1 **Effects of leaf litter extracts from four tree species on aquatic invertebrates: an**
2 **ecotoxicological risk assessment approach**

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Abstract

Upper reaches of temperate streams and oligotrophic lakes depend trophically on the organic matter coming from the surrounding watershed. These aquatic ecosystems accumulate particulate and dissolved organic matter, and chemical compounds liberated from decomposing leaves or from the leachates of leaf litter. These materials supply a wide range of chemical compounds, which may serve as food, but also cause ecotoxicological effects on aquatic organisms. However, this ecotoxicological risk has been amply ignored, especially for freshwater invertebrates. The aims of our study are: 1) to assess the direct toxicity of aqueous extracts of leaf litter from two native (*Populus alba*, *Fraxinus angustifolia*) and two tree species exotic to Europe (*Robinia pseudoacacia*, *Ailanthus altissima*) on a pelagic and a benthic species of model aquatic invertebrates: *Daphnia magna* (Cladocera) and *Potamopyrgus antipodarum* (Mollusca) and 2) to estimate the ecotoxicological risk of the leaf litter extracts to lacustrine ecosystems using different simulated scenarios of shallow lakes. For both invertebrate species, we assessed the effective concentration of leaf extract causing an effect on 50% of the population (EC50) by means of laboratory bioassays. Our results show that the extracts of leaf litter from all the tested trees have a potential ecotoxicological risk in some scenarios, which is independent from the geographic origin of the tree species.

Keywords: behaviour; exotic trees; leaf litter; lakes; risk assessment; aquatic toxicity

Introduction

Riparian forests are an important source of organic matter for aquatic ecosystems (Cummins et al. 1989; France 1995; Allan and Castillo 2007). In upper reaches of temperate streams and oligotrophic lakes this allochthonous material constitutes the main support of trophic webs, as primary autochthonous productivity is very scarce (Cummins et al. 1989; Allan and Castillo 2007; Glaz et al. 2012).

Anthropogenic activities in floodplains have deeply altered species composition of the riparian forests (Pozo et al. 1998; Allan and Castillo 2007). This alteration can modify the quality and quantity of the leaf litter entering the aquatic ecosystems, subsequently causing an impact in the structure and functioning of ecosystems. The quality of the leaf litter entering the aquatic ecosystems is a key factor controlling its processing by microbes and invertebrates (Pozo et al. 1998; Richardson et al. 2004; Alonso et al. 2010). For instance, leaf litter from N-fixing species can alter the nitrogen cycle (MacKenzie et al. 2013) or leaf litter with high content of secondary compounds can reduce the microbial activity in soils and sediments (Scholes and Nowicki 1998; Castro-Díez et al. 2012). By contrast, leaves with a high amount of calcium may be beneficial for crustacean communities (Ohta et al. 2014). Previous studies on the effects of leaf litter in aquatic ecosystems have mainly focused on the structure and function of the invertebrate and the microbial community (Cummins et al. 1989; Abelho and Graça 1996; Alonso et al. 2010; Bottollier-Curtet et al. 2011; Medina-Villar et al. 2015a; Santschi et al. 2018). Less attention has been paid to the potential toxicological effects of leaf litter to particular species of aquatic organisms (Kamara and Pflugmacher 2007; Krevš et al. 2013). For instance, Timofeyev et al. (2006) showed that an increase of natural organic matter may

increase mortality in several species of amphipods (*Gammarus tigrinus*,
Chaetogammarus ischnus and *Eulimnogammarus cyaneus*), changing their peroxidase
activity and increasing the expression of heat shock protein under laboratory conditions.
On the contrary, Hofmann et al. (2012) found beneficial effects of leaf litter extracts on
cladocerans. These contradictory studies indicate that further research is necessary to
assess the effects of leaf litter to aquatic ecosystems.

An additional impact of terrestrial vegetation on aquatic ecosystems may occur when the
native trees are replaced by exotic ones (Naiman and Decamps 1997; Pozo et al. 1998;
Richardson et al. 2007; Castro-Díez et al. 2014). These exotic species may differ from
natives not only in the quantity of leaf litter that enter in the aquatic system, but also in
its chemical composition. Riparian floodplains and lowland areas are among the most
susceptible ecosystems to plant invasions in Europe (Chytrý et al. 2009). Examples of
common invasive exotic trees in European riparian and lacustrine forests are tree of
heaven (*Ailanthus altissima* (Mill.) Swingle (Simabouracea)) and black locust (*Robinia
pseudoacacia* L., (Fabaceae)) (Sanz Elorza et al. 2004; Swan et al. 2008; DAISIE 2015).
Both species have been found to exert allelopathic and antibiotic effects on terrestrial
organisms (Heisey 1996; Nasir et al. 2005; Small et al. 2010; Huo et al. 2012; Albouchi
et al. 2013; Catalán et al. 2013; Marinas et al. 2014; Cioch et al. 2017; Poljuha et al 2017).
However, their potential to have toxicological effects to aquatic animals have been largely
ignored. Studies on other tree species, either native or exotic to the study site, suggest a
potential toxicity of leaf litter of some species on aquatic animals. For instance, Beleza et
al. (2019) showed that extract from exotic *Eucalyptus* sp. reduced growth and
development rates –among others– in the freshwater diptera *Chironomus riparius* in
comparison with native plant extracts. Earl et al. (2012) showed that extracts from the

native *Quercus rubra* reduced tadpole survival. Given that *A. altissima* and *R. pseudoacacia* can locally replace native species in Europe, comparing the effects of their leaf litter extracts with those of native trees on aquatic invertebrates is a relevant component of their impact assessment.

Lacustrine ecosystems, especially small ones, are highly influenced by the organic matter coming from the surrounding watershed than fluvial ecosystems, because of their low rate of water turnover (France 1995; Glaz et al. 2012; Martin and Blossey 2013). Small lakes accumulate natural organic matter, dissolved organic matter and chemical compounds liberated from decomposing leaves or from leaf litter leachates from the shoreline (France 1995; Pflugmacher et al. 2001; Mulholland 2003; Earl et al. 2012). These materials are a source of a wide range of chemical compounds, including nutrients, tannins, monoterpenes, aromatic hydrocarbons or N-alkanes. Many of them can exert physiological stress to aquatic organisms at relatively high concentrations (Bakker and Alvarado 2006; Kamara and Pflugmacher 2007; Earl et al. 2012; Martin and Blossey 2013; Krevš et al. 2013; Beleza et al. 2019). Leaf litter increases the amounts of dissolved compounds in the water column, which may cause direct toxicity to pelagic and benthic invertebrates. In temperate areas, the ecotoxicological effects could increase during autumn, when deciduous forests shed high quantities of leaf litter (Cummins et al. 1989; Allan and Castillo 2007; Castro-Díez et al. 2014). However, the likely toxicological effects of those compounds on aquatic organisms have been scarcely studied.

The aims of our study are: 1) to assess the direct toxicity of aqueous extracts of leaf litter from trees native and exotic to Europe on two ecotoxicological model organisms: the freshwater cladoceran *Daphnia magna* Straus, 1820 and the aquatic snail *Potamopyrgus*

antipodarum Gray, 1843, and 2) to estimate and compare the ecotoxicological risk of the native and exotic leaf litter in simulated lacustrine scenarios. We expect the extracts of the two exotic trees to be more toxic to the aquatic invertebrates, given the allelopathic and antibiotic effects already reported on other groups of terrestrial organisms (Heisey 1996; Nasir et al. 2005; Small et al. 2010; Huo et al. 2012; Albouchi et al. 2013; Catalán et al. 2013; Marinas et al. 2014; Cioch et al. 2017; Poljuha et al 2017). The results would contribute to understand the ecological risk associated to the replacement of native by exotic trees in floodplains of Europe.

Material and methods

Species selection and leaf collection

Two native and two exotic riparian tree species were selected for the study. The two exotics (*Ailanthus altissima* and *Robinia. pseudoacacia*) are among the 100 most aggressive invasive exotic species in Europe and Spain (GEIB 2006; DAISIE, 2015) and they frequently invade riparian forests in Central Spain (Castro-Díez et al. 2014; Castro-Díez and Alonso 2017). As natives, *Populus alba* L., (Salicaceae) and *Fraxinus angustifolia* Vahl, (Oleaceae) were selected, because they naturally occur in the riparian habitats invaded by *A. altissima* and *R. pseudoacacia* in Central Spain (Castro-Díez et al. 2014; Medina-Villar et al. 2015a, 2015b), where the leaf litter sampling sites were located.

Leaf litter was collected from riparian forest of the Henares River (Central Spain). Senescent leaves (i.e. those with detach **that** a gentle touch) collected from trees were

gathered in autumn from at least five different individuals per species from four separated localities distributed along a stretch of 30 km (localities of Guadalajara, Cabanillas del Campo, Meco and Alcalá de Henares). In this way we diluted potential intraspecific differences of leaf properties associated to specific environmental properties. For each species, leaves were pooled and dried at laboratory temperature (20-22°C).

Extract preparation, toxicity bioassays and toxicological parameters

Four short-term (96 hours) toxicity bioassays (one for each tree species) were conducted for each test species: the freshwater invertebrate *Daphnia magna* (Straus, 1820) and the aquatic snail *Potamopyrgus antipodarum* (Gray, 1843). *D. magna*, individuals were obtained from a commercial kit (DAPHTOX Kit F magna, MicroBio Tests Inc., Nazareth, Belgium), and *P. antipodarum* from our own laboratory culture, initiated with individuals collected at the upper watershed of the Henares River (Guadalajara, Spain) in January 2009. For all bioassays, a stock leaf extract from leaf litter of each tree species was prepared using 10 g of lab-dried entire leaves, to mimic field conditions, in 1 l of standardized water (see below for details of standardized water). These stock solutions were prepared in darkness 24 h before the bioassays and with a light stirring in a magnetic stirrer (Selecta® Agimatic-N, [Abrera, Spain](#)). After 24 h, the stock solutions were filtered using a 1 mm pore and serial dilutions were prepared (100%, 50%, 25%, 12.5%, 3.1% and 0.75% for *D. magna* or 100%, 50%, 12.5%, 3.1% and 0.75% for *P. antipodarum*), which are equivalent to a gradient of litter fall spanning from 10 to 0.075 g l⁻¹ of air-dried leaf. Those values were selected to ensure toxic effects in the higher concentrations for both test species. The *D. magna* bioassays were conducted in a climatic chamber at 21°C (ToxKits™, [EBPI, Burlington, Canada](#)) with standardized freshwater (64.75 mg

NaHCO₃; 294 mg CaCl₂·H₂O; 246.5 mg MgSO₄·7H₂O; 5.75 mg KCl in 1 liter of deionized water) (DAPHTOX Kit F magna). *P. antipodarum* bioassays were conducted in a climatic chamber at 15°C with standardized freshwater enriched with calcium carbonate (96 mg NaHCO₃, 60 mg CaSO₄·2H₂O, 4 mg KCl, 122 mg MgSO₄·7H₂O, 10 mg CaCO₃ per liter of deionized water) (U.S. Environmental Protection Agency 2002).

For each tree species a short-term (96 h of exposure) toxicity bioassay was conducted with neonates of *D. magna* (<24 h) using the standardized crustacean toxicity screening test of freshwater DAPHTOX Kit F magna. A control (with standardized water) and serial dilutions of each leaf litter extract were used in triplicate, using 8 neonates in each replicate (10 ml). Every 24 h the number of immobile and death animals was counted, and death animals were removed by means of a small plastic pipette. In *P. antipodarum* bioassays, a control and serial dilutions were used for each leaf species with 14 replicates per dilution including control, using one animal in each replicate. The monitoring variable was the behavioral activity (Alonso and Camargo 2004). This variable was the time (in seconds) spent by each snail to start the normal movement after manipulation. Each animal was taken up with forceps and placed in the center of the experimental glass vessel (25ml); thereafter the animal retracted its soft body inside the shell and the time spent to sliding movement was recorded. This endpoint was monitored at 96 h of exposure. An animal was considered as immobile if after 360 s it did not move and as dead if after a slightly touch of the operculum with forceps no reaction was observed.

The cumulative number of immobile plus death animals was used to estimate the Effective Concentration 50 (EC50, i.e. the concentration that caused the monitored effect in the 50% of the exposed population) for *D. magna* at 96 h for each leaf species extract

and their respective 95% confidence intervals. In the case of *P. antipodarum*, the EC50 was estimated using the behavioral activity (seconds) at 96 h of exposure. EC50 values were calculated by means of logistic models performed with the software package “drc” for analysis of multiple dose response curves in R (R Development Core Team 2004; Knezevic et al. 2007; Ritz et al. 2015). Log logistic models were selected to calculate EC values, with four parameters (upper and lower limits, slope and EC50 value). Concentrations of extracts were log transformed. The upper and lower limits of *D. magna* models were fitted with a range of mortality between 0 and 100%, and for *P. antipodarum* behavioral activity was fitted between 3.72 and 360 seconds (i.e. the lowest and upper behavioral activity recorded). Statistical differences ($P < 0.05$) in 96h EC50 across tree species were tested by means of ratio test (Wheeler et al. 2006; Knezevic et al. 2007).

Chemical characterization of leaf extracts

During the bioassays several chemical water properties were monitored: dissolved water oxygen, electrical conductivity, pH, water temperature, nitrates and total ammonia. Oxygen and temperature were measured by means of a Crison OXI45+ oximeter, conductivity with a Crison CM35+ conductivimeter and pH with a pHmeter Crison microPH 2001. Nitrate and total ammonia were measured using the spectrophotometric standardized method Spectroquant® Merck Kit for dilution samples. Samples with no color at naked eye were selected for analysis (i.e. samples with dilution of $\leq 12.5\%$). Additionally, all stock solutions were kept frozen (-20°C) until performing chemical analyses to determine the concentrations of Al, B, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn, and total phenolic content. Metal contents were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) with a Varian 720-ES. The

concentration of total phenolic compounds was determined by the colorimetric method using the Folin-Ciocalteu reagent (Singleton et al. 1999). Absorbance was measured after 120 min at 760 nm in a UV-visible Cary 50 Probe Spectrophotometer, using gallic acid (Acros Organics, Thermo Fisher Scientific, Madrid, Spain) as a reference standard for quantification.

Leaf litter production of the four studied species, concentrations in aquatic scenarios and ecotoxicological risk assessment

To estimate the leaf litter production of the four tree species a bibliographic search was performed using Web of ScienceTM, without any restriction of publication year. The search formulae was (*Ailanthus altissima* OR *Robinia pseudoacacia* OR *Populus alba* OR *Fraxinus angustifolia*) AND (Leaf litter OR Litterfall OR Leaf production OR Riparian OR River OR Stream OR Lake OR Aquatic ecosystem OR Leaching OR Lateral transport OR Organic matter). In the retrieved papers we searched the maximum monthly production for each tree species to estimate the worst-case scenario of leaf production in a riparian or shoreline forest. Then, litter inputs to simulated lacustrine scenarios were estimated in two ways: 1) intermittent scenarios, assuming that all leaf litter production in a day (i.e. monthly production divided by 30) reached the lacustrine ecosystems and 2) continuous scenarios, assuming that all production in one month reached the lacustrine ecosystems (see below). The concentration of the litter extracts in the simulated lacustrine scenarios was expressed as g l⁻¹ of air-dried leaf.

The risk of leaf extracts to aquatic ecosystems was assessed using a risk quotient approach (TGD 2003). This approach uses the quotient between the Predicted Environmental

Concentration (PEC) and Predicted no-effect concentration (PNEC). When this ratio is higher than 1 it means there is a likely ecotoxicological risk, when less than 1 there is an unlikely risk (TGD 2003). The lowest value of EC50 among the two test animals was selected for each tested tree species to simulate the worst-case scenario (i.e. the most sensitive species for each tree extract). Two PNECs for each tree species were calculated as the EC50 divided by a safety factor. The first PNEC for an intermittent exposure ($PNEC_{int}$) was calculated as EC50 divided by a safety factor of 100, which is recommended for substances with intermittent release (TGD 2003). However, given that the leaf litter input could occur continuously during the autumn, a second PNEC for continuous exposure ($PNEC_{con}$) was also calculated with a safety factor of 1000, which is recommended for continuous exposures (TGD 2003). PECs were estimated for different shallow lakes scenarios, which are the aquatic ecosystems with the lowest depth of water column, normally less than 3 m (Scheffer 2004). These ecosystems present a slower water turnover than lotic systems, which make them propitious to be affected by the toxicity of leaf litter extracts. We simulated a range of shallow lake scenarios by combining three average depths (0.5, 1.0 and 1.5 m), three lake surfaces (1.5, 3.0 and 6.0 ha), three widths of shoreline forest (1, 5 and 10 m, leading to forested surfaces from 434.2 to 8683.2 m²), four monocultures of each of the tree target species and two ways of litter input (continuous and intermittent) (Table S1). For simplicity, all simulated lakes were circular, but they may also represent any other keeping the same volume/edge ratio. The selected average depth and surface encompass real shallow lakes (such as Alderfen Broad Lake in England (average depth 0.8 m and a surface of 5 ha) and Lake Zwemlust in the Netherlands (average depth 1.5 m and a surface of 1.5 ha) (Scheffer 2004). For all scenarios the amount of water in the lakes were calculated, ranging from 7,500 to 90,000 m³. The ratio between water volume in the lakes and the forested surface in the shoreline

was also calculated for each scenario. To estimate the amount of leaf litter that can potentially reach the scenarios, the monthly leaf production reported in bibliography for each tree species and that parameter divided by 30 were used to estimate the amount of leaf litter in one month and one day, respectively. On the bases of these values, as well as the water volume, we estimated the concentration of extract in each simulated lacustrine scenario (PEC_{int} and PEC_{con}) (as $g\ l^{-1}$ of air-dried leaf). The risk assessment was conducted for each scenario (3 lake depths x 3 lake surfaces x 3 shoreline width x 4 species composition x 2 ways of litter input) as the ratio between PEC and the calculated PNEC (both $PNEC_{int}$ for one day and $PNEC_{con}$ for one month). If this risk quotient was higher than 1 it was considered a scenario with an ecotoxicological risk for the aquatic ecosystem.

Results

The mean water temperature was $21.2^{\circ}C \pm 0.39$ ($n=39$) for *D. magna* bioassays and $16.4^{\circ}C \pm 0.87$ ($n=347$) for *P. antipodarum* bioassays. In all bioassays, pH and dissolved oxygen declined and conductivity increased at increasing extract concentrations (Table 1). The leaf extract of *F. angustifolia* showed the highest concentration of nitrate whereas that of *A. altissima* showed the highest concentration of ammonium and phenolic compounds (Table 1 and 2). In the case of metals, the extract from *P. alba* presented the highest Zn concentration, *F. angustifolia* had the highest concentration of Fe and Mn, and *R. pseudoacacia* the highest concentration of B (Table 2).

The response of each invertebrate species to the gradient of extracts of each tree species, along with the log logistic models fitted to each concentration are presented in Figure 1.

The effective concentrations 50 (EC50) at 96 h are presented in Table 3. The most toxic extract for *D. magna* was that of *P. alba* with an EC50 of 1.77 g l⁻¹ of air-dried leaf, which was significantly more toxic than the others (P<0.05; Ratio test) (Table 3). The extract from *R. pseudoacacia* was the second most toxic while *F. angustifolia* and *A. altissima* presented a similar toxicity to *D. magna* (Table 3) (P>0.05; Ratio test). In the case of *P. antipodarum*, the most toxic extracts were *P. alba* and *F. angustifolia*, and the less toxic was the extract from *A. altissima* (P<0.05; Ratio test) (Table 3).

The mean annual (g m⁻² y⁻¹) and maximum monthly (g m⁻² month⁻¹) leaf litter production reported in bibliography are presented in Table 4. *A. altissima* shed over 65% of its total annual leaf litter in November (208 g m⁻²) in a riparian forest of Central Spain (González-Muñoz et al. 2013). In a nearby area, *P. alba* also presented the highest monthly litter production in November (166 g m⁻²) (Medina-Villar et al. 2015b). *R. pseudoacacia* showed a maximum monthly leaf litter production of 135 g m⁻² in a plantation in China (Tateno et al. 2007). Finally, *F. angustifolia* showed a maximum monthly leaf litter production of 96 g m⁻² in a riparian ecosystem in Central Spain (Medina-Villar et al. 2015b).

The PECs, PNECs and the risk assessment for the different scenarios are summarized in Figure 2. Only scenarios with a continuous exposure to tree extract (one month) presented ecotoxicological risk to the tested aquatic species (Figure 2, Table S1). The Risk Quotient (RQ) for continuous exposure were higher than 1 for all species and forested shorelines of 5 and 10 m of width (with a maximum of 68.6 for *P. alba*) (data not shown). In the case forested shoreline 1 m width, only *P. alba* showed a potential risk for aquatic biota

in continuous exposure. For the intermittent scenarios, no risk was detected for any of the tree extracts, as all RQs were less than 0.221 (Figure 2, Table S1).

Discussion

This study applies the approach of the ecotoxicological risk assessment to different scenarios to assess whether extracts of leaf litter from different trees may have toxic effects to aquatic organism. As argued in the introduction, we expected higher toxicity in exotic than in native tree leaf litter extracts. However, we found that extracts from the exotic trees are not more toxic than those from European native trees, *P. alba* being the most toxic. This unexpected result may be attributed to the fast decomposition/degradation of the allelopathic and antibiotic compounds, which in the case of *A. altissima* are quickly degraded (Heisey 1996). The stronger toxic effect of *P. alba* leachates may be attributed to the possession of allelopathic and antibiotic compounds that cannot be degraded or are more recalcitrant than those of the exotics (e.g. the phenolic compounds of *A. altissima*). A potential explanation, argued below, may be the higher content of Zn found in *P. alba* leachates, compared with the leachates of the other species, as this metal can cause toxicity to aquatic invertebrates (see below).

Our simulations reveal that only the continuous exposure (i.e. all the leaf litter produced in the month when leaf abscission peaked) may pose a risk to aquatic animals. Shallow and heavily forested small lakes are the most susceptible to toxicity by leaf leachates, but the likely toxicological consequences for invertebrates in deeper lakes should be less harmful for short-term effects. Additionally, the long-term toxicological effects of leaf extracts in shallow lakes may be diluted by water renewal, which tends to be faster in

lakes with high drainage area to surface area ratios (Mullholand 2003). However, this dilution effect may be negligible at short-term exposures.

Our scenarios cover a wide range of situations in shallow lakes, but only the massive input of leaf litter represents an ecotoxicological risk. However, under natural conditions this scenario is quite unlikely, given that the litter enter tends to be sequential, and that part of the litter production would decompose in the ground. On the other hand, leaf litter may also liberate non-soluble compounds that accumulate in the sediment, representing a risk for benthic animals. However, the soluble compounds (aqueous fraction) in the water column are affecting both benthic and pelagic animals. In any case, future studies should focus on the actual ecotoxicological effects of long-term leaf litter inputs under natural aquatic ecosystems and mesocosm studies.

Few studies have assessed the toxicity of whole leaf extract on aquatic invertebrates (Earl et al. 2012; Hofmann et al. 2012; Manusadžianas et al. 2014; Li et al. 2015; Beleza et al. 2019). Most of them have used cladocerans as experimental animal (Hofmann et al 2012; Manusadžianas et al. 2014; Li et al. 2015). Manusadžianas et al. (2014) compared the toxicity of litter extracts from the exotic tree boxelder maple (*Acer negundo*) and the native *Alnus glutinosa* to the freshwater crustaceans *Thamnocephalus platyurus* and *D. magna*, and also to algae and bacteria, and found that the litter of the exotic tree had higher toxicity. Beleza et al. (2019) also found a higher toxicity of exotic extracts on the dipteran *Chironomus riparius*. Other studies found contrasting effects of leaf litter extracts on aquatic biota, from beneficial effects on growth and reproduction of the cladoceran *Moina macrocopa* (Hofmann et al. 2012) to adverse toxic effects on four species of tadpoles (*Pseudacris maculate*, *P. crucifer*, *Bufo americanus* and *Hyla versicolor*), the fish

Oreochromis niloticus and the insect *Aedes albopictus* (Earl et al. 2012; Li et al. 2015). However, our study is the first attempt to conduct an ecotoxicological risk assessment of whole leaf litter extracts on aquatic scenarios.

Although we used fresh extracts from leaf litter, during the decomposition of leaf litter a variety of reactive organic compounds are released to the aquatic environment causing direct toxicity on aquatic biota (Steinberg et al. 2003; Hofmann et al. 2012). Therefore, the natural biodegradation of leaf leachates can increase their initial toxicity to different aquatic groups. Thus, the risk of the extracts of these species could be higher during the biodegradation period. Manusadžianas et al. (2014) found for several aquatic groups (bacteria, algae, invertebrates) that the degradation time had a significant effect on the toxicity of the leaf leachates and that the highest toxicities appeared between 30 to 90 days since the beginning of leaf leachate degradation. The effects of these biodegradation products on invertebrates need further research to get a long-term ecotoxicological risk assessment in aquatic ecosystems. In any case, our risk assessment may help to bridge the gap between the laboratory toxic bioassays and the effects of leaf extracts in natural scenarios. Additionally, leaf litter extracts can be not lethal for invertebrates but affect their fitness in the long term. In fact, a recent study of Beleza et al. (2019) has shown that leachates from *Eucalyptus* leaf litter impaired the growth and development rates of the aquatic larvae of the dipteran *Chironomus riparius*. These leachates affected key biochemical pathways, including energetic and neurophysiological activities, suggesting a long-term effect of leachates to invertebrates and likely to other animals.

Leaf extracts are complex mixtures of several organic and inorganic compounds (Timofeyev et al. 2004; Martin and Blossey 2013; Barbara et al. 2014; Li et al. 2015).

Dissolved natural organic matter (NOM) can form several external reactive oxygen species (e.g. hydroxyl radical) that may alter biological membranes. Organisms take up NOM and generate internal reactive oxygen species (Paul et al. 2004; Timofeyev et al. 2004; Hofmann et al. 2012). Additionally, the mineralization of organic matter under low dissolved oxygen can increase the concentrations of ammonia (NH_4^+), which in its unionized form (NH_3) presents high toxicity to aquatic invertebrates (Camargo and Alonso 2006; Krevš et al. 2013). In our study, the total measured ammonia concentrations ranged from less than 0.05 to 0.36 mg N- NH_4 l⁻¹, but it could be up to eight times higher in the highest extract concentration (0.36 x 8=2.88 mg N- NH_4 l⁻¹ in the treatment of 10 g l⁻¹ of air-dried leaf for *P. antipodarum* and *A. altissima* extract). In this case, as temperature (16.4°C) and pH (5.3) were low, the concentration of the toxic form of ammonia (unionized form) was less than 0.0001 mg N- NH_3 l⁻¹, which is much lower than the safe concentration for this toxicant (0.02 mg N- NH_3 l⁻¹) (Alonso and Camargo 2011). However, under natural scenarios the ammonia toxicity may decline due to nitrification processes (oxidation of ammonia to nitrite and nitrate), which are more likely to occur under aerobic conditions (Smorczewski and Schmidt 1991; Rodríguez-Martínez 2010; Wu et al. 2019). In addition, ammonia toxicity may decline due to assimilation by autotrophic bacteria at very low concentration (Dobson and Frid 2009). Our results show that during the bioassays the dissolved oxygen concentrations were very low at the highest leaf extract concentrations (<3 mg l⁻¹), this oxygen level is lower than the validation criterion of the short-term standardized test with *D. magna* (OECD 2004). However, most of the other leaf extract concentrations were within the validation criterion for dissolved oxygen, and still caused lethality on *D. magna*. This is indicative of the contribution of other factors/compounds to the extract toxicity. For instance, several authors (Barbara et

al. 2014; Li et al. 2015) showed that some plant species contain compounds toxic to insects.

In the case of *P. alba* its low EC50 is especially remarkable. Among the measured compounds of this leaf extract, zinc presented a very high concentration (mean value of 0.208 mg l⁻¹ for the stock solution-100%). This element is highly toxic to *D. magna*, according to the reported EC50 values of 0.10-0.72 mg Zn l⁻¹ after two days of exposure (Okamoto et al. 2014). In our study, the EC50 value for Zn corresponding to the EC of leaf extract of *P. alba* was 0.037 mg Zn l⁻¹ at 96 hours (data not shown). Although this value is lower than that of Okamoto et al. (2014), our longer exposure time (4 days vs 2 days), along with the low dissolved oxygen in the highest concentrations may contribute to the high toxicity of the leaf extract of *P. alba*. Other studies have shown that other compounds, such as tannins and saponins, also cause adverse effects to animals (Martin and Blossey 2013). In fact, saponins decrease the surface tension between water and gills in fish (Sparg et al. 2004). Additionally, the microbial decomposition of organic matter also causes a significant change in pH, contributing to exacerbate the deleterious effects of leaf extract (Manusadžianas et al. 2014). This effect, together with the depletion of dissolved oxygen, may only occur under massive input leaf litter, which rarely happens under natural scenarios. However, some tree species shed their leaves more synchronically than others so their impact on adjacent water ecosystems can be highly species-dependent (Manusadžianas et al. 2014). In fact, synchronic peaks of leaf fall have been reported from both *A. altissima* and *R. pseudoacacia* (Medina-Villar et al. 2015b).

The adverse effects of leaf litter extract on aquatic biotas represent a multi stress process. These extracts alter several key parameters, such as pH, conductivity or dissolved oxygen,

which are essential for the homeostasis of animals. These parameters and the leachate of several organic and inorganic compounds from the leaf litter may cause combined deleterious effects on animals. Our procedure has assessed the whole toxicity of leachates from leaf litter (including changes in environmental parameters such as conductivity or dissolved oxygen) under laboratory conditions, and these results have been the basis for the extrapolation to natural scenarios. In fact, environmental parameters have a fundamental influence on toxicity. For instance, the combination of low dissolved oxygen and high ammonia caused increased mortality in crustaceans (Maltby 1995). Pilgrim and Burt (1993) showed the combined lethal effects of low pH and temperature variations on amphipods. The increase of conductivity can cause contrasting effects on aquatic invertebrates depending on the specific conductance of the affected ecosystems (Clements and Kotalik 2016). It is an evidence that the combined effects of multiple stressors on biotas cannot tackled as a simple result of their individual effects, and their interactions need a further study in order to understand the likely consequences of leaf litter leachates to freshwater biotas.

The ecotoxicological approach used here to assess the potential risk of leaf litter extracts to aquatic invertebrates of shallow lakes has the advantage of using easy-to-assess standard techniques, which may be helpful to support management decisions. However, our simulations cover ideal situations, which, as discussed above, presents limitations if results are to be extrapolated to natural ecosystems. In such ecosystems, the specific conditions may alter the response of invertebrates to litter extracts (water renewal, water properties) and long-term effects may be much more complex.

We conclude that leaf litter extracts from native and exotic trees may cause toxicity on two contrasting species of aquatic invertebrates (benthic and pelagic), the native *P. alba* showing the highest toxicity. Some of the simulated scenarios suggest an ecotoxicological risk of the leaf litter extracts of the studied tree species at short-term exposures for invertebrates. Our findings, along with other evidences of laboratory, show that ecotoxicological effects of leaf extracts are likely to occur under natural conditions. However, other factors such as microorganism activity, degradation, uptake, water renewal, etc. could modulate that risk in natural ecosystems (Mullholand 2003; Dobson and Frid 2009; Rodríguez-Martínez 2010).

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Figure 1. Effects of leaf litter extracts of each tree species on the proportion of immobile plus death animals for *Daphnia magna* (left panel) and on the time (in seconds) spent to start normal movement of *Potamopyrgus antipodarum* (right panel) adjusted to log logistic models.

Figure 2. Ecotoxicological risk assessment of leaf litter extracts of *Robinia pseudoacacia*, *Ailanthus altissima*, *Populus alba* and *Fraxinus angustifolia* for invertebrates of shallow lake systems. The upper and lower panels represent simulated scenarios of intermittent and continuous exposure to litter inputs, respectively. The X-axis represents the ratio between the lake water volume and the surface of forested area around the lake and the Y-axis the concentration of leaf litter per liter. Dots represent each of the 27 simulated scenarios resulting from combining three lake surfaces (1.5; 3 and 6 hectares), three widths of the forested area around the lake (1; 5 and 10 meters from the shoreline) and three lake depths (0.5; 1 and 1.5 meters) (Table S1 for details). The colored curves represent the predicted environmental concentration of leaf litter (PEC) along a gradient of water volume/forest area ratios for each tree species. The horizontal dashed lines represent the predicted no-effect concentration (PNEC) for an intermittent (i.e. the lowest EC50 divided by 100, PNEC_{int}) (upper panel) and continuous (i.e. the lowest EC50 divided by 1000, PNEC_{cont}) (lower panel) exposures for each tree species. Scenarios where PEC exceeds the dashed line with the same color (PNEC) have an ecotoxicological risk for invertebrates (Risk quotient >1).

Table 1 Mean (±SD, n= 3-28) chemical characteristics of the leaf litter extracts from the exotic (RP=*Robinia pseudoacacia* and AA= *Ailanthus altissima*) and native leaf litter (PA= *Populus alba* and FA= *Fraxinus angustifolia*) for each toxicity test concentration and control.

Sps	Extract concentration (g l ⁻¹ of air-dried leaf)	pH		Conductivity (µS cm ⁻¹)		Dissolved oxygen (mg l ⁻¹)		NO ₃ -N (mg l ⁻¹)		NH ₄ -N (mg l ⁻¹)	
		<i>D. magna</i>	<i>P. antipodarum</i>	<i>D. magna</i>	<i>P. antipodarum</i>	<i>D. magna</i>	<i>P. antipodarum</i>	<i>D. magna</i>	<i>P. antipodarum</i>	<i>D. magna</i>	<i>P. antipodarum</i>
	Control	7.7±0.06	7.7±0.06	846±73	413.5±21.8	8.3±0.25	9.9±0.16	<0.5	<0.5	<0.05	<0.05
RP	10	5.9±0.02	5.6±0.01	1703±4.2	1071±1.7	2.1±0.5	2.3±0.5	n.m	n.m	n.m	n.m
	5	6.6±0.04	6.6±0.06	1343±7.8	720±1	2.7±0.6	3.3±0.5	n.m	n.m	n.m	n.m
	2.5	6.9±0.05	-	1122±4.5	-	4.5±0.4	-	n.m	-	n.m	-
	1.25	7.2±0.05	7.1±0.05	997±4.8	472±2	6.7±0.5	7.6±0.7	1.47±0.06	1.53±0.15	0.08±0.02	0.13±0.06
	0.31	7.6±0.08	7.6±0.02	896±4.4	412±0.6	8.0±0.4	9.4±0.5	<0.5	<0.5	0.08±0.01	0.12±0.04
AA	0.075	7.8±0.04	7.8±0.02	875±4	383±0.6	8.3±0.4	9.9±0.6	<0.5	<0.5	0.07±0.04	0.09±0.01
	10	5.3±0.02	5.3±0.02	1533±31.2	1022±0.7	2.2±0.4	2.6±0.4	n.m	n.m	n.m	n.m
	5	6.7±0.33	6.4±0.02	1246±14.3	718±0.6	3.7±0.9	4.4±1.0	n.m	n.m	n.m	n.m
	2.5	7.1±0.08	-	1094±6.5	-	4.4±0.3	-	n.m	-	n.m	-
	1.25	7.5±0.37	7.2±0.04	994.8±10	503±0.7	6.4±0.3	8.1±0.9	1.02±0.08	1.00±0.1	0.29±0.08	0.36±0.02
PA	0.31	7.5±0.04	7.5±0.03	919.3±12.5	448±0.6	8.0±0.2	9.3±0.3	<0.5	<0.5	0.17±0.03	0.15±0.005
	0.075	7.7±0.03	7.7±0.06	906±7.1	437±0.6	8.1±0.2	9.4±0.3	<0.5	<0.5	0.07±0.01	0.07±0.07
	10	5.7±0.03	5.7±0.03	1051.3±29.3	800±6.4	2.4±0.3	2.6±0.4	n.m	n.m	n.m	n.m
	5	6.5±0.01	6.5±0.01	887.3±22.7	614±4.9	3.0±0.2	3.2±0.3	n.m	n.m	n.m	n.m
	2.5	6.8±0.15	-	871.8±22.9	-	4.3±0.1	-	n.m	-	n.m	-
FA	1.25	7.2±0.02	7.1±0.02	867.3±31.2	481±1.2	6.1±0.4	7.3±0.9	3.2±0.36	n.m	0.10±0.01	0.11±0.01
	0.31	7.6±0.03	7.6±0.03	859.8±41.2	445±1.5	7.6±0.4	9.3±0.5	<0.5	<0.5	0.07±0.01	0.06±0.005
	0.075	7.8±0.06	7.8±0.06	848.5±42.2	442±1.5	8.1±0.1	9.5±0.2	<0.5	<0.5	<0.05	<0.05
	10	5.9±0.05	6.0±0.13	1707±6.7	1327±1.5	2.3±0.7	2.4±0.7	n.m	n.m	n.m	n.m
	5	6.6±0.14	6.5±0.05	1286±5.1	907±1	2.5±0.7	2.8±0.6	n.m	n.m	n.m	n.m
	2.5	6.9±0.01	-	1002±2.6	-	5.0±0.3	-	n.m	-	n.m	-
	1.25	7.2±0.04	7.2±0.05	877.8±3.2	529±1.5	7.0±0.6	8.0±0.7	n.m	n.m	n.m	n.m
	0.31	7.6±0.07	7.5±0.04	861.3±2.6	430±0.6	7.6±0.3	9.7±0.7	1.33±0.15	1.31±0.16	0.12±0.01	0.11±0.01
	0.075	7.8±0.02	7.8±0.02	843.5±13.4	388±2	8.2±0.2	9.9±0.7	0.53±0.06	0.57±0.06	0.11±0.01	0.10±0.01

n.m = not measured

- = treatment was not performed

Table 2 Chemical parameters of the leaf litter extracts (10 g of lab-dried entire leaves in 1 litre of bioassay water) from the exotic (RP=*Robinia pseudoacacia* and AA= *Ailanthus altissima*) and native leaf litter (PA= *Populus alba* and FA= *Fraxinus angustifolia*). Values are means ($\pm$ SD, n= 3). For the parameters of Cd, Co, Cr, Mo and Se all extracts showed a concentration below than the detection limit (detection limits of 0.01 mg l⁻¹ for Mo and 0.05 mg l⁻¹ for the others) (data not shown). Water from control showed a concentration below the detection limit for all parameters.

Species	Total phenolics (mg l ⁻¹)	Al (mg l ⁻¹)	B (mg l ⁻¹)	Cu (mg l ⁻¹)	Fe (mg l ⁻¹)	Mn (mg l ⁻¹)	Ni (mg l ⁻¹)	Zn (mg l ⁻¹)
RP	119.5 $\pm$ 9.2	0.07 $\pm$ 0.05	1.24 $\pm$ 0.03	0.017 $\pm$ 0.003	0.083 $\pm$ 0.044	0.270 $\pm$ 0.016	<0.005	0.089 $\pm$ 0.011
AA	468.6 $\pm$ 39.6	<0.02	0.72 $\pm$ 0.1	0.008 $\pm$ 0.002	0.033 $\pm$ 0.003	0.185 $\pm$ 0.018	<0.005	0.051 $\pm$ 0.019
PA	227.8 $\pm$ 35.5	<0.02	0.77 $\pm$ 0.06	0.009 $\pm$ 0.003	0.046 $\pm$ 0.002	0.274 $\pm$ 0.025	<0.005	0.208 $\pm$ 0.062
FA	203.2 $\pm$ 10.9	0.08 $\pm$ 0.03	0.03 $\pm$ 0.00	0.023 $\pm$ 0.007	0.107 $\pm$ 0.019	0.385 $\pm$ 0.018	0.008 $\pm$ 0.001	0.066 $\pm$ 0.004

Table 3 Effective concentration 50 (EC50) at 96 hours for each tree extract on *Daphnia magna* and *Potamopyrgus antipodarum*. 95% confidence limits are shown in parentheses. Different letters show significant differences between EC50 values across the different tree extracts for each animal species (p<0.05; Ratio test).

Leaf extract	EC50 (g l ⁻¹ of air-dried leaf)	
	<i>D. magna</i>	<i>P. antipodarum</i>
<i>Robinia pseudoacacia</i> [#]	4.29 (3.58-4.99) ^b	6.11 (0.47-11.7) ^{b,c}
<i>Ailanthus altissima</i> [#]	10.1 (6.08-14.2) ^c	6.94 (6.28-7.59) ^c
<i>Populus alba</i> [¶]	1.77 (1.54-1.99) ^a	1.45 (1.21-1.67) ^a
<i>Fraxinus angustifolia</i> [¶]	9.50 (5.58-13.4) ^c	2.02 (0.61-3.43) ^{a,b}

[#]exotic to Europe [¶]native to Europe

Table 4 Maximum mean annual production ($\text{g m}^{-2} \text{ year}^{-1}$) and monthly production ($\text{g m}^{-2} \text{ month}^{-1}$) of leaf litterfall reported in bibliography for the tree species.

Species	Mean annual production ($\text{g m}^{-2} \text{ year}^{-1}$)	Maximum monthly production ($\text{g m}^{-2} \text{ month}^{-1}$)	Ecosystem	Reference
<i>Robinia pseudoacacia</i>	310	135*	Tree plantation	Tateno et al. (2007)
<i>Ailanthus altissima</i>	305	208	Degraded riparian ecosystem	González-Muñoz et al. (2013)
<i>Populus alba</i>	412	166	Degraded riparian ecosystem	Medina-Villar et al. (2015b)
<i>Fraxinus angustifolia</i>	263	96	Degraded riparian ecosystem	Medina-Villar et al. (2015b)

*Estimation based on the percentage of leaf litter shed in the month with the maximum litterfall