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Effects of temperature and salinity on the production of cell biomass, chlorophyll-*a*, Intra and extracellular NOD and nodulopeptin 901 produced by *Nodularia spumigena* KAC 66

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Abstract

Nodularia spumigena is one of the dominant toxic cyanobacteria, which produces blooms in the Baltic Sea, a brackish water body, particularly in late summer. *Nodularia* spp. are known to produce hepatotoxic nodularins (NOD) and other bioactive peptides such as spumigins and nodulopeptins. In a recent study, three new nodulopeptins with a molecular weight of 899, 901 and 917 Da were characterized from *N. spumigena* KAC 66. To gain further insight into the effects of environmental stress on growth and production of bioactive metabolites in *N. spumigena* KAC 66, two parameters were investigated: temperature and salinity. It was found that growth conditions have a considerable effect on biomass and toxin levels of *N. spumigena* KAC 66. Increasing temperature had a profound effect on NOD production with an increase from 22 to 25°C resulting in a 50% decrease in intracellular NOD levels. At 30°C little or no NOD was detected. In contrast, whilst concentrations of nodulopeptin 901 decreased with increasing temperature, it was still detectable throughout the growth cycle at elevated temperatures. The light intensity of 13 μmol photons m^2 s^{-1}, 22°C and 11-20 ‰ salinity were optimal growth conditions to obtain maximum biomass, intra and extracellular peptide production. This is the first report to evaluate the effects of selected environmental parameters on NOD/nodulopeptin 901 productions which ultimately may be helpful to explain the distribution, control of natural blooms and toxin levels of *N. spumigena* in the Baltic Sea, as well as in laboratory based experiments.

Keyword: *Nodularia spumigena,* cyanobacteria, hepatotoxic nodularins, bioactive metabolites, temperature and salinity.

Introduction

The nitrogen-fixing filamentous brackish water cyanobacterium, *Nodularia spumigena* KAC 66 produces hepatotoxic nodularin (NOD) (Dahlmann et al., 2001). In addition to producing nodularin, this species produces many other bioactive compounds such as spumigins, nodulopeptins Fujii et al., 1997; Mazur-Marzec et al., 2013) and recently three new nodulopeptins A-C (Schumacher, et al., 2012) have been characterized.

Nodularin (NOD) is the most common hepatotoxin that is known to be produced by *N. spumigena* (Falconer, 2001). According to the World Health Organization (WHO) a tolerable intake of NOD is 0.04 mg · kg^{-1}, but at lower levels this peptide can still cause severe health problems in humans (Karlsson, 2003; Schumacher et al., 2012).

The second largest semi-enclosed brackish water body, the Baltic Sea, has special environmental, geographical, and oceanographic, and physiochemical conditions. Water quality in the Baltic Sea is under pressure from agriculture run-off along with other human activities leading to excessive nutrients and the potential impact of greenhouse effects. It also receives the rainwater catchment from 14 countries (HELCOM, 2007). Due to its heavy nutrient load, the Baltic Sea is under the influence of eutrophication (Mazur-Marzec et al., 2005; Report on Estonia, 2005; Lilover and Stips, 2008) which has resulted in the occurrence of dense toxic cyanobacterial blooms. In late summer the dominant and toxic strain is *N. spumigena* (Kankaanpaa et al., 2002) and along with the potentially toxic *Aphanizomenon flos-aquae* and *Anabaena* spp., produce massive and lethal blooms in many areas of the Baltic Sea (Sivonen et al.,1989a; Ibelings et al., 2007; Lilover and Stips, 2008; Suikkannen et al., 2010).

Many cases have been reported of animal poisoning along the coasts of the Baltic Sea (Persson et al., 1984; Edler et al., 1985) and historically, *N. spumigena* was reported to produce lethal blooms in Lake Alexandrina, Australia (Rinehart et al., 1988). In the report of HELCOM (2007) it mentions that the level of nutrients is much higher than previous reports dating from 1950. In recent decades, increasing nutrient enrichment has provided suitable conditions for bloom formations.

The mass culturing of cyanobacterial strains under suitable laboratory conditions, provide an opportunity to produce a high amount of cells to isolate bioactive compounds. Under favourable conditions *N. spumigena* produces a significant amount of toxin within the cells (Lehtimäki et al., 1997) and also released into the growth medium. The end of a log phase, together with other environmental conditions, is the best time to ensure the highest biomass along with extracellular and intracellular hepatotoxins in cyanobacteria (Vezie et al., 2002).

The growth and toxin production in cyanobacteria is affected by many environmental factors *i.e.* temperature, light, salinity, phosphate, availability of nitrates and carbon dioxide (Sivonen, 1996). These abiotic factors are also helpful to control the dominance of specific strains in the natural cyanobacterial blooms as well as in laboratory based experiments.

The concentrations of toxin, produced by *N. spumigena,* may increase or decrease under specific environmental parameters. Some work has been done on changes in growth and toxin levels in response to factors *i.e.* growth duration (Gupta et al., 2002), light (Lehtimäki et al., 1997; Stal et al., 1999 and Hobson and Fallowfield, 2003), temperature (Lehtimäki et al., 1997; Hobson and Fallowfield, 2003) and salinity (Blackburn et al., 1996; Hobson et al., 1999; Moisander et al., 2002; Hobson and Fallowfield, 2003; Musial and Plinski, 2003; Mazur-Marzec et al., 2005).

Temperature plays an important role in the formation of blooms and production of toxins. The combined effects of temperature and irradiance influences growth, dominancy, toxin production and survival of strains and may vary from species to species (Lehtimäki et al., 1997; Hobson and Fallowfield, 2003).

The effects of temperature on cyanobacterial growth and biological process are widely-studied parameters. Higher temperature supports cyanobacterial growth compared with other phytoplankton. Temperature together with irradiance affects growth and toxin production by *N. spumigena* in the aquatic ecosystems (Hobson and Fallowfield, 2003). At higher temperature (30°C) with 30 µmol photons m^2s^{-1}, *N. spumigena* produces high cell biomass and intracellular NOD toxins (Hobson and Fallowfield, 2003).

Cyanobacteria are also salt tolerant organisms and can survive varying salinity ranges. The salinity also affects the growth and NOD produced by *N. spumigena.* However, extremes of salinity do not support growth of *N. spumigena* but promotes akinetes development with a decrease in heterocytes observed at 35 % (Mazur-Marzec et al., 2005). The fluctuation in salinity levels in the Baltic Sea depend on freshwater supply and temperature, precipitation and climate change (Dailidienė and Davulienė, 2008). These factors also affect the development of blooms (Moisander et al., 2002; Dailidienė and Davulienė, 2008). Salinity combined with light intensities affects the production of NOD (Hobson and Fallowfield, 2001; Mazur-Marzec et al., 2005), photosynthetic activities and N_2 fixation by *Nodularia* spp. (Hobson and Fallowfield, 2001; Moisander et al., 2002).

There is a knowledge gap regarding the effect of such environmental factors on the production of NOD and nodulopeptin 901. No research has been done on the effects of environmental

factors on chlorophyll-*a* concentration, cell biomass, extra and intracellular nodulopeptin 901 concentrations produced by *N. spumigena* KAC 66. In this study Chl-*a* was also estimated to evaluate photosynthetic capacity of *N. spumigena* KAC 66 during experiment under normal and stressed conditions, achieved by modifying either salinity or temperature.

