INVESTIGATION OF THE PRODUCTION AND ISOLATION OF BIOACTIVE COMPOUNDS FROM CYANOBACTERIA

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A thesis submitted in partial fulfilment of the requirements of the Robert Gordon University for the degree of Doctor of Philosophy

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DECLARATION

This thesis, which is submitted for consideration for the degree of Doctor of Philosophy, is a record of research carried out in the School of Pharmacy and Life Sciences, The Robert Gordon University, under the supervision of Prof. Linda Lawton and Dr. Christine Edwards.

I declare that the work presented in this thesis is my own, except where used published or unpublished work, which has been acknowledged. My work is believed to be original and has not been previously submitted by me in any other institution for any degree.

SHAISTA HAMEED

DEDICATED

To my beloved mother, who could not stay to see it.

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ABBREVIATIONS

16S rDNA	16S ribosomal deoxyribonuclic acid
ADP	Adenosine diphosphate
ABPN	Anabaenopeptins
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl deca-4,6-dienoic acid
ANA	Anabaenopeptin A
ANB	Anabaenopeptin B
AnTx-a	Anatoxin-a
AnTx-a(s)	Anatoxin-a (salivation)
Aph	3-amino-6-hydroxy-piperidone
Asp	Aspartic acid
ASM	Growth medium
ASTM	Growth medium
ATP	Adenosine triphosphate
BAPNA	N-α-benzol-DL-arginine-*p*-nitroanilide
BG-11	Blue green algal growth medium
BSA	Bovine serum albumin
Chl-*a*	Chlorophyll-*a*
CH	Chalkley's medium
CYN	Cylindrospermopsin
Da	Dalton
D-Lys	D-Lysine
DNA	Deoxyribonucleic acid
ESI+	Positive ion electro-spray mode
Glu	Glutamic acid
HAB	Harmful algal blooms
HELCOM	Helsinki Commission (Baltic Marine Environment Protection Commission)
HIV virus	Human immunodeficiency virus
Hpla	Hydroxy-phenyl lactic acid
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
ISOC-HAB	International Symposium on Cyanobacterial Harmful Algal Blooms

KAC	Kalmar Algal Collection
lb	Pounds
LC-MS	Liquid chromatography-mass spectrometry
LC_{50}	Half maximal inhibitory lethal concentration
L-MeAla	L-Methyl alanine
LNOD	Linear nodularin
L-Phe	L-Phenylalanine
-LR	Leucine-arginine
L-Tyr	L-Tyrosine
L-Val	L-Valine
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
MC	Microcystin
Mdha	N-methyldehydroalanine
Mdhb	2-(*N*-methyleamine)-2-dehydrobutyric acid
MeAsp	D-erythro-β-methylaspartic acid
MeOH	Methanol
M+H	Molecular mass of the neutral amino acid residue + hydrogen
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass-to-charge ratio
N_2 fixing	Nitrogen fixing
NeoSTx	Neosaxitoxin
NRC	National Research Council, Canada
NOD	Nodularin
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
PDA	Photodiode array detector
pNPP	*para*-nitrophenyl phosphate
PP1	Protein phosphatase type 1
PP2A	Protein phosphatase type 2A
PSP	Paralytic Shellfish Toxins
PSU	Practical salinity units
rpm	Revolutions per minute

RNA	Ribonucleic acid
RPFC	Reversed phased flash chromatography
SDC	Serial dilution culture method
STx	Saxitoxin
TFA	Trifluoroacetic acid
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol hydro cholroic acid
UPLC-PDA-MS	Ultra performance liquid chromatography-photodiode array-mass spectrometry
UV	Ultraviolet
v/v	Volume/volume
VFDF	Very fast death factor
wt/vol	Weigh/volume
-YA	Tyrosine-alanine
-YM	Tyrosine-methionine
-YR	Tyrosine-arginine
Z8	Growth medium

ABSTRACT

Name: Shaista Hameed
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Title: Investigation of the production and isolation of bioactive compounds from cyanobacteria

Due to heavy nutrient load and adverse climate change the occurrence of toxic cyanobacterial blooms have significantly increased during the last decades. *Nodularia spumigena* is one of the dominant toxic cyanobacteria which produces massive and inherent blooms in brackish water body, the Baltic Sea, particularly in late summer. *Nodularia* spp. are known to produce nodularins (NOD) and a range of other bioactive peptides such as spumigins and nodulopeptins, all of which have unclear function. In a recent study, three new nodulopeptins with molecular weight of 899, 901 and 917 were characterised from *N. spumigena* KAC 66.

In the present study, *N. spumigena* KAC 66 was fractionated by reversed phase flash chromatography and their toxicity was determined by their lethality to *Daphnia pulex* and *D. magna* along with inhibition of protein phosphatase 1 assay (PP1). All fractions showed lethality to Daphnids and inhibitory activity against PP1, the toxicity was due to additional compounds as NOD and nodulopeptin 901 were only detected in 7 fractions. Pure NOD was lethal to *D. pulex* and *D. magna* LC_{50}= 8.4 µg/mL and 5.0 µg/mL, respectively. The newly characterised nodulopeptin 901 was also tested against *D. magna* (LC_{50}=>100 µg/mL). NOD and nodulopeptin 901 inhibited PP1 with IC_{50} 0.038 µg/mL and 25 µg/mL, respectively.

In common with many studies, the maximum amount of NOD was retained within the cells during the seven week growth experiment. In contrast, as much as ~50% of nodulopeptin 901 was detected in the growth media throughout the duration of experiments.

To gain further insight on the effects of environmental stress on growth and production of bioactive metabolites in *N. spumigena* KAC 66, a range of parameters were investigated which included; temperature, salinity, nitrate and phosphorus.

In the present study it was investigated that extreme growth conditions have a considerable effect on biomass and toxin levels by *N. spumigena* KAC 66.

The light intensity ranged from 17.35-17.47 µmol/s/m^2, 22°C and 11-20 ‰ of salinity were the optimal growth conditions to obtain maximum biomasses, intra and extracellular peptide contents. At 6.5 mg/L nitrate the maximum growth, as indicated by Chl-*a* and maximum concentrations of intracellular NOD and nodulopeptin 901 were detected found in week 5 and 4, respectively.

Temperature had the greatest effect on peptide production. Whilst growth was similar at 22°C, 25°C and 30°C, increase in temperature had a profound effect on NOD production in that an increase from 22°C to 25°C resulted 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, they were still detected at consistent levels suggesting they play an important role.

The results from phosphate experiment showed Chl-*a*, cell biomass and peptide production did not show clear dependency on availability of PO^{-3}_4.

This is the first study to evaluate the effects of selected environmental parameters on NOD/nodulopeptin 901 production which ultimately may be helpful to explain the distribution, control of natural blooms and toxin levels of *N. spumigena* in the Baltic Sea and as well as laboratory based experiments.

In an attempt further exploit cyanobacterial diversity, 20 strains were isolated from the Dian Lake and 6 from the Dead Sea. The UPLC-PDA-MS analysis of isolates, *Microcystis* spp. from Dian Lake, China indicated the presence of several peptides namely MC-LR, cyanopeptolin A and aerucyclamides A-D. These new isolates will be examined for biological activity and chemical characterisation in future studies.

Keywords: *Nodularia spumigena* KAC 66, the Baltic Sea, fractions, *Daphina* assay, protein phosphatase 1 assay, environmental factors, biomass, nodularin (NOD), nodulopeptin 901, the Dian Lake, the Dead Sea

CHAPTER 1

INTRODUCTION

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1.1. INTRODUCTION

1.1.1. Cyanobacterial occurrence

Cyanobacteria (Cyanoprokaryota/Cyanophyta/blue-green algae) belong to the oldest group of photosynthetic organisms (Catling *et al.,* 2001; Kasting, 2001). The name blue-green has been given due to the presence of two photosynthetic pigments; chlorophyll-*a* and phycocyanins (Mur *et al*., 1999).

They evolved in the middle of the Pre-Cambrian era, approximately 3.5-3.8 billion years ago (Brasier *et al.,* 2002). They are Gram-negative (Gerba *et al.,* 2000) and photoautotrophic prokaryotes (without nucleus, Puschner and Jean, 2007) having higher plant-type oxygenic photosynthesis (Whitton and Potts, 2000).

Cyanobacteria are commonly found in an extremely wide range of environments including water columns (i.e. *Aphanizomenon flos-aquae, Nodularia spumigena* and *Anabaena circinalis* (Syn. Genus *Dolichospermum;* Ralfs ex Bornet et Flahault, comb.nova) in the Baltic Sea; Kutser *et al*., 2008), sediments (i.e. *Rivularia* sp. and *Gleotrichia* sp.; Limaye *et al.,* 2010), hot springs (i.e. *Phormidium* sp., *Oscillatoria* sp., *Spirulina* sp., and *Synechococcus* sp.; Stal, 2012; Krienitz *et al*., 2003), cold lakes (belonging to orders Chroococcales, Oscillatoriales, and Nostocales; Singh and Elster, 2007), soils (i.e. genera *Phormidium, Oscillatoria, Lyngbya, Anabaena, Nostoc, Scytonema* and *Calothrix*; Bhatnagar *et al.,* 2008) and other terrestrial environments (i.e. *Nostoc* sp., *Microcoleus* sp*., Chroococcidiopsis* sp*. and Chroococcus* sp.; Stal, 2012). Many species of cyanobacteria are capable of surviving in the

extreme hypersaline environments (*Anabaena* sp., *Synechococcus* sp., *Calothrix* sp., *Synechocystis* sp., *Gloeothece* sp*., Synechococcus* sp*.* and *Synechocystis* sp*.;* Stal, 2012; Mazur-Marzec *et al.,* 2005; Mackay *et al*., 1984), sea bottoms (Lopez-Cortes *et al.,* 2001), alkaline lakes (*Arthrospira* sp., *Anabaenopsis* sp., *Spirulina* sp., and *Phormidium* sp.; Ballot *et al.,* 2005) and habitats of fresh (Golubic *et al.,* 2009) and marine waters (Miller and Castenholz, 2000). Cyanobacteria also have the ability to survive in a wide range of temperatures, (-10 to 72ºC; Lopez-Cortes *et al.,* 2005; Singh and Elster, 2007) and are neutral to alkaline conditions (pH 7.0-10; Stal, 2012).

Cyanobacteria mainly contain photosynthetic pigments chlorophyll-*a,* phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin), xanthophylls and *β*-carotene, which carry out photosynthesis with the production of oxygen (Whitton and Potts, 2000). Cyanobacteria produce different kinds of cells. Under favorable growth conditions some produce photosynthetic cells/vegetative cells, under harsh environment conditions some form akinete cells/spores and under appropriate conditions some form thick walled heterocytes. Several cyanobacterial genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Nodularia* and *Nostoc* (Mur *et al.,* 1999) have evolved specialised cells for nitrogen fixation (heterocytes), which contain nitrogenase enzyme to fix nitrogen.

A dilemma exists as to whether cyanobacteria should be classified under the International Code of Botanical Nomenclature or the International Code of Nomenclature of Bacteria (Skulberg *et al.,* 1993). On the basis of their morphological and genetic characteristics cyanobacteria are placed in a separate and distinct group of algae: Class Cyanophyceae

(Anagnostidis and Komarek, 1985; Rippka *et al.,* 1979). Oren (2004) also proposed further integration of the cyanobacteria under the Bacteriological Code.

1.1.2. Ecological importance of cyanobacteria

Cyanobacteria are involved in oceanic primary production and give a total primary production on earth *c.* 10^{16} g C y^{-1} (Kaiser *et al.,* 2005) and certain strains of cyanobacteria like *Prochlorococcus* and *Synechococcus,* have the highest rank among photosynthetic organisms on earth (Penno *et al.,* 2006).

$$6CO_2+12H_2O \xrightarrow{\text{Sun light}} C_6H_{12}O_6+6O_2+6H_2O$$

Cyanobacteria are involved in the global nitrogen and carbon cycles. Through photosynthesis they fix atmospheric carbon dioxide (CO_2) and form organic compounds. In addition, they (i.e. a brackish water cyanobacterium *Nodularia spumigena*; Mazur-Marzec *et al.,* 2005) also play an important role in dinitrogen fixation, and help in providing biologically available nitrogen to the environment.

This nitrogen is used by higher plants (Paerl, 2000) and mangroves (Bashan *et al.,* 1998). Some cyanobacterial species i.e. *Oscillatoria salina, Plectonema terebrans and Aphanocapsa* sp. are also known to play an important role in the biodegradation of oil in environments affected by oil spills (Raghukumar *et al.,* 2001).

Cyanobacteria have an impressive ability to produce useful biochemicals (Fatima and Venkataraman, 1999) and a variety of toxic/bioactive

compounds, known as the 'cyanotoxins' (van Apeldoorn *et al.,* 2007; Welker and von Döhren, 2006). Some of these compounds are toxic to many microscopic organisms and to higher vertebrates (Carmichael, 2001; Codd *et al.,* 1997) including human beings (Osborne *et al.,* 2008; Fleming *et al.,* 2002). Some may have the ability to kill tumour cells, viruses (Review by Dittmann and Wiegand, 2006) and an anti-HIV protein (cyanovirin-N) has been isolated from *Nostoc ellipsosporum* (Gustafson *et al.,* 1997). Several reports have suggested that these natural compounds could be utilised in drug industries (Tan, 2007; Proksch *et al.,* 2002) and are being marketed directly for human consumption (i.e. *Aphanizomenon flos-aquae*, *Arthrospira maxima* and *Arthrospira platensis*; Singh *et al.,* 2011).

1.1.3. Cyanobacterial blooms

Under favourable conditions several aquatic cyanobacterial strains are capable of growing in abundance and often form blooms or toxic blooms, associated with eutrophication and environmental factors (Larsson *et al*., 2001; Wasmund, 1997; Kononen *et al*., 1996).

In many cases blooms can be toxic with records suggesting that 50% of cyanobacterial blooms maybe toxic. Approximately, 40 cyanobacterial genera produce cyanobacterial toxins the main ones are *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia, Nostoc*, *Lyngbya* and *Planktothrix.* Cyanobacterial blooms and their toxins are responsible for lethal effects on domestic/wild animals and human beings.

Cyanobacterial blooms occur in temperate lakes (ISOC-HAB, 2008; Hudnell *et al.,* 2008), coastal waters (Dietrich *et al.,* 2008; Albert *et al.,* 2005; Watkinson *et al.,* 2005) and especially in freshwaters (Kanoshina *et al.,* 2003). In the past these blooms were considered a natural phenomenon, but in more recent years their frequency has increased considerably. Agricultural run off and other pollutants to aquatic environments have resulted in increased nutrient enrichment thus providing favourable conditions for the growth of toxic cyanobacteria (Codd *et al.,* 2005; Sivonen and Jones, 1999). Many cyanobacterial species i.e. *Aphanizomenon flos-aquae*, *Anabaena* spp. and *Nodularia spumigena* are known to produce cyanobacterial blooms in the world's second largest brackish water body, the Baltic Sea (Sukikkanen *et al.,* 2010).

1.1.4. Types and nature of cyanotoxins

On the basis of mode of action cyanobacterial toxins can be divided into different categories. Some cyanobacterial strains produce intracellular hepatotoxins (microcystins, nodularin and cylindrospermopsin), neurotoxins (anatoxins and saxitoxins) and skin irritants (lipopolysacchride endotoxins). The details of hepatotoxins and their target sites are shown in Table 1.1.

Table. 1.1. General features and targets of cyanobacterial toxins (selected references).

Toxic group	Primary target cells/organs in mammals	Microalgal genera and species	References
Cyclic peptides (hepatotoxin)			
Microcystins (MC)	Liver Gastrointestinal illness	*Microcystis aeruginosa*	Lahti, (1997), Bateman *et al.,* (1995), Azevedo *et al.,* (1994), Craig *et al.,* (1993), *Sivonen et al* ., (1992a,b,c), Kaya and Watanabe, (1990), Stoner *et al* ., (1989), Gathercole and Thiel (1987), Botes *et al* ., (1984 and 1985), Elleman *et al* ., (1978), Watanabe *et al.,* (1988), Lippy and Erb, (1976), Konst *et al* ., (1965),
		Microcystis spp.	Lahti, (1997), Namikoshi *et al* ., (1995), Luukkainen *et al.* , (1994), Namikoshi et al., (1992), Yu *et al., (* 1988)
		Anabaena sp.	Sivonen *et al., (* 1992a), Namikoshi *et al., (* 1992), Harada *et al.* , (1991b), Namikoshi *et al., (* 1995), Namikoshi *et al., (* 1998)
		Microcystis wesenbergii	Luukainen *et al.* , (1993), Carmichael *et al.* , (1988), Botes *et al., (* 1985)
		Microcystis viridis	Barco *et al.,* (2002), Harada *et al.,* (1990)
		Planktothrix, Nostoc, Hapalosiphon,	Briand *et al.* , (2003), Sivonen and Jones, (1999), Lawton *et al., (* 1994), Ressom *et al.,* (1994)
		Anabaen, Planktothrix, Anabaenopsis milleri	Agrawal *et al.* , (2006), Diehnett *et al* ., (2005), Sivonen and Jones, (1999), Chorus and Bartram, (1999), Namikoshi *et al.,* (1998), Namikoshi *et al.,* (1992)
		Planktothrix agardhii	Luukkainen *et al* ., (1993), Krishnamyrthy *et al., (* 1989)
		Anabaena flos-aquae *Nostoc* sp.	Sivonen *et al* ., (1992c), Harada *et al.,* (1991a) Beattie *et al.* , (1998), Sivonen *et al., (* 1990a), Namikoshi *et al.,* (1990), Sivonen *et al., (* 1992c)
		Nodularia spumigena AV1	Fujii *et al.,* (1997)
		Nodularia spumigena	Baker and Humpage, (1994), Jones *et al* ., (1994), Sivonen *et al* ., (1989b), Runnegar *et al.,* (1988), Rinehart *et al.,* (1988), Eriksson *et al* ., (1988), Carmichael *et al.* , (1988), Edler *et al.* , (1985), Persson *et al.* , (1984), Main *et al* ., (1977), LindstrØm, (1976), Francis, (1878)
Nodularin (NOD)	Liver		

Contd.....

Toxic group	Primary target cells/organs in mammals	Microalgal genera and species	References
Guanidine alkaloid (hepatotoxin)		*Nodularia* PCC 7804	Beattie *et al.,* (2000)
Cylindrospermopsin (CY)	Liver	*Cylindrospermopsis rasciborscii*	Hawkins *et al.,* (1985)
		Anabaena bergii	Schembri *et al.,* (2001)
		C. ovalisporum	Shaw *et al.,* 1(999)
		Planktothirx, Raphidiopsis, Microcystis, Anabaena	Al-Lay *et al.,* (1988), Carmichael *et al.,* (1988), Carmichael, (1978)
		Nodularia, Lyngbya and *Nostoc*	Harada *et al.,* (1991a and b, 1994), Ohtani *et al.,* (1992), Krishnamurthy et al., (1989) Fastner *et al.,* (2003), Li *et al.* , (2001a and b), Schembri *et al* ., (2001), Banker *et al., (* 1997), Hawkins *et al* ., (1997)
		Lyngbya wollei	Seifert *et al.,* (2007)
		Aphanizomenon onalisporum	Shaw *et al.,* (1999)
		Aphanizomenon ovalisporum	Shaw *et al.* , (1999), Banker *et al.,* (1997)
		Aphanizomenon flos-aquae	Preußel *et al.* , (2006)
		Cylindrospermopsis raciborskii	Hawkins *et al.,* (1985 and 1997), Törökné, (1997)
		Umezakia natans	Harada *et al., (* 1994)
Alakloids (Neurotoxin)			
Anatoxin-a (AnTx)	Nerve synapse Depolarize neuromuscular blocking agent	*Anabaena palnktonica, Cylindrospermum* sp.,	Carmichael *et al.,* (1975 and 1990), Devlin *et al.,* (1977), Gorham *et al.* , (1964)
		Phormidium favosum,	Edwards *et al.* , (1992), Sivonen *et al* ., (1989a), Carmichael and Bent, (1981)
		Anabaena planktonica,	Bruno *et al.,* (1994), Rapala *et al.,* (1993), James *et al.,* (1997a, b)
		Raphiidiopsis mediterranea,	Gugger *et al., (* 2005), Ballot *et al., (* 2005), Viaggiu *et al.* , (2004), Namikoshi *et al.,* (2003)
		Planktothrix rubescens, Anthrospira fusiformis	Viaggieu *et al.,* (2004)
		Anabaena flos-aquae	Devlin *et al.,* (1977), Carmichael *et al.* , (1975), Hurber, (1972), Rapala *et al* ., (1993), Carmichael, (1992), Carmichael and Bent., (1981) Carmichael, (1992), Carmichael *et al., (* 1975)
		Anabaena spp.	Sivonen *et al.,* (1989a), James *et al.,* (1997a , b)
		Anabaena blooms	
		Anabaena planctonica bloom	Bruno *et al., (* 1994), Sivonen *et al., (* 1989a)
		Anabaena circinalis	James *et al.,* (1997a, b), Bruno *et al.* , (1994), Sivonen *et al.* , (1989a)
		Anthrospira fusiformis	Ballot *et al.,* (2005)
		Aphanizomenon sp.	Codd *et al.,* (1997), Sivonen *et al.,* (1989a)
		Aphanizomenon blooms	Bumke-Vogt, (1999)
		Anabaena spiroides	Carmichael, (1992)
		Cylindrospermum sp.	Sivonen *et al.,* (1989a)

Contd……

Toxic group	Primary target cells/organs in mammals	Microalgal genera and species	References
		Microcystis sp. *Oscillatoria* spp. *Planktothrix* sp. *Phormidium favosum*	Codd *et al.,* (1997) James *et al.* , (1997a, b), Edwards *et al.,* (1992) Sivonen *et al.,* (1989a) Gugger *et al.,* (2005)
Anatoxin-a(s), (AnTx-a(s)	Nerve synapse Antichlorinesterase	*Anabaena flos-aquae, Anabaena lemmermannii* *Anabaena flos-aquae* *A. lemmermannii*	Onodera *et al.,* (1997), Matsunaga *et al.,* (1989) Mahmood and Carmichael, (1986 and 1987) Matsungaga *et al.,* (1989) Matsunaga *et al., (* 1989), Henriksen *et al.* , (1997), Mahmood and Carmichael, (1986) Onodera *et al.,* (1997)
Homoanatoxin-a (HAnTx)	Nerve synapse	*Oscillatoria rubescens* *Phormidium* sp. *Planktothrix formosa*	Aas *et al.,* (1996) Wood *et al.,* (2007) Skulberg *et al.,* (1992)
Saxitoxins (STx) (Red-tide algae) (paralytic shellfish poisonings; PSPs)	Nerve axons Sodium channel blocker	*Aphanizomenon flos-aquae,* *Lyngbya wollei* and *Cylindrospermopsis, raciborskii,* *Cylindrospermopsis* and marine dinoflagellates *Anabaena circinalis* *Aphanizomenon flos-aquae* *Cylindrospermopsis raciborskii* *Lyngbya wollei*	Kao and Walker, (1982) Negri *et al* ., (1997), Negri and Jones, (1995), Humpage *et al* ., (1994) Mahmood and Carmichael (1986), Ikawa *et al.,* (1982) Lagos *et al.,* (1997) Carmichael *et al., (* 1997), Onodera *et al., (* 1997)
Neosaxitoxin (NeoSTx)	Sodium channel blocker	*Aphanizomenon flos-aquae,* *Anabaena circinalis*	Ikawa *et al* ., (1982) Negri *et al* ., (1995), Humpage *et al.,* (1994)
Dermatoxic alkaloids **Skin irritants**			
Aplysiatoxin	Skin, protein kinase C activator Gastrointestinal illness	*Lyngbya, Oscillatoria* *Schizothrix calcicola*	Mynderse *et al.,* (1978), Fujiki *et al.,* (1990) Mynderse and Moore, (1977)
Lyngbayatoxin-a	Skin, gastrointestinal potent tumour promter Inflamatory agent, severe oral and gastrointestinal inflamatory agent	*Schizothrix, Oscillatoria, Lyngbya majuscula* *Lyngbya majuscula*	Aimi *et al.* , (1990), Fujiki *et al.* , (1990), Fujuki *et al.,* (1984), Cardellina *et al.* , (1979), Mynderse *et al* ., (1978) Izumi and Moore, (1987), Serdula *et al., (* 1982) Fujiki *et al.,* (1990)
Debromoaplysiatoxin	Skin Inflamatory activator	*Oscillatoria, Schizothrix* and *Oscillatoria nigroviridis*	Mynderse *et al.,* (1977), Moore *et al., (* 1984), Fujiki *et al., (* 1984)
Lipopolysaccharides (LPS)	Endotoxin, potential irritant; affects any exposed tissues	*Anacystis nidulans, Schizothrix calcicola,* *Oscillatoria brevis,* *Anabaena flos-aquae, Oscillatoria tenuis,* *M. aeruginosa, Anabaena variabilis*	Ressom *et al., (* 1994), Weise and Drews, (1970)
Cyclic guanidine alkaloids Cytotoxins	Cell line	*Cylindrospermopsis raciborskii* *Umezakia natans* *Aphanizomenon ovalisporum*	Hawkins *et al., (* 1985 and 1997) Harada *et al., (* 1994) Banker *et al.,* (1997)

1.4.1.1. Hepatotoxins

Several freshwater, brackish water and marine cyanobacterial species of the genera *Microcystis, Anabaena, Nodularin, Planktothrix, Nostoc* and *Hapalosiphon* (Terrestrial genera) produce hepatotoxins (Rinehart *et al.,* 1994). These toxins include cylindrospermopsins (CYN), nodularins (NOD) and microcystins (MCs).

1.1.4.1.1. Cylindrospermopsins (CYN) are guanidine tricyclic alkaloid toxins (MW 415 Daltons; van Apeldoorn *et al.,* 2007, Fig. 1.1) and have been isolated from several cyanobacterial species and strains (Table 1.1). They inhibit protein synthesis (Wiegand and Pflugmacher, 2005) and potent hepatotoxin results in fatty liver and central globular necrosis in laboratory mice when injected intraperitonially (Kinnear, 2010). CYN is hepatotoxic but does not inhibit PP1 and PP2. Blooms of *Cylindrospermopsis raciborskii* are reported from Australia, North and South America and Europe.

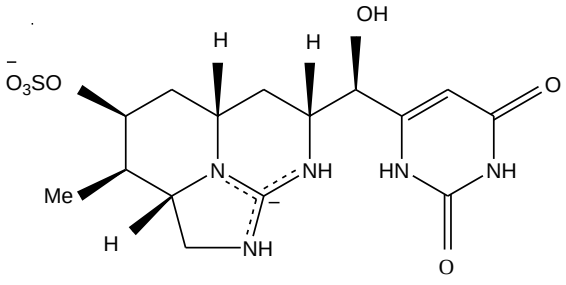


Figure 1.1. Chemical structure of cylindrospermopsin

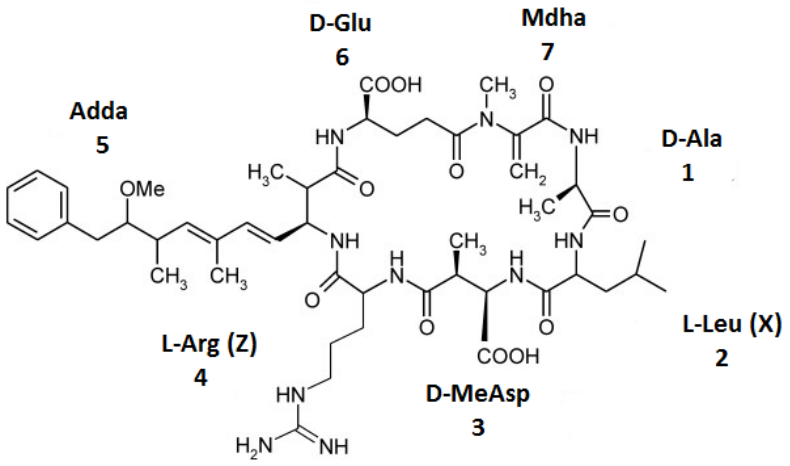
1.1.4.1.2. Cyclic hepatotoxic peptides - microcystins and nodularin

1.1.4.1.2.a. Microcystins (MC) are cyanobacterial monocyclic hepatotoxic peptides (Kuper-Goodman *et al.*, 1994; Fig. 1.2A). They were first isolated from the cyanobacterium, *Microcystis aeruginosa* (Bishop *et al.,* 1959). MCs have a similar structure to NOD, although it has 7 amino acids where NOD has 5. The main differences between both hepatotoxins are the replacement of Mdha in MC with Mdhb in NOD. Another difference is lack of amino acids D-Ala and L-Leu (X position; Rantala *et al*., 2004) in NOD structure.

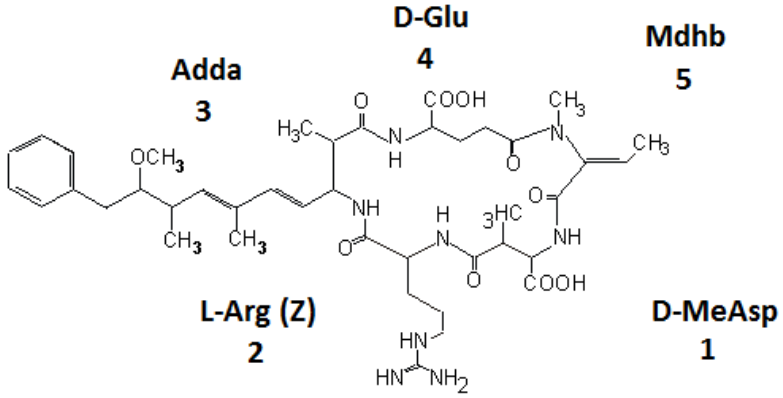
MCs contain two variable L-amino acids, three D-amino acids (alanine, methylaspartic acid and glutamic acid) and two unusual amino acids Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and Mdha (*N*-methyldehydroalanine; Botes *et al.,* 1985). More than 80 MCs and their variants have been isolated from strains of cyanobacteria (Welker and von Döhren, 2006). Some MCs and their amino acids variations are shown in Table 1.2.

Table 1.2. Variation of amino acids on X and Z positions.

MC variant	X- amino acid	Z- amino acid	Molecular weight (Daltons)
MC–WR	Tryptophan	Arginine	1068
MC–YR	Tyrosine	Arginine	1045
MC–RR	Arginine	Arginine	1038
MC–LW	Leucine	Tryptophan	1025
MC–LY	Leucine	Tyrosine	1002
MC-LR	Leucine	Arginine	995
MC–LF	Leucine	Phenylalanine	986
MC- LA	Leucine	Alanine	910



A



B

Figure. 1.2. General chemical structures of microcystin-LR (**A**) and nodularin-R (**B**).

[**D-Ala**= D–Alanine, **L-Leu**= X- Variable L-amino acid (L-Leucine), **D-MeAsp**= Methyl Aspartic acid, **L-Arg**= Z- Variable L-amino acid (L-Arginine), **Adda**= (3-amino-9-methoxy-2,6,8-trimethyl-10-pheny ldeca-4,6-dienoic acid, **D-Glu**= D-Glutamic acid, **Mdha** (*N*-methyldehydroalanine) and **Mdhb** [2-(*N*-methyleamine)-2-dehydrobutyric acid]

The toxicities of these MCs do not vary greatly. *M. aeruginosa* is the most frequently studied organism to produce hepatotoxic MC-LR.

The Adda amino acid is only found in hepatotoxic cyanobacterial toxins such as NOD and MCs.

1.1.4.1.2.b. Nodularin (NOD) is a mono cyclic pentapeptide hepatotoxin (m/z 825 Daltons; Fig. 1.2B; Sivonen and Jones, 1999). *Nodularia spumigena* and *Nostoc* sp. (Gehringer *et al*., 2012) is the only one cyanobacterial strain which produces NOD. The chemical structure of NOD is cyclo-(D-MeAsp^{1}-L-arginine^{2}-Adda^{3}-D-glutamate^{4}-Mdhb^{5}), in which Mdhb is 2-(*N*-methyleamine)-2-dehydrobutyric acid. D-MeAsp is D-methylaspartic acid. Adda is 3-amino-9-methoxy-2,6,8-tri-methyl-10-phenyldeca, 4,6-dienoic acid. This toxicosis caused death of cattle, sheep, dogs, horses and pigs. Death can occur within a few hours to a few days, followed by coma, muscle and general pains (Galey *et al.,* 1987). In humans *Nodularia spumigena* causes flu-like symptoms (result of inhaling organism with LPS endotoxin), allergic reactions and skin irritations (Henriksen, 2005). NOD has LD_{50} of 50-150 µg/kg in mice when injected intraperitoneally (Lehtimäki, 2000).

Several NOD variants have been isolated from *N. spumigena* (Table 1.3).

Table 1.3. Some NOD variants isolated from *N. spumigena*.

NOD variants	**Molecular weights $[M+H]^+$**	**References**
Nodularin	825	Mazur-Marzec *et al.* , (2006)
Linear NOD	843	
Nodularin-R	825	
[DMAdda^3]NOD	811	
[Glu^4(OMe)]NOD	839	
[dhb^5]NOD	811	
[MeAdda^3]NOD	839	
Glu^4(OMe)NOD	839	
[D-Asp^1]NOD	811	
Demethylated-Adda form	811	
[L-Har^2]NOD	839	Beattie *et al.* , (2000)
[L-valine^2]NOD or motuporin		Mazur-Marzec *et al* ., (2006); Namikoshi *et al* ., (1993)

(Har=homoarginine)

Motuporin or [L-valine^2]NOD has also been isolated from a marine sponge, *Theonella swinhoei*, which has symbiotic relationships with cyanobacteria (DeSilva *et al.,* 1992).

1.1.4.1.2.c. Toxic effects of hepatotoxic nodularin an Microcystins

Cyanobacterial toxins are indirectly or directly harmful to animals and human beings through recreational and drinking water supplies. In 1878, the first report of cyanobacterial poisoning was reported from Lake Alexandria, Australia by Frances. The cause was, drinking of scum containing *Nodularia spumigena.* The cyclic peptide MCs and NOD are specific in causing liver toxicology in mammals. The chronic exposure to high doses of toxins results in liver haemorrhage or failure and promotes

the size of liver and forms tumours followed by death. MCs and NOD may also cause heart failure, hemodynamic shock and subsequently death. In laboratory animals they affect the lungs, intestines and kidneys (Carmichael and Bent, 1981).

In humans, fatality has only been observed as a result of intravenous exposure to dominant toxin MC-LR, through renal dialysis at a haemodialysis centre in Caruaru, Brazil (Jochimsen *et al.,* 1998).

Few incidents of animal toxicology by *Nodularia* spp. and NOD have been reported compared with *Microcystis* and MCs. The first report relates to the toxicity of cyanobacterial blooms as presented by Francis (1878). In his report he pointed out the death of domestic animals i.e. sheep, cattle, dogs, horses and pigs around the estuary of the Murray River, Australia. The main cyanobacterium responsible for these deaths, was *Nodularia spumigena*. In Finland (Perrson *et al.,* 1984) and Germany (Edler *et al*., 1985) death of dogs have been reported, which were also caused by *Nodularia spumigena*.

Hepatotoxic MCs and NOD were also found to be inhibitors of eukaryotic protein serine/threonine phosphatase 1 and 2 (see section 1.1.8.2).

1.1.4.1.3. Other cyanobacterial cyclic and linear peptides

Cyanobacteria are known to produce toxic and non toxic peptides. A detailed description of peptides, their target cell/organs and their producing cyanobacteria is shown in Tables 1.1 and 1.4.

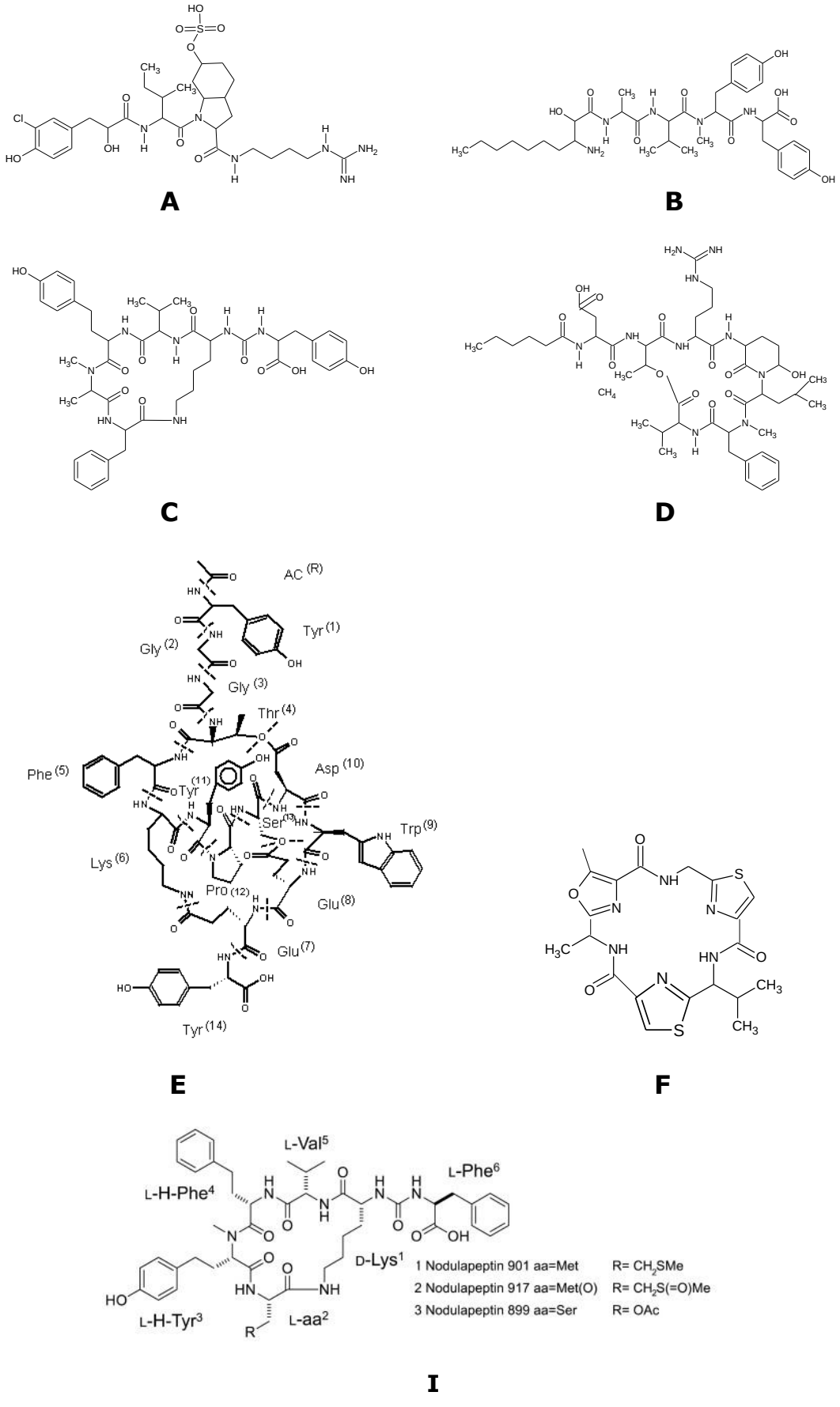


Figure 1.3. General chemical structures of cyanobacterial peptides.
A:-Aeruginosin 98-A, **B**:- Microginin, **C**:- Anabaenopeptin A,
D:- Cyanopeptolin, **E:-** Microviridin A, **F:-** Nostocyclamide and
G:- Polypeptide (eg. nodulopeptin A-C; R=functional group)

On the bases of molecular structures and mode of action cyanobacterial peptides can be divided into the following main classes (Schumacher *et al.*, 2012; Welker and von Döhren, 2006).

1.4.1.3.a. Aeruginosins are linear peptides and are characterised by an arginine derivative and a derivative of hydroxy-phenyl lactic acid (Hpla) (Welker and von Döhren, 2006; Fig. 1.3A).

1.1.4.1.3.b. Microginin is a class of linear peptides and was first described by Okino *et al.,* (1993a). It is characterised by two tyrosine units at the C-terminus and a decanoic acid derivative, 3-amino-2-hydroxy-decanoic acid (Ahda; Fig. 1.3B).

1.1.4.1.3.c. Anabaenopeptins (ABPN) are cyclic peptides and have been isolated from freshwater (Harada *et al.,* 1995), brackish water cyanobacteria (Fujii *et al.,* 1997) and terrestrial habitats (Reshef and Carmeli, 2002; Fig. 1.3C).

1.1.4.1.3.d. Cyanopeptolins are cyclic peptides and have high structural variability. They have been isolated from Chroococcales, Oscillatoriales and Nostocales (Fig. 1.3D).

1.1.4.1.3.e. Microviridins have a main peptide ring consists of seven amino acids with an ester bond (Fig. 1.3E, Ishitsuka *et al.,* 1990) and are isolated from many cyanobacterial strains.

1.1.4.1.3.f. Cyclamides also have various structures and isolated from several strains of cyanobacteria (Fig. 1.3F).

1.1.4.1.3.g. Polypeptide (A-C) is a new class of peptides (Fig. 1.3G) and has been isolated from filamentous cyanobacterium, *Nodularia*

spumigena KAC 66, namely nodulopeptin A (899 Daltons), B (901 Daltons) and C (917 Daltons; Schumacher *et al.,* 2012).

In addition to these cyanobacterial peptides a variety of more rare peptides have been reported from various species of cyanobacteria listed in Table 1.4.

Table. 1.4. General features of cyanobacterial peptides (selected references).

Oliopeptides	Type of peptide	Microalgal origin	References
1. Main classes of peptides			
Aeruginosins	Linear	*Microcystis, Planktothrix, Nodularia*	Murakami *et al.,* (1995)
a. Suomilide		*Nodularia*	Fujii *et al.,* 1997
b. Banyaside		*Nostoc*	Ploutno and Carmeli, (2002)
Microginins	Linear	*Microcystis, Planktothrix, Nostoc*	Okino *et al.,* (1993), Ishida *et al., (* 1998 and 2000)
a. Nostoginin		*Nostoc*	Ploutno and Carmeli, (2002)
Anabaenopeptins	Cyclic	*Anabaena, Aphanizomenon. Microcystis, Planktothrix*	Harada *et al.,* (1995); Reshef and Carmeli, (2002); Fujii *et al.* , (1995 and 1997)
Cyanopeptolins	Cyclic	*Anabaena, Lyngbya, Microcystis, Planktothrix, Scytonema, Symploca, Cdroococcales, Oscillatoriale, Nostocales*	Harrigan *et al.,* (1999); Martin *et al.,* (1993); Okino *et al.,* (1993b); Tsukamoto *et al., (* 1993)
Microcystins	Cyclic	*Microcystis, Anabaena, Planktothrix, Nodularia Nostoc, Hapalosiphon, Anabaenopsis*	Botes *et al.,* (1984; see more references in Table 1.3)
Nodularins	Cyclic	*Nodularia*	Sivonen *et al.,* (1989b; see more references in Table 1.3)
Microviridins	Multicyclic	*Microcystis, Planktothrix, Nostoc*	Ishitsuka *et al.,* (1990)
Cyclamides	Cyclic hexa		
a. Nostocyclamide		*Lyngbya, Microcystis, Nostoc, Oscillatoria,*	Todorova *et al.,* (1995)
b. Westiellamide		*Stigonema, Westelliopsis*	Prinsep *et al.,* (1992)
		Nostoc	
Polypeptide (A-C)	Linear	*Nodularia spumigena* KAC 66	Schumacher *et al.,* (2012)
II. Other peptides			
Crytophycins	Cyclic desi	*Noctoc*	Schwartz *et al.,* (1990)
Microcolins	Linear	*Lyngbya*	Koehn *et al., (* 1992)
Mirabimids	Linear	*Scytonema*	
Tantazoles	Linear tetra	*Scytonema*	Carmeli *et al.,* (1990 and 1991)
Mirabazoles	Penta	*Scytonema*	Carmeli *et al.,* (1990 and 1991)
Other peptides			
a. Aeruginosinamide	Linear tetra		Lawton *et al.,* (1999)
b. Barbamide	Linear tetra	*Lyngbya*	Orjala and Gerwich, (1996), Williamson *et al.,* (1999)
c. Lyngbyabellin B	Cyclic hexa	*Lyngbya*	Luesch *et al.,* (2000a)
d. Apramides	Linear non		Luesch *et al.,* (2000b)
e. Wewekazole	Cyclic undeca	*Lyngbya*	Nogle *et al.,* (2003)
f. Puwainaphycin	Cyclic deca- and undeca	*Anabaena*	Gregson *et al.,* (1992)
h. Laxaphycin	Lipo	*Anabaena*	Frankmolle *et al.,* (1992a and b)
i. Lobocyclamide			MacMillan *et al* ., (2002)
j. Calophycin		*Calothrix*	Moon *et al.,* (1992)
k. Kawaguchipeptin	i. Cyclic deca- and undeca		
	ii. Undeca	*Microcystis*	Ishida *et al.,* (1996 and 1997)
l. Oscillatorin	Cyclic deca		Sano and Kaya, (1996)
m. Radiosumin			Matsuda *et al., (* 1996)
n. Aeruginoguanidin	Tri		Ishida *et al.,* (2002)
o. Kasumigamide	Linear penta		Ishida and Murkakami, (2000)
p. Antanapeptin		*Lyngbya*	Nogle and Gerwich, (2002)
q. Malevamide C	Mono-cyclic		Horgen *et al.,* (2000)
r. Yanucamide		*Lyngbya/Schizothrix* as asemblage	Sitachitta *et al.,* (2000)

(based on Welker and von Dohren, 2006)

1.1.4.1.3.h. Linear nodularin (LNOD) is a non-toxic precursor of toxic nodularin (Fig. 1.4; Rinehart *et al.*, 1994) and first isolated during the biosynthesis of toxic NOD. During biosynthesis the non-toxic NOD is produced but the linear form is also detected as a bacterial degradation product (Rinehart *et al.*, 1994). The toxic strain, *Nodularia* strain AV1 is also known to produce linear and cyclic peptides, nodulopeptins and spumigins (Fujii *et al.*, 1997).

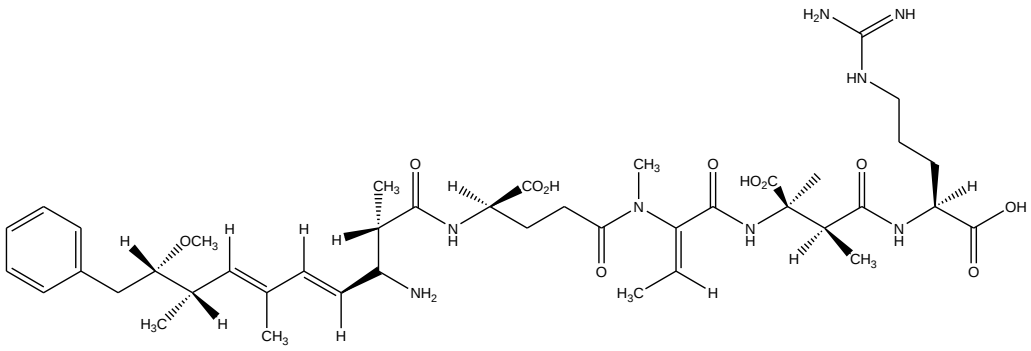


Figure 1.4. Chemical structure of linear nodularin (LNOD)

1.1.4.1.4. Neurotoxins - toxic alkaloids

These cyanotoxins are alkaloid in nature and only five neurotoxins have been studied in detail (Table 1.1). About 46 cyanobacterial species are able to produce neurotoxins, e.g. anatoxin-a, anatoxin-a(s), Homoanatoxin-a, saxitoxin and Neosaxitoxin (Ernst *et al.,* 2006).

1.1.4.1.4.a. Anatoxin-a (AnTx-a) and Homoanatoxin-a (HAnTx). Anatoxin-a formerly called 'very fast death factor' (VFDF) is a bicyclic secondary amine of alkaloid origin (Carmichael *et al*., 1975; Figs. 1.5A). This toxin was first described in the freshwater cyanobacterium *Anabaena*

flos-aquae NRC 44-1. AnTx-a is a low molecular weight alkaloid (MW=165 Da, *m/z*; $C_{10}H_{15}NO$) a secondary amine, 2-acetly-9-azabicyclo (4.2.1) non-2-ene. It is a potent post-synaptic neuromuscular blocker (Carmichael *et al.,* 1997). Homoanatoxin-a is a unique and potent neuromuscular blocking agent and has been reported from *Planktothrix rubescens* and *Phormidium formosa* (Fig. 1.5B).

1.1.4.1.4.b. Anatoxin-a (s) (AnTx-a(s)) is a low molecular weight phosphate ester (MW=252 Da *m/z*, $C_7H_{17}N_4O_4P$) of a cyclic N-hydroxy-guanidine methyl phosphate ester. It has been reported in blooms and isolated from strains of *Anabaena lemmermannii*. When injected into laboratory mice it produces marked salivation. (Fig. 1.5C).

1.1.4.1.4.c. Saxitoxin (STx) and Neosaxitoxin (NeoSTx) Saxitoxins are a group of carbamate alkaloid neurotoxins (Figs. 1.5D and E). This group of toxins is produced by several cyanobacteria and certain genera of marine dinoflagellates (red-tide algae). They are also known as paralytic shellfish poisonings (PSPs; Hallegreaff, 1993) in the freshwater mussel *Alathyria condola* (Negri and Jones, 1995).

The variation in structure of saxitoxins depends on the addition of double sulphated (C-toxins) or sulphated (gonyautoxins – GTx; Table 1.5).

Table 1.5. Structural variation of saxitoxins (R=functional group).

Toxin variant	R1	R2	R3	R4	R5
Saxitoxin					
STx	H	H	H	$CONH_2$	OH
Neosaxitoxin					
NeoSTx	OH	H	H	$CONH_2$	OH
Gonyautoxins					
GTx1	OH	H	OSO_3^-	$CONH_2$	OH
GTx2	H	H	OSO_3^-	$CONH_2$	OH
GTx3	H	OSO_3^-	H	$CONH_2$	OH
GTx4	OH	OSO_3^-	H	$CONH_2$	OH
GTx5	H	H	H	$CONHSO_3^-$	OH
GTx6	OH	H	H	$CONHSO_3^-$	OH
C-toxins					
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH
Decarbamoyl					
dcSTx	H	H	H	H	OH
dcGTx2	H	H	OSO_3^-	H	OH
dcGTx3	H	OSO_3^-	H	H	OH

(Adopted from Van Apeldoorn et al., 2007)

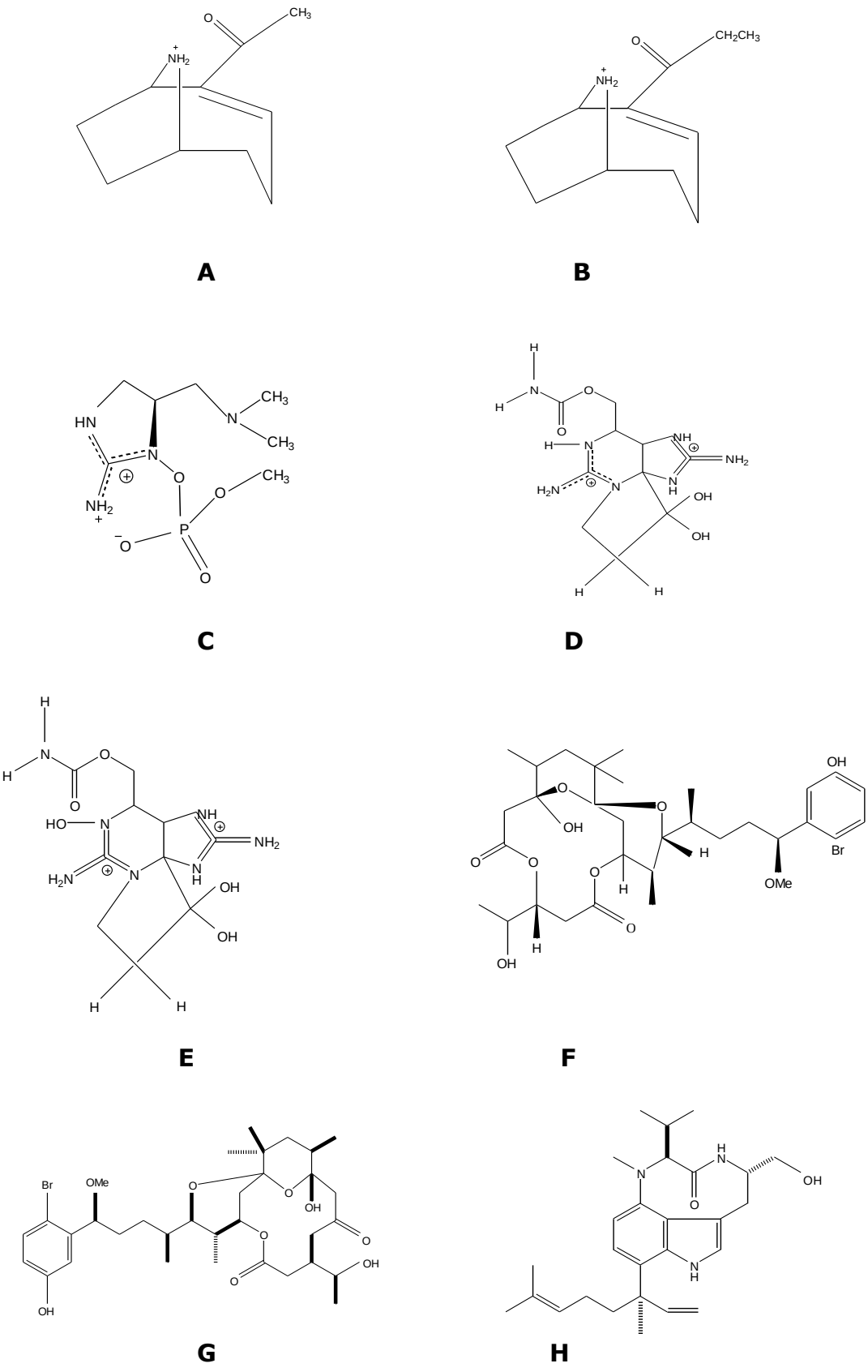


Figure 1.5. General chemical structures of cyanobacterial neurotoxins. **A**:- Anatoxin-a, **B**:- Homoanatoxin-a, **C**:- Anatoxin-a (s), **D**:- Saxitoxin and **E**:- Neosaxitoxin and skin irritants: **F**:- Aplysiatoxin, **G**:- Debromoaplysiatoxin and **H**:- Lyngbyatoxin

1.1.4.1.5. Skin irritants

Some marine cyanobacteria contain dermatotoxic alkaloids (skin irritants, Table 1.1) like lyngbyatoxins and aplysiatoxins. The marine genera *Lyngbya, Oscillatoria* and *Schizothrix* produce toxins i.e. aplysiatoxins (Fig. 1.5F), debromoaplysiatoxins (Fig. 1.5G) and lyngbyatoxin (Fig 1.5H). Lipopolysacchride (Fig. 1.6) was first isolated from the cyanobacterium, *Anacystis nidulans* (Weise and Drews, 1970) and found in the outer membrane of the cell wall of cyanobacteria.

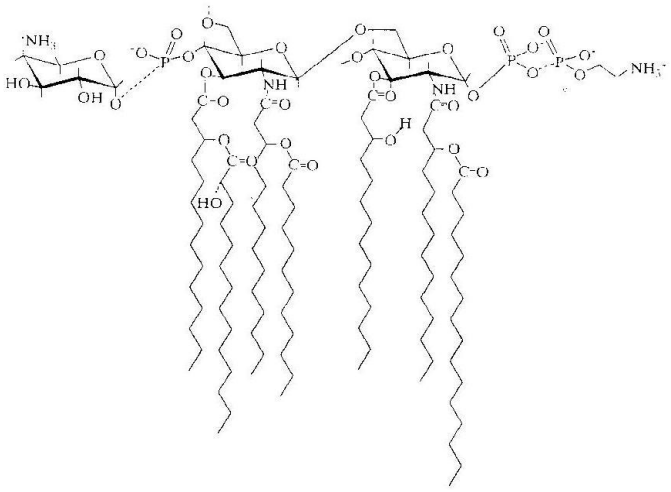


Figure 1.6. General chemical structure of lipopolysaccharide endotoxin

1.1.5. Allelopathy and allelochemicals

Some strains of cyanobacteria have the ability to produce secondary metabolites known as allelochemicals/signalling compounds (Smith and Doan, 1999). Through the production of such allelochemicals cyanobacteria can communicate with other organisms and reduce (negative allelopathy) or promote (positive allelopathy) the growth/photosynthesis, growth rate, N_2 fixing, inhibit the

replication/synthesis of DNA/RNA of their co-occurring cyanobacteria and/or phytoplankton species (Leao *et al.,* 2009a and b; Ganter *et al.,* 2008; Suikkanen *et al.,* 2004).

Allelopathy is a biological phenomenon and is also important in the control of toxic blooms of cyanobacteria (Leao *et al*., 2009a), macro-algae (Leao *et al.,* 2009a), algal succession and bloom formation (Vardi *et al.*, 2002; Keating, 1977) and can also target angiosperms (Leao *et al.,* 2009a).

The research on allelopathic activities of cyanobacteria was started in the 1980s. Despite this relatively little is known about the type of signalling compounds produced by cyanobacteria and how these compounds affect other organisms within that environmental compartment. Few studies have been done on the effects and control of cyanobacterial toxins by allelochemicals. Some studies have attempted to demonstrate that toxin production (anatoxin and microcystin) by a free-living freshwater cyanobacterium *Anabaena flos-aquae,* is regulated in part by the presence of extracellular products of a eukaryotic green alga, *Chlamydomonas reinhardtii* (Kearns and Hunter, 2000).

Table 1.6. Some allelopathic cyanobacteria and their mode of actions.

Species	Mode of action		References
	Beneficial	Detrimental	
Anabaena spp.	Increase N2 fixing		Suikkanen *et al.,* (2004); De Nobel *et al* ., (1998)
Aphanizomaenon flos-aquae		Growth inhibitor	Leao *et al.* , (2009a), Suikkanen *et al* ., (2004)
Cylindrospermopsis rasciborskii		Photosynthesis inhibitor	Figueredo *et al* ., (2007)
Calothrix spp.		Growth inhibitor RNA and DNA replication inhibitor	Doan *et al.* , (2001) Doan *et al.* , (2001)
Fischerella spp.		Growth inhibitor RNA and DNA replication inhibitor	Leao *et al.* , (2009b); Doan *et al* ., (2001) Doan *et al.* , (2001)
Fischerella sp. CENA19		Growth inhibitor	Etchegaray *et al* ., (2004)
Fischerella sp. strain 52-1		Growth inhibitor Photosynthesis inhibitor	Gantar *et al* ., (2008) Gantar *et al* ., (2008)
Leptolyngbya foveolarum		Growth inhibitor	van der Grinten *et al* ., (2005)
Microcystis sp.		Photosynthesis inhibitor	Wiegand *et al* ., (2002)
Microcystis aeroginosa		Growth inhibitor	Yamasaki, (1993)
Nodularia harveyana		Cytotoxic	Flores and Wolk (1986); Keating, (1977, 1978); Volk, (2005)
Nodularia spumigena	Growth promotor	Growth inhibitor	Suikkanen *et al* ., (2004)
Nostoc insulare		Cytotoxic	Volk, (2005)
Nostoc 31		Growth inhibitor	Juttner *et al* ., (2001)
Nostoc spp.		Growth inhibitor	Leao *et al* ., (2009b)
Oscillaroria sp.		Growth inhibitor	Leao *et al* ., (2009a)

In another study, Engelke *et al.,* (2003) investigated elevated microcystin and nodularin levels in cyanobacteria growing in spent medium of *Planktothrix agardhii* CYA 29. They found that the presence o*f P. agardhii* and its spent medium increased the toxin level in *Microcystis aeruginosa* and *Nodularia* sp. Engelke, (1998) also applied spent media of *Oscillatoria agardhii, Microcystis aeruginosa, Synechococcus* sp., *Chlorella vulgaris*, and *Nodularia* sp. to various cyanobacteria to note the growth rate and toxin level although no significant effects were observed. Berry *et al.,* (2008) and Zupla *et al.,* (2003) also worked on intra and extracellular allelochemicals produced by some cyanobacterial strains (Table 1.6).

1.1.6. Description of *Nodularia spumigena* KAC 66

The nitrogen-fixing filamentous brackish water cyanobacterium, *Nodularia spumigena* KAC 66 (Fig. 1.7) is known to produce a variety of

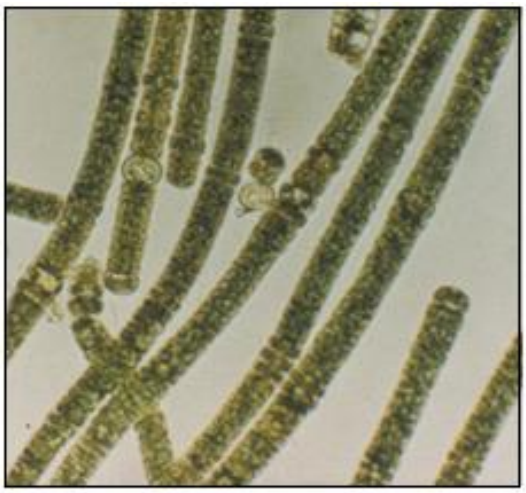


Figure 1.7. Light micrograph of *Nodularia spumigena* KAC 66 used in the present study.

hepatotoxic nodularins and cyclic hepatopeptides (Fujii *et al*., 1997; Dahlmann *et al.,* 2001).

