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Title: New knowledge on gene expression in Baltic Sea cyanobacteria relative to growth under changing environmental conditions

Work package: 3

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Responsible contact person:
Jarone Pinhassi, Linnaeus University (LNU), Sweden

Introduction

Cyanobacteria encounter several environmental stressors in the Baltic Sea, such as nutrient limitation, UV-radiation and varying salinity, that cause unfavorable conditions for growth, limit survival, and affect species distributions (Lehtimäki et al., 1997; Kopf et al., 2015). Massive cyanobacterial blooms in the Baltic Sea are toxic and cause harm for recreational users and animals every summer. Blooms are dominated by nitrogen-fixing (diazotrophic) species from different genera, *Nodularia spumigena*, *Aphanizomenon* sp. and *Dolichospermum* sp. (former name *Anabaena* sp.) of which *N. spumigena* and *Dolichospermum* may produce toxins; nodularins and microcystins (Sivonen et al., 2007). Due to the capability to use atmospheric nitrogen gas dissolved in seawater, phosphorus is usually the primary nutrient that limits the growth of *Dolichospermum* sp. and *N. spumigena* in the Baltic Sea. Salinity (referring to the concentration of dissolved NaCl), in turn, is an important abiotic factor suggested to influence the distribution of different cyanobacteria species (Sivonen et al., 2007; Pade & Hagemann, 2014). Climate change model analyses indicate that the salinity in the Baltic Sea will decrease in the future due to increased riverine runoff within the catchment area (resulting from predicted increases in precipitation) and rarer saline water pulses via the Danish Straits (Kjellström & Ruosteenoja, 2007; BACC II Author Team, 2015; Graham, 2016). Cyanobacteria, like other living organisms, have developed several mechanisms to protect themselves against adverse changing environmental conditions by reconstruction of their metabolism and biochemical structure. Nevertheless, knowledge about linkages between genome structure of cyanobacteria and their physiological and ecological function under varying environmental conditions is still scarce.

In the work package 3 we have studied how changing environmental conditions affect the gene expression of toxic Baltic Sea cyanobacteria. Our aim was to gain better understanding of the relationship between cyanobacterial genome structure and their function in a changing environment as well as unravel competitive advantages of different genera in cyanobacterial blooms. Effects of high light and oxidative stress on the toxic Baltic Sea *N. spumigena* were studied earlier (Kopf et al., 2015) and thus we focused on two other important stressors, varying salinity (manuscript under preparation) and phosphorus limitation (Teikari et al., 2015). We found that all genome sequenced

N. spumigena isolates from the Baltic Sea carry a gene cluster responsible for phosphonate transport and assimilation. Thus, the capability of phosphonate utilization by *N. spumigena* was further studied (manuscript under preparation) to resolve whether this genetic character may serve an ecological advantage in phosphorus limited blooms. Ultimately knowledge into such adaptations can prove valuable to inform about the environmental status of the Baltic Sea.

Methodological workflow

A summary of the workflow used in this study is presented in Figure 1. All cyanobacterial strains used in this study are axenic and they belong to the University of Helsinki Culture Collection (UHCC). To study effects of salinity on *Nodularia spumigena* UHCC 0039 and *Dolichospermum* sp. UHCC 0315, Z8X medium with a salinity gradient from 0 to 9 g NaCl L^{-1} was used (Kotai, 1972). Growth at different salinities was monitored and gene expression was analyzed after 16 days of growth. Effects of high and low phosphorus on *Dolichospermum* sp. UHCC 0090 (former name *Anabaena* sp. 90) was studied after 4 days of incubation in normal Z8X (5.5 mg/L of phosphorus) and Z8X containing 0.05 mg/L of phosphorus. To study the role of phosphonates as a sole phosphorous source for *Nodularia spumigena* UHCC 0039, 5.5 mg/L of methylphosphonate, ethylphosphonate or 2-aminomethylphosphonate were added to the Z8XS-P (Z8XS without phosphorus) medium. Growth was followed by measuring the concentration of chlorophyll *a* every fourth day and cells for RNA-sequencing and RT-qPCR were harvested after 12 days in medium containing the studied phosphorus sources. Medium without phosphorus was used as a negative control and normal Z8XS medium was used as a positive control. For all phosphorus related studies, cells were starved in non-phosphorus medium for 7 days before the experiments to empty their cellular phosphate storages.