MATERIALS AND METHODS

For the present study the cyanobacterial strain, *N. spumigena* KAC 66 was obtained from Kalmar Collection Centre, Department of Marine Sciences, Kalmar University, Sweden. This stain had been isolated from samples collected from Askö, Baltic Sea. To culture *N. spumigena* KAC 66 nitrate-free Allen's blue green algal medium, BG-11 prepared in 1/5 strength Instant Ocean artificial seawater (equivalent to 20‰ salinity; Allen and Stanier, 1968 modified by Stanier et al., 1971) was autoclaved (Astell Scientific, UK) for 15 minutes (volumes upto 2 L) and 1 h (volumes over 2 L) at 1 x 10^5 Pascals and 121°C (Kawachi and Noël, 2005). For the current investigation two different studies were performed, namely, temperature and salinity.

Temperature

To determine the effect of temperature on the chlorophyll-*a*, cell biomass, intra and extracellular NOD and nodulopeptin 901 production, three temperatures 22, 25 and 30°C were selected, which covered the range of the thermal optimum conditions for *N. spumigena* in the Baltic Sea (Sivonen et al., 1989b; Lehtimäki et al*.,* 1997; Musial and Plinski, 2003) at which the blooms frequently occur. Before starting the experiment the temperature controlled water baths were setup for one week and monitored to confirm a constant temperature adjusted at 22, 25 and 30°C. During the experiment the level of water baths was kept constant by adding Milli-Q water. Erlenmeyer flasks (500 mL) were filled with 350 mL BG-11(20‰ salinity) and autoclaved. After cooling 35 mL of one month old stock culture of *N. spumigena* KAC 66 was inoculated in each flask (cell biomass 43 $\mu g \cdot mL^{-1}$ and Chl-*a* 0.033 $\mu g \cdot mL^{-1}$). Triplicate flasks were incubated in respective temperature controlled water baths. The Erlenmeyer flasks had optimal salinity 20‰ at 22°C, was considered as controls in temperature experiment.

Salinity

In order to measure the effect of salinity (2, 7, 11, 20 and 25 ‰) a range of salinities was selected, based on optimal growth conditions of *N. spumigena* in the Baltic Sea (Wasmund, 1997; Musial and Plinkski, 2003; Gasiunaite et al*.,* 2005; Mazur-Marzec et al., 2005; MURSYS Reports 2003, 2005 and 2006). All treatments were prepared in triplicate and inoculated with

35 mL of one month old stock culture of *N. spumigena* KAC 66 (cell biomass 43 µg · mL^{-1} and Chl-*a* 0.033 µg · mL^{-1}). All experimental Erlenmeyer flasks containing different salinities were kept in temperature controlled room at 22°C. For salinity experiment the test flaks had optimal salinity 20‰, were considered as controls.

All experimental flasks for temperature and salinity studies were supplied by continuous slow aeration and constant illumination from two cool white fluorescent tubes (36 W) delivering 13 µmol photons m^{-2} s^{-1}.

Sampling procedure

The sampling procedure and analysis protocols for both experiments were same. The sampling was done on the day of inoculation and on a weekly basis for 6 weeks (0 and 1-6).

The samples were taken from Erlenmeyer flasks were sampled for 6 weeks to determine cell biomass, chlorophyll-*a*, intra and extracellular peptide levels (NOD and nodulopeptin 901).

To determine the cell biomass, 20 mL of the culture was filtered through pre-weighed GF/C glass microfiber filter discs (55 mm Ø, Whatman, UK). The filtrate was used for the determination of extracellular toxin levels.

The filter papers were freeze dried overnight in a freeze dryer (HSC 500, Modulyo, Edwards, UK) at -45ºC and 10^{-1} m bar. The next day filters were transferred to a desiccator overnight and weighed three times to obtain constant weight. The initial weight of the filter was subtracted to obtain the dry weight of cells, and the data converted to µg · mL µg · mL^{-1}. For chlorophyll-*a* estimation the weighed filter papers were transferred to -4°C until extraction. All filters were analysed within 2 months.

Chlorophyll-*a* measurement is a commonly used method to determine biomass (Lawton *et al.*, 1999). The amount of chlorophyll-*a* present within the samples on filter papers was determined spectrophotometrically by extracting in 5 mL of 100% methanol (Rathburn, Walkerburn, UK) in 25 mL universal bottles. The bottles were vortexed (Fisons, Whirlimixer, UK) and left in the dark for 1 hour to extract chlorophyll-*a*. Extracts were placed in centrifuge tubes (25 mL) and centrifuged in a refrigerated centrifuge (ALC4237, Italy) at 4°C for 10 min at 2000 *g*. To estimate the amount of Chl-*a*, absorbance was noted on a spectrophotometer (Spectrophotometer, UK) at a wavelength (λ) of 665 nm. Methanol was used as a blank and the amount of Chl-*a* was calculated (Murphy *et al.,* 2005 and 2009).

To measure extracellular and intracellular peptide levels 20 mL filtrate/spent medium (extracellular) of the sample was freeze-dried for analysis by UPLC-MS-PDA to detect the

presence and concentrations of any toxins released into the surrounding growth medium. The freeze dried spent medium was re-suspended in 1 mL 80% MeOH: H_2O (80:20, v/v) for one hour.

For intracellular peptide analysis, 1.5 mL was transferred into a micro-centrifuge tube (Eppendorf Centrifuge 5410, Germany) and centrifuged at 10,800 *g* for 10 min. The supernatant was discarded and the pellet vortexed with 150 µl MeOH (80%) and extracted for one hour. All extracts were stored at -20°C until HPLC- PDA-MS analyses.

High Performance Liquid Chromatography Photodiode Array Mass Spectrometry (HPLC-PDA-MS)

Identification and quantification of NOD and nodulopeptin 901 was performed using HPLC-PDA-MS. All extracts were centrifuged at 10,800 *g* for 10 min and 100 µl supernatant was transferred into an LC-MS vial. The system combined a Waters Alliance 2695 solvent delivery system, photodiode array detector (PDA, model 2996) and mass detector (ZQ 2000 MS), all supplied by Waters (Elstree, UK). The separation of peptides was achieved on a Sunfire C_{18} column (Waters, Elstree, UK; 5 µm particle size; 2.1 mm i.d. 150 mm long) maintained at 40°C. The mobile solvent phase A was Milli-Q water with 0.05% (v/v) trifluoroacetic acid (TFA; Fisher Scientific, UK) and mobile solvent phase B was acetonitrile (Fisher Scientific, UK) with 0.05% TFA (v/v). Samples and standards were separated using a gradient increasing from 15 to 60% B for 25 minutes at a flow rate of 0.3 mL · min^{-1} followed by ramp up to 100% B and re-equilibration after 10 minutes. Mass spectrometry was performed in positive ion electro-spray mode (ESI+), scanning from m/z 100 to 1200 with a scan time of 2 seconds and inter-scan delay of 0.1 second ion source parameters. The sprayer voltage was set at 3.07 kV and cone voltage 80 V. The source temperature and desolvation temperatures were 100°C and 300°C, respectively. MassLynx software v4.0 was used to control the instrument for data acquisition and processing. The photo diodearray (PDA) was set to a resolution of 1.2 nm and data acquired from 200 to 400 nm. The injection volume for standards and samples was 10 and 20 µl, respectively. Quantification of peptides was based on calibration with external standards, NOD at 238 nm and nodulopeptin 901 at 210 nm.