Several other bioactive and non-bioactive compounds (Table 1.7) and allelochemicals (Table 1.8) have also been reported from *Nodularia* spp*.* In addition to producing hepatotoxins, this species produces many other bioactive compounds such as spumigins, nodulopeptins (Mazur-Marzec *et al.,* 2013) and recently three new nodulopeptins A-C (Schumacher, *et al.,* 2012) have been characterised.

Table 1.7. Toxic and non-toxic compounds produced by *Nodularia* spp.

Strain	Compounds	References
N. spumigena KAC 66	Polypeptide 899, 901 and 917	Schumacher *et al.*, (2012)
N. spumigena	cyclic pentapeptide nodularin	Carmichael *et al.*, (1988)
N. spumigena AV1	Nodulapeptins A and B, spumigan A, B and C	Fujii *et al.*, (1997)
N. spumigena	Nodularin (NOD)	Jones *et al.*, (1994), Baker and Humpage, (1994), Sivonen *et al.*, (1989b), Runnegar *et al.*, (1988), Rinehart *et al.*, (1988), Carmichael *et al.*, (1988), Eriksson *et al.*, (1988), Edler *et al.*, (1985), Persson *et al.*, (1984), Main *et al.*, (1977), Lindstrøm, (1976), Francis, (1878)
Nodularia sp.	Cylindrospermopsin (CY)	Fastner *et al.*, (2003), Schembri *et al.*, (2001), Li *et al.*, (2001a and b), Banker *et al.*, (1997), Hawkins *et al.*, (1997), Harada *et al.*, (1991a and b), Ohtani *et al.*, (1992), Krishnamurthy *et al.*, (1989)
Nodularia PCC 7804	Cylindrospermopsin	Beattie *et al.*, (2000)
N. spumigena Huebel 1988/306	Nodularin	Kruger *et al.*, (2009)
N. spumigena NSGG01	Nodularin	Kruger *et al.*, (2009)
N. spumigena NSGG0205	Nodularin	Kruger *et al.*, (2009)
N. spumigena Wilhelmshaven	Nodularin	Kruger *et al.*, (2009)
N. spumigena AV2	Cyclic pentapeptide nodularin	Martin *et al.*, (1990)
Nodularia sp.	Diarrhetic toxin	Quilliam *et al.*, (1999), Draisci *et al.*, (1998)
N. spumigena	ADDA nodularin, nodularia toxin, spumigen, suomilide	Namikoshi *et al.*, (1993),
N. spumigena	nodularin-R	Carmichael *et al.*, (1988)
Nodularia spumigena BY1	Anabaenopeptin B	Fujii *et al.*, (1995)
N. spumigena	Linear peptide 2	Choi *et al.*, (1993)
N. spumigena KAC10, KAC12, KAC66, KAC68	Nodularin	Dahlmann *et al.*, (2001)
N. spumigena	Nodularia-R (cyclic nonribosomal peptide)	Sivonen *et al.*, (1989b)
N. spumigena	Spumigins, anabaenopeptins, aeruginosins and nodularins	Mazur-Marzec *et al.*, (2013)

Table 1.8. Allelochemicals and their effects produced by *N. spumigena.*

Target species	Mode of action		References
	Beneficial	Detrimental	
Thalassiosira weissflogii		Growth inhibtor	Suikkanen *et al.,* (2004, 2005, 2006)
Rhodomonas sp.		Growth inhibtor	Suikkanen *et al.,* (2004, 2005, 2006)
Prymnesium parvum	No effect	No effect	Suikkanen *et al.,* (2004)
Cryptophytes		Growth inhibtor	Suikkanen *et al.,* (2004, 2005, 2006)
Snowella spp.	Growth promoter		Suikkanen *et al.,* (2005)
Pseudanabaena spp.	Growth promoter		Suikkanen *et al.,* (2005)
Anabaena spp.	Growth promoter		Suikkanen *et al.,* (2005)
Oocystis sp.	Growth promoter		Suikkanen *et al.,* (2005)
Macoma balthica		Acetylcholinesterase increase	Lehtonen *et al.,* (2003)

Nodularin (NOD) is the only hepatotoxin that is known to be produced by *N. spumigena* (Falconer, 2001). According to the world Health Organisation (WHO) a tolerable intake of nodularin is 0.04 mg/kg, but at lower levels this peptide can cause severe health problems in human beings (Schumacher *et al.*, 2012; Karlsson, 2003).

1.1.7. The Baltic Sea and *N. spumigena*

Due to special environmental, geographical, oceanographic and physiochemical conditions, second largest semi-enclosed brackish water body is the Baltic Sea (Fig. 1.8). The water body is under pressure from agriculture run off, human activities, excessive nutrients and greenhouse effects. It also receives the rainwater catchment from 14 countries (HELCOM, 2006). Due to its heavy nutrient load, the Baltic Sea is under the influence of eutrophication (Lilover and Stips, 2008; Report on

Figure 1.8. Map showing the location of the Baltic Sea
(Google map used with permission)

Estonia, 2005; Mazur-Marzec *et al.,* 2005) which has resulted in the occurrence of heavy 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., this produces massive and lethal blooms in many areas of the Baltic Sea (Suikkannen *et al.,* 2010; Lilover and Stips, 2008; Ibelings *et al.,* 2007; Sivonen *et al.,* 1989b). The blooms of *N. spumigena* are more common in low nitrogen and phosphorus deficient parts of the Baltic Sea. They rarely occur in the Kattegaat, the Baltic Sea, which has low N:P ratios and high salinity. Probably high salinity does not promote the bloom formation of *N. spumigena* (Lehtimäki, 2000).

Many cases have been reported to cause animal poisoning along the coasts of the Baltic Sea (Edler *et al.,* 1985; Persson *et al.,* 1984). *N. spumigena* has also been reported to have lethal blooms in Lake Alexandrina, Australia (Rinehart *et al.,* 1988). In the report of HELCOM (2006) it has mentioned that the level of nutrients is much higher than previous years reported in 1950. In past decades increasing nutrient enrichment is providing 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 compounds. Under favorable conditions *N. spumigena* produces a high amount of toxins within the cells (Lehtimäki *et al.,* 1997) and also release in the growth medium. The end of log phase and starting lag phase, together with other environmental factors, is best time to get the highest amount of cells and extracellular and intracellular microcystins (Vezie *et al.,* 2002).

1.1.8. Bioassays

1.1.8.1. *Daphnia* assays

Cyanobacteria are known to produce toxic compounds, which can be lethal to several zooplanktons and phytoplanktons. The toxins can affect growth, feeding rates, feeding habits, survivorship, population size and fecundity of zooplanktons. Small planktonic caladocerans, *Daphnia* spp. (water fleas; (whole organism assay/*in vivo*) are a useful tool to note the lethality of any known and unknown toxins. Several studies have mentioned that they inhibit feeding rates and increase mortality of

daphnids. The toxin producing strains *N. spumigena* (DeMott, 1991), *Microcystis aeruginosa* (Lurling and van der Grinten., 2003; Nizan *et al.,* 1996), *Microcystis* PCC7806 (Jungmann, 1992), *Planktothrix* spp. (Oberhaus *et al.,* 2007) and *Cylindrospermopsis raciborskii* (Nogueira *et al*., 2004) have been tested against lethality to daphnids. The lethality to daphnids depends on mode of exposure, species of daphnids and dose of toxins.

1.1.8.2. Protein phosphatase 1 assay (PP1)

Protein phosphatases are a group of several enzymes that catalyse the dephosphorylation of hepatocytes phosphoproteins (Fig. 1.9).

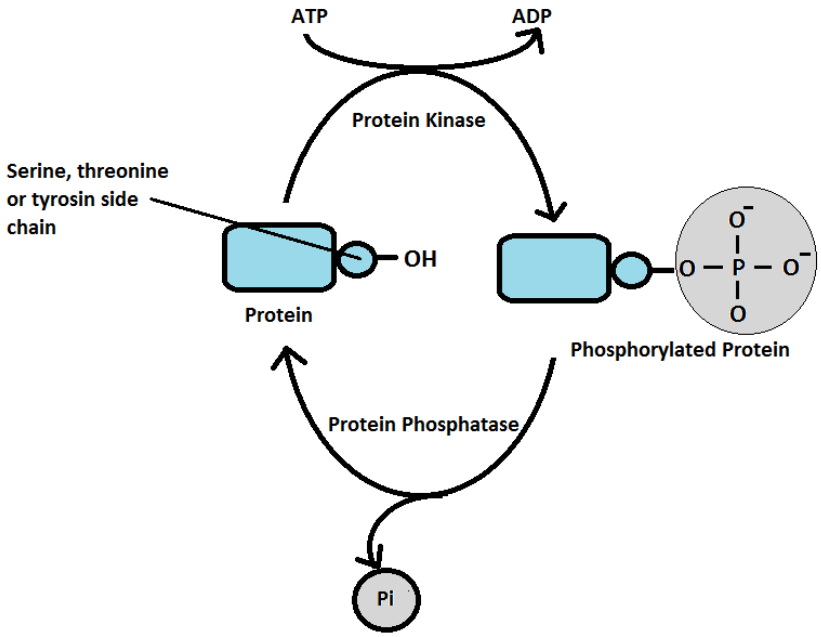


Figure 1.9. Mechanism of protein kinase and protein phosphatase
(ATP:- Adeno triphosphate, **ADP**:- Adeno diphosphae, **PO_4**:- phosphate, **OH**:- hydroxyl group, **Pi**:- inorganic phosphate)
(Adopted from: http://www.scq.ubc.ac/protein=phosphorylation-a-regulator-ofcellular-activity/)

Hepatotoxic peptides, the MCs and NOD, are potent protein phosphates (PP1 and PP2A) inhibitors, which are responsible for the dephosphorylation of phosphoproteins (MacKintosh *et al.,* 1993). Protein phosphatases (biochemical assay/*in vivo*) play an important role in mammalian liver cells. In hepatocytes regulatory proteins are phosphorylated on their amino acids namely serine and threonine residues. Serine/threonine-specific protein phosphatases regulate several cellular activities like cell proliferation and cellular processes (Ikehara *et al*., 2008). Both amino acids have similar side-chains and can be phosphorylated by a single enzyme.

In addition the inhibition of PP1 and PP2A relate to the hepatotoxicity of MCs and NOD, which leads to inhibition of PP activities in the cytoplasmic matrix of mammalian liver and attach with protein phosphatases 1 and 2A. This process leads to increase of phosphoproteins (hyperphosphorylation) and also disturbs the structure and homeostasis of hepatocyte results in liver necroses, haemorrhage and tumour formation in liver (Fig. 1.10), which leads to death (Yoshizawa *et al.,* 1990).

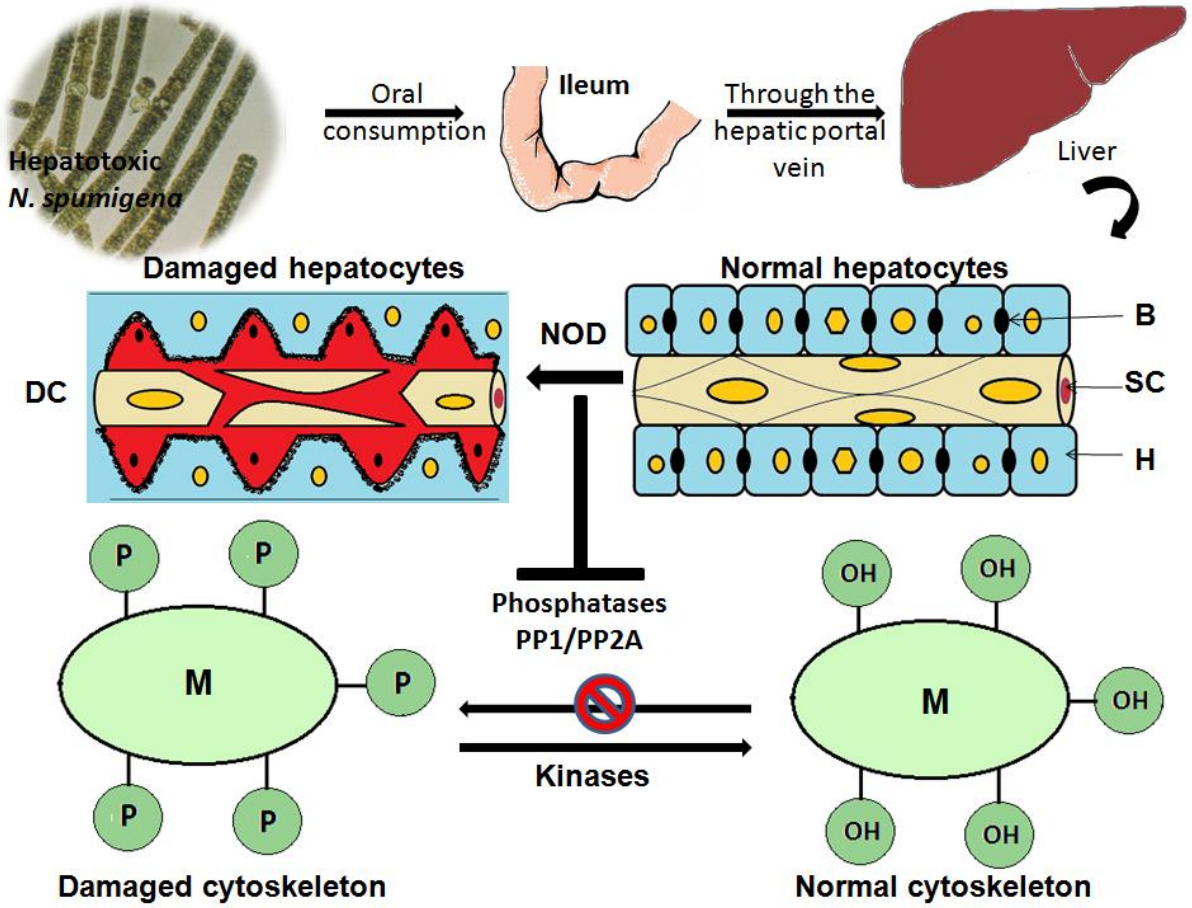


Figure 1.10. Route, mode of action, lethality and inhibition of protein phosphatase (PP1 and PP2A) by hepatotoxic NOD.

(**B**:- Bile duct, **SC**:- sinusoidal capillary, **H**:- hepatocytes, **DC**:- damaged capillary, **NOD**:- hepatotoxic nodularin, **PP1**:- protein phosphatase 1 **PP2A**:- protein phosphatase 2A, **M**:- microtubules, microfilaments, intermediary filaments, **P**:- phosphate group, **OH**:- hydroxyl group)
(Adopted from Carmichael, 1994 and Menezes et al., 2013)

MCs and NOD have similar toxicity mechanism to hepatocytes, except that NOD has Mdhb (2-(*N*-methyleamine)-2-dehydrobutyric acid), which does not bind covalently with protein phosphatases (Dawson, 1998). The Adda group (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) interact with hydrophobic catalytic site of protein phosphatase results in inactivation of protein phosphatase (Wiegand and

Pflugmacher 2005). The PP1 LD_{50} value for NOD is 30-50 µg/kg in the mouse (Chorus and Bartram, 1999).

Due to the hepatotoxic nature of microcystins and NOD, there are several reports available on the protein phosphatase inhibition activities isolated from *M. aeruginosa* (Gesner-Apter and Carmeli, 2009; Yoshizawa *et al.,* 1990; Honkanen *et al*., 1990)*, M. viridis* (Yoshizawa *et al.,* 1990)*, Planktothrix rubescens* (Grach-Pogrebinsky *et al.,* 2003), *Anabena flos-aquae, Oscillatoria agardhii* and *N. spumigena* (Yoshizawa *et al.,* 1990; Chapter 2; Table 2.1).

1.1.9. Effect of environmental parameters and *N. spumigena* KAC 66

The bloom formation in natural environments is controlled by abiotic and biotic factors. These environmental factors affect toxic bloom formation concerns namely, temperature, salinity, pH, light intensity and nutrient availability, especially nitrogen and phosphors (Sivonen, 1996). Much work has been done on the effects of temperature (Lehtimäki *et al.,* 1994 and 1997), salinity (Musial and Plinkski, 2003; Hobson and Fallowfield, 2003; Mosiandar *et al*., 2002; Lehtimäki *et al.*, 1997), nitrogen (Vuorio *et al*., 2005; Stal *et al*., 2003; Lehtimäki *et al.,* 1997) and phosphorus (Lehtimäki, 2000; Kononen *et al*., 1996) on the growth and toxin production by *N. spumigena*.

1.1.9.1. Temperature

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

The effects of temperature on cyanobacterial growth and biological process have been widely studied parameters. Higher temperature supports the cyanobacterial growth compared with other phytoplanktons. 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/s/m^2 *N. spumigena* produces high cell biomass and intracellular NOD toxins (Hobson and Fallowfield, 2003).

1.1.9.2. Salinity

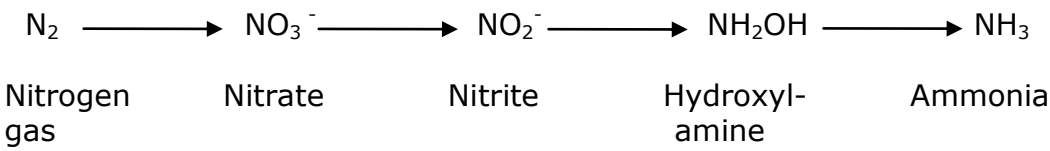
Cyanobacteria are salt tolerant organisms and can survive at extreme low and high salinity ranges. The salinity concentrations also affect the growth and nodularin production by *N. spumigena.* The extreme high and low salinities do not support growth of *N. spumigena* but promotes akinetes development with a decrease in heterocytes at 35 psu (Mazur-Marzec *et al.,* 2005).

The fluctuation and an increase and decrease 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 development of blooms (Dailidienė and Davulienė, 2008) and growth (Moisander *et al.,* 2002). Salinity combined with light intensities affect the production of NOD (Hobson and Fallowfield, 2001; Mazur-Marzec *et al.,* 2005), photosynthetic activities and N_2 fixation by *Nodularia* spp. (Moisander *et al.,* 2002; Hobson and Fallowfield, 2001).

1.1.9.3. Nitrate

Nitrogen gas (N_2) is a key part of atmosphere (80%) and nitrogen (N) is one of the primary nutrients, which is an important component for the survival of all living organisms. Although, dinitrogen gas (N_2) is available in high amount in the atmosphere, higher plants and animals are not able to utilise nitrogen gas.



In general, several cyanobacterial strains are able to use nitrate (NO_3^-), nitrite (NO_2^-), urea ($CO(NH_2)_2$), ammonium (NH_4^+), or some amino acids. In cyanobacteria, nitrogen plays an important part in synthesis of proteins and amino acids (for deoxyribonuclic acid, DNA) and production of other biologically important compounds like hepatotoxins, neurotoxins and lipopolysaccharide endotoxins. Heterocyts are thick walled specialised cells, which contain an enzyme nitrogenase for nitrogen

fixation. The diazotrophic/heterocytous (nitrogen fixing) and toxic *N. spumigena* and non toxic *A. flos-aquae* from blooms in nitrogen deficient areas of the Baltic Sea (Mur *et al.,* 1999; Lehtimäki *et al.,* 1997) and have ability to convert dinitrogen gas into nitrogen. Later nitrogen converts into ammonia (NH_3), thus can be utilised by primary producers like plants. The process of conversion of dinitrogen gas into biological nitrogen is termed as nitrogen fixation.

1.1.9.4. Phosphate

Phosphorus is also an important nutrient and available in dissolved form for aquatic organisms.

Since the last few decades human activities i.e. urbanisation, industrial and agricultural development, have contributed a major role in increasing cyanobacterial blooms in the Baltic Sea. In general, cyanobacteria require N:P ratio as 7:1 which depends and varies from species to species. The diazotrophic bacteria are primarily limited by low N:P ratio (Mazur-Marzec *et al.,* 2005; Stal *et al.,* 2003; Mur *et al.,* 1999). In the Baltic Sea low nitrogen and high phosphorus favour the blooms of *N. spumigena* and *A. flos-aquae* (Lehtimäki, *et al.,* 1997). The bloom development in the Baltic Sea is associated with low nitrogen (Lehtimäki *et al.,* 1997) and high phosphorus concentrations at low N:P ratios (Mazur-Marzec *et al.,* 2006) with moderate salinity (5–13 PSU; Kabir and Mandal, 2012; Mazur-Marzec *et al*., 2006). An increase or decrease in nitrogen and phosphorus concentrations affects cyanobacterial growth, community structure and toxin production (Lehtimäki, *et al.,* 1997).

1.1.10. Isolation of cyanobacteria and detection of peptides from different environments

It has been mentioned earlier that cyanobacteria are rich in production of secondary metabolites, which can be used in biological screening/drug discovery programmes, agricultural industry and to control a number of viruses, bacteria, fungi and other microorganisms (Mundt *et al.,* 2001). There is a need to isolate cyanobacteria from various water bodies to discover new compounds, which can also provide a comparison on geographical distribution and toxin production ability among genetically exact strains found in different habitats. The freshwater Dian Lake or Dianchi Lake or Kunming Lake, China and the Dead Sea are an interesting source to target new strains of cyanobacteria and also provide a comparison between same species toxicology isolated from fresh and hypersaline waters. The Dianchi Lake is famous for toxic blooms of *Microcystis aeruginosa*, *M. viridis* and *A. flos-aquae* (Wu *et al.,* 2009; Mei *et al.,* 2006 and Liu *et al*., 2006).

For several decades the Dead Sea has been under the influence of high salinity with the water level seeing a drop of more than 20 m (Oren *et al*., 2008). The increasing precipitation resulted in high salinity, which diminished a number of strains. In 1963 at 27 psu (Volcani, 1944, see Oren, 1999) several strains of cyanobacteria (Table 4.1) existed compared to the current time at 34-40 psu (Oren, 2008). However, under these intensive conditions the Dead Sea is dominated by viruses, bacteria, fungi and the increasing blooms of green alga, *Dunaliella pawa* (Oren, 2008 and 2000).

1.1.11. Aims and objectives

A number of studies have examined the effects of environmental factors (temperature, light, salinity, nitrate and phosphate) on the growth of *N. spumigena* KAC 66 but no work has been done on the effects of abiotic factors on freeze dried cell biomass, Chl-*a*, extracellular NOD and recently characterised nodulopeptin 901 produced by *N. spumigena* KAC 66. The present study has provided new knowledge about *N. spumigena* KAC 66 and the effects of environmental factors on the production of freeze dried cell biomass, Chl-*a*, extracellular NOD and intra and extracellular concentrations of newly discovered nodulopeptin 901 (Schumacher *et al.,* 2012). The lethality of fractions of *N. spumigena* has not been evaluated. The present research also provided knowledge on lethality of fractionated extracts of *N. spumigena* to *Daphnia pulex, D. magna* and inhibition of protein phosphatase 1 assay. The present study also provided information on compounds found in toxic cyanobacterial strains, which were isolated from freshwater Dian Lake and hypersaline lake, the Dead Sea.

Main goals of this study were:

1.1.11.1. Evaluate the toxicity of bioactive components of *N. spumigena* by daphnids and inhibition of protein phosphatase assays

Daphnia spp. and protein phosphatase 1 assays are more frequently using bioassays to determine the lethality of compounds or whole cells. This study is the first of its kind to determine the lethality of fractions of *N. spumigena* KAC 66 collected from the reversed phase flash

chromatography. The fractions were tested against two strains of daphnids, *Daphnia pulex* and *D. magna* and for inhibition of protein phosphatase 1.

1.1.11.2. Evaluate the optimal growth conditions for *N. spumigena* KAC 66 in two different culture vessels

Mass culturing plays an important role to obtain highest cell biomass to isolate new compounds. The present study has provided knowledge about optimal growth conditions to obtain maximum cell biomass, NOD and nodulopeptin 901. For this purpose *N. spumigena* was grown in 8 L Perspex columns and 10 L glass flasks under laboratory conditions at 22°C.

1.1.11.3. Investigate the effects of environmental factors on growth and toxin production by *N. spumigena*

The production of cell biomass, Chl-*a* and peptides can be affected by extreme environmental conditions. The extreme low or high environmental conditions can be helpful to increase or decrease the production of cell biomass and peptides. The control on availability of nutrients can also be helpful to control cyanobacterial blooms and their toxin production in natural water bodies as well as production of high amounts of peptides for laboratory based experiments. The nutrient concentrations can also alarm the developing blooms. A number of studies have been done on the effects of abiotic factors (light, temperature, salinity, phosphate and nitrate) on the production cell biomass and intracellular NOD production. There is no information available regarding the effects of the nutrients on the production of

extracellular NOD and intra and extracellular levels of recently characterised nodulopeptin 901. This study has provided the information on the effects of temperature, salinity, phosphate and nitrate on the production of NOD and newly characterised nodulopeptin 901.

1.1.11.4. Isolation of cyanobacterial strains and identification of bioactive compounds from freshwater Dian lake, China and hypersaline lake, the Dead Sea

In 1944 due to less salinity the Dead Sea was inhabited by a number of microorganisms, which do not exist at the present time (Oren, 2008). The present study has provided a recent update of occurrence of cyanobacterial strains from Israeli side, Quedem, the Dead Sea. During this research some strains have been isolated and analysed on UPLC-PDA-MS.

In contrast bloom samples from freshwater Dian Lake, China were also isolated and analysed on UPLC-PDA-MS. Therefore, it is interesting to know that two strains namely *Microcystis* spp., showed the presence of several toxic peptides during UPLC-PDA-MS analysis.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF PEPTIDES FROM *NODULARIA SPUMIGENA* KAC 66

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2.1. INTRODUCTION

Cyanobacteria have been found to produce a wide range of bioactive compounds which can be of interest in drug discovery or due to their impact on both natural ecosystems and human health. One reason why interest continues in that, cyanobacteria can be found in a wide range of habitats, which is thought to lead to a wide diversity of interesting active metabolites.

More than 120 natural products have been isolated from the cyanobacteria and most of them are secondary metabolites. These metabolites have showed cytotoxic, antimalarial, anticancer, anti-HIV, antifungal and antimicrobial activities (Burja *et al.,* 2001). Schrader *et al.,* (2002) isolated several bioactive compounds from cyanobacteria and algae, which showed lethality against herbs, algae, insects, molluscs and fungus related to control of agriculture invertebrate pests and insects. The nitrogen fixing marine cyanobacterium, *Nodularia harveyana* isolated from the Mediterranean Sea expressed antibiotic, allelopathic activity and toxicity against a wide range of organisms such as chlorophytes, cyanobacteria, eubacteria, rotifers and crustaceans (Pushparaj *et al.,* 1998).

Nodularia spp. are found primarily in brackish water but can tolerate a wide range of salinities. They are also well known as producers of the potent cyanotoxin, nodularin (NOD). *Nodularia spumigena* KAC 66 was isolated from the Baltic Sea and known to produce other compounds. This study explores the purification and biological activity of compounds

produced by *N. spumigena* KAC 66. Two bioassays were selected to screen to the samples. Firstly the *Daphnia* bioassay was performed, which is a generic test for aquatic toxins. The other assay used was protein phosphatase inhibitory assay, which is known to detect both NOD and microcystins (MCs) was employed to evaluate the presence of other compounds related to this group.

The wide distribution and toxicological effects of hepatotoxic *Nodularia spumigena* on food chain, has caused significant attention towards the effects of the species. The present study will investigate further *N. spumigena* to detect the effects of peptides it produces on PP1 and *Daphnia* spp.

In the present study two well established bioassays, *Daphnia* as a generic aquatic toxicity test and protein phosphatase inhibitory assay as a specific induction at the action of NOD and MCs (Gulledge, *et al*., 2002; Yoshizawa *et al*., 1990) were used.

2.1.1. Bioassays

2.1.1.a. *Daphnia* bioassay

A number of simple bioassays can be performed to test toxicity, ranging from molecular assays to whole-organism assays (McLaughlin, 1991).

Daphnia (Daphnids or water fleas) are members of aquatic crustaceans (Order Cladocera and Class Crustacea). They are found in freshwater ponds. Daphnids can tolerate varying levels of environmental factors, and common biology experiment conditions. They are considered a useful tool

for preliminary assessment of cytotoxicity. The neonates or adult daphnids are used for *Daphnia* lethality assay. Under favourable conditions daphnids produce parthenogenicity, while under stressed conditions they reproduce sexually and produce dark brown/black saddle-shaped resting egg cases known as ephippia.

Bioactive natural compounds are often toxic to daphnids (Reinikainen *et al*., 2002 and 2001). Hence *in vivo* lethality to daphnids can be used as a rapid and simple preliminary screening of bioactive compounds during the isolation of natural products.

Daphnia assays have also been used for the detection of fungal toxins and their active peptide metabolites (Czarneck, *et al.,* 2006) along with polyunsaturated fatty acids and microcystin (Reinlkainen *et al.,* 2000).

It has been suggested that the *Daphnia* assay can determine the lethality of materials indicating the ability of compound to kill cancer cells (Olvera-Ramírez, *et al.,* 2010). The ingestion or direct exposure to cyanobacterial toxins may affect on population, feeding and filtrating rates of daphnids. There are several reports available on the effects of *N. spumigena, M. aeruginosa* and *Microcystis* PCC 7806 on the survival and feeding behaviour of *D. pulex, D. hyaline, D. pullicaria* and *D. magna* (DeMott, 1991; Reinikainen *et al.,* 1994, Lauren-Maatta, *et al.,* 1997; Jungmann, 1992 and Reinikainen *et al.,* 2001). Hence the *Daphnia* bioassay was established and used to screen toxicity of compounds/fractions of *N. spumigena* KAC 66.

2.1.1.b. Protein phosphatase assay (PP1)

MCs and NOD are named as tumour promoters and inhibit PP1 and PP2A, which set to regulate liver function (see 1.8.2). This assay was developed on the basis of the ability of MC and NOD to inhibit serine/threonine protein phosphatase enzymes (Table 2.1).

Table 2.1. Inhibition of PP1 (IC_{50}) by microcystin-LR and NOD

MC-LR	NOD	References
0.03 nM	-	Ward *et al.,* (1997)
-	1.8 nM	Honkanen *et al.,* (1990)
-	0.7nM	Yoshizawa *et al.,* (1990)
1.4 nM		Yoshizawa *et al.,* (1990)
1.6 nM		Yoshizawa *et al.,* (1990)
1.7 nM	-	Honkanen *et al.* (1990)
0.3 nM	-	Ana and Carmichael, (1994)
0.01 nM	-	Fontal *et al* ., (1999)
~0.1 nM	-	Mackintosh *et al.,* (1990)

MCs and NOD are known tumour producers due to their inhibitory activity on protein phosphatase PP1 and PP2A, which act to regulate cell function.

Due to this potent inhibitory activity it has been possible to develop an enzyme inhibition assay which determines both MCs and NOD along with related compounds. The assay vary in their determination methods with early methods relying as radio labelled phosphate where as now most researchers employ as colour reaction in a 96 well plate format.

PP1 consists of a targeting subunit or a specific protein inhibitor (see section 1.8.2). The *para*-nitrophenyl phosphate (*p*NPP) hydrolysis in the presence of phosphatase and form *para*-nitrophenol and under alkaline conditions it converts into *para*-nitophenolate, with a strong yellow colour detectable at 405 nm (Fig. 2.1).

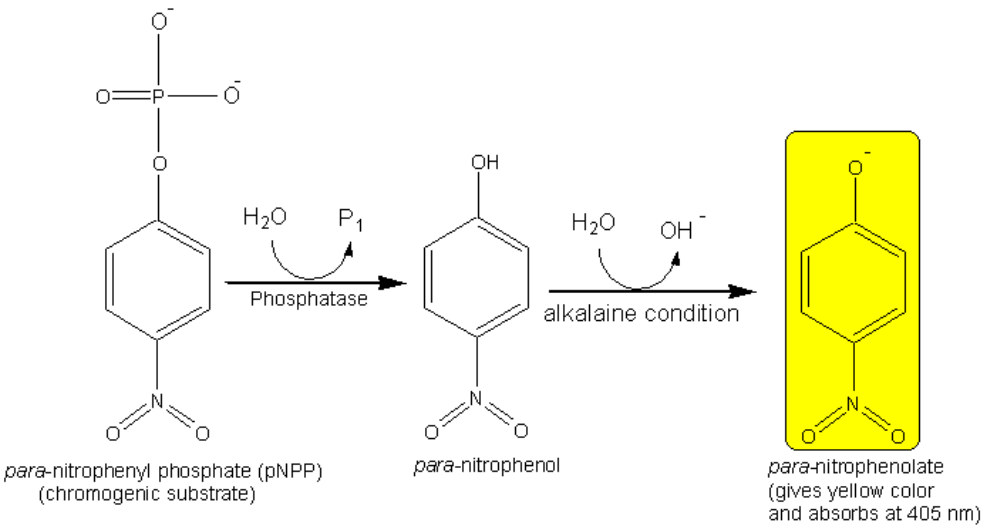
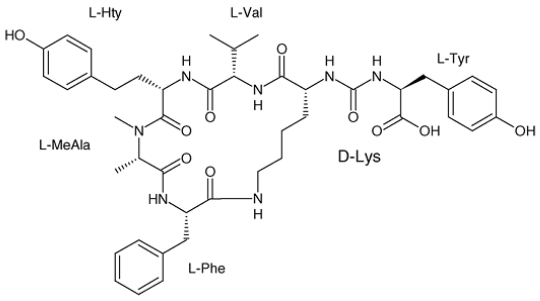


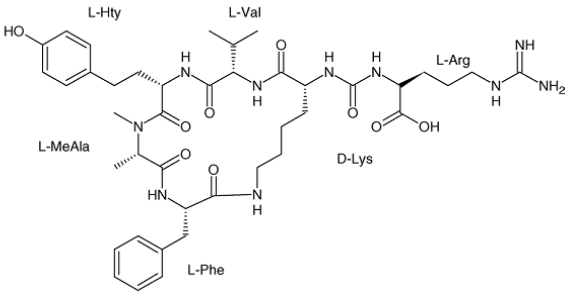
Figure 2.1. Chemical reaction of hydrolysis of *para*-nitrophenyl phosphate (*p*NPP) resulted in *para*-nitrophenol and than *para*-nitophenolate (which gives yellow colour; (**Pi**= phosphate group or inorganic phosphate)
(adopted from *http://www.gbiosciences.com/PhosphataseAssay-desc.aspx*).

PP1 assay has several advantages for the laboratory strains and bloom samples tested. PP1 has better advantages as compared to the HPLC as the screening method is more sensitive; whilst the expensive equipment, sample unit cost and level of expertise required are markedly lower (Ward *et al.,* 1997).

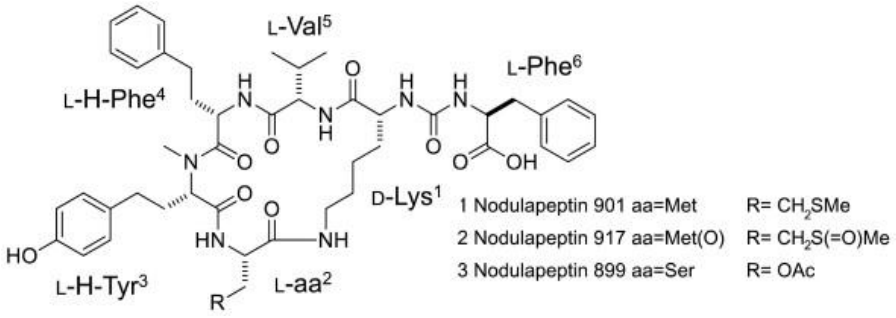
The aim of this study was to determine the lethality against *Daphnia pulex, D. magna* and PP1 inhibitory activity of NOD and recently discovered nodulopeptin 901 present in the aqueous fractions of brackish water cyanobacterium, *N. spumigena* KAC 66. Anabaenopeptolin A, B and linear peptides were also used to determine their inhibitory activity against protein phosphatase. The anabaenopeptin A and B were used as reference peptides as they have similarity in structure with newly characterised nodulopeptin 901 (Figs. 2.2; Table 2.2).



A



B



C

Figure 2.2. General chemical structures of cyanobacterial PP1 inhibitory peptides.
A:- Anabaenopeptin A, **B**:- Anabaenopeptin B,
C:- Nodulopeptin 901

Table 2.2. Inhibition percentage of PP1 by anabaenopeptins and anabaenopeptilides (Gkelis *et al*., (2006)

Peptides	% Inhibition
Anabaenopeptin A	45-60%
Anabaenopeptin B	5-75%
Anabaenopeptilides	5-35%
Anabaenopeptilides	5-23%

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Fisher Scientific, Leicestershire, UK. HPLC-grade methanol and acetonitrile were purchased from Rathburn, Walkerburn, UK. Pure water was obtained from a Milli-Q system (Millipore, Watford, UK).

Anabaenopeptin A and B were purified from batch cultures of *Microcystis aeruginosa* as previously described for microcystins (Edwards and Lawton, 2009 and Edwards *et al*., 1996). NOD, nodulopeptin 901 and LNOD were obtained from the cultures of *N. spumigena* KAC 66 following the same protocols.

2.2.2. Large scale culture of *N. spumigena* KAC 66

N. spumigena KAC 66 was grown in BG-II with 20% instant ocean water in 10 L Pyrex flasks or 8 L Perspex columns (Mazur-Marzec *et al*., 2006). Cultures were sparged with sterile air (0.22 µm filter, Millipore, UK) and exposed to continuous illumination generated by cool white Osram

fluorescent tubes (58 W) 20 µmol/s/m^2 (Li-Cor intelligent light meter Li-250) in a temperature controlled (22°C ± 2) room.

2.2.3. Harvesting and extraction of *N. spumigena* KAC 66

Cyanobacterial cultures were harvested by centrifugation (2,500 x rpm, 4°C) for 30 min and stored at -20 °C prior to being thawed for extraction.

Freeze-thawed wet cells were extracted for 1 h in 50% (v/v) aqueous methanol and centrifuged (1500 rpm, 30 min). Supernatants were decanted and the pellets were re-extracted a further two times. The aqueous extracts were diluted to 20% (v/v methanol) and loaded into C18 cartridge for concentration and clean-up using reversed phase flash chromatography (Edwards *et al*., 1996).

2.2.4. Reversed Phase Flash Chromatography (RPFC)

Concentration and clean-up of nodularin and other peptides was achieved by application of the 20% (v/v) aqueous methanol extracts onto a preconditioned C18 cartridge (40 mm I.D. x 75 mm long; 40-63 µm particle size) at 40 mL/min, using the Biotage Horizon™ flash chromatography system (Cardiff, UK). Compounds were eluted with a gradient from 0 to 100% methanol, in 10% increments with 240 mL per step/fraction. Fractions were analysed by HPLC-PDA-MS and those containing target peptides were further purified by preparative HPLC.

2.2.5. Preparative HPLC

Preparative HPLC separations were achieved using a Phenomenex (Macclesfield, UK) Luna C18 column (21 mm I.D. x 250 mm long; 10 µm

particle size). Mobile phase was Milli-Q water (A) and acetonitrile (B) both containing 0.05% TFA. Samples from flash purification were redissolved in 50% (v/v) aqueous methanol and 0.5 mL injected per run. The "sandwich" solvent, used to surround the sample in the loop and prevent precipitation, was also 50% (v/v) aqueous methanol. Polar compounds, such as nodularin, were eluted using a gradient increasing from 25% to 50% B over 30 min at a flow rate of 20 mL/min. Less polar compounds such as nodulopeptin 901 were eluted with a gradient increasing from 45% to 75% B over 30 min at the same flow rate. Fractionation was based on volume and eluent was collected in deep well microtitre plates (48 x 5 mL). Fractions were analysed by HPLC-PDA-MS and those of acceptable purity were pooled, desalted, eluted in methanol and dried and weighed to constant weight (Welgamage, 2012).

2.2.6. HPLC-PDA-MS analysis

For identification and quantification of nodularin and nodulopeptin 901 was performed using HPLC-PDA-MS (Lawton *et al.,* 1994). 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 (5 µm particle size; 2.1 mm i.d. 150 mm long) adjusted at 40°C using a Waters temperature control module.

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 followed by ramp up to 100 % B and re-equilibration after 10 next 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 ºC and 300ºC, respectively. MassLynx software v4.1 was used to control the instrument for data acquisition and processing. The photo diode array (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. Compounds were identified on the basis of retention time, characteristic UV and mass spectra compared to standards. Quantification of peptides was based on calibration with external standards, nodularin at 238 nm and nodulopeptin 901 at 210 nm.

2.2.6.1. Calibration curves

Purified NOD was re-dissolved in 80% (v/v) aqueous methanol to a concentration of 200 µg/mL. A concentration range from 0.1 to 200 µg/mL was analysed on HPLC-PDA-MS. Since the absorbance maximum is 238 nm, chromatograms were extracted at this wavelength and peak area determined.

Nodulopeptin 901 was prepared in 80% (v/v) aqueous methanol at a concentration of 100 µg/mL. A concentration range from 0.1 to 100

µg/mL was analysed by HPLC-PDA-MS. The chromatograms were extracted at wavelength 210 nm and peak area response determined.

2.2.7. Extraction and fractionation for bioactivity evaluation

Six hundred mL cells of *N. spumigena* KAC 66 extracted in 600 mL of methanol (100%) in a flask. The flask was shaken well and left for the whole night for extraction. Next day 1800 mL of Milli-Q water was added and passed through filtration unit. A sample of filtrate was taken for UPLC-PDA-MS analysis to note the presence of any NOD and nodulopeptin 901. Six hundred mL was kept for second extraction (Fig. 2.3).

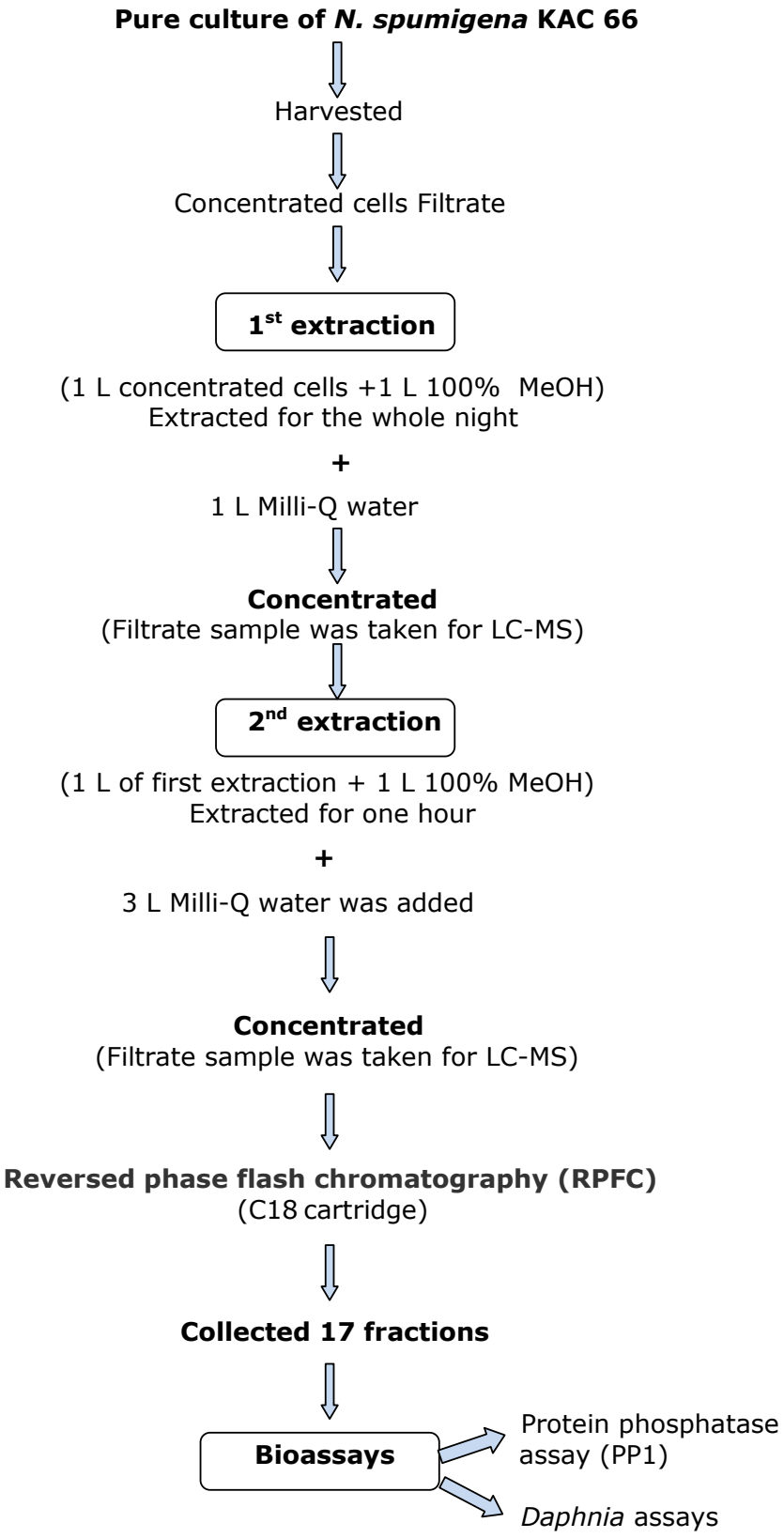


Figure 2.3. Summary of extraction and fractionation method used for *N. spumigena* KAC 66.

For fractionation an Isolera Flash chromatography system (Biotage, Cardiff, UK) was used. The SNAP C18 cartridge (60 g, 39 mm I.D. x 157 mm long; particle size 37-70 µm) was pre-conditioned with 200 mL of 100% methanol followed by 300 mL of Milli-Q water. The aqueous extracts were diluted to 20% (v/v methanol) and loaded onto the cartridge at a flow rate of 40 mL/min. Compounds were eluted with a linear gradient from 0 to 100% methanol. Fractions were collected on the basis of volume (60 mL/fraction) and monitored by UV at 254 nm and 220 nm.

Fractions were analysed by UPLC-PDA-MS to detect the presence of any NOD and nodulopeptin 901. The spent media from first and second extracts were also analysed.

Aliquots (2 mL) of fractions were dried at 45°C under nitrogen and stored at -20°C prior to use in bioassays.

2.2.8. Analysis by UPLC-PDA-MS

The system comprised a Waters Acquity Ultra performance LC coupled to a photodiode array and a Xevo quadrupole time of flight mass spectrometer (Waters, Elstree, UK). Samples were separated on a BEH C18 column (100 x 2.1 mm; 1.7 µm particle size) which was maintained at 40°C. Mobile phase was Milli-Q Water plus 0.1% formic acid (A) and acetonitrile plus 0.1 % formic acid (B). Separation was achieved using a gradient increasing from 20% B to 70% B over 10 min, followed by a 100% B wash step and re-equilibration. Autosampler was maintained at 6°C at all times.

Data was acquired in positive ion electrospray scanning from *m/z* 50 to 2000 with a scan time of 2 s and inter-scan delay of 0.1 s. Ion source parameters; capillary and sampling cone were 2.9 V and 25 V respectively; desolvation temperature, 300ºC; and source temperature, 80ºC. Cone gas and desolvation gas flows were 50 L/h and 400 L/h respectively. Sodium iodide (2 µg/µl in 50% aqueous propan-2-ol (v/v)) was used as the calibrant with leucine-enkephalin (0.5 mg/L in 50% aqueous methanol (v/v)) as the lockspray. Instrument control, data acquisition (centroid) and processing were achieved using MassLynx v4.1.

2.2.8.1. Calibration curves

Nodularin and nodulopeptin 901 standards were prepared in 80% (v/v) aqueous methanol at a concentration of 100 µg/mL. For each compound a range of concentrations from 0.1 to 100 µg/mL were analysed on UPLC-PDA-MS. Chromatograms were extracted at 238 and 210 nm for quantification of nodularin and nodulopeptin 901, respectively.

2.2.9. *Daphnia* bioassay (*in vivo*)

For comparison of toxicological effects of fractions from KAC 66 and purified NOD on two species of daphnids i.e. *Daphnia pulex* and *D. magna* were used.

For this assay daphnids were provided by Yorkshire Brine Shrimp suppliers (Bradford, UK). The Daphnids were acclimatised in 2 different population culture tanks in a temperature controlled room under continuous fluorescence light (0.77-1.21 µmol/s/m^2) at 22ºC for 24 h.

Two litres of Chalkley's (CH) and ASTM media were used for culturing of *D. pulex* and *D. magna*, respectively. *Chlorella vulgaris* (50 mL) was used as food (≈17x10^7 cells/mL) and added to each tank. Each day dead daphnids were removed from the tanks by 10 mL pipette. Ten healthy daphnids females were selected from culture tanks using dissecting microscope.

2.2.9.a. Chalkley's medium (CH) for *D. pulex*

For cultivation of *D. pulex* Chalkley's medium was used (Table 2.3). Five mL of each nutrient was added in 1 L of deionised water and autoclaved at 15 psi for 15 minutes.

Table 2.3. The chemical composition of growth medium Chalkley's medium (CH) use for cultivation of *D. pulex*

Nutrients	**Stock solution (g/500 ml)**	**Final conc. (g/L)**
NaCl	2	0.04
KCl	0.08	0.0016
$CaCl_2$	0.12	0.0024

2.2.9.b. ASTM medium for *D. magna*

D. magna were cultivated in artificial freshwater ASTM hard water (Table 2.4). Ten mL of each nutrient used in one litre of deionised water autoclaved at 15 psi for 15 minutes.

Table 2.4. ASTM hard water growth medium for cultivation of *D. magna.*

Nutrients	**Stock solution (g/500 ml)**	**Final con. (g/L)**
$NaHCO_3$	9.6	0.19
$CaSO_4.2H_2O$	6	0.12
$MgSO_4.7H_2O$	12.8	0.25
KCl	0.4	0.008
Seaweed extract	0.5 µl	1 ppm/L

pH was 7.6-8.0
Hardness (as mg/L of $CaCO_3$): 160-180 g/500 ml

2.2.9.c. Culturing of *Chlorella vulgaris*

Ten percent of one month old pure isolate of *C. vulgaris* was inoculated in a two litre Erlenmeyer flask containing 1 L BG-11. The culture was grown for 2 weeks under white fluorescent light (17.67-17.91 µmol/s/m^2) in temperature controlled room at 22ºC. After 2 weeks the culture was scaled up to 1800 mL with BG-11 and 50 mL of culture was used as food for daphnids culture tanks containing 10 L CH/ASTM media. The cell count was performed on Sedgewick rafter counting cell slide (Fisher Scientific, Roskilde, Denmark) under light microscope (x4 magnification; Olympus, UK).

2.2.9.d. Preparation of fractions, NOD and nodulopeptin 901 for daphnid assays

The same methods, preparation of toxins and fractions dilutions were used to determine the lethality of toxins and fractions against *D. pulex*

and *D. magna*. Daphnids were tested for the lethal effects of standard NOD and nodulopeptin 901 and 17 fractions.

Purified NOD was diluted in CH and ASTM media for *D. pulex* and *D. magna,* respectively, to give a concentration range of 0.1-100 µg/mL. The pure nodulopeptin 901 was diluted in ASTM medium for *D. magna* to give a concentration range of 0.1-120 µg/mL.

2.2.9.e. Stock solution for 17 fractions of *N. spumigena* KAC 66

Aliquots (2 mL) of each flash fraction were evaporated under nitrogen at 45ºC for 3-4 h. The dried fractions were re-suspended in 2 mL of CH/ASTM media for *D. pulex* and *D. magna,* respectively.

Fractions were diluted x2 and x4 and tested in duplicate. NOD at a concentration of 100 µg/mL was used as a positive control and media alone was used as negative control.

2.2.9.f. Experimental setup

One mL of each test sample and 1 mL of daphnids (10-15) were placed in triplicate in 24 well Nunclon plates (Thermo Fisher Scientific, Roskilde, Denmark; Fig. 2.4).

The Nunclon plates were covered with lids and incubated in a temperature controlled room under continuous fluorescent light (0.78-0.99 µmol/s/m^2) at 21ºC for 24 h. Next day both dead and living daphnids were counted and LC_{50} values were calculated.

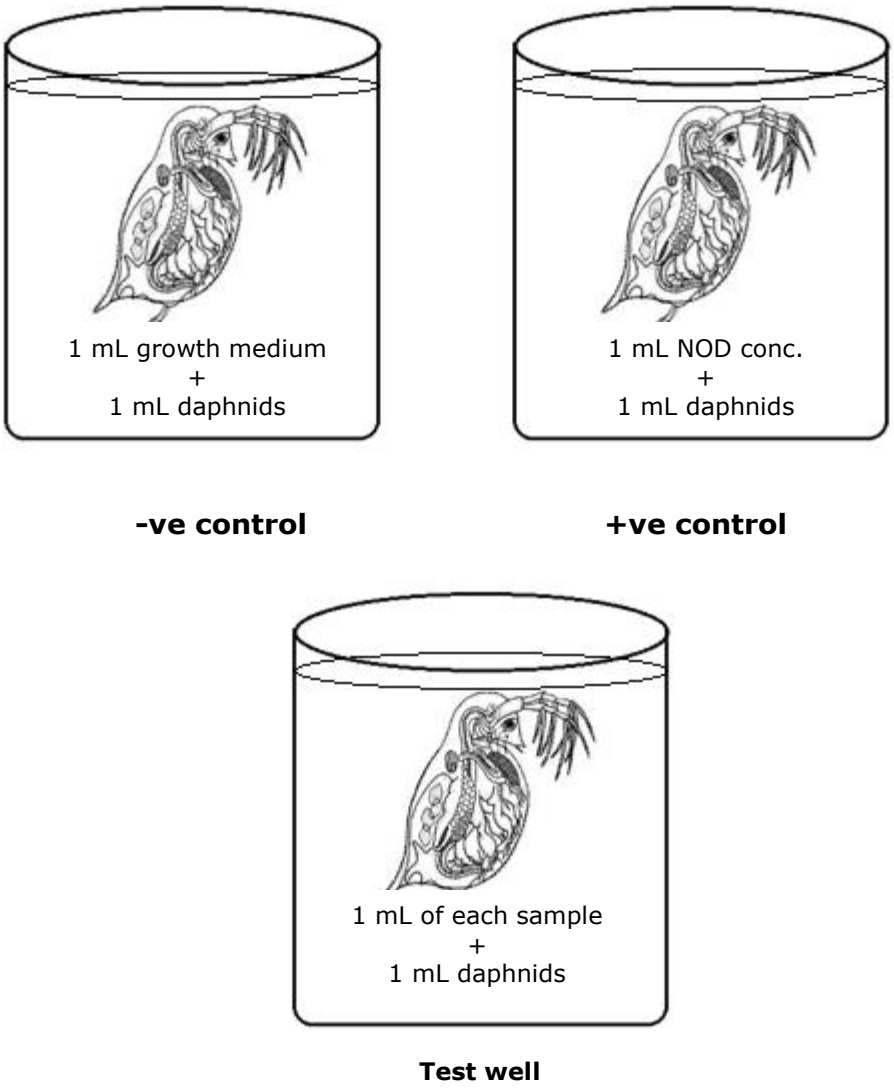


Figure 2.4. Experimental design of *Daphnia* assay to detect the lethality of toxins against *D. pulex* and *D. magna.*

2.2.10. Protein phosphatase inhibition assay (PP1; *in vitro*)

To determine the inhibitory activity against PP1, NOD, nodulopeptin 901, 17 fractions of *N. spumigena*, anabaenopeptin A (ANA), anabaenopeptin B (ANB) and linear NOD (LNOD) were used.

Protein phosphatase inhibition assay was performed using a modification of previously reported colourimetric procedures (Ana and Carmichael, 1994 and Ward *et al.,* 1997 and Liu *et al.,* 2002). The assays were performed in triplicate.

Buffer A and B were prepared in 500 mL Milli-Q water. All chemicals and the enzyme were obtained from Sigma, Poole, UK.

Buffer A contained 50mM Tris-HCl, BSA (Bovine serum albumin; 0.5 g/500 mL), $MnCl_2$ 1.0mM and Dithiothreitol 2.0mM. The buffer was adjusted to pH 7.4 using freshly prepared 1M NaOH.

Buffer B contained 50mM Tris-HCl, $MgCl_2$ 20mM, $MnCl_2$ 0.2mM and *p*-nitrophenyl phosphate (phosphate substrate) 5mM. The buffer was adjusted to pH 8.0 using freshly prepared 1M NaOH. The light sensitive buffer B was wrapped in aluminium foil to prevent from light. All buffers were freshly prepared before use.

Buffer C contained protein phosphatase 1 (PP1) was diluted with buffer A to obtain a working concentration of 5.0 µg/mL.

NOD was double diluted to obtain a concentration range of 0.009 to 10 µg/mL, which would enable determination of IC_{50}.

Other test peptides (nodulopeptin 901, linear NOD, ANA and ANB) were prepared at a broader concentration range (0.009 to100 µg/mL).

2.2.10.a. Test fractions: 17 fractions of *N. Spumigena* KAC 66

Seventeen fractions of *N. Spumigena* KAC 66 collected from reversed phase flash chromatography (RPFC) were tested to determine the inhibitory activity of NOD and nodulopeptin 901 against PP1.

Two mL of each methanolic fraction was evaporated under nitrogen at 45 ºC and re-suspended in 200 µl of Milli-Q water (stock solution) and vortexed thoroughly to dissolve the material giving x10, x100 and x1000 concentration sample. Each fraction was tested for protein phosphatase inhibition.

Concentrated fractions were diluted 10 fold (equivalent to undiluted flash fraction), 100 fold and 1000 fold.

2.2.10.b. Experimental setup

All working solutions were freshly prepared and kept at 4ºC prior to use. Microtiter plates were prepared by adding enzyme (10 µl; 5.0 µg/mL) followed by test toxins (10 µl) in three replicate wells. Substrate (180 µl) was added to the wells using 22-200 µl multichannel pipette (Anachem, Luton, UK).

The plate was incubated and shaken (30 rpm) in a shaking incubator (Stuart Orbital Incubator, Staffordshire, UK) overnight (14 h) at 37ºC immediately (Table 2.5). After 14 h the plate placed in a reader at 405

Table. 2.5. Details of method used in PP1 assay.

Conditions	Step 1	Step 2	Step 3	Step 4
Blank	10 µl buffer A	190 µl MQ	After adding substrate the plate was shaken (30 rmp) and incubated overnight (14 h) at 37 ºC.	After 14 h the plate placed in a reader at 405 nm.
Control	10 µl MQ	190 µl buffer B/substrate		
+ ve control (0 µl fractions)	10 µl enzyme/buffer C (5.0 µg/mL) + 10 µl NOD (10 µ/mL)	180 µl substrate		
Samples (peptides)	10 µl enzyme/buffer C (5.0 µg/mL) + 10 µl fractions/peptides	180 µl substrate		

nm on Microplate Spectrophotometer (Biotek, Vermont, USA) using Gen 5 programme.

Protein phosphatase was detected by colour change reaction through the action on *para*-nitrophenol phosphate. The developed yellow colour showed no enzyme inhibition (Fig. 2.5).

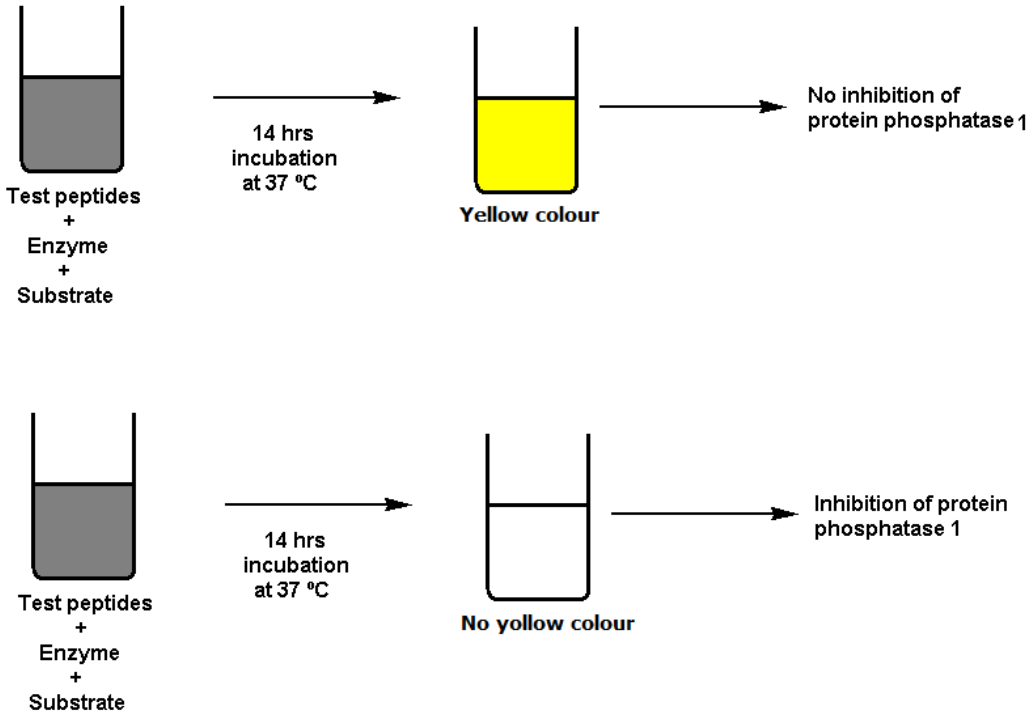


Figure 2.5. Development of colouration indicates no inhibition of PP1 and no colour indicates inhibition of PP1.

