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Morphological, toxicological and molecular characterization of a benthic *Nodularia* isolated from Atlantic estuarine environments

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Abstract

A polyphasic study of a benthic *Nodularia* isolate (LEGE06071) from an Atlantic environment, specifically salt pans, was performed. LEGE06071 resembled both type strains of *Nodularia sphaerocarpa* and *Nodularia harveyana*, while ACOI00729 (purchased isolate) was identified as *N. sphaerocarpa*. The length and width of vegetative cells varied from 3.10 to 3.15 μm and from 3.71 to 4.25 μm, respectively, while heterocyts were 3.91–4.89 μm long and 4.20–4.74 μm wide. None of the isolates had aerotopes. The sequencing of the 16S rRNA gene from the two *Nodularia* isolates indicated that they belonged neither to *Nodularia spumigena* nor *N. harveyana*. Nodularin and other cyanotoxin synthesis-associated genes could not be detected, nor could nodularin production be detected by ELISA. However, MALDI-TOF analysis of LEGE06071 revealed the presence of other compounds, namely, glycolipids. Hence, toxicological screenings against *Artemia* nauplii, *Escherichia coli* and *Salmonella typhimurium* were performed. Toxic effects could only be observed against *Artemia,* with 48 h-LC_{50} values for the aqueous and crude extract of methanol of 53.21 mg ml^{-1} and 17.81 mg ml^{-1}, respectively. This study presents the first evidence of a non-nodularin-producing *Nodularia* isolate in Atlantic salt pan ecosystems and its potential ecotoxicity against *Artemia* sp.

Keywords: Atlantic ecosystems; Ecotoxicity; Estuary; *Nodularia* sp.; 16S ribosomal RNA

1. Introduction

The diversity, ecology and toxic properties of benthic *Nodularia* species, and in particular those from Atlantic environments, namely Portuguese estuaries, have been poorly studied. The few existing studies on benthic *Nodularia* have been mainly restricted to the Baltic Sea [14]. *Nodularia* distribution is reported in marine and brackish waters from a few European countries as well as the United States, Australia and New Zealand [4,32].

Nodularia habitats range from slightly brackish coastal and saline waters to inland alkaline lakes, but are only rarely found in freshwater reservoirs and in soil [6,14,19]. The genus *Nodularia* comprises diazotrophic filamentous cyanobacteria, which can occur as planktonic, benthic and terrestrial species. It is composed of four defined planktonic species, *Nodularia spumigena, Nodularia littorea, Nodularia crassa* and *Nodularia baltica*, and three benthic ones, *Nodularia harveyana*, *Nodularia sphaerocarpa* and *Nodularia willei*. Differences in size and shape of the planktonic species diverge from those of benthic species as well as their ability to form aerotopes and to produce nodularin [15].

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Planktonic *Nodularia* species occur mainly in marine and brackish biotopes, saline coastal and inland waters, and swamps ranging from temperate to subtropical areas. An uncommon benthic case is *N. willei,* which occurs in mats covering plants or muddy bottoms, mostly rice fields and brackish swamps throughout the tropics. The benthic *N. sphaerocarpa* is a freshwater alkaliphilic species existing mainly in temperate zones (Europe), littorals of lakes and on limestone substrates. *N. harveyana* is found in saline environments and has a cosmopolitan occurrence [14,19].

The taxonomy of *Nodularia* species based on morphological characters has never been simple. Occasionally, diacritical markers, for example, the presence of gas vesicles, may change during culture conditions, rendering species classification extremely complex [14]. Studies using phylogenetic markers such as small subunit ribosomal RNA (16S rRNA), internal transcribed spacers (ITSs) and phycocyanin genes, among others, are common and helpful tools for assessing the diversity and evolutionary relationships of cyanobacteria. The most popular marker for phylogenetic relationships among cyanobacteria is 16S rRNA, though it may be less informative for intrageneric level analysis [3]. According to Wilmotte [39], analysis of the 16S rRNA gene is not usually congruent with morphological features. Despite its limitations, Lehtimaki et al. and Lyra et al. [18,19] showed that studies involving 16S rRNA gene sequences are helpful for differentiating toxic from non-toxic strains of *Nodularia* and for pointing out its close relatedness.