RESULTS

a. Temperature

The Chl-*a* concentrations for *N. spumigena* at 20°C was very low (0.1 µg · mL^{-1}) in week 1 but as time progressed the concentration of chlorophyll-*a* increased and by week 6 it had risen to 0.19 µg · mL^{-1}. Total Chl-*a* concentrations during the experiment at 25°C showed an increase in weeks 3 and 4 (0.17 µg · mL^{-1}) and then declined by week 6 (0.13 µg · mL^{-1}). At the highest temperature (30°C) the maximum concentration was observed at week 4 (0.21 µg · mL^{-1}) and a considerable decline noted by week 6 (0.12 µg · mL^{-1}; Fig. 1D-1F). The cell biomass of *N. spumigena* KAC 66 was high during week 3 at 22°C (76.7 µg · mL^{-1}) and 25°C (71.7 µg · mL^{-1}; Figs. 1A and 1B). A decline (60.0-31.7 µg · mL^{-1}) in biomass was noted at the highest temperature 30°C from week 3 to week 6 (Fig. 1C).

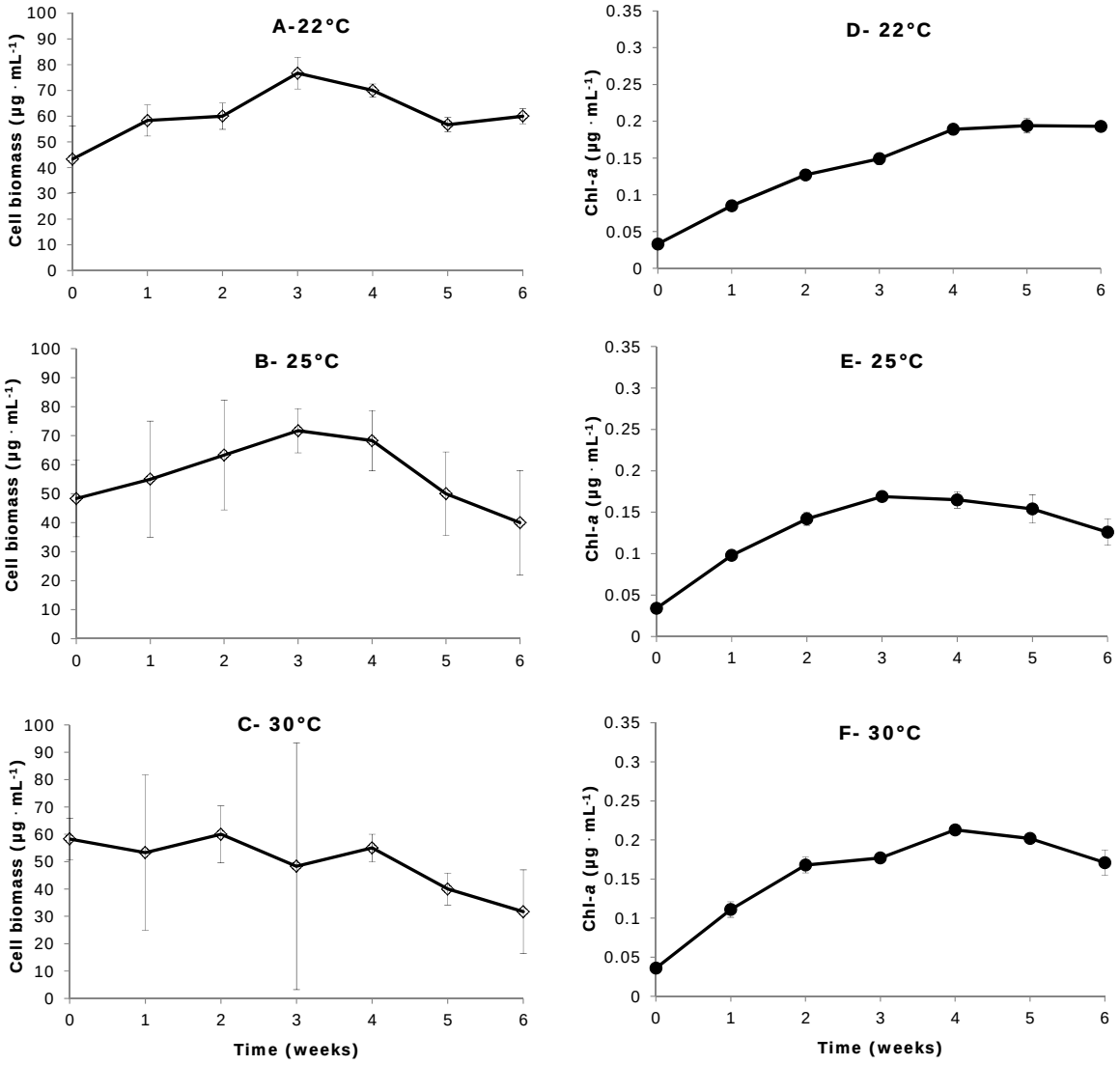

Fig. 1. Cell biomass (A-C) and Chlorophyll-*a* (D-F) concentrations at different temperatures for cultures of *N. spumigena* KAC 66 grown for 6 weeks (n=3, bars=1 SD).

In comparison, both 22°C and 25°C supported the high production extra and intracellular NOD (intracellular) and nodulopeptin 901 (see supplementary data; Fig. S1).

No extracellular NOD was observed regardless of the growth temperature. The production of intracellular NOD at 22°C was low during week 2 and 3 (472-851 ng · mL^{-1}), after which the strain grew better in week 4 and produced the maximum amount of intracellular NOD (1,061 ng/mL), while the maximum values for 25°C (473 ng · mL^{-1}) and 30°C (46.7 ng · mL^{-1}) were observed at week 3. At 30°C from week 4 to 6 a complete disappearance of intracellular NOD contents was noted (Fig. 2A-2C).

The lowest extra and intracellular nodulopeptin 901 levels were noted from week 1 to 3 at all temperatures. The nodulopeptin 901 levels were increased in the surrounding medium and within the cells at later time points. At 25°C, week 5, the highest amounts of extra (347 ng · mL^{-1}) and intracellular (488 ng · mL^{-1}) nodulopeptin 901 were observed (Fig. 2D-2F).

In general, at elevated temperatures (25 and 30°C) lower NOD was recorded as compared to 22°C. A considerable decrease in the production of nodulopeptin 901 was noted at high temperature (30°C) in both extra and intracellular levels. During the experiment no NOD was released in growth medium. The highest proportion of nodulopeptin 901 found intracellularly, occurred at the lowest test temperature. The maximum percentages of intracellular nodulopeptin 901 were recorded in week 3 at 22°C (84%) and 25°C (70%), while the highest percentage was noted at week 2 at 30°C (55%).