The percent inhibition was calculated by following formula. Inhibition concentration (IC_{50}) for all peptides and 17 fractions were calculated from graphs.

$$\text{Inhibitory \%} = \frac{\text{Normal activity - inhibitory activity}}{\text{Normal activity or +ve control}} \times 100$$

2.3. RESULTS

2.3.1. Isolation and purification of peptides from *N. spumigena* KAC 66

The 20% methanol fraction contained linear nodularin and nodularin as determined by UV (λ max 234 and 238 nm) and mass spectra ($[M+H]^+$ *m/z* 843 and 825; Figs. 2.6 ad 2.7; Appendices 1-4).

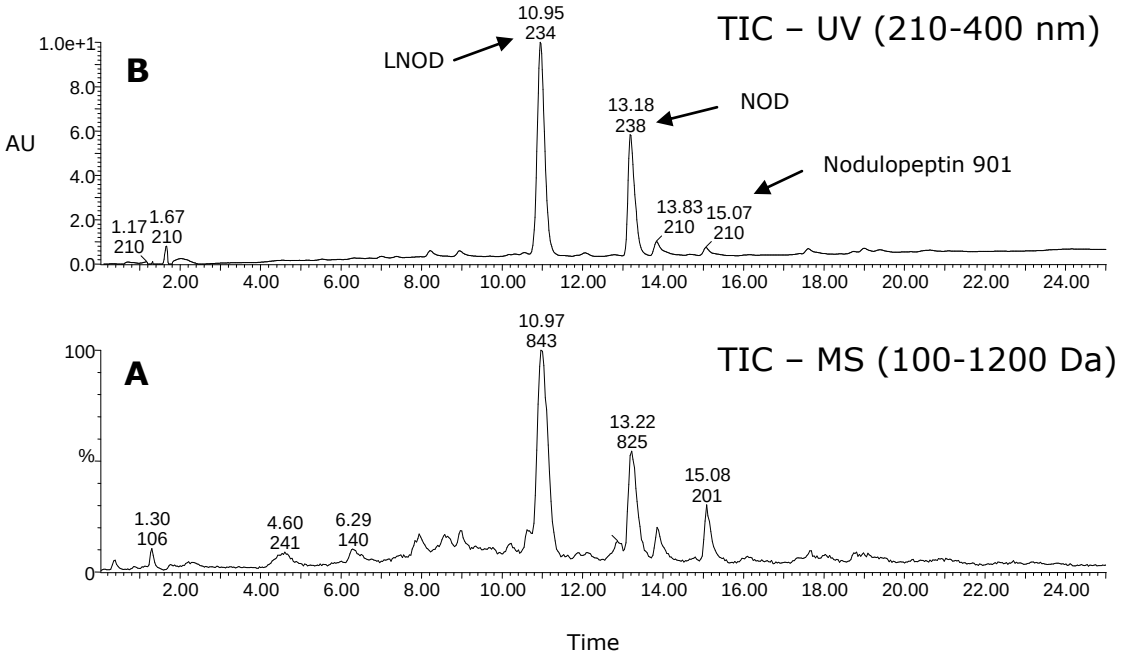


Figure 2.6. UV and MS of 20% methanol fraction from flash chromatography

The 40% methanol fraction contained nodulopeptin 901 as determined by $[M+H]^+$ at *m/z* 902 (Appendices 5-7). Both flash fractions were processed and further purified by preparative HPLC resulting in linear NOD (4 mg), NOD (3 mg) and nodulopeptin 901 (3 mg). The purified peptides further used as standards and experimental compounds.

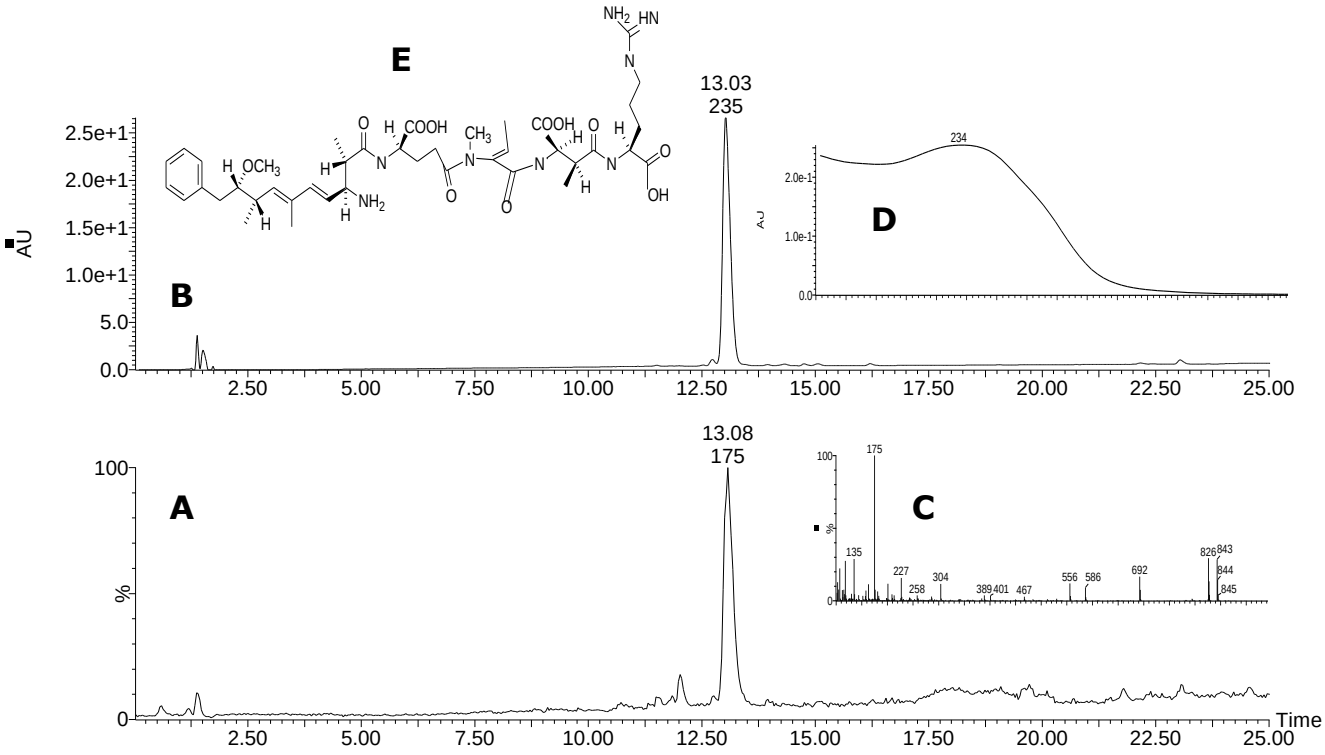


Figure 2.7. Typical absorption spectrum of linear NOD (LNOD) with presence of peptide at 235 UV spectrum (*m/z*; **A** and **B**), mass spectrum (**C**), absorption spectrum (**D**) and chemical structure of LNOD (**E**).

2.3.2. HPLC-PDA-MS analysis

2.3.2.a. Standard curve of NOD

The standard curve of NOD was prepared between the concentrations of NOD against peak area response by HPLC-PDA-MS. The UV chromatogram of NOD was extracted at its highest absorption 238 nm and continued a correlation coefficient of R^2=0.9998 (Fig. 2.8; Appendix 8).

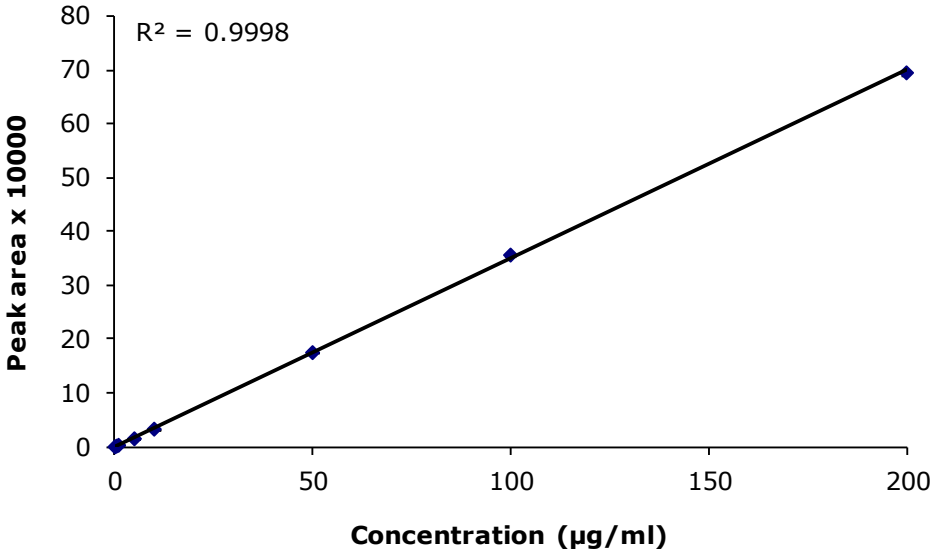


Figure 2.8. Standard curve for NOD quantified on HPLC at the wavelength of 238 nm with diode array at 12.00 min retention time. SD was less than 5% mean of n=3 (n=3, bars= 1 SD).

2.3.2.b. Standard curve of nodulopeptin 901

The standards curve of nodulopeptin 901 was plotted as the concentration of nodulopeptin 901 against peak area responded by HPLC-PDA-MS (Fig. 2.9; Appendix 9). The standard curve of nodulopeptin 901 gave a correlation coefficient of R^2=0.9996.

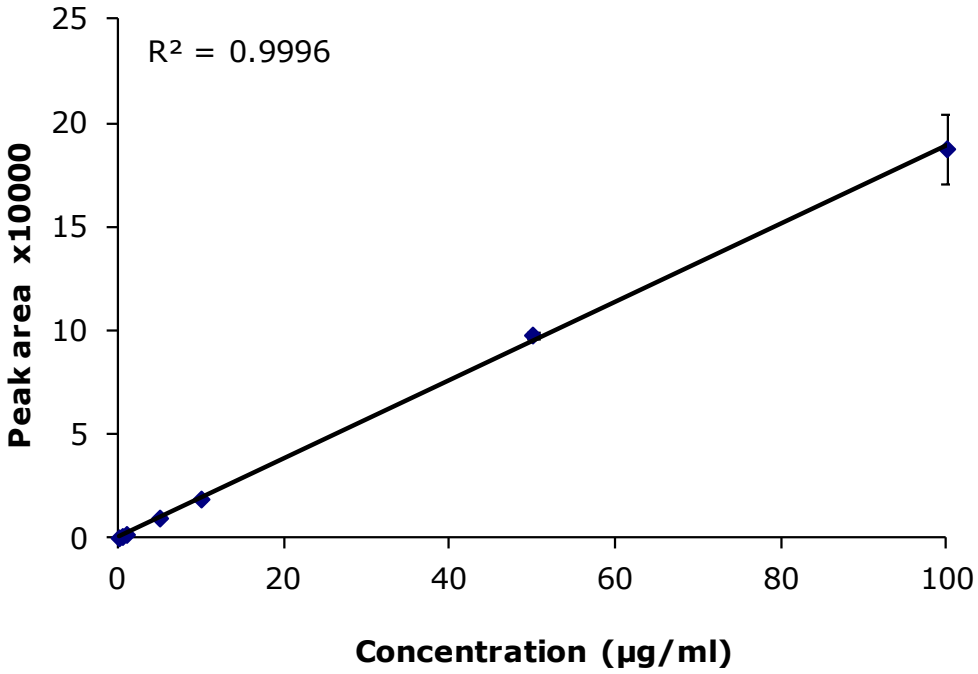


Figure 2.9. Standard curve for nodulopeptin, based on nodulopeptin 901, quantified on HPLC at the wavelength of 210 nm with diode array at 18.00 min retention time. SD was less than 5% mean of n=3 (n=3, bars= 1 SD).

2.3.2.c. Analysis of NOD and nodulopeptin 901 on HPLC-PDA-MS

Nodularin (2.10A) was quantified at the wavelength of 238 nm and at 12:00 min retention time. The full scanned chromatograms and absorbance spectrum are shown in Fig. 2.10.B and Fig. 2.10C, respectively. The nodulopeptin 901 was quantified at 210 nm wavelength at 18:00 min retention time (Figs. 2.11A-D).

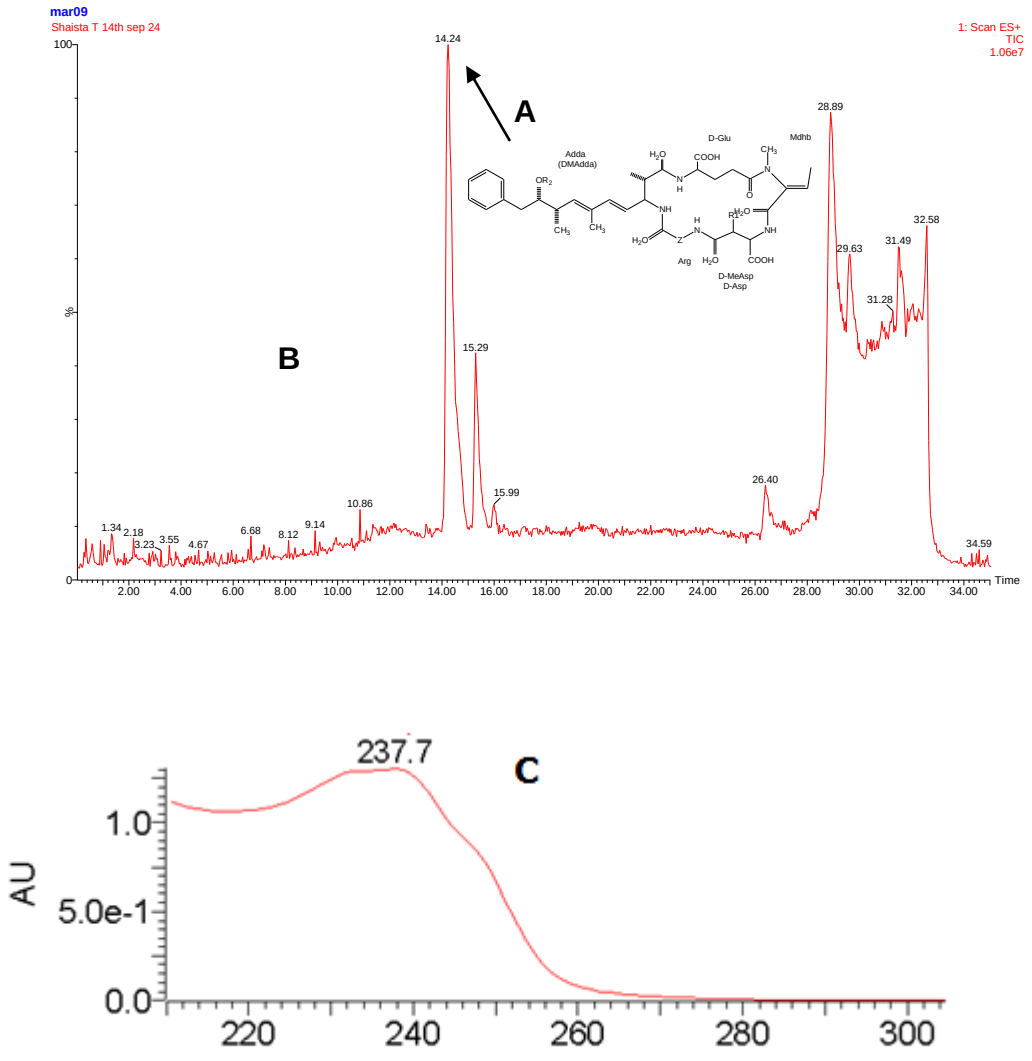


Figure 2.10.
A:- Chemical structure of NOD
B:- Full scanned chromatogram of NOD
C:- Typical absorbance spectrum of NOD at 238 nm

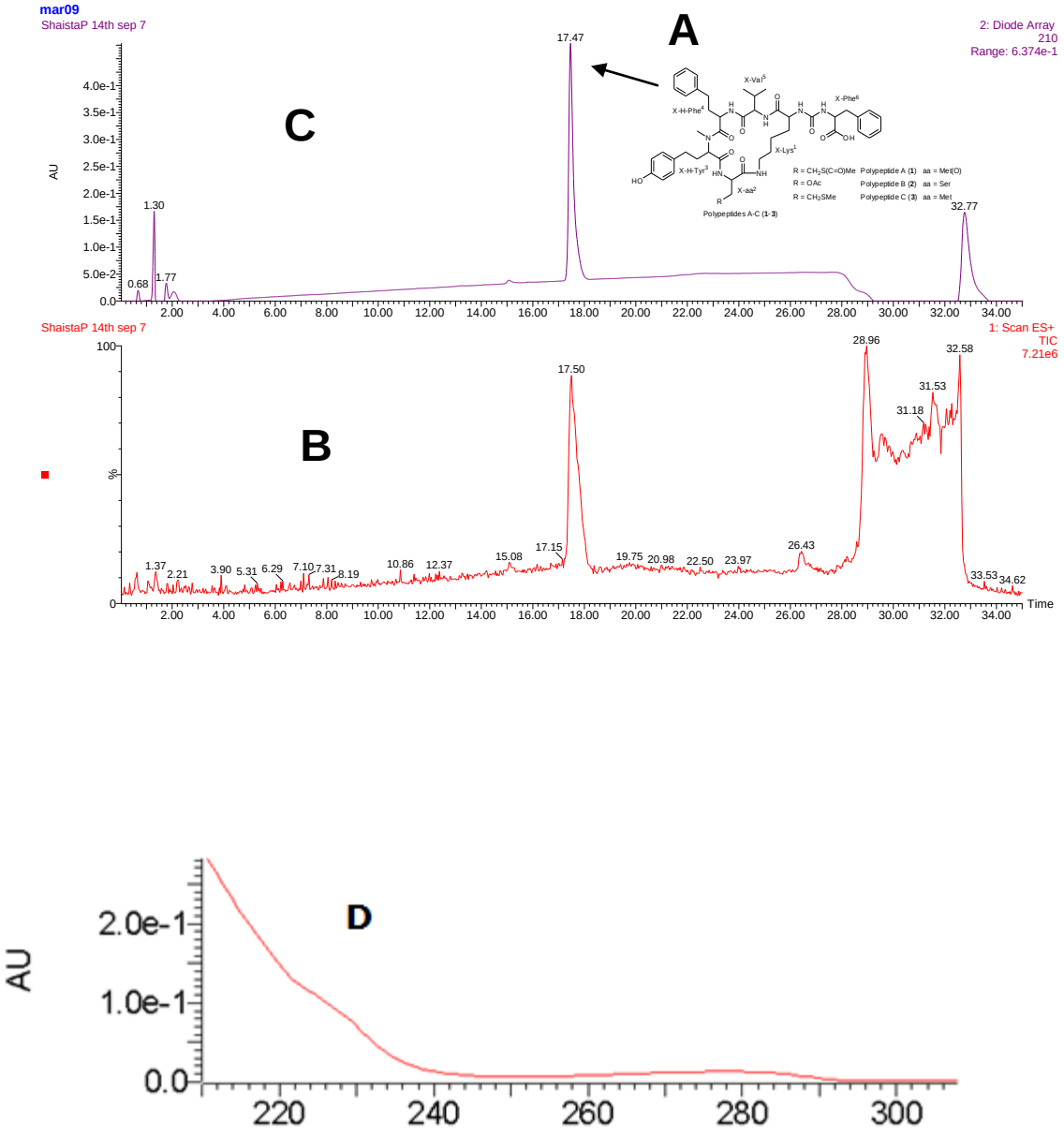


Figure 2.11.

A:- Chemical structure of nodulopeptin 901

B:- Full scanned chromatogram of nodulopeptin 901

C:- At 210 nm

D:- Typical absorbance spectrum of nodulopeptin 901 at 210 nm

2.3.3. UPLC-PDA-MS analysis

2.3.3.a. Standard curve of NOD

A range of NOD concentrations (Appendix 10) was used to plot a standard curve of pure NOD. The calibration curve of pure NOD was plotted as concentration of NOD against peak area's response by UPLC-PDA-MS. The pure NOD showed a linear line and maintained a correlation R^2 = 0.9993 (Fig. 2.12).

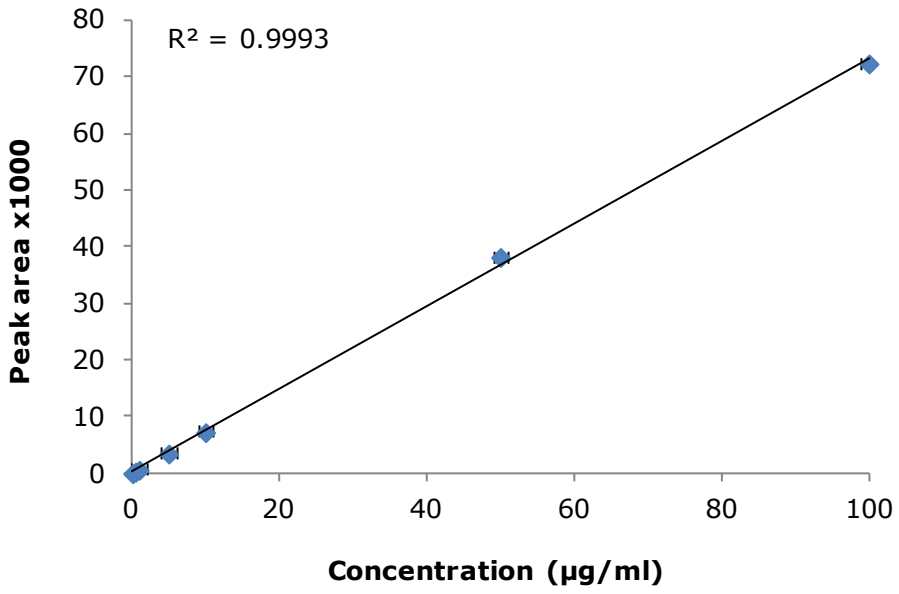


Figure 2.12. Standard curve for NOD, quantified on UPLC at the wavelength of 238 nm with diode array at 6.05-6.13 min retention time (n=3, bars= 1 SD).

2.3.3.b. Standard curve of nodulopeptin 901

The typical calibration graph for nodulopeptin 901 showed a linear correlation between peak area and concentration of nodulopeptin 901 response by UPLC-PDA-MS chromatograms. The pure nodulopeptin 901 indicated a linear response at UV-210 nm and maintained a correlation coefficient R^2= 0.9972 (Fig. 2.13; Appendix 11).

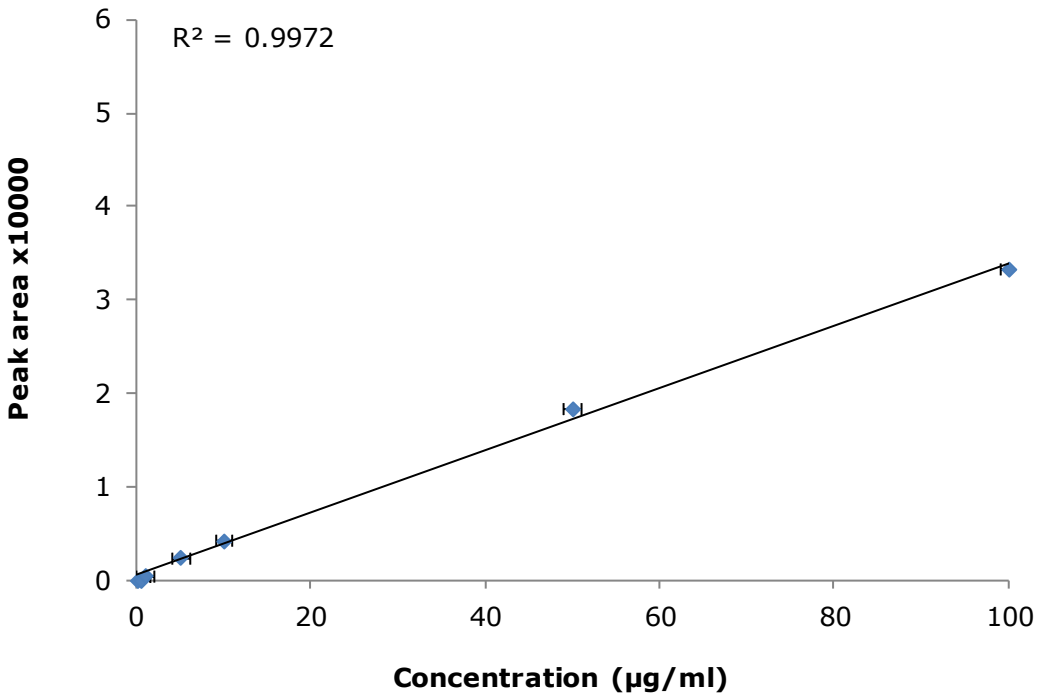


Figure 2.13. Standard curve for nodulopeptin 901, based on nodulopeptin 901, quantified on UPLC at the wavelength of 210 nm with diode array at 7.88-7.90 min retention time (n=3, bars= 1 SD).

2.3.3.c. Analysis of NOD and nodulopeptin 901 on UPLC-PDA-MS

Nodularin was quantified at the wavelength of 238 nm at 6.19 min retention time (Fig. 2.14). The nodulopeptin 901 was quantified at 210 nm wavelength at 7.99 min retention time (Fig. 2.15).

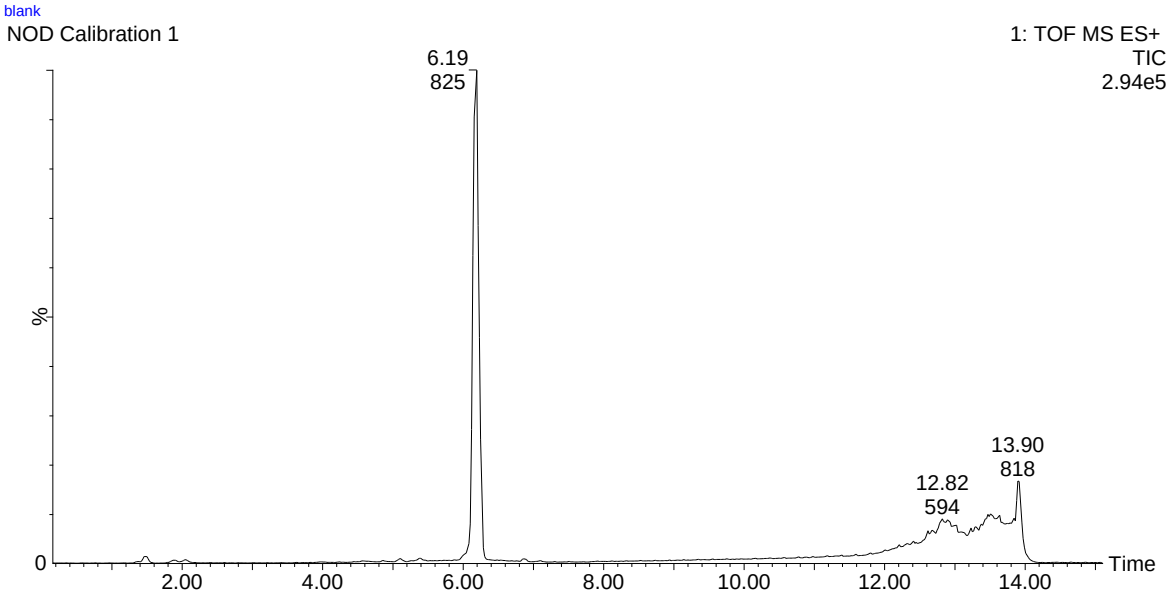


Figure 2.14. Full scanned chromatogram of NOD at 6.19 min retention time.

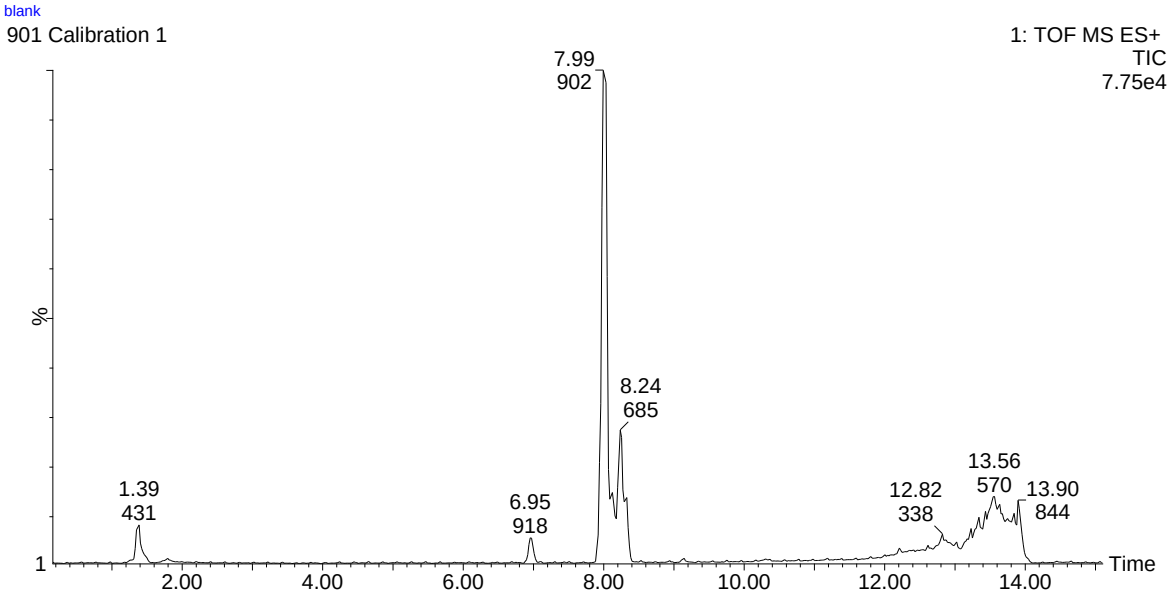


Figure 2.15. Full scanned chromatogram of nodulopeptin 901 at 7.99 min retention time.

2.3.4. Bioactivity evaluation of fractions from *N. spumigena* KAC 66

The extract from *N. spumigena* KAC 66 was separated by reversed phase flash chromatography (RPFC) using a linear gradient and yielded 17 fractions. Fractions were analysed by UPLC-PDA-MS using standards of NOD and nodulopeptin 901 to identify and quantify the toxins in the fractions.

The peptides were identified in fractions of *N. spumigena* KAC 66 on the basis of their wavelength and retention time (NOD 238 nm wavelength with retention time 5.53 min and nodulopeptin 901, 210 nm wavelength with retention time 7.71 min).

A series of methanolic fractions (10-100%) of *N. spumigena* KAC 66 were also analysed on UPLC-PDA-MS. NOD was only eluted in five fractions

Table 2.6. Amount of NOD and nodulopeptin 901 in fractions of *N. spumigena* KAC 66 analysed on UPLC-PDA-MS. (NOD 238 nm wavelength and retention time 5.53 min; and nodulopeptin 901, 210 nm wavelength and retention time 7.71 min (n.d. not detected).

Fraction	NOD (µg/ml)	NOD (mg/fraction)	Nodulopeptin 901 (µg/ml)	Nodulopeptin 901 (mg/fraction)
1	-	-	-	-
2	-	-	-	-
3	47.76	2.87	-	-
4	20.86	1.25	-	-
5	14.43	0.87	-	-
6	11.47	0.69	-	-
7	1.64	0.09	5.44	0.33
8	-	-	6.45	0.39
9	-	-	4.42	0.27
10	-	-	n.d.	n.d.

(F3-F7) while nodulopeptin 901 was eluted in three fractions (F7-F9), respectively (Table 2.6). Fractions 3, 4 and 5 contained NOD at 90% purity whereas fractions 6 and seven were more complex and NOD was a minor component. Nodulopeptin 901 was detected in fractions 7, 8 and 9, although these fractions were complex.

Fractions 1, 2, and 10-17 did not contain any known compounds in UV or MS chromatograms.

2.3.5. Bioassays

2.3.5.a. *Daphnia* bioassays - *D. pulex*

Determination of the toxicity of NOD towards *D. pulex* gave an LC_{50} of 8.4 µg/mL (Figure 2.16; Appendix 12). When *D. pulex* were exposed to undiluted fractions, mortality was 100% in F2-F6 and F8-F15 fractions (Appendix 13). However, F1 and F16 showed less mortality 21 and 5.8%,

respectively. These fractions were eluted in low (F1) methanol and high methanol (F16) where NOD and nodulopeptin 901 were not eluted hence toxicity against *Daphnia* was observed (Table 2.7). The UPLC-PDA-MS analysis indicated that NOD and nodulopeptin 901 were only present in fraction F3-F9, while in undiluted approximately all fractions showed lethality to *D. pulex*. This maybe due to the presence of other toxic compounds in the extracts, which killed daphnids.

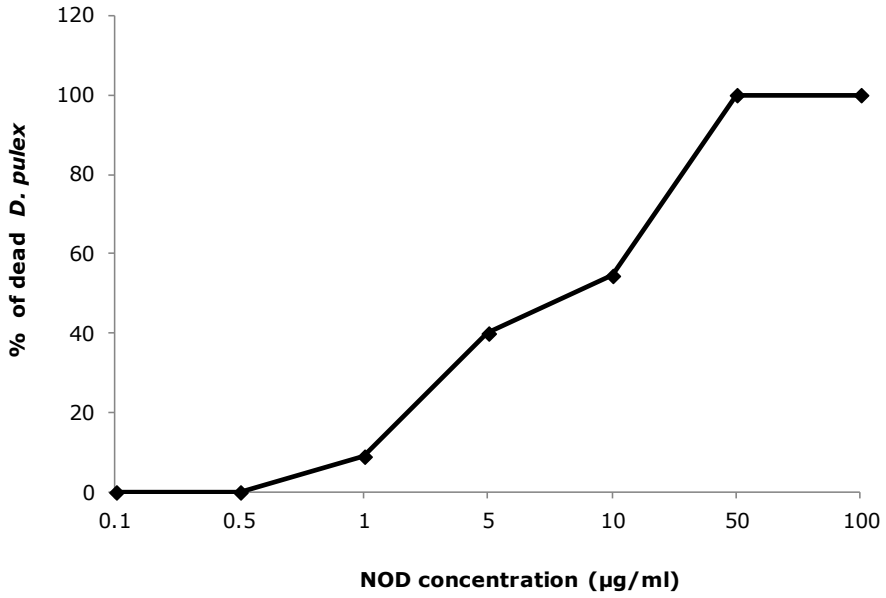


Figure 2.16. LC_{50} value (8.4 µg/mL) determination for standard NOD against *D. pulex*. NOD was serially diluted in CH medium. SD was less than 5% mean of n=3 (n=3, bars= 1 SD).

Table 2.7. Lethality of 17 fractions of *N. spumigena* KAC 66 collected from RPFC for *D. pulex* (the daphnids exposed for 24 h with each undiluted fraction, CH= Calkley's medium)

Fractions of *N. spumigena* KAC 66 (2 ml dried fraction dissolved in 1 ml CH)	***D. pulex***			
	Total number	**Alive**	**Dead**	**% of dead**
F1	14	11	3	21
F2	9	-	9	100
F3	15	-	15	100
F4	15	-	15	100
F5	15	-	15	100
F6	14	-	14	100
F7	12	6	6	50
F8	10	-	10	100
F9	11	-	11	100
F10	16	-	16	100
F11	15	-	15	100
F12	15	-	15	100
F13	10	-	10	100
F14	15	-	15	100
F15	12	-	12	100
F16	17	16	1	5.8
F17	11	11	-	0
-ve Control	13	13	-	0

Further dilution (x2 diluted fraction) of fractions indicated that in fractions F1, F7, F8 and F16 mortality rate was slightly lower than undiluted fractions, ranging from 8-70% daphnids were killed (Table 2.8; Appendix 14). At x2 diluted fraction several fractions (F3, F4, F6, F10, F11, F12, F13 and F14) demonstrated 100% lethality to *D. pulex*, which further decreased in the dilution factor x4 diluted fraction (Appendix 15). In fraction numbers F3, F4 F5, F6, F7, F11 and F12 high mortality (10-45%) was observed. Although no mortality was recorded in F1, F2, F8, F9, F10 and F13-F17 (Table 2.8). In undiluted fraction number 3 contained the highest amount of NOD (24 µg/mL) and killed 100% of *D. pulex*, which was gradually decreased till F6 (0.8 µg/mL of NOD) and 38% mortality was recorded (Table 2.8).

Table 2.8. Mortality (%) of *D. pulex* when treated with undiluted and diluted fractions of *N. spumigena* KAC 66. The values in brackets represent amount of NOD and nodulopeptin 901, calculated on the basis of amount of peptides collected from reversed phase flash chromatography.

Fractions	Dead *D. pulex* (%)			Dead *D. pulex* (%) and lethailty of NOD			Dead *D. magna* (%) and lethailty of nodulopeptin 901		
	Undiluted	Diluted		Undiluted	Diluted		Undiluted	Diluted	
		(x2)	(x4)		(x2)	(x4)		(x2)	(x4)
	Fractions do not contain NOD and nodulopeptin 901			Fractions contain NOD			Fractions contain Nodulopeptin 901		
F1	8	8	0	-	-	-	-	-	-
F2	100	78	0	-	-	-	-	-	-
F3				100 (24 µg/ml)	100 (11.9 µg/ml)	45 (5.97 µg/ml)	-	-	-
F4				100 (10.4 µg/ml)	100 (5.2 µg/ml)	30 (2.61 µg/ml)	-	-	-
F5				100 (7.2 µg/ml)	80 (3.6 µg/ml)	9 (1.8 µg/ml)	-	-	-
F6				100 (5.7 µg/ml)	100 (2.9 µg/ml)	30 (1.43 µg/ml)	-	-	-
F7				38 (0.8 µg/ml)	40 (0.4 µg/ml)	8 (0.21 µg/ml)	38 (2.72 µg/ml)	40 (1.4 µg/ml)	8 (0.7 µg/ml)
F8	70	9	0	-	-	-	70 (3.2 µg/ml)	9 (1.6 µg/ml)	0 (0.8 µg/ml)
F9	100	73	0	-	-	-	100 (2.2 µg/ml)	73 (10.5 µg/ml)	0 (0.6 µg/ml)
F10	100	100	0	-	-	-	-	-	-
F11	100	100	18	-	-	-	-	-	-
F12	100	100	10	-	-	-	-	-	-
F13	100	100	0	-	-	-	-	-	-
F14	100	100	0	-	-	-	-	-	-
F15	100	36	0	-	-	-	-	-	-
F16	100	18	0	-	-	-	-	-	-
F17	8	10	0	-	-	-	-	-	-
-ve Control	8								
+ ve Control (NOD 100 µg/ml)	100								

Due to high amount of NOD and high mortality rate the undiluted fraction was further diluted. At x2 diluted fraction dilution 40-100% daphnids were killed in F3-F7 fractions containing 0.4-12.0 µg/mL NOD. At high dilution a smaller number of daphnids were killed compared with undiluted and x2 fraction dilution, which ranged from 8-45% with 0.21-6.0 µg/mL NOD (Table 2.8).

The fraction number 9 had 2.2 µg/mL nodulopeptin 901, which killed 100% daphnids. The daphnid's mortality was gradually decreased (0-8) as dilution factor was increased (0.6-0.8 µg/mL).

2.3.5.b. *D. magna* assay

i. Lethality of standard NOD

A series of dilutions were performed to determine the lethal activity of NOD against *D. magna* with 5.0 µg/mL (Fig. 2.17; Appendix 16).

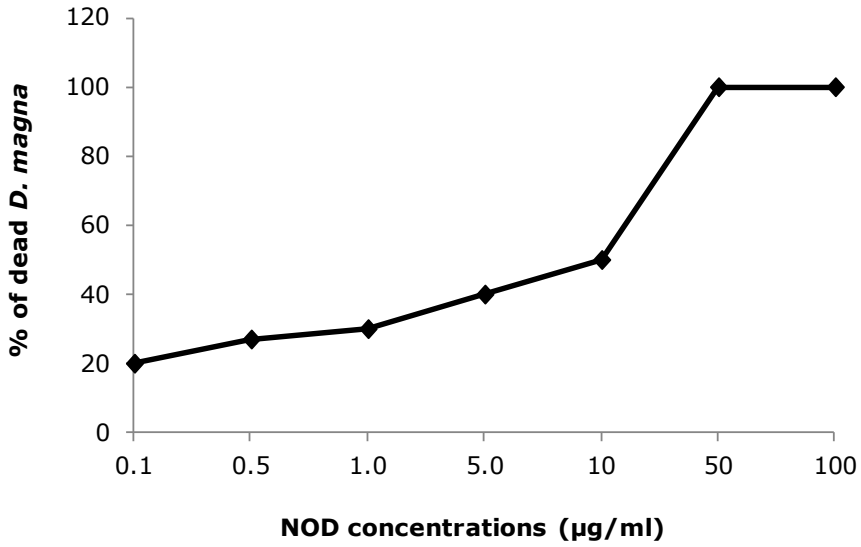


Figure 2.17. LC_{50} value (5.0 µg/mL) determination for standard NOD against *D. magna*. NOD was serially diluted in ASTM medium. SD was less than 5% mean of n=3 (n=3, bars=1 SD).

ii. Lethality of standard nodulopeptin 901

Pure nodulopeptin 901 showed lethal activity against *D. magna* with 116 µg/mL (Fig. 2.18; Appendix 17).

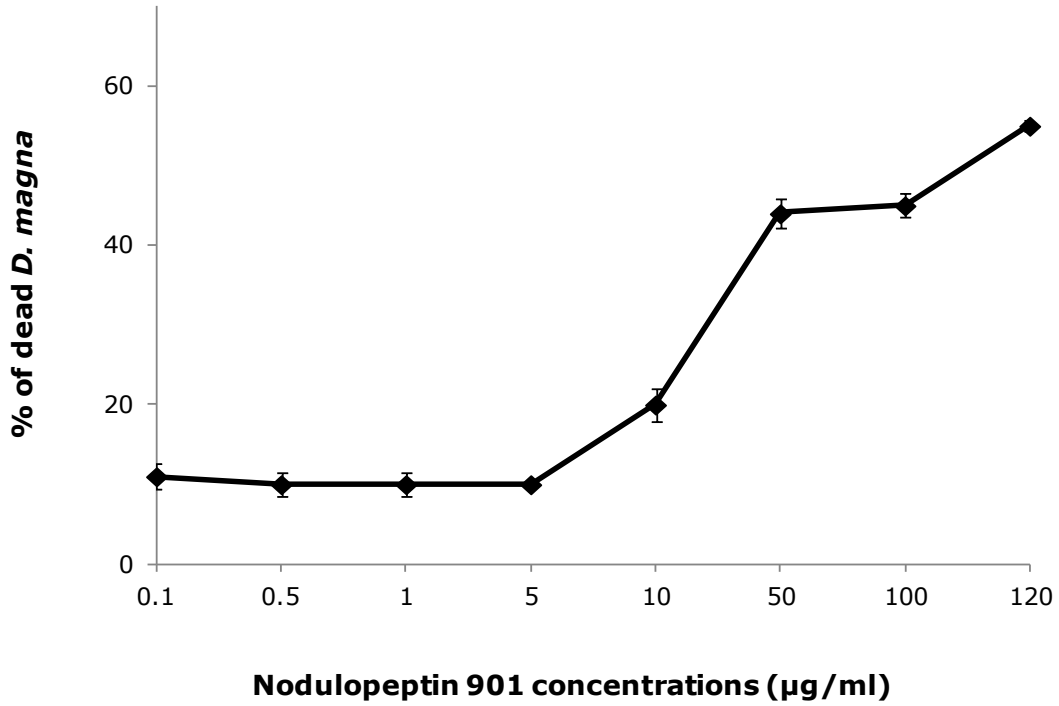


Figure 2.18. LC_{50} value (116 µg/mL) determination for standard nodulopeptin 901 against *D. magna*. NOD was serially diluted in ASTM medium (n=3, bars=1 SD).

In general it is observed that NOD toxic to *D. magna* compared with nodulopeptin 901.

Only in undiluted fraction numbers F3 and F16 100% mortality of *D. magna* were observed (Appendix 18). Due to high mortality rate the fractions were diluted further. The fraction obtained at 100% MeOH showed lowest mortality (18%). At x2 diluted fraction due to presence of

other toxic compounds rather than NOD and nodulopeptin 901 F16 represented 100% mortality of *D. magna* (Appendix 19). In comparison with UPLC-PDA-MS data, the F3 showed highest amount of NOD. The toxicity of NOD (23.9 µg/mL NOD; Table 2.9) was also confirmed by the 80% mortality of *D. magna* in the same fraction. At x4 diluted fraction no mortality was observed in undiluted F2, F11 and F17, however, in F16 total 91% daphnids were dead. Fractions F3 to F9 confirmed the lethality of NOD and nodulopeptin 901, ranged from 9-30% (Table 2.9; (Appendix 20).

UPLC-PDA-MS analysis showed that nodulopeptin 901 were eluted in fraction numbers F7-F9 and in same fractions 9-70% mortality was recorded. In undiluted fractions the highest mortality of daphnids (70%) was found in F8 with 3.2 µg/mL nodulopeptin 901, which was gradually decreased with an increase in dilutions (Table 2.9).

Table 2.9. Mortality (%) of *D. magna* when treated with undiluted and diluted fractions of *N. spumigena* KAC 66. The values in brackets represent amount of NOD and nodulopeptin 901, calculated on the basis of amount of peptides collected from reversed phase flash chromatography.

Fractions	Dead *D. magna* (%)			Dead *D. magna* (%) and lethailty of NOD			Dead *D. magna* (%) and lethailty of nodulopeptin 901		
	Undiluted	Diluted		Undiluted	Diluted		Undiluted	Diluted	
		(x2)	(x4)		(x2)	(x4)		(x2)	(x4)
	Fractions do not contain NOD and nodulopeptin 901			Fractions contain NOD			Fractions contain Nodulopeptin 901		
F1	33	20	10	-	-	-	-	-	-
F2	40	20	0	-	-	-			
F3				100 (23.9 µg/ml)	82 (11.9 µg/ml)	10 (6 µg/ml	-	-	-
F4				50 (10.4 µg/ml)	40 (5.2 µg/ml)	20 (2.6 µg/ml)	-	-	-
F5				60 (7.2 µg/ml)	20 (3.6 µg/ml)	10 (1.8 µg/ml)	-	-	-
F6				60 (5.7 µg/ml)	45 (2.9 µg/ml)	30 (1.4 µg/ml)	-	-	-
F7				36 (0.8 µg/ml)	27 (0.4 µg/ml)	18 (0.2 µg/ml)	36 (2.7 µg/ml)	27 (1.4 µg/ml)	18 (0.7 µg/ml)
F8	70	30	22	-	-	-	70 (3.2 µg/ml)	30 (1.6 µg/ml)	22 (0.8 µg/ml)
F9	30	22	9	-	-	-	30 (2.2 µg/ml)	22 (1.1 µg/ml)	9 (0.6 µg/ml)
F10	30	20	20	-	-	-	-	-	-
F11	90	40	0	-	-	-	-	-	-
F12	60	20	20	-	-	-	-	-	-
F13	90	50	20	-	-	-	-	-	-
F14	90	56	10	-	-	-	-	-	-
F15	40	27	20	-	-	-	-	-	-
F16	100	100	91	-	-	-	-	-	-
F17	18	10	0	-	-	-	-	-	-
-ve Control	10	10	10						
+ ve Control (NOD 100 µg/ml)	100	100	100						

A comparison of lethality of standard NOD against *D. pulex* and *D. magna* was also performed (Table 2.10). It shows that from 5-100 µg/mL of NOD concentration has same lethal effects on both species of daphnids (100-40%). At 1.0 µg/mL of NOD was 9% and 30% lethal to *D. pulex* and *D. magna*, respectively. At 0.5 and 0.1 µg/mL NOD concentration the *D. magna* showed sensitivity to NOD compared to *D. pulex,* 27 and 20% mortality was observed, respectively. It shows that the lowest concentrations are toxic to *D. magna* and *D. pulex* survived on those NOD concentrations.

Table 2.10. Concentrations and lethality of standard toxin, NOD for *D. pulex* and *D. magna* (the daphnids exposed for 24 h with NOD)

NOD concentrations (µg/ml)	*D. pulex*			*D. magna*		
	Mean total ($\bar{x}$)	Mean dead ($\bar{x}$)	Dead (%)	Mean total ($\bar{x}$)	Mean dead ($\bar{x}$)	Dead (%)
100	10	10	100	12	12	100
50	10	10	100	12	12	100
10	11	6	55	10	5	50
5	10	4	40	10	4	40
1	11	1	9	10	3	30
0.5	11	-	0	11	3	27
0.1	10	-	0	10	2	20
-ve Control	15	-	0	10	1	10

Typically undiluted and x2 diluted fraction dilution all fractions showed higher lethality to *D. pulex* compared to *D. magna*. In undiluted fractions only *D. magna* were found to be more sensitive to F1 (33%) and F17 (18%) than *D. pulex*. Generally, more *D. pulex* were killed at x2 diluted fraction dilution and survival rate increased at x4 diluted fraction dilution as might be expected. It is concluded that *D. magna* were more sensitive to all fractions compared to *D. pulex*.

2.3.6. Protein phosphatase assay (PP1)

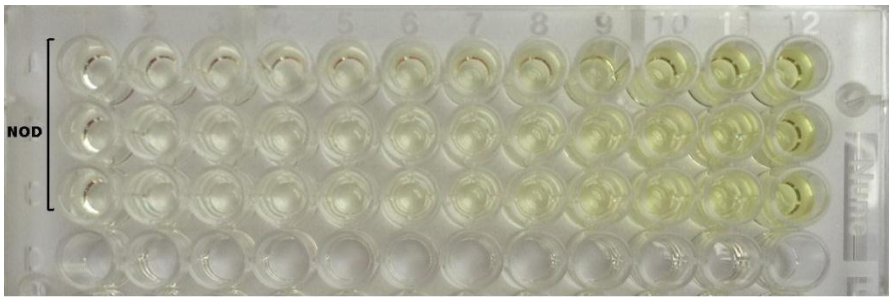
2.3.6.a. PP1 inhibition by standard peptides

A number of purified peptides NOD, ANA, ANB, linear NOD and recently characterised nodulopeptin 901 were tested for their inhibitory activity against protein phosphatase 1. The colouration in microtiter plates showed the inhibitory activities of peptides against PP1 after 14 h incubation at 37°C. The plates were read at 405 nm (Fig. 2.19A-C).

The results indicated that NOD inhibited the PP1 with IC_{50} 0.038 µg/mL, which was the highest inhibitory activity among all other tested peptides (Fig. 2.20A; Appendix 21). Increasing NOD concentrations resulted in increasing % inhibition.

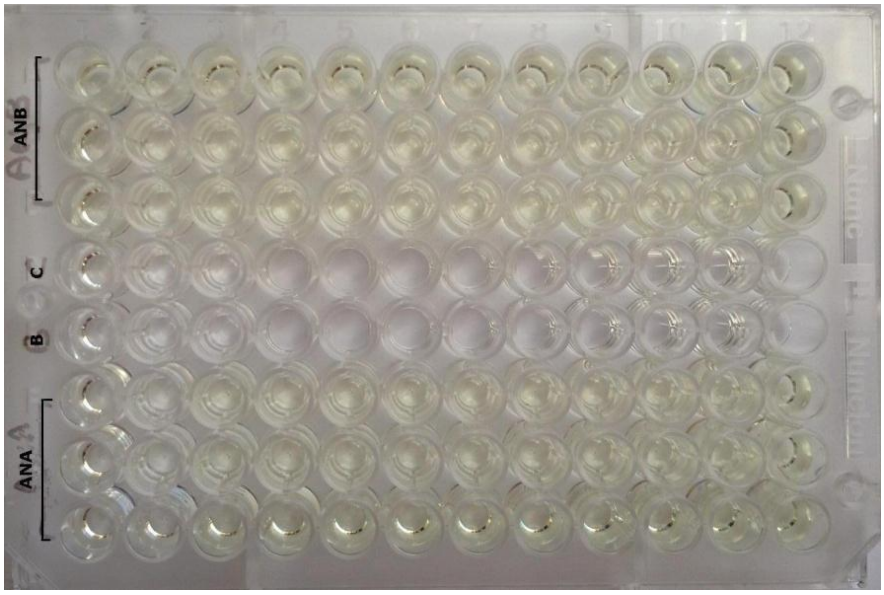
Linear NOD inhibited PP1 with IC_{50} 20 µg/mL. Only at highest concentration (100 µg/mL) very small inhibitory activity was noted (0.225%, Fig. 2.20B; Appendix 22). ANA and ANB inhibited PP1 with IC_{50} 70 and 100 µg/mL, respectively (Figs. 2.20C and D; Appendix 23). Nodulopeptin 901 inhibited PP1 with IC_{50} 20 µg/mL (Fig. 2.20E; Appendix 24).

A



B

C



D

E

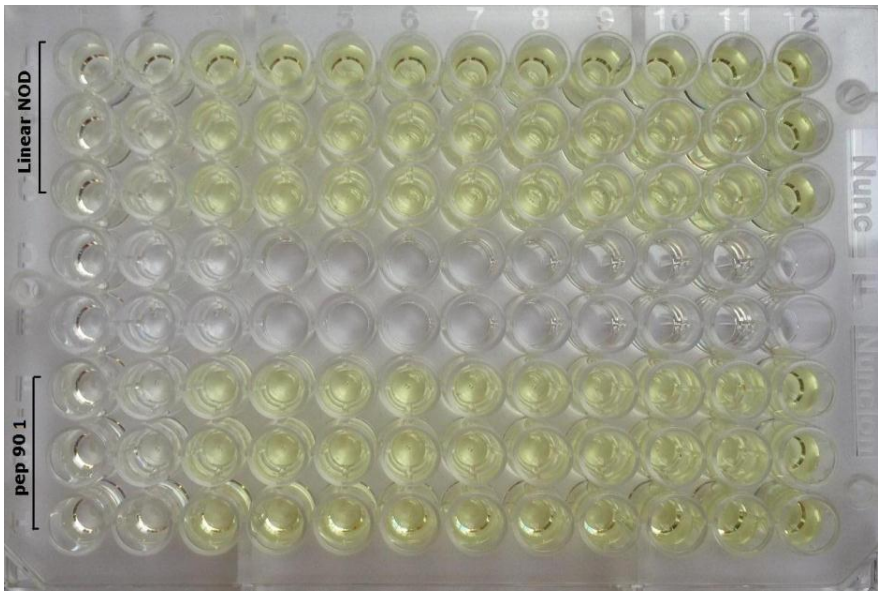


Figure 2.19. Microtiter plates showing the colouration of inhibitory activities of peptides against PP1 after 14 h incubation at 37°C. The plates were read at 405 nm (**A**:- NOD, **B**:- ANB, **C**:- ANA, **D**:- linear NOD and **E**:- nodulopeptin 901)

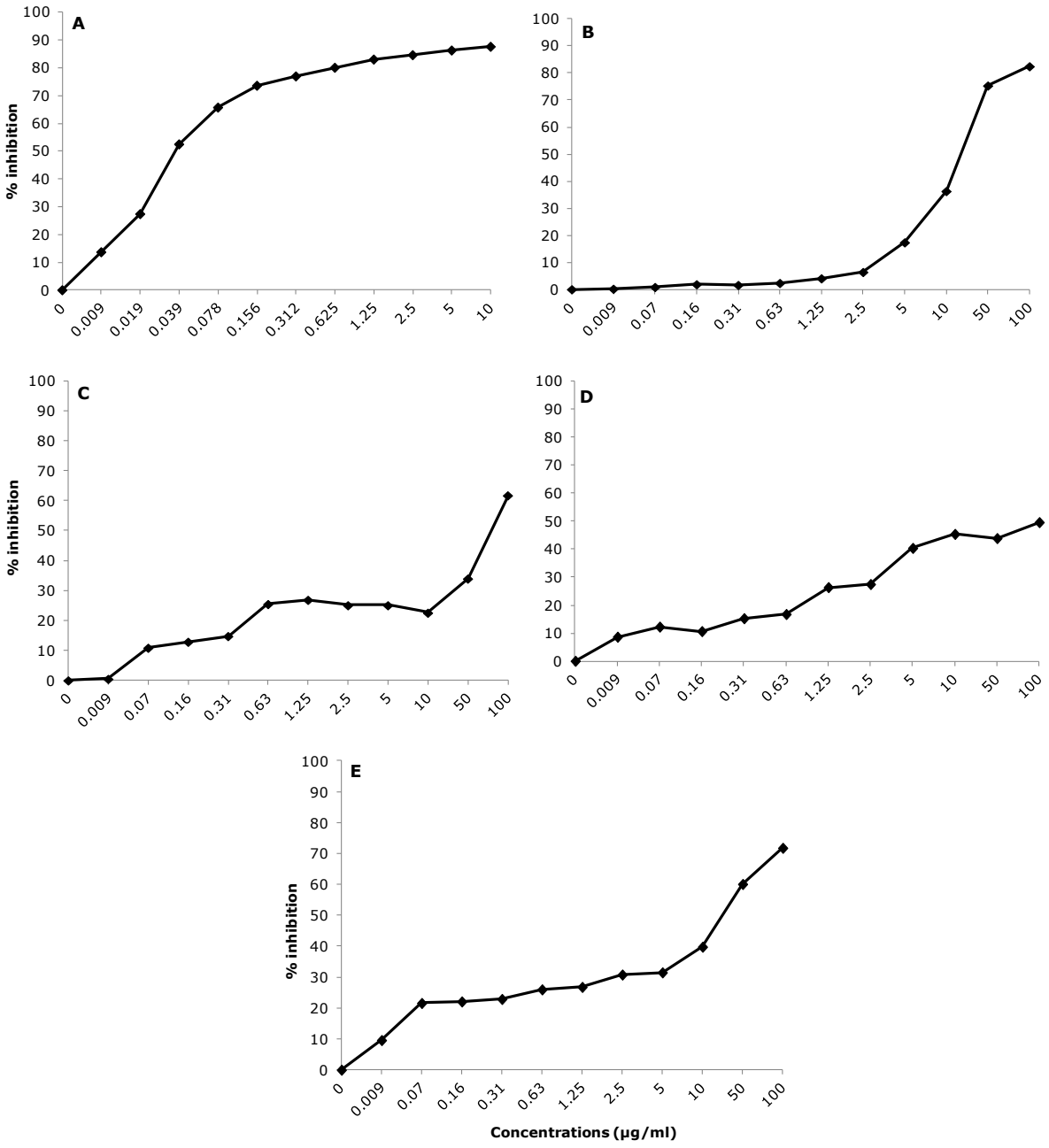


Figure 2.20. PP1 inhibitory activity and IC_{50} values of standard peptides after 14 h incubated at 37 °C. The plates were read at 405 nm. IC_{50} values: **A**:- NOD 0.038 µg/mL, **B**:- LNOD 20 µg/mL, **C**:- ANA 70 µg/mL, **D**:- ANB 100 µg/mL and **E**:- nodulopeptin 901, 25 µg/mL SD was less than 5% mean of n=3 (n=3, bars= 1 SD).

2.3.6.b. PP1 inhibition by undiluted 17 fractions

The undiluted fractions collected from RPFC indicated PPI inhibition ranged from 63.2%-90% (Fig. 2.11; Appendix 25). NOD 144.3-208.7 µg/mL (73.4%-86.8%; F3-F7) and nodulopeptin 901 44.2-64.5 µg/mL (77.6-82.6%; F8 and F9) inhibited PP1.

All undiluted fractions inhibited protein phosphatase, relatively NOD and nodulopeptin 901 were only eluted in F3-F9 fractions during RPFC (Fig. 2.21). This maybe due to the presence of other eluted toxic compounds present in fractions.

Due to high % inhibition the undiluted fraction was further diluted up to x10, x100 and than x1000.

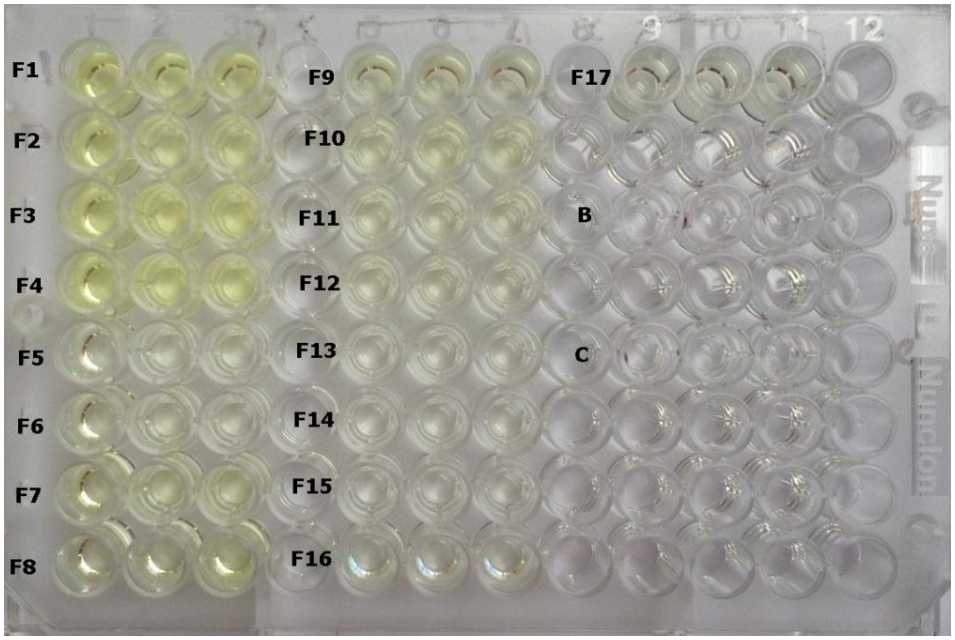


Figure 2.21. Microtiter plate showing the colouration of inhibitory activities of diluted fractions against PP1 after 14 h incubation at 37°C. The plates were read at 405 nm

Table 2.11. Comparison of PP1 inhibitory activity of undiluted and diluted fractions of *N. spumigena* KAC 66. The values in brackets represent IC_{50} of NOD and nodulopeptin 901, calculated on the amount of peptides collected from reversed phase flash chromatography.