Nodularia cyanobacteria are known to form blooms which can be toxic and cause chronic effects at several levels of the food chain [5,11]. The genus *Nodularia* is well-known due to the toxicity of *N. spumigena,* which produces nodularin, a hepatotoxin [19]. Nodularin was first reported in Australia, where it caused animal poisoning [7]. Nodularin is a cyclic heptapeptide with a cyclo-(D-MeAsp-L-arginine-Adda-D-glutamate-2-(methylamino)-2-dehydrobutyric-acid) structure [33]. It is non-ribosomally synthesized by a large multienzyme complex containing non-ribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) domains, as well as putative transposases and tailoring enzyme coding genes [10,24,25]. The toxicity of nodularin hepatotoxin is linked to inhibition of protein phosphatases 1 and 2A. Other toxic effects not linked to nodularin have been described in *Nodularia* against a free-living nematode, *Cephaloboides oxycerca* [27]. Moreover, the allelopathic activity of lipophilic secondary metabolites of a *N. harveyana* extract, as well as the antibacterial, antifungal and antibiotic activity of other *Nodularia* extracts, have been demonstrated [28].

To our knowledge, no previously published study reported the presence of *Nodularia* strains in European Atlantic estuarine environments. In this study, we characterized, at the morphological, toxicological and molecular level, a *Nodularia* isolate from a mainland estuary in Portugal. We also compared the morphometric characteristics of our isolate with a purchased isolate originally collected on the Madeira Island. The 16S rRNA phylogenetic relationships of those two Atlantic *Nodularia* isolates were compared with others collected worldwide. Furthermore, we analyzed the presence or absence of the nodularin synthetase gene as well other genes involved in production of common cyanotoxins. Finally, we assessed the ecotoxic, genotoxic and mutagenic activity of these Atlantic *Nodularia* to evaluate their potential environmental risk.

2. Materials and methods

2.1. Cyanobacterial isolation and culture

The strains of *Nodularia* used in this study were isolated from a water sample collected in the River Vouga estuary located in central Portugal (40°38′32.87″N, 8°39′47.85″O; LEGE06071); the Madeira Island strain (32°45′38.55″N; 16°57′34.10″W; ACOI0729) was purchased at the Collection of Algae of Coimbra. LEGE06071 was isolated by a micromanipulation technique and grown in $Z8^{0}_{10}$ medium [16] containing NaCl (10 g l^{-1}) and lacking nitrogen and the purchased isolate was maintained in Z8 medium. They were grown in the laboratory at 25 °C an under light intensity of 20.8–27.4 × 10^{-6} E m^{-2} s^{-1} with a light/dark cycle of 14/10 h. Cultures were unicyanobacterial and non-axenic. After 4–8 weeks of growth, cyanobacteria cells were extracted from the culture medium by filtration, washed once with bidistilled water, frozen at −20 °C and freeze-dried.

2.2. Phenotypic characterization and identification

For phenotypic analysis, the length and width of vegetative cells, heterocysts and, when present, akinetes of 4-week-old cultures were measured. Examination was carried out using a Leica Q500IW microscope with a digital camera, and LEICA QWIN standard V 2.3 image analysis software. Fifty cells were measured, but when akinetes were scarce, measurements were done on 25 cells. The presence or absence of gas vesicles was recorded. Morphological description was carried out according to the current classification system [6,14].

2.3. Statistical analyses

The average and standard deviations of the sizes of cells were calculated for each *Nodularia* isolate using Statistica software system, version 7 [34]. One-way analysis of variance (ANOVA) using Statistica was employed to evaluate differences in length and width of vegetative cells and heterocysts between isolates. We used the Kolmogorov–Smironov as goodness-of-fit procedure. Since normal distribution was not assessed, logarithmic transformation was applied to the data. We consider the data significantly different if their p-values were less than or equal to 0.05.

Principal components analysis (PCA) of the mean lengths and widths of vegetative cells and heterocysts of the two *Nodularia* isolates from Portugal plus cell dimensions of *Nodularia* strains described previously in Laamanen et al. [17] was performed using Statistica.