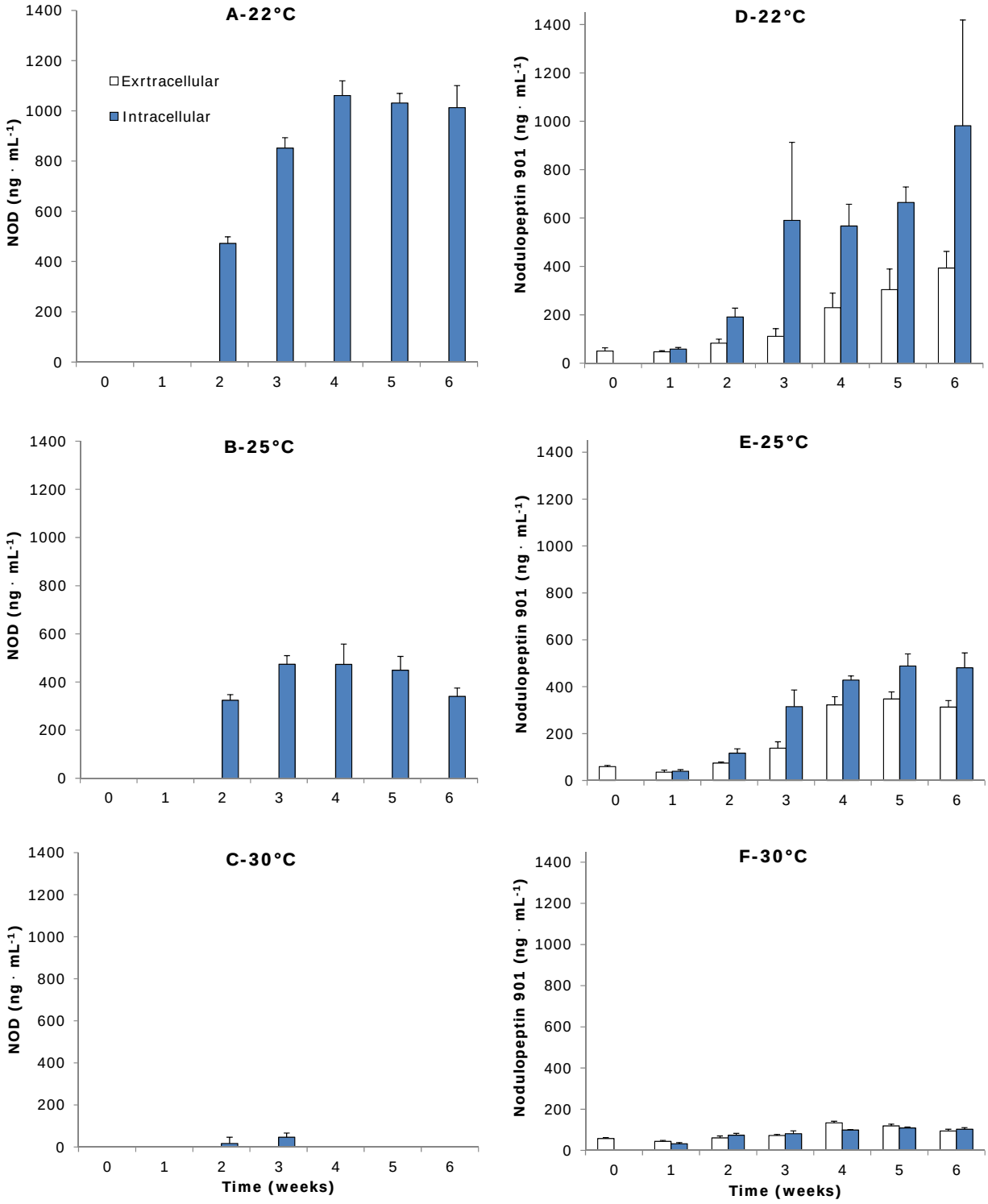

Fig. 2. The intra and extracellular levels of NOD (A-C) and nodulopeptin 901 (D-F) at different temperatures for cultures of *N. spumigena* KAC 66 grown for 6 weeks (n=3, bars=1 SD).

b. Salinity

In general, there was little difference between cell biomass and chlorophyll-*a* concentration at different salinities (Fig. 3).

At 2, 11 and 20‰ cell biomass were found to have a relation with time, which showed a normal growth trend and a gradual increase of biomass was observed during growth. In weeks 5 and 6 the highest cell biomass was recorded for 2, 7, 11 and 20‰ salinity, ranged from 1,207-1,740 $\mu g \cdot mL^{-1}$. Elevated salinity (25‰) suppressed the growth of this strain and the highest cell biomass (973 $\mu g \cdot mL^{-1}$) was noted in week 3, which was the lowest cell biomass among all salinities (Fig. 3A-3E). It seems that variations in salinities have a substantial effect on cell biomass.

At all salinities, chlorophyll -*a* concentrations started increasing from weeks 1 to 4, and then declined by week 5 and 6. The results show that the concentrations of chlorophyll-*a* at 2, 11, 20 and 25‰ was decreased during the growth period except at 7‰ (Fig. 3F-3J).

The effect each salinity condition had on NOD production is represented in Fig. 4. The intracellular NOD showed an increased level in cultures subjected to 2, 7 and 11 and 25‰ in week 5 and declined by week 6, only cultures at 20‰ showed a maximum level of NOD from week 3 to week 5. Increasing salinity from 2 to 11 ‰ was found to have a pronounced effect on the total amount of intracellular NOD production, but the highest amount was found in 2‰ salinity during weeks 4 and 5, ranging from 1.5-1.7 $\mu g \cdot mL^{-1}$. In weeks 1 and 2 a relatively low amount of intracellular NOD was observed in all cultures, while the lowest detectable amount (205 $ng \cdot mL^{-1}$) was recorded in week 6 at 25‰. The extracellular NOD contents varied from week 3 to 6 at all salinities. In this experiment relatively low extracellular NOD was detected in the elevated salt conditions (25‰). During the whole course of the experiment all salinities tested had a substantial effect on the production of intra and extracellular nodulopeptin 901 (Fig. 5).

At 25‰, in week 5, maximum concentrations of the intracellular nodulopeptin 901 were found (447 $ng \cdot mL^{-1}$) and declined in week 6 (344 $ng \cdot mL^{-1}$). However, culture conditions which supported the best yield of intracellular nodulopeptin 901 were salinities of 7 and 11‰

Due to the low concentration of cells in newly inoculated cultures no undetectable intracellular peptides were observed. During the experiment at different salinities the peptides were mainly intracellular, with 89-100 % of NOD from week 1 to 6 and from week 2-6, 50-68% nodulopeptin 901 was intracellular. In week 2 at elevated salt concentration (25‰) an equilibrium between intra and extracellular nodulopeptin 901 (50:50%) was observed.