Fractions	Undiluted	Diluted			Undiluted	Diluted			Undiluted	Diluted		
		(x10)	(x100)	(x1000)		(x10)	(x100)	(x1000)		(x10)	(x100)	(x1000)
	Fractions do not contain NOD and nodulopeptin 901				Fractions contain NOD				Fractions contain Nodulopeptin 901			
F1	77.6	85.2	81.9	1.4	-	-	-	-	-	-	-	-
F2	63.2	88.5	83.6	38.9	-	-	-	-	-	-	-	-
F3					85.1 (477.6 µg/ml)	90.7 (47.8 µg/ml)	90.2 (4.8 µg/ml)	76.4 (0.5 µg/ml)	-	-	-	-
F4					86.8 (208.7 µg/ml)	90.5 (20.87 µg/ml)	87.9 (2.09 ug/ml)	65.5 (0.21 µg/ml)	-	-	-	-
F5					73.4 (144.3 µg/ml)	90.4 (14.43 µg/ml)	89.9 (1.44 µg/ml)	75.9 (0.14 µg/ml)	-	-	-	-
F6					83.9 (114.7 µg/ml)	90.4 (11.47 µg/ml)	90.5 (1.15 µg/ml)	76.8 (0.11 µg/ml)	-	-	-	-
F7					81.5 (16.4 µg/ml)	90.0 (1.64 µg/ml)	90.0 (0.16 µg/ml)	60.7 (0.02 µg/ml)	81.5 (54.4 µg/ml)	90.0 (5.44 µg/ml)	90.0 (0.54 µg/ml)	60.7 (0.05 µg/ml)
F8					-	-	-	-	82.6 (64.5 µg/ml)	90.6 (6.45 µg/ml)	89.1 (0.65 µg/ml)	61.1 (0.06 µg/ml)
F9					-	-	-	-	77.6 (44.2 µg/ml)	87.5 (4.42 µg/ml)	89.0 (0.44 µg/ml)	53.7 (0.04 µg/ml)
F10	78.7	88.0	84.3	47.4	-	-	-	-	-	-	-	-
F11	75.8	86.2	85.5	53.7	-	-	-	-	-	-	-	-
F12	79.2	89.4	86.1	50.2	-	-	-	-	-	-	-	-
F13	86.1	89.8	83.6	53.7	-	-	-	-	-	-	-	-
F14	88.5	88.8	80.0	52.6	-	-	-	-	-	-	-	-
F15	89.9	85.2	51.8	54.5	-	-	-	-	-	-	-	-
F16	86.2	45.8	42.0	52.5	-	-	-	-	-	-	-	-
F17	83.3	23.9	38.2	58.0	-	-	-	-	-	-	-	-

2.3.6.c. PP1 inhibition by diluted 17 fractions

In x10 diluted fractions the inhibition ranged from 24.0%-90.7% (Table 2.11; Appendix 26). PP1 was greatly inhibited by 1.64-47.8 µg/mL NOD (90.0%-90.7%) present in F3-F6 and by 6.45 µg/mL nodulopeptin 901 in F8 (90.6%; Table 2.11). Fraction 7 also inhibited PP1 (90%), which is supposed to be inhibited by nodulopeptin 901 as it was found in high amounts (5.44 µg/mL) compared to NOD (1.64 µg/mL) in F7 during UPLC-PDA-MS analysis.

At x100 dilution the % inhibition ranged from 38.2-90.5%. NOD 0.16-4.8 µg/mL greatly inhibited PP1 (88.9-90.5%) present in fractions F3-F7. In fractions F7-F9 Nodulopeptin 901 4.42-6.45 µg/mL also inhibited PP1 (89-90%; Appendix 27).

At x1000 dilution all fractions showed PP1 inhibition. NOD 0.02-0.5 µg/mL (F3-F7) inhibited PP1 ranged from 65.5%-76.4%. Nodulopeptin 901 0.04-0.06 µg/mL (F8 and F9) inhibited PP1 ranged from 53.7-61.1%. The 60.7% (0.02 µg/mL) inhibition for NOD in F7 was near to standard NOD % inhibition (0.038 µg/mL; Table 2.11; Appendices 28 and 29).

2.3.7. A comparison among benchtop bioassays

UPLC-PDA-MS analysis indicated presence of NOD in F3-F7 and nodulopeptin 901 in F7-F9. In PP1 and daphnid assays all dilutions of fractions gave positive results. It shows that *N. spumigena* KAC 66 produces other toxic compounds which affected/inhibited PP1 and killed *D. pulex* and *D. magna* (Table 2.12).

Table 2.12. PP1 inhibitory activity and lethal effects on daphnids by aqueous methanolic fractions collected from *N. spumigena* KAC 66.

Fractions of *N. spumigena* KAC 66 collected from flash chormatography	UPLC-PDA-MS analysis		Benchtop bioassays		
	NOD	Nodulopeptin 901	*Daphnia pulex* assay	*Daphnia magna* assay	PP1 inhibition assay
F1	-	-	+	+	+
F2	-	-	+	+	+
F3	+	-	+	+	+
F4	+	-	+	+	+
F5	+	-	+	+	+
F6	+	-	+	+	+
F7	+	+	+	+	+
F8	-	+	+	+	+
F9	-	+	+	+	+
F10	-	-	+	+	+
F11	-	-	+	+	+
F12	-	-	+	+	+
F13	-	-	+	+	+
F14	-	-	+	+	+
F15	-	-	+	+	+
F16	-	-	+	+	+
F17	-	-	+	+	+

2.4. DISCUSSION

Several studies have demonstrated that cyanobacteria are rich in natural products, which can be used in pharmaceutical and agricultural industries (Burja *et al*., 2001). This study provided an additional investigation towards the lethal effects of aqueous methanolic fractionation of hepatotoxic *N. spumigena* against protein phosphatase 1 and a comparison of toxicological effects of fractions on the two species of daphnids, *D. pulex* and *D. magna*.

2.4.1. Fractionation of *N. spumigena* KAC 66

At 20%, 30%, 40% and 50% methanolic fractions NOD and nodulopeptin 901 were eluted from the extract. It shows the polar and semi-polar nature of hepatotoxins. It was also noted that pure methanol did not extract NOD and nodulopeptin 901 quantitatively from extract of *N. spumigena.* Fastner *et al.* (1998) also reported that pure methanol does not extract microcystins from lyophilised cyanobacterial samples.

Several species of *Nodularia* spp. are known to produce toxins. The present study indicated that the methanolic fractionation contained NOD and nodulopeptin 901. These fractions were found to be very toxic to daphnids and strongly inhibited PP1. Another *Nodularia*, *N. harveyana* has showed activity against a number of organisms. The acetone extracts of *N. harveyana* represented allelopathic and toxicological effects on several organisms such as Chlorophyceae, cyanobacteria (*Anabaena* spp*., Nostoc* sp., *Spirulina platensis* and *Nodularia harveyana* itself*),* eubacteria, plant fungal pathogens, rotifers and crustaceans (Pushparaj *et al.,* 1999).

2.4.2. *Daphnia* bioassay

The NOD is produced by *N. spumigena* and highly toxic to daphnids (Reinikainen *et al.,* 2002) and copepods (DeMott *et al.,* 1991). The copepods can resist up to 2 µg/mL of dissolved NOD (Reinikainen *et al.,* 2002). The present study showed that at 10 µg/mL of pure NOD more than half of the tested population of *D. pulex* and *D. magna* was killed. In concentrations above 10 µg/mL, 100% mortality occurred when both

species were exposed to pure NOD. Below 10 µg/mL *D. magna* showed sensitivity to NOD compared to *D. pulex* and no single individual of *D. pulex* was killed. It has been suggested that this maybe due to different species of daphnids having different levels of tolerance. DeMott *et al.,* (1991) worked on the effects of hepatotoxins isolated from *N. spumigena* (NOD) and *M. aeruginosa* (MC-LR), on three species of *Daphnia* (*D. pulex, D. hyaline* and *D. pulicaria*) and a copepod (*Diaptomus birgei*). The copepods were exposed for 48 h to NOD the LC_{50} was approximately 4–10 µg/mL. The present study also confirms that the *D. pulex* exposed for 24 h with NOD the LC_{50} =8.4 µg/mL was recorded, while *D. magna* were more sensitive to NOD (LC_{50} =5.0 µg/mL).

DeMott *et al.,* (1991) also noted the different LC_{50} values when different zooplanktons were exposed to MC-LR for 48 h. They further investigated that the copepod *D. birgei* was most sensitive to MC-LR (0.45-1.00 µg/mL) compared to *D. pulicaria* (LC_{50} = 21.4 µg/mL), *D. hyalina* (LC_{50}= 11.6 µg/mL) and *D. pulex* (LC_{50}= 9.6 µg/mL) and each zooplankton species responded the same when exposed to hepatotoxic MC-LR and NOD, as both toxins have similar hepatotoxicity and chemical structures.

In the present study both tested species behaved differently when exposed to pure standard NOD, fractions containing NOD and nodulopeptin 901. In general, *D. pulex* showed tolerance to pure NOD (44.5%) and were killed when concentrations of pure NOD were high, while *D. magna* showed sensitivity to all NOD concentrations (55.5%).

Approximately, it seems that *D. pulex* is more resistant than *D. magna* when exposed to fractions (Table 1.13).

The previous studies indicated that several cladoceran species behave differently to cyanobacterial exposure. A planktonic water flea, found near shoreline of ponds and lakes, *Bosmina longirostris* was more tolerant to cyanobacterial toxins than other water fleas, *D. parvula* and *Moina micrura* (Fulton, 1988). Species of same genus of *Daphnia* also showed different feeding behaviour when exposed to *Microcystis* sp. (Hietala *et al.,* 1995).

In comparison to standard NOD the newly characterised nodulopeptin 901 (Schumacher *et al.,* 2012) was also found to be less toxic against *D. magna* (116 µg/mL). Due to newly discovered nodulopeptin 901 no data is available to compare the lethality of peptide to *D. magna*.

The positive results of all fractions against daphnids indicated that *N. spumigena* is rich in a new source of toxic compounds. There is a further need to discover compounds, which can be useful in agricultural and pharmaceutical industries.

2.4.3. Protein phosphatase assay (PP1)

Microcystins and nodularin are known hepatotoxins and inhibit protein phosphatase activity (PP1 and PP2A; Gulledge, *et al*., 2002; Yoshizawa *et al*., 1990). The positive results of *Daphnia* assay can also be confirmed by PP1 and UPLC-PDA-MS. The initial screening of fraction or extracts with daphnids and PPI assays are helpful to determine the presence of hepatotoxins. Due to hepatotoxic effects of microcystin-LR

(MacKintosh, 1990) and NOD (Yoshizawa *et al*., 1990) much work has been done on PP1 inhibition on both toxins. In the present study IC_{50} 0.038 µg/mL for pure standard NOD was recorded.

A NOD variant, [L-Har^2]NOD and NOD isolated from freshwater cyanobacterium, *Nodularia* PCC 7804, inhibited PP1 with an IC_{50} of 0.005 µg/mL and 0.006 µg/mL, respectively (Beattie *et al.,* 2000). These values are lower than % inhibition (0.038 µg/mL) of enzyme by standard NOD used in this study. The IC_{50} of enzyme inhibition for MC-LR was 0.0035 µg/mL (Beattie *et al.,* 2000).

In the present study nodulopeptin 901 inhibited PP1 (IC_{50} 25 µg/mL) while Schumacher *et al*., (2012) indicated that recently characterised three nodulopeptins 901, 917 and 899, isolated from *N. spumigena* KAC 66, do not inhibit PP1. Nodulopeptin 901 has similarity in structure with anabaenopeptolin A and B (Gkelis *et al*., 2006) and showed high inhibitory activity than anabaenopeptolin A and B.

The inhibitory activities of *N. spumigena* KAC 66 and *Nodularia* PCC 7804 indicated that *N. spumigena* KAC 66 is rich in the production of hepatotoxins. However, NOD is the most abundant hepatotoxin compared to other analogues of NOD.

Almost all stock solutions, undiluted and diluted fractions of *N. spumigena* KAC 66, collected from RPFC inhibited PP1. It suggests that the extracts were green colour and that the green colour interfered with OD values of fractions it or maybe that some other minor toxic compounds were also present (produced by *N. spumigena* KAC 66) in fractions, which have yet to be discovered. Beattie *et al*., (2000)

reported that *Nodularia* strains, PCC 7804, although containing NOD, also produces several unreported nodularia variants.

2.5. CONCLUSION

The present study proved that nitrogen fixing cyanobacterium, *N. spumigena* KAC 66 expressed toxic and inhibitory activities against daphnids and PP1, respectively. The results from fractions also indicated that *N. spumigena* possessed compounds, which are lethal to daphnids. There is a need to investigate the presence of other toxic compounds, produced by *N. spumigena* KAC 66.

CHAPTER 3

EFFECT OF ENVIRONMENTAL FACTORS ON THE GROWTH AND PEPTIDE PRODUCTION BY *NODULARIA SPUMIGENA* KAC 66

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3.1. INTRODUCTION

The growth and toxin production of 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,* maybe increased or decreased 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 (Hobson and Fallowfield, 2003; Stal *et al*., 1999 and Lehtimäki *et al*., 1997), temperature (Hobson and Fallowfield, 2003; Lehtimäki *et al*., 1997), salinity (Mazur-Marzec *et al.,* 2005; Hobson and Fallowfield, 2003; Musial and Plinski, 2003; Moisander *et al.*, 2002; Hobson *et al*., 1999 and Blackburn *et al*., 1996) and nitrate and phosphorus ratios (Lilover and Stips, 2008; Mazur-Marzec *et al.,* 2005; Stolte *et al.*, 2002; Repka *et al.,* 2001; Stal *et al*., 1999 and Lehtimäki *et al.*, 1994).

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* concentrations, cell biomass and nodulopeptin 901 concentrations within the cells and in growth medium produced by *N. spumigena* KAC 66. To fill this knowledge gap the ecologically important and toxin producing cyanobacterium, *N. spumigena* KAC 66 was selected for the present

study, obtained from Kalmar Collection Centre, Dept. of Marine Sciences, Kalmar University, Sweden. The strain was isolated by Gisselson L Å in 1996 from Askö, Baltic Sea (7 ‰).

Due to the ecological importance of this cyanobacterium and the hazards to human and animal health it is important to extract all intracellular and extracellular toxins from *N. spumigena* KAC 66 to characterise and quantify the production of NOD and nodulopeptin 901. These toxins can be analysed and quantified using high performance liquid chromatography photo diode array mass spectrometry (HPLC-PDA-MS) technique in laboratory cultures. LC-MS is a sensitive technique to detect the presence of cyanobacterial toxins especially microcystins (MC; Rohrlack *et al.,* 2003; Lawton *et al*., 1994), nodulopeptin 901, 917 and 899 (Schumacher *et al.*, 2012), nodularins (NOD; Anjos *et al.,* 2006; Diehnelt *et al.,* 2005; Zhang *et al.,* 2004) and other secondary metabolites/allelochemicals (Puddick and Prinsep, 2008).

Chlorophyll-*a* concentrations and cell biomass are also important factors and are frequently used as indicators to determine the biomass of *N. spumigena* blooms in the Baltic Sea as well as in the laboratory cultures.

The aims of this study were to highlight important and new knowledge about effects of environmental factors (temperature, salinity, nitrate and phosphate) on the growth, chlorophyll-*a*, cell biomass and production of nodularin and the recently characterised nodulopeptin 901 produced by *N. spumigena* KAC 66.

3.2. MATERIALS AND METHODS

In this study the influence of abiotic factors (temperature, salinity, nitrate and phosphate) on the production of extra and intracellular nodularin and nodulopeptin 901 in the continuous batch cultures of toxic cyanobacterium, *Nodularia spumigena* KAC 66 was investigated. The biomass was determined using freeze dried cells and Chl-*a* concentrations. All experiments were carried out in triplicate.

3.2.1. Preparation of growth medium BG-11

In the laboratory, culturing of cyanobacterial species requires conditions similar to those in the natural environments in which they are normally found. The cultivation and growth of cyanobacteria can be difficult, as different strains require specific environmental conditions. The requirements of cyanobacteria for vitamins, organic and inorganic nutrients are species dependant (Andersen and Kawachi, 2005).

Additionally culturing and growth of different species requires a variety of other conditions to be optimised such as pH, salinity, temperature, phosphate, nitrate, CO_2, light and aeration.

For culturing and experiments of *N. spumigena* KAC 66 the growth Allen's blue green algal medium, BG-11 (20 ‰; Allen and Stanier, 1968 modified by Stanier *et al.,* 1971) was prepared by following SOP/PtR/003a (Table 3.1). The small volume and large volumes of media were autoclaved for 15 minutes and 1 h, respectively, at 15 psi of pressure and 120°C (Kawachi and Noël, 2005) in an autoclave (Astell Scientific, UK). The stock solutions were prepared (in Milli-Q water) and

Table 3.1. The chemical composition of growth medium BG-11.

Nutrients	**Concentraion (g/L)**
$NaNO_3$	0.75
K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Na_2CO_3	0.02
Citric acid	0.006
$FeSO_4.7H_2O$	0.006
EDTA (disodium)	0.001
Trace element solution	1 ml/L
Trace element solution	
H_3BO_3	2.68
$MnCl_2.4H_2O$	1.81
$NaMoO_4$ $2H_2O$	0.39
$ZnSO_4.7H_2O$	0.222
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.049

The pH was 7.1 after sterilisation.

stored in plastic bottles at room temperature. Only trace metals were kept in fridge (4°C).

3.2.2. Sub culturing and maintenance of *N. spumigena* KAC 66 cultures

The unialgal and purified culture of *N. spumigena* KAC 66 was used in the present study. *N. spumigena* KAC 66 underwent routine culture maintenance on a monthly basis for sub culturing and ongoing research

experiments. For routine maintenance 10% (10 mL, Sivonen *et al.,* 1989a) of old stock culture was transferred into 6 x 250 mL Erlenmeyer flasks (250 mL) containing 100 mL of BG-11 (20 ‰) to give a final culture volume of ~110 mL/flask. To avoid any contamination, transfer of cultures was performed under axenic conditions in a laminar flow hood (Microflow, Biological Safety Cabinet, UK). The flasks were kept on a shelf and grown in a temperature controlled room (22°C). All cultures received continuous cool white illumination (0.80 µmol/s/m^2; LI-250A, light meter, USA) and allowed to grow photoautotrophically. The flasks were shaken twice a week manually and grown for one month to obtain sufficient cultures for experiments.

3.2.3. Investigation and experimental set up for growth and peptide production

N. spumigena KAC 66 was grown in 10 L flat bottom round glass flasks and 8 L Perspex columns to evaluate the effect of time on the growth conditions, to find suitable culture vessels and to assess the effect of these factors on the production of peptide levels. Harvested cultures were used for fractionationand to obtain pure LNOD, NOD and nodulopeptin 901 (see Chapter 2).

3.2.3.a. Growth in 10 L glass flasks

Three 10 L flat bottom round flasks were filled with 8 L of BG-11 (20 ‰). The glass flasks were supplied by 2 silicon tube outlets. One tube was stopped by gate clamp and syringe for future sampling. The second tube was connected to a heap filter to produce sterile aeration (Fig. 3.1; Jun aquarium air-pump,

Figure 3.1. Culturing of *N. spumigena* KAC 66 in triplicate 10 L glass flasks for 7 weeks at 22°C.

ACO-5503, Japan). The flasks were autoclaved for 1 h at 15 lb of pressure and 120°C. All autoclaved flasks were left at room temperature for cooling. Cells from one month old stock cultures of *N. spumigena* (3 x 1 L) were mixed together in a 5 litre autoclaved Erlenmeyer flask. An autoclaved measuring cylinder was used to transfer the culture into three separate flasks (700 mL/flask). All flasks were kept at 22°C under continuous illumination from two cool white fluorescent tubes (36 W) delivering 17.35-17.47 µmol/s/m^2 (Table 3.2; Fig. 3.1). Samples were taken once per week for 7 weeks for cell biomass, chlorophyll-*a*, intra and extracellular peptide levels.

Table. 3.2. Light conditions for the growth of *N. spumigena* KAC 66 in glass flasks grown for 7 weeks at 22°C.

Flask nos.	light intensity ($\mu mol/s/m^2$)
1	17.35
2	17.47
3	17.36

3.2.3.b. Growth in 8 L Perspex columns

Five x 7 L BG-11 medium (20 ‰) was autoclaved for 1 h at 15 psi of pressure and 120°C and allowed to cool at room temperature. Next day 5 Perspex columns (140 cm x 10 cm) were washed with 2 L distilled water and purged with air to clean the air stones. Two liter of autoclaved BG-11 medium (0 ‰) was then used to wash columns and columns were left for 4 h to clean air stones. After 4 h columns were emptied and each column was filled with autoclaved BG-11 medium (7 L/column, 20 ‰).

Figure 3.2. Culturing of *N. spumigena* KAC 66 in 8 L Perspex columns for 5 weeks at 22°C.

In a 5 L autoclaved flask one month old (5 x 1000 mL) stock cultures were mixed and equally divided into five volumes (900 mL/column) using an autoclaved measuring cylinder. The culture was inoculated into each column. All columns were aerated continuously from the bottom of column by air-stones and the top sealed with a sterile foam bung.

All five columns were kept in a temperature controlled room (22°C) under continuous illumination from two cool white fluorescent tubes (36 W; Fig. 3.2). Sampling was carried out once per week for 5 weeks to determine cell biomass, chlorophyll-*a*, intra and extracellular peptide levels. The intensity of light was also measured at different positions on columns (Table 3.3). Column number 1 and 2 were one foot away from

Table. 3.3. Light conditions for the growth of *N. spumigena* KAC 66 in Perspex columns at different points down the column.

Distance from the top of the column (cm)	Light intensity (µmol/s/m^2)				
	Away from light source		Near to light source		
	1	2	3	4	5
0	1.6	1.4	2.5	16.9	12.9
30	1.7	1.5	2.4	12.5	12.0
60	1.6	2.0	5.6	33.9	14.1
90	1.7	3.3	7.1	42.6	17.1
120	1.6	2.1	6.1	40.4	18.0

the light source while column 3, 4 and 5 were near (≈ 8 inches) to the fluorescent tubes.

3.2.4. Investigation and experimental setup for effect of environmental parameters on growth and peptide production

3.2.4.a. Temperature

In this experiment 22, 25 and 30°C temperatures were selected, which covered the range of the thermal optimum conditions for *N. spumigena* in the Baltic Sea (Musial and Plinski, 2003; Lehtimäki *et al.,* 1997; Sivonen *et al.,* 1989c) at which the blooms frequently occur.

To note the effect of temperature on growth and toxin production three temperature controlled water baths were used. Water baths 1, 2 and 3 were adjusted at 22, 25 and 30°C, respectively and then placed under continuous illumination from cool white fluorescent tubes (36 W) delivering 13.36-13.49 µmol/s/m^2 (Table 3.4).

Table 3.4. Light conditions for *N. spumigena* KAC 66 grown for 7 weeks at different temperatures.

Water bath no.	Temperature (°C)	Light intensity (µ mol/s/m^2)
1	22	12.36
2	25	13.32
3	30	13.49

The water baths were setup for one week and monitored to confirm a constant temperature. During the experiment the water level was kept constant by adding Milli-Q water.

In this experiment 9 x 500 mL Erlenmeyer flasks were used, three for each temperature (22, 25 and 30°C). Each flask contained 350 mL of BG-11 (20 ‰) and was supplied by 2 silicon tube outlets. All flasks were autoclaved (15 minutes at 15 lb of pressure at 120°C) and cooled at room temperature. After cooling 35 mL (10%) of one month old stock culture of *N. spumigena* KAC 66 was inoculated into each flask.

Three Erlenmeyer flasks were kept in a temperature-controlled water bath number 1 (22°C), three flasks in water bath number 2 (25°C) and three in water bath number 3 (30°C; Fig 3.3) under continuous

Figure 3.3. Experimental set up for *N. spumigena* KAC 66 for 7 weeks at different temperatures.

illumination from two cool white fluorescent tubes (36 W) delivering 13.36 to 13.49 µmol/s/m^2 (Table 3.4). All flasks were supplied by continuous and slow aeration by silicon tubing. Cultures were sampled every week for 7 weeks for cell biomass, chlorophyll-*a* and intra and extracellular peptide levels.

3.2.4.b. Salinity

In order to measure the effect of salinity on chlorophyll-*a*, biomass and peptide production, a range of salinities were selected based on optimal growth conditions of *N. spumigena* in laboratory and salinity conditions of the Baltic Sea (Gasiunaite *et al.,* 2005; Mazur-Marzec *et al.,* 2005; Musial and Plinkski, 2003; Wasmund, 1997). Gasiunaite *et al.,* (2005) the Baltic Sea receives freshwater from many rivers results in a variation in salinity in different areas (0-25.5 psu; ≈0-25.5 ‰). Salinity range between 3.8-

11.5 psu (≈3.8-11.5 ‰) is important for the distribution of *N. spumigena* blooms in the Baltic Sea (Wasmund, 1997). According to Mazur-Marzec *et al.,* (2005) grew *N. spumigena* NSGG-1 strain at various salinities (0, 3, 7, 18 and 35 psu; ≈0, 3, 7, 18 and 35 ‰). They used lower salinities because the Baltic Sea is a semi-enclosed brackish water body and receives less water from Kattegat, North Sea (Kullenberg, 1981).

To carry out the experiment the Erlenmeyer flasks (15 x 500 mL) were prepared with 350 mL of BG-11 medium. The salinity in each flask was adjusted to the required concentrations (2, 7, 11, 20 and 25 ‰; Table 3.5) using sodium chloride (Fisher Scientific, UK).

Table 3.5. Different saline BG-11 medium for growth of *N. spumigena* KAC 66 at 22°C.

Salinity (‰)	**BG 11 medium (ml)**	**Amount of inoculum (ml)**
2	350	35
7	350	35
11	350	35
20	350	35
25	350	35

All flasks were supplied by 2 silicon tube outlets (see section 3.2.4.a, where appropriate). Prior to the inoculation of the cultures all flasks were autoclaved for 15 minutes at 15 lb pressure at 120 °C and left to cool at room temperature. The following day one month old stock cultures (9 x 100 mL) of inoculum were transferred into each flask (35 mL/flask).

This experiment was carried out in a temperature controlled room (22°C). All flasks were kept under constant illumination from two cool white fluorescent tubes (36 W) delivering 13.3-13.5 µmol/s/m^2; Fig. 3.4). Samples were taken once per week for 6 weeks for cell biomass, chlorophyll-*a*, intra and extracellular peptide levels.

Figure 3.4. Experimental set up for *N. spumigena* KAC 66 for 6 weeks at different salinities at 22°C.

3.2.4.c. Nitrate (NO^-_3)

Cyanobacteria are key organisms involved in the uptake and reduction of nitrate to ammonium by photosynthesis. Like higher plants and algae they use nitrates for their growth. The annual nitrogen fixation in the Baltic Sea occurs due to extensive blooms of heterocystic cyanobacteria *N. spumigena* along with *Aphanizomenon,* which is approximately equal to the total nitrogen input from atmospheric deposition, river run off and agricultural lands (Schneider *et al.,* 2004), because of nitrogen fixation ability *N. spumigena* has a high ecological importance for the Baltic Sea.

Therefore, the current experiment was performed to note the effects of nitrate on the biomass and peptide production. For this purpose various concentrations of nitrate were used on the basis of NO^-_3-NO^-_2 (nitrate-nitrite) and nitrate levels measured at different places in the Baltic Sea (Table 3.6).

Table 3.6. Various levels of NO^-_3 and NO^-_3-NO^-_2 at different places in the Baltic Sea.

Locations	Nitrate levels (µM/L)	Nitrate-Nitrite level (µM/L)	References
North Sylt Wadden Sea	40-50	-	MURSYS Report 2003
North Sylt Wadden Sea (summer)	<0.1	-	
Salzhaff	95	-	
Outer coastal waters of Mecklenburg-Vorpommern (Aug 2003)	<0.10-0.08	-	
Outer coastal waters of Mecklenburg-Vorpommern (Sept 2003)	0.17-1.30	-	
Mecklenburg	-	0.07	
Central Gotland Sea	-	0.49	
Inner coastal waters of Mecklenburg-Vorpommern (July 2003)	<0.01-1.20	-	
Inner coastal waters of Mecklenburg-Vorpommern (Sept 2003)	1.5	-	
German Bight (2005)	0.1	-	MURSYS Report 2005
Coastal waters of the German Bight (2005)	-	19.4±6.0	
Offshore waters	-	8.7±5.7	
Kiel Bight to the northern Gotland Basin (2006)	2.2-4	-	MURSYS Report 2006
Outer coastal waters of Mecklenburg-Vorpommern,	1.0	-	
Lower Warnow	260	-	
Barther Bodden	98	-	

Eighteen Erlenmeyer flasks (500 mL) were prepared with 350 mL of BG-11 (20 ‰) medium and adjusted at different concentrations of sodium nitrate ($NaNO_3$, Fisher Scientific, UK; Table 3.7). The flasks were supplied by 2 silicon tube outlets (see section 3.2.4.a, where appropriate) and autoclaved for 15 minutes at 15 lb of pressure at 120°C.

Table 3.7. Amount of $NaNO_3$ used to obtain different concentrations of nitrate for growth of *N. spumigena* KAC 66 in 1 L BG-11 medium (20 ‰) grown at 22°C.

Amount of $NaNO_3$ (mg/L)	**Amount of $NaNO_3$ (mM/L)**	**$NaNO_3$ (%)**
0	0	0
3.5	56	9.85
6.5	105	18.3
7.5	121	21.12
8.5	137	23.94
9.5	153	26.76

Thirty five mL of one month old culture of *N. spumigena* KAC 66 was inoculated into an Erlenmeyer flask and then placed under constant illumination by fluorescent tubes delivering 13.4-13.7 µmol/s/m^2. Cultures were sampled every 6 days to determine the cell biomass, Chl-*a*, intra and extracellular peptide levels.

3.2.4.d. Phosphate

Like other nutrients phosphorus also plays an important part in increasing cyanobacterial biomass in the Baltic Sea. Enhanced phosphorus input from increased river run off since the early 1970s has lead to the high phosphorus concentrations in the surface layers of the Baltic proper (Eilola *et al.,* 2009) and resulted in an increase in intensity and duration of the *N. spumigena* blooms. In this experiment, a range of phosphate concentrations were tested to monitor the effects of phosphorus on the production of Chl-*a*, cell biomass and peptides produced by

N. spumigena. These concentrations were based on phosphate levels recorded from the different locations of the Baltic Sea (Table 3.8).

To determine the effects of phosphorus on biomass and peptide production the experiment was performed in the Erlenmeyer flasks (21 x 500 mL) prepared containing 350 mL of BG-11 medium (20 ‰). The phosphate concentrations in each flask were adjusted to the required concentrations (0, 0.1, 10, 40, 70, 100 and 120 mg/L; Table 3.9) using potassium phosphate (Fisher Scientific, UK).

Before the experiment was started, the cultures were grown in BG-11 (20 ‰) for one month. The Erlenmeyer flasks were supplied 2 silicon tube outlets, one for sampling and the other to provide constant aeration (see section 3.2.4.a, where appropriate). Prior to the inoculation of the cultures all flasks were autoclaved for 15 minutes at 15 lb of pressure at 120°C and left to cool at room temperature. The following day one month old stock cultures (9 x 100mL) of inoculum were transferred into each flask (35 mL/flask). The experiment was conducted in a temperature controlled room at 22°C. All flasks were kept under constant illumination from two cool white fluorescent tubes (36 W) delivering 13.5-14.5 μmol/s/m^2). Samples for peptide analysis, Chl-*a* and cell biomass were taken on the day of inoculation and on a weekly basis for 5 weeks.

Table 3.8. Various levels of phosphate at different locations in the Baltic Sea.

Locations	Phosphate levels (µM/L)	References
North Sylt Wadden Sea	1.2	
North Sylt Wadden Sea (summer)	0.1-1	
Outer coastal waters of Mecklenburg-Vorpommern (July 2005)	0.04-0.52	
Central Gotland Sea (October-November)	0.20	
Pomeranian Bight (October-November)	0.84	
Inner coastal waters of Mecklenburg-Vorpommern (September)	0.25-4.79	MURSYS Report 2005
German Bight (2005)	0.02-0.15	
Coastal waters of the German Bight (2005)	0.66±0.24	
Offshore waters	0.66±0.24	
Elbe estuary	1.09±0.25	
Darsser Ort	0.16	
Outer coastal waters of Mecklenburg-Vorpommern	0.02	
Inner coastal waters of Mecklenburg-Vorpommern	0.2-0.3	
Kiel Bight to the northern Gotland Basin (2006)	0.1-0.35	
Coastal water of North Sea (January 2006)	1.19±0.14	
German Bight (January 2006)	0.77±0.13	MURSYS Report 2006
Central North Sea	0.64±0.13	
Near shore waters of North Sea	0.97±0.20	
German Bight	0.64±0.2	
der Central North Sea	0.53±0.2	

Table 3.9. Amount of K_2HPO_4 for getting different Concentrations of phosphate for the growth of *N. spumigena* KAC 66 at 22°C.

Phosphate conditions	Amount of K_2HPO_4 (mg/L)	K_2HPO_4 (%)
0	0	0
0.1	0.1	4.8
10	10	9.5
40	40	14
70	70	19
100	100	24
120	120	29

3.2.5. Analytical methods and sampling procedures for cell biomass and chlorophyll-*a* (Chl-*a*)

The sampling procedure and sample analysis protocols for all experiments were the same. The sampling was done on the day of inoculation and on weekly basis (T0 and T1-T7). Before sampling all flasks were well shaken by hand and 25 mL of culture removed T0 (time zero) and weekly thereafter from each column/flask in separate plastic beakers (20 mL was used for cell biomass, chlorophyll-*a* estimation and extracellular peptide levels while 1.5 mL was used for determination of intracellular peptide levels). A detailed sampling procedure to determine the biomasses and the intra and extracellular peptide levels is shown in Fig. 3.5.

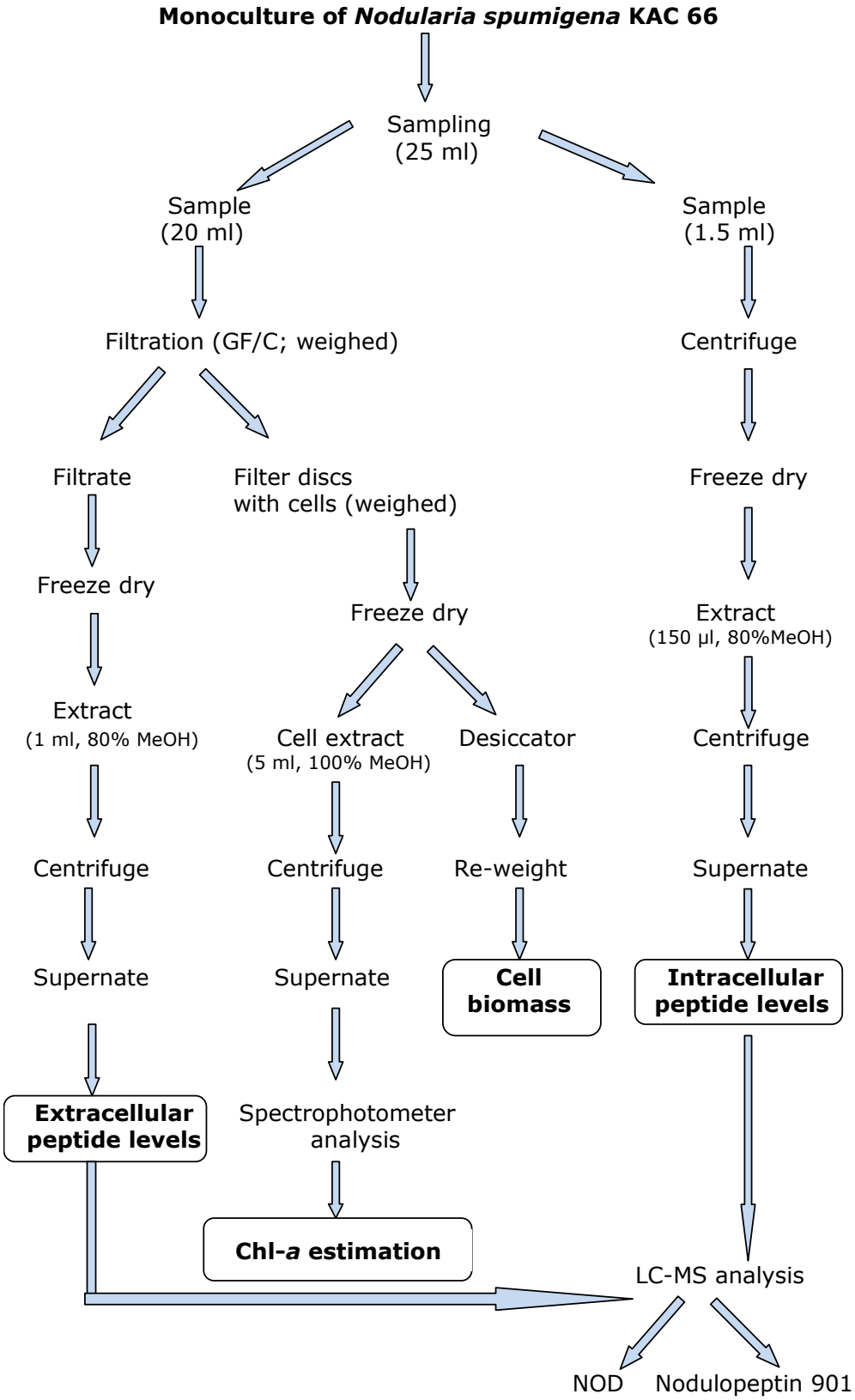

Figure 3.5. A flow diagram for sampling procedure to observe the effects of environmental factors on the growth parameters and toxin production by *N. spumigena* KAC 66.

3.2.5.1. Cell biomass determination

To determine the cell biomass 20 mL sample cultures of each treatment were used. To obtain the constant weight, GF/C glass microfiber filter discs (55mm Ø, Whatman, UK) were placed in pre-labelled individual plastic Petri plates and kept in a desiccator for one week. All filter discs re-weighed three times to obtain constant weight and then 20 mL culture was filtered through the pre-weighed filter discs. The filtrate was used for the determination of extracellular toxin levels.

The filter papers were freeze dried over night in a freeze dryer (HSC 500, Modulyo, Edwards, UK) at -45 ºC and 10^{-1} m bar. The next day filters were transferred to a decicator overnight and reweighed three times to obtain constant weight. The initial weight was subtracted from the weight of with cells to obtain dry weight of cells (mg/20 mL), later the data was converted into µg/mL. For Chl-*a* estimation the weighed filter papers were transferred to -4 ºC until extraction. All filters were analysed within 2 months.

3.2.5.2. Chlorophyll-*a* estimation

Like higher plants and algae, cyanobacteria contain chlorophyll-*a*, a major photosynthetic pigments which helps in photosynthesis by harvesting light.

Chlorophyll-*a* estimation 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. These 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 4000 rpm. To estimate the amount of Chl-*a*, absorbance were noted on a spectrophotometer (Spectrophotometer, UK) at a wavelength (λ) of 665 nm. Methanol was used as a blank. The amount of Chl-*a* was calculated by the following equation as mentioned by Murphy *et al.,* (2005 and 2009).

$$\text{Chlorophyll-}a\ (\mu g/mL) = (13.0 \times A \times v)/(d \times V)$$

Where:

13.0= Constant for methanol
A= absorbance at 665 nm
v= solvent in mL (5 mL MeOH)
V= initial filtered sample volume (20 mL)
d= Path length of cuvette in cm (1 cm)

3.2.5.3. Correlation between cell biomass and chlorophyll-*a*

To determine the correlation between cell biomass and chlorophyll-*a,* 6 x 3 GF/C filter papers were dried in a desiccator (see section 3.2.5.1, where appropriate). Prior to sample analysis a serial dilution (Table 3.10) of one month old culture of *N. spumigena* KAC 66 was prepared to calibrate the cell biomass and Chl-*a*. Culture in BG-11 (20 ‰) was diluted using BG-11 (20 ‰) to obtain the following dilutions 1, 5, 10, 25,

Table 3.10. A serial dilution of *N. spumigena* KAC 66 culture with BG-11 (20 ‰) to find a relation between cell biomass and Chl-*a* contents.

Amount of culture (ml)	**Amount of BG-11** (ml)
100	0
50	50
25	75
10	90
5	95
1	99

50 and 100%. From each dilution 20 mL was removed for Chl-*a* calibration. All experiment was performed in triplicate.

3.2.5.4. High Chl-*a* absorbance on spectrophotometer

Samples containing the high concentrations of chlorophyll-*a* was confirmed by a serial dilution (100, 50 and 25%) as these were out with the linear range of the spectrophotometer. Two mL of 100% extract was added in 2 mL MeOH to get 50% extract. These dilutions were read on spectrophotometer at 665 nm. The same procedure was used for all concentrated chlorophyll-*a* samples, however the dilution factor was taken into account where calculating the Chl-*a* concentrations.

3.2.6. Analytical methods and sampling procedures for extra and intracellular peptide levels

3.2.6.1. Extracellular peptide level analysis

Twenty mL filtrate/spent medium (extracellular) of sample was used for analysis by LC-MS to detect the presence and concentrations of any toxins released into the surrounding growth medium. The spent medium

was freeze dried and re-suspended in 1 mL 80% MeOH:H_2O (80:20, v/v) for one hour and all extracts were transferred into 1.5 mL microcentrifuge tube. To measure extracellular peptide levels all extracts were stored at -20ºC until LC-MS analyses. All extracts were analysed within 2 months.

3.2.6.2. Intracellular peptide level analysis

From 25 mL of the culture sample 1.5 mL was transferred into an microcentrifuge tube and centrifuged at 13,000 rpm (Eppendorf Centrifuge 5410, Germany) for 10 min. The supernate was discarded and the palette vortexed with 150 µl MeOH (80%) and extracted for one hour.

To measure intracellular peptide levels all extracts were stored at -20ºC until LC-MS analyses. All extracts were analysed within 2 months.

For HPLC-PDA-MS analysis all extracts were centrifuged at 13,000 rpm for 10 min. One hundred µl supernate was carefully transferred into an LC-MS vial.

3.2.6.3. Analysis of nodularin and nodulopeptin 901 on HPLC-PDA-MS

The samples from growth experiment preformed in Perspex columns and 10 L glass flasks and effects of environmental factors (temperature, salinity and nitrate) on the cell biomass, Chl-*a*, intracellular and extracellular peptide productions produced by *N. spumigena* KAC 66 were analysed on HPLC and UPLC-PDA-MS.

For identification and quantification of nodularin and nodulopeptin 901 was performed using HPLC-PDA-MS. 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 (5 µm particle size; 2.1 mm i.d. 150 mm long) meaasured at 40°C. The mobile solvent phase A was Mili-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 followed by ramp up to 100% B and re-equilibration after 10 next 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 100ºC and 300ºC, respectively. MassLynx software v4.0 was used to control the instrument for data acquisition and processing. The photo diode array (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, nodularin at 238 nm and nodulopeptin 901 at 210 nm.

3.3. RESULTS

3.3.1. Growth medium BG-11 and maintenance of *N. spumigena* KAC 66

In this study *N. spumigena* KAC 66 was successfully grown and tested in BG-11 growth medium (20 ‰). The observations showed that 22°C and low light intensity (0.80 µmol/s/m^2) are good for long term maintenance of *N. spumigena* KAC 66.

3.3.2. A correlation between cell biomass and chlorophyll-*a*

The amount of chlorophyll-*a* at different dilutions of cells of *N. spumigena* KAC 66 was quantified by plotting a correlation graph (Fig. 3.6.; Appendices 30 and 31).

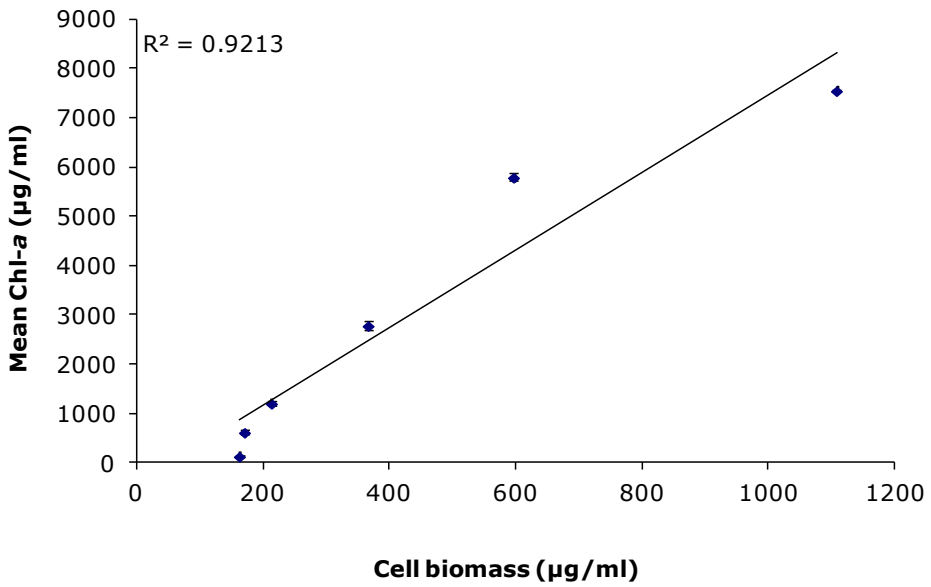

Figure 3.6. A correlation between cell biomass and Chl-*a* (n=3, bars=1 SD).

It was noted that as the cell biomass of *N. spumigena* increases the amount of Chl-*a* also increases. At the highest cell biomass (1,108 µg/mL) the highest Chl-*a* value (7,554.1 µg/mL) was observed, which gradually decreased as the dilution was increased (Appendix 32).

3.3.3. High Chl-*a* absorbance on spectrophotometer

The highest absorbance values (2.32, 2.32 and 2.32) for Chl-*a* were observed in a 100 mL dilution while plotting a correlation graph between Chl-*a* and cell biomass (Fig. 3.7; Appendix 33), which was closed to the highest readable absorbance limit (2.5) of the spectrophotometer. The results show that the Chl-*a* concentration was high and could not be correctly read by spectrophotometer. The results showed a difference of 0.66 absorbance between 100% and 50% diluted extracts (Fig. 3.7) indicating typical plateau effect observed when using spectrophotometer.

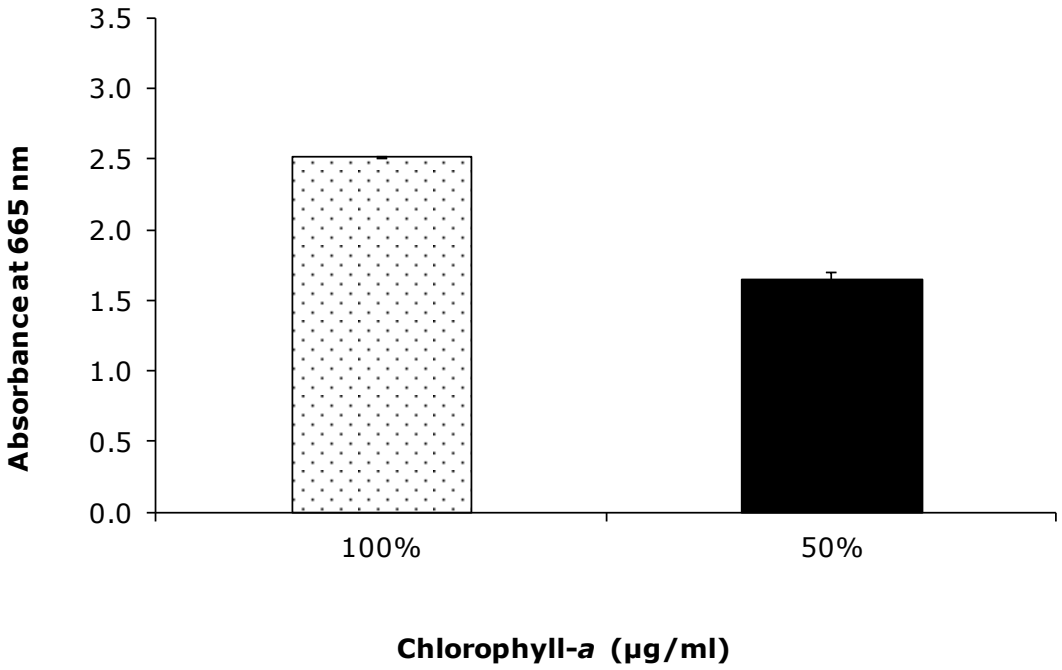

Figure 3.7. Difference between absorbance of Chl-*a* at 100% and 50% dilutions at 665 nm wavelength (n=3, bars=1 SD).

3.3.4. Investigation on growth and peptide production

3.3.4.1. Growth in 10 L glass flasks

3.3.4.1.a. Cell biomass and chlorophyll-*a*

N. spumigena KAC 66 cell biomass and Chl-*a* concentrations were maintained for 7 weeks and ranged from 6,387 to 6,917 µg/mL and 0.05 to 0.2 µg/mL, respectively. A relationship between Chl-*a* concentration and time was observed as would be expected, as the time passed Chl-*a* was also increased with a slight decline in week 7 (0.19 µg/mL; Fig. 3.8; Appendix 34).

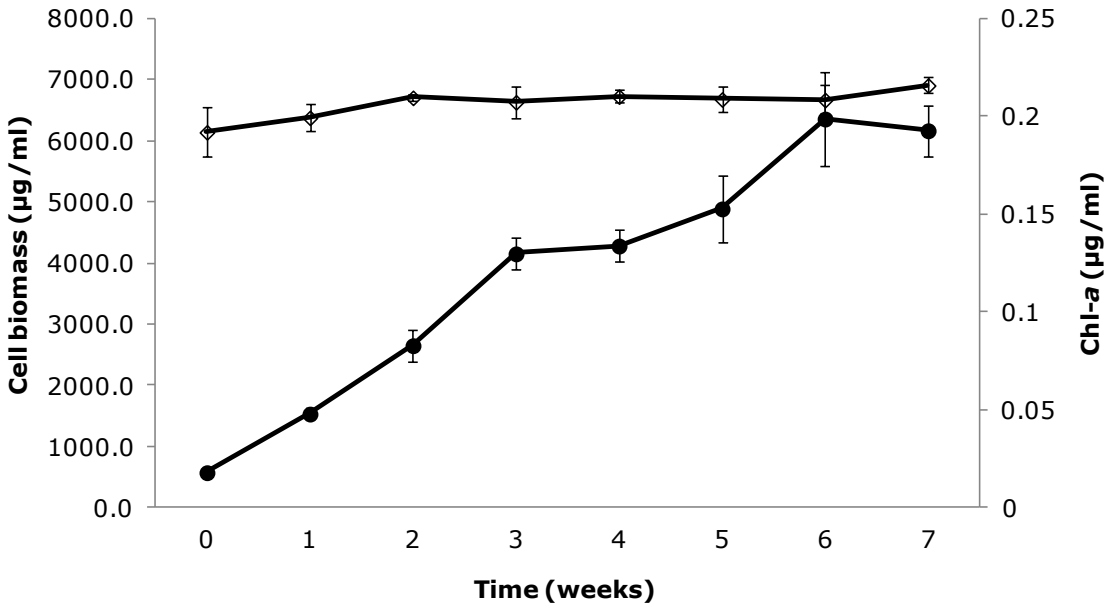

Figure 3.8**.** Chl-*a* concentrations and cell biomass for cultures of *N. spumigena* KAC 66, grown in glass flasks for 7 weeks at 22°C (n=3, bars=1 SD) (Chlorophyll-*a* ●, cell biomass ◇)

In week 1 the lower Chl-*a* concentration (0.05 µg/mL) and cell biomass (6,387 µg/mL) was recorded but mostly high concentrations exhibited in last weeks of experiment, which showed a normal growth pattern. Due to error in measurement the cell biomass did not show any considerable change from week 2 to 6 and continued with an increase (6,917 µg/mL; Fig. 3.8) in week 7. This would suggest a problem with the accuracy of the dry weight measurements.

3.3.4.1.b. Extra and intracellular peptide levels

Nodularin and nodulopeptin 901 were produced in high amount *by N. spumigena* (Appendix 35). The UPLC-PDA-MS analyses showed that *N. spumigena* retain high amounts of NOD within the cell. In the case of

nodulopeptin 901 both the intra and extracellular levels increased as time passed (Figs. 3.9A and B; Appendix 35).

During the 7 weeks it was observed that nodulopeptin 901 concentrations were rich significant in the extracellular fraction. At T0 (first day of inoculation) no traces or undetectable amount of extracellular peptides observed.

The intracellular NOD levels increased between weeks 1 to 6 (137-390 ng/mL) and decreased (304 ng/mL) in week 7 (Fig. 3.9A; Appendix 36). From week 1 to 4 the cyanobacterium started to release NOD in to the surrounding medium and concentrations ranging from 7.0-36 ng/mL were detected (Fig. 3.9B; Appendix 36).

In the case of nodulopeptin 901 a gradual increase both in extra and intracellular concentrations was observed during growth over time. The highest concentration (282 ng/mL) of extracellular nodulopeptin 901 was recorded in week 6. After this in week 7 the nodulopeptin 901 level decreased (226 ng/mL) due to the commencement of death phase of the cyanobacteria and toxin release rate into the growth medium was reduced. Intracellular concentrations of nodulopeptin 901 gradually increased until the last week of harvesting (91-370 ng/mL). The week 6 is the optimum time to harvest the cyanobacterium to get the highest amount of extra and intracellular nodulopeptin 901 and intracellular NOD (Fig. 3.9B; Appendix 63). During the growth experiments the relative properties of extra and intracellular concentrations were calculated. In week 5 to 7 undetectable amount of NOD was released in medium and 100% toxin remained within the cells (Table 4.1). Comparatively, it

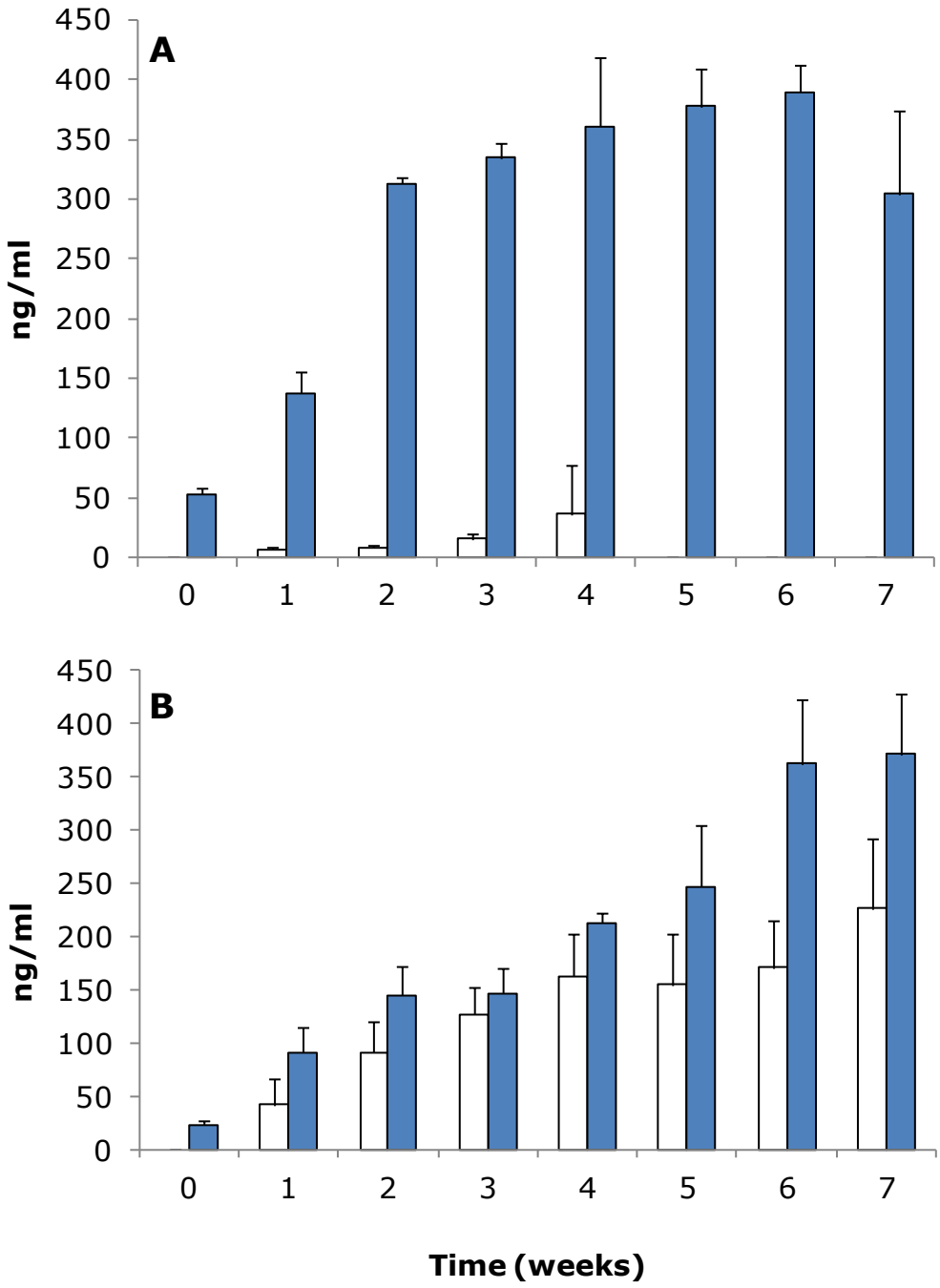

Figure 3.9. The Intra and extracellular levels of peptides for cultures of *N. spumigena* KAC 66 grown in glass flasks for 7 weeks at 22°C (n=3, bars=1 SD). **A**:- NOD, **B**:- nodulopeptin 901 (Extracellular □, intracellular ■).

Table 3.11. Percentages of peptides in extra and intracellular levels in weekly samples of *N. spumigena* KAC 66 cultures grown in glass flasks for 7 weeks at 22°C (n.d. = not detected, the data based of mean values given in Appendix 36).

Time (weeks)	Extracellular (%)	Intracellular (%)
NOD		
T0	n.d.	100
T1	4	96
T2	3	97
T3	4	96
T4	9	91
T5	n.d.	100
T6	n.d.	100
T7	n.d.	100
Nodulopeptin 901		
T0	n.d.	100
T1	31	69
T2	38	62
T3	46	54
T4	43	57
T5	40	60
T6	40	60
T7	38	62

seems that during the whole experiment the high amount of NOD remained within the cells, ranged from 91-100%.

The highest percentage (100%) of nodulopeptin 901 was recorded within the cells and no detectable amount was recorded at the day of inoculation. From week 1 to 7 a considerable amount of intracellular nodulopeptin 901 was recorded (54-69%) in surrounding medium (31-46%; Table 3.11). The highest percentages of extracellular nodulopeptin 901 was noted in weeks 3 and 4 (Table 3.11). Intracellular NOD

concentrations were 91 % of the total NOD detection with much lower (9%) extracellular concentrations. Intracellular concentrations of nodulopeptin 901 were consisting higher (approximately 66%) than extracellular nodulopeptin (34%).

In general the cell biomass, Chl-*a* contents and peptide production levels were considerably co-related with each other and all parameters were recorded on their maximum levels in week 6 (except extracellular NOD levels).

3.3.4.2. Growth in 8 L Perspex columns

3.3.4.2.a. Cell biomass and chlorophyll-*a*

The variation in cell biomass and Chl-*a* concentrations/week in five Perspex columns are summerized in Figs. 3.10 and 3.11, respectively. Due to poor growth of *N. spumigena* in three columns, the experiment was only performed for 5 weeks.

As expected in all columns the lowest cell biomass was found at the day of inoculation, when cell concentration was low (Appendix 37).

