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The endocrine disruptor nonylphenol induces sublethal toxicity in vascular plant development at environmental concentrations: A risk for riparian plants and irrigated crops?☆

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ABSTRACT

In recent years, there is a growing concern among the scientific community about the presence of the so-called emergent pollutants in waters of different countries, especially endocrine-disrupting compounds (EDCs) that have the ability to alter the hormonal system. One of the substances found almost ubiquitously and in higher concentrations is the alkylphenol nonylphenol. Albeit this compound is included in priority lists as a probable risk for human health and the environment, little is known about its effects on developing plants. The aim of this work is to assess the acute and sub-chronic toxicity of environmental concentrations of nonylphenol in riparian vascular plant development using spores of the fern *Polystichum setiferum* and a biomarker-based approach: mitochondrial activity (cell viability), chlorophyll (plant physiology) and DNA content (growth). Mitochondrial activity and DNA content show that nonylphenol induces acute and sub-chronic toxicity at 48 h and after 1 week, respectively. Significant effects are observed in both parameters in fern spores at ng L^{-1} but chlorophyll autofluorescence shows little changes. The inhibition of germination by natural allelochemicals has been reported to be related with the active hydroxyl group of phenolic compounds and largely independent of the structural nucleus to which it is attached. Results presented in this study suggest that environmental concentrations of nonylphenol could interfere with higher plant germination development by mimicking natural allelochemicals and/or phytohormones acting as a "phytoendocrine disruptor" likely posing ecophysiological risks.

1. Introduction

Nonylphenol is among the 33 priority substances, which present a significant risk to the aquatic environment, as established in the Directive 2008/105/EC of the European Parliament and of the Council on Environmental Quality Standards in the field of water policy. Nonylphenol belongs to the family of synthetic chemicals called alkylphenols and is produced from cyclic intermediates in oil refining and tar. It is formed by alkylating phenol with isomeric nonenes in the presence of an acid catalyst. The resulting product is a mixture of various isomers of nonylphenol and 4-nonylphenol and traces of 2,4-dinonylphenol (U.S. EPA, 2005). This compound is widely used in many fields, such as textile products, detergents, plasticizers, cosmetics, food packaging and other formulated products in our daily lives (Zhang et al., 2014).

Nonylphenol is an endocrine-disrupting compound (EDC). EDCs are substances that supplant natural hormones and interfere with the normal processes of reproduction and development of living beings. In particular, EDCs can act in three different pathways on the endocrine system by mimicking the function of hormones, blocking this operation or both (Rahman et al., 2009). EDCs

Abbreviations: ATP, adenosine triphosphate; EC_{50}, effective mean concentration; LOEC, lowest observed effect concentration; NMDRC, non-monotonic dose response curve; NOEC, no observed effects concentration; TPF, triphenylformazane; TTC, 2,3,5-triphenyltetrazolium chloride.

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comprise very different chemicals - from alkylphenols to organochlorines - whose effects vary from one species to another (Rahman et al., 2009) and are a real problem since biological fitness relies on a healthy intercellular communication to reproduce and develop normally (Bergman et al., 2012) but more studies are needed to understand their mechanisms of action.

In animals, the most sensitive exposure stage to EDCs is during critical periods of development like fetal stage and puberty because the tissues and organs are being formed (Bergman et al., 2012). Developmental exposures can cause changes that are not evident at birth but can increase incidence of diseases throughout life. While developmental effects occur at low concentrations in the offspring, effects in adults are generally only observed at high concentrations (Bergman et al., 2012). Some of the effects that have been observed resulting from the action of EDCs in organisms are: reduced fertility, feminization, changes in sexual behavior and changes in the reproductive organs of aquatic organisms such as fishes, algae, frogs and others (Pal et al., 2010). Also the EDCs possess the potential to induce multiple hormone-like activities has moved the focus from only reproductive endpoints to other endocrine regulated systems including the immune, cardiovascular and neuroendocrine systems (Lyche et al., 2011). Besides, current studies suggest that the risks posed by mixtures of EDCs are likely to be accumulative (Bergman et al., 2012).

Regarding nonylphenol, several studies have reported detectable levels in water bodies: wastewaters (Bergé et al., 2012; Soares et al., 2008), surface waters (Bergé et al., 2012; Esteban et al., 2014a; Quednow and Püttman, 2009; Soares et al., 2008; Stasinakis et al., 2012; Writer et al., 2010; Xu et al., 2014), groundwaters (Careghini et al., 2015; Félix-Cañedo et al., 2013; Tao et al., 2011) and drinking waters (Amiridou and Voutsa, 2011; Carvalho et al., 2015; Esteban et al., 2014b). In particular, European and international surface water studies (Esteban et al., 2014a; Quednow and Püttmann, 2009; Stasinakis et al., 2012) detected similar concentrations of nonylphenol in the $\mu g\ L^{-1}$ range.

Concerning the ecotoxicological effects of nonylphenol, studies have been mostly performed on fishes (Writer et al., 2010), fungi (Bärlocher et al., 2011) and algae (Gao and Tam, 2011; Liu et al., 2013; U.S. EPA, 2005; Zhou et al., 2013), and other review articles describe the estrogenicity of nonylphenol on animals. For example, the study of Zhang et al. (2014) in rats showed that when exposure to nonylphenol occurs during the critical period of development (fetal and/or early postnatal periods), permanent alterations in adipose tissue and then obesity can be produced. Many of these studies use aquatic species as models, which provide results for molecular or biochemical variables, such as induction or egg protein vitellogenin (U.S. EPA, 2005) or that have shown the impacts of nonylplhenol in the environment including feminization of aquatic organisms and decrease in male fertility [8]. Furthermore, nonylphenol is moderately bioaccumulative in aquatic organisms (Careghini et al., 2015; OEHHA , 2009; U.S. EPA, 2005).

Plants are also used as sensitive targets in ecotoxicity bioassays (Silva et al., 2003), because of their key role in terrestrial and aquatic ecosystems and their importance in primary productivity and the recycling of nutrients. In particular, vascular plants are essential in a regular ecosystem and new bioassays based on these organisms are important in the evaluation of potential impacts of pollutants. While some studies have addressed the effects of nonylphenol on vegetable crops due to its bioaccumulation and risk for human health (Lu et al., 2015; Soares et al., 2008), there are no studies on the possible toxic effects of nonylphenol in vascular plants with ecological relevance. Actually, all bioassays were conducted on crops plants such as beetroot (*Beta vulgaris*), barley (*Hordeum vulgare*) tomato (*Lycopersicon esculentum*) (Bokern and Harms, 1997), lettuce (*Lactuca sativa*) (Lu et al., 2015) basil plant (*Ocimum basilicum*) (Capota et al., 2004) or thale cress (*Arabidopsis thaliana*) (Chen and Yen, 2013). All of these studies showed a reduction in plant growth and other effects such as changes in pigmentation or an impairment of the photosynthetic process (Capota et al., 2004). Moreover, nonylphenol was shown to be phytotoxic to 14 different species of plants studied by Bokern and Harms (1997) with EC_{50} values varying from 11 to 220 mg L^{-1}. Free living microalgae show similar sensitivity to animals. In this case, nonylphenol affected the vegetative growth (Gao and Tam, 2011; Qian et al., 2011; Zhou et al., 2013). But plants and algae show very different sensitivity to pollutants and the latter should not be taken as a surrogate for plant toxicity (Wang, 1991).

Some years ago a new bioassay of phytotoxicity based on fern spores has been developed. This bioassay provides the use of an organism with ecological relevance and the capacity to assess the impact on natural riparian ecosystems (Catalá et al., 2009). This new approach uses the combination of the advantages of bioassays based on unicellular or isolated cells with the ecological importance of a higher plant (Catalá et al., 2009; Feito et al., 2012). Recently, this bioassay has been successful in the determination of the toxicity and ecotoxicological risks of micropollutants as the pharmaceuticals diclofenac and venlafaxine for vascular plants (Feito et al., 2012, 2013), showing increased sensitivity compared to validated bioassays (Esteban et al., 2013).

The aim of this study is to evaluate the acute and sub-chronic toxicity of the nonylphenol in vascular plant development using spores of *Polystichum setiferum* and a biomarker-based approach.

2. Material and methods

2.1. Nonylphenol

A logarithmic range of concentrations of nonylphenol (IUPAC name: 4-(2,4-dimethylheptan-3-yl)phenol, Sigma Aldrich) from 0.002 $\mu g\ L^{-1}$ to 20 $\mu g\ L^{-1}$ was used. A commercial mixture of various nonylphenol isomers was used in order to mimic the mixture shed to natural waters from urban discharges (Fig. 1): isononylphenol (Nº CAS: 11066-49-2), 4-nonylphenol (branched) (Nº CAS: 84852-15-3), nonylphenol (Nº CAS: 25154-52-3) and 4-*n*-nonyphenol (Nº CAS: 104-40-5). This concentration range was chosen according to the concentrations found in the environment (Esteban et al., 2014a; Quednow and Püttman, 2009; Stasinakis et al., 2012). A stock solution of 1 g L^{-1} of the nonylphenol mix in methanol was prepared. Working dilutions with a maximal methanol concentration of 0.001% were made in spore culture medium and autoclaved. Solvent controls containing 0.001% methanol were used in every experiment to account for solvent effect.

2.2. Biological material

Polystichum setiferum spores, a typical fern of riparian habitats, were sampled in northwest Spain, A Coruña province, in the Sar River or nearby. Fragments of leaf were collected with mature but closed sporangia. In the laboratory fragments were dried on smooth paper for a week to promote the release of spores. Spores were stored dry at 4 °C in darkness until use. Spores were sieved and suspended in gametophyte culture medium, sterilized and counted as detailed in Feito et al. (2012).

2.3. Toxicity in plant development

Acute toxicity was analyzed at 48 h by measuring two different parameters: mitochondrial activity and DNA content. Sub-chronic toxicity was assessed after a week and three parameters were used: mitochondrial activity, DNA content and chlorophyll