Harmful Algae 78 (2018) 69–74

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Insights into cyanophage-mediated dynamics of nodularin and other non-ribosomal peptides in *Nodularia spumigena*

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ARTICLE INFO

Keywords:
Aquatic viruses
Baltic Sea
Cyanotoxins
Filamentous cyanobacteria
virus-bacterium interactions

ABSTRACT

The effect of cyanophage infection and lysis on the dynamics of the hepatotoxin nodularin (NOD) and other non-ribosomal peptides (NRPs) produced by cyanobacteria is poorly understood. In this study, changes in concentration of NOD and other NRPs during cyanophage infection of the filamentous cyanobacteria *Nodularia spumigena* were assessed using incubation experiments. Viral infection and lysis were associated with a significant reduction (93% at the 96 h post infection) of *N. spumigena* cell density. While no correlation between *N. spumigena* abundance and total concentration of NOD (ng mL^{-1}) within the infected cells was observed, cellular NOD quota (ng $cell^{-1}$) gradually increased in the remaining cyanophage resistant *N. spumigena* subpopulation. Lysis of *N. spumigena* cells resulted in a substantial increase (> 57 times) of dissolved NOD concentration in the culture medium. The relative concentration of other cyclic (anabaenopeptins) and linear (aeruginosins, spumigins) NRPs produced by *N. spumigena* also increased in response to cyanophage addition. This study highlights the importance of cyanophage infection on the population toxicity of filamentous cyanobacteria and demonstrates a significant contribution of virus-mediated cell lysis on the conversion of NOD from the particulate to dissolved phase.

1. Introduction

The filamentous cyanobacterium *Nodularia spumigena* form extensive blooms in fresh- and brackish water environments, including the Baltic Sea (Akcaalan et al., 2009; Sivonen et al., 1989). These blooms can be characterized by a large chemical diversity of non-ribosomally produced cyanopeptides (NRPs) (Fewer et al., 2013; Mazur-Marzec et al., 2015, 2016), including hepatotoxin nodularin (NOD), which may have a detrimental impact on aquatic organisms and adversely affect ecosystem functioning (Pearson et al., 2010; Bownik, 2016). The production and concentration of NOD within the *N. spumigena* cells depends on the expression level of toxin synthetase genes, rate of peptide biosynthesis, and its excretion/uptake from/to the cells (Repka et al., 2001; Jonasson et al., 2008; Pearson et al., 2010). Another important aspect in the dynamics of NOD and other NRPs is their conversion from the particulate (cell-bound) to the dissolved phase. It could be hypothesized that cyanophages can significantly influence the concentration of NOD within the cells by metabolic reprograming of the infected cells, through the up/down regulation of NOD and other NRPs-synthesis associated genes as well as via rewiring of their biosynthetic pathways (Rosenwasser et al., 2016; Howard-Varona et al., 2016). Further, cyanophages may influence the concentration of dissolved NOD and other NRPs by releasing cellular content into the surrounding environment during cell lysis (Steffen et al., 2017). Cyanophage infection and lysis have been implicated in the control of proliferation and termination of toxic cyanobacteria blooms (Manage et al.,1999; Tucker and Polland, 2005; Simis et al., 2005; Honjo et al., 2006). Yet changes in concentration and profiles of NOD and other NRPs during viral infection as well as release of NOD and other NRPs following cyanophage lysis have so far not been thoroughly investigated. The lack of this

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https://doi.org/10.1016/j.hal.2018.07.004
Received 27 February 2018; Received in revised form 9 July 2018; Accepted 10 July 2018

knowledge precludes our understanding of the dynamics of noxious compounds produced by ecologically relevant cyanobacteria and prevents us from deciphering their functional implications for food web dynamics and ecosystem functioning.

To address the effect of cyanophage infection and lysis on the dynamics of NOD and other NRPs, a short-term (156 h) laboratory experiment was conducted, in which *N. spumigena* strain KAC68 (hereafter KAC68) was infected by a lytic cyanophage vB_NodS-kac68v162-1 (hereafter 68v162-1). Changes in cyanobacterial population density, particulate and dissolved concentration of NOD and other NRPs as well as biodegradation of NOD were monitored through the experiment, thereby allowing for an assessment of virus-mediated intracellular dynamics of cyanopeptides and their transition from particulate to dissolved phase.

2. Materials and methods

2.1. Experimental organisms and culture conditions

The toxic cyanobacterium *Nodularia spumigena* strain KAC68 was isolated from coastal waters at Askö, Sweden (58° 35´ N, 18° 14´ E) in 1996 (Janson and Granéli, 2002), and is available at the Kalmar Algae Collection (Linnaeus University, Kalmar, Sweden). The strain is maintained as a unialgal, yet non-axenic culture. The strain belongs to the chemotype cluster CT_B sub-group 3, which is characterized by anabaenopeptins (carboxypeptidase A inhibitors) with a conserved Phe^1 + CO + Lys^2 + Val^3 amino acid sequence (Mazur-Marzec et al., 2016). The culture was grown in f/2 medium (Guillard, 1975) prepared using pre-filtered and autoclaved Baltic Sea water (salinity 7 practical salinity units [PSU]) under controlled conditions at a temperature of 20 °C ± 2 °C and light intensity of approximately 100 µmol m^{-2} s^{-1} with a light-dark cycle of 16:8 h.

Cyanophage vB_NodS-kac68v162-1 (68v162-1) was isolated from the Baltic Sea (Baltic proper; 56° 40´ N, 19° 18´ E) in 2016 using *N. spumigena* strain KAC68 as a host organism, following descriptions given in Šulčius et al. (2015). Briefly, *N.spumigena* strain KAC68 was inoculated with serially diluted (10-fold dilutions over 10 orders of magnitude) Baltic Sea water concentrates (prepared using tangential flow filtration system VivaFlow 200 [Sartorius, Germany] equipped with 30.000 kDa PES cartridge) in 96-well microtiter plates until lysis occur (well clearance). Clonal isolate of 68v162-1 was obtained by repeatedly applying dilution-to-extinction procedure (Šulčius et al., 2015). Cyanophage morphological analysis and lytic cycle characterisation was performed as described in Šulčius et al. (2015). High titre cyanophage stocks for the infection experiment (see below) were prepared using exponential growth phase *N. spumigena* cultures under the same conditions as described above. The cyanophage-cyanobacterium system is available at the Linnaeus University Center for Ecology and Evolution in Microbial model Systems (EEMiS, Kalmar, Sweden).

2.2. Infection experiment

Cyanobacterial inoculum was derived from late exponential phase culture and added to 150 mL of f/2 medium in Erlenmeyer flasks (Schott Duran, Mainz, Germany) at a density of ~5.5 × 10^5cells mL^{-1} (± 0.5 × 10^5cells mL^{-1}). Cyanophage suspension was added to cyanobacteria culture at the multiplicity of infection (MOI) of ~ 1 to minimize host cell infection by more than one phage and at the same time increasing the likelihood of the majority of cells to be infected in the first infection cycle. Control treatment was amended with filter-sterilized f/2 medium instead of cyanophage suspension. The characterization of cyanophage 68v162-1 infection cycle demonstrated that it takes about 12 h until most of cyanophages (~80%) were adsorbed to the cells (Fig S1B). Therefore, the first samples from both control and infected treatments were taken 12 h post inoculation of either cyanophage suspension (infected treatment) or culture medium (control treatment) and the time of the first sampling was set to 0 h post infection (correspond to 12 h post inoculation), highlighting the start and duration of viral replications during the experiment (rather than time post inoculation of cyanophages). Further sampling was defined based on the analysis of *N. spumigena* generation time before the experiment (data not shown). The calculated doubling time of *N. spumigena* was 0.16 d^{-1}, indicating that population doubles in its size approximately every 6 days. Therefore, samples were taken every 48 h at 0, 48, 96 and 144 h post infection (hpi), assuming that 4 sampling points taken within one *N. spumigena* generation will be sufficient enough to detect any changes in the experiment. The total duration of incubation experiment was 156 h (12 h post inoculation + 144 h post infection).

2.3. Sample collection and processing

Samples (1 mL) for determination of *N. spumigena* cell density were fixed with 0.2 µm filtered acidified Lugol's iodine solution (to give a final concentration of ~1%) and kept in the dark at + 4 °C until further analysis. Cells (vegetative and akinetes) of *N. spumigena* were enumerated under a light microscope using the Fuchs-Rosenthal counting chamber and examining no less than 600 units.

Samples for determination of particulate nodularin and other NRPs (Table S1) were taken by filtering 10 mL of either control or infected culture onto Whatman GF/C filters (pore size - 1.2 µm, Ø 25 mm). The filters were frozen and stored at −20 °C for 2 weeks until further processing. The material collected on filters was extracted with 1 mL of 75% methanol in MilliQ water, centrifuged for 10 min at 10,000 × g to remove cell debris, transferred to chromatographic vial and analyzed by LC–MS/MS. We note that in using this approach, materials that remains associated with cell debris after lysis will be denoted as particulate.

Samples for determination of nodularin and other NRPs in the culture medium were taken by filtering 30 mL of either control or infected culture through Milipore PES filters (pore size - 0.2 µm, Ø 25 mm) to remove *N. spumigena* cells. The filtrates were subjected to a solid phase extraction on 1 g Sep-Pak Vac tC18 cartridges (Waters, Milford, MA, USA). The fraction eluted with 90% methanol was evaporated to dryness in vacuum, re-dissolved in 75% methanol and submitted to LC–MS/MS analysis.

2.4. LC–MS/MS analysis

Chromatographic separation of nodularin and other NRPs was performed using Agilent 1200 (Agilent Technologies, Germany) with a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm; Agilent Technologies, USA). Gradient elution with a mobile phase composed of 5% acetonitrile in Milli-Q water (A) and acetonitrile (B), both containing 0.1% formic acid, was used.

The mass spectrometer (5500 QTRAP, AB Sciex) was operated in positive mode, with a turbo ion spray (550 °C) at a voltage of 5.5 kV. For the detection and identification of cyanobacterial metabolites, the information dependent acquisition method (IDA) was used. For ions with m/z in a range 500–1000 and signal above the threshold of 500,000 cps, fragmentation spectra were collected. Concentration of NOD and determination of relative amounts of its degradation products and other peptides in the samples were estimated based on the peak area in the MRM chromatogram (Table S1).

2.5. Statistical analysis and calculations

The differences in *N. spumigena* cell densities and NOD concentration were compared between treatments over the time course of the experiment using a repeated-measures ANOVA (RM-ANOVA), with treatments (Control vs Infected) as a within-subjects factor. To comply with assumptions of normality and homogeneity of variance (tested using Kolmogorov–Smirnov and Levene's test, respectively) the RM-