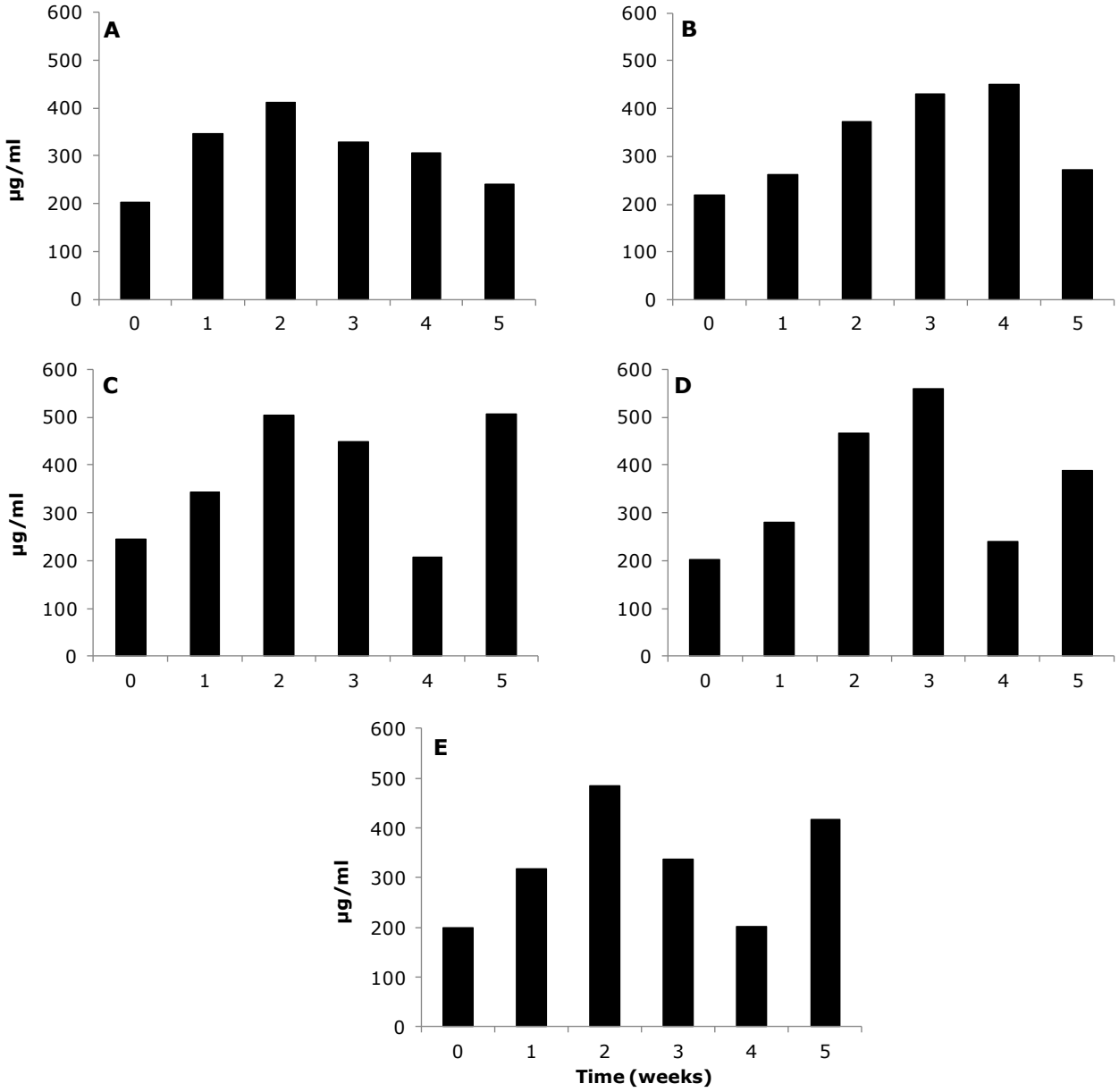

Figure 3.10. Cell biomass for cultures of *N. spumigena* KAC 66, grown in columns for 5 weeks at 22°C (**A**:- column 1, **B**:- column 2, **C**:- column 3, **D**:- column 4 and **E**:- column 5)

Among five columns the highest cell biomass was observed in column 4 in week 3 (559 µg/mL; Fig. 3.10). The columns 1 and 2 were slightly further away from the light source and showed healthy growth pattern over time (Figs. 3.10A and B). The cell biomass reached its maximum 410.5 and 450.0 µg/mL in week 2 and 4, respectively (Figs. 3.10A and B). A decrease in cell biomass was observed in column 1 (241.5 µg/mL) and 2 (273.0 µg/mL) in week 5.

Columns 3, 4 and 5 were closed to light source. Columns 3 and 5 represented an increasing growth pattern from the first day of inoculation (T0) to week 2 ranged from 198.5-505.0 µg/mL (Figs. 3.10C and E). While column 4 showed increase in cell biomass till week 3 (559.0 µg/mL; Fig. 3.10D).

In week 5 in coulmns 3-5 a sudden decrease in cell biomass ranged from 202.5-239.5 µg/mL with an increase (387.0-506.0 µg/mL) was observed (Figs.3.10C-D).

The Chl-*a* contents in all 5 Perspex columns represented a same pattern as cell biomass showed (Fig. 3.11; Appendix 38). Column 1 and 2 (away from light source) showed an increase in Chl-*a* concentrations in weeks 2 and 3 (Fig. 3.11A and B). The Chl-*a* concentration of column 3, 4 and 5 was reached on its maxima in week 2 (ranged from 0.22 to 0.26 µg/mL) and started to decline from week 4 to 5 (Figs. 3.11C, D and E).

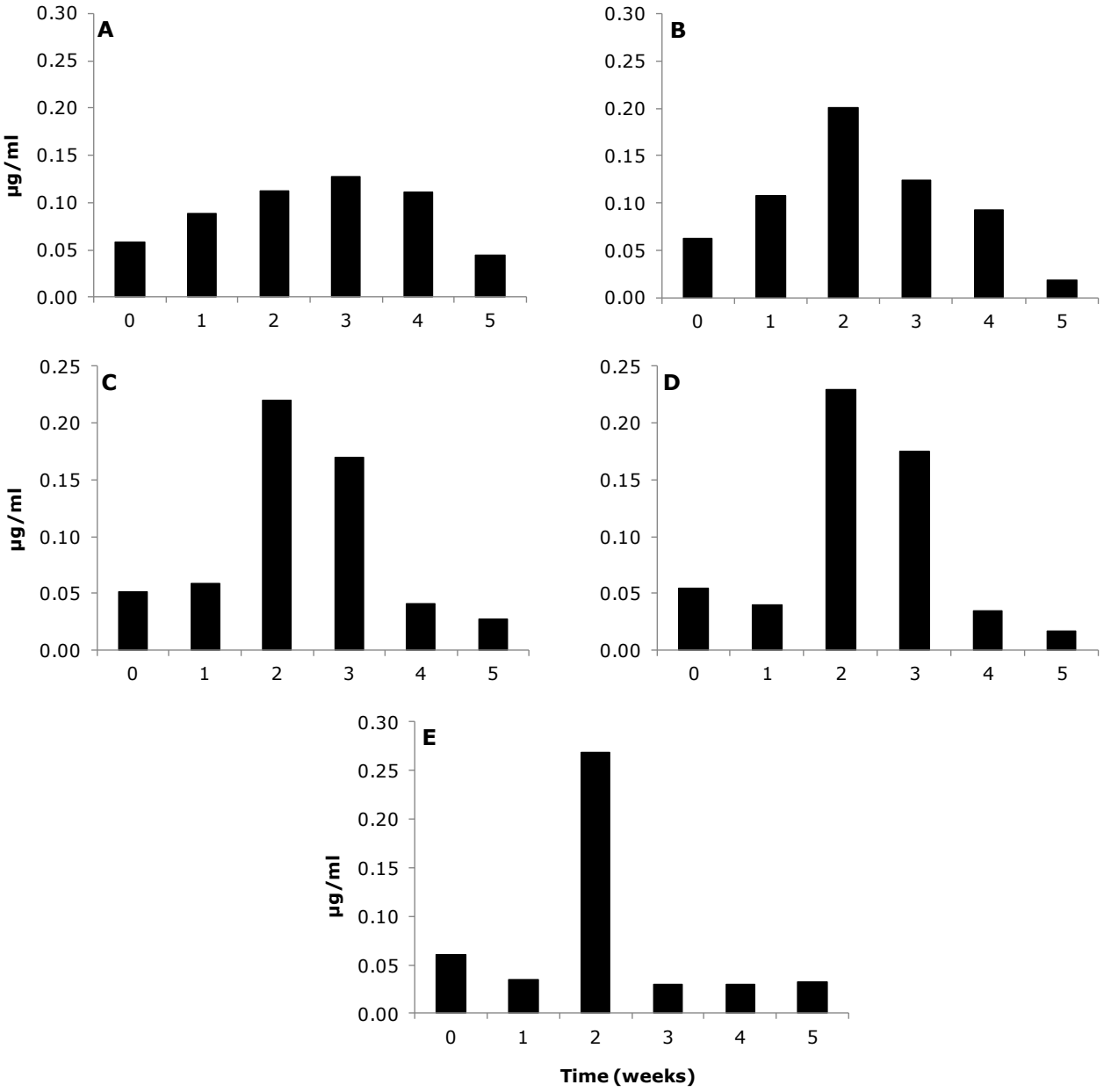

Figure 3.11. Chl-*a* concentrations for cultures of *N. spumigena* KAC 66, grown in columns for 5 weeks at 22°C (**A**:- column 1, **B**:- column 2, **C**:- column 3, **D**:- column 4 and **E**:- column 5)

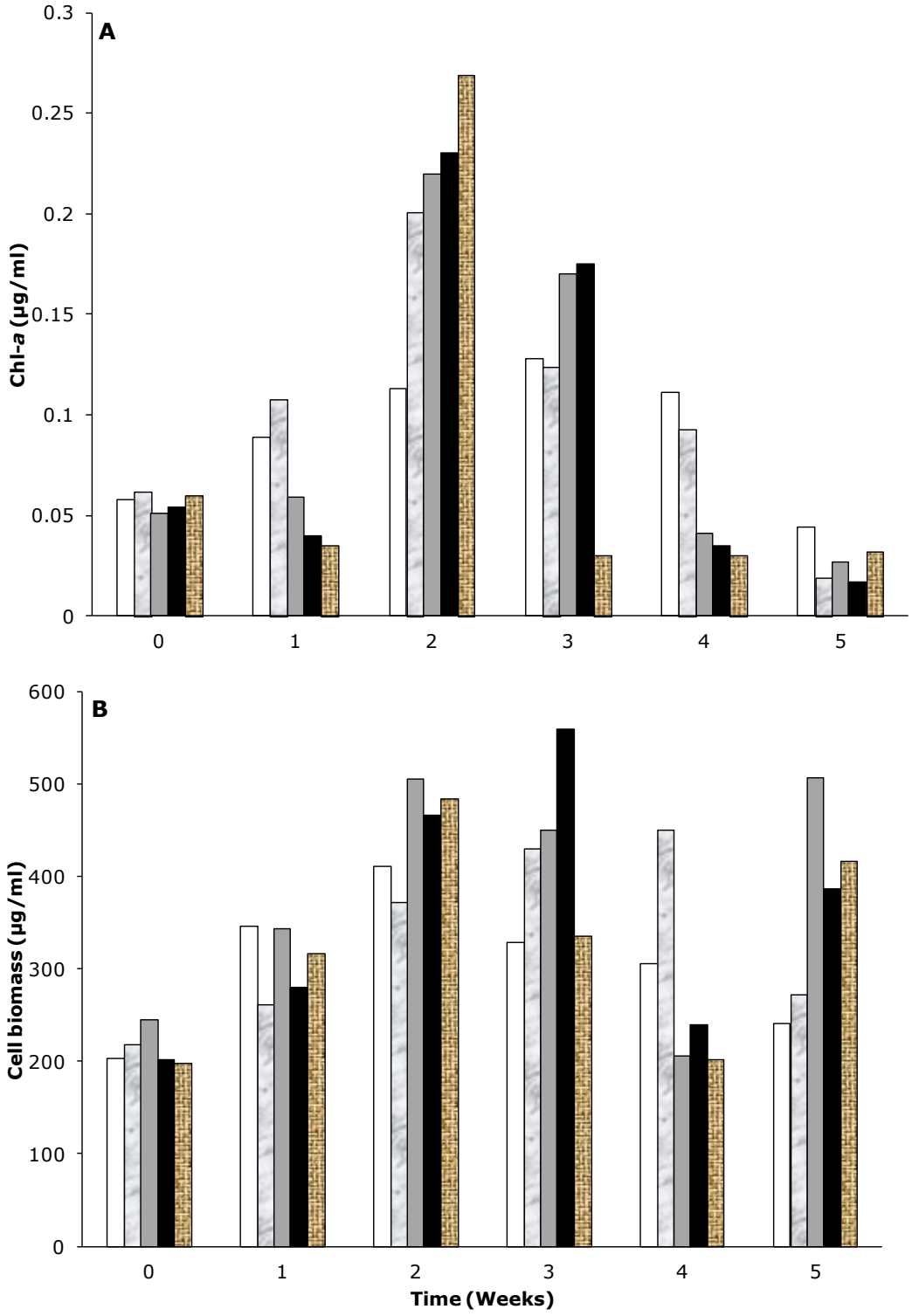

Figure 3.12. A comparison of biomasses for cultures of *N. spumigena* KAC 66, grown in individual columns for 5 weeks at 22°C (**A**:- Chl-*a* concentrations, **B**:- cell biomass; column 1=□, column 2= ▫, column 3= ▩, column 4= ■ , column 5= ▩).

A comparative analysis of Chl-*a* contents and cell biomass among 5 columns was also done (Figs. 3.12A and B). It was also noted that those columns (3, 4 and 5) were near to light source started to die first compared to column 1 and 2, which were away from high irradiance. After death of cell the colour of cultures was turned from green to pale yellow, which affected the biomass (Figs. 3.12A and B).

3.3.4.2.b. Extra and intracellular peptide levels

The results relating to the production of peptides are summarized in Figs. 3.13 and 3.14; Appendix 39). The amount of intra and extracellular peptides in ng/mL are summarized in Appendix 40.

The HPLC-PDA-MS analysis show that in all columns undetectable amount or no release of NOD was noted in week 1, but release occurred between weeks 2 to 5. In column 5 at the day of inoculation (T0) and in week 1 no traces of intracellular and extracellular NOD were detected. In week 3 the highest intracellular NOD (1,300.3 ng/mL) was recorded which started decline till week 5 (1.9 ng/mL). The highest intracellular NOD levels were noted in column 1 in week 4 (1,457.5 ng/mL) and lowest in column 2 in week 5 (2.5 ng/mL; Fig. 3.13).

In all columns no extracellular nodulopeptin 901 was detected during the whole experiment. The highest intracellular nodulopeptin 901 concentrations were noted in column 4 and 5 in week 2 (236 and 324 ng/mL, respectively). The lowest concentration of intracellular nodulopeptin 901 was recorded in column 3 (11.5 ng/mL, Fig. 3.14C; Appendix 40). Columns 1, 2 and 4 did not show any traces of intracellular nodulopeptin 901 from week 3-5 (Figs. 3.14A, B and D).

Results in the present experiment show considerable effects of irradiance and the death of cells on the production of extra and intracellular peptide levels. Both factors suppressed the production intra and extracellular nodulopeptin 901 concentrations resulted in no or undetectable amount of nodulopeptin 901 was released in the surrounding growth medium.

The percentages in columns 1 and 3 the extracellular NOD concentrations were high in week 3 (96% and 79%, respectively; Table 3.12). The highest extracellular NOD concentrations were recorded in columns 2, 4 and 5 in week 5 ranged from 69-99%. Intracellular NOD concentrations were highest in columns 1-4 in week 2 (100%) and in column 5 in weeks 2-4 (94-99%). During the whole growth duration nodulopeptin 901 was not release into medium and 100% nodulopeptin 901 retained within the cells.

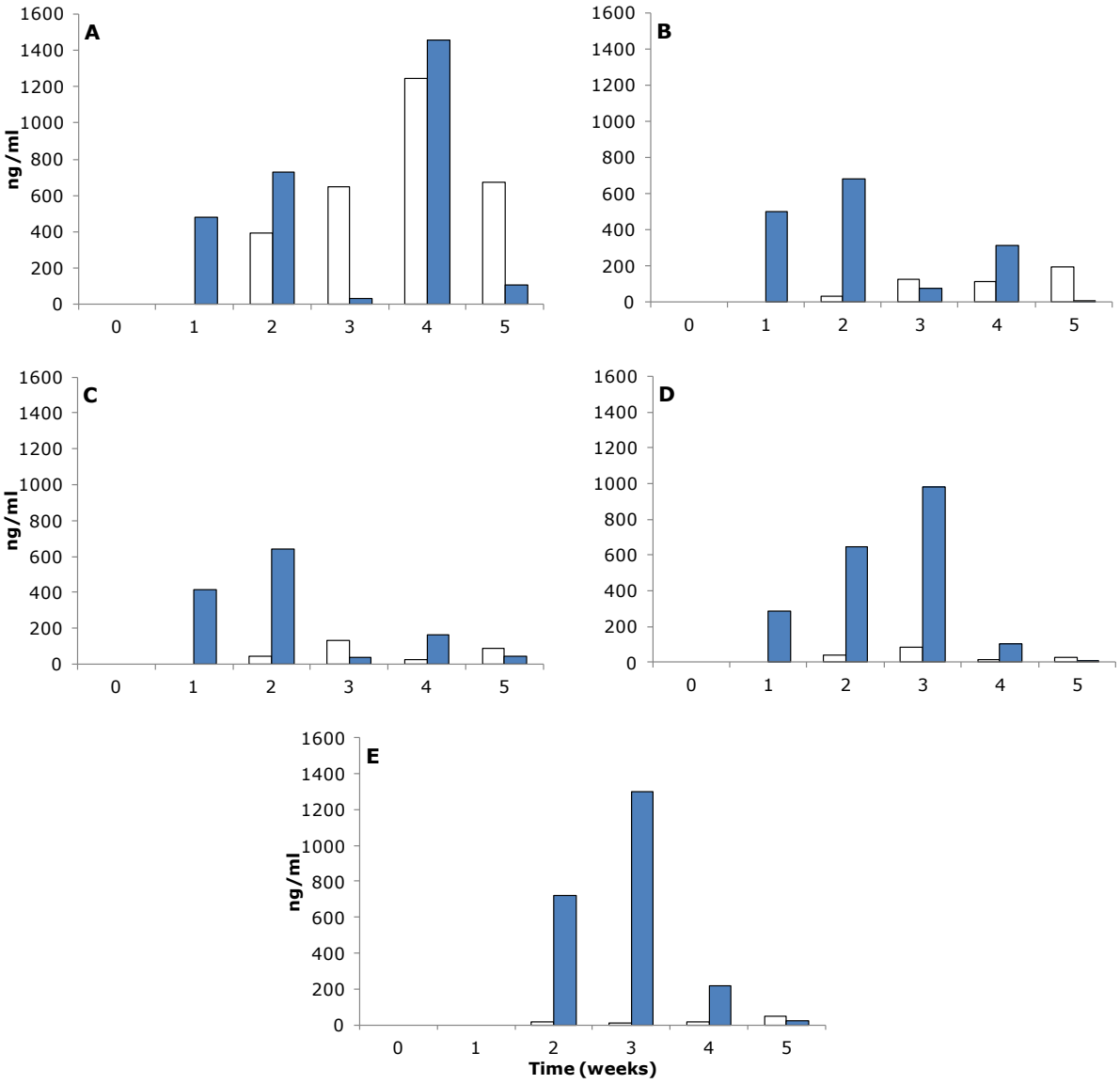

Figure 3.13. The Intra and extracellular levels of NOD for cultures of *N. spumigena* KAC 66 grown in columns for 5 weeks at 22 °C. **A**:- column 1, **B**:- column 2, **C**:- column 3, **D**:- column 4, **E**:- column 5 (Extracellular □, intracellular ■)

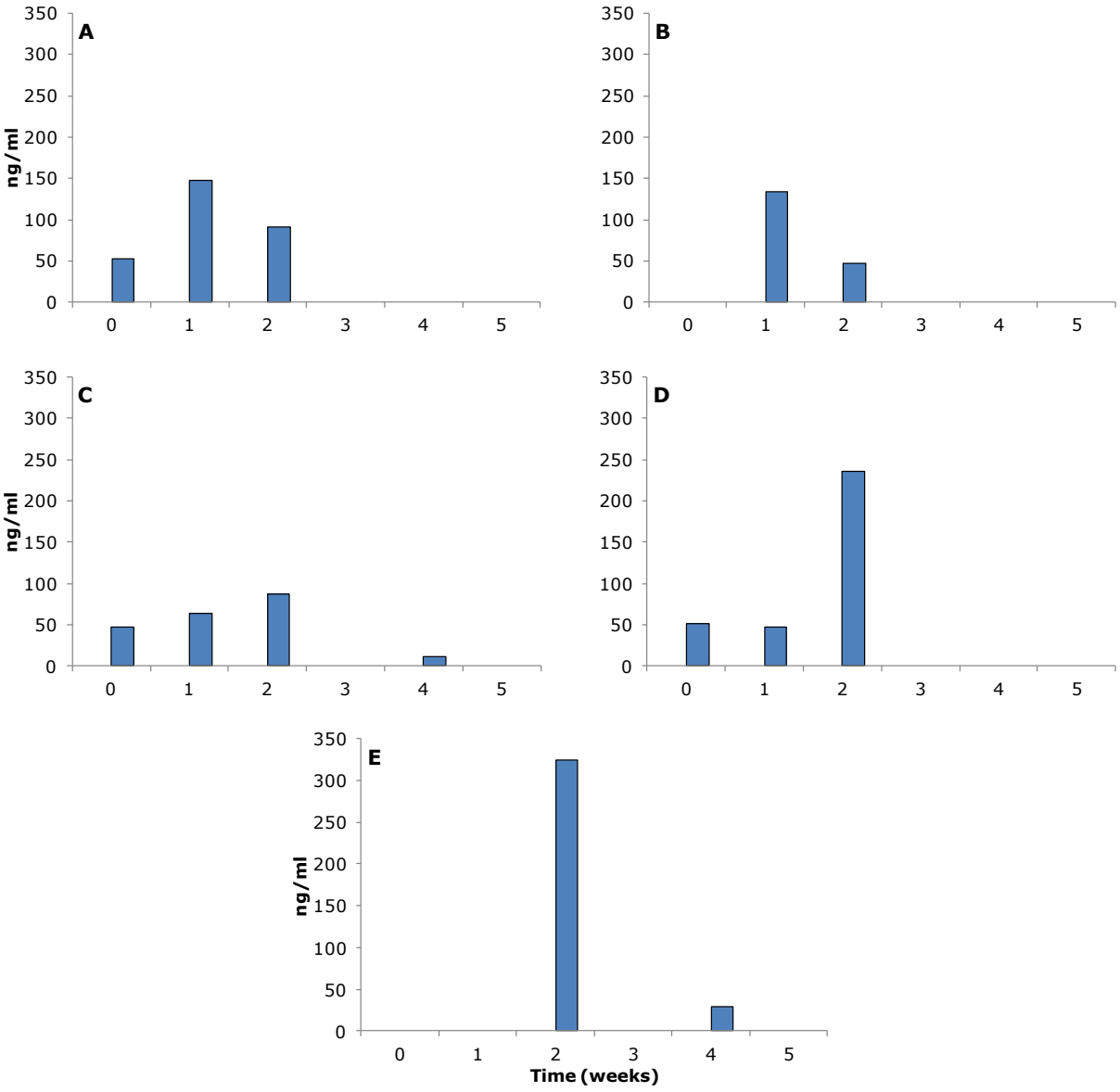

Figure 3.14. The Intracellular levels of nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown in columns for 5 weeks at 22°C. **A**:- column 1, **B**:- column 2, **C**:- column 3, **D**:- column 4, **E**:- column 5 (intracellular ▢)

Table 3.12. Percentages of NOD and nodulopeptin 901 in extra and intracellular levels for cultures of *N. spumigena* KAC 66 grown in columns for 5 weeks at 22°C (n.d = not detected, the data is based on mean values of Appendix 40).

Columns	NOD (%)						Nodulopeptin 901 (%)					
	Time (weeks)						Time (weeks)					
	T0	T1	T2	T3	T4	T5	T0	T1	T2	T3	T4	T5
Extracellular												
1	n.d	n.d	35	96	46	1	n.d	n.d	n.d	n.d	n.d	n.d
2	n.d	n.d	5	62	0	99	n.d	n.d	n.d	n.d	n.d	n.d
3	n.d	n.d	7	79	12	68	n.d	n.d	n.d	n.d	n.d	n.d
4	n.d	n.d	6	8	10	96	n.d	n.d	n.d	n.d	n.d	n.d
5	n.d	n.d	3	1	6	69	n.d	n.d	n.d	n.d	n.d	n.d
Intracellular												
1	n.d	100	65	4	54	99	100	100	100	n.d	n.d	n.d
2	n.d	100	95	38	100	1	n.d	100	100	n.d	n.d	n.d
3	n.d	100	93	21	88	32	100	100	100	n.d	100	n.d
4	n.d	100	94	92	90	34	100	100	100	n.d	n.d	n.d
5	n.d	n.d	97	99	94	31	n.d	n.d	100	n.d	100	n.d

3.3.5. Investigation on effect of environmental factors on growth and peptide production

3.3.5.1. Temperature

3.3.5.1.a. Cell biomass and chlorophyll-*a*

Chl-*a* concentrations for *N. spumigena* KAC 66 grown in Erlenmeyer flasks for 6 weeks at 22°C, 25°C and 30°C.

At 22°C the concentration of Chl-*a* was very low in week 1 (0.1 µg/mL) but as time progressed the concentration of Chl-*a* was increased by week 6 (0.19 µg/mL; Appendix 41).

A total of Chl-*a* concentrations during the experiment at 25°C, showed an increase in weeks 3 and 4 (0.17 µg/mL) and then declined by week 6 (0.13 µg/mL). At the highest temperature (30°C) the maximum concentration was observed in week 4 (0.21 µg/mL) and there was a considerable decline was noted in week 6 (0.12 µg/mL; Fig. 3.15A-C). Due to difficulties with dry weights containing freeze dried cells the data could not be included.

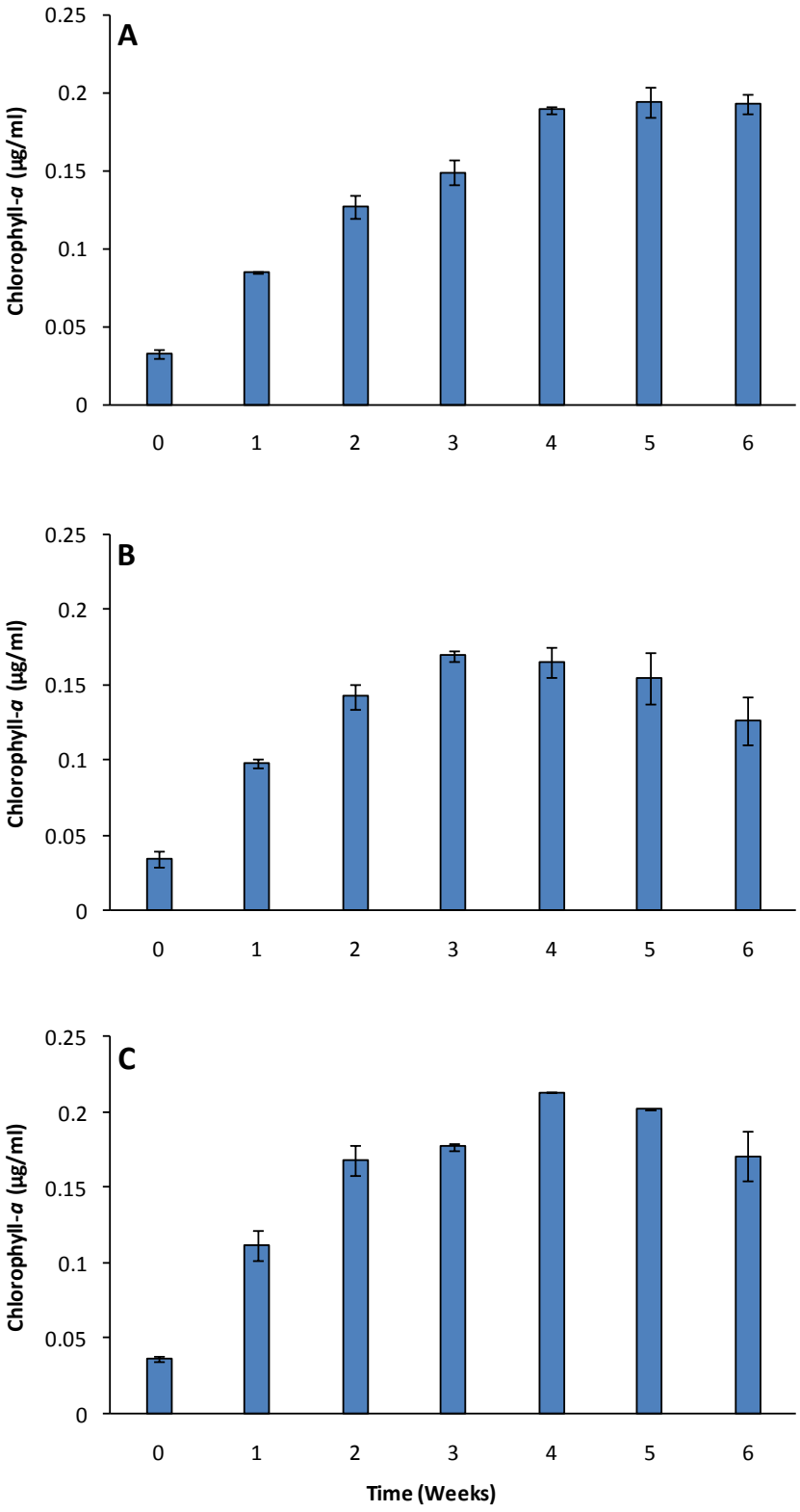

Figure 3.15. Chlorophyll-*a* concentrations at different temperatures for cultures of *N. spumigena* KAC 66 grown for 6 weeks (n=3, bars=1 SD). **A**:- 22°C **B**:- 25°C and **C**:- 30 °C.

3.3.5.2. b. Extra and intracellular peptide levels

It was observed that extra and intracellular peptide concentrations decreased as temperatures increased (Figs. 3.17 and 3.18; Appendices 42 and 43). The amount of intra and extracellular NOD and nodulopeptin 901 are shown in Appendices 44 and 45, respectively.

In comparison firstly 22°C and secondly 25°C supported the high production extra and intracellular of NOD (intracellular) and nodulopeptin 901 (Fig. 3.16).

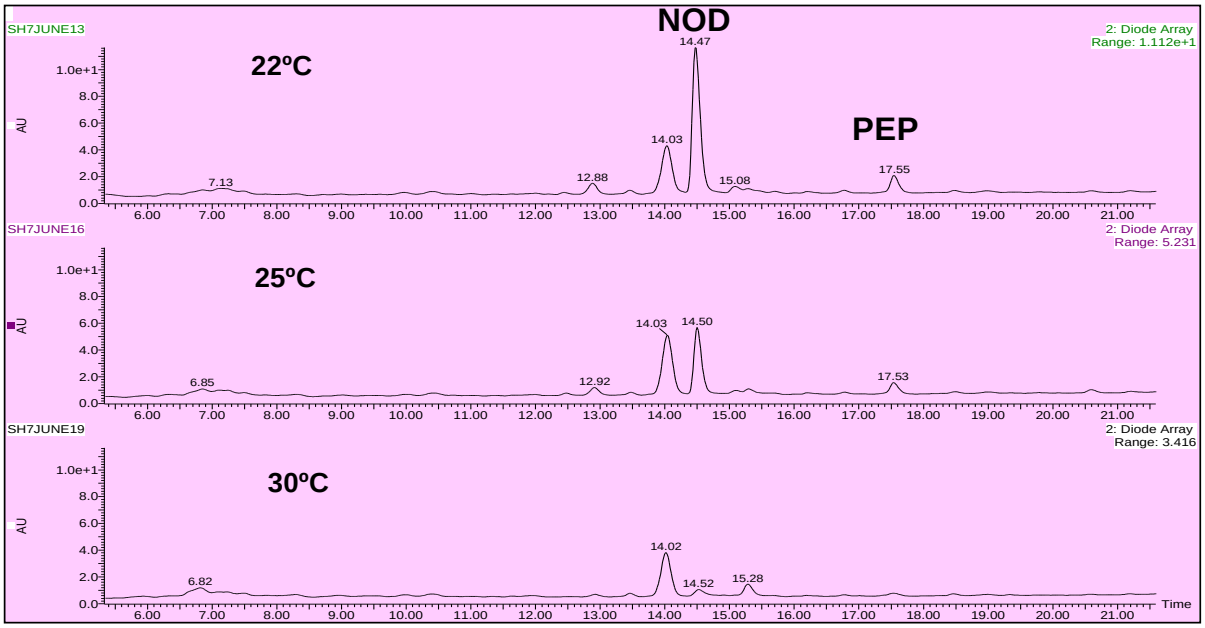

Figure 3.16. The chromatograms show the effects of different temperatures on the production of intracellular NOD and nodulopeptin 901 for *N. spumigena* KAC 66 grown for 6 weeks.

No extracellular NOD was observed regardless of the growth temperature. In the case of the typical culturing temperature (22°C) no traces or undetectable amount of extracellular NOD were recorded (the same was also observed in growth experiment performed in 10 L glass

flasks). The production of intracellular NOD levels at 22°C was low during the week 2 and 3 (472-851 ng/mL), after which the strain grew best in week 4 and produced maximum amount of intracellular NOD (1,061 ng/mL), while the maximum values for 25°C (473 ng/mL) and 30°C (46.7 ng/mL) were observed at week 3. At 30°C from week 4 to 6 a complete disappearance of intracellular NOD contents was noted (Appendix 44).

The lowest extra and intracellular nodulopeptin 901 levels were noted from week 1 to 3 at all temperatures, as time progressed. The nodulopeptin 901 levels were increased in the surrounding medium and within the cells. Likewise, it was noted that 22°C enhanced the production of nodulopeptin 901 in cultures both in extra and intracellular nodulopeptin 901 concentrations. At 25ºC at week 5 the highest amounts of extra (347 ng/mL) and intracellular (488 ng/mL) nodulopeptin 901 were observed (Appendix 45).

In general, at elevated temperature (25ºC and 30ºC) lower NOD was recorded as compared to 22ºC. A considerable decrease in the production of nodulopeptin 901 was noted at high temperature (30°C) in both extra and intracellular levels (Appendix 43). During the experiment no NOD was released in growth medium (Table 3.13). 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 22ºC (84%) and 25ºC (70%), while the highest percentage was noted in week 2 at 30ºC (55%). In week 1 the elevated percentages were observed at 22, 25 and 30ºC (45-58%).

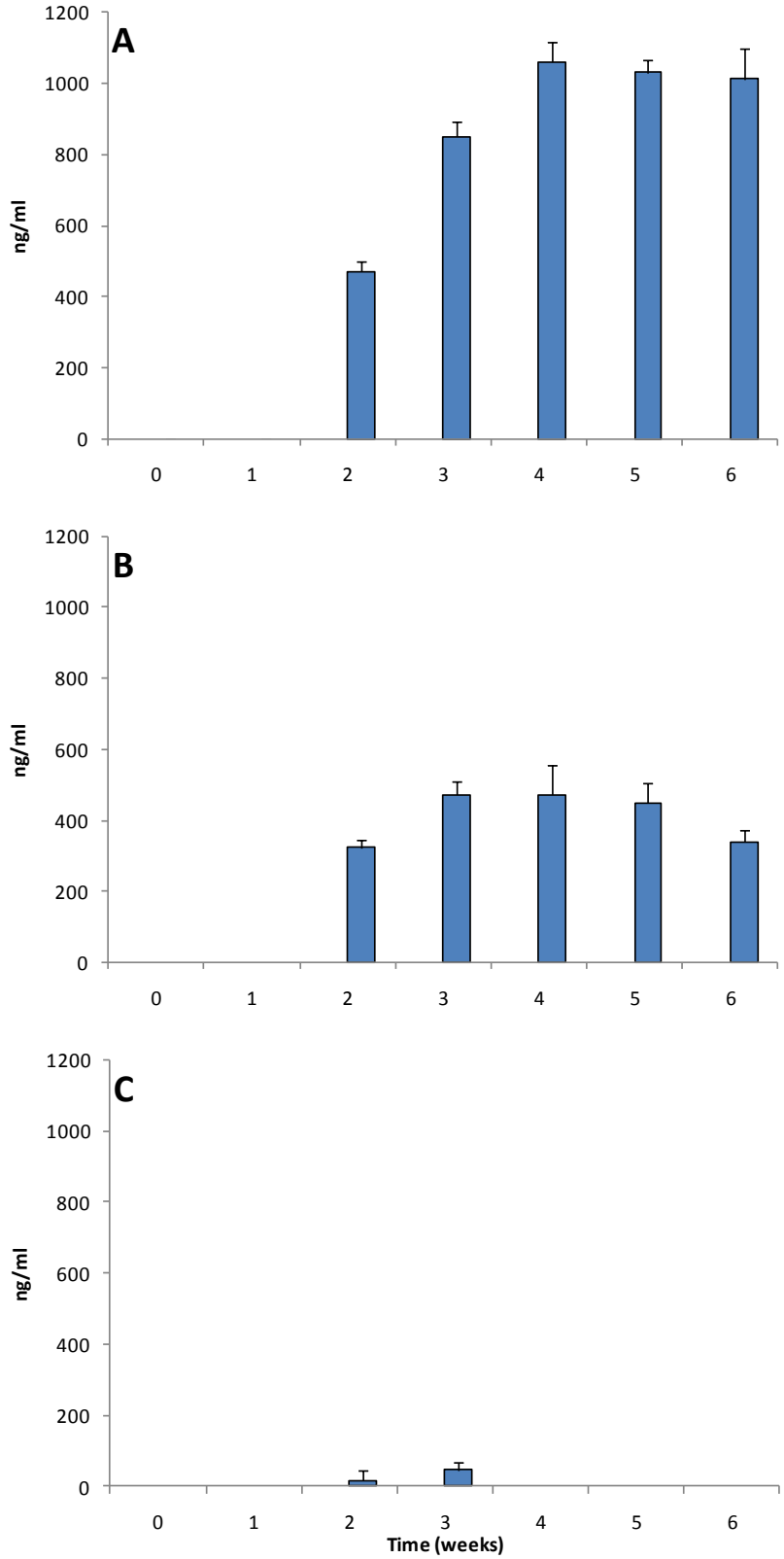

Figure 3.17. The Intra and extracellular levels of NOD at different temperatures for cultures of *N. spumigena* KAC 66 grown for 6 weeks (n=3, bars=1 SD). **A**:- 22°C **B**:- 25°C and **C**:- 30°C. (Extracellular □ and intracellular ■)

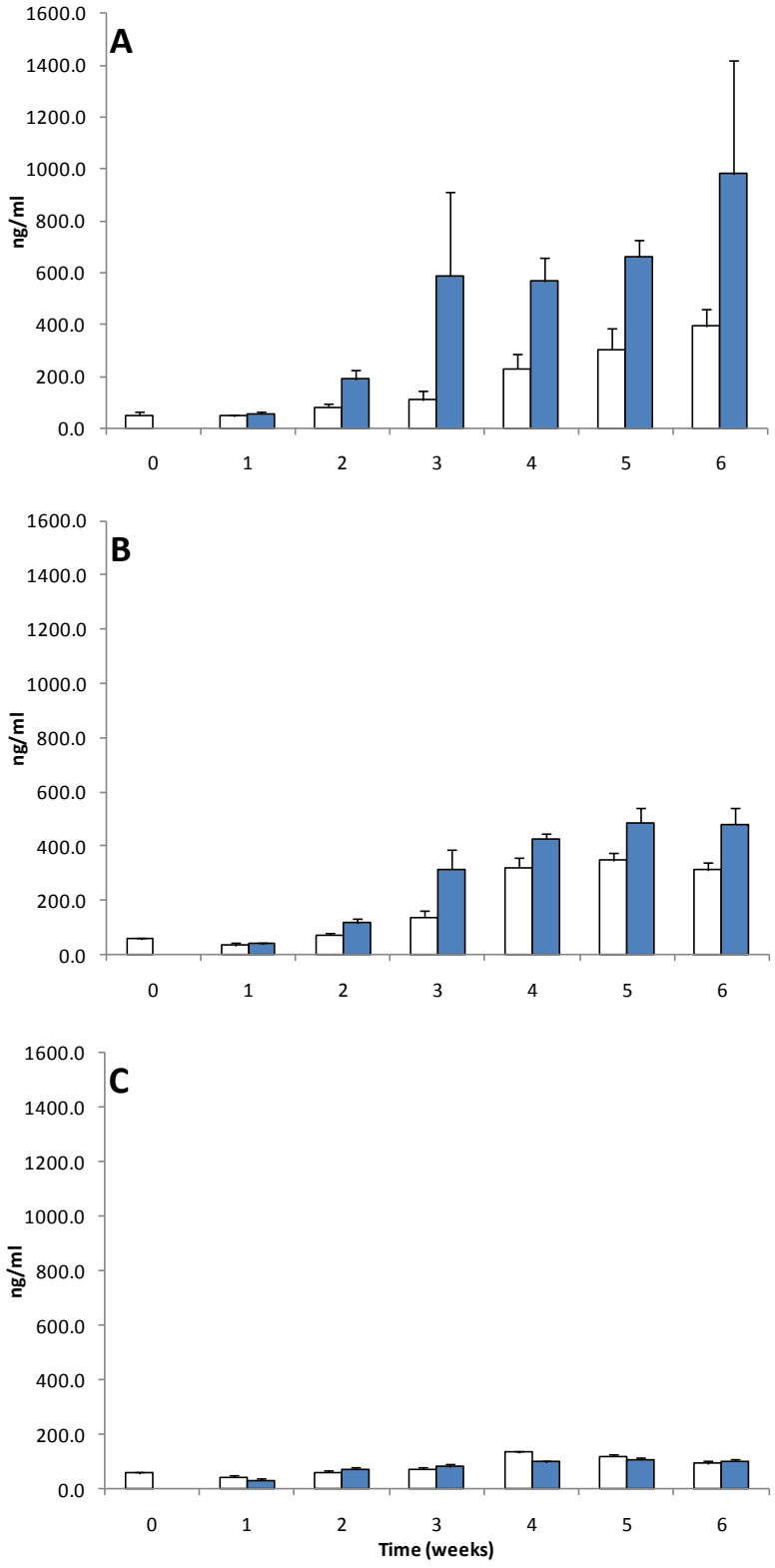

Figure 3.18. The Intra and extracellular levels of nodulopeptin 901 at different temperatures for cultures of *N. spumigena* KAC 66 grown for 6 weeks (n=3, bars=1 SD). **A**:- 22°C, **B**:- 25°C and **C**:- 30°C. (Extracellular ☐ and intracellular ■)

Table 3.13. Percentages of extra and intracellular NOD and nodulopeptin 901 levels for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different temperatures (n.d= not detected, the data is based on mean values of Appendices 44 and 45).

Time (weeks)	Temperature conditions (°C)	NOD		Nodulopeptin 901	
		Extracellular (%)	Intracellular (%)	Extracellular (%)	Intracellular (%)
T0		n.d	n.d	100	n.d
T1		n.d	n.d	45	55
T2		n.d	100	30	70
T3	**22**	n.d	100	16	84
T4		n.d	100	29	71
T5		n.d	100	31	69
T6		n.d	100	29	71
T0		n.d	n.d	100	n.d
T1		n.d	n.d	47	53
T2		n.d	100	39	61
T3	**25**	n.d	100	30	70
T4		n.d	100	43	57
T5		n.d	100	42	58
T6		n.d	100	39	61
T0		n.d	n.d	100	n.d
T1		n.d	n.d	58	42
T2		n.d	100	45	55
T3	**30**	n.d	100	47	53
T4		n.d	100	57	43
T5		n.d	n.d	52	48
T6		n.d	n.d	48	52

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3.3.5.2. Salinity

3.3.5.2.a. Cell biomass and chlorophyll-*a*

The relationship between cell biomass and Chl-*a* at different salinities is presented in Fig. 3.19 (Appendices 46 and 47). The concentration of intra and extracellular peptides are shown Appendices 48 and 49.

In general, no correlation between cell biomass and Chl-*a* concentrations was observed. Except 20 ‰ all salinities decreased the production of

Chl-*a* of treated cultures, as time progressed. At all salinities, Chl-*a* concentrations started increasing from weeks 1 to 4, and then declined by week 5 and 6. The results show that the concentrations of Chl-*a* at 2, 11, 20 and 25 ‰ was decreased during the growth period except at 7 ‰.

At 2, 11 and 20 ‰ cell biomass was 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 at 2, 7, 11 and 20 ‰, ranged from 1,207-1,740 µg/mL. The elevated salinity (25 ‰) suppressed the growth of strain and the highest cell biomass (973 µg/mL) was noted in week 3, which was lowest cell biomass among all salinities. It seems that variations in salinities have substantial effect on cell biomass and Chl-*a* concentrations.

3.3.5.2.b. Extra and intracellular peptide levels

The effect of each condition had on NOD production is represented in Fig. 3.20. 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 maximum level of NOD from week 3 to week 5. Increasing salinity from 2 to 11 ‰ was found to have a pronounced effect on total amount of intracellular NOD production, but the highest amount was found at 2 ‰ in weeks 4 and 5, ranged from 1,511-1,666 ng/mL. In weeks 1 and 2 a relatively low amount of intracellular NOD were observed in all tested cultures, while lowest detectable amount (205 ng/mL) 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 ‰; Appendix 50). The results of effects of salinity on the production of intra and extracellular nodulopeptin 901 levels by *N. spumigena* are shown in Fig. 3.21A-C. During the whole course of experiment all salinities tested had substantial effect on the production of intra and extracellular nodulopeptin 901.

At 25 ‰ the intracellular nodulopeptin 901 concentrations were found maximum in week 5 (447 ng/mL) and declined in week 6 (344 ng/mL; Appendix 51). In case of intra and extracellular nodulopeptin 901 concentrations all salinities supported the production of toxins.

Due to low concentration of cells in newly inoculated cultures no traces or undetectable intracellular peptides were observed and 100 % peptides found in the surrounding medium, which maybe already present in stock culture. During the experiment at different salinities it was noted that from week 1 to 6 NOD (89-100 %) and from week 2-6 nodulopeptin 901 were predominantly (50-68 %) intracellularly. In week 2 at elevated salt concentration (25‰) an equilibrium between intra and extracellular nodulopeptin 901 (50:50%) was observed (Table 3.14).

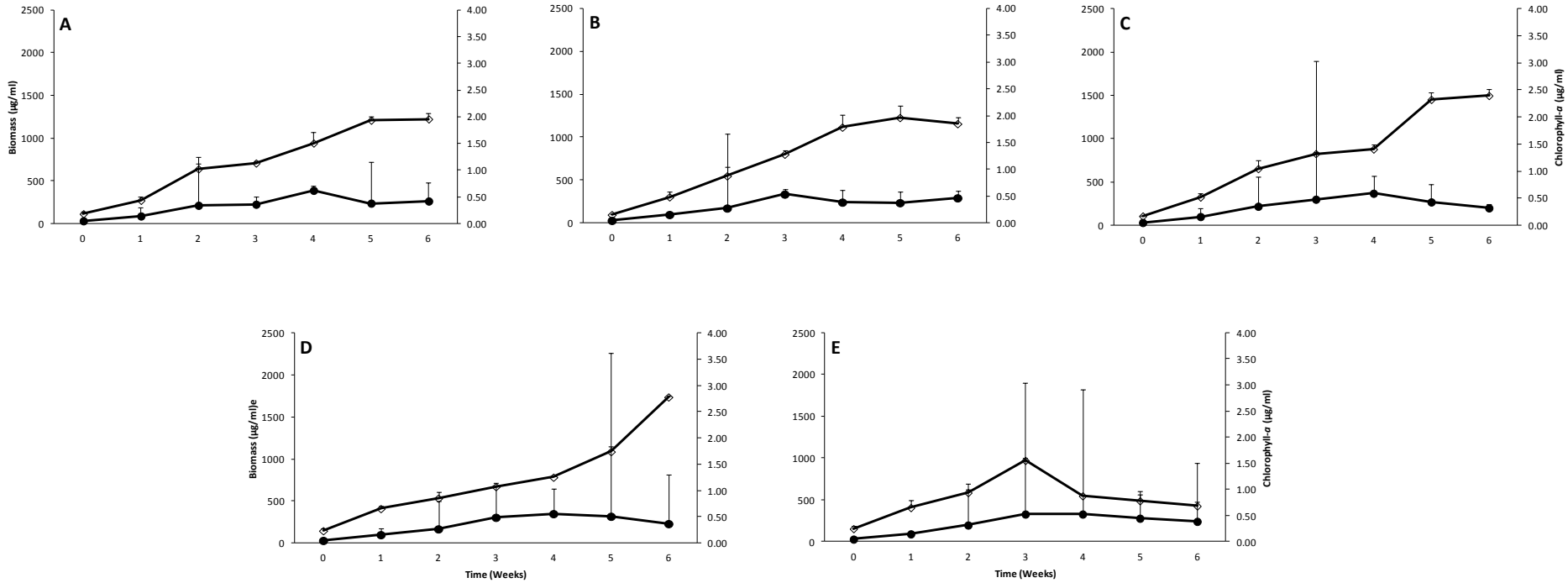

Figure 3.19. Chl-*a* concentrations and biomass for cultures of *N. spumigena* KAC 66, grown at different salinities for 6 weeks at 22°C. (n=3, bars=1 SD). **A**:- 2 ‰, **B**:- 7‰, **C**:- 11 ‰, **D**:- 20 ‰ and **E**:- 25 ‰.
(Chlorophyll-*a* ●, cell biomass ◇)

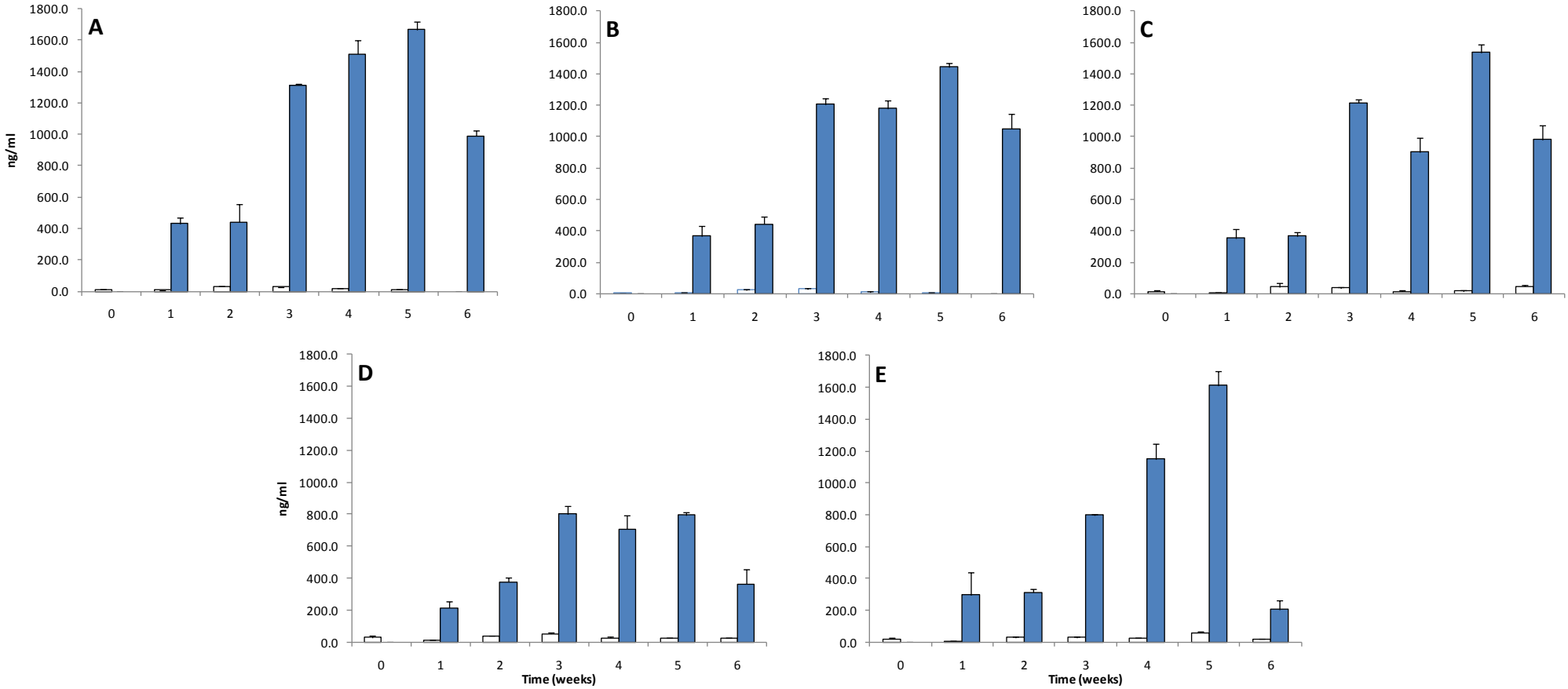

Figure 3.20. The Intra and extracellular levels of NOD at different salinities for cultures of *N. spumigena* KAC 66 grown for 6 weeks at 22 °C (n=3, bars=1 SD). **A**:- 2 ‰, **B**:- 7 ‰, **C**:- 11 ‰, **D**:- 20 ‰ and **E**:- 25 ‰ (Extracellular□ , intracellular■).

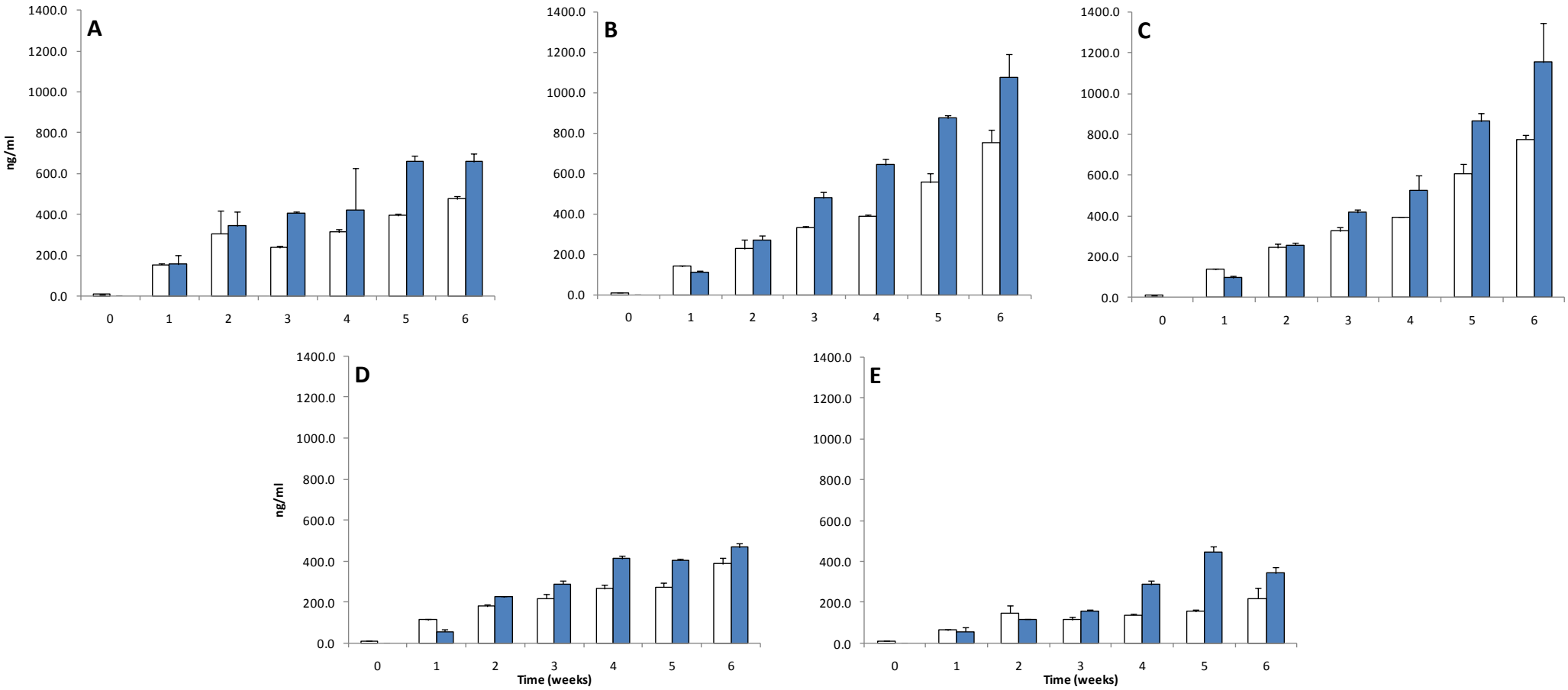

Figure 3.21. The Intra and extracellular levels of nodulopeptin 901 at different salinities for cultures of *N. spumigena* KAC 66 grown for 6 weeks at 22 °C (n=3, bars=1 SD). **A**:- 2 ‰, **B**:- 7‰, **C**:- 11 ‰, **D**:- 20 ‰ and **E**:- 25 ‰ (Extracellular □ , intracellular ■).

Table 3.14. Percentages of NOD and nodulopeptin 901 in extra and intracellular levels for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different salinities (n.d= not detected, the data is based on mean values of Appendices 50 and 51).

Time (weeks)	Salintiy conditions (‰)	NOD		Nodulopeptin 901	
		Extracellular (%)	Intracellular (%)	Extracellular (%)	Intracellular (%)
T0		100	n.d	100	n.d
T1		2	98	50	50
T2		6	94	47	53
T3	**2**	2	98	37	63
T4		1	99	37	63
T5		1	99	37	63
T6		n.d	100	42	58
T0		100	n.d	100	n.d
T1		2	97	56	44
T2		5	95	46	54
T3	**7**	3	97	41	59
T4		1	99	38	62
T5		1	99	39	61
T6		n.d	100	41	59
T0		100	n.d	100	n.d
T1		3	98	59	41
T2		11	89	49	51
T3	**11**	3	97	44	56
T4		2	98	43	57
T5		1	99	41	59
T6		4	96	40	60
T0		100	n.d	100	n.d
T1		5	95	68	32
T2		9	91	44	56
T3	**20**	6	94	43	57
T4		4	96	39	61
T5		3	97	40	60
T6		6	94	45	55
T0		100	n.d	100	n.d
T1		2	98	53	47
T2		8	92	50	50
T3	**25**	4	96	42	58
T4		2	98	32	68
T5		4	96	26	74
T6		9	91	39	61

3.3.5.3. Nitrate

3.3.5.3.a. Cell biomass and chlorophyll-*a*

At all nitrate concentrations a linear relationship was observed between cell biomass and Chl-*a* contents*,* as time passed the cell biomass and Chl-*a* also increased (Fig. 3.22; Appendices 52 and 53). The results indicated that the *N. spumigena* can easily grow both in the absence and at high concentrations of nitrate. Combining the observations an increased biomass (183.3 to 1068.3 µg/mL) production was noted at $NaNO_3$ free experiment.

At all concentration the maximum cell biomass was observed in week 5 ranged from 1,068.3-2,223.3 µg/mL. At 7.5 mg/L less cell biomass (1,160.7 µg/mL) was recorded compared to 6.5, 8.5 and 9.5 mg/L (1,826.7, 1973.3 and 2,223.3 µg/mL, respectively). It showed that a rise in nitrate concentration increased the cell biomass production.

At all nitrate concentrations, Chl-*a* showed a linear increase versus progressed time (Fig. 3.30). Throughout the experiment, in nitrate free medium the Chl-*a* concentrations were low (0.19 µg/mL) compared with other concentrations (Fig. 3.22). In week 5 at 3.5 mg/L 7.5 mg/L, approximately the highest Chl-*a* concentrations (0.27 and 30.0 µg/mL, respectively) were recorded (Figs. 3.30B and D). In week 5 the elevated concentrations of nitrate (6.5, 8.5 and 9.5 gm/L) showed the highest values of Chl-*a* 0.48, 0.44 and 0.39 µg/mL, respectively (Figs. 3.22C, E and F).

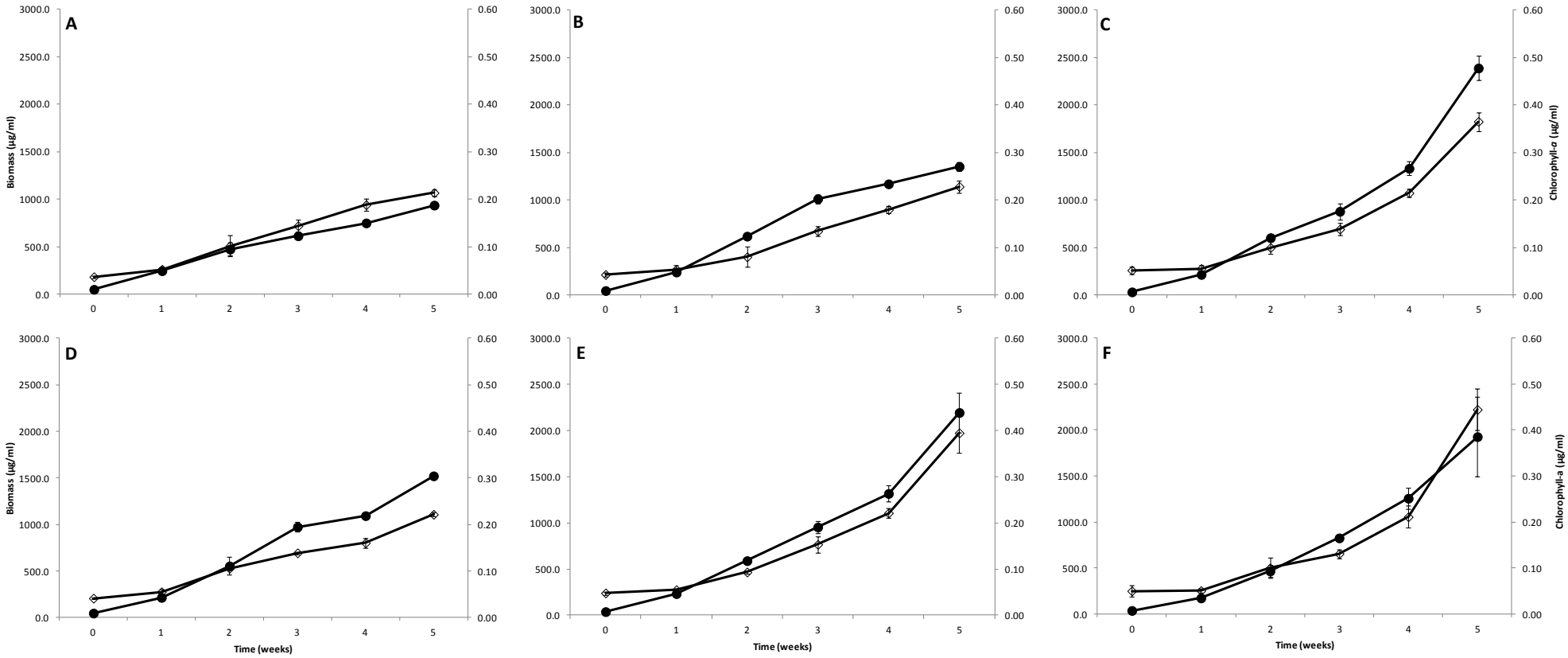

Figure 3.22. Chl-*a* concentrations and cell biomass for cultures of *N. spumigena* KAC 66, grown at different concentrations of nitrate for 5 weeks at 22°C. (n=3, bars=1 SD). **A**:- 0, **B**:- 3.5, **C**:- 6.5, **D**:- 7.5, **E**:- 8.5 and **F**:- 9.5 mg/L. (Chlorophyll-*a* ●, biomass ◇)

Analyzing nitrate concentration and time together showed that the 6.5 mg/L favoured the high biomass and (1826.7 µg/mL) Chl-*a* concentrations (0.48 µg/mL) in week 5.