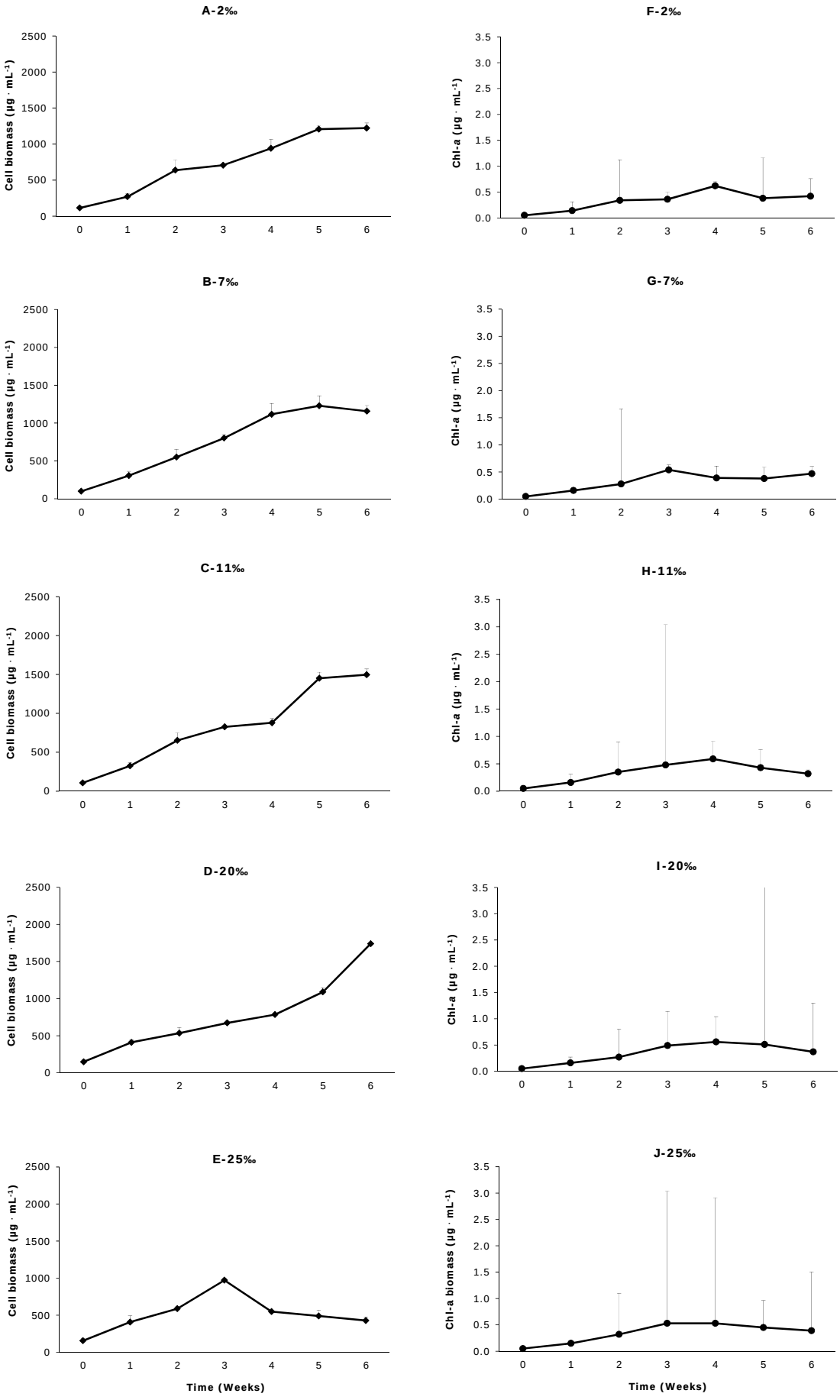

Fig. 3. Cell biomass (A-E) and Chl-*a* (F-J) for cultures of *N. spumigena* KAC 66, grown at different salinities for 6 weeks at 22°C (n=3, bars=1 SD).

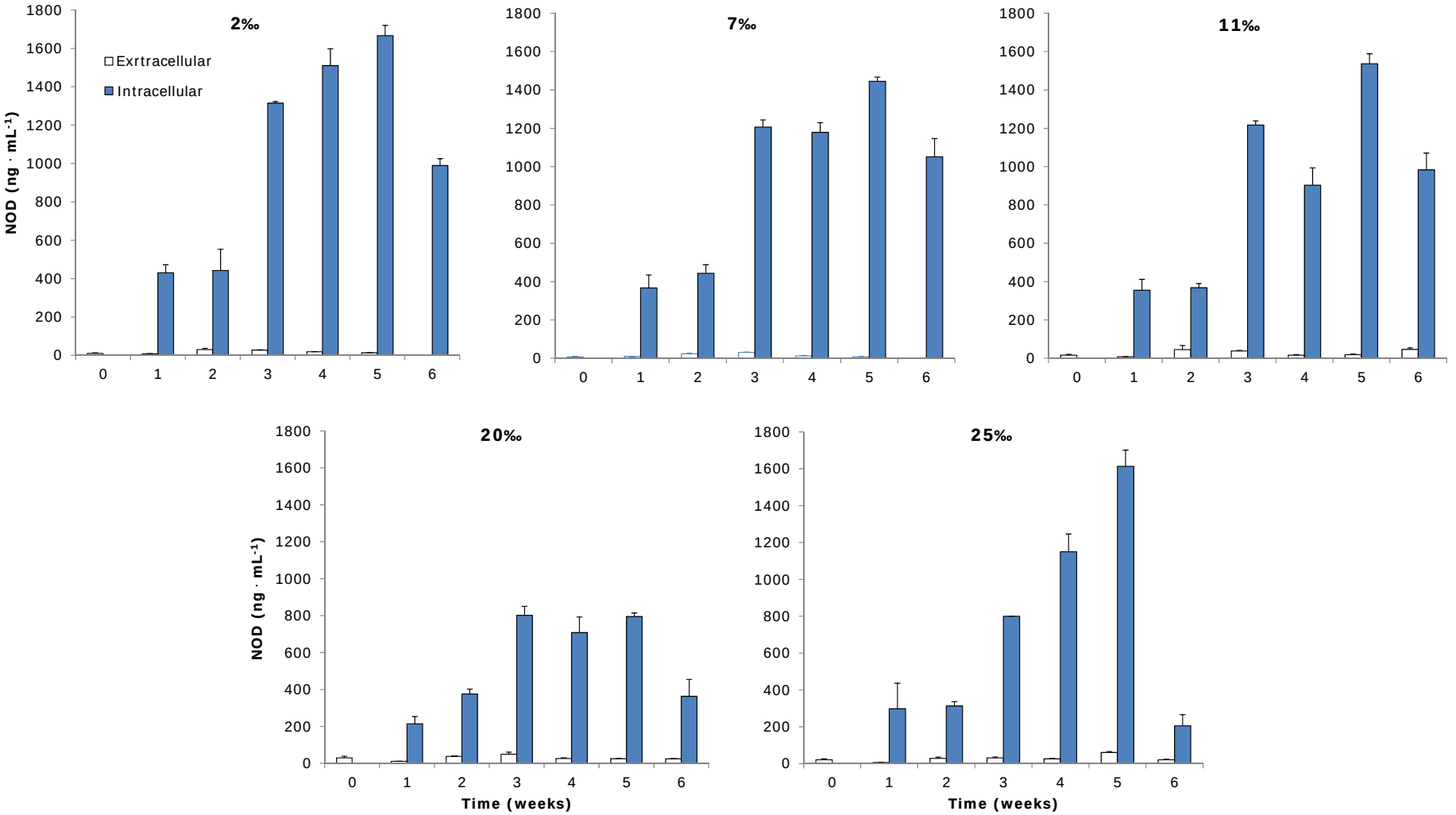

Fig. 4. The Intra and extracellular levels of NOD at different salinities for cultures of *N. spumigena* KAC 66 grown for 6 weeks at 22°C (n=3, bars=1 SD).