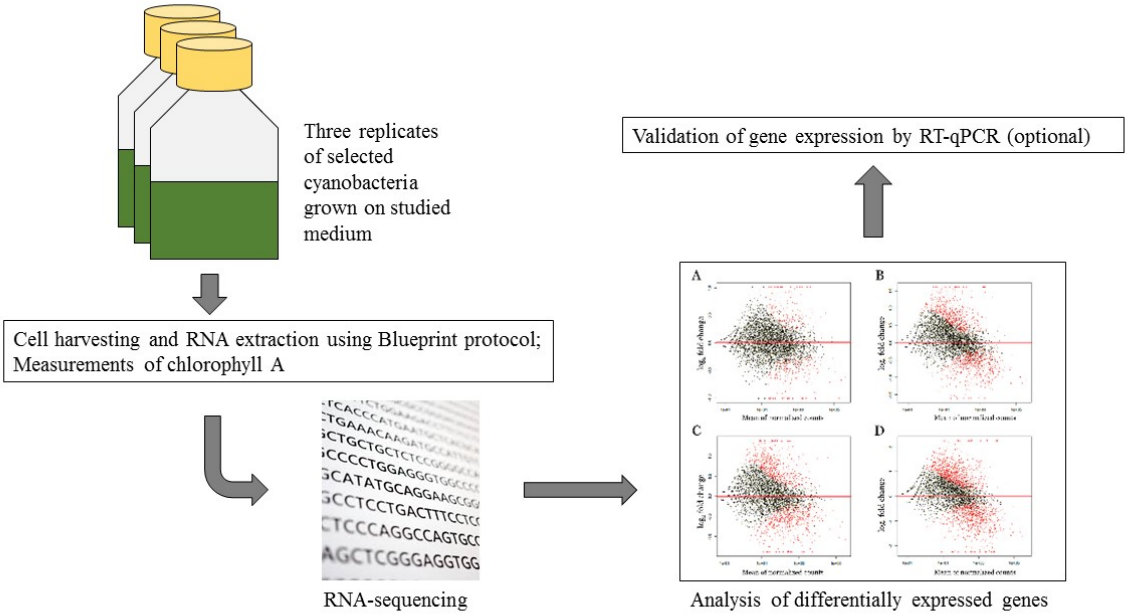

Figure 1. Methodological workflow used in this study. RNA samples were treated and extracted according to BONUS Blueprint protocol (Bennke and Beier, 2014 unpublished) and cDNA libraries were sequenced using SOLiD or Illumina HiSeq2500 sequencer.

Results and Discussion

Salinity

N. spumigena UHCC 0039 was able to grow in all tested salinities (0-9 g NaCl L^{-1}) but growth was heavily arrested at 0 g NaCl L^{-1}. On the contrary, the highest salinity (9 g NaCl L^{-1}) turned out to be fatal for *Dolichospermum* UHCC 0315 and already 6 g NaCl L^{-1} inhibited its growth. Due to the scarce genomic information available for the Baltic Sea cyanobacteria, genomes of *N. spumigena* UHCC 0039 and *Dolichospermum* sp. UHCC 0315 were sequenced to achieve good reference genomes for RNAseq data analysis. Genomic analysis unraveled that, despite the differences in the salinities of the isolation regions, newly sequenced *N. spumigena* UHCC 0039 isolated from Gulf of Finland share many genetic regions (including the gene clusters of synthesis of natural products and trehalose) with the previously sequenced *N. spumigena* CCY 9414 originating from Baltic Proper (Voss et al., 2013). Interestingly, assembly of the *Dolichospermum* sp. UHCC 0315 genome unraveled that our culture consists of two substrains, one of which carries a deletion of five genes. Further single-filament analysis showed that these five hypothetical genes may play a role in formation of gas vacuoles and thus participate in regulating buoyancy of cyanobacteria. Deletion may be a consequence of the loss of selection pressure with mixing in the laboratory conditions. Based on RNA-seq analysis, unfavorable salinity arrested growth of cyanobacteria by affecting electron transport through the photosynthetic apparatus but also influencing amino acid synthesis, transcription and translation. Lowering salinity induced expression of transcripts in *N. spumigena* UHCC 0039 responsible for cell wall structure and biosynthesis of glycogen, which is known to be a precursor molecule for trehalose synthesis. Interestingly, a huge genetic region suggested to be responsible of two new, yet unidentified, natural products in *N. spumigena* UHCC 0039, was drastically down-regulated in low salinity, suggesting that maintenance of transcription of this region is energetically expensive for this cyanobacterium under stress induced by low salinity. Our results indicate that salinity strongly regulates the intensity in, and species distribution of, toxic cyanobacterial blooms in the Baltic Sea.