3.3.5.3.b. Extra and intracellular peptide levels

At all concentrations of nitrate the levels of intracellular NOD and nodulopeptin 901 showed an increase from week 1 to week 5 (Fig. 3.23; Appendices 54 and 56). In contrast to the results at nitrate free cultures (0 mg/L; 159.2-561.4 ng/mL and at high nitrate level (9.5 mg/L) the lowest amount (157-712.2 ng/mL) of intracellular NOD was recorded (Figs. 3.23A and F). At 6.5 mg/L the highest amount (1,833.5 ng/mL) of intracellular NOD was observed by week 4, which decreased in week 5 (985.0 ng/mL; Fig. 3.23C). In comparison, at all other nitrate concentrations the week 3 and 4 supported the highest amount of intracellular NOD, which gradually decreased by week 5 (Fig. 3.23). In nitrate free cultures, the extracellular NOD was higher in week 1 (7.4 ng/mL), which gradually decreased till week 3 (2.9 ng/mL) and increased again in week 4 (3.2 ng/mL). In week 5 no traces or an undetectable amount of extracellular NOD was observed (Fig. 3.23A; Appendix 55).

The media containing 3.5 and 6.5 mg/L nitrate showed similar pattern of extracellular NOD production, as time passed the amount of NOD increased ranged from 6.6-9.3 ng/mL from week 1-3 (Figs. 3.23B and C). This amount was found to be high (16.7 ng/mL) in week 2 at 6.5 mg/L nitrate level (Fig. 3.23D). The amount of extracellular NOD at 9.5 mg/L was minimum in week 1 and 3, 8.2 and 8.3 ng/mL, respectively. This amount was maximum (11.1 ng/mL) in week 2 (Fig. 3.23F). At all nitrate

conditions, except in nitrate free cultures, no traces of extracellular NOD were observed in week 4 and 5.

Nodulopeptin 901 was found intra and intracellularly and intracellularly in all nitrate conditions (0-9.5 mg/L; Fig. 3.24), which increasing as time progressed.

The observations indicated that at all nitrate concentrations the higher intracellular nodulopeptide 901 levels were recorded in week 4 (ranged from 559.3-1,231.8 ng/mL), except at 7.5 mg/L (555.3 ng/mL). Analyzing all combinations of nitrate concentrations, nitrate free cultures and 6.5 mg/L leaded to a higher intracellular nodulopeptin 901 concentration 1039.6 and 1231.8 ng/mL, respectively in week 4 (Figs. 3.24A and C).

Combining the observations of increased extracellular nodulopeptin 901 production in all nitrate conditions, showed an exponential increase from week 1 to week 4, with a decrease in week 5 (Fig. 3.24). The extracellular nodulopeptin 901 concentrations in nitrate free medium (72.5-359.7 ng/mL) and at 7.5 mg/L (42.6-133.7 ng/mL), showed same trend but less amount was recorded in 7.5 mg/L (Figs. 3.32A and D). At 3.5 mg/L (130.2 ng/mL) and 6.5 mg/L (126.7 ng/mL) nitrate conditions a slight decrease in extracellular nodulopeptin 901 was recorded in week 3 (Figs. 3.24B and C). The nitrate free medium supported the highest amount of extracellular nodulopeptin (359.7 ng/mL) in week 4 (Fig. 3.24A).

It is suggested that the highest amount of intracellular NOD (1,833.5 ng/mL) and nodulopeptin 901 (1,231.8 ng/mL) can be obtained at 6.5-7.5 mg/L of nitrate in week 4 (Appendix 57).

At all concentrations, at the day of inoculation and in week 5 no traces or undetectable amount of extracellular NOD was observed and 100% NOD was retained within the cells (Table 3.15). During the whole experiment period low percentage of extracellular NOD was recorded from week 1 to week 4 ranged from 1-5%. Between 3-29% of total extracellular nodulopeptide 901 was found under all conditions. The percentage composition of nodulopeptin 901 represented that 71-97% retained intracellularly (Table 3.15).

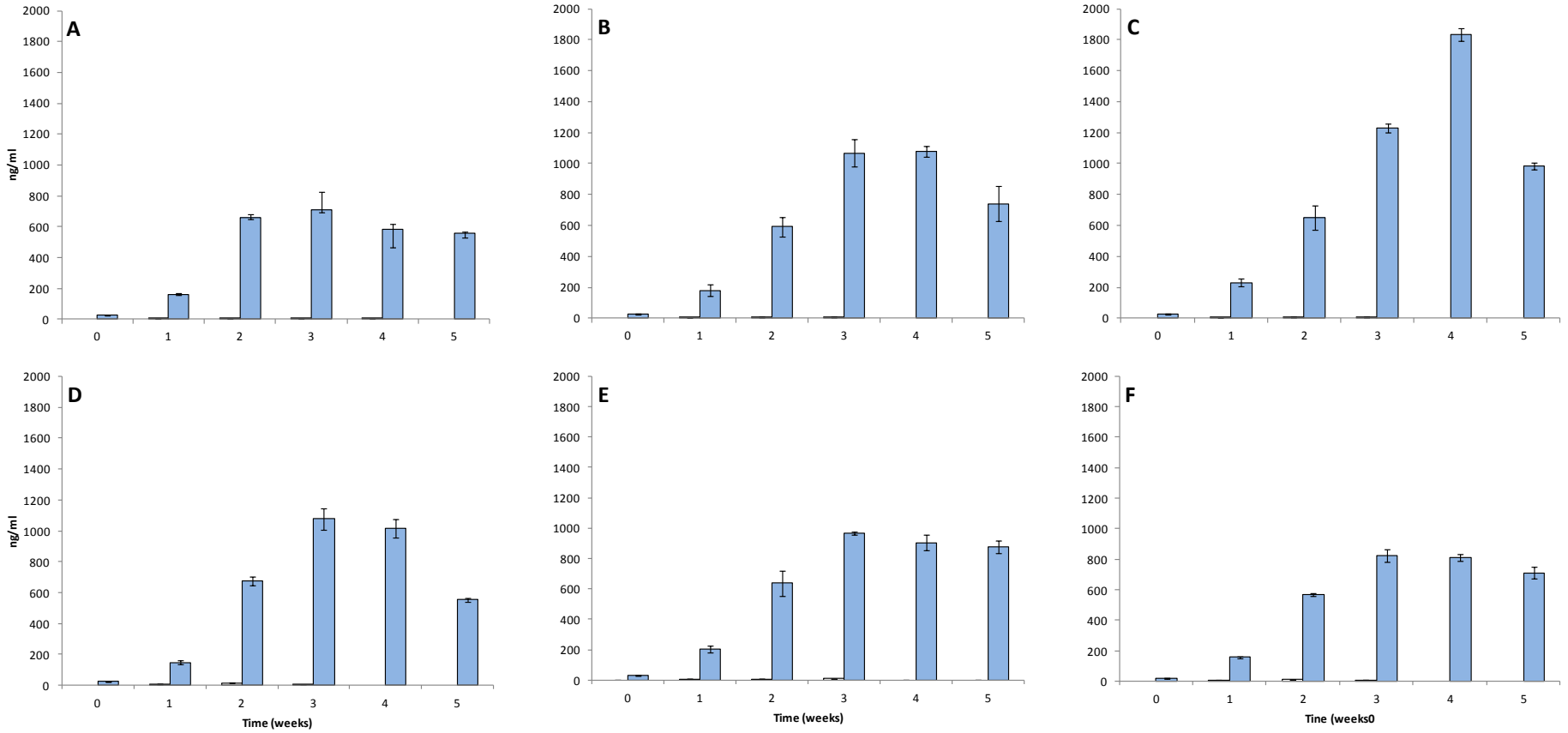

Figure 3.23. The Intra and extracellular levels of NOD at different concentrations of nitrate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 °C (n=3, bars=1 SD). **A**:- 0, **B**:- 3.5, **C**:- 6.5, **D**:- 7.5, **E**:- 8.5 and **F**:- 9.5 mg/L (Extracellular □ , intracellular ■)

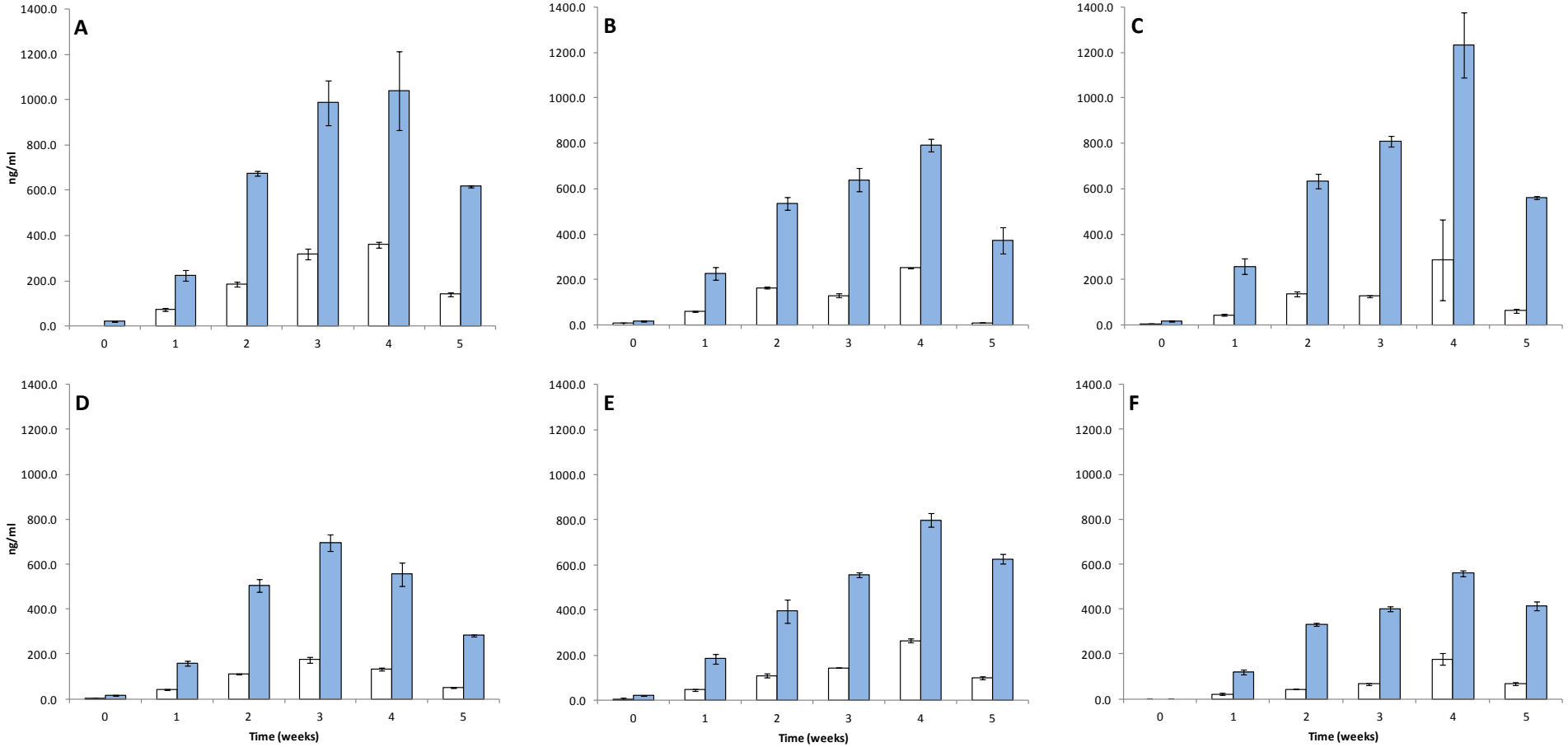

Figure. 3.24. The Intra and extracellular levels of nodulopeptin 901 at different concentrations of nitrate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 °C (n=3, bars=1 SD). **A**:- 0, **B**:- 3.5, **C**:- 6.5, **D**:- 7.5, **E**:- 8.5 and **F**:- 9.5 mg/L (Extracellular □, intracellular ■).

Table 3.15. Percentages of NOD and nodulopeptin 901 in extra and intracellular levels for cultures of *N. spumigena* KAC 66 grown for 5 weeks at different nitrate concentrations of nitrate (n.d= not detected, the data is based on mean values of Appendices 55 and 56).

Time (weeks)	Nitrate conditions (mg/L)	NOD		Nodulopeptin 901	
		Extracellular (%)	Intracellular (%)	Extracellular (%)	Intracellular (%)
T0	**0**	n.d.	100	n.d.	100
T1		4	96	24	76
T2		1	99	22	78
T3		0	100	24	76
T4		1	99	26	74
T5		n.d.	100	19	81
T0	**3.5**	n.d.	100	29	71
T1		4	96	21	79
T2		2	98	23	77
T3		1	99	17	83
T4		n.d.	100	24	76
T5		n.d.	100	3	97
T0	**6.5**	n.d.	100	21	79
T1		3	97	15	85
T2		1	99	18	82
T3		1	99	14	86
T4		n.d.	100	19	81
T5		n.d.	100	10	90
T0	**7.5**	n.d.	100	17	83
T1		5	95	21	79
T2		2	98	18	82
T3		0	100	20	80
T4		n.d.	100	19	81
T5		n.d.	100	15	85
T0	**8.5**	n.d.	100	24	76
T1		4	96	20	80
T2		1	99	22	78
T3		1	99	20	80
T4		n.d.	100	25	75
T5		n.d.	100	14	86
T0	**9.5**	n.d.	100	n.d.	n.d.
T1		5	95	16	84
T2		2	98	12	88
T3		1	99	14	86
T4		n.d.	100	24	76
T5		n.d.	100	14	86

3.3.5.4. Phosphate

3.3.5.4.a. Cell biomass and chlorophyll-*a*

As discussed previously, a number of phosphate concentrations were used to determine their effects on biomass and toxin productions. Figure 3.25 represents a correlation among Chl-*a*, cell biomass and growth time.

At 0, 0,1, 40, 70 and 100 mg/L phosphate concentrations a similar trend was observed, following an[ui896 initial increase, later samples of cell biomass decreased (Figs. 3.25A, B, D, E and F; Appendix 58). However, in contrast at 10 and 120 mg/L the amount of cell biomass was increase throughout the growth period (Figs. 3.25C and G). In PO_4^{-3} free cultures the lowest cell biomass values were recorded ranged from 200-470 µg/mL (Fig. 3.25A) and 120 mg/L supported the maximum cell (275.0-931.7 µg/mL) in week 5 (Fig. 3.25G). The combination of observations showed that Chl-*a* represented similar pattern as normal growth curve showed (Fig. 3.25).

In phosphate free cultures death phase occurred around in week 4 and 5 amount (0.003 µg/mL) Chl-*a* recorded (Fig. 3.25A). None the less of the relatively low phosphate level at 10 mg/L the highest Chl-*a* concentration was observed in week 2 followed by week 3 (0.18 µg/mL; Fig. 3.25C). Except for 70 and 120 mg/L, at all other PO_4^{-3} conditions, the maximum Chl-*a* levels were recorded in week 2 ranged from 0.12-0.18 µg/mL (Appendix 59).

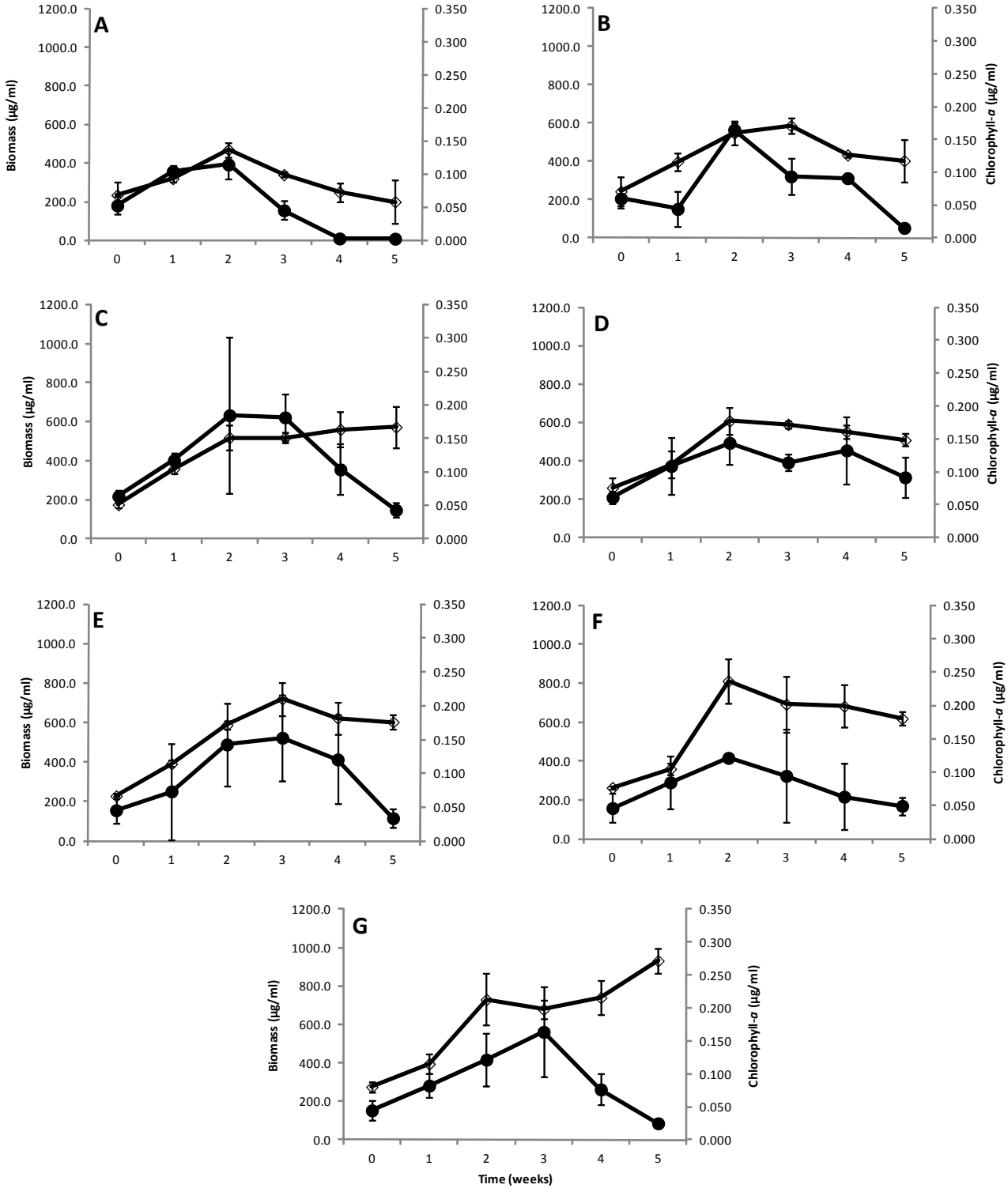

Figure 3.25. Chl-*a* concentrations and biomass for cultures of *N. spumigena* KAC 66, grown at different concentrations of phosphate for 5 weeks at 22°C. (n=3, bars=1 SD). **A**:- 0, **B**:- 0.1, **C**:- 10 , **D**:- 40, **E**:- 70, **F**:- 100 and **G**:- 120 mg/ L (Chlorophyll-a ●, cell biomass ◇)

3.3.5.4.b. Extra and intracellular peptide levels

At all concentrations, the highest amount of NOD was always located intracellularly (Fig. 3.2.6; Appendices 60 and 61). The intracellular NOD concentration in phosphate deficient medium, was relatively low in week 4 and 5 (23.5-28.3 ng/mL; Fig. 3.26A). In all experimental conditions in week 2 the intracellular NOD concentrations were found to peak (1,829.2-2,469.3 ng/mL; Fig. 3.26), which then gradually decreased untll week 5. Combining the observation of increased extracellular NOD production at medium containing high amount of phosphate from 40 mg/L to highest elevated concentration of phosphate (120 mg/L), increased extracellular NOD levels were recorded (2,274.9- 2,469.3 ng/mL; Figs. 3.26D-G).

The relative proportion of extracellular NOD was very low compared to concentrations of NOD within the cell. At 0 mg/L, 10 mg/L and the highest (120 mg/L) phosphate conditions, the concentration of this extracellular NOD was lowest in last three weeks ranged from 1.3-5.2 ng/mL (Figs. 3.26AC and G). In phosphate free cultures an increase (23.5 ng/mL) of extracellular NOD was observed till week 3 followed by a decrease in week 4 (3.1 ng/mL) and 5 (3.3 ng/mL; Fig. 3.26A). In 40 mg/L this peptide started decrease in week 3 and 4 with a slight increase in week 5 (Fig. 3.34D). At 100 mg/L 28.3 ng/mL (Fig. 2.34A) *N. spumigena* released the highest amount of NOD in surrounding medium (Fig. 3.26F; Appendix 61), although levels were still relatively low.

Comparing all conditions of phosphate levels of intracellular nodulopeptin 901 was almost the same regardless of phosphate concentrations upto

week 2, followed by a decrease (Fig. 3.27; Appendix 62). At all concentrations an elevated level of this peptide was noted in week 2 ranged from 303.5-502.4 ng/mL, with a slight increase in week 5 at 40 mg/L (173.8 ng/mL; Fig. 3.27D).

The levels of this extracellular peptide showed the same trend and decreased as time passed. In general week 3 and 4 supported the maximum release of nodulopeptin 901 in growth medium.

In phosphate free condition was nodulopeptin 901 was highest (156.5 ng/mL) in week 3 (Fig. 3.27A). Forty mg/L supported an increase in extracellular nodulopeptin 901 as time passed (Fig. 3.27D). At 0, 70, 100 and 120 mg/L phosphate conditions the cultures demonstrated the same pattern with an increase in 3 and 4 (86.9-114.1 ng/mL; Figs. 3.27C, E, F and G; Appendix 63).

Between 96-100% of total NOD was found intracellularly under all conditions and it was not release in surrounding medium (Table 3.16). Extracellular nodulopeptin 901 released in surrounding medium (11-49%) at all phosphate conditions and much amount retained (51-89%) within the cells. At 0, 0.1 and 70 mg/L conditions an equilibrium were observed between extra and intracellular nodulopeptin 901 concentrations in week 4.

The highest values of cell biomass, Chl-*a* and peptide production during environmental factors experiments are summarized in Table 3.17.

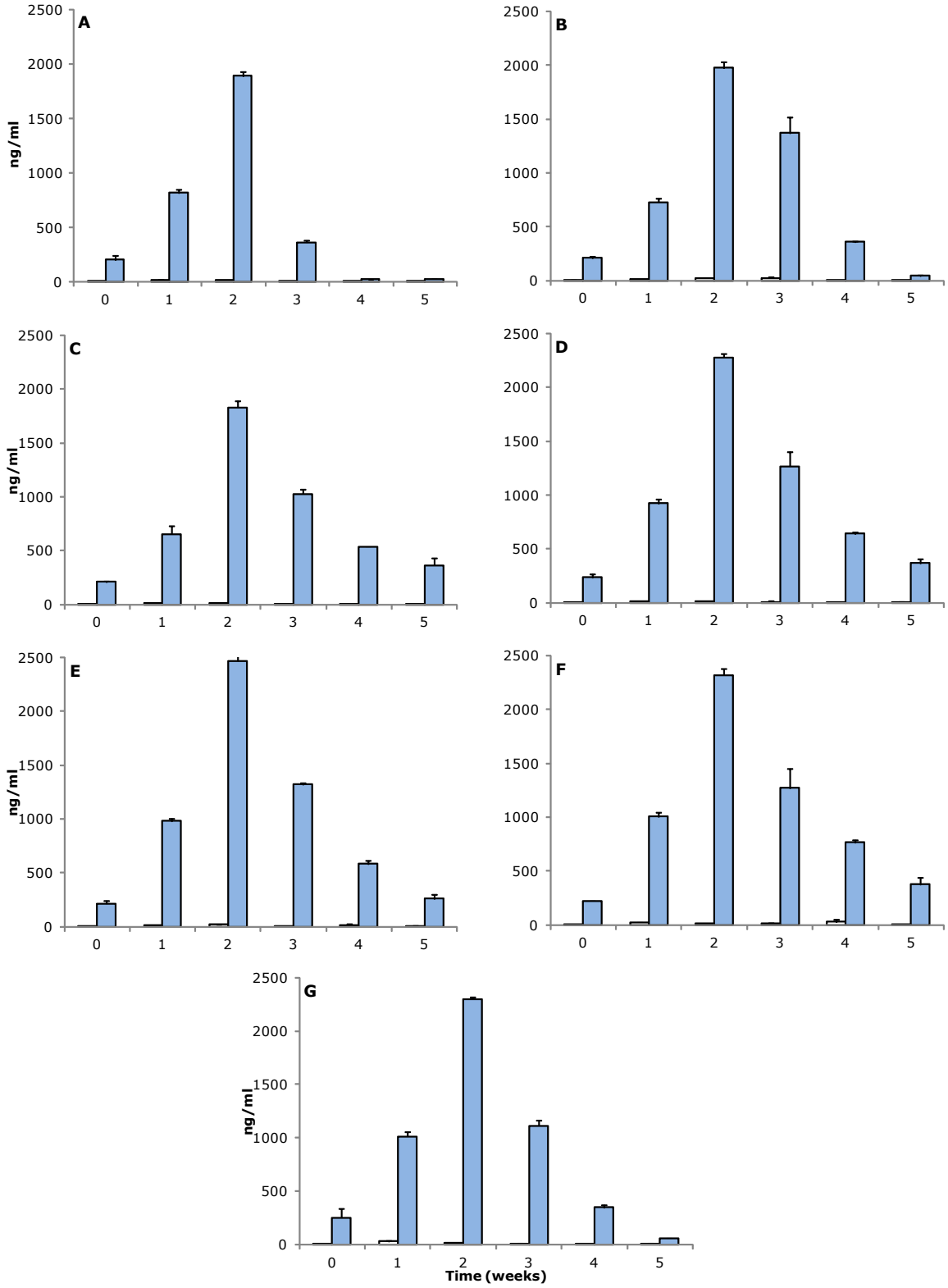

Figure 3.26. The intra and extracellular levels of NOD at different concentrations of phosphate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 °C (n=3, bars=1 SD). **A**:- 0, **B**:- 0.1, **C**:- 10, **D**:- 40, **E**:- 70 **F**:- 100 and **G**:- 120 mg/L (Extracellular□ , intracellular ■).

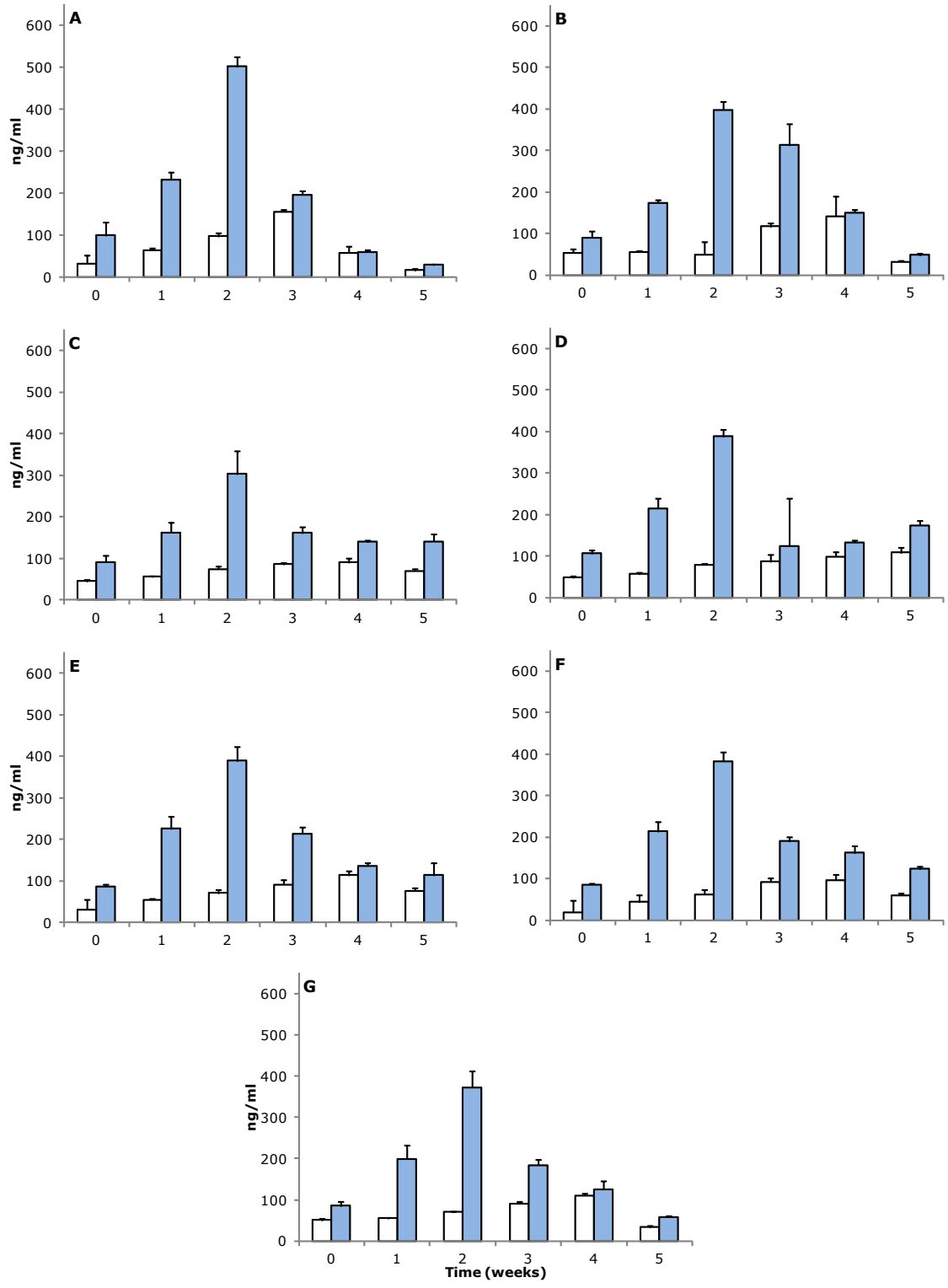

Figure 3.27. The intra and extracellular levels of nodulopeptin 901 at different concentrations of phosphate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 °C (n=3, bars=1 SD). **A**:- 0, **B**:- 0.1, **C**:- 10, **D**:- 40, **E**:- 70, **F**:- 100 and **G**:- 120 mg/L (Extracellular □, intracellular ■).

Table 3.16. Percentages of NOD and nodulopeptin 901 in extra and intracellular levels for cultures of *N. spumigena* KAC 66 grown for 5 weeks at different phosphate concentrations of nitrate (n.d= not detected, the data is based on mean values of Appendices 61 and 63).

Time (weeks)	Phosphate conditions (mg/l)	NOD		Nodulopeptin 901	
		Extracellular (%)	Intracellular (%)	Extracellular (%)	Intracellular (%)
T0	**0**	3	97	25	75
T1		2	98	22	78
T2		1	99	16	84
T3		1	99	44	56
T4		6	94	49	51
T5		13	87	36	64
T0	**0.1**	2	98	37	63
T1		2	98	24	76
T2		1	99	11	89
T3		2	98	27	73
T4		1	99	48	52
T5		3	97	40	60
T0	**10**	3	97	33	67
T1		2	98	26	74
T2		1	99	20	80
T3		0	100	35	65
T4		0	100	40	60
T5		0	100	33	67
T0	**40**	3	97	31	69
T1		2	98	21	79
T2		8	92	17	83
T3		1	99	41	59
T4		0	100	42	58
T5		1	99	39	61
T0	**70**	3	97	27	73
T1		2	98	20	80
T2		1	99	15	85
T3		0	100	30	70
T4		2	98	45	55
T5		1	99	40	60
T0	**100**	3	97	19	81
T1		2	98	17	83
T2		1	99	14	86
T3		1	99	33	67
T4		4	96	37	63
T5		0	199	33	67
T0	**120**	4	96	37	63
T1		3	97	22	78
T2		1	99	16	84
T3		0	100	33	67
T4		4	96	47	53
T5		8	92	37	63

Table 3.17. A summary of the highest values of cell biomass, Chl-*a*, and peptides obtained during effects of environmental factors on the growth of *N. spumigena* KAC 66.

Measured parameters	Environmental factors																					
	Temperature (°C)						Salinity (‰)						Nitrate (mg/mL)					Phosphate (mg/mL)				
	weeks						weeks						weeks					weeks				
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	1	2	3	4	5
Cell biomass	-	-	-	-	-	-	-	-	-	-	-	20	-	-	-	-	9.5	-	-	-	-	120
Chl-*a*	-	-	-	30	30	-	-	-	-	0	-	-	-	-	-	-	6.5, 8.5, 9.5	-	10	10, 120		-
Intracellular NOD	-	-	-	22	22	-	-	-	-	-	0	-	-	-	-	6.5	-	-	70, 100, 120	-	-	-
Extracellular NOD	-	-	-	-	-	-	-	-	-	-	25	-	-	7.5	-	-	-	120	-	-	100	-
Intracellular nodulopeptin 901	-	-	-	-	-	22	-	-	-	-	-	11	-	-	-	6.5	-	-	0	-	-	-
Extracellular nodulopeptin 901	-	-	-	-	-	22	-	-	-	-	-	11	-	-	-	0	-	-	-	-	-	0

3.4. DISCUSSION

This chapter emphasized the effects of various environmental factors on the growth and toxin production by *N. spumigena* KAC 66.

3.4.1. Growth medium BG-11 and maintenance of *N. spumigena* KAC 66

In this study BG-11 (20 ‰; Allen and Stanier, 1968) growth medium was used for culturing of *N. spumigena* KAC 66 to obtain good growth. Many studies have used different media and salinity for the culturing of *N. spumigena*, the choice depending on the meteorological conditions of their collection sites. Likewise, Sivonen *et al.,* (1989c) used Z8 medium with 7.5 ‰, which was identical to the salinity of the Southern Baltic Sea, while Carmichael *et al.,* (1988) used Tris-buffered bold basal medium containing 1% NaCl to maintain toxic *N. spumigena,* isolated from Lake Ellesmere Lake, New Zealand. Hobson and Fallowfield, (2003) used ASTM medium for culturing of *N. spumigena* 001E, Australia. All cultivated *N. spumigena* successfully but BG-11 is highly recommended growth media for the cultivation of study strains (Musial and Plinski, 2003).

Cyanobacteria generally have lower maximal growth rates compared to other algal species (Reynolds, 1984). Due to their slow growth rate cyanobacteria require a longer time period and extra nutrients to form a mass development (Mur *et al.,* 1999; Reynolds, 1984). In this study BG-11 (20 ‰) was used for the growth of *N. spumigena* KAC 66. Due to presence of Mg^{+}, Na^{+}, and Ca^{+} ions BG-11 provided favourable conditions for the studied strain (Oliver and Ganf, 2000). Without addition of nutrients and trace metals, the yield of cyanobacteria becomes too low

for laboratory experiments (Harrison and Berges, 2005). Trace metals also play an important role in the culturing of cyanobacteria and without addition of these, the growth rate decreases and the concentration of cyanobacteria becomes too low for laboratory experiments (Harrison and Berges, 2005). The effect of varying trace metal concentrations on the regulation of peptides has been investigated by several researcher groups. The effect of metal concentrations on toxin production by *Microcystis aeruginosa* was found not to have any effect on algal growth and toxin production Lukae *et al.,* (1993).

During the present study all stock cultures and experiments (except for temperature effect experiment) were performed at 22°C in a temperature controlled culture room. It was noted that 22°C is the best growth temperature for *N. spumigena* KAC 66. Some culture collection centres maintain their cultures at 20°C. Previous studies have indicated that most common planktonic cyanobacterial strains (Mur *et al.,* 1999) including *N. spumigena* (Musial and Plinski, 2003) grow best at around 20°C. Further studies by Robarts and Zohary (1987) showed that 25°C is a suitable temperature for many cyanobacterial strains to achieve their maximum growth rates, however a temperature higher than 22°C combined with a high light intensity can damage the cells and increase the evaporation rate of growth medium (Lorenz *et al.,* 2005).

3.4.2. Growth and peptide production in 10 L glass flasks

Cyanobacterial biomass and toxin yield also varies between strains and species, and commonly the highest toxin production rates are observed under optimum growth conditions. During the growth experiment

conducted in 10 L glass flasks the biomass and Chl-*a* did not show any clear relationship, while Chl-*a* contents showed a clear and normal growth pattern as time passed. It is suggested that to determine the biomass of strain the pigment estimation gives more reliable results as compared to cell biomass. The intracellular NOD followed the same pattern as Chl-*a.* During the study period intracellular NOD concentration was high from week 2 to 6, maybe as a result of high concentration of cells. In comparison extracellular NOD concentrations were lower and toxins were not released in the surrounding medium or may be they degraded by bacteria present in medium. This resulted in very low amount of extracellular NOD, which maybe due to no cell lyses. Berg *et al.,* (1987) indicated that cyanobacterial toxins remain inside the cell and are released into the surrounding medium only when the cells start dying and lysing. Several studies have shown that under favourable environmental conditions hepatotoxins (Gupta *et al.,* 2002; Sivonen, 1990a; Kiviranta *et al.,* 1991,) and anatoxin-a (Gupta *et al.,* 2002) are mostly retained within the cells and start to be released in the surrounding medium when cells die. In this experiment an increase of extracellular NOD was observed in week 3, also confirmed by Sivonen *et al.*, (1999a). They mentioned that during growth experiments *Oscillatoria agardhii* from Finnish lakes, extracellular hepatotoxins were detected in the end of week 3-4.

The production of nodulopeptin 901 within the cells and in the surrounding medium showed a similar trend to Chl-*a* concentrations. But intra and extracellular nodulopeptin 901 concentrations did not show any relation with cell biomass. Gupta *et al.,* (2002) and Carmichael *et al.,*

(1988) showed that toxin production by *N. spumigena* did not relate to biomass increase but was correlated with Chl-*a* concentrations. Gupta *et al.,* (2002) stated that toxin production was related to the primary energy processes within cells. It is recommended that the best time to harvest cells to obtain the highest amount of toxins, cell biomass and Chl-*a* contents for *N. spumigena* KAC 66 grown in 10 L flasks is week 6 at 22°C.

3.4.3. Growth and peptide production in 8 L Perspex columns

During the present study the effect of growth duration on cell biomass, Chl-*a* concentrations and the production of intra and extracellular peptides was also observed in Perspex columns. In this experiment in all columns the optimum cell biomass and Chl-*a* contents were observed in week 2, which declined as time passed. The cell biomass and Chl-*a* may vary according to the availability of light intensity and temperature. During the growth in columns, the cells started to decay and the colour of cultures was turned from green to pale yellow, probably due to high light intensity or maybe due to not proper cleaning of columns. This type of culturing system cannot be sterilized hence there is always the potential for increased variability and cell death due to contamination. It seems that less or over illumination was the main reason of poor or unhealthy cultures resulted in damaged or death of cells, low production of biomass and toxin production (Sivonen, 1990; Lehtimäki, *et al.,* 1997).

Hobson and Fallowfield (2003) suggested that 30 µmol/s/m^2 is the best light intensity for culturing of *N. spumigena* isolated from Lake Alexandrina, Australia. Other researchers have also investigated the

effect of light on the growth and toxin production by other cyanobacterial strains and mentioned that production of peptides is directly influenced by intensity of light (Weidner *et al.,* 2003; Utkilen and Gjolme, 1992; Lehtimäki *et al.,* 1997; Watanabe and Oishi, 1988b). Hobson and Fallowfield (2003) noticed that due to photoinhibition at high irradiance *N. spumigena* 001E decreases its high production ability of intracellular hepatotoxins.

In previous studies light intensity was found to be a factor effecting the toxin production in many cyanobacterial strains i. e. *Oscillatoria* (Sivonen, 1990), *Microcystin* (Wiedner *et al.,* 2003; Utkilen and Gjølme, 1992), *Anabaena, Aphanizomenon* (Rapala *et al.,* 1997 and 1993), *Lyngbya* (Yin *et al.,* 1997) and *Nodularia* (Lehtimäki *et al.,* 1997 and 1994).

In this study irradiance also had an apparent effect on the production of extra and intracellular peptide levels. Growth duration was also shown to effect peptide production. In general in this experiment intracellular NOD concentrations were high before the death of cell in columns while the extracellular NOD concentration kept increasing after the cell death, probably as a result of a considerable release of toxin into the medium from the cells (Berg *et al.,* 1987). It was noted that due to stress as a result of over illumination or the presence of bacteria, nodulopeptin 901 was not released or detected into the surrounding medium and high amounts of toxins retained within the cells or they maybe degraded by bacteria (Berg *et al*., 1987). The conclusions of previous studies showed that the highest amount of intracellular hepatotoxins is produced under favourable growth conditions (Vezie *et al.*, 2002; Watanabe and Oshi, 1985).

A comparison between biomass of *N. spumigena* KAC 66, cultured in different vessels, was also carried out. It was mentioned earlier that light plays an important role in culturing of cyanobacterial strains. In this study it was observed that 17.35-17.47 µmol/s/m^2 was ideal light intensity for growth experiments of *N. spumigena* KAC 66 in glass flasks. The high concentrations of cell biomass, Chl-*a* and peptides were observed in glass flasks compared to that found in columns. This was because all flasks were receiving equal light intensity and were not shaded by side walls. Columns were received different illumination at different lengths (ranged from 1.4 to 42.6 µmol/s/m^2), which affected the growth and production of peptides. Flasks produced approximately ~15 fold higher cell biomass compared to columns. It is concluded that to get best results, mass cultures of *N. spumigena* KAC 66 should be prepared in 10 L glass flasks.

3.4.4. Effect of temperature on growth and peptide production

The release and production of hepatotoxins were also 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* (Stolte *et al.,* 2002; Sivonen and Jones, 1999). Among all parameters temperature is major factor to control the growth and toxin production ability of cyanobacteria. The growth of many cyanobacterial strains, such as *Anabaena* (Rapala *et al.,* 1993), *Microcystis* (Utkilen and Gjolme, 1992), *Nodularia* (Lehtimäki *et al.,* 1994) and *Oscillatoria* (Sivonen, 1990) is controlled by temperature. In the present study

temperature was observed to influence Chl-*a* concentrations and peptide productions. In this experiment *N. spumigena* grew well at all temperatures while considering Chl-*a,* at 30°C the highest Chl-*a* concentration was recorded. According to Lehtimäki *et al.* (1997) *N. spumigena* can grow fast at temperature of 25 to 28°C and show tolerance to survive at much higher temperatures and slower growth at temperature below than 16°C (Lehtimäki *et al.,* 1997).

This experiment has shown that the extreme temperatures do not influence on the release of nodulopeptin 901 but affected on the total amount of toxins. The low temperature (22°C) supported the highest concentrations extra and intracellular nodulopeptin 901. Similar results were reported by Sivonen, (1990), who observed that at high temperature (30ºC) *Oscillatoria agardhii* does not leak extracellular hepatotoxins in the surrounding medium but affect on the amount of toxins.

The findings indicate that the higher temperature does not completely diminish the presence of extra and intracellular toxins but it can have an effect on their amounts.

Hobson and Fallowfield (2003) worked on combined effects of light and temperature on hepatotoxin and biomass produced by *N. spumigena* 001E. They showed that low light intensity (30 µmol/s/m^2) combined with high temperature (30°C) and high irradiance (80 µmol/s/m^2) with low temperature (20°C) enhance the production of high intracellular toxins and biomass. Van der Westhuizen and Eloff (1985) studied the effect of temperature and light on the toxicity and growth of *Microcystis*

aeruginosa. They recorded that *M. aeruginosa* produces high intracellular toxin contents at 145 µmol/s/m^2 and high biomass at 205 µmol/s/m^2 at 20°C. Sivonen and Jones (1999) also suggested that at 18-25°C usually 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 toxin production. The high temperature (30°C) did not support high production of intra and extracellular NOD concentration, probably due to the environmental conditions the strain did not release recordable amount of toxins in the surrounding medium.

Lehtimäki *et al.* (1997), Van der Westhuizen *et al.* (1986 and 1985); Watanabe *et al.* (1985) also observed that the high temperature cause stress and affect on the production of toxins and production of nodularin (Lehtimäki *et al.,* 1994). The high temperature inhibits/slows the growth, production of toxins and metabolic activities results in cells go under stressed conditions (Graham, 2007) and a large amount of hepatotoxins retained within the cells, although leakage of nodulopeptin 901 increased towards the end of experiment. 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 same response against biotic and abiotic factors on the hepatotoxin production (Sivonen, 1990). At high temperature most of hepatotoxins kept within the cells and leakage of hepatotoxins occurred when cells die or cell lysis.

It is suggested that 22°C is the best temperature to obtain maximum amount of intracellular NOD and intracellular and extracellular

nodulopeptin 901. It is also recommended that 10 L flasks are suitable culture vessels to obtain maximum amount of biomass and peptides.

3.4.5. Effect of salinity on growth and peptide production

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 at higher salt concentration (25 ‰) decreased the growth rate. Many cyanobacterial strains have acclimation mechanisms to survive under stenohaline and hypersaline conditions (Mazur-Marzec *et al.*, 2005). The results obtained from the present study showed that all salinities had a considerable effect on biomass and the total amount of NOD produced as time progressed. This has been previously described in the cyanobacterium, *Microcystis aeruginosa* (Moisander *et al.*, 2002). In the present study the high intracellular NOD contents were found at 2-11 ‰. Lehtimäki *et al.* (1997) worked on *N. spumigena* BY1 collected from the Baltic Sea. They indicated that the maximum growth rate of strain BY1 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, from where they collected.

In this study at the lowest salinity (2 ‰) the highest concentration of Chl-*a* was observed. This observation was also supported by Lehtimäki *et al.,* (1997). They observed that at low salinity (10 ‰) *N. spumigena* produced the highest amount of Chl-*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 11 ‰.

At all salinities Chl-*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/Chl-a ratios and found that increased salinity affect on increases NOD and Chl-*a* concentrations.

In this study *N. spumigena* KAC 66 also showed that an increase in cell biomass did not support toxin production but showed correlation with Chl-*a* concentrations (Carmichael *et al.,* 1988). Lehtimäki *et al.,* (1997) worked on effect of salinities (0-30 ‰) on Chl-*a* contents and dried cell biomass of *N. spumigena.* They noticed that dried cell biomass does not have any correlation with salinity, while Chl-*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 high salinities (20 and 25 ‰) the cultures went under stressed conditions and high amount of NOD could not be produced (Blackburn *et al.*, 1996) or degraded. 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 Fallowfield (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 noted that salinity has effect on the cell numbers, optical density, Chl-*a* concentration, dry biomass and growth rate. They also speculated that *N. spumigena* NSG 0897 grows considerably 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 another study Musial and Plinski (2003) worked on effects of a range of salinities (4, 8, 12, 16, 24, 30, 35 psu; (≈4, 8, 12, 16, 24, 30, 35 ‰) on growth of *N. spumigena* NSG 0897 collected from the Gulf of Gdansk, Baltic Sea. In another study Burja *et al.,* (2001) cultured *Lyngbya majuscula* in 3 different media having different salinity, pH and trace elements. They suggested that varying the culture conditions under which *Lyngbya majuscula* was grown had the greatest effect on secondary metabolite production. Orr *et al.,* (2004) carried out a study using a laboratory culture of *Microcystis aeruginosa* and found that *M. aeruginosa* is more tolerant at high salinity and continue producing microcystin.

It is concluded that the biological activity of cyanobacteria appears to be depended upon growth conditions and therefore the performance of toxin producing organisms maybe improved by altering the cultural conditions. It is suggested that 11 to 20 ‰ is the best salinity range to harvest culture to obtain high amount of extra and intracellular peptides and biomass.

3.4.6. Effect of nitrate on growth and peptide production

The observations from the present study showed that in nitrate free and all other nitrate conditions Chl-*a*, cell biomass and intracellular peptides levels increased with time. However, during the later phase of growth the toxin levels often dropped. These results can be associated with that where growth nutrients are in limitations, in stressed conditions the strain decreased the production of peptides because biosynthesis of peptides consumes significant energy and cells use limited nutrient sources to survive. Vezie *et al.,* (2002) did a comparative study between toxic and non toxic *Microcystis* spp. under variable nitrogen (0.84-84 mg/L) and phosphorus (0.05-5.5 mg/L) conditions. They noted that in nutrient limited conditions toxic strains reduce the production of toxins and use resources for their growth only. Therefore, non toxic *Microcystis* strain grew better than toxic strain. It might be that the biosynthesis of hepatotoxin microcystin requires additional energy consumption during toxin production process.

Thus, the effect of increased nutrients and low N/P ratio on the blooms of *N. spumigena* in the Baltic Sea is still a debated topic. According to Oliver and Ganf (2000) low nitrogen source also affect the growth of cyanobacterium. In the present study the highest cell biomass was recorded in week 5 at highest concentrations of nitrates. Vintila and El-Shehawy (2012) mentioned that *N. spumigena* strains isolated from the Baltic Sea, did not respond considerably to nitrate rich cultures. They suggested that *N. spumigena* strains do not seem to be efficient to uptake dissolved inorganic nitrogen (DIN) compared with other nitrogen

fixing cyanobacteria. In the Baltic Sea *N. spumigena* produces blooms in N limited areas (Stal *et al*., 2003) and seem to be affected by other factors i.e. salinity, temperature and phosphorus (Vintila and El-Shehawy, 2012). It has suggested that maybe due to geographical distribution and variability in the genetic background within the species *N. spumigena* strains respond differently (Vintila and El-Shehawy, 2012).

Stal *et al.,* (1999) and Kivi *et al.,* (1993) speculated that in late summer *N. spumigena* forms blooms in nutrient limited conditions in the Baltic Sea. According to them it is an assumption that low N:P ratio in the Baltic Sea water promotes the cyanobacterial growth. However, it seems to be apposite in this laboratory based study the highest cell biomass was observed at high concentrations of $NO^-_{3.}$ Stal *et al*., (2003) suggested that abundance of N_2-fixing cyanobacteria in the Baltic Sea is due to low N:P ratios.

At lower nitrate conditions and control cultures, an increased intracellular NOD levels were observed, this hypothesis is supported by one of laboratory based experiments that *Anabaena* spp. (Rapala *et al.,* 1997) and *N. spumigena* (Lehtimäki *et al*., 1997) demonstrated an increase in microcystin and nodularin productions under N limited conditions, respectively. In another study made by Vuorio *et al*., (2005), who performed an experiment on the effect nitrogen to phosphorus ratio on the phytoplankton community structure in mesocosm conducted in Archipelago Sea, Northern Baltic Sea. In the end of 3 weeks experiment they found that microcystin and nodularin increased with increasing biomass of *Anabaena* spp. and *N. spumigena,* respectively.

3.4.7. Effect of phosphate on growth and peptide production

Cyanobacterial strains have ability to store phosphorus and can maintain their growth and toxin production under phosphorus deficient conditions (Karjalainen, 2005). This hypothesis was approved in this study that *N. spumigena* was grown under all phosphorus conditions and cell biomass and Chl-*a* concentrations were high maintained for several weeks but when fully depleted results in decline. At the highest phosphorus level a linear increase in cell biomass was recorded compared with other conditions. It shows that the high phosphorus concentration supported the growth of *N. spumigena*. The blooms samples of *Aphanizomenon flos-aquae* and *N. spumigena* collected from the Gulf of Finland, Baltic Sea, grew in the high biomass in under high phosphorus and low N:P ratios. (Kononen *et al*., 1996). Phosphorus studies on both phosphorus starved inocula of strains, represented the slow growth by hepatotoxic *N. spumigena* and stimulated growth by non-toxic *A. flos-aquae,* from the Baltic Sea. It represented that the deficient medium non toxic strains grow well because they do not spend energy on the biosynthesis of hepatotoxins (Lehtimäki *et al.,* 1997).

In present study the high cell biomass, Chl-*a* and intra and extracellular NOD and nodulopeptin 901 concentrations were found in the end of log phase and in beginning of stationary phase in control to high phosphorus conditions, which followed by a decline. Microcystins, anabaenopeptilides and anabaenopeptins, produced by *Anabaena* strain 90, showed the highest peptide concentrations in the medium phosphate levels (Repka *et al.,* 2004). The same results were found for microcystins at the middle of

the growth period (Sivonen and Jones, 1999). In general, in this study, at all phosphorus conditions extracellular NOD and nodulopeptin 901 (except in control medium) a decline of toxins was observed with incubation time. Lehtimäki, (2000) reported that at different phosphorus conditions extracellular NOD concentrations increased with increasing time.

In the present study salinity, nitrate and phosphate experiments the much amount of NOD retained within the cells during late log phase and early stationary growth phases. This hypothesis was also supported by several investigations. Under favourable conditions anatoxin-a (Gupta *et al*., 2000) and microcystin (Rapala *et al.,* 1997 and Sivonen, 1990) mostly retained within the cells. Vezie *et al.,* (2002) also noted that the toxic *Microcystin* cultures were at late logarithmic or early stationary phases, the much amount of intracellular microcystin retained within the cell, while growing in different N and P concentrations. In the present study at high concentrations of nitrate the lower amounts of intra and extracellular peptides were recorded. Lehtimäki *et al.,* (1997) found that at high inorganic N concentrations lower amount of intracellular NOD was found in nitrogen fixing *N. spumigena.*

Due to recently discovered nodulopeptin 901 no data is available on the effects of environmental factors on its intra and extracellular levels. It is concluded that the biological activities of *N. spumigena* KAC 66 appear to be dependent upon suitable growth conditions. They also affect the ability to produce less or high amounts of biomass and toxins, which can be controlled by altering the culture conditions.

In all experiments, Chl-*a* contents showed a positive correlation with extra and intracellular NOD production, as Chl-*a* concentrations decreased NOD levels were also decreased. Only columns showed that only intracellular NOD levels had positive correlation with Chl-*a* contents, it maybe due to the cell death in lag phase in columns and toxins could not be released in the surrounding media, while nodulopeptin Chl-*a* contents showed a negative correlation, a decrease of Chl-*a* contents showed an increase of extra and intracellular nodulopeptin 901 contents. Carmichael *et al.* (1988) and Lehtimäki *et al.,* (1997) mentioned that Chl-*a* contents of *N. spumigena* collected from the Baltic Sea, showed a positive correlation with extra and intracellular toxins.

In this study in all experiments a fluctuation in cell biomass was observed, probably due to start of death phase of cultures and died cells were measured as cell biomass. The second reason was that the salt and nitrate contents (in salinity and nitrate experiments) remained on filter discs, used for filtration to determine cell biomass, which may cause variation in cell biomass (Hobson and Fallowfield, 2003; Lehtimäki *et al.,* 1997). Problem with residual salt contributing to dry weight shows that cell biomass is not a reliable measure to determine biomass of cultures. Many scientists also recommended that Chl-*a* pigments are good to determine biomass of growing strains (Murphy *et al*., 2005; Gupta *et al.,* 2002; Lawton, 1999; Lehtimäki *et al.,* 1997; Becker, 1994).

3.5. CONCLUSION

It is concluded that the environmental factors have considerable impact on the biomass and toxins production. Recent years due to heavy load of

agricultural runoff and other pollutants to the Baltic Sea, have resulted in increase of nutrients. The global warming is also providing favourable conditions for spreading of toxic blooms of *N. spumigena* in the Baltic Sea and its adjacent areas and other freshwater and brackish water bodies. If water temperature rises in the Baltic Sea, this study suggests, more rapid growth of *N. spumigena* but lower NOD levels release into environment. It has also been predicted that N_2 fixation by *N. spumigena* might be increased by 67±50% in the next 100 years, which can promote the blooms of *N. spumigena.* This is first study on the factors influencing on the production of newly characterised nodulopeptin 901.

The results from present study give physiological and chemical evidences that which conditions are favourable and unfavourable for the production of biomass and toxin levels, which maybe helpful to explain the distribution of *N. spumigena* in the Baltic Sea. According to present study these abiotic factors will also helpful to control the dominance of strain in the natural blooms in the Baltic Sea and as well as laboratory based experiments to produce high amount of peptides for research purposes.

The investigations on the growth limiting N or P nutrients and the response of the *N. spumigena* are still being unexplored. It was observed that ≈40-50% nosulopeptin 901 released extracellularly. It is suggested that there are further investigations required to note the role of nodulopeptin 901 as a signalling compound.

To obtain highest amount of cell biomass, Chl-*a,* and intracellular and extracellular peptides, there is need to make some changes in recipe of BG-11 and time to harvest *N. spumigena* KAC 66 cultures.

CHAPTER 4

TAXONOMY AND CHARACTERISATION OF COMPOUNDS FROM ISOLATES OF FRESHWATER AND HYPERSALINE ENVIRONMENTS

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4.1. INTRODUCTION

Natural products play an important role in the development of medicines. Cyanobacteria are most promising organisms to produce bioactive and novel compounds. They represent a wide biological diversity from terrestrial habitats to water bodies and are a rich source for potential discovery of new biochemicals, including enzymes, pharmaceuticals and novel bioactive compounds. In the past 30-40 years marine (Burja *et al*., 2001; Tan, 2007) and freshwater (Singh *et al.,* 2011) cyanobacteria have been targeted to explore bioactive and novel compounds for drug discovery programmes.

In recent reviews (Singh *et al.,* 2011; Butler, 2005; Burja *et al*., 2001), mentioned that several members of cyanobacterial genera belong to *Microcystis, Synechocystis*, *Lyngbya*, *Oscillatoria*, *Phormidium*, *Anabaen*a, *Cylindrospermopsis, Nodularia* and *Nostoc* have been known to produce a number of antibacterial, antifungal, anticancer, antimalarial, anti-HIV, cytotoxic, antiviral, anti-protozoal, and enzyme inhibitors.

A literature survey was done on published bioactive cyanobacterial compounds in Journal of Natural Products (2007-2008; Singh *et al.,* 2011) and represented that in one year 38 new compounds have been isolated from cyanobacteria (Fig. 4.1A), among which 82% of compounds were antiprotozoal, antibacterial, antiviral, cytotoxic, protease inhibitors and Ca^{2+} channel inhibitors. In another review Burja *et al.,* (2001) analysed 550 research papers published during 1996-2001. They revealed that a total of 424 compounds were isolated from marine cyanobacteria and most of them showed activity against cell lines, cancer

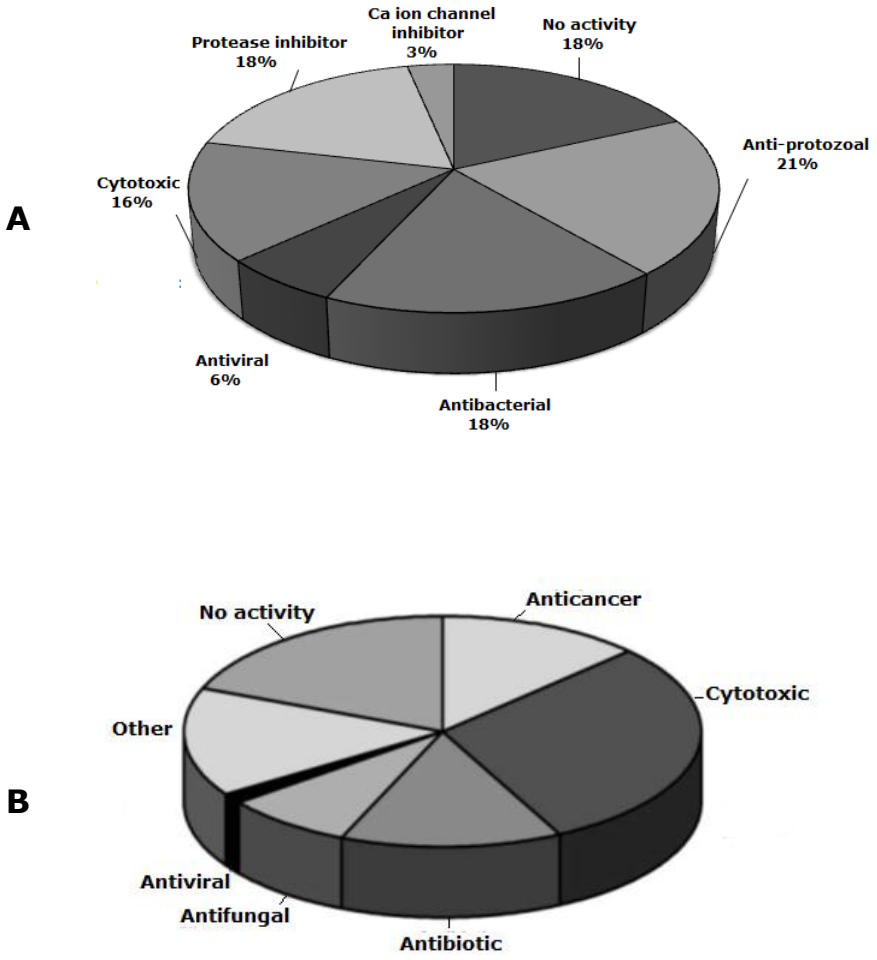

Figure 4.1. Reported biological activities of cyanobacterial compounds

A:- Cyanobacterial compounds (38 new compounds; *adopted from Singh et al., 2011*)
B:- Marine cyanobacterial compounds (424 compounds; *adopted from Burja et al., 2001*)

cells, antibiotics, fungi and viruses (Fig. 4.1B).

