.11	0.79 ± 0.15	0.49 ± 0.14
Caffeic acid	0.17 ± 0.03	nd	nd	nd
4-hydroxy-3-methoxybenzoic acid	1.25 ± 0.11	0.12 ± 0.08	3.42 ± 0.004	nd
***P*-coumaric acid**	nd	1.46 ± 0.22	nd	0.70 ± 0.001
Piceatanol	nd	0.13 ± 0.06	nd	0.10 ± 0.02
Benzoic acid	0.25 ± 0.98	0.50 ± 0.18	0.30 ± 0.10	0.16 ± 0.03
Coumarin	0.08 ± 0.02	0.08 ± 0.02	0.22 ± 0.06	0.12 ± 0.03
Total	4.64	6.92	11.17	4.93

nd – Not identified

Figure 1: Litter production of *P. taeda* and *A. angustifolia* evaluated in summer and winter. The levels are present as mean ± standard error. Results are expressed in g/m^2.

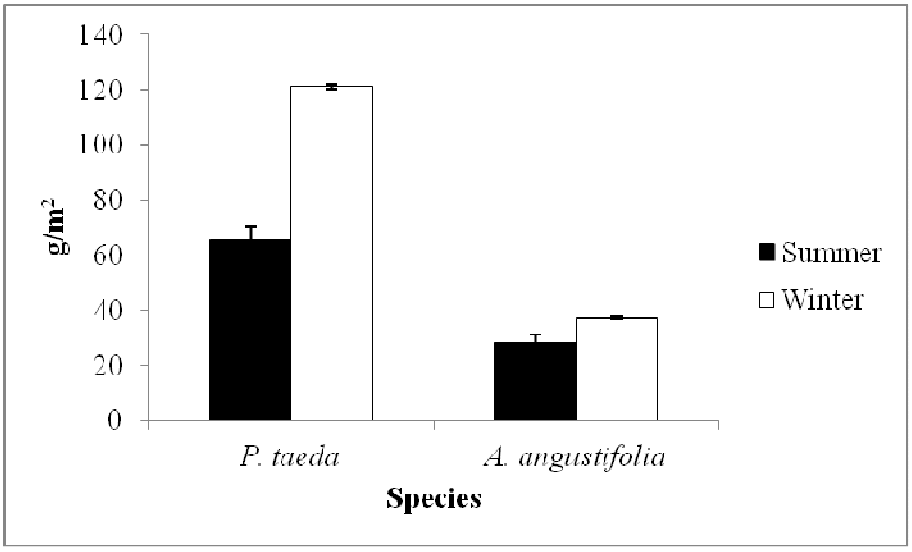


Figure 2: Levels of hydrosoluble phenolic compounds released from *Pinus taeda* or *Araucaria angustifolia* by leaching. Results are expressed in mg/g DW.

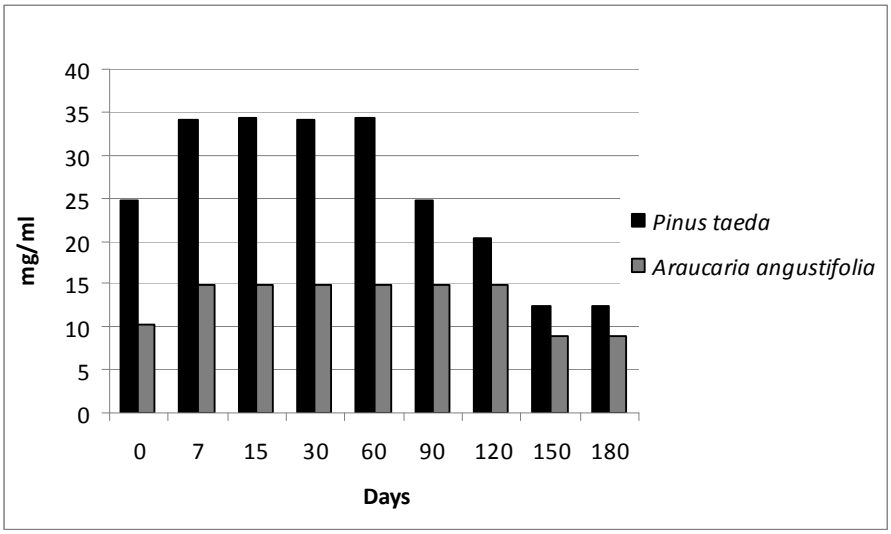
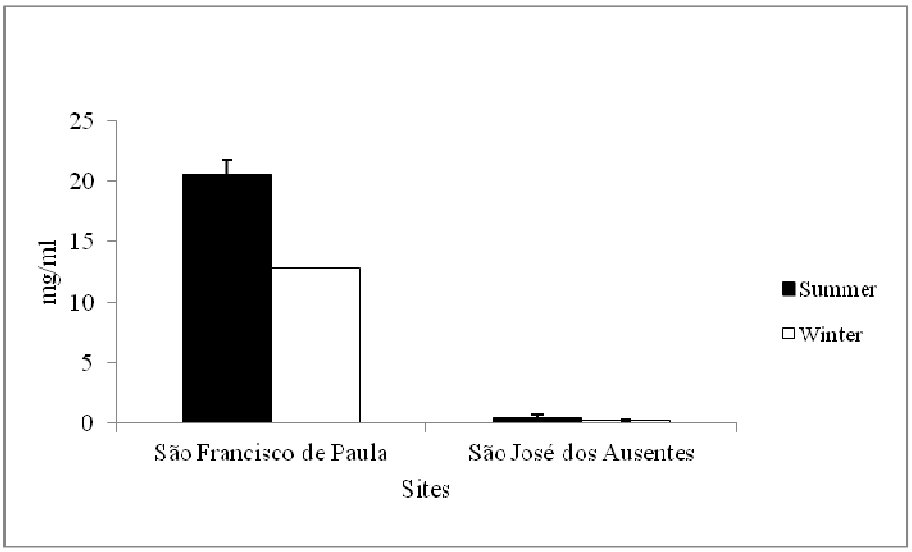


Figure 3: Levels of hydrosoluble phenolic compounds in water samples collected in water bodies localized near (São Francisco de Paula) and far (São José dos Ausentes) from commercial culture with *P. taeda* or *A. angustifolia.* The levels are present as mean ± standard error. Results are expressed in mg/ml.



Capítulo 3

Hydrosoluble compounds of exotic and native Coniferae species interfere in the activity of the respiratory electron transport system of *Hyalella castroi*

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ABSTRACT

The aim of the present study was to evaluate the effect of plant dry material of two conifers, *Pinus taeda*, an exotic species, and *Araucaria angustifolia*, a native species in the activity of the respiratory electron transport system (ETS) of *Hyalella castroi*. Amphipods and leaves of *A. angustifolia* and *P. taeda* were collected in summer and winter, in Rio Grande do Sul, Brazil. After seven days of exposition, animals were used for determining the ETS. The radical scavenging activity of the plant aqueous extracts was also evaluated. The results of this study allow us to suggest that hydrosoluble compounds produced by extract of coniferae species have different antioxidant potentials and affect the amphipods in a divergent form in terms of the ETS. This pattern of response can help to explain how exotic species of conifers such as *P. taeda*, modify the natural environment and cause severe alterations in freshwater ecosystem.

Keywords: ETS, DPPH, *Pinus taeda*, *Araucaria angustifolia*, Amphipoda

1. INTRODUCTION

Allelochemicals produced by different species of plant play an important role in natural ecosystems. These effects are seen in terrestrial and phytoplankton succession, inhibition of nitrogen fixation, nitrification and others (Kohli et al. 1997). Among plant allelochemicals, phenolics represent a widely group of compounds with known properties of inhibiting plant growth (Inderjit 1996). These compounds have high solubility in water (Inderjit 1996) and affect photosynthesis, protein synthesis, synthesis of chlorophyll, as well as alter membrane permeability and water balance in plants (Rice 1984; Sasikumar et al. 2001).

Conifers are known to produce and release phytotoxins/allelochemicals (predominantly phenolic compounds) in the environment from the fallen litter layers (Singh et al. 1999). Among coniferous, the genus *Pinus* contains high levels of phenolics compounds, with known toxic activity in biological systems (Arise et al. 2009). Extensive *Pinus* spp. plantation is associated with environmental impact due to the ability to invade native areas and produce allelochemical compounds (Richardson and Higgins 1998). Although this genus is exotic in Brazil, the cultures with *Pinus* have been increased since 1954, replacing the south native forests characterized by *Araucaria angustifolia*, another coniferous species.

Another prominent biological property of the phenolics' group is its radical scavenging ability. According to the hypothesis of Apak et al. (2007), phenolic compounds are synthesized probably as a result of antioxidative strategies evolved by respirative organisms starting from precursors of cyanobacteria. Plant polyphenols in general are multifunctional and can act as reducing agents, hydrogen donor and singlet O_2 quenchers exhibiting their antioxidant activity, via hydrogen atom transfer, electron donation, through

metal chelation, interaction with other antioxidants (co-operative actions) or by localization and mobility of the antioxidant (Pai et al. 2010).

Simcic & Brancelj (1997) working with five species of *Daphnia* described that the activity of the respiratory electron transport system (ETS) is a biochemical parameter for evaluating the effect of exogenous compounds in the metabolic activity. The ETS is localized in the mitochondrial inner membrane and acts as bridge between the oxidizing organic matter and O_2. This multienzyme complex contains flavoproteins, metallic proteins and cytochromes which are arranged in a complete biochemical redox system for transporting electrons from the coenzymes NADH, NADPH and succinate, arriving from the Krebs' cycle, to the terminal electron acceptor – O_2 (G.-Toth et al. 1995). Among crustacean amphipods, *Hyalella castroi* is an indicator species for ecotoxicological studies of freshwater ecosystems (Dutra et al. 2008, 2009, 2011). Thus, the exposition of organisms to pollutants may increase the energy requirement of animals and hence result in reduced energy available for growth and reproduction (Olsen et al. 2008; Dutra et al. 2011).

Our hypothesis was that exotic cultures with *Pinus taeda* produce hydrosoluble compounds that disturb the metabolic energy of the native crustacean amphipods, *Hyalella castroi*. To address this hypothesis, we evaluated the effect of plant dry material of two conifers, *Pinus taeda*, an exotic species, and *Araucaria angustifolia*, a native species, in activity of the respiratory electron transport system (ETS) of *H. castroi*.

2. MATERIAL AND METHODS

Animals were collected and maintained in accordance with Brazilian laws (N°. 23378-1- SISBIO/IBAMA) and were used with approval from the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) (License 002/09).

2.1 *Plant material*

Green leaves of *Pinus taeda* and *Araucaria angustifolia* were collected from trees older than 20 years old cultivated in a commercial culture in São Francisco de Paula Municipality (29°23'36.2"S – 50°22'50.7"W; 900 m a.s.l.), Rio Grande do Sul, Brazil. Leaves were stored in a paper bag and dried in oven at 40ºC for 72h. The dry material was processed in a knife grinder and stored at -20ºC until use. The levels of hydrosoluble phenolic compounds were used as parameter for preparing four different concentrations of plant material to be supplemented in aquariums.

2.2 *Hyallela castroi*

Animals were collected in the summer of 2009/2010 (December, January and February) and winter 2009/2010 (June, July and August); along with macrophytes *Callitriche rimosa* from their habitat with fish traps. *H. castroi*, 330 males and 330 females by each season, were collected in São José dos Ausentes Municipality (28°47'00"S – 49°50'53"W; 1200 m a.s.l.), Rio Grande do Sul, Brazil.Animals were transported on cooled water in insulated containers to the Laboratory of Conservation Physiology of PUCRS. Twenty animals of each sex were immediately cryoanesthetized, in order to assess whether there were any differences between the animals collected in the wild (control group) and the animals that received diet *ad libitum* for 7 days (Diet 7) or 14 days (Diet 14) in cultivation aquariums and the others that received this diet for 7 days and were then exposed to plant material for 7 days.

The animals were fed a combination of commercial feed for fish and the macrophyte (*Callitriche rimosa*), presented 351.99 Kcal/100g to total caloric value, as standardize by Gering et al. (2009).

In order to establish the profile of variation in the respiratory electron transport system (ETS) activity in the amphipods, individuals of *H. castroi* were exposed to plant dry

material containing four different concentrations of phenolics (0.10, 0.25, 0.50 and 0.75 mg/L). Concentrations were standardized according the amount of hydrosoluble phenolic compounds in the plant material. These concentrations were chosen based on previous bioassays made in our laboratory with *H. castroi* exposed to plant material containing hydrosoluble phenolics in concentrations equal or higher than 1.0 mg/L (for *Pinus taeda*) which showed mortality higher of 70%.

2.3 *Experimental procedure*

Adult animals were kept submerged in aerated aquariums (20 L), divided with netting in order to maintain chemical contact but to prevent any physical interaction between males and females (the water passed through both sides of the aquarium). Previous studies in our laboratory demonstrated that this arrangement is important to keep the animals alive (Gering et al. 2009). The mean temperature was 23 ± 1ºC and the photoperiod was 12 hours light. The animals were acclimated in the aquariums for seven days, during which they received only food (macrophytes and artificial diet) *ad libitum*, daily during periods when most of the animals were active (Gering et al. 2009). After this acclimation period, 20 animals of each sex were cryoanesthetized (Diet 7) for determination of all biochemical parameters.

After acclimation, amphipods were sorted in and fed *ad libitum* with the same diet for seven days. The experimental groups consisted in: (1) Animals that received only the diet for more 7 days (diet 14); amphipods exposed to compounds released from the *Pinus taeda* material with the final hydrosoluble phenolic concentration of 0.10 mg/L (Group 2), 0.25 mg/L (Group 3), 0.50 mg/L (Group 4) and 0.75 mg/L (Group 5). Likewise amphipods exposed to compounds released from the *Araucaria angustifolia* material with the final hydrosoluble phenolic concentration of 0.10 mg/L (Group 6), 0.25 mg/L (Group 7),

0.50 mg/L (Group 8) and 0.75 mg/L (Group 9). All amphipods from groups 2 to 9 were exposed to the plant material for a period of 7 days.

All amphipods were cryoanesthetized and weighed at the end of experiments, and stored at -80°C until the biochemical analysis. All experiments were analyzed through three independent repetitions.

2.4 *Determination of the antioxidant capacity*

Radical scavenging activity of the aqueous extracts (1g dry material/ml water) of *P. taeda* and *A. angustifolia* was established by measuring the decrease in absorbance of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical according to Choudhury et al. (2006). Various concentrations of the aqueous extracts were added to 100µM ethanol solution of DPPH. The bleaching of DPPH was measured at 517 nm. Control solution containing equal volume of DPPH and MeOH was used as blank. The results are presented as percent inhibition of free radical.

2.5 *Respiratory electron transport system (ETS) activity*

ETS activity was measured using the method developed by Owens & King (1975) with some modifications. The buffer for homogenization, substrate solution, reagent solution and stopping solution were prepared just before the experiments to avoid substrate decomposition and bacterial contamination. The determination were done in total homogenate of each animal, in a total of five males and five females for each point (each experimental group) and homogenized in ice cold homogenization buffer in a homogenizer (potter) for 3 min. The homogenate was centrifuged for 10 minutes at 3,000 rpm at 4ºC and stored subsequently on ice. After 0.05 ml of the homogenate (in triplicate) was incubated in 0.150 ml substrate solution with 0.05 ml reagent solution for 30 min at 30ºC. The reaction was stopped by adding 0.05 ml stopping solution. ETS activity was measured as the rate of tetrazolium dye reduction to formazan, and converted to equivalent oxygen utilized per wet

mass per hour (μlO_2 $mgWW^{-1}$ h^{-1}), as described by Kenner & Ahmed (1975). The formazan production was determined spectrophotometrically from the absorption of the sample at 490 nm.

2.6 *Statistical Analysis*

The results are expressed as mean ± standard error, and all parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). A three-way ANOVA test was used for statistical analysis followed by a Bonferroni test. The significance level adopted was 5%. Tests were performed with the program Statistical Package for the Social Sciences (SPSS 11.5) for Windows.

3. RESULTS

Aqueous extracts of *A. angustifolia* and *P. taeda* showed antioxidant activity (Figure 1 A and B). The highest antioxidant activity was observed in extracts of *A. angustifolia* compared with the extracts of *P. taeda*. In this species, the antioxidant activity showed an inverse pattern when compared to the A. angustifolia extracts.

There were no differences in the activity of respiratory electron transport system (ETS) between the samples of *H. castroi* collected in the environment and maintained on artificial diet for 7 or 14 days (Figure 2). Moreover, supplementation of plant material of *A. angustifolia* did not promoted any difference ($p>0.05$) in ETS comparing females and males of *H. castroi*, independently of plant concentration (Figure 2A).

Plant material of *P. taeda* promoted the reduction in the activity of ETS of approximately 3.36 times, independently of the gender. *P. taeda* was effective in ETS reduction ($p<0.05$) compared to *A. angustifolia* where animals presented a same pattern of ETS activity that a control groups (Figure 2B).

4. DISCUSSION

Antioxidant capacity of aqueous extracts of *P. taeda* showed an inverse relation between concentration of phenolic and free radical inhibition. The antioxidant activity was reduced when concentration of phenolics increased (Figure 1B and 2B). However, there was a positive dose-dependent response between concentration of phenolic and the antioxidant capacity in *A. angustifolia* extracts. (Figure 1A and 2A). Cortés et al. (2010) studying *Pinus radiata* reported a high antioxidant activity of extracts varying from 6 to 12 $\mu g \cdot mL^{-1}$, concentrations much lower than those used in the present work. In the present study, the highest free radical inhibition was observed in *A. angustifolia*, compared with *P. taeda*. *P. taeda* is an exotic species in Brazil and their phenolic composition may differ according to environmental factors, such as temperature, water source and UV radiation. (Globo-Neto and Lopes 2007).

Several studies have evidenced that simple phenolic compounds of low molecular weight are responsible for the toxic effects on seed germination (Aliotta et al. 2002), aquatic organisms (Paixao et al. 1999; Yesilada et al. 1999; Fiorentino et al. 2003), and bacteria (Yesilada and Sam 1998). Some phenolic acids, such as ρ-coumaric acid and vanillic acid, were reported to increase the levels of lipid peroxidation in aquatic organisms by the elevation in the O_2^- and malondialdehyde content (Zhang et al. 2010). Moreover, Rikans and Hornbrook (1997) reported that lipid peroxidation is considered to be a major mechanism, by which oxyradicals can cause tissue damage, leading to impaired cellular function and alterations in physico-chemical properties of cell membranes, which in turn disrupt vital functions.

According to data reported by Dutra et al. (2012), which used the same experimental methodology, soluble compounds of *P. taeda* promoted high levels of lipoperoxidation in both gender of *H. castroi*, whereas no effect was observed when animals

were treated with *A. angustifolia* (Table 1A). In the same work, when these crustaceans were exposed to hydrosoluble compounds of *P. taeda*, the levels of activity of the Na^+/K^+ATPase decreased approximately 44 times in all concentrations tested. However, no significant difference was observed in the Na^+/K^+ATPase activity between control and animals treated with *A. angustifolia* material (Table 1A and B). These responses can be a consequence of the minor antioxidant activity observed using *P. taeda* and reinforced the hypothesis of toxic potential of this exotic tree in relation to the native species (*A. angustifolia*).

Although respiratory electron transport system (ETS) is not frequently used for analyzing toxic compounds, we showed that this parameter was adequate for this purpose. Unfortunately, there is a lack of works correlating the antioxidant potential of the phenolic compounds with lipid peroxidation or Na^+/K^+ATPase and ETS activity. A significant decrease in ETS activity in *H. castroi* exposed to material from *P. taeda* may be correlated to an intense decrease of antioxidant capacity observed in this species. According to Lukancic et al. (2010) the physiological responses of two freshwater crustaceans, *Asellus aquaticus* and *Gammarus fossarum* following *in vitro* exposure to two toxicants (atrazine and imidacloprid) showed lower levels of ETS activity after 1 h exposure to concentrations of up to 10 mg L^{-1} in both test species. Similarly, a negative correlation was observed between ETS activity and toxicant concentrations of insecticide (triazophos), herbicide (butachlor) and fungicide in the soil (Subhani et al. 2002).

The results of this study allow us to suggest that hydrosoluble compounds produced by *P. taeda* and *A. angustifolia* have different antioxidant potential and affect the amphipods in a divergent form in terms of the respiratory electron transport system. This pattern of response can help to explain how exotic species such as *P. taeda* modify the natural environment and cause severe alterations in freshwater ecosystem.

5. ACKNOWLEDGEMENTS

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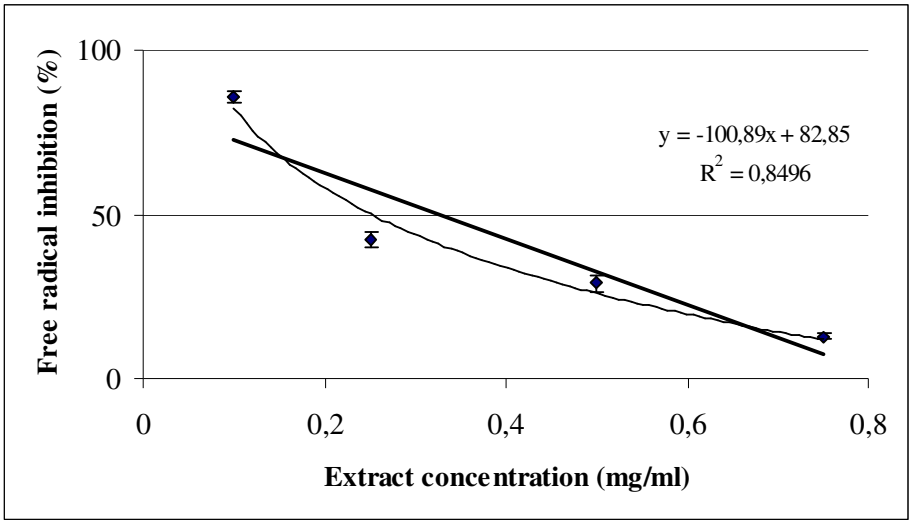
Figure 1: Radical scavenging activity of the aqueous extracts of *Araucaria angustifolia* (A) and *Pinus taeda* (B). The results are expressed as % inhibition of free radical.

A.



B.

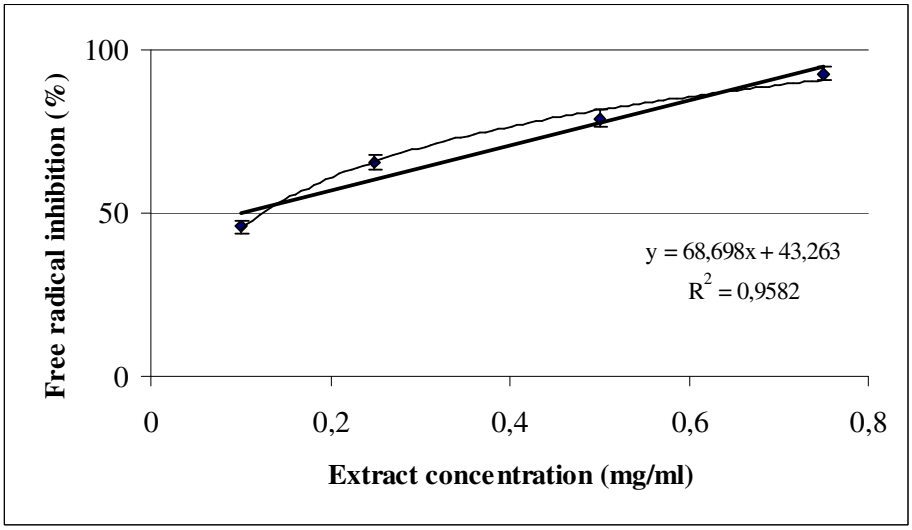
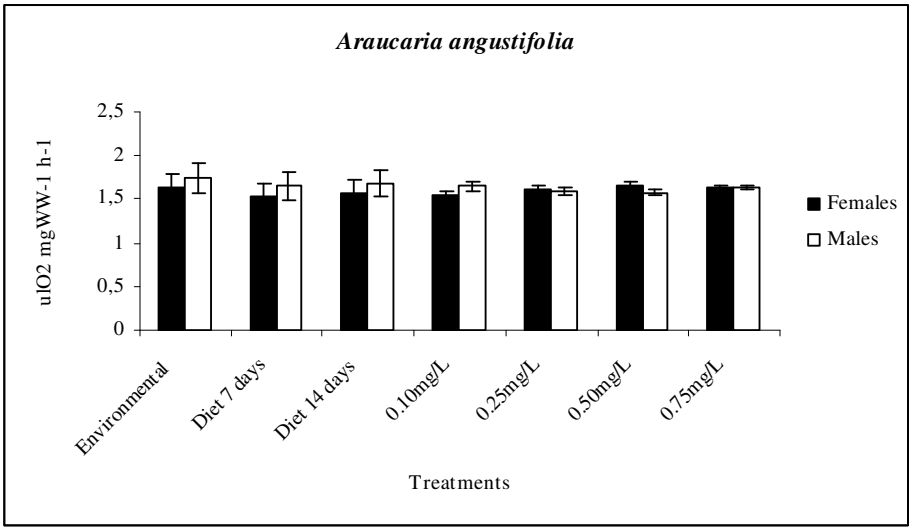


Figure 2: Levels of electron transport system activity of *Hyalella castroi* exposed to aqueous extract from *Araucaria angustifolia* (A) and *Pinus taeda* (B). The results are expressed as mg O_2.mg $WW^{-1}.h^{-1}$. The results are present as mean ± SD.

A.



B.

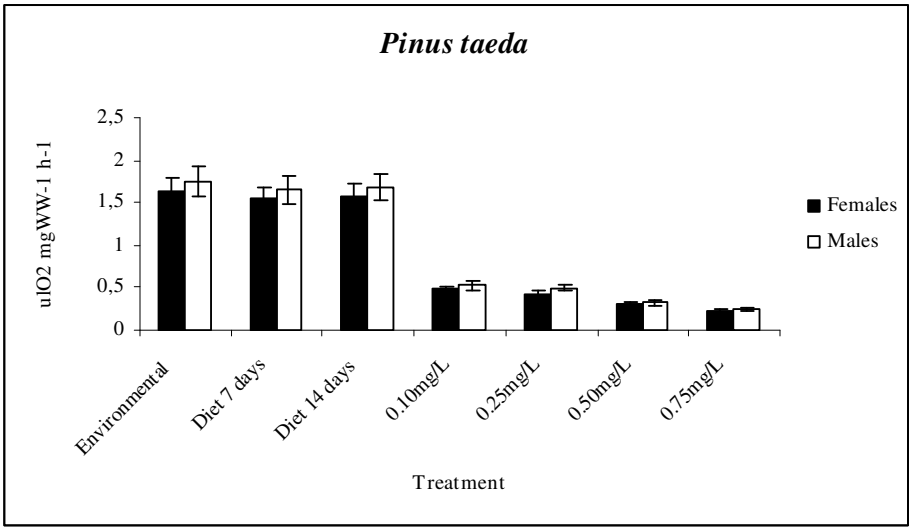


Table 1: Levels of lipoperoxidation and Na^+/K^+ATPase of *Hyalella castroi* exposed to aqueous extract from leaves of *Araucaria angustifolia* (A) of *Pinus taeda* (B). The results are presented as mean ± SD. These results were extracted from Dutra et al. 2012 (authorized by the authors).

A.

		Lipoperoxidation	*Na^+/K^+ATPase*
Environmental	**M**	23.60±0.99	7.58±0.28
	F	19.90±1.12	7.05±0.03
Control 7d	**M**	26.18±0.56	6.88±0.22
	F	22.38±1.14	6.66±0.09
Control 14d	**M**	24.46±0.33	6.55±0.33
	F	20.84±1.32	6.20±0.31
0.1mg/L	**M**	11.81±0.76	6.26±0.98
	F	11.71±0.15	6.14±0.42
0.25mg/L	**M**	14.46±0.85	6.15±0.47
	F	11.17±0.17	6.18±0.51
0.5mg/L	**M**	14.66±0.98	6.17±0.15
	F	11.28±0.23	6.44±0.09
0.75mg/L	**M**	16.08±0.60	6.13±0.62
	F	10.76±0.16	6.39±0.85

B.

		Lipoperoxidation	*Na^+/K^+ATPase*
Environmental	**M**	23.60±0.99	7.58±0.28
	F	19.90±1.12	7.05±0.03
Control 7d	**M**	26.18±0.56	6.88±0.22
	F	22.38±1.14	6.66±0.09
Control 14d	**M**	24.46±0.33	6.55±0.33
	F	20.84±1.32	6.20±0.31
0.1mg/L	**M**	82.85±1.55	0.78±0.05
	F	42.40±1.19	0.85±0.01
0.25mg/L	**M**	83.60±1.61	0.59±0.05
	F	65.79±4.66	0.50±0.03
0.5mg/L	**M**	80.31±2.34	0.48±0.03
	F	72.27±3.05	0.26±0.01
0.75mg/L	**M**	81.47±2.78	0.09± 0.01
	F	95.12±1.70	0.18±0.01

Capítulo 4

Evaluation of the effects of *Pinus taeda* in body water in Brazilian highlands

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ABSTRACT

Pinus plantation has emerged as a solution to replace the source of feedstock for production of furniture, paneling, particle board, paper, cellulose, among others, and economically viable due to the use of fast-growing species. The concern for the development of this activity is the consequences of the use of exotic species and the practice of monoculture on the local ecosystem. In this work we study the changes physical-chemical parameters and hydrosoluble phenolics in one body water near and another distant from the plantations of *Pinus taeda*. We collected samples in two body waters: one in São José dos Ausentes Municipality (28°47'00"S – 49°50'53"W; 1200 m a.s.l.) distant of the *Pinus taeda* plantation, and other in São Francisco de Paula Municipality (29°23'36.2"S – 50°22'50.7"W; 900 m a.s.l.) near the *P. taeda* plantation, both in Rio Grande do Sul, Brazil during summer and winter of 2009 and 2010. The parameters measured were total levels of phenolic compounds, total and fecal coliforms, hardness, nitrite, nitrate, total solids, sulphate, biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen, pH and water temperature. Out of the parameters analyzed in the present work only BOD, oxygen dissolved and pH seemed to change by the presence of the *Pinus taeda* plantation near of the body water and results suggest that the alteration are related with the presence of the needles as well as the high concentration of the phenolic compounds in the body water. These changes in body water may have consequences in aquatic ecosystems.

Keywords: *Pinus taeda*; Aquatic ecosystem; physical-chemical parameters

1. INTRODUCTION

Commercial culture with *Pinus* has emerged as an alternative wood source for production of furniture, paneling, particle board, paper, cellulose, and others (Guimarães et al. 2010). Wide areas with this exotic species may degrade local ecosystem.

Plantations with *Pinus* spp. have been associated with environmental impact due their ability to invade native areas and produces allelochemical compounds (Richardson and Higgins 1998).The environmental impact of *Pinus* in water quality has been reported in areas planted with this genus such as color change, increase in oxygen demand and concentration of bicarbonate, higher hardness and chloride concentration, compared to water from areas with natural herbaceous vegetation (McKee and Wolf 1963). In spite of conifers impact in water sources, Pierce (1965) has observed that this group of plants has been preferred for the culture areas around the reservoirs, leading to cause various changes in water composition.

Bruckert and Tout Ain (1971) reported the presence of simple and polymerized organic compounds in the rain water collected in cultures with *Fagus sylvatica* and *Pinus sylvestris*.

According to Dunger and Voigtländer (2005) practices used for managing commercial cultures may disrupt the natural process of nutrient cycling, resulting in a direct increase in the concentration of nutrients in the water.

Cultures in Brazil with exotic species as *Pinus taeda* and *Pinus elliottii* have contributed to increase the pH, electric conductivity, turbidity and nitrate (Guimarães et al. 2008; 2010). Moreover, the cultivation of *Pinus* in micro watersheds with steep relief led to the increase of sediment and loss of soil nutrients.

The aim of this study was to evaluate the potential changes in physical-chemical parameters of water promoted by *Pinus taeda* cultivated in commercial cultures.

2. MATERIAL AND METHODS

Body waters were localized in two sites: *i*) São José dos Ausentes Municipality (28°47'00"S - 49°50'53"W; 1200 m a.s.l.) – distant from a commercial cultures with *P. taeda*, and *ii*) in São Francisco de Paula Municipality (29°23'36.2"S - 50°22'50.7"W; 900 m a.s.l.) – near a commercial culture, both in Rio Grande do Sul, Brazil, during summer and winter of 2009 and 2010. In São José dos Aunsentes the samples were collected in a tank of trout culture with water from the Rio das Antas which is part of the Rio das Antas basin, already in Sção Francisco de Paula the samples were collected in a dam that receveid water of affluent of Rio dos Sinos basin.

Water samples (500 ml) were collected in both sites according to the Guide to Collection and Preservation of Water Samples (CETESB 1988) and analyzed in accordance with Standard Methods for the Examination of Water and Wastewater (APHA 1995). Samples were analyzed immediately or stored under refrigeration at 4 ± 2°C up to 24 hours.

2.1 Parameters analyzed

a. Total levels of phenolic compounds were evaluated using the Folin-Ciocaulteau method (Poiatti et al. 2009). The results are presented as mg/L.

b. Total and fecal coliforms: fecal analysis was done using the Colilert method (Method Substrate Chromogen). This method is based on Defined Substrate Technology; the product contains nutrients that develop indicators staining and / or fluorescence when the culture medium is metabolized by bacteria. The results are expressed as most probable number – MPN/100mL.

c. Hardness: The hardness analysis was performed by titration of EDTA-Na. By the addition of EDTA-Na to the solution color, is the formation of a stable complex and not dissociated from the EDTA-Na and Ca^{++} and Mg^{++}, separating the dye. The results are expressed in mg/L.

d. Nitrite: was determined through the formation of a reddish purple color complex at pH 2 to 2.5, by diazotization of sulphanilic acid with dichloride N-(1-naphthyl) - ethylenediamine. The results are expressed in mg/L.

e. Nitrate: we used the method Fenoldissulfônico acid (Roller & Mc Kaige 1939) where the intensity of yellow color is proportional to the concentration of nitrates. Readings are made in Nessler tubes in a spectrophotometer at 400nm. The results are expressed in mg/L.

f.Total solids: were determined by gravimetria (ABNT / NBR 10664 1989), in a porcelain capsule an oven at (550 ± 50) °C for 1 hour, followed by cooling in a desiccator and weighing. The contents of the capsule was transferred to 200 mL of the sample, measured in a test tube in a water bath and evaporated to dryness. After evaporation of the sample, we dry the dish with residue in an oven at 103-105 ° C for 1 hour, let it cool in a desiccator at room temperature and then weighed to an accuracy of up to 0.1 mg. The results are expressed in mg/L.

g. Sulphate: The method developed by Tabatabai (1974) is based on the measurement of turbidity formed by the reaction of barium chloride with sulphate present in the sample, forming barium sulfate, which remains suspended in the solution muddying. The results are expressed in mg/L.

h. Biological Oxygen Demand (BOD): The determination of the BOD consists of measurements of dissolved oxygen concentration in the samples before and after the incubation period of 5 days at 20 ° C. The results are expressed in mg/L.

i. Chemical Oxygen Demand (COD): The determination of COD used the open reflux titrimetric method that is based on the oxidation of reducing by the addition of dichromate-Cr2O72 in excess. By convention, the amount of chromium-III that forms is equivalent to the amount of dichromate reduced; the amount is equivalent to the chemical oxygen demand. The results are expressed in mg/L.

j. Dissolved oxygen: the measurement was made with aid of a portable termoxymeter (OXI 330/SET-WTW). The results are expressed in mg/L.

k. pH: the measurement was made with a portable pH meter.

l. Water temperature: made with a thermometer of internal scale. The results are expressed in ºC.

2.2. *Litter fall estimation*

The litter fall of *P. taeda* and *A. angustifolia* trees was seasonally estimated using 6 collectors with 1 m^2 of area installed at the perimeter of each plantation. Accumulated litter fall was collected during summer and winter in 2009 and 2010. Samples were sorted and only leaves were collected. Other components (bark, twigs, debris) were discarded. Leaves were oven-dried at 40°C for 72h and dry mass was determined. The results are presented as g/m^2.

2.3 Statistical Analysis

The results are expressed as mean ± standard error. T test of Student for the independent sample was used for statistical analysis to compare the data obtained from different collecting sites and seasons. When no significant differences were observed between samples collected in 2009 and 2010, results were analyzed independently of the year. The significance level adopted was 5%. Tests were performed with the program Statistical Package for the Social Sciences (SPSS 11.5) for Windows.

3. RESULTS

Water samples collected in the site near commercial culture with *P. taeda* (NPt) presented levels of phenolic compounds ranging from 12.82 mg/L in winter to 20.56mg/L in summer (Table 1). On the other hand, samples from sites distant from commercial areas (DPt) presented levels ranging from 0.23 mg/l winter to 0.46 mg/l summer.

The levels of total coliforms measured in the site NPt were 2 times higher in the summer than in the winter. In contrast, the level of coliforms was higher in winter in samples collected on the site DPt (table 2). Both sites near (NPt) and distant (DPt) from commercial culture with *P. taeda* presented similar levels of total coliforms in summer, but these levels increased 3.8 times in DPt samples during the winter.

Samples collected in NPt and DPt sites showed levels of fecal coliforms of 55 and 3 times higher in winter than in the summer, respectively.

Both NPt and DPt samples collected in summer presented higher levels of hardness than those collected in winter (Table 2). The highest level of water hardness was observed in samples collected in the site DPt during the summer.

In both bodies water were not detected levels of nitrite, independently of the season. However, the levels of nitrate were 4 times higher in NPt samples collected in the summer than in winter. Moreover, the levels of nitrate were 3.5 times higher in NPt than in DPt.

The highest levels of total solids were observed in samples collected in the winter for both DPt and NPt sites.

On the other hand, the levels of sulphate were in average 2 times higher in the water collected in summer than in the winter, independently of the site, but the levels of

sulphate were higher in the winter for NPT site In general, the levels of BOD were higher in samples from DPt than NPt, independently of season.

The levels of COD showed low variation between seasons in water samples collected in NPt (Table 2).

The levels of dissolved oxygen were higher in samples collected in the winter than in the summer, independently of the site. Moreover, the levels of dissolved oxygen were higher in DPt than in NPt.

In general, the lowest pH values were observed in water samples collected in NPt, independently of season. Moreover, the pH from NPt samples was drastically reduced during the winter (pH 2.03±0.02).

In both sites analyzed, the average temperature of water was higher in the summer than in the winter and there were no significant differences in temperatures between sites.

The leaching experiments indicated that the maximum level of hydrosoluble compounds in both P. *taeda* and *A. angustifolia* was released in seven days (Figure 2). However, needles from *P. taeda* remain releasing high amount of phenolics for three months. In contrast, these levels in A. *angustifolia* were lower than in *P. taeda* and remain liberating phenolics for four months (figure 1).

4. DISCUSSION

Natural water contamination with fecal material has been already reported in cultures with *Pinus*, with values ranging from 16.5 to 67.5 NMP/100mL (Guimarães et al. 2008). It was attributed to the presence of livestock or to the sewage generate by residents.

The authors observed that in the month in which the use of animals is substantially decreased, the concentration of coliforms was also reduced. The increase

in the levels of fecal coliforms during the winter may be attributed to the increment in tourists, since the winter represents the high season for tourism in this area.

According to the standards of water quality for human consumption (Ordinance No. 518 of 25 March 2004), the desirable maximum is 500 mg $CaCO_3$/L. Custodio & Lamas (1983) report that the waters can be classified in terms of hardness (mg CaCO3/ L), as "bland" (<50), "somewhat hard" (50-100), "hard" (100 - 200) and "very hard" (> 200). The values observed in the present work classified the water as bland, independently of the site, and suggest that the *Pinus* plantation has no effect on this parameter.

The fact of nitrite has not been observed in the samples was expected because this compound occurs naturally as trace concentration (Rudorff 2005). Nitrite is quickly oxidized to nitrate in the environment by nitrifying bacteria. According to Alaburda and Nishihara (1998), concentrations above 3 mg/L NO^{3-} are indicative of contamination due to anthropogenic activities.

Santos (2000) reported that natural waters generally have nitrate levels between 0.1 and 10 mg/L, but in polluted waters, the levels can reach 1,000 mg/L. According to the National Resolution (CONAMA, Nº 357), the nitrate limit established for classes of freshwater is 10 mg/L N-NO^{3-}. The values observed in both collecting sites in the present work respected this limited and suggest that the *Pinus* plantation had no effect on this parameter.

Bubb et al. (2001) studying body waters near and distant from *Pinus* plantations verified concentrations of solids ranged from <10 to 264 mg/L. In the present work the levels ranged from 31 to 132.25mg/L.

The sulfate anion is very common in nature and is present in varied concentrations in natural waters. The USEPA sets a standard for the concentration of

sulfate in drinking water of 250 mg/l as higher concentrations affect the smell and taste of water. The values observed in both collecting sites in the present work respected this limited and suggest that the *Pinus* plantation not interfere in this parameter.

Guimarães et al. (2010) reported that the variations in the levels of BOD can be linked to variations in rainfall in each region, which provides the carrying of sediments and organic matter to water bodies. Chaves and Corrêa (2005) found that the organic matter content in soil is generally found in smaller quantities in areas with *Pinus* species than in natural environments of tropical forests, due to the slow decomposition of the needles. This explains the lower BOD recorded in the body of water nearby pine plantations in this study.

Guimarães et al. (2010) verified the body water close to plantations of *Pinus* monitored showed optimal conditions of oxygenation of the water during the study. In the present work the levels of dissolved oxygen were considered satisfactory only in the body of water distant from the *Pinus* plantations, as in the water body low oxygen levels can affect the quality of life of aquatic species. According the resolution of the CONAMA 357 (2005) the body water should have more than 5mg/L of the dissolved oxygen.

Bubb et al. (2001) verified that the water pH varied between 4.0 and 6.5 and this pattern can be linked with rainfall and runoff, as well as decomposition processes associated with stagnant and organic-rich conditions. In the present work the pH in DPt did not varied, whereas in NPT the pH varied between 2 and 3.5, demonstrating a clear acidification of the water.

In most natural bodies of water, the pH is influenced by the dissolution of carbonic acid or by the discharge of domestic and industrial effluents or by weathering of rocks and erosion of agricultural areas with the use of lime and fertilizer (Conte et al.

2000). Most of the groundwater has a pH between 5.5 and 8.5. In exceptional cases the values may vary between 3 and 11 (Santos 2000). Brazilian law provides pH values between 6.5 and 8.5 for water intended for human consumption, and between 6.0 and 9.0 for all classes of freshwater.

According to Hem (1970) the most natural waters (unpolluted) the pH ranges from 6.0-8.5. In a study of the effect of plantations of *Pinus* and *Eucalyptus* on the water, it was reported that pH was around 5.6 (Lima and Barbin 1975). On the other hand, lower values of soil pH were observed in cultures of pine compared to tropical forests (Souza and Souza 1981; Chaves and Corrêa 2005). Similarly, we verified an acidification in the body water near the plantations of *Pinus taeda*, and suggest that this effect is relative to the substances liberated by the needles in the environmental. This hypothesis is reinforced by the levels of hydrosoluble phenolics found in environmental, because some these compounds have an acid character.

According Bubb et al. (2001) the water temperatures were similar at both stations, ranging from a minimum 10°C in winter to a maximum 28°C in summer. The authors reported that values found corroborated with the expected, which was no effect of harvesting, because the retained native vegetation adjacent to the stream provided shading. Likewise, in our work the temperature found corroborated with the values expected for the region (Dutra et al. 2007).

There is a lack of information considering not only the levels of phenolic compounds in *P. taeda* and *A. angustifolia*, but also their relation with leaching (Figure 1). Leaching experiments are even after death, the allelopathic substances are still in their tissues, where they are released by evaporation, if volatile products, or by leaching through dew and rain, if they are soluble in water, being dragged the ground, where,

upon reaching the necessary concentration, may influence the development of microorganisms and plants found therein (Neves 2005).

Out of all parameters analyzed in the present work, BOD, oxygen dissolved and pH seems to be changed by the presence of the *Pinus taeda* plantation near of the body water. Likely, these alterations are related to the presence of the needles as well as the high concentration of the phenolic compounds in the body water and thus, these changes in body water may have consequences in aquatic ecosystems because the drastic alterations can lead an extinction of the native aquatic species of the fauna and flora.

5. ACKNOWLEDGEMENTS

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Table 1: Levels of hydrosoluble phenolic compounds present in water samples collected near (NPt) and distant (DPt) from commercial culture with *P. taeda*. The levels are present as mean ± standard error. Results are expressed in mg/L.

	São José dos Ausentes	São Francisco de Paula
Summer	0.46 ± 0.03	20.56 ± 1.23
Winter	0.23 ± 0.01	12.82 ± 0.34

Figure 1: Litter production of *P. taeda* and *A. angustifolia* evaluated in summer and winter. The levels are present as mean ± standard error. Results are expressed in g/m^2.

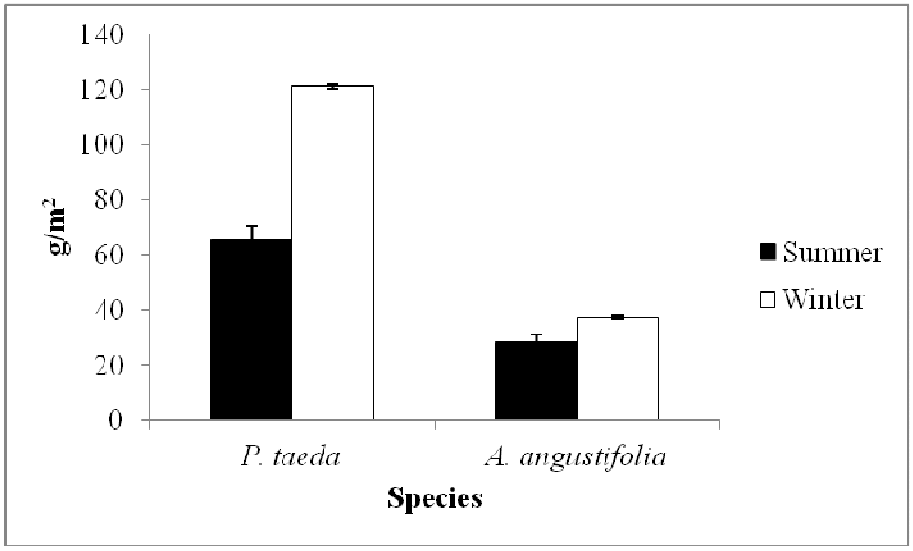


Table 2: Parameters of water quality evaluated in samples collected near (NPt) and distant (DPt) sites from commercial culture with *P. taeda,* during summer and winter. The levels are present as mean ± standard error. BOD, Biological Oxygen Demand; COD, Chemical Oxygen Demand.

	DPt		NPt	
	Seasons		Seasons	
	Summer	**Winter**	**Summer**	**Winter**
Total Coliforms (MPN/100mL)	1435.67 ± 573.07	2980.72 ± 1922.71	1534.50 ± 723.01	786.97 ± 423.39
Fecal Coliforms (MPN/100mL)	34.43 ± 16.67	96.27 ± 20.39	3.50 ± 0.40	192.50 ± 136.71
Hardness (mg $CaCO_3$/L)	2.00 ± 1.53	0.00 ± 0.00	0.35 ± 0.28	0.00 ± 0.00
Nitrite (mg/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Nitrate (mg/L)	0.08 ± 0.04	0.020 ± 0.001	0.08 ± 0.03	0.07 ± 0.01
Total Solids (mg/L)	31.67 ± 8.96	132.25 ± 8.81	32.18 ± 0.81	56.50 ± 29.86
Sulphate (mg/L)	1.75 ± 1.28	0.00 ± 0.00	2.24 ±0.01	1.05 ±0.04
BOD (mg/L)	0.83 ± 0.16	0.83 ± 0.14	0.50 ± 0.04	0.37 ± 0.04
COD (mg/L)	26.67 ± 16.68	13.16 ± 7.28	31.67 ± 20.85	44.03 ± 13.51
Dissolved oxygen (mg/L)	6.24 ± 0.37	7.24 ± 0.07	4.51 ± 0.64	5.65 ± 0.23
pH	6.87 ± 0.04	6.38 ± 0.26	3.26 ± 0.24	2.03 ± 0.02
Temperature (ºC)	19.03 ± 3.08	11.03 ± 3.08	21.09 ± 2.59	13.09± 1.45

Conclusões Gerais

Conclusão final

O conjunto de dados aqui apresentados demonstram que entre os compostos hidrossolúveis extraídos de *Pinus taeda,* uma espécie exótica, encontra-se uma alta concentração de fenólicos totais aliada a uma capacidade antioxidante diminuída em relação ao extrato obtido da espécie nativa *Araucaria angustifolia.* Tais características aliadas a um possível sinergismo dos componentes hidrossolúveis determinam uma resposta diferenciada tanto em *Hyalella castroi* como nas sementes de *Lactuca sativa* submetidos a estes extratos. Contudo, ambos os organismos indicaram a existência de efeitos deletérios relacionados aos compostos solúveis produzidos por *P. taeda* e a inexistência destes efeitos nos tratamentos utilizando *A. angustifolia*.

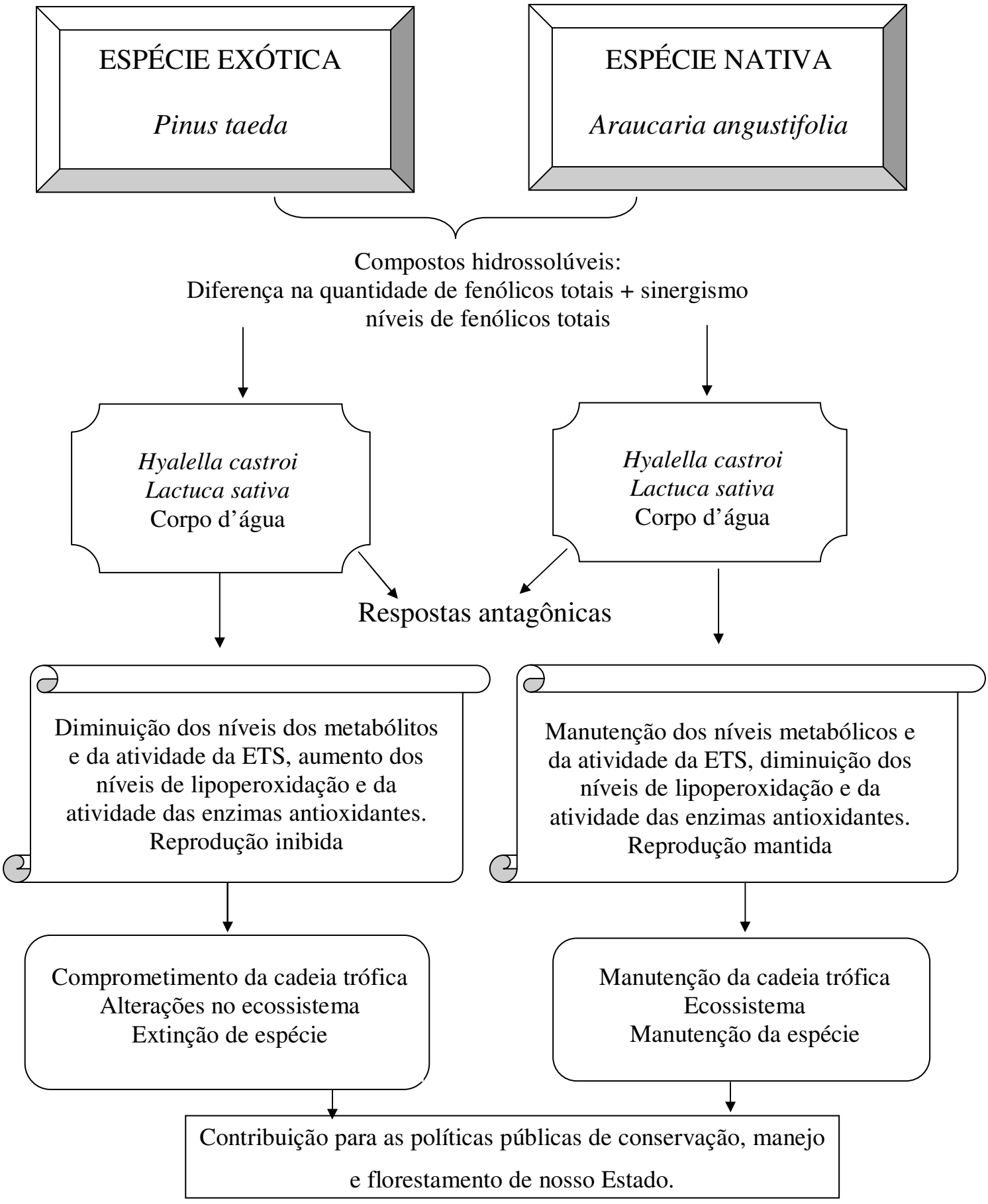
Em *H. castroi,* exposta a compostos hidrossolúveis de *P. taeda,* verificou-se alterações no padrão metabólico do animal, com uma diminuição das reservas energéticas analisadas e dos níveis de colesterol, estando estas aliadas a uma diminuição da atividade da Na^+/K^+ATPase e ETS. Quando analisamos o balanço oxidativo dos animais observamos um aumento nos níveis de estresse oxidativo, traduzidos por um incremento da lipoperoxidação, apesar do aumento das enzimas antioxidantes. Este conjunto de respostas conduz, provavelmente, a um comprometimento reprodutivo da espécie o que pode determinar no futuro uma alteração da estrutura trófica destes ambientes límnicos visto que os anfípodos são um importante elo na cadeia alimentar destes ecossistemas e em ambiente natural estes compostos hidrossolúveis são liberados de forma conjunta.

Cabe salientar que embora na água em ambiente natural tenha sido verificada a acidificação da água em condições controladas esta alteração não foi verificada. Outra consideração importante é uma vez que os compostos hidrossolúveis são impalatáveis estes podem estar rementendo os animais a um estado de jejum.

A luz da literatura especializada e do conjunto de resultados aqui observados os fenólicos hidrossolúveis constituem-se nos principais candidatos determinantes de tais modificações. Contudo, não podemos descartar a possibilidade de outras substâncias, como terpenos, e até mesmo o sinergismo entre estas de estarem determinando os resultados aqui encontrados.

A análise físico-química do corpo d'água próximo à plantação de *P. taeda* também mostrou alteração em alguns parâmetros físico-químicos (DBO, oxigênio dissolvido e pH) que associada aos altos níveis de fenólicos podem ter conseqüências importantes para o ecossistema aquático, como perda de diversidade e impossibilidade de uso deste curso.

Acreditamos que este conjunto de resultados possa contribuir de forma relevante nas políticas públicas de conservação e manejo de nosso Estado.


ESPÉCIE EXÓTICA
Pinus taeda
ESPÉCIE NATIVA
Araucaria angustifolia
Compostos hidrossolúveis:
Diferença na quantidade de fenólicos totais + sinergismo
níveis de fenólicos totais
Hyalella castroi
Lactuca sativa
Corpo d'água
Hyalella castroi
Lactuca sativa
Corpo d'água
Respostas antagônicas
Diminuição dos níveis dos metabólitos e da atividade da ETS, aumento dos níveis de lipoperoxidação e da atividade das enzimas antioxidantes.
Reprodução inibida
Manutenção dos níveis metabólicos e da atividade da ETS, diminuição dos níveis de lipoperoxidação e da atividade das enzimas antioxidantes.
Reprodução mantida
Comprometimento da cadeia trófica
Alterações no ecossistema
Extinção de espécie
Manutenção da cadeia trófica
Ecossistema
Manutenção da espécie
Contribuição para as políticas públicas de conservação, manejo e florestamento de nosso Estado.

Apêndices

Apêndice I

***Oecologia* Author Instructions – General**

Aims and scope

Oecologia publishes innovative ecological research of general interest to a broad international audience. We publish several types of manuscripts in many areas of ecology:

Categories:	Manuscript Types:
Physiological ecology	Concepts, Reviews, and Syntheses
Behavioral ecology	Views and Comments
Population ecology	Special Topics
Plant-animal interactions	Original Research Papers
Community ecology	Methods
Ecosystem ecology	
Global change ecology	
Conservation ecology	

In general, studies that are purely descriptive, mathematical, documentary, and/or natural history will not be considered.

In the *Concepts, Reviews and Syntheses* section, we seek papers on emerging issues in ecology, especially those that cross multiple boundaries in ecology, provide synthesis of important bodies of work or delve into new combinations of theory and observations with the potential to create new paradigms or challenge existing paradigms. These papers are usually invited, but we welcome unsolicited contributions. In the *Views and Comments* section we seek short papers with the intent to provide contrary and/or broader perspectives on papers recently published in Oecologia. Alternatively, pairs of short papers which present opposing views on a topic of high interest in the ecological research community will be published in this section, with the intent to stimulate open debate. In both cases, the papers must be relatively short (up to 5 printed pages in the case of opposing view pairs of papers, or up to 3 printed pages in the case of comments on previously-published work), and to contain not only an opinion or criticism on methods or statistics, but also relevant data or original analyses that support the opposing view or comment. Manuscripts or letters intended for the Views and Comments section will be reviewed by one of the Editors-in-Chief and a Handling Editor in the field appropriate to the submission. *Special Topics* are a collection of integrated papers on a critical topic of broad interest. Proposals for Special Topics should be submitted to one of the Editors-in-Chief. *Methods* are papers that outline new approaches that address standing questions in the discipline. *Original Research Papers* provide the core of our journal and represent original investigations that offer new insights into ecological systems.

Legal and ethical requirements

Submission of a manuscript implies that the work described has not been published before (except in the form of an abstract or as part of a published lecture or thesis); that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities - tacitly or explicitly - at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation. In addition,

- Plagiarism will not be tolerated. All text should represent contributions of the authors unless material is quoted and attributed to its original source. Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) and to include evidence that such permission has been granted when submitting their papers.
- All animal experiments must have been conducted in conformity with the "Guiding principles in the care and use of animals" approved by the Council of the American Physiological Society. Evidence of the adherence to these principles should be apparent
- All human studies must have been performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and reviewed by the appropriate ethics committee. The text should clearly state that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects should be omitted.
- Authors must include a declaration that experiments comply with the current laws of the country in which the experiments were performed
- Authors must indicate whether or not they have a financial relationship with the organization that sponsored the research. If no conflict exists, authors should state: The authors declare that they have no conflict of interest.

The Editors-in-Chief reserve the right to reject manuscripts that do not comply with the above-mentioned requirements. The author(s) will be held responsible for false statements or for failure to fulfill the above-mentioned requirements.

Manuscript submission

Authors must submit their articles to "Oecologia" online. Electronic submission substantially reduces the editorial processing, review and publication time. After passing a pre-review assessment for journal eligibility by an Editor-in-Chief and a Handling Editor, submitted manuscripts are subject to peer review and copy editing. Please log directly onto the link below and upload your manuscript following the on-screen instructions. For the review process, the manuscript may be submitted as one single file (PDF, Microsoft Word or Rich Text Format with embedded illustrations, tables, etc.). If the manuscript is accepted, original files (not pdf or html) of the final version of the manuscript must be uploaded for production. Online appendices (Electronic Supplementary Material, ESM) must be submitted in a separate file. There is a total

file size limit of 60 MB for a manuscript submission, including ESM. If ESM (e.g., video) exceeds this size, please contact the appropriate Editor-in-Chief directly.
Submit Online

Manuscript preparation

- The length of articles should not exceed 10 printed pages (equivalent to approximately 35 submitted pages) including all references, tables, figures, and figure legends. Views and Comments submissions must be limited to 3 to 5 printed pages. One printed page corresponds to approximately 3 submitted pages, 850 words text, or 3 illustrations with their legends, or 55 references. There will be a charge of 100 €, plus 19% VAT, for each page exceeding this limit. Editors typically return manuscripts prior to review that are likely to exceed the page limit.
- Manuscripts must be written in English and double-spaced throughout (including references) with at least 2.5 cm (1 inch) margins. Please write in the active voice using the past tense only for methods and results sections.
- Page numbers are required for the manuscript but should not be included on tables and figures. Pages in Electronic Supplementary Material (ESM) should be numbered separately.
- Line numbers for text should run consecutively throughout the text, from the title page through the figure legends. Do not number lines in tables or figures.
- Use a normal, plain font (e.g. Times New Roman) for text. Genus and species names should be in italics. The common names of organisms should not be capitalized
- Abbreviations should only be used for terms repeated at least 3 times. Abbreviations should be defined at first mention in the abstract and again in the main body of the text and used consistently thereafter.
- Format dates as day-month-year with months abbreviated: e.g., 01-Jan-2008
- Use the equation editor of a word processing program or MathType for equations. (Note: If you use Word 2007, do not create equations with the default equation editor but use the Microsoft equation editor or MathType instead.) Symbols for parameters should be *italicized*.
- Report values in equations, tables, figures and statistics with the number of digits that matches the precision of the data.
- Please always use Unicode (http://www.unicode.org) font for non-roman characters. Use internationally accepted signs and symbols following the Standard International System of Units (SI, http://physics.nist.gov/cuu/Units/units.html) throughout the manuscript (in the text, tables and figures). Avoid complex units wherever possible (e.g use "no. m^{-2}" instead of "no. per 16 m^{2}"). Units should use exponent notation and avoid multiplication and division symbols (e.g., "*", "/", "x"): i.e., "no. m^{-2}" and not "no./m^{2}").
- Footnotes should not be used, except on the title page or in Tables.
- For indents, use tab stops or other commands, not the space bar.

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ECOLOGICAL INDICATORS

Integrating Sciences for Monitoring, Assessment and Management

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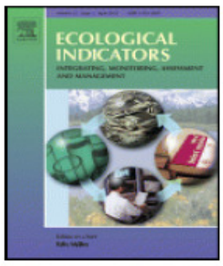

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Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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ENVIRONMENTAL POLLUTION

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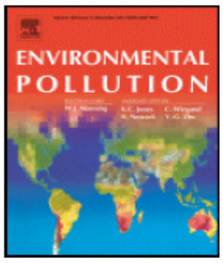

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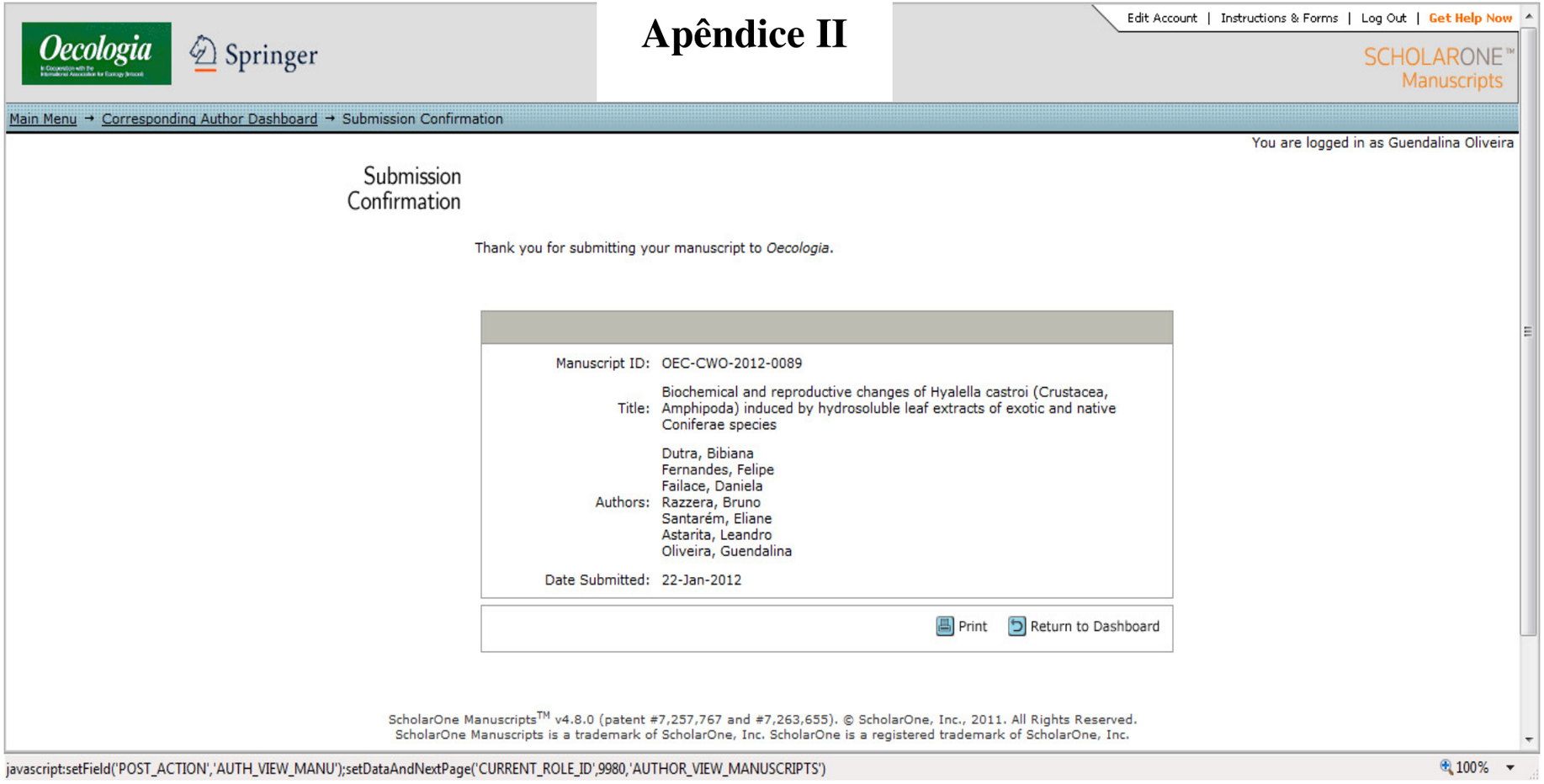
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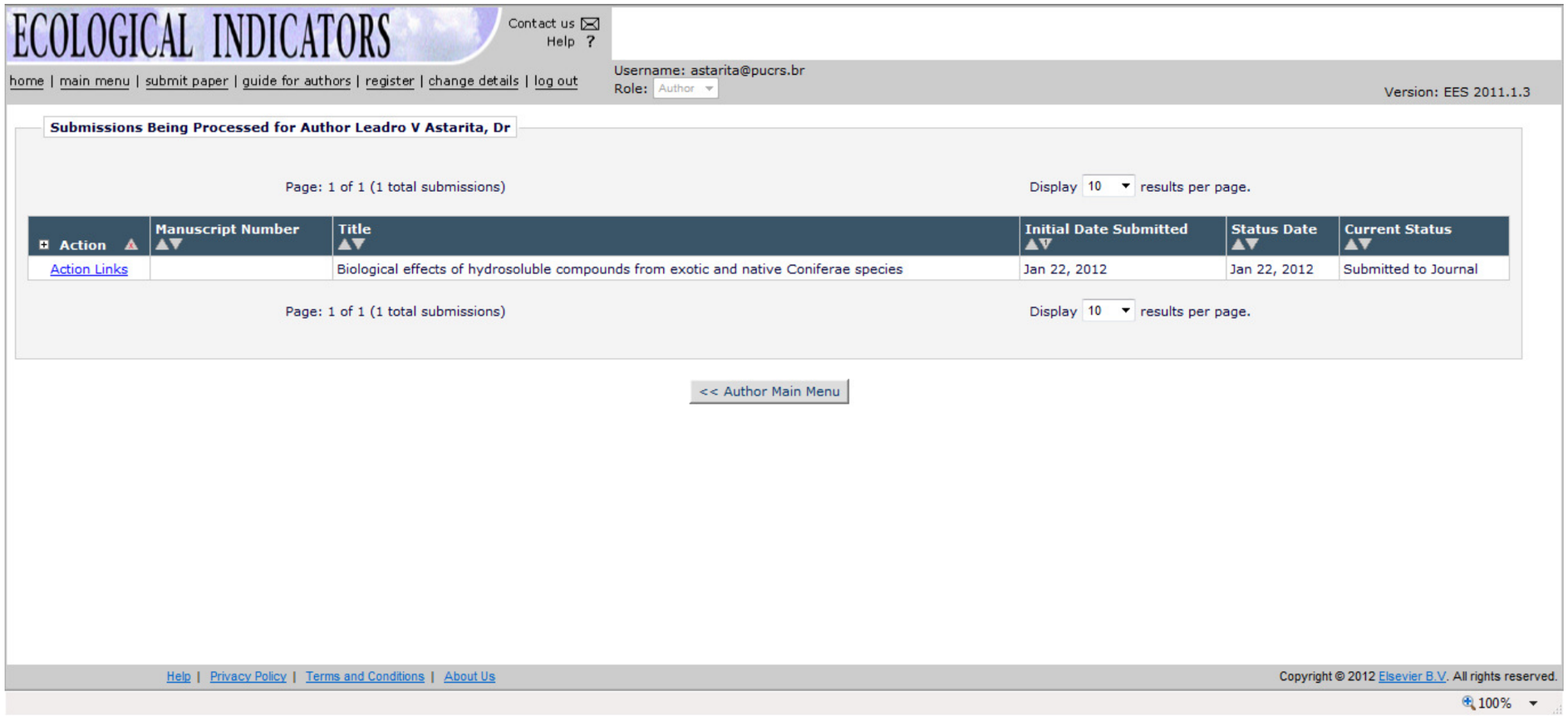
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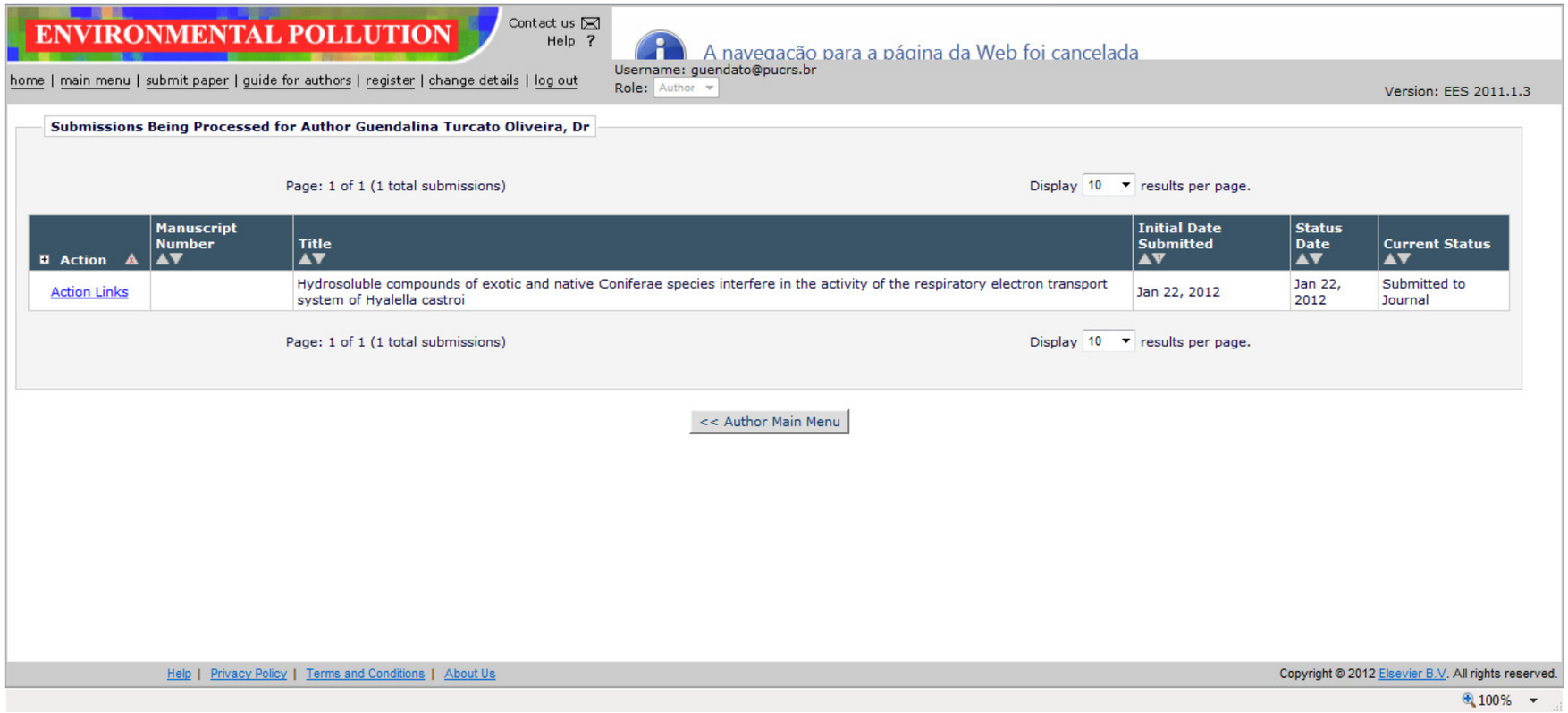
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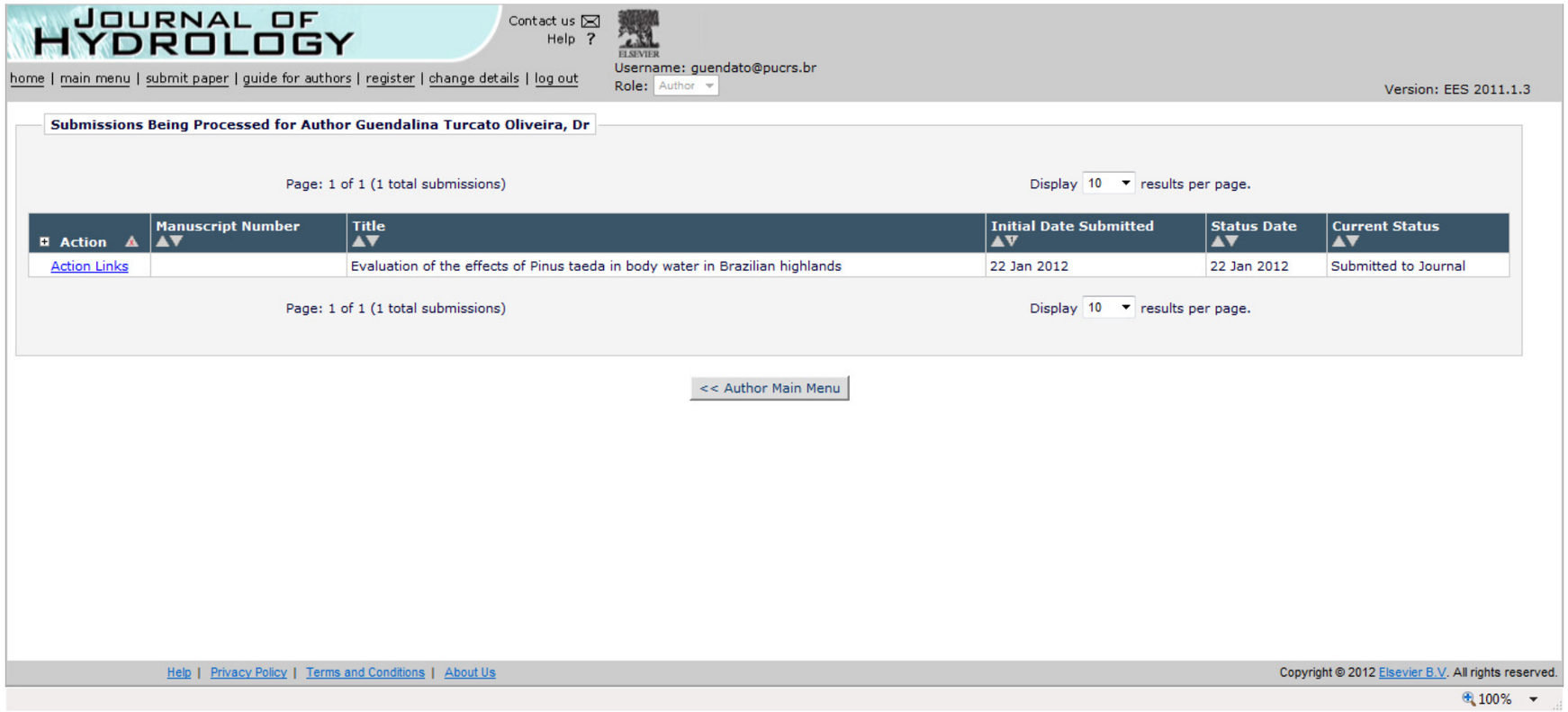
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