Phosphorus limitation and additional phosphorus sources

In the diazotrophic cyanobacterial blooms, inorganic phosphorus is usually the primary growth-limiting factor. However, cyanobacterial genomes carry genes for production of alkaline phosphatases - enzymes capable of cleaving phosphate from organophosphate compounds - and the activity of this particular enzyme is widely used as an environmental marker for inorganic phosphorus scavenging. In the study of *Dolichospermum* sp. UHCC 0090, we found that measurement of the gene expression of alkaline phosphatase is not suitable for monitoring purposes because of its insignificant variation of expression under prolonged phosphorus limitation (Teikari et al., 2015). In contrast, upregulation of the *pstS* gene, a part of the high affinity phosphate transporter complex *pstABCS*, remained high – indicating that the *pstS* gene is a potent indicator of P-stress.

Phosphonates, organophosphorus compounds containing bonds between carbon and phosphorus, is another interesting group with potential to act as a phosphorus source for cyanobacteria. It has been suggested that phosphonates constitute 10% of dissolved high molecular weight organophosphates

and they have previously been shown to serve as an additional phosphorus reservoir for *Trichodesmium* cyanobacteria (Dyhrman et al., 2006; Dyhrman, et al., 2009). Based on genomic information, we found that all sequenced *N. spumigena* genomes carry genes for phosphonate transport and degradation (*phn* gene cluster) and, in addition to the brackish water environment with intermediate salinities, this character may constitute a competitive advantage for *N. spumigena* in the summer blooms. Based on the growth experiments and enzyme activity tests we found that naturally produced methylphosphonate is a good source of phosphorus for *N. spumigena*. Ethylphosphonate and 2-aminomethylphosphonate (a degradation product of glyphosate) can also be a source of phosphorus but to a lesser extent. The *phn* gene cluster is under the pho-regulon, which is activated under low inorganic phosphorus. Induction of the *phn* gene cluster in non-phosphorus and three phosphonate conditions were tested by RT-qPCR, showing that the *phnD* gene, responsible of phosphonate transport, was highly expressed in all treatments (including no-phosphorus conditions). In contrast, expression of the *phnJ* gene was induced only in the presence of phosphonates. This finding suggests that *phnJ* may be a good marker for studying the availability of phosphonates in aquatic ecosystems. This is important because direct chemical analysis of phosphonates in natural water sources have showed to be highly demanding.

Methylphosphonate as a source of phosphorus was studied in detail because it is naturally produced by aquatic microbes and, based on our results, it seems to be a good source of phosphorus for *N. spumigena* in the Baltic Sea. Methane is released into the environment as a degradation product of methylphosphonate, and production of methylphosphonate may thus play a role in regulating aerobic methane release in the Baltic Sea. This may also partially explain identified late-summer methane peaks in the Baltic Sea (Bange et al., 1994). Interestingly, by using the RNA-seq approach, the *phn* gene cluster was shown to be heavily upregulated when methylphosphonate was a sole source of phosphorus. We also found that the expression of the gene cluster encoding synthesis of a cryptic natural product was heavily repressed in low salinity but, that its expression increased substantially in the presence of methylphosphonate. The gene cluster showed similarities to the siderophore synthetase found in *Agrobacterium tumefaciens* and searches to find this putative siderophore are in progress (Rondon et al., 2004). Our findings emphasize that methylphosohonate can be a good source of phosphorus for cyanobacteria.

As a summary, salinity was found to affect the distribution of the cyanobacterial species in the Baltic Sea, with high salinity favoring the proliferation of *Nodularia spumigena*. In addition, inorganic phosphorus is the preferred form of phosphorus for cyanobacteria but capability to utilize organophosphates, namely phosphonates, also allow the cyanobacteria access to the dissolved organic phosphorus pool. To monitor the status of the total phosphorus in the Baltic Sea, we suggest that in addition to the alkaline phosphatase gene, the *pstS* and *phnJ* genes could successfully be applied to monitoring programs to inform about the bioavailability of inorganic and organic phosphorus, respectively. These findings indicate the feasibility to utilize cyanobacterial gene expression patterns to contribute to assessments of the environmental status of the Baltic Sea.

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