The bloom forming and hepatotoxin producing strain *Microcystis aeruginosa* is known to produce variety of toxic compounds. It produces cyclic heptapeptide microcystin (tumour promoter) and cyclic desipeptide cyanopeptolins i.e. aeruginosin (enzyme inhibitor),

microcystilide (cell differentiation), micropeptin (endotoxic; Burja *et al.,* 2001). Cyanopeptolins are also known as micropeptins, aeruginopeptins and microcystilides and widely distributed in cyanobacteria (Chroococcales, Oscillatoriales and Nostocales). They have more than 100 variants and have a diverse mass range (770 – 1181 Da). They are inhibitors of serine proteases of crustaceans and mammals (Gademann *et al*., 2010).

Cyanopeptolins are characterised by 3-amino-6-hydroxy-piperidone (Aph) and cyclisation occurring with the formation of an ester bond between the *β*-hydroxy group of threonine with the carboxy group of the terminal amino acid. Attached to the amino group of threonine is a side chain of variable length and composition. Common side chains include one or two amino acids and an aliphatic fatty acid such as octanoic acid or glyceric acid (Fig. 4.12).

Due to continued medicinal importance there is a need to isolate new strains of cyanobacteria from different habitats, which may never been sampled. Sampling from different environments can also provide information on geographical distribution of species and toxin production under extreme environments. The present study provides an opportunity to isolate cyanobacterial strains and toxic compounds from two different water bodies, the hypersaline water body, the Dead Sea and freshwater Dian Lake, China.

4.1.1. Dian Lake, Dianchi Lake or Kunming Lake, China

Dian Lake is the sixth largest hypereutrophic lake and main water source of Kunming, Yunnan Province of southwestern China, which is divided

Figure 4.2. Map showing the location of Dianchi Lake, China. *(Google map, used with permission)*

into two sections, Waihai and Caohai (Fig. 4.2). It covers 298 km^2 area of land (Liu *et al.,* 2013) and is 1,886.5 m above than sea level.

Due to industrialisation and urbanization it receives a heavy nutrient load, which causes development of frequent cyanobacterial blooms (Liu *et al*., 2013). According to a survey made in 2006-2007, the total phosphorus (0.12-0.35 mg/L), total nitrogen (1.40-5.06 mg/L) and chlorophyll-*a* (0.047-0.336 mg/L) concentrations were high (Wang *et al.,* 2011), which provided favourable eutrophic conditions for cyanobacterial

blooms.

In spring and summer blooms (September 2001 to July 2002) *Microcystis* (35.7%-88.0%) and *Aphanizomenon* (0.46%-30.75%) were dominant strains among the total concentration of phytoplankton (Yuan *et al*., 2005; Mei *et al.,* 2006). In another report (Liu *et al*., 2006) the main bloom forming cyanobacteria were *Microcystis aeruginosa*, *Microcystis viridis* (Lirong *et al.,* 1999) and *Aphanizomenon flos-aquae*. Wu *et al.,* (2009) also indicated that microcystin-LR and MC-RR were the dominant hepatotoxins, reported from bloom samples of Dian Lake.

4.1.2. The Dead Sea

The Dead Sea, Sea of Death or the Salt Sea is a unique, athalassohaline, exposed to extreme environmental stresses, inland hypersaline lake located near the Syrian–African Rift Valley, on the border between Jordan and Israel (Fig. 4.3). It is 377 m deep and the earth's lowest elevation on land. The surface of the lake is 423 m below sea level. The main sources of water input in the Dead Sea are winter rain floods and only the Jordan River, bringing water from Lake Kinneret, Israel.

Figure 4.3. Map showing the location of the Dead Sea
(Google map, used with permission)

Currently, the pH of the Dead Sea is about 6 (Oren, 2000) and the salinity is around 34-40 PSU (Kis-Papo *et al*., 2003; Oren, 2008). In the lake the cation concentrations is exceptionally very high (Cl^- > Br^- >Mg^{++} >Na^+> Ca^{++}>K^+; Khlaifat *et al.,* 2010).

The biological monitoring of the Dead Sea was started in 1936 and first time Benjamin Elazari-Volcani reported the presence of algal and cyanobacterial communities (Oren, 2008) when salinity was much lower than at present time.

The microorganisms, bacteria, diatoms, green algae and fungi (Table 4.1) are best adapted to this hypersaline environment. In the salt lake

the halophilic phototrophic primary producer is the unicellular green alga *Dunaliella pawa* which forms dense blooms followed by halophilic Archaeal belong to family Halobacteriaceae. The blooms of these microorganisms occur after rain when rain and fresh water from Jordan River cause remarkable dilution in the upper layers of water (Oren, 2000). As a result of evaporation, the upper layer of water becomes unfavourable and the dense blooms of *Dunaliella* and Archaea disappear slowly.

Some cyanobacteria are able to adapt to salinity stress (see 1.1.9.2) and can survive in hypersaline environments like other halophilic microorganisms. However, in the Dead Sea ecosystem the cyanobacteria do not play a significant ecological role, although several cyanobacterial strains have been identified (Volcani, 1944; Oren, 2008; Nevo and Wasser, 2000).

Table 4.1. A list of micro-organisms reported from various habitats of the Dead Sea.

Genera/species	References
Virus-like particles	Oren *et al.* , (1997)
Anaerobic bacteria *Halobacteroides halobius* , *Orenia marismortui* *Selenihalanaerobacter shriftii, Sporohalobacter lortetii*	Oren *et al.* , (2004)
Aerobic bacteria *Chromohalobacter marismortui* , *Halomonas halmophila* , *Chromohalobacter israelensis, Salibacillus marismortui*	Oren *et al.* , (2004)
Fungi 77 fungal species	Kis-Papo *et al.* , (2003) Oren, (2005)
Protozoa	Volcani, (1944)
Diatoms *Melosira, Navicula, Gomphonema, Cymbella, Pinnularia,* *Eunotia, Synedra*	see Oren, (2008)
Dinoflagellates *Exuviella*	see Oren, (2008)
Cyanobacterial genera *Aphanocapsa* , *Aphanothece* , *Microcystis* (?), *Oscillatoria* , *Phormidium, Plectonema*	Volcani, (1944)
From freshwater springs *Scytonema, Homoeothrix, Phormidium, Cylindrospermum*	Oren, (2008)
From brackish water bodies *Chroococcus, Gloeocapsa, Gloeothece, Gomphosphaeria,* *Johannesbaptista, Merismopedia, Nodularia Oscillatoria,* *Phormidium* and *Schizothrix*	Oren, (2008)
From terrestrial environments *Entophysalis, Schizothrix, Microcoleus* and *Nostoc*	Oren, (2008)
Experimental solar ponds *Aphanothece, Aphanocapsa, Gomphosphaeria, Gloeocapsa,* *Chroococcus, Chlorogloea, Chroococcidiopsis, Phormidium,* *Spirulina* and *Oscillatoria*	Dor and Ehrlich, (1987)
Green algae *Dunaliella pawa, Scenedesmus, Pediastrum, Ulothrix*	Nevo and Wasser, (2000) (see Oren, 2008)

(based on Oren, 2008)

4.1.3. Isolation and identification of cyanobacteria

Photosynthetic cyanobacteria belong to morphologically and developmentally diverse group of prokaryotes and range from unicellular to filamentous forms. About 2000 cyanobacterial strains have been reported (Mundt *et al*., 2001) and found in variety of habitats from aquatic to terrestrial but their taxonomic diversity is not different. In comparison to their diversity the identification of cyanobacteria is only restricted to comparatively few representatives. The methodology used for isolation and purification of cyanobacteria is quite different compared to other bacteria. The traditional isolation techniques used to isolate cyanobacteria may restrict the number of strains, which can be important for production of biochemicals and can act as useful industrial and research tools.

The identification and distribution of cyanobacteria play an important role in the field of research especially in bloom samples. Rapid microscopic identification of cyanobacteria can be helpful to detect community composition and presence of harmful species. Microscopy can be readily used to identify organisms to genera level, which is often adequate to predict possible bloom hazards and initial precautions to control blooms in drinking or recreational water bodies. It is also useful to count cell numbers i.e. how much number of cyanobacteria may be hazardous to health.

A number of studies have been done on the isolation and purification of cyanobacteria (Allen and Stanier, 1968; Guillard, 1973; Rippka, *et al.,* 1988; Hallegraeff, *et al.,* 1995, Rippka 1979; Lawton, *et al.,* 1999).

4.1.4. Microscopic examination

Cyanobacteria can be identified on the basis of their morphologically and can be divided into non filamentous (solitary cells, aggregated, colonial or surrounded by mucilage), filamentous with specialised cells (akinete, heterocysts) and filamentous non specialised cells (hormogonia). They are also capable of reproduction by binary fission and show true branching, which can be useful to identify certain strains under microscopic examination. There are several techniques which have been developed to identify cyanobacterial species including light, compound, inverted, epifluorescence microscopes and methods comparing DNA-sequences. Most cyanobacterial strains can be easily differentiated from other phytoplanktons and microalgae under the microscope on the basis of their morphological characteristics. Recently, Hoffmann *et al.,* (2005) proposed classification system of cyanobacteria based on the genetic relationships, mainly 16S rDNA gene sequence, morphology and thylakoid arrangements.

4.1.5. Axenic cultures

Field samples contain a variety of contaminants and are difficult to separate from cyanobacterial strains. To grow single species of cyanobacteria it is essential to obtain axenic cultures. Several methods have been suggested to obtain axenic cultures from heavily contaminated natural field samples (Cho *et al.,* 2002 and Choi *et al.*, 2002). Antibiotic treatment is a successful and widely used method to achieve axenic cyanobacterial strains (Choi *et al.*, 2002 and Cho *et al.*, 2002; Vazquez-Martinez *et al.,* 2004). In order to obtain bacteria free cultures of

cyanobacteria, a range of antibiotics or combination of antibiotics i.e. cycloheximide, kanamycin, nystatin, erythromycin, mixture of streptomycin, β-lactum, penicillin and chlorophenicol, ampicilin, carbenicllin and chlorampehnicol (Vazquez-Martinez *et al*., 2004) are added to the medium to inhibit the growth of Gram +ve and Gram –ve rods and cooci bacteria.

In some cases due to the presence of a mucilaginous envelop around the cells/filaments, antibiotic treatments do not show any effectiveness. Mucilaginous covering protects cyanobacteria from any antibiotic treatments (Cho *et al*., 2002). High concentration of antibiotic treatment can also harm to cyanobacteria. To avoid these difficulties several physical methods are available to isolate cyanobacteria from mixed and natural field samples i.e. sample crushing between two glass slides, filtration, homogenization in a homogenizer, serial dilution, streaking over agar plates and capillary methods. Some strains can be isolated by gliding and phototaxis of motile cyanobacteria (Vaara *et al*., 1979). Antibiotic treatment is often the last method to obtain bacteria free isolates to get rid of bacterial contaminants well attached with mucilaginous sheaths and among bunch of cells/colonies.

4.1.6. Isolation techniques

Isolation and purification of cyanobacteria are relatively restricted due to the difficulties faced during isolation and purification of a single strain. Several liquid and solid media have been developed by Allen and Stainer (1968), Rippka (1979) and Waterbury and Stainer (1981) to obtain purified and isolated colonies or single cells. Traditionally streaking over

agar plates and capillary methods are suggested to isolate cyanobacterial strains. Commercially available agar is used as solidifying agent to obtain single strain, but it is known to contain impurities, which may harm cyanobacterial growth or inhibits the growth of certain strains of cyanobacteria. A number of studies have been done to reduce the negative effect of impurities present in agar by means of separate sterilization of agar and nutrient solutions in separate Erlenmeyer (Allen and Stanier, 1968), lower agar concentration (Shirai *et al.*, 1989), glass fibre filters (Ferris and Hirsch, 1991) and washing of agar (Krieg and Gerhardt, 1981).

The main aim of the current study was to provide a new knowledge about cyanobacterial diversity and to detect presence of any novel compound/s from two different water bodies, the Dian Chi Lake and the Dead Sea. These novel bioactive compounds may be used for pharmaceutical drug discovery programmes.

4.2. MATERIALS AND METHODS

In the present study cyanobacteria were isolated from diverse environments including fresh, marin, brackishwaters, eurythermal, stenohaline and euryhaline habitats. The natural samples from the Dead Sea were provided by Dr. Iain Douglas, University of Aberdeen, United Kingdom. The field samples were collected from various localities of the Israeli side of the lake, the samples were either collected from the shore, the spring themselves, the water table and from the thermal springs of Quedem. Fifty three samples were received from the Dead Sea and divided into seven groups on the basis of salinity which ranged from 4-

>32 ‰ (Appendix 64). The salinity of samples was checked by refrectometer.

The bloom samples from the Dian Lake**,** China were provided by Mr. David Van Alstyne, Director (St. Cyrus, Scotland), Scottish Bioenergy. The samples were identified microscopically on the basis of their morphology, colour, shape and size.

In this study the classification and identification of species are based on classification schemes of Komarek and Anagnostidis (1999 and 2005). The present study was initiated with a review to isolate cyanobacterial strains collected from blooms samples of Dianchi Lake, China and the Dead Sea samples.

The following methods were used to obtain isolated and purified single clone or colony (Fig. 4.4).

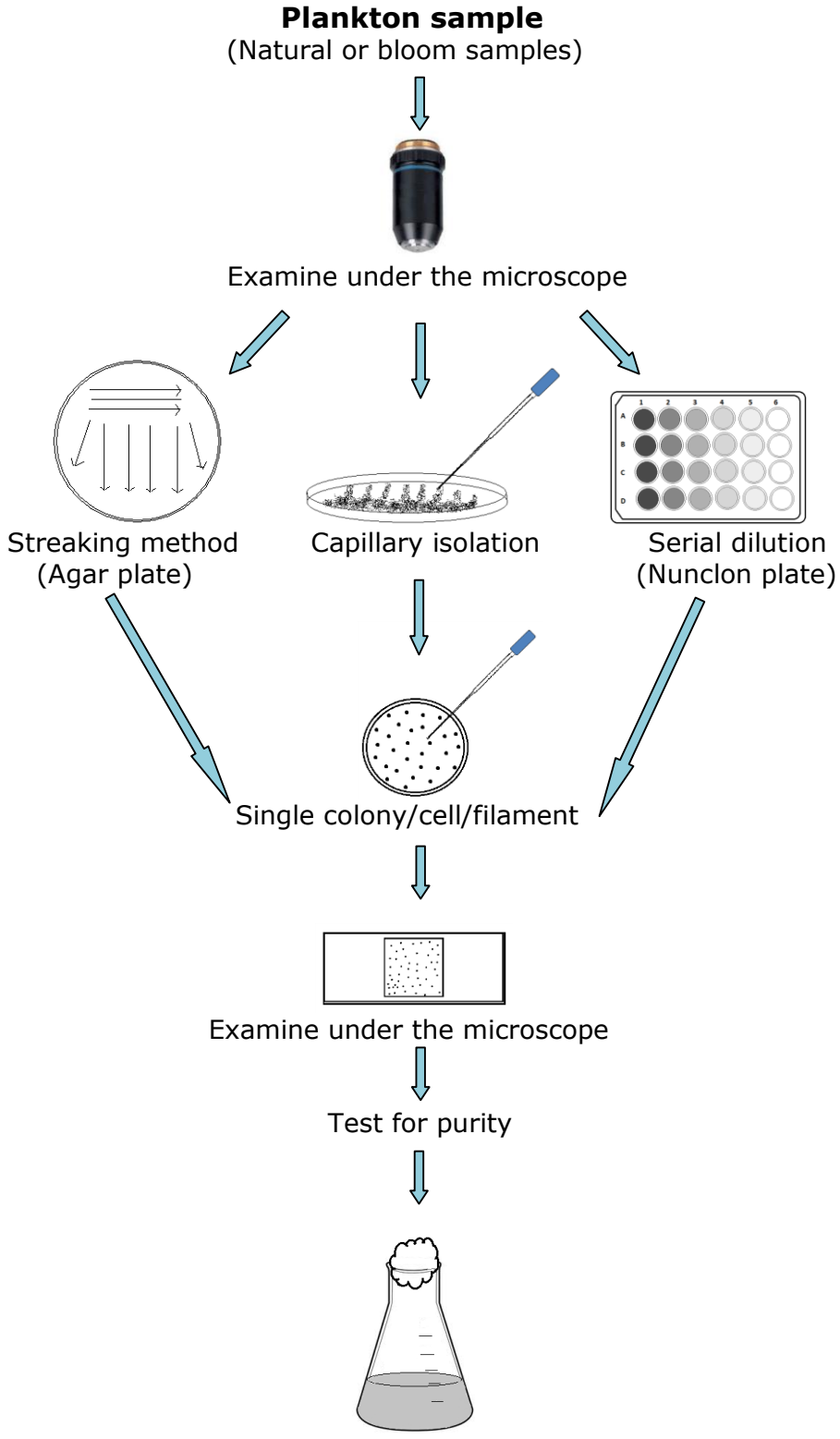

Figure 4.4. A method to obtain a single cell or colony of cyanobacteria/microalgae from natural sample.

4.2.1. Preparation of agar plates

In an Erlenmeyer flask (500 mL) 23 g of agar (Oxoid, Hampshire, UK) was dissolved in 400 mL distilled water and heated up to transparency, equilibrated up to room temperature and cut into small cubes (~1 cm). The agar cubes were dipped into distilled water for 3-4 days with several replacement of distilled water to remove its toxicity (Waterbury *et al.*, 1981; Rippka, 1988).

In another Erlenmeyer flask (1000 mL) 600 mL double-strength medium (BG-11; 0 ‰) was prepared. Both flasks were autoclaved at 15 lb of pressure at 121 ºC for 15 min. After cooling, both solutions were mixed together (Rippka, 1988) and the lukewarm medium was poured into Petri plates (~25 mL agar/Petri plate).

This procedure was repeated for another set of Petri dishes containing antibiotic. In this study cycloheximide (Sigma, Poole, UK) was used to inhibit protein biosynthesis in eukaryotic organisms. The cooled molten agar was supplemented with aqueous stock solution of cycloheximide (25 µg/mL; wt/vol), prepared freshly and sterilized by filtration syringe driven filter unit (33 mm; pore size 0.45 µm) (Millex, Millipore, Ireland). The molten agar was poured over Petri dishes (≈25mL agar/Petri plate) and allowed to set at room temperature in a laminar flow hood to avoid any contamination.

4.2.2. Streaking method

The naturally contaminated and benthic cyanobacteria, present in bloom samples, were isolated by streaking method (Hoshaw and Rosowski, 1973) on pre-prepared solidified agar plates. After solidification of agar in

Petri plates, cyanobacterial sample was crushed between two glass slides and a drop of crushed cyanobacteria streaked over solidified medium with an autoclaved bent Pasteur pipette (Rippka, 1988). The isolation by streaking method was done in two sets of Petri dishes. One set was contained agar and another with agar contained the antibiotic, cycloheximide.

The sides of all Petri plates were sealed with Parafilm and incubated for 4-5 weeks under continuous low white cool illumination (0.08-0.90 μmol/s/m^2) at 22ºC in order to obtain best growth of cyanobacterial species. All Petri dishes were covered for 3 days with muslin to protect new colonies from high light intensity. The plates were examined at weekly intervals to observe any new colony of cyanobacteria under dissecting microscope at lower magnification (60x, Olympus SZ40, Japan). The purity of newly isolated cyanobacterial strains were checked. Those cyanobacteria were glided much further from streaking lines and rapidly separated from their contaminants were picked by Pasteur micropipette and transferred into 250 mL flash containing 100 mL BG-11. In some cases motile and filamentous cyanobacteria were self-purified on agar plates by gliding away from other cyanobacterial strains and contaminants. Those filamentous cyanobacteria were directly picked up by capillary method.

4.2.3. Capillary method

Several cyanobacterial and green algal strains were easily isolated by capillary direct picking method at the first attempt using Pasteur micropipette (Rippka, 1988). The Pasteur pipette is an ideal tool to pick

single filament or unicellular cells. A drop of fresh bloom or natural samples was placed over a glass slide and a single cell or colony of cyanobacteria was picked by capillary method under dissecting microscope (Andersen and Kawachi, 2005). Once getting single cell or colony was obtained, it was checked for its purity and then transferred into 250 mL Erlenmeyer flasks containing 100 mL BG-11 (0 ‰).

4.2.4. Serial dilution culture (SDC) method

Serial dilution method is used to isolate planktonic cyanobacteria as described by Guillard (1973) modified by Andersen and Throndsen (2003). Two or three drops of sample were crushed between two glass slides to separate different strains with each other. For serial dilution 24 well Nunclon plate (Thermo, Fisher Scientific, Roskilde, Denmark) containing 1 mL growth medium (BG-11, 0 ‰) was used. Serial dilution was done by transferring 1 mL into first well, second well to last well (10x-1,00,000x). The pates were incubated under constant fluorescence light (16.62-17.23 µmol/s/m^2) for 1-2 weeks at 22 ºC. All wells were supplied by fresh medium to prevent the new mini-cultures from desiccation. After 1-2 weeks each well was observed under dissecting microscope to obtain any single filament or colony. The isolates were examined for purity and transferred to 250 mL Erlenmeyer flask containing 100 mL liquid growth BG 11 medium for identification and mass culturing.

4.2.5. Test for purity of cultures

Several methods are available to check the purity of samples (Vaara and Niemela, 1979; Rippka, 1988; Ferris and Hirsch, 1991). The purity of newly isolated colonies was checked as described by Rippka (1998). Few drops of the liquid culture and isolated colony from agar plates/capillary/serial dilution methods were placed on agar plates supplemented with BG-11, casamino acid (Sigma, Poole, UK; 0.02-0.05%, w/v) and glucose (Fisher Scientific, Loughborough, UK; 0.5%, w/v). The plates were incubated in dark for 2-3 days at 22°C, typical for the growth of sample's cyanobacteria, since if any bacteria are still present they would be expected to grow at that temperature. The contaminated strains were again streaked over fresh agar plates containing antibiotic, cycloheximide. If no contamination was observed, the cultures were assumed axenic. The presence of any bacteria in cultures was also determined by phase-contrast microscope and/or under oil immersion lens.

4.2.6. Culturing and maintenance of cyanobacterial strains

After one month the purified cyanobacterial cells/colonies were inoculated into 250 mL flasks containing 100 mL of growth medium (BG-11). The flasks were kept under constant illumination of cool white fluorescent light (18.37-18.57 µmol/s/m^2) and the cyanobacteria were allowed to grow photoautotrophically. In 15 days, an adequate growth of cyanobacteria were obtained, later which were used for UPLC analysis.

To maintain cyanobacterial strains the growth medium was regularly changed on monthly basis. Ten mL (10%) of old culture was transferred

into 100 mL of growth media.

4.2.7. Identification and morphological characterisation

Most cyanobacteria can readily be distinguished from other phytoplankton algae and particles under the microscope on the basis of their morphological features i.e. size, cell structure, shape and filamentous and non-filamentous morphology.

For identification of strains small drop of fresh and pure culture was spread on a glass slide and covered with a glass cover slip. A drop of immersion oil was placed over the cover slip and the slide observed at 1000x magnification under florescence microscope lighting system (DMLS; Leica, Wetzlar, Germany). The measurements were done in µm by using a computer based program (available with microscope) and pictures taken by a digital sight camera (DFC 300 FX, Leica, Wetzlar, Germany). For unicellular strains 30 cells were measured and the length and width were noted. For filamentous strains the length and width of 30 individual cells were measured. The strains were indentified on the bases of their morphological characteristics. The literatures used for identification were: Komárek and Anagnostidis (1999 and 2005).

4.2.8. Extraction and analysis of isolates

A total of 26 extracts of isolates from the Dead Sea and Dian Lake were analysed on analytical Ultra High Performance Liquid Chromatography-Photo Diode Array-Mass Spectrometer-Mass Spectrometry (UPLC-PDA-MS).

Aliquots (1 mL) bloom material was centrifuged at 13,000 rpm for 10

min. The supernatant was discarded and the pellet was extracted in 80% aqueous methanol (v/v) for 1 h, with intermittent vortexing. Extracts were centrifuged at 13, 000 rpm and supernatant analysed by UPLC-PDA-MS as described in section 2.2.8.

One hundred µl of each extract was taken in UPLC vials and analyzed on UPLC (Waters Acquity) coupled to a Xevo Quadrupole Time of Flight Mass Spectrometer (Xevo QToF-MS). For separation of extracts a BEH C18 column (100 x 2.1 mm, 1.7 µm particle size) maintained at 40 ºC was used. Milli-Q water and 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as mobile phase. Analytical gradient reagents, acetonitrile and methanol were obtained from Rathburn, Walkersburn, UK.

UPLC chemicals formic acid and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich, Dorset, UK and Leicestershire, UK, respectively. Milli-Q pure water was obtained from a Milli-Q system purified to 18.2 MΩ (Millipore, Watford, UK).

Samples were separated using a gradient increasing from 20% to 70% B over 20 min followed by ramp up to 100% B wash step and re-equilibration over the next 20 min. Autosampler was constantly maintained at 6 ºC. Analysis by mass spectrometry was acquired in positive ion electro-spray mode, scanning from *m/z* 50 to 2000 Da with a scan time of 2 s and inter-scan delay of 0.1 s. The ion source parameters; capillary and sampling cone were 2.9 V and 25 V, simultaneously. The desolvation temperature and source temperature were 300 °C and 80 °C, respectively. The desolvation gas and cone gas

flows were 400 L/h and 50 L/h, respectively. Leucine-enkephalin (0.5 mg/L in 50% aqueous methanol; v/v) was used as the lockspray and sodium iodide (2 µg/µl in 50% aqueous propane-2-ol; v/v) as the calibrant. Instrumental control, data acquisition (centroid) and processing were obtained using Masslynx v4.1. ToF analysis was performed on low and high energy voltages, 6 v and 25-40 v, respectively.

MS/MS was performed on predominant ion *m/z* 993.5 $[M+H]^+$ during UPLC separation. MS/MS conditions: source was as standard (see 2.2.8) mass range was from 50-1000 Da, scan time was 0.2 sec with interscan 0.025 sec, collision energy was ramped from 40-50 eV. Purified compounds were identified on the basis of their molecular weights.

4.3. RESULTS

A total of 26 cyanobacterial and green algal strains were isolated from natural samples of Dian Lake and the Dead Sea (Table 4.2). The cyanobacterial strains belonged to two orders: Chroococcales and Oscillatoriales, and green algae belonged to three orders: Chlorellales, Chlorosarcinales and Sphaeropleales.

Out of 26 strains 5 unicellular cyanobacterial and green algal strains were isolated by serial dilution belonging to genera *Synechococcus* (3 strains) and *Chlorella* (2 strains), respectively. A total of 19 filamentous and non-filamentous cyanobacterial and algal strains were purified by streaking and capillary method or a combination of both techniques belonging to the following genera: *Microcystis* (2 strains), *Phormidium* sp. (1 strain), *Chlorogleaopsis* (1 strain), *Oscillatoria* (2 strains), *Lyngbya* (2 stains), *Chlorosarcinopsis* (2 strains), *Desmodesmus* (3 strains), *Pseudoanabaena*

(3 strains) and *Phormidium* (3 strains). Only 2 non filamentous strains were were isolated by capillary method (Table 4.2).

The cycloheximide was found to be an effective against all contaminants. All strains were grown well on both agar plates with and without cycloheximide except *Microcystis* spp. They isolated from the agar plates, which were not supplemented by cycloheximide.

Table. 4.2. Isolation techniques and culturing conditions of strains isolated from Dian Lake and the Dead Sea grown in BG-11 under continuous light (18.3-18.57 µmol/s/m^2) at 22 °C.

Collection sites	Isolated strains	Sapmle codes	Isolation techniques	Salinity (‰)	References
Dian Lake's bloom isolates	**Phylum Cyanoprokaryota**				
	Order Chroococcales				
	Synechococcus sp.	P5	Serial dilution	0	Komarek and Anagnostidis, (1999)
	Synechococcus sp.	P12	Serial dilution	0	
	Synechococcocus sp.	P16	Serial dilution	0	
	Microcystis sp.	P18	Streaking and capillary method	0	
	Microcystis sp.	P19	Streaking and capillary method	0	
	Order Oscillatoriales				
	Phormidium sp.	P7	Capillary method	0	Komarek and Anagnostidis, (2005)
	Phormidium sp.	P9	Streaking and capillary methods	0	
	Chlorogleopsis sp.	P20	Streaking and capillary methods	0	
	Oscillatoria sp.	P10	Streaking and capillary methods	0	
	Oscillatoria sp.	P11	Streaking and capillary methods	0	
	Oscillatoria sp.	P17	Capillary method	0	
	Lyngbya sp.	P1	Streaking and capillary methods	0	
	Lyngbya sp.	P8	Streaking and capillary methods	0	
	Phylum Chlorophyta				
	Class Chlorophyta				
	Order Chlorellales				
	Chlorella sp.	P3	Serial dilution	0	Canter-Lund and Lund, (1995)
	Chlorella sp.	P15	Serial dilution	0	
	Order Chlorosarcinales				
	Chlorosarcinopsis sp.	P13	Streaking and capillary methods		Trainor and Hilton, (1967)
	Chlorosarcinopsis sp.	P14	Streaking and capillary methods		
	Order Sphaeropleales				
	Desmodesmus sp.	P2	Streaking and capillary methods	0	Canter-Lund and Lund. (1995)
	Desmodesmus sp.	P4	Streaking and capillary methods	0	
	Desmodesmus sp	P6	Streaking and capillary methods	0	
Dead Sea	**Phylum Cyanoprokaryota**				
	Order Oscillatoriales				
	Pseudoanabaena sp.	D11	Streaking and capillary methods	20	Komarek and Anagnostidis, (2005)
	Pseudoanabaena sp.	D15	Streaking and capillary methods	25	
	Pseudoanabaena sp.	D16	Streaking and capillary methods	22	
	Phormidium sp.	D12	Streaking and capillary methods	22	
	Phormidium sp.	D39	Streaking and capillary methods	4	
	Phormidium sp.	D49	Streaking and capillary methods	4	

4.3.1. Identification of cyanobacterial strains isolated from bloom samples from Dian Lake

All strains were identified by traditional microscopy using oil immersion lens on the basis of their cell size, form, cell structures, mucilaginous envelopes and colonial characteristics.

4.3.1.1. Taxonomic description of cyanobacterial strains

Out of 20 isolates 13 cyanobacterial strains were isolated from the bloom samples of the Dian Lake, which belong to two orders i.e. Chroococcales and Oscillatoriales. The identification was based on Komárek and Anagnostidis (1999). The detailed morphology of pure isolates of cyanobacteria and green algae isolated from the Dian Lake have presented in Plates 1-3.

4.3.1.1.1. Chroococcales

4.3.1.1.1.a. *Synechococcus* sp. Nageli 1849 (P5) Cells solitary, cylindrical, slightly curved, two to several times longer than wide, without mucilage and arranged irregularly. They form irregular microscopic clusters of colonies. Cells 1.5-2.5 µm wide and 2.7-3.3 µm long (Plate 1A). In cultures they formed green sheaths attached with the bottom of culturing flasks. Cells divided by binary fission into two isomorphic daughter cells.

4.3.1.1.1.b. *Synechococcus* sp. Nageli 1849 (P12) Cells solitary, cylindrical, straight, two to several times longer than wide, without mucilage and arranged irregularly (Plate 1B). They form irregular microscopic clusters of colonies. Cells 1.6-1.8 µm wide and 2.5-3.0 µm long and form green granules on the bottom of culturing flasks. Cells divided by binary fission into two isomorphic daughter cells.

4.3.1.1.1.c. *Synechococcus* sp. Nageli 1849 (P16) Cells bright green, cylindrical, rounded from corners, solitary and longer (6.0-3.7 µm) than wide (2.1-2.3 µm). The cells were without mucilage homogenous in

the growth medium (Plate 1C).

4.3.1.1.1.d. *Microcystis* sp. Kutzing ex Lemmermann 1907 (P18)

In newly obtained bloom samples the cells were enveloped in transparent mucilage. But this characteristic was lost in old cultures and cells became solitary. The old cultures formed irregular lobate and irregularly arranged colonies and packed with cells. The colonies were packed in colourles mucilage. The solitary cells were pale blue-green, microscopic and more or less spherical (3.5-3.8 x 3.3-3.8 µm; Plate 1D). Within the cell numerous aerotopes can easily be seen under microscope. Bright green solitary cells were homogenous in the growth medium.

4.3.1.1.1.e. *Microcystis* sp. Kutzing ex Lemmermann 1907 (P19)

The blue-green cells form irregular, elongated and lobate colonies enveloped by indistinctly slime in fresh samples and cells were densely packed in the mucilaginous colonies. The isolated pure cultures lost colonial characteristics and cells were observed in solitary form. The cells more or less spherical in diameter (3.5-3.8 x 3.3-3.8 µm) with numerous aerotopes. In newly obtained bloom samples the cells were enveloped in a transpatrent mucilage (Plate 1E). The old and isolated cells lost their mucilaginous covering and become homogenous in the growth medium.

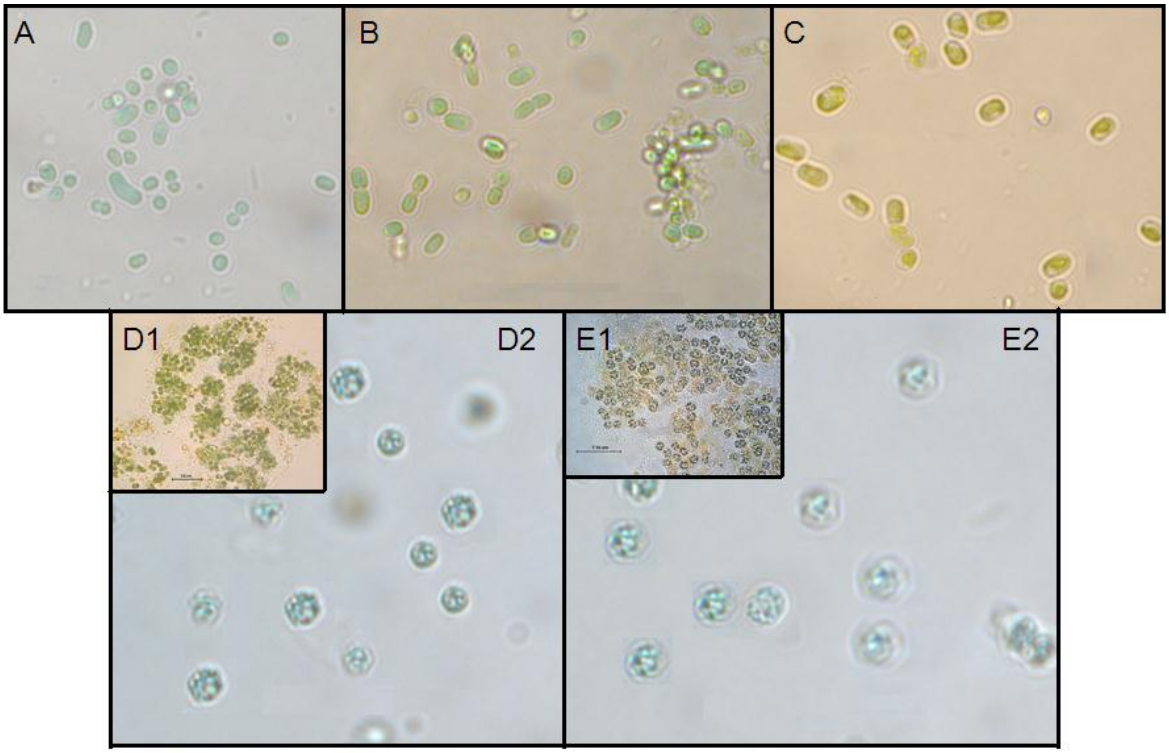

Plate 1. Light micrographs of isolated Chroococcales from blooms samples of Dian Lake

A:- *Synechococcus* sp., **B:-** *Synechococcus* sp., **C:-** *Synechococcus sp.,* **D2:-** *Microcystis* sp. (**D1:-** mucilaginous colony), **E2:-** *Microcystis* sp. (**E1-** mucilaginous colony)

4.3.1.1.2. Oscillatoriales

The identification was based on Komárek and Anagnostidis (2005).

4.3.1.1.2.a. *Phormidium* sp. Kützing ex Gomont 1892 (P7) The filaments of *Phormidium* sp. formed green sheaths on the surface of growth medium. Single trichome was enclosed in a single, gelatinous, firm and thick sheath, which was opened from both ends (Plate 2A). No false branching was observed. Trichomes mainly straight and containing cells (2.6-3.2 µm wide and 4.5-4.8 µm long). The cells were clearly motile inside the sheaths. The gas vesicles were spread over the whole

cell contents.

4.3.1.1.2.b. *Phormidium* sp. Kützing ex Gomont 1892 (P9) Filaments formed dark green mats on the surface of growth medium. Single trichome enclosed in single, gelatinous, firm and thick sheath, opened at the both ends (Plate 2B). No false branching was observed. Trichomes mainly very long, straight and containing cells (2.6-3.0 µm wide and 2.0-2.3 µm long). The cells were clearly motile inside the sheaths. The gas vesicles were spread over the whole cell contents.

4.3.1.1.2.c. *Chlorogleaopsis* sp. Desikachary (P20) The filamentous thallus was dark green and contained 3-6 cells. Sheath was absent. The cells were non-motile, 5.0-1.8 µm broad and 2.7-3.7 µm long (Plate 2C). Apical cells were non-capitate. In laboratory cultures they grew on the bottom of culturing flasks.

4.3.1.1.2.d. *Oscillatoria* sp. Vaucher ex Gomont 1892 (P10) The blue-green trichomes form brownish red mats over the surface of growth medium. Trichomes straight, single, trembling and clearly constricted at cross-walls. Fully grown trichomes were with identical ends. Cell longer (2.8-3.2 µm) than wide (1.3-1.8 µm). Sheath lacking (Plate 2D).

4.3.1.1.2.e. *Oscillatoria* sp. Vaucher ex Gomont 1892 (P11) The blue-green trichomes form brownish red mats over the surface of growth medium. Trichomes straight, single, trembling and clearly constricted at cross-walls. Fully grown trichomes were with identical ends. All cells in the trichome were longer (2.5-3.5 µm) than wide (1.3-1.5 µm), usually several times. Sheath lacking. Apical cells without calyptras (Plate 2E).

4.3.1.1.2.f. *Oscillatoria* sp. Vaucher ex Gomont 1892 (P17) The blue-green trichomes form brownish red mats over the surface of growth medium. Trichomes were straight, single, trembling and clearly constricted at cross-walls. Fully grown trichomes were with identical ends. All cells in the trichome were longer (2.8-3.0 µm) than wide (1.6-2.1 µm), usually several times. Sheath lacking. Apical cells without calyptras (Plate 2F).

4.3.1.1.2.g. *Lyngbya* sp. C. Agardh ex Gomont 1892 (P1) Filaments were straight, solitary and enveloped in sheaths. The filaments were mainly forming thick, compact and dark green sheaths in culturing flasks. Sheaths yellow-brown in colour containing one straight trichome. The sheath opened from both ends. Cells were short and wider (2.0-2.3 µm) than length (1.9 µm). Apical cells identical, more or less rounded without any thickening (Plate 2G).

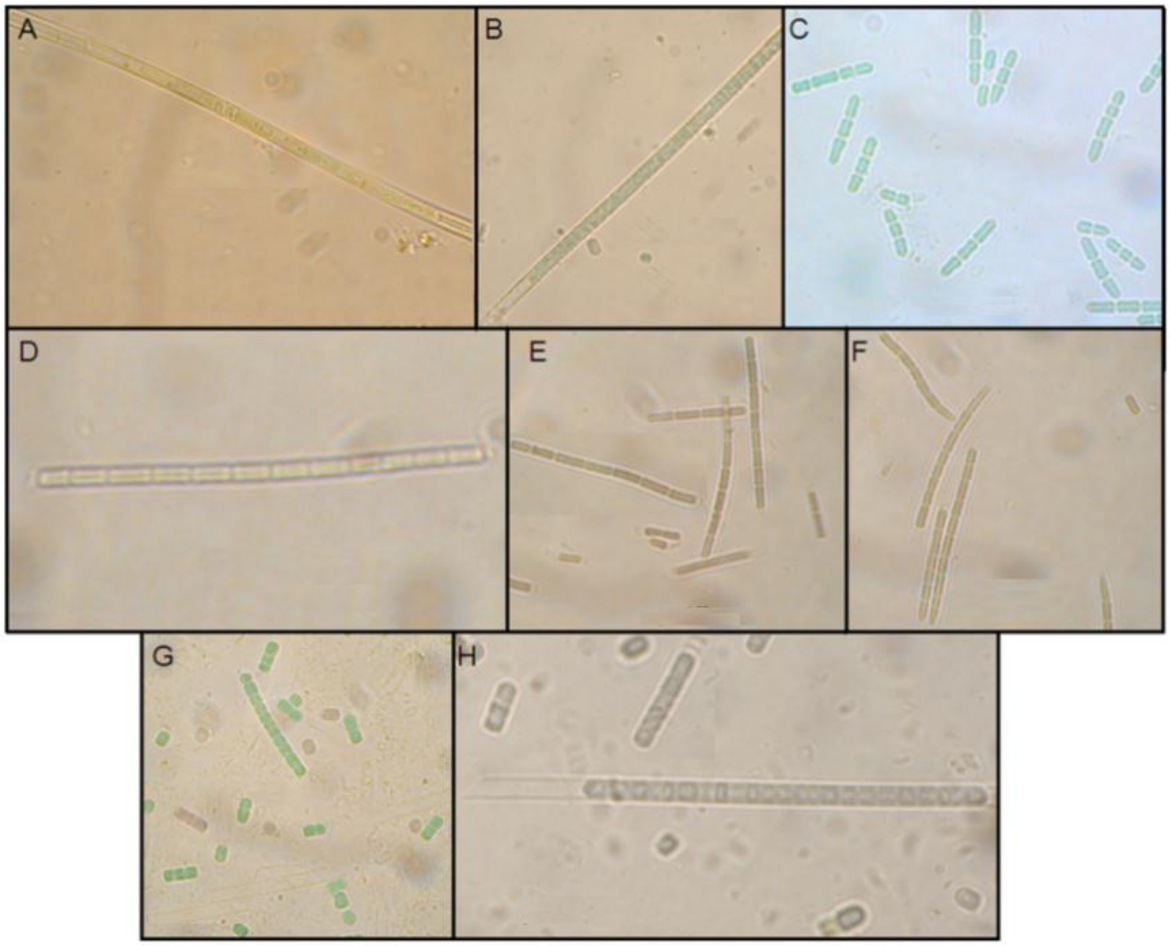

Plate 2. Light micrographs of isolated Oscillatoriales from blooms samples of Dian Lake

A:- *Phormidium* sp., **B:-** *Phormidium* sp., **C:-** *Chlorogleaopsis* sp., **D:-** *Oscillatoria* sp., **E**:- *Oscillatoria* sp., **F:-** *Oscillatoria* sp., **G:-** *Lyngbya* sp*.* **H:-** *Lyngbya* sp*.*

4.3.1.1.2.h. *Lyngbya* sp. C. Agardh ex Gomont 1892 (P8) Filaments long, solitary, straight, non-motile and form dark green large and think sheaths in the growth medium. Fully grown trichomes were morphologically identical and with more or less rounded apical cells. All brownish red cells in the trichomes were solitary, straight, shorter (1.8-2.3 µm) than wide (2.6-2.7 µm) and regularly enveloped in brownish sheaths. Sheaths were gelatinous, thin and firm and cylindrical with open ends (Plate 2H).

4.3.1.2. Taxonomic description of chlorophytes

Out of 20 isolates 7 chlorophytes were isolated from the bloom samples, which belong to three orders i.e. Chlorellales, Chlorosarcinales and Sphaeropleales. These strains were identified by traditional microscopy using oil immersion lens (Olympus, Japan) on the basis of their cell size, form, cell structures and colonial characteristics.

4.3.1.2.1. Chlorellales

The identification was based on Canter-Lund and Lund (1995) and Graham and Wilcox (2000).

4.3.1.2.1.a. *Chlorella* sp. Beijerinck 1890 (P3) The bright green coloured cells of *Chlorella* sp. are free living or endosymbionts. The cells were found solitary, planktonic and homogenous in the growth medium and 3.1-4.0 x 3.1-3.4 µm in diameter. The shape of cells varied from spherical, subspherical, semi-spherical or irregular-rounded forms (Plate 3A). The chloroplast is partietal. Asexual reproduction is by the formation of four autospores.

4.3.1.2.1.b. ***Chlorella*** **sp. Beijerinck 1890 (P15)** The cells were green to olive green in colour. They formed microscopic, regular or more or less spherical green cells 3.5x3.5 µm in diameter (Plate 3B). The bright green cells were homogenous in stagnant medium. The cells divided by binary fission.

4.3.1.2.2. Chlorosarcinales

4.3.1.2.2.a. ***Chlorosarcinopsis*** **sp. Herndon 1958 (P13)** This freshwater green alga has solitary, very adherent and spherical cells tightly packed in colonies. The cells and colonies packed in firm gelatinous material. Cells were 7.3-9.7 µm in diameter with adjacent sides flattened when in colonies (Plate 3C). The cells contain uni-nucleate and granular cytoplasm with no vacuoles in vegetative cells. The colonies settled on the bottom of culturing flask in the form of small green balls. Cell divided by binary fission and form tetrads.

4.3.1.2.2.b. ***Chlorosarcinopsis*** **sp. Herndon 1958 (P14)** This freshwater unicellular freshwater green alga form small rounded and floating colonies on the bottom of culturing flask. Individual cells were spheres (5.7-11.4 µm in diameter), solitary, tightly packed together into grape like groups with smooth regular to irregular cell wall. The cells and colonies packed in firm gelatinous material (Plate 3D). The cells contained uni-nucleate and granular cytoplasm with no vacuoles in vegetative cells. Cell divided by binary fission and form tetrads.

4.3.1.2.3. Sphaeropleales

4.3.1.2.3.a. ***Desmodesmus*** **sp.** **Meyen 1829 (P2)** *Desmodesmus* is commonly found in the plankton of freshwater rivers, ponds, and lakes,

and sometimes in brackish habitats. The free living or epiphytic cells of *Desmodesmus* sp. formed small balls, settled on the bottom of culturing flasks. They formed microscopic and irregular colonies (11.8-16.3 x 6.9-7.8 µm). The multicellular colonies were packed in packet-like, 2-4 celled groups (2.1-16.3 x 6.9-7.8 µm). The terminal serrated cells had long and slightly curved spines (2.6-3.5 µm). No middle spines were observed. The cells contained green homogenous granules with a central or sub-central vacuole (Plate 3E).

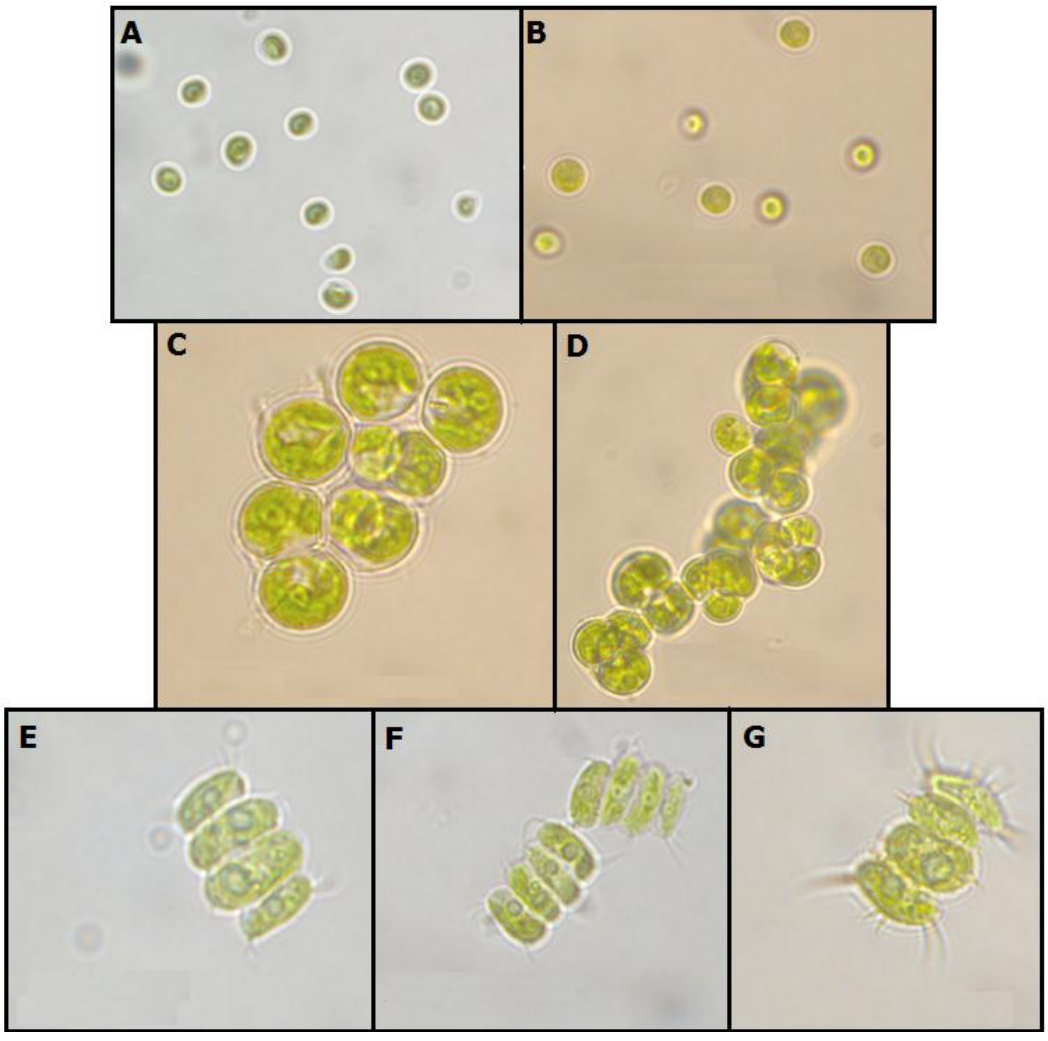

Plate 3. Light micrographs of isolated Chlorophyceans from blooms samples of Dian Lake

A:- *Chlorella* sp., **B:-** *Chlorella* sp., **C:-** *Chlorosarcinopsis* sp., **D:-** *Chlorosarcinopsis* sp., **E:-** *Desmodesmus* sp., **F:-** *Desmodesmus* sp., **G:-** *Desmodesmus* sp.

4.3.1.2.3.b. *Desmodesmus* sp. Meyen 1829 (P4) The free living bright green cells of *Desmodesmus* sp. appeared as a free living group of 2-4 cells (4.3-5.0 x 7.5-9.5 µm) and arranged in a row. The serrated terminal cells had long and slightly curved spines (3.5-4.2 µm) on each corner of the cell. No middle spines were observed in the middle of the outer cell. The species form scattered colonies (12.8-16.1x7.5-9.5 µm) on the bottom of culturing flasks. The cells contained green homogenous granules and a central or sub-central vacuole (Plate 3F).

4.3.1.2.3.c. *Desmodesmus* sp. Meyen, 1829 (P6) The free living or epiphytic and bright green cells formed small, irregular and rounded colonies (13.4-18.6x7.0-8.5 µm) settled on the bottom of culturing flasks. The multicellular colonies were packed in packet-like, 2-4 celled groups (3.6-4.5 x 7.2-8.9 µm). The terminal cells had long and slightly curved spines (4.3-5.8 µm) and 2-4 middle and small spines. The cells contained green homogenous granules with a central or sub-central vacuole (Plate 3G).

The species composition of Dian Lake isolates was dominated by genera *Synechococcus, Oscillatoria* and *Desmodesmus*. Some other diatoms (*Navicula* sp., *Surirella* sp., *Pinnularia* sp.), cyanobacterium (*Chroococcus* sp.) and green microalgae (*Pediastrum* sp.) were also observed under microscopic examination (Fig. 4.5).

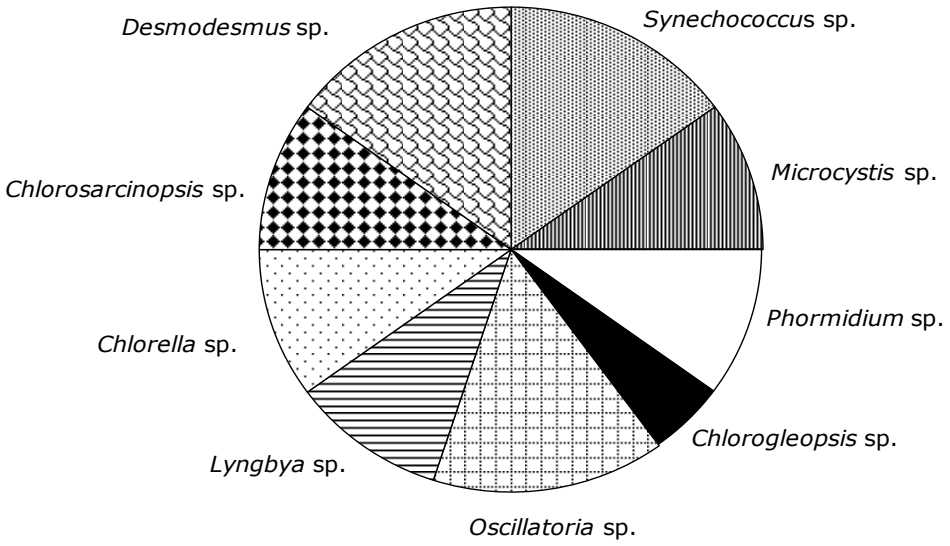

Figure.4.5. The relative abundance of cyanobacterial and microalgal isolates from the Dian Lake, China.

4.3.2. Identification of cyanobacterial strains isolated from the Dead Sea

Six cyanobacterial strains were isolated from the Dead Sea samples. These strains belonged to one order Oscillatoriales. These strains were identified by traditional microscopy using oil immersion lens (Olympus, Japan) on the basis of their cell size, form, cell structures, mucilaginous envelopes and colonial characteristics. Some unidentified organisms were also isolated from the samples of the Dead Sea.

4.3.2.1. Taxonomic description of cyanobacterial strains

4.3.2.1.1. Oscillatoriales

The identification was based on Komárek and Anagnostidis (2005).

4.3.2.1.1.a. *Pseudoanabaena* sp. Lauterborn (D11) Green, solitary and motile, trichomes. Old cultures form planktonic fine mats. Cylindrical

trichomes contain several cells with clear constrictions at cross-walls. Trichomes without sheaths. Slow gliding motility. Cells cylindrical with rounded and identical ends, longer than wide almost double in length (5.38-5.48 µm) than wide (1.01-1.06 µm; Plate 4A).

4.3.2.1.1.b. *Pseudoanabaena* sp. Lauterborn (D15) Trichomes, solitary, green and motile on their places. Filaments without sheaths, short consisted of few cells with remarkable constrictions at cross-walls. The clusters of trichomes formed benthic sheaths on the bottom of culturing Erlenmeyer flask. Cylindrical cells longer (1.85-1.86 µm) than wide (1.18-1.68 µm; Plate 4B).

4.3.2.1.1.c. *Pseudoanabaena* sp. Lauterborn (D16) Trichomes green, straight or slightly curved, strongly constructed, motile on their places consisted of very few to several cells. Filaments without sheath. Cell cylindrical, longer (2.19 -2.23 µm) than wide (1.52-1.64 µm). The cells were usually rounded cylindrical with rounded corners. Apical cells were rounded and identical, without calyptras and thickened outer cell wall. The clusters of trichomes formed benthic sheaths (Plate 4C).

4.3.2.1.1.d. *Phormidium* sp. Kützing ex Gomont 1892 (D12) Thallus bright green, large filaments that were somewhat curved and showed gliding movements. The cells were broad 1.35-1.67 µm and 1.52-2.52 µm long. Cells non-capitate, longer than broad, constricted at the ends and at cross walls (Plate 4D). Thin and simple sheaths were present. Under laboratory condition they were grown on the bottom of flasks in the form of thick and compact colonies.

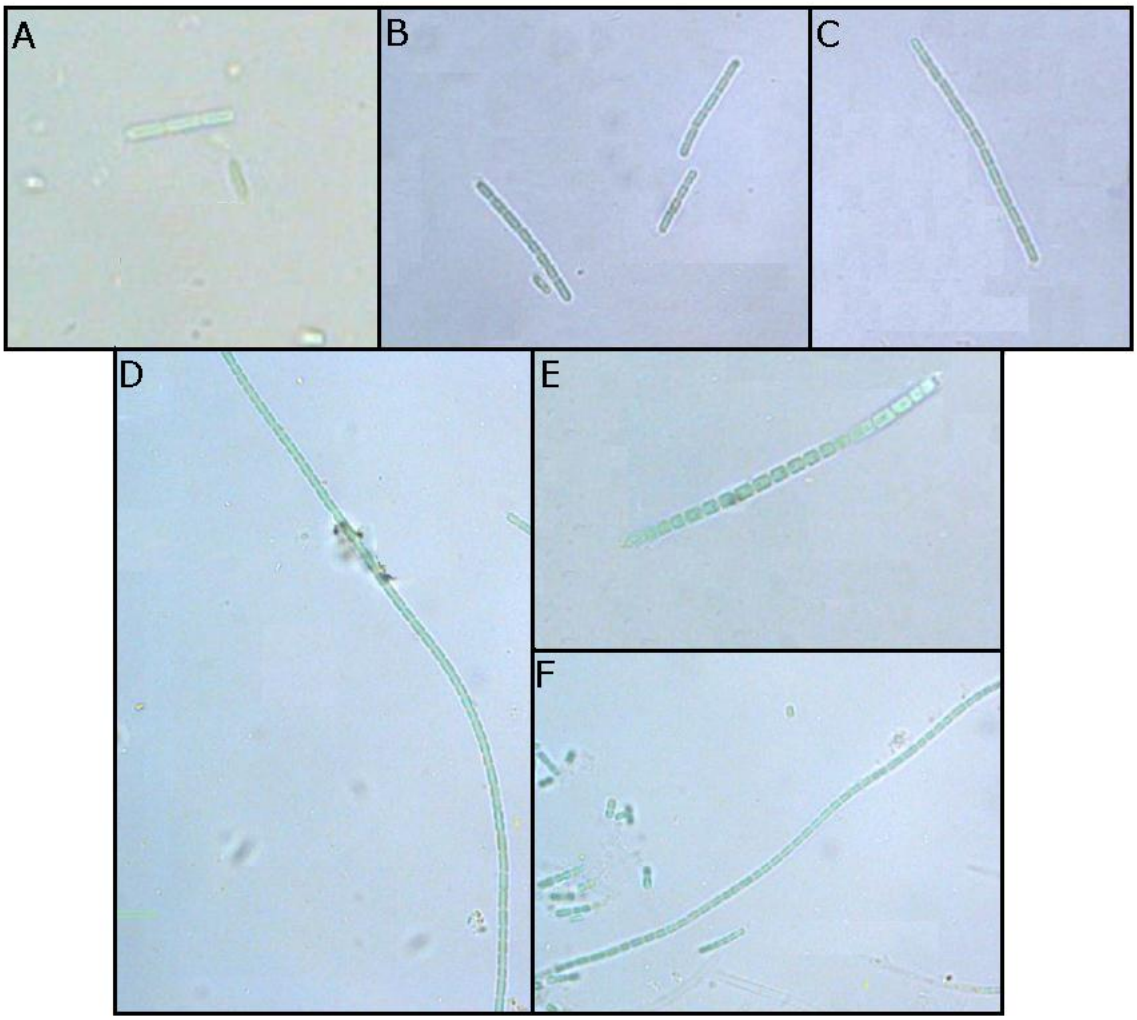

Plate 4. Light micrographs of isolated Oscillatoriales from the Dead Sea samples

A:- *Pseudoanabaena* sp., **B:-** *Pseudoanabaena* sp.,
C:- *Pseudoanabaena* sp., **D:-** *Phormidium* sp.,
E:- *Phormidium* sp., **F:-** *Phormidium* sp.

4.3.2.1.1.e. *Phormidium* sp. Kützing ex Gomont 1892 (D39) Cells were bright-green in colour, mucilaginous sheath present, beaded appearance as cells slightly separated from each other, formed small colonies, 2.10-212 µm broad and 1.67-2.86 µm long. Filaments were long and non-motile. Apical cell was non-capitate and rounded (Plate 4E). Under laboratory conditions they were grown on bottom of flasks in the form of dense sheaths. On shaking they were mixed thoroughly in

culturing medium.

4.3.2.1.1.f. *Phormidium* sp. Kützing ex Gomont 1892 (D49)

Trichomes solitary, planktonic, green in colour, form sheaths, non motile, beaded appearance and separated from each other. Filaments without sheaths form dense colonies on the bottom of culturing flask. Cells longer (2.35-2.36 µm) than wide (1.34-1.35 µm; Plate 4F).

The isolates of the Dead Sea showed that the genera *Pseudoanabaena* and *Phormidium* were commonly occurring and easy to isolate strains. In the fresh samples of the Dead Sea several other microorganisms were also observed belonged to bacteria (Order Halobacteriales), Pennate diatoms (*Synedra* sp., *Pseudo-nitzschia* sp., *Navilcula* spp., *Pinnularia* sp., *Cymbella* sp.), cyanobacterium (*Chroccoccus* sp.) and some new and unidentified strains.

4.3.3. Analysis of pure isolates

Aqueous methanolic extracts of isolated cyanobacterial, micro green algal and natural bloom samples of the lake were analysed on UPLC-PDA-MS to detect presence of any compound/s. Only three isolates i.e. *Microcystis* sp. (P18) and *Microcystis* sp. (P19) from the Dian lake and *Pseudoanabaena* sp. (D15) from the Dead Sea showed presence of peaks.

4.3.4. Identification of compounds in bloom samples of Dian Lake

The generic RP method was used for cyanobacterial peptides analysis. The UPLC – TIC ESI+ of extracts of bloom samples showed several peaks of cyanopeptolins and MC-LR (Fig. 4.6A-D). The fragments of compounds

observed in the mass spectra represented presence of two cyanopeptolins with *m/z* 1020 (Fig. 4.7A) and *m/z* 992 (Fig. 4.7B).

The extracts showed clear fragments indicative of Aph moiety. The positive ion electrospray chromatograms also indicated the presence of compounds (Fig. 4.8). The structural fragments of MS/MS of pk *m/z* 993.53 confirmed presence of two fragments *m/z* 150.1 (MeTyr), 243.13 (Aph-Phe-H_2O) and 420.22 (Aph-Phe-Metyr; Fig.4.8). The extracted ion chromatogram at *m/z* 150 (MeTyr) indicated several cyanopeptolins and other five minor cyanopeptolins (Fig. 4.9).

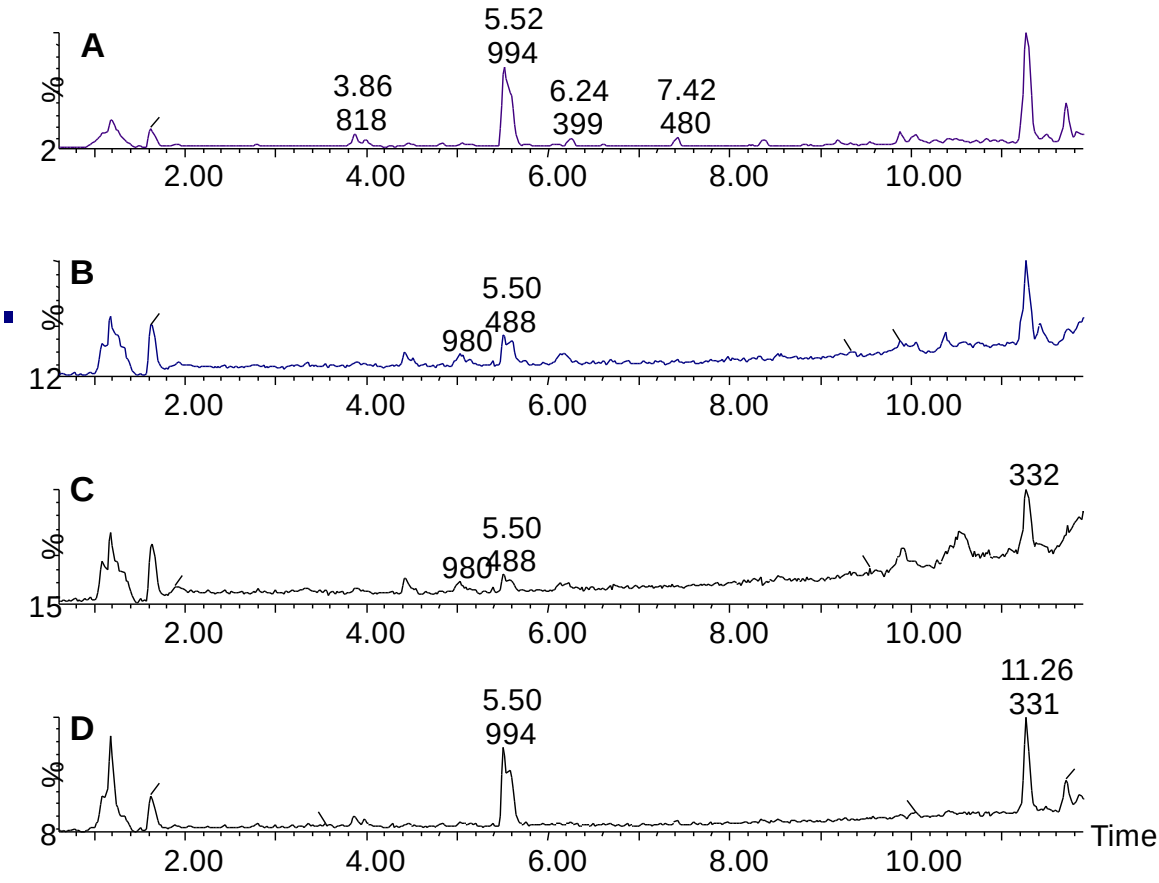

Figure 4.6. UPLC - TIC ESI+ of extracts of bloom material from 4 locations at Dian Lake

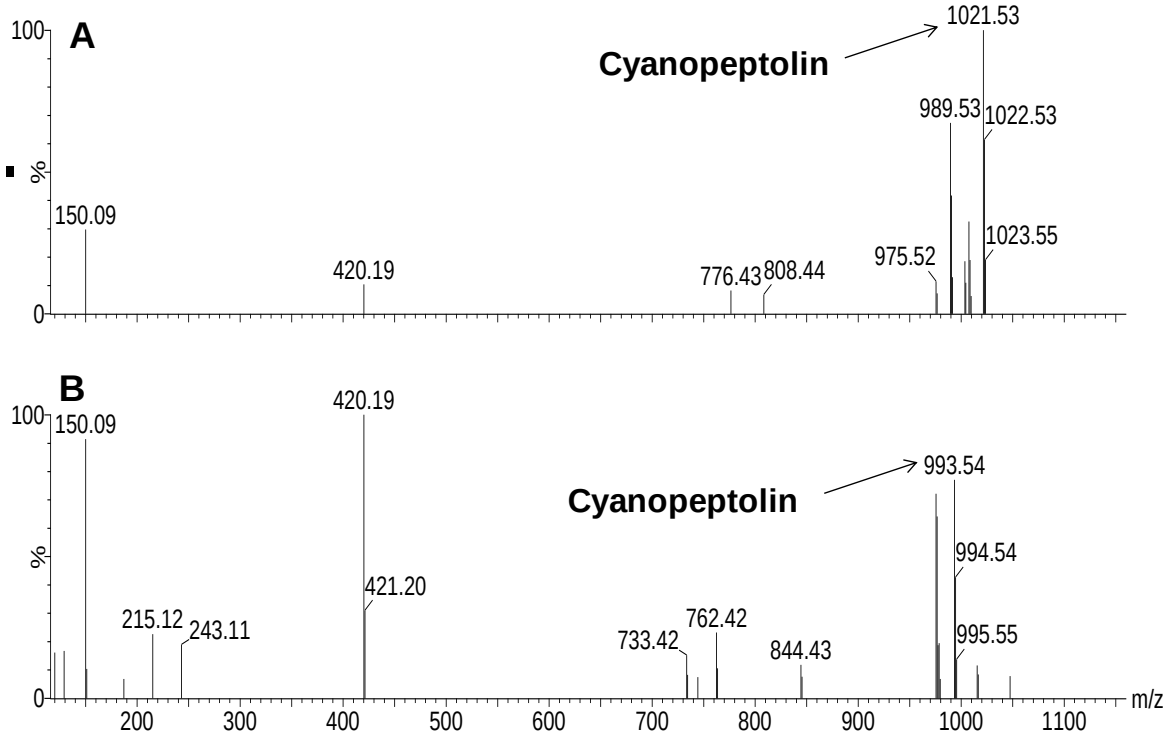

Figure 4.7. Mass spectra of predominant compounds (unresolved by UPLC gradient) in extracts from *Microcystis* bloom material from Lake Dian, China

Characteristic fragments suggest tentative identification as

A:- cyanopeptolin MW 1020, $[M+H]^+$ at *m/z* 1021 (could be a or b) and

B:- cyanopeptolin MW 992, $[M+H]^+$ at *m/z* 993

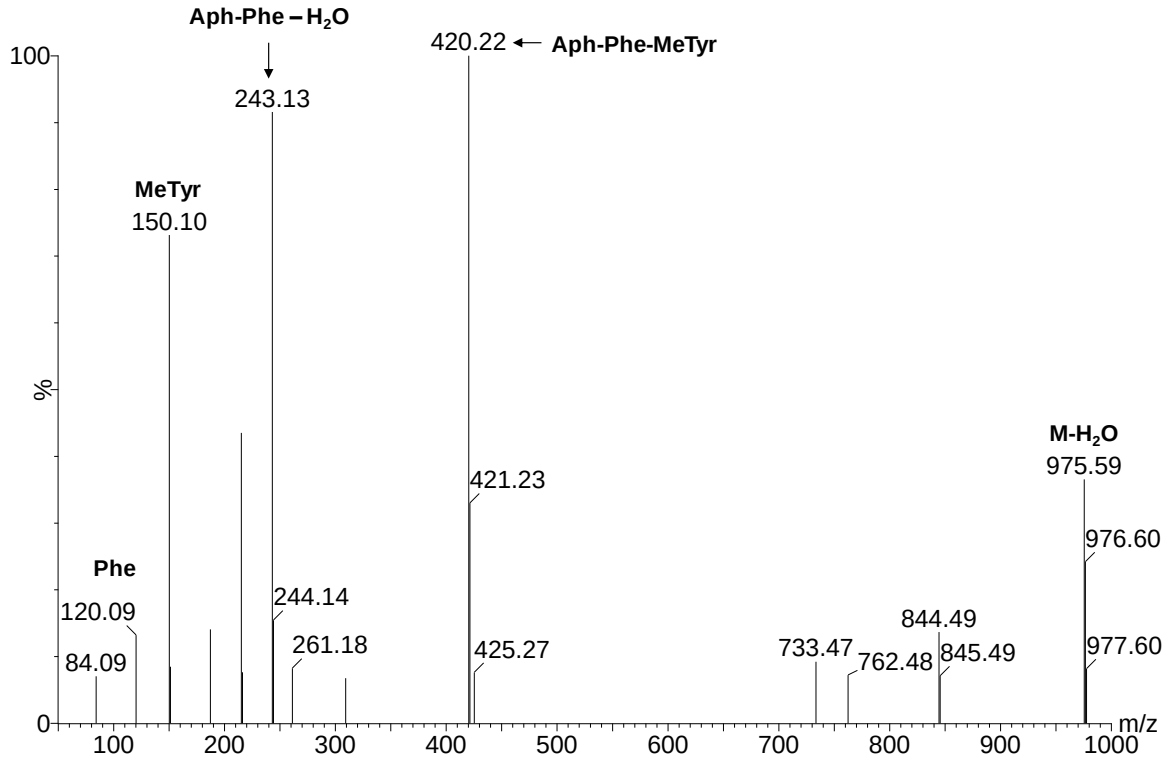

Figure 4.8. MS/MS confirmation of major compound in bloom material from Lake Dian with common fragments associated with the unusual amino acid – 3-amino-6-hydroxy-piperidone, which is characteristic of this large group of compounds.
(NOTE: all fragments are protonated)
Cyanopeptolin 992 [Lys-Aph-Phe-MeTyr-Val-O]-Glu-HA

A number of other minor cyanopeptolins were also found in the isolates and only cyanopeptolin 1006A ([Arg-Aph-Phe-MeTyr-Val-O]-Glu-OA could identified (Fig. 4.9). Other cyanopeptolins are new unreported variants of this large peptide family where there is high variability in amino acid and side chain composition and Aph is highly conserved (Fig. 4.9).

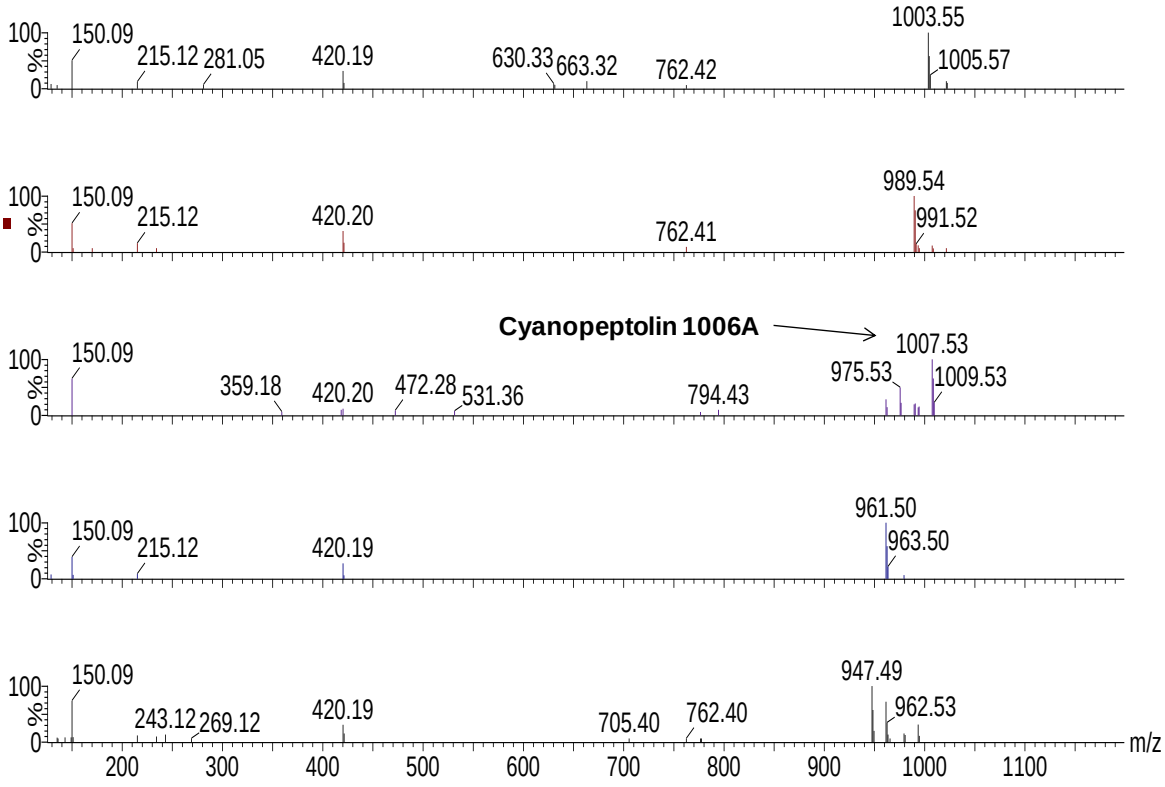

Figure 4.9. Presence of other minor cyanopeptolins as indicated by fragments at *m/z* 150 and 420 (MeTyr and Aph-Phe-MeTyr)

4.3.5. Identification of bioactive peptides in isolates

4.3.5.1. *Microcystis* sp. (P18): The pure isolate of *Microcystis* sp. (P18) showed 5 clear peaks of compounds ranged from 6.61-11.10 min (Fig. 4.10)

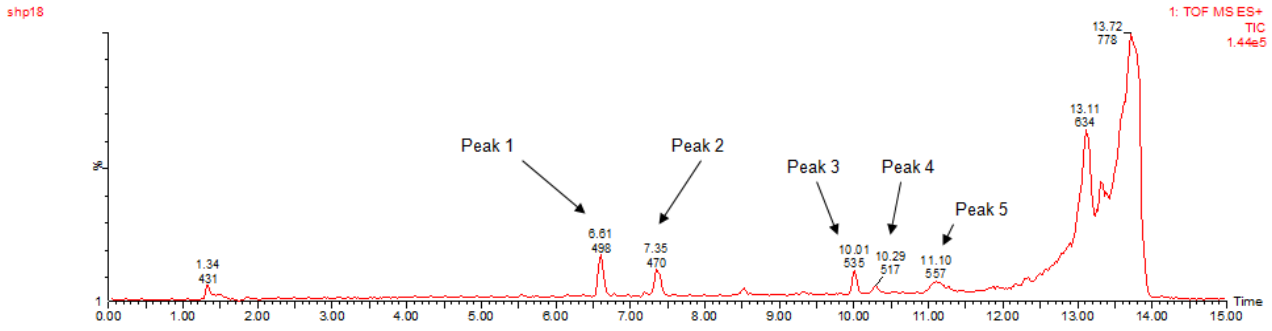

Fig. 4.10. Chromatogram of *Microcystin* sp. (P18) indicated the presence of five peaks in the bloom material.

4.3.5.2. *Microcystis* sp. (P19): The pure and isolated culture of *Microcystis* sp. (P19) also showed presence of a number of compounds. The mass spectra revealed five major peaks ranged from 6.58–10.31 min (Fig. 11).

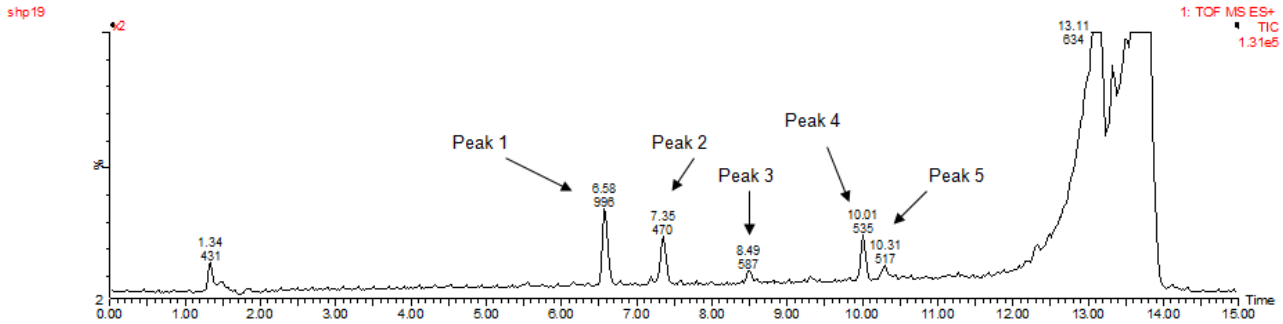

Fig. 4.11. Chromatogram of *Microcystin* sp. (P19) indicated the presence of five peaks.

4.3.5.3. Time of flight mass spectrometry (ToF)

The extracted ion chromatograms of methanolic extracts of both isolates from Dian Lake *Microcystis* spp. (P18 and P19) indicated the clear peaks of one microcystin-LR (MW 995 Da), one desipeptide, cyanopeptolin A (MW 957 Da) and four hexacyclopeptides, aerucyclamide A (MW 957 Da), B (MW 533 Da), C (MW 517 Da) and D (MW 587 Da; Fig. 4.12). The TIC ESI+ spectrum at high (Fig. 4.13A) and low (Fig. 4.13B) energy also confirm the presence of compounds (Fig 4.14). For further confirmation of aerucyclamides A-D peaks were analysed by ToF MS ESI+ (Figs. 4.15-4.18).

A detailed summary of compounds identified in *Microcystis* spp. is shown in Table 4.3.

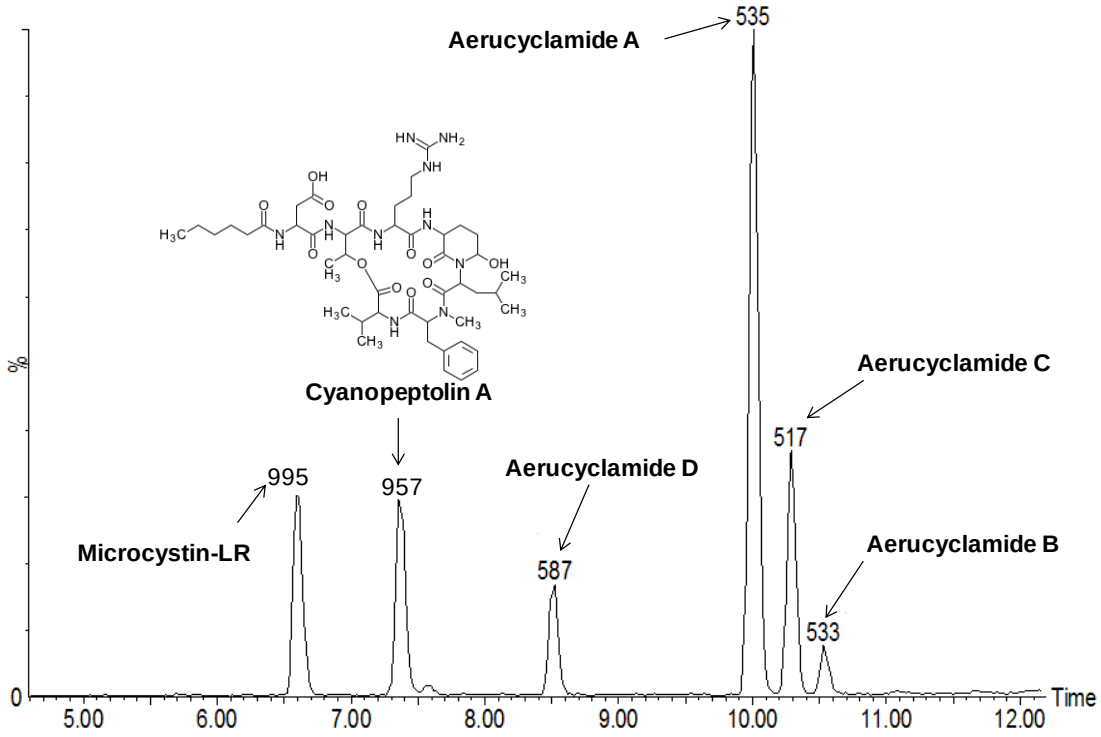

Figure 4.12. Extracted ion chromatogram of major compounds at low energy

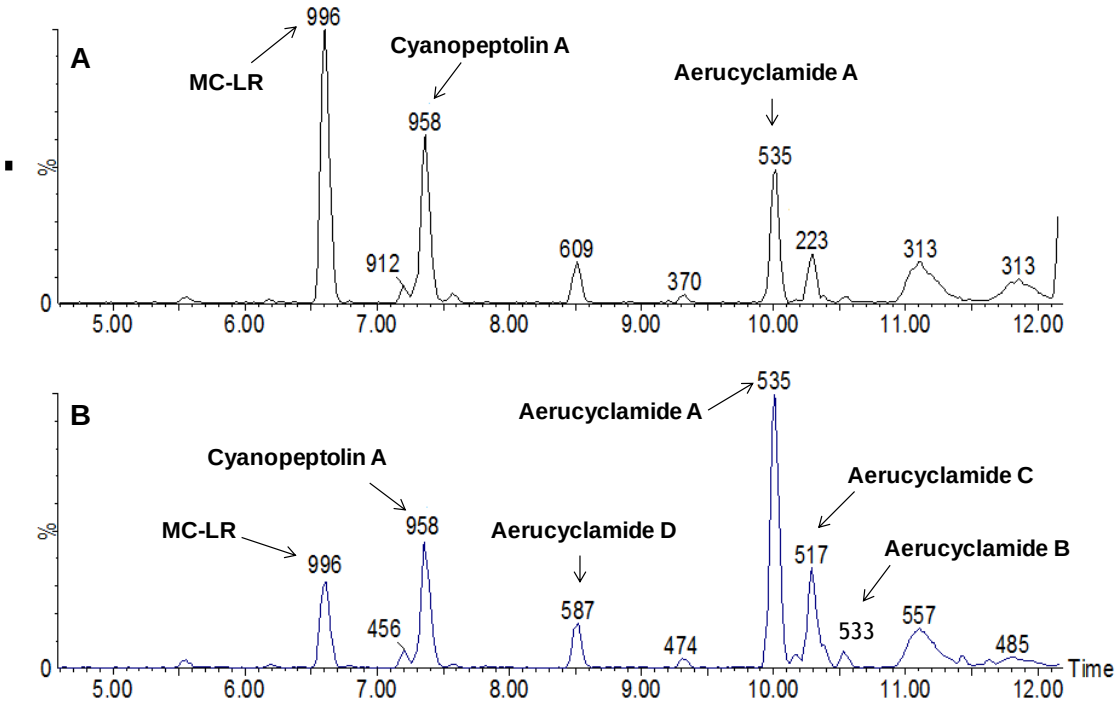

Figure 4.13. TIC ESI+ spectrum of compounds identified in *Microcystis* spp. (P18 and P19) at *m/z* 50-12000
A:- at high energy; **B:-** at lower energy
(MC-LR MW 995, $[M+H]^+$ at *m/z* 996; cyanopeptolin A MW 957 $[M+H]^+$ at *m/z* 958; aerucyclamide A MW 535; aerucyclamide B MW 533 aerucyclamide C MW 517 aerucyclamide D MW 587)

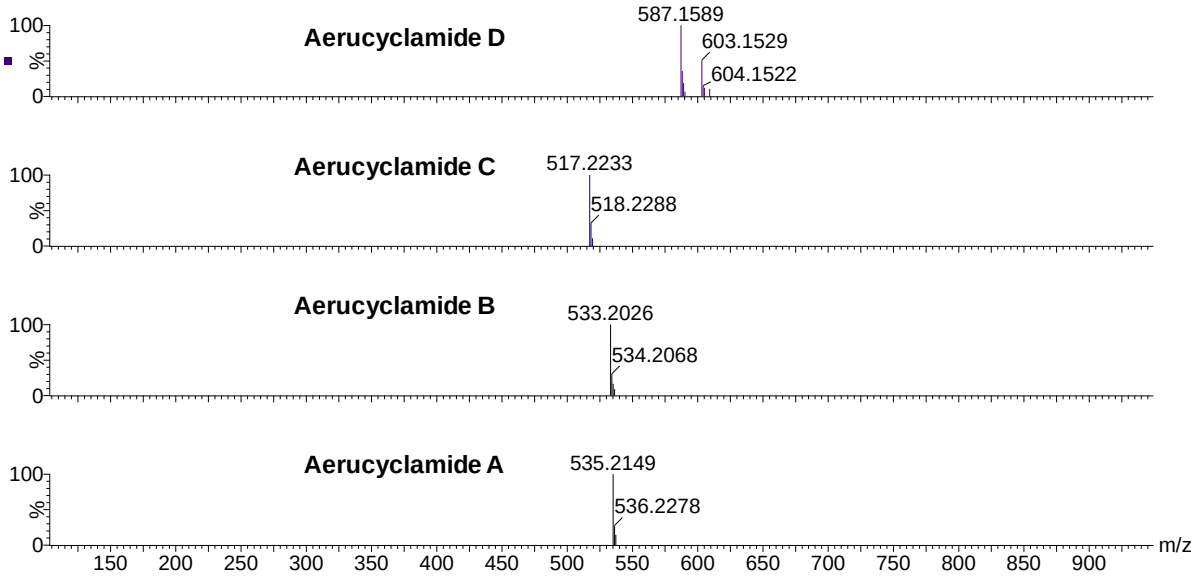

Figure 4.14. Chromatograms of *Microcystis* spp. isolated from bloom samples of Dian Lake, show the four aerucyclamides (A-D)

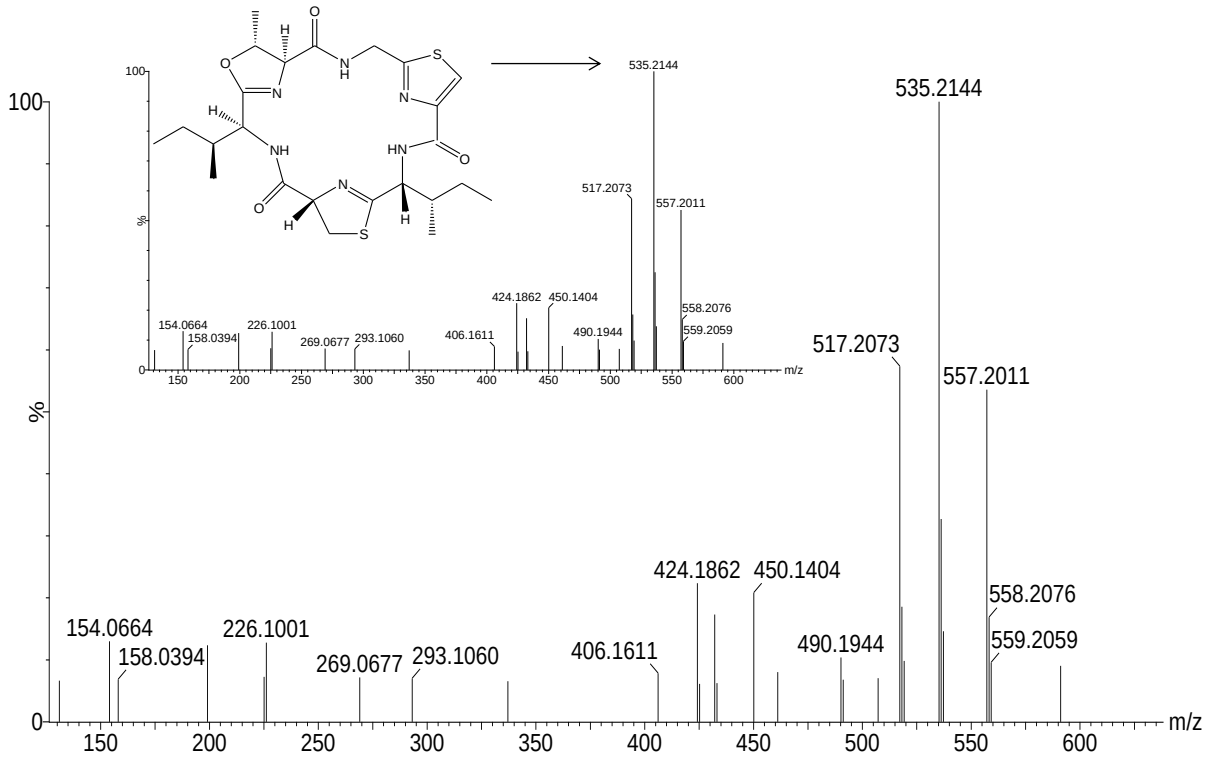

Figure 4.15. ToF MS ESI+ spectrum of aerucyclamide A, *m/z* 535 (**A**), extracted spectrum (**B**) and chemical structure of aerucyclamide A (**C**).

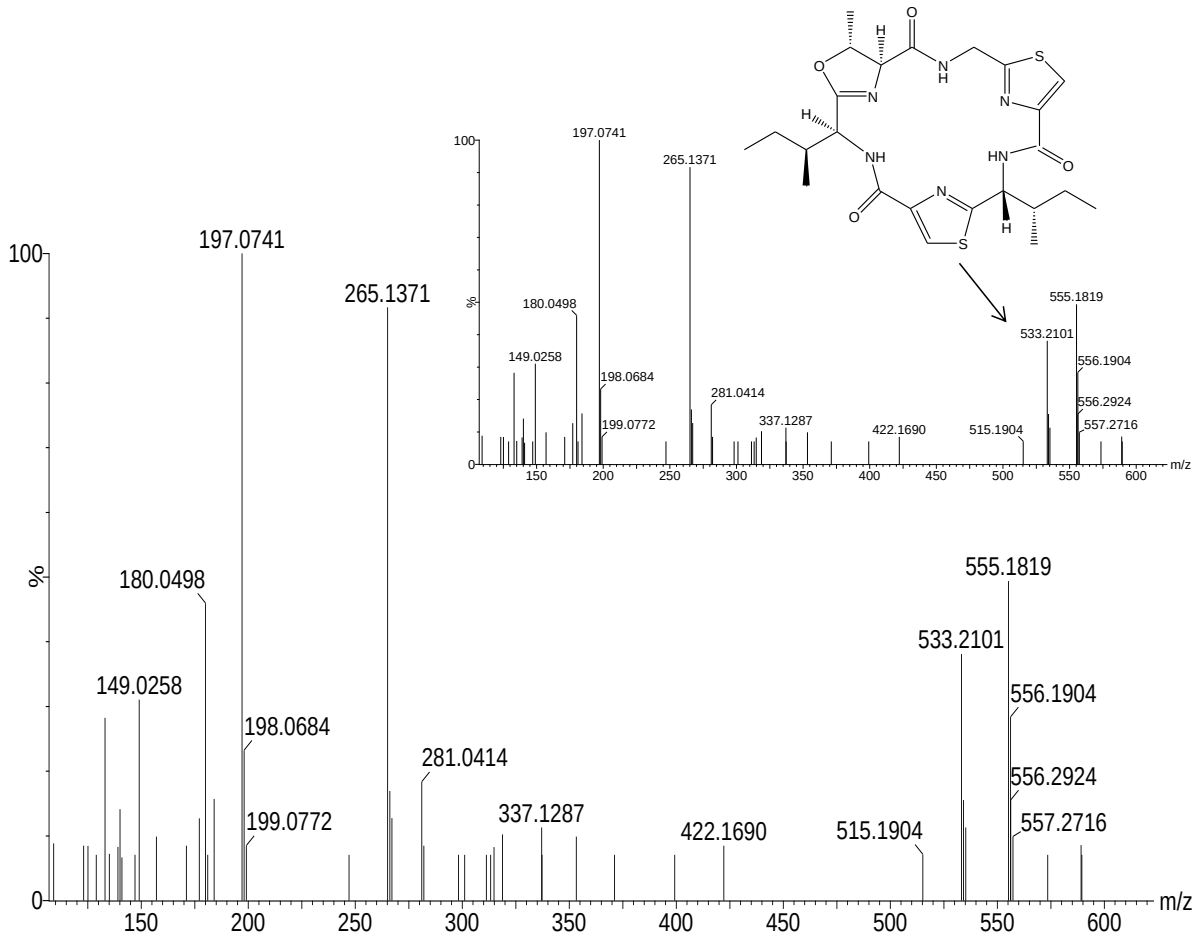

Figure 4.16. ToF MS ESI+ spectrum of aerucyclamide B, *m/z* 533 (**A**), extracted spectrum (**B**) and chemical structure of aerucyclamide A (**C**).

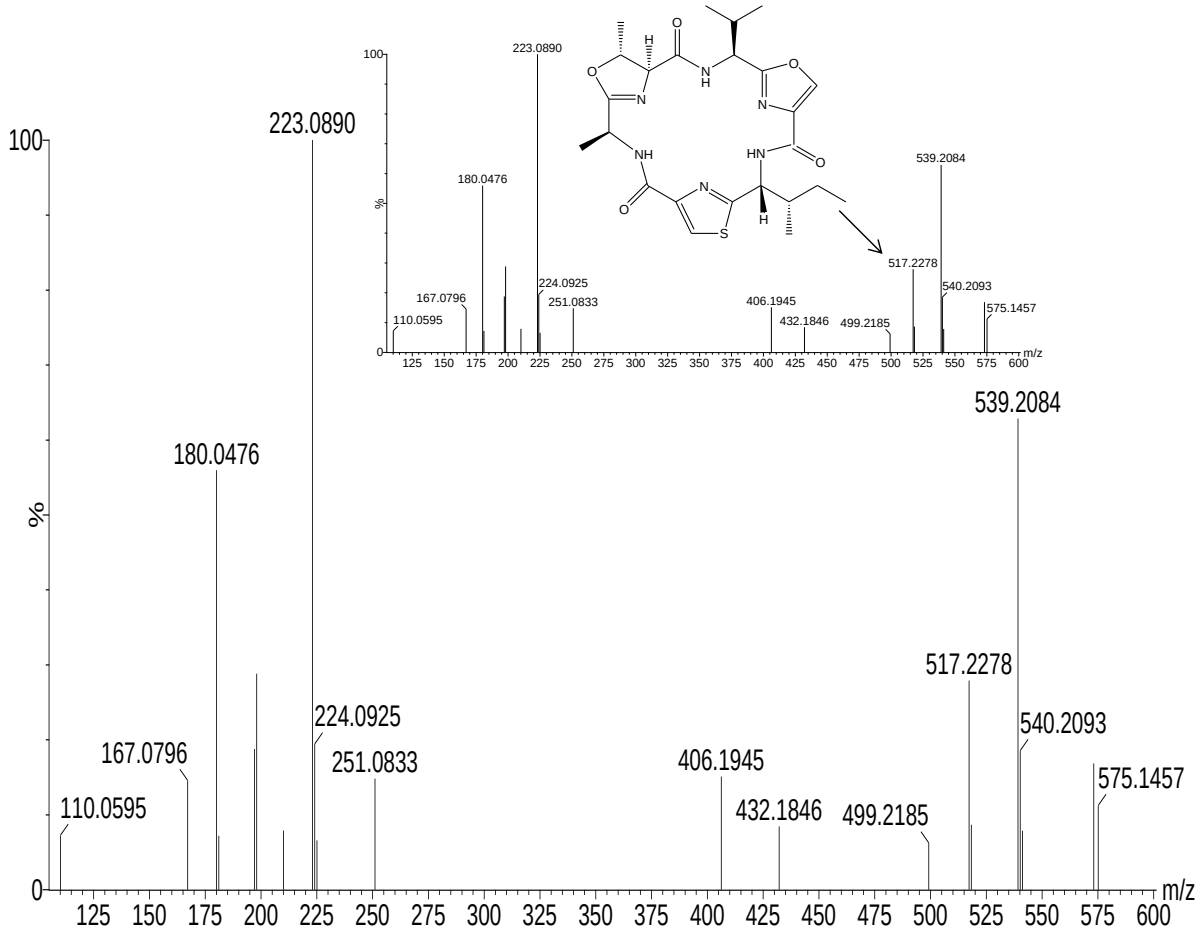

Figure 4.17. ToF MS ESI+ spectrum of aerucyclamide C, *m/z* 517 (**A**), extracted spectrum (**B**) and chemical structure of aerucyclamide A (**C**).

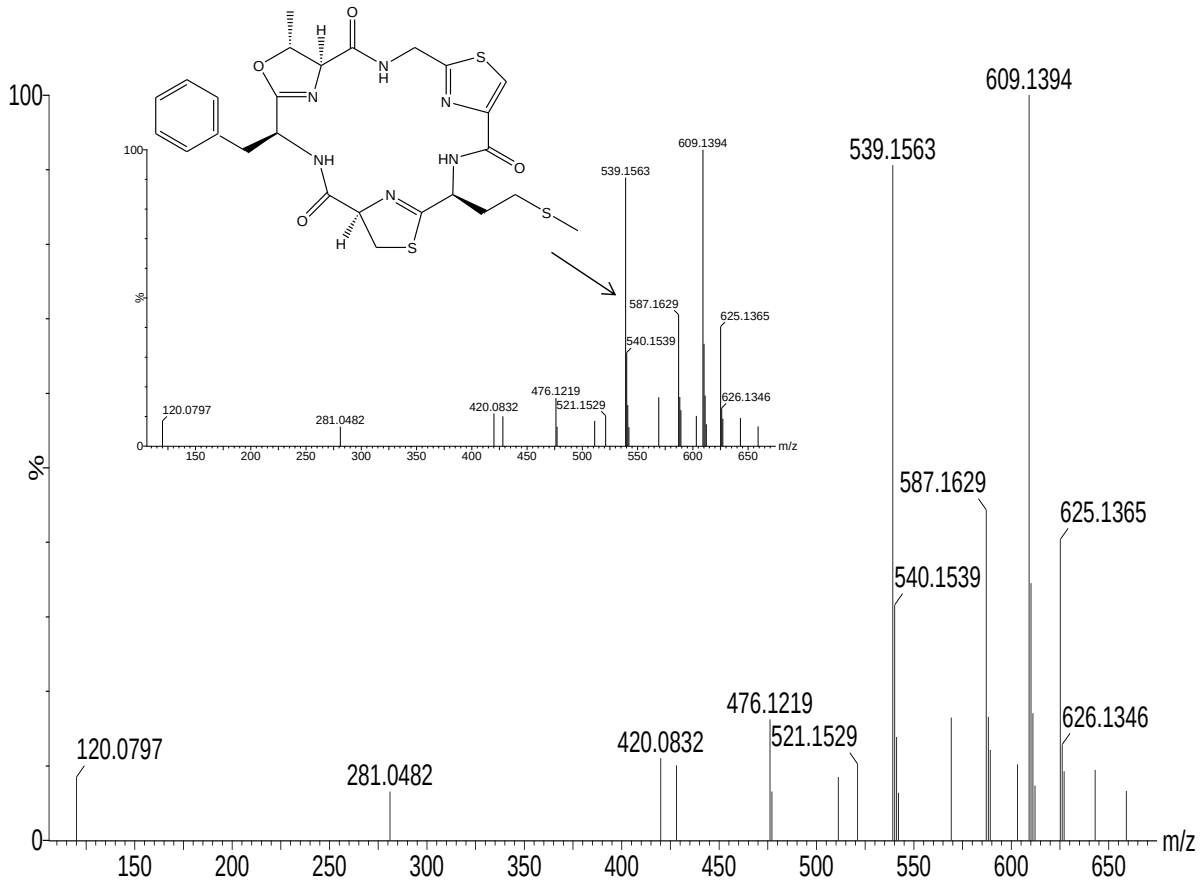

Figure 4.18. ToF MS ESI+ spectrum of aerucyclamide D, *m/z* 587 (**A**), extracted spectrum (**B**) and chemical structure of aerucyclamide A (**C**).

Table 4.3. Identification of MC-LR (standard reference), cyanopeptolin A (standard reference), aerucyclamides (A-D) in *Microcystis* spp. using elemental composition tool in MassLynx 4.1

Compound	Chemical formula	Calculated mass [M+H]+	Calculated mass [M+H]+(Δppm)	References
Microcystin–LR	$C_{49}H_{74}N_{10}O_{12}$	995.56		Welker *et al* . (2006)
Cyanopeptolin A	$C_{46}H_{72}N_{10}O_{12}$	957.54		Martin *et al.,* (1993) Welker *et al* ., (2006)
Aerucyclamide A	$C_{24}H_{34}N_6O_4S_2$	535.2161	535.2149 (2.2)	Portmann *et al.,* (2008a)
Aerucyclamide B	$C_{24}H_{33}N_6O_4S_2$	533.2005	533.2026 (3.9)	Portmann *et al.,* (2008a)
Aerucyclamide C	$C_{24}H_{33}N_6O_5S$	517.2233	517.2233 (0)	Portmann *et al.,* (2008b)
Aerucyclamide D	$C_{26}H_{31}N_6O_4S_3$	587.1569	587.1589 (3.4)	Portmann *et al.,* (2008b)

4.3.6. Identification of compounds in the Dead Sea isolates

4.3.6.1. *Pseudoanabaena* sp. (D15):

The chromatogram of *Pseudoanabaena* sp. showed the presence of two peaks (Fig. 4.19). The ToF MS IES+ spectra of peak one was showed *m/z* 561 at low and high energy voltages (Fig. 4.20). The analysis of ToF MS IES+ spectra of peak 2 was showed *m/z* 575 at low and high energy voltages (Fig. 4.21). Due to new nature of compounds no data was available to identify peaks.

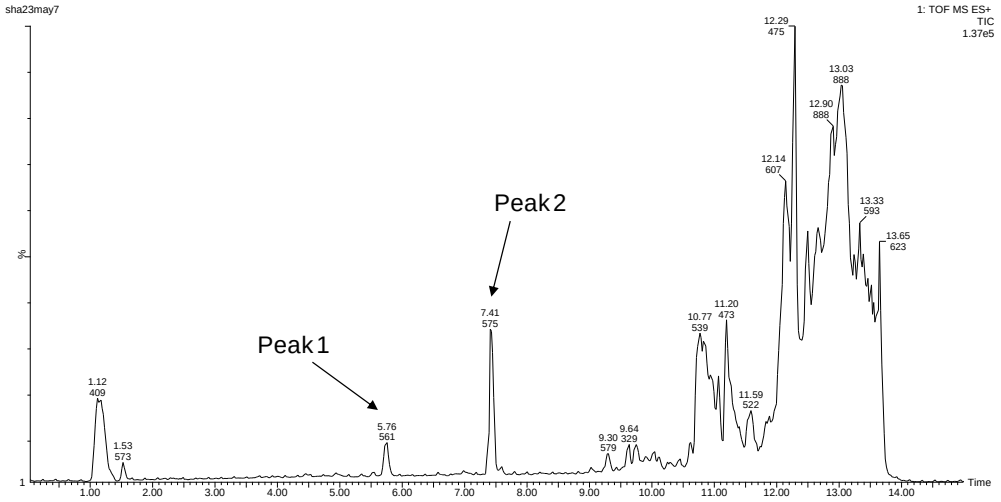

Figure 4.19. Chromatogram of pure isolate of *Pseudoanabaena* sp. (D15) indicates two clear peaks

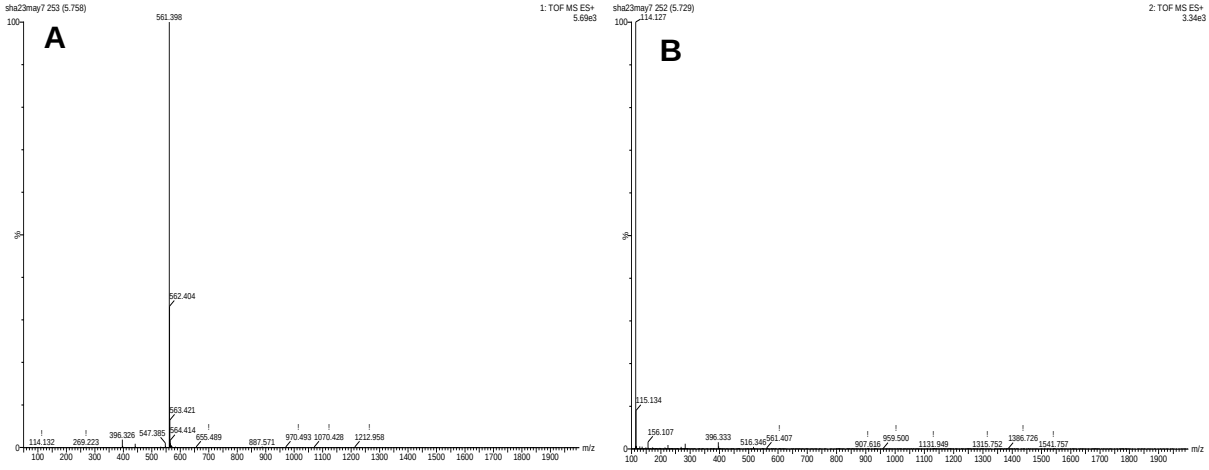

Figure. 4.20. ToF MS IES+ spectra of peak one *m/z* 561 at low (**A**) and high (**B**) energy voltages

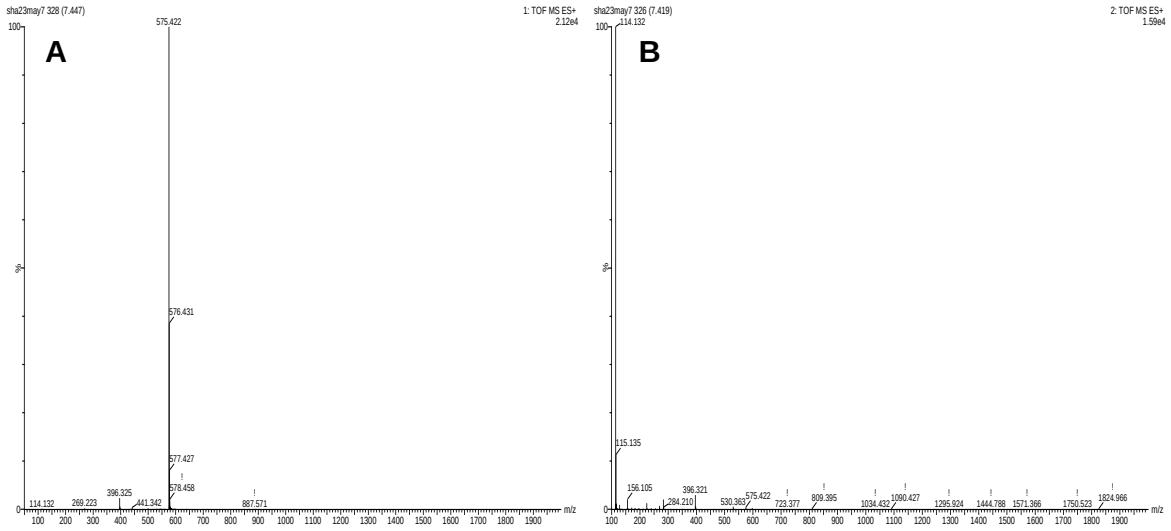

Figure 4.21. ToF MS IES+ spectra of peak 2 *m/z* 575 at low (A) and high (B) energy voltages

4.4. DISCUSSION

4.4.1. Isolation and identification of cyanobacteria

In this study double strength BG-11 was used to prepare agar plates because cyanobacterial growth rate is very slow and they take 4-5 weeks to grow. The slow rate of strains results in depletion of nutrients present in the agar as previously recommended by Yuvakkumar *et al.,* (2008). The antibiotic, cycloheximide was found to be very effective against all the contaminants of cyanobacterial and algal isolates, and thus it appears to be the drug choice to obtain axenic cultures (Yuvakkkumar, *et al*., 2008).

A selection of methods was used to optimize the isolation of benthic and planktonic cyanobacteria from natural samples. Serial dilution method was successful technique to isolate unicellular strains i.e. coccoid and green microalgae, *Chlorella* spp., as previously described by Andersen

and Kawachi (2005). Streaking and capillary methods or combination of both methods were found to be useful techniques to isolate some green algae and filamentous cyanobacteria (Andersen and Kawachi, 2005).

Due to close association of contaminating bacteria with compact and gelatinous colonies of *Microcystis* spp., it was very difficult to obtain axenic isolates of *Microcystis* strains by standard plating method (Bolch and Blackburn, 1996). However, in this study *Microcystis* spp. were successfully isolated by combination of both streaking and capillary methods as before streaking mixed cultures were crushed between two glass slides to separate mixed cell to each other. The unicellular non filamentous strains, *Synechococcus* spp. and *Chlorella* spp. was isolated by streaking method and by serial dilution. But results showed that *Synechococcus* spp. do not grow well on solid media, but satisfactory results were obtained by serial dilution (Waterbury *et al*., 1981).

Microscopic based identification of strains requires time and skills. Sometimes it is very difficult to differentiate between certain strains of the same genus, toxic and non-toxic strains (Scholin *et al.,* 2003), morphological characteristics due to different culturing conditions (Castenholz and Waterbury, 1989) and morphologically identical strains (Wilmotte and Herdman, 2001). Lehtimäki *et al.* (2000) and Gugger *et al.,* (2002) also mentioned that the phenotype of heterocytous cyanobacteria change their morphology during laboratory cultivation which makes the identification of strains difficult. Traditionally microscopic technique was convenient to identify cyanobacterial strains but not for all strains especially in the case of *Pseudoanabaena* and *Phormidium* strains. In the

present study during microscopic characteristics, based on morphology, it was difficult to distinguish between genera of *Pseudoanabaena* and *Phormidium.* It seemed that all morphological characteristics of filamentous benthic cyanobacteria, *Pseudoanabaena* and *Phormidium* strains, were more or less same but they were only distinguished on the basis of their motility (Komárek and Anagnostidis 2005) and aggregation of trichomes (John *et al.,* 2002). Komárek and Anagnostidis (2005) mentioned that *Phormidium* spp. show clear motile movements; gliding, creeping, waving, trembling while *Pseudoanabaena* spp. did not show clear motility. They also mentioned that the taxonomy of *Phormidium* belongs to the most difficult cyanoprokaryotic genera as it comprises of numerous morphotypes with many closely similar forms. According to John *et al.,* (2002) many trichomes of *Phormidium* spp. form gelatinous or leathery mats while filaments of *Pseudoanabaena* spp. do not gather. During the present investigation two strains of *Phormidium* were isolated from Dian Lake. Three strains of *Phormidium* spp. and three species of *Pseudoanabaena* were isolated from different localities of the Dead Sea. It shows that *Phormidium* spp. are more common species as compared to *Pseudoanabaena* spp. (personal communication with Prof. Jan Rueness, University of Oslo, Norway). From the spring waters of Ein Boqeq and the water springs of Hamei Zohar, the Dead Sea, only two species of *Phormidium* were identified (Oren, 2008). According to the NIVA Culture Collection of Algae a total of 30 strains of *Phormidium* and 7 strains of *Pseudoanabaena* have isolated from marine and freshwaters of the North Sea, Norway. Only 8 species of marine *Phormidium* was reported from Swedish waters and 8 species from Oslofjord, Norway (Wiik, 1981). Both

of them did not find any species of *Pseudoanabaena* from marine Norwegian waters. These observations show that *Phormidium* spp. are more common in aquatic environments compared to *Pseudoanabaena* spp.

It is suggested that identification of strains by traditional microscopic is not adequate to identify to species levels. For confirmation to species levels modern techniques are available. In recent years DNA analysis is one of the most reliable methods to identify the strains by using oligonucleotide primers/probes and it also helps to identify bloom forming cyanobacteria (Neilan *et al.,* 1995). The phylogenetic analysis is a reliable method to differentiate between toxic and non-toxic strains (Lyra *et al.,* 2001), which is it not possible by microscopic identification. The combination of habitat, size of strains, molecular biological methods and microscopy can make it possible to identify strains correctly and up to species levels to understand the rapid microbial diversity of cyanobacteria (Rajaniemi-Wacklin *et al.,* 2008; Oren, 2008).

4.4.2. Dead Sea isolates

From the beginning of 19th century several reports have been published on the occurrence of microbial communities in the Dead Sea and a number of cyanobacteria and diatoms have also been reported. In the present study out of 53 samples only 6 cyanobacterial strains were isolated from the Dead Sea dominated by *Pseudoanabaena* and *Phormidium* spp. It may be due to reduced in flow of fresh water from the Jordan River and the Sea of Galilee resulted in the decrease of water level (Gavrieli *et al.*, 2002), increase salinity and low pH (Oren, 1999). Oren (2000) suggested that at low pH (6 pH) cyanobacteria do not found in abundance, result in the

disappearance of many strains, which were reported from in early studies.

Finding of new strains from various habitats especially from hypersaline waters provide additional targets of bioactive compounds. Only one isolate, *Pseudoanabaena* sp. from the Dead Sea showed 2 peaks of new and unknown compound. Due to lack of information of unknown compound the peaks could not be identified.

4.4.3. Dian Lake isolates and bloom samples

Microcystis aeruginosa and *Aphanizomenon flos-aquae* are common bloom forming cyanobacteria in the Dian Lake (Yuan *et al.,* 2005). The UPLC-PDA-MS chromatograms of mixed natural bloom samples from Dian's Lake showed presence of cyanopeptolin, which may have been produced by either *Microcystis* sp. or *Aphanizomenon* sp. Pure isolated strains from the lake did not show presence of detectable cyanopeptolin, which was observed in natural bloom's chromatograms. Suggesting that the strains providing this class of compounds were lost during isolation or strains were died during culturing and unsuitable environmental conditions.

4.4.4. Identification of peptides from cyanobacteria isolated from the Dian Lake

The results show that toxic MC-LR, cyanopeptolin A and aerucyclamides A, B, C and D were present in the isolates of *Microcystis* spp. and have previously been identified in *Microcystis aeruginosa* PCC 7806. *Microcystis aeruginosa* is known to produce microcystins. Sivonen and Jones (1999) depicted that the freshwater *M. aeruginosa, Anabaena* sp., *A. flos-aquaes, M. viridis, Oscillatoria agardhii* and *Nostoc* sp. and salt water

Synechococcus spp. (Carmichael and Li, 2006) have potential to be a significant source of MC-LR. MC-LR extensively studied and is a commonly occurring hepatotoxic peptide and a potent and specific protein phosphatases 1 and 2A inhibitor (Mackintosh *et al.,* 1990).

During UPLC-PDA-MS analysis a peak of cyanopeptolin A was detected, which had same molecular weight as observed in *Microcystis aeruginosa* PCC 7806 (Martin *et al.,* 1993). They have reported the presence of several cyanopeptolins A-D from *Microcystis aeruginosa* PCC 7806 and they possess identical structures except a basic variable amino acid.

Microcystins, anabaenopeptins and anabaenopeptilides (have similar structure of cyanopeptolins), are also produced by *Anabaena* 90 (Tonk *et al.,* 2009). They mentioned that cyanopeptolins have ability to exhibit protease-inhibitory (trypsin) activity which may have pharmaceutical research application (Martin *et al*., 1993).

The chromatograms showed that *Microcystis* spp. were rich in aerucyclamides. The same aerucyclamides have been previously reported by Portmann *et al.,* (2008a, b). They isolated four aerucyclamide A-D from freshwater cyanobacterium *Microcystis aeruginosa* PCC 7806 and tested toxicity of aerucyclamides A and B against freshwater crustacean *Thamnocephalus platyurus* with LC_{50} values 30.5 and 33.8 µM, respectively.

In another publication Portmann *et al.,* (2008b) found aerucyclamide B was toxic to protozoan malarial parasite *Plasmodium falciparum* and aerucyclamide C lethal to flagellate protozoan *Trypanosomas brucei*.

The Dian Lake is a major source of water supply to the city and occurs in the Yunnan Province, which is famous for huge phosphorus mines (Chen *et al.,* 2002) and input of nitrogen from agricultural land (Liu *et al.,* 1995). The leaching of phosphorus into the lake causes eutrophication and results in frequent bloom formation of *Microcystis* spp. Several reports have been published on the regularly increasing *Microcystis* spp. blooms in the Dian Lake (Sheng *et al.,* 2012). According to Hou *et al.,* (2011) since 1985 the lake has faced an increase in cyanobacterial blooms dominated by *Microcystis* spp. results in disappearance of molluscan communities (Li-Na *et al.,* 2011; Zhang *et al.,* 2012) and there is a risk to humans who consume muscles and gonads of molluscs.

The present study reflects that cyanobacteria are rich in bioactive compounds and existing compounds need further screening, chemical and biological characterisation.

4.5. CONCLUSION

This chapter has described that capillary and agar plate methods supplemented with antibiotic cycloheximide are appropriate tools to obtain most of cyanobacterial and algal isolates. Cyanobacterial diversity from two different environments (the Dian Lake and the Dead Sea areas) demonstrated that in this instance and as might be expected freshwater habitat supports a wider range of microbial biota than hypersaline environment. It was also noted that the samples collected from the hypersaline locations (>32 ‰) of the Dead Sea gave no indication of the presence of cyanobacteria or algae. All six cyanobacteria strains were isolated from location which had salinity ranged from 4-25 ‰. It was

also showed that the cyanobacteria do not contribute a major role in the ecology of the Dead Sea as compared to diatoms.

The presence of toxic MC-LR, cyanopeptolin and aerucyclamides in frequently occurring blooms in economically important Dian Lake, it is important to monitor the concentrations of peptides more intensively and nutrient concentrations. It is concluded that the presence of the peptide co-occurrence and in same pattern in *Microcystis* spp. helps to know about the cosmopolitan distribution of same *Microcystis* strains in various aquatic environments and can also be served as a useful tool to find geographical distribution of the same strain.

Cyanobacteria are known to produce a variety of compounds. The findings from the present study showed that cyanobacteria from both habitats are a potential source of new active/inactive compounds that could be useful for future research, biochemical and pharmacological industries. To find more peptides and other compounds there is a need to increase concentration of extracts, extraction and detection methods as a number of novel compounds could have gone unreported in the current study either because they were not extracted or detected. Further studies of all isolates will now be performed under a range of extraction/detection methods. .

This investigation provides an updated of current understanding of the microalgal and cyanobacterial diversity in Dian Lake and around the Dead Sea. It is suggested that the further studies are required in other freshwater, salt lakes and salt-stressed ecosystems to identify and isolate

new strains of cyanobacteria and diatoms, their importance and detection of any possible compound/s.

CHAPTER 5

GENERAL DISCUSSION

Discussion

The main aims of the present investigation were:

1. To fractionate, purify and evaluate the bioactivity of compounds produced by *Nodularia spumigena* KAC 66.
2. Effects of abiotic factors on the production of biomass and peptide production.
3. Isolation and taxonomy of cyanobacteria from blooms of freshwater Dianchi Lake, China and the Dead Sea.

The elucidation of NOD and nodulopeptin 901 with MeOH:H_2O fractions F3 to F9 (20%-80% MeOH) showed polar and semi-polar nature of hepatotoxins. Almost all polar, semi polar and non polar diluted fractions were significantly toxic to daphnids and inhibited PP1, when compared to standard peptides. Different mortality percentages proved that lethality of pure NOD and fractions, containing NOD and nodulopeptin 901, varied from species to species. The purified NOD caused dose-dependent mortality in *D. pulex* and *D. magna,* while *D. magna* were found to be more sensitive to NOD concentrations than *D. pulex.* Previous studies have also been approved that different daphnid species have different levels of sensitivities to toxins (DeMott *et al*., 1991; Hietala *et al*., 1995). Approximately, all purified peptides, ANA, ANB, linear NOD, nodulopeptin 901 and fractions inhibited PP1 showing a range of inhibiting effects. As reported previously hepatotoxic NOD is a potent protein inhibitor and contains Adda group. However, purified ANA, ANB and nodulopeptin 901 also inhibited PP1, which do not contