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Solid lipid nanoparticles affect microbial colonization and enzymatic activity throughout the decomposition of alder leaves in freshwater microcosms

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ABSTRACT

Solid lipid nanoparticles (SLNs) are used as carriers for drug delivery, and are high biocompatible and designed to endure in the host organism. Despite its current industrial production is low, many of these substances are available on the market, and much more are in the production pipeline. As a result, many of them will end in aquatic systems raising the question whether they can pose a risk to aquatic biota and the associated ecological processes. Microbial decomposers of plant litter, play a key role in forested streams being responsible for the energy flow between terrestrial and aquatic environments. Here, we investigated the effects of SLNs on alder leaf litter decomposition by aquatic microbes. Alder leaves were immersed in a stream of Northeast Portugal to allow microbial colonization before being exposed in microcosms of two types of SLNs at two concentrations for 42 days. Results showed that rates of leaf decomposition decreased with exposure to SLNs. Bacterial biomass was not inhibited by SLNs, and cultivable fungi densities remained constant (SLN-A) or increased (SLN-C) compared with control microcosms. The type and concentration of SLNs influenced differently the leaf colonization by fungi as well as fungal sporulation rate. These effects were accompanied by changes in the community extraenzymatic profile: the activities of alkaline phosphatase, acidic phosphatase, Naphthol-AS-BI-phosphohydrolase (P cycle) and lipases increased in the SLNs microcosms. This study provided the first evidence of the adverse effects of the release of SLNs to streams on leaf litter decomposition. Those effects seem to depend on the composition and concentration of SLNs, as well on the microbial target group, or enzyme. Thus, prior to massive industrial production of these nanomaterials, some measures should be taken to avoid environmental impact affecting the microbial communities responsible for detritus decomposition.

1. Introduction

Engineered nanoparticles (ENPs) find use in a variety of different areas such as electronic devices, fabrics, agro-food, biomedical, pharmaceutical, composites, cosmetic, energy, environmental, catalytic and material applications (Nowack and Bucheli, 2007; Piccinno et al., 2012). Among the ENPs, are the Lipid Nanoparticles (LNs) used as carriers for delivery of anti-cancer, anti-fungal and antibiotic drugs, delivery of gene-based therapeutics, and the delivery of anesthetics and anti-inflammatory drugs (Allen and Cullis, 2013). Although not produced in high quantities when compared with metallic nanoparticles, several LNs are commercialized since the 90's of the last century, and many more are in the pipeline (Allen and Cullis, 2013). Despite their biodegradability, which depends greatly on lipid composition, they are design to persist into the host organisms, and delivery the drug into cells. Although the increased use of LNs, they are not listed by OEDC (2010). But, due to their routes of administration (e.g. parenteral, oral, topical, dermal and transdermal), most of them end in aquatic systems, through wastewater treatment plants or industrial discharges (Bekersky et al., 2002; Scown et al., 2010; Gottschalk and Nowack, 2011). Their toxicity depends on the chemical composition, particle size and coating (for a review see Kahru and Ivask (2013). Also, because LNs are high biocompatible (Doktorovova et al., 2011) could have unintended impacts when released into natural ecosystems even at low concentrations. When released to the environment ENPs can undergo numerous transformations that depend on the type of ENPs and the receiving medium (Colman et al., 2012; Bartley et al., 2013), such transformations may include adsorption to organic matter,

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aggregation, participation on redox reactions (mainly with the metal nanoparticles), reaction with other molecules present on the medium that may be inert or present toxic effects (Souto et al., 2009; Bartley et al., 2013; Doktorovova et al., 2014b). Among the ENPs the LNs, such as solid lipid nanoparticles (SLNs) and the nanostructured lipid carriers (NLCs), use biodegradable and biocompatible raw materials and are already in use in pharmaceutical and cosmetic products, most of them of GRAS (generally regarded as safe) status (Doktorovova et al., 2014b), but their properties may change when produced as a nanoscale system. SLNs are formulated in aqueous medium and are composed of lipids that are solid at room and body temperature (e.g. cetyl palmitate, myristyl myristate, tripalmitate, waxes) and stabilized with surfactants or emulsifiers (e.g. tween, cholic acid sodium salt) in order to maintain their superficial properties (Doktorovova et al., 2014b).

Plant litter decomposition is a key process in freshwater systems, responsible for the energy flow between terrestrial and aquatic environments. It is a complex process, involving, and being influenced by, abiotic (chemical and physical) and biotic (microbial and invertebrate) characteristics of the environment (Sampaio et al., 2004, 2007, 2008; Pascoal et al., 2005; Menéndez et al., 2011; Fidalgo et al., 2013). Fungi and bacteria have been recognized to play a role in litter decomposition and energy transference to higher trophic levels such as invertebrates (Graça, 2001). Several studies demonstrated that litter decomposition is sensitive to water physic-chemical parameters (Ferreira and Chauvet, 2011; Menéndez et al., 2011), and leaf litter breakdown rate was proposed as a functional measure of the freshwater ecosystems health (Pascoal et al., 2003; Woodward et al., 2012).

There are a few studies on the aquatic effects of metal nanoparticles, produced in larger quantities, but to the best of our knowledge there are no studies on the effect of LNs on aquatic systems. Studies involving the effect of metal nanoparticles showed the influence of the nanoparticle titanium oxide ($nTiO_2$) on biofilm growth of freshwater algae (Kulacki et al., 2012), the impact on litter decomposition and the associated biota, both microorganisms and invertebrates (Pradhan et al., 2011, 2012). More recently, it was demonstrated that metal and organic nanoparticles not only influenced fungal growth rates but also induced changes in the chemical composition of the white-rot fungi mycelium (Galindo et al., 2013). Nevertheless, the understanding of the impact of ENPs, particularly SLNs, on biotic processes like leaf litter decomposition in freshwater is still scarce. In this context and in the absence of studies on the effects of organic ENPs, such as SLNs, on alder leaf litter breakdown the aim of this work is to fulfill this gap. For that, we will mimic alder decomposition in freshwater using microcosms with or without SLNs, to test the effect of the particle size and lipid composition, using two different SLNs with the same coating composition, but different particle size and lipid composition. Along the experiment, leaf mass loss, microbial colonization, fungal conidia production and microbial enzyme activities will be measure.

2. Material and methods

2.1. Leaf-discs conditioning in freshwater

The leaves of black alder (*Alder glutinosa* (L.) Gaertn.), collected from the trees in autumn (before starting the project), were air-dried at 30 °C and stored until use. We chose this species because it is one of the most common in the riparian forests in Portugal and is worldwide distributed. Before the experiment, the leaves were rehydrated in deionized water and cut into discs (12 mm diameter). A set (30) of leaf discs were put in fine mesh bags (n=72, 20×15 cm, 0.5 mm mesh size), to prevent invertebrate colonization, and immersed in the "Tourinhas" stream (N 41°17′25.9, W 7 °43′42.0, altitude 450 m, in Vila Real, Portugal) in 20th March, for 7 days for microbial colonization. To determine the initial leaf mass weight, four leaf bags were randomly retrieved from the stream after 1 h of immersion.

Table 1
Composition of cationic SLN tested in the present work and respective physicochemical characterization. Z-ave (d nm): average diameter particle size; PdI: Polydispersity Index; ZP: Zeta potential; TSA: Total surface area (1 mg mL^{-1}).

	SLN-A	SLN-C
Composition		
Lutrol F68	0.25	0.25
CTAB	5.0	0.5
Compritol 888 ATO	–	0.5
Imwitor 900 P	5.0	–
Characterization		
Z-ave (d nm)	141.0 ± 0.1	222.2 ± 25.1
PdI	0.421 ± 0.001	0.354 ± 0.086
ZP (mV)	55.0 ± 1.44	72.5 ± 1.63
TSA (m^2)	4.0527×10^{-2}	2.5740×10^{-2}

Temperature, water conductivity and pH were measured in situ with a multiprobe field water equipment (Horiba U-10, Kyoto, Japan). Water samples were collected to determine the phosphate, nitrate and nitrite concentrations. Also, we collected 30 L of stream water for the making and maintenance of the microcosms.

2.2. Preparation and characterization of SLNs

The two tested SLNs had distinct formulations (Table 1), with a different lipid core, but were stabilized by the same surfactant, as described before (Doktorovova et al., 2014a). Both SLNs had cetyltrimethylammonium bromide (CTAB) as surfactant, a compound that has shown high toxicity in vitro studies (Fangueiro et al., 2014), but it is present at the same concentration in these formulations. The other compounds have been reported to have low toxicity. The SLNs were characterized in terms of particle size, distribution and surface charge (Table 1). Because both SLNs contain cationic lipids and surfactant, they have a positive Zeta Potential (ZP). The magnitude of the ZP values is relevant to minimize interaction between particles and thus aggregation of SLN. Also, a positive ZP value of SLNs allows the interaction of SLNs with the surface of cell membranes (that has a negative charge) and permits its internalization by the cells. SLN-C are bigger, with higher capacity to interfere with cell membrane, than SLN-A. However, SLN-A have a higher total surface area (TSA).

2.3. Leaf litter microcosm assay

After bags retrieval from the stream, the discs from each bag were rinsed with deionized water and placed into 150-mL sterile Erlenmeyer flasks (n=60) with 60 mL of filtered (0.45 μm) and autoclaved stream water. At the time of the incubation of the leaf bags in stream, the water had a pH of 7.3, 13 °C of temperature, conductivity of 34 μS cm^{-1}, 11 mg L^{-1} of nitrate, 0.03 mg L^{-1} of nitrite, 0.06 mg L^{-1} of phosphate.

The filtered and sterilized stream each water microcosms (n=48) was supplemented with the respective SLN (SLN-A or SLN-C), in two concentrations (1 μg mL^{-1} and 3 μg mL^{-1}). Along the article and with the purpose to simplify, these concentrations will be designated by 1 and 3, respectively, after the abbreviations SLN-A and SLN-C. We have chosen these SLNs concentrations taking into account their toxicity to animal cells (they begin to be toxic from 100 μg mL^{-1}) obtained by Doktorovova et al. (2014a) and its dispersion / dilution in the environment. The remaining microcosms (n=12) were kept without SLNs (control). All microcosms were kept under shaking (120 rpm.) at a constant temperature (18 °C) in the dark, and water/SLNs suspensions renewed every 7 days. After 14, 28 and 42 days microcosms were sacrificed (three replicates of each treatment per time) for further analyses.

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