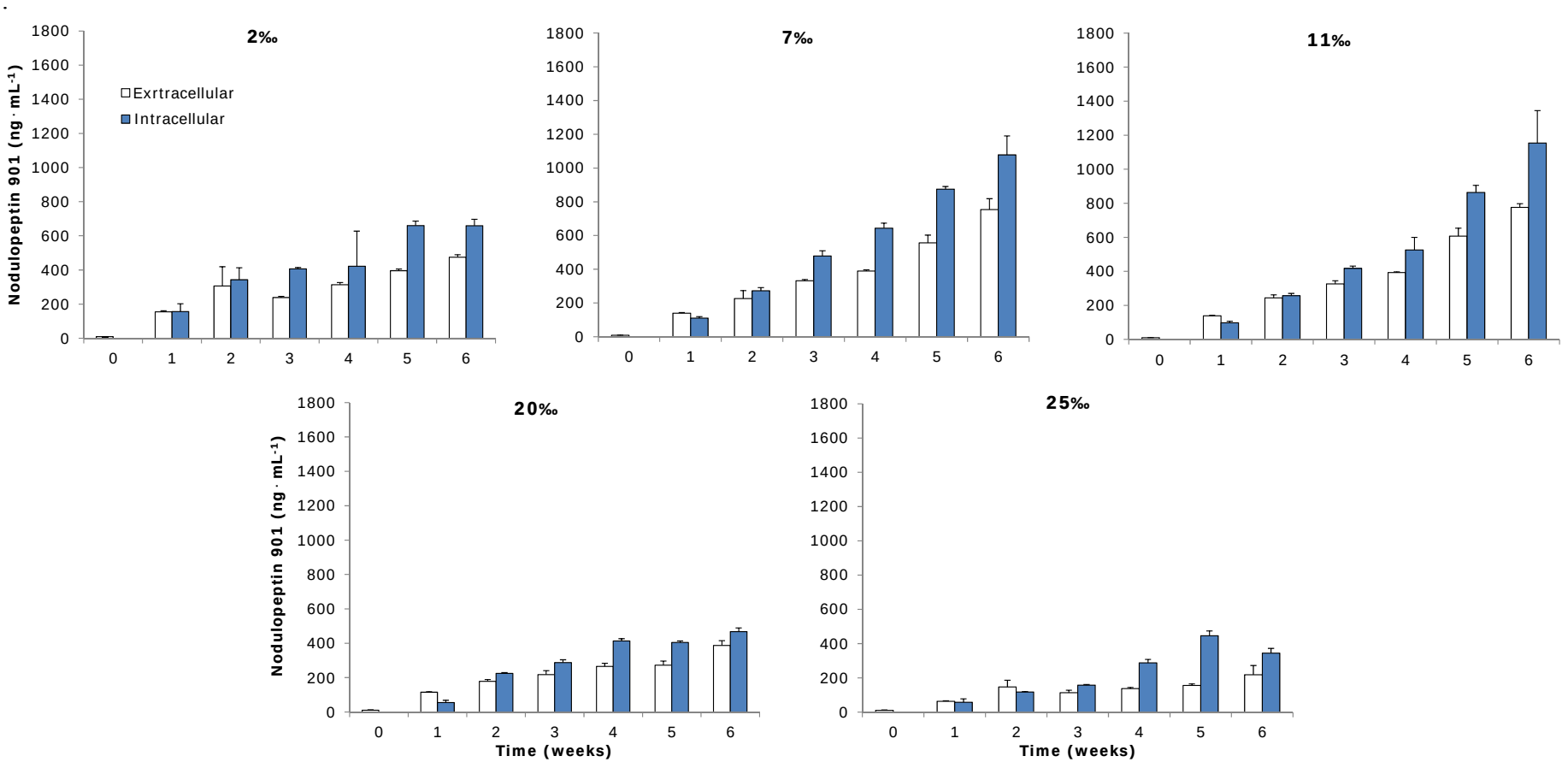

Fig. 5. The Intra and extracellular levels of nodulopeptin 901 at different salinities for cultures of *N. spumigena* KAC 66 grown for 6 weeks at 22°C (n=3, bars=1 SD).

DISCUSSION

The release and production of cyanobacterial peptides is affected by meteorological and physiological conditions (Veize et al., 2002). Salinity, light and temperature are commonly studied environmental parameters and are thought to affect the concentration of toxins produced by *N. spumigena* (Sivonen and Jones, 1999 and Stolte et al., 2002). Among all parameters, temperature is a major factor to control the growth and toxin production ability of cyanobacteria. In the present study temperature was observed to influence chlorophyll-*a* concentrations and peptide productions. In this investigation *N. spumigena* grew well at all temperatures and at 30°C the highest chlorophyll-*a* concentration was recorded. According to Lehtimäki et al. (1997) *N. spumigena* can grow fast at a temperature of 25 to 28 °C and shows a tolerance to survive at much higher temperatures with slower growth at temperatures below 16 °C (Lehtimäki et al., 1997).

This study has shown that the high temperatures do not influence the release of nodulopeptin 901 but do have an effect on the total amount of peptide. The lowest temperature (22°C) supported the highest concentrations of extra and intracellular nodulopeptin 901.

Hobson and Fallowfield (2003) worked on the combined effects of light and temperature on total NOD and biomass produced by *N. spumigena* 001E. They showed that high temperature (30°C) and low temperature (20°C) enhance the production of high intracellular NOD and biomass. Sivonen and Jones (1999) also suggested that at 18-25 °C several cyanobacterial strains produce most toxins. The results from the present study have shown that the optimum growth temperature (22°C) is associated with high intracellular NOD and nodulopeptin 901 productions. The high temperature (30°C) did not support high production of NOD concentration.

Watanabe et al. (1985), Van der Westhuizen et al. (1986 and 1985) and Lehtimäki et al. (1997) also observed that the high temperature causes stress having an effect on the production of toxins and production of NOD (Lehtimäki et al, 1994). Higher temperature inhibits/slows the growth and production of toxins and metabolic activity results in cells being stressed, (Graham, 2007). It is observed that high temperature had negative effects on extracellular NOD and positive on intra and extracellular nodulopeptin 901. The filamentous cyanobacterium, *O. agardhii* also showed the same response towards biotic and abiotic factors influencing hepatotoxin production (Sivonen, 1990). At high temperature most of microcystin-RR was retained within the cells and leakage of hepatotoxins only occurred when cells die and there is cell lysis.

Salinity is also an important environmental factor to control the growth and toxin production of all cyanobacterial strains. In the present study, 11 and 20‰ supported the growth of *N. spumigena,* while it decreased the growth rate at higher salt concentration (25‰). Many cyanobacterial strains have acclimation mechanisms to survive under hypersaline conditions (Mazur-Marzec et al., 2005). The results obtained from the present study showed that all salinities had an effect on biomass and the total amount of NOD produced over time. The investigation found the high intracellular NOD contents at 2-11‰. Lehtimäki et al., (1997) indicated that the maximum growth rate of *N. spumigena* BY1, collected from the Baltic Sea, was found at 10 psu (≈10‰) while 15 psu (≈15‰) favoured the highest production of intracellular NOD at 15 psu (≈15‰). Mosiandar et al., (2002) demonstrated that *N. spumigena* FL2f collected from the Baltic Sea, did not show any change in growth rate when treated with salinities, ranged from 0-20 psu (≈0-20‰) NaCl. Horstman (1975) indicated that optimal growth of *N. spumigena* from the Baltic Sea was recorded at 5-15 psu (≈5-15‰). It is concluded that the salt tolerance of *N. spumigena* varies and depends on strains, and on where they collected.

In this study at the lowest salinity (2‰) the highest concentration of chlorophyll-*a* was observed. Lehtimäki et al., (1997) also supported this result that at low salinity (10‰) *N. spumigena* produced the highest amount of chlorophyll -*a*. Blackburn et al., (1996) worked on six strains of *N. spumigena* collected from various locations of Australia and noticed that the maximum growth of strains was recorded at 12‰. This study also confirms that *N. spumigena* KAC 66 produces the highest biomass at 1‰.

At all salinities chlorophyll -*a* contents and increasing intracellular NOD levels increased initially and then decreased as time progressed. These results are supported by Stal et al., (1999). They estimated NOD contents by NOD/ chlorophyll-*a* ratios and found that increased salinity has an effect on increased NOD and chlorophyll -*a* concentrations.

When *N. spumigena* KAC 66 was treated with different salinities an increase in cell biomass was observed. Increasing salinity did not support toxin production and also showed correlation with chlorophyll -*a* concentrations (Carmichael et al., 1988). Lehtimäki et al., (1997) worked on effect of salinities (0-30‰) on chlorophyll-*a* contents and dried cell biomass of *N. spumigena.* They noticed that dried cell biomass does not have any correlation with salinity, while chlorophyll-*a* has positive correlation with salinity. Stal *et al.* (1999) found considerable effects of salinity on growth and NOD production. The maximum growth was recorded between 7-18 psu (≈7-18‰) and lower

at 3 and 24 psu (≈3 and 24‰). While the growth was strongly inhibited at 0 and 35 psu (≈35‰). At higher salinities (20 and 25‰) the cultures underwent stressed conditions and a high amount of NOD was not produced (Blackburn et al., 1996) or degraded. In the current study it was noticed that the low salinities (2 to 11‰) enhanced the production of extra and intracellular nodulopeptin 901 and intracellular NOD levels. Hobson and Fallow field (2003) indicated that *N. spumigena* from Lake Alexandria, Australia produces high amount of intracellular hepatotoxins at salinities ranged from 0.36 and 26.4 ‰. They also observed that salinity has an effect on cell numbers, optical density, chlorophyll-*a* concentration, dry biomass and growth rate. They also speculated that *N. spumigena* NSG 0897 grows well in salinities ranged from 4-16 psu (≈4-16‰), while 8 psu (≈8‰) was the best salinity to obtain the optimal growth of this strain.

In conclusion, the biological activity of cyanobacteria appears to be dependent upon growth conditions and therefore, the performance of toxin producing organisms may be improved by altering the cultural conditions. It is recommended that 22°C is the best temperature to obtain maximum amount of intracellular NOD and intracellular and extracellular nodulopeptin 901. The culturing of *N. spumigena* KAC 66 in salinity ranging from 11 to 20‰ was found to yield a high amount of extra and intracellular peptides and biomass. It is also predicted that when the temperature and salinity of the Baltic Sea reaches to 22°C and 11 to 20‰ some precautions should be taken to control prospective blooms of toxic stain of *N. spumigena*.

References

Allen MM, Stanier RY (1968) Growth and division of some unicellular blue green algae. J Gen Microbiol 51:199-202.

Blackburn SI, McCausland MA, Bolch CJS, Newman SJ, Jones GJ (1996) Effect of salinity on growth and toxin production in cultures of the bloom-forming cyanobacterium *Nodularia spumigena* from Australian waters. Phycologia 35:511–522.

Carmichael WW, Escedor JT, Patterson GM, Moore RE (1988) Toxicity and partial structure of a hepatotoxic peptide produced by the cyanobacterium *Nodularia spumigena* Mertens emend. L575 from New Zealand. Appl Environ Microbiol 54: 2257–2263.

Dahlmann J, RuÈhla, A., Hummerta, C., Liebezeit, G., Carlssonc, P. and GraneÂli, E., (2001) Different methods for toxin analysis in the cyanobacterium *Nodularia spumigena* (Cyanophyceae). Toxicon, 39, 1183-1190.

Dailidienė I, Davulienė L (2008) Salinity trend and variation in the Baltic Sea near the Lithuanian coast and in the Curonian Lagoon in 1984-2005. J Marine Syst 74:S20-S29.

Edler L, Fernö S, Lind NG, Lundberg R, Nilsson PO (1985) Mortality of dogs associated with a bloom of the cyanobacterium *Nodularia spumigena* in the Baltic Sea. Ophelia 24:103-109.

Falconer IR (2001) Toxic cyanobacterial bloom problems in Australian waters: risks and impacts on human health. Phycologia 40:228-233.

Fujii K, Sivonen K, Adachi K, Noguchi K, Sano H, Hirayama K, Suzuki M, Harada K-I (1997) Comparative study of toxic and non-toxic cyanobacterial products: novel peptides from toxic *Nodularia spumigena* AV1. Tetrahedron Lett 38:5525–5528.

Gasiunaite ZR, Cardoso AC, Heiskanen A-S, Henriksen P, Kauppila P, Olenina I, Pilkaityt_R, Purina I, Razinkovas A, Sagert S, Schubert H, Wasmund N (2005) Seasonality of coastal phytoplankton in the Baltic Sea: Influence of salinity and eutrophication. Estuar Coast Shelf Sci 65:239-252.

Graham DJL (2007) An investigation into factors influencing the production and degradation of microcystis. Dissertation, The Robert Gordon University, Aberdeen, UK Pp 204.

Gupta N, Bhaskar ASB, Lakshmana RPV (2002) Growth characteristics and toxin production in batch cultures of *Anabaena flos-aquae*: effects of culture media and duration. World J Microbiol Biotechnol 18:29–35.

HELCOM (2007) Climate Change in the Baltic Sea Area – HELCOM Thematic Assessment in 2007. Balt. Sea Environ. Proc. No. 111, p 48

Hobson P, Fallowfield H (2001) Effect of salinity on photosynthetic activity of *Nodularia spumigena.* J Appl Phycol 13:493-499.

Hobson P, Fallowfield HJ (2003) Effect of irradiance temperature and salinity on growth and toxin production by *Nodularia spumigena.* Hydrobiologia 493:7–15.

Hobson P, Burch M, Fallowfield HJ (1999) Effect of salinity on photosynthetic activity of *Nodularia spumigena.* J Appl Phycol 13:493–499.

Horstmann U (1975) Eutrophication and mass occurrence of blue-green algae in the Baltic Merentutkimuslaitoksen Julk (Havsforskningsinst Skr) 239:83-90.

Ibelings BW, Chorus I (2007) Accumulation of cyanobacterial toxins in freshwater ‘‘seafood’’ and its consequences for public health: A review. Environ Pollut 150:177-192.

Kankaanpaa H, Vuorinen PJ, Sipia V, Keinanen M (2002) Acute effects and bioaccumulation of nodularin in sea trout (*Salmotruttam Trutta* L) exposed orally to *Nodularia spumigena* under laboratory conditions Aquatic. Toxicology 61:155e168.

Karlsson K, Sipia V, Kankaanpaa H, Meriluto J (2003) Mass spectrometric detection of nodularin and desmethylnodularin in mussels and flounders. J Chromatogr B 784:243-253.

Kawachi M, Noël MH (2005) Sterilization and sterile technique In: R A Andersen (Ed) Algal culturing techniques, Elsevier Academic Press, UK, Pp 565.

Lawton AL (1999) Determination of cyanobacteria in the laboratory. In I Chorus and Bartram J (Eds) Toxic cyanobacteria in water: A guide Toxic cyanobacteria in water: a guide to their public health consequences monitoring and management, E & FN., Spon, London Pp 347-366.

Lehtimäki J, Moisander P, Sivonen K, Kononen K (1997) Growth nitrogen fixation and nodularin production by two Baltic Sea cyanobacteria. Appl Environ Microbiol 63:1647–1656.

Lehtimäki J, Sivonen K, Luukkainen R, Niemela SI (1994) The effects of incubation time temperature light salinity and phosphorus on growth and hepatotoxin production by *Nodularia* strains. Arch Hydrobiol 13:269–282.

Lilover MJ, Stips A (2008) The variability of parameters controlling the cyanobacteria bloom biomass in the Baltic Sea. J Marine Syst 74: S108–S115.

Mazur-Marzec H, Żeglińska L, Pliński M (2005) The effect of salinity on the growth toxin production and morphology of *Nodularia spumigena* isolated from the Gulf of Gdańsk southern Baltic Sea. J Appl Phycol 17:171-179.

Mazur-Marzec H, Kaczkowska MJ, Blaszczyk A, Akcaalan R, Spoof L, Meriluoto J (2013) Diversity of Peptides Produced by *Nodularia spumigena* from Various Geographical Regions. Mar Drugs 11:1-19.

Moisander PH, McClinton E, Paerl H (2002) Salinity effects on growth photosynthetic parameters and nitrogenase activity in estuarine planktonic cyanobacteria. Microb Ecol 43:432-442.

Murphy RJ, Underwood AJ, Pinkerton MH, Range P (2005) Field spectrometry: New methods to investigate epilithic micro-algae on rocky shores. J Exp Mar Biol Ecol 325:111–124.

Murphy RJ, Tolhurst TJ, Chapman MG, Underwood AJ (2009) Seasonal distribution of chlorophyll on mudflats in New South Wales Australia measured by field spectrometry and PAM fluorometry Estuar Coast Shelf Sci 84:108–118.

MURSYS (2003) North Sea and Baltic Sea - Report 2.
(http://www.bsh.de/en/Marine_data/Observations/MURSYS_reporting_system/mursys1203.jsp)

MURSYS 2005- North Sea and Baltic Sea - Report 2.
(http://www.bsh.de/Vorlagen/ressources/Druckversion.jsp?_PRINTPAGE_=yes&_PRINTOID_=51997&)

MURSYS 2006- North Sea and Baltic Sea - Report 2.
(http://www.bsh.de/en/Marine_data/Observations/MURSYS_reporting_system/mursys0207.jsp)

Musial A, Plinski M (2003) Influence of salinity on the growth of *Nodularia spumigena* Mertens. Oceanol Hydrobiol St 2:45-52.

Persson P-E, Sivonen K, Keto J, Kononen K, Niemi M, Viljamaa H (1984) Potentially toxic blue-green algae (cyanobacteria) in Finnish natural waters. Aqua Fenn 14:147-154.

Rinehart KL, Harada K, Namikoshi M, Chen C, Harvis CA, Munro MHG, Blunt JW, Mulligan PE, Beasley VR, Dahlem AM, Carmichael WW (1988) Nodularin microcystin and the configuration of ADDA. J Am Chem Soc 110:8557-8558.

Schumacher M, Wilson N, Tabudravu J, Edwards C, Lawton L, Mottid C, Wright T, Diederich M, Jaspars M (2012) Novel polypeptides from *Nodularia Spumigena* KAC66. Tetrahedron 68:1622-1628.

Sivonen K (1996) Cyanobacterial toxins and toxin production Phycologia 35:12-24.

Sivonen K, Jones G (1999) Cyanobacterial Toxins In I Chorus and Bartram J (Eds) *Toxic* Cyanobacteria in Water A guide to their public health consequences monitoring and management E & FN Spon Press London, Pp 41-111.

Sivonen K (1990) Effects of light temperature nitrate orthophosphate and bacteria on the growth of the hepatotoxin by *Oscillatoria agardhii* strains. Appl Environ Microbiol 56:2658-2666.

Sivonen K, Kononen K, Carmichael WW, Dahlem AM, Rinehart KL, Kiviranta J, Niemelä SI (1989a) Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and the structure of the toxin. Appl Environ Microbiol 55:1990-1995.

Sivonen K, Kononen K, Esala AE, Niemelä SI (1989b) Toxicity and isolation of the cyanobacterium *Nodularia spumigena* from the Southern Baltic Sea. Hydrobiologia 185:3-8.

Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular bluegreen algae (Order Chroococcales) Bacteriol Rev 35:171205.

Stal LJ, Staal M, Villbrandt M (1999) Nutrient control of cyanobacteria blooms in the Baltic Sea Aquat Microb Ecol 18:165–173.

Stolte W, Karlsson C, Carlsson P, Graneli E (2002) Modelling the increase of nodularin content in Baltic Sea *Nodularia spumigena* during stationary phase in phosphorus-limited batch cultures FEMS Microb Ecol 41:211–220.

Suikkanen S, Kaartokallio H, Hällfors S, Huttunen M, Laamanen M (2010) Life cycle strategies of bloom-forming filamentous cyanobacteria in the Baltic Sea. Deep Sea Res Part II Top Stud Oceanogr 57:199-209.

Utkilen HA, GJolme N (1992) Toxin Production by *Microcystis aeruginosa* as a function of light in continuous cultures and its ecological significance. Appl Environ Microbiol 58:1321-1325.

Van der Westhuizen AJ, Eloff JN (1985) Effect of temperature and light on the toxicity and growth of the blue-green alga *Microcystis aeruginosa* (UV-006). Planta 163:55-59.

Van der Westhuizen AJ, Eloff JN, Krüger GH (1986) Effect of temptature and light (fluence rate) on the composition of the toxin of the cyanobacterium *Microcystis aeruginosa* (UV-006) Arch Hydrobiol 108:145-54.

Vezie C, Rapala J, Vaitomaa J, Seitsonen J, Sivonen K (2002) Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microb Ecol 4:443-454.

Wasmund N (1997) Occurrence of cyanobacterial blooms in the Baltic Sea in relation to environmental conditions. Intern Revue Hydrobiol Hydrogr 82:169–184.

Watanabe MF, Oishi S (1985) Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. Appl Environ Microbiol 49:1342-1344

Supplementary data

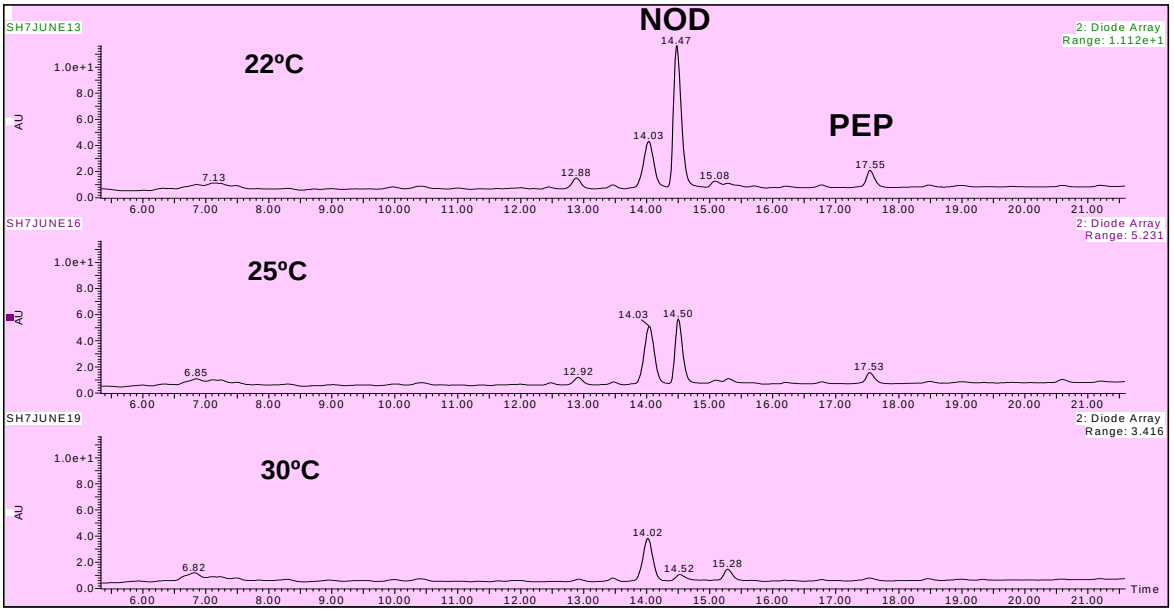

Fig. S1: The chromatograms show the effects of different temperatures on the production of intracellular NOD and nodulopeptin 901 for *N. spumigena* KAC 66 grown for 6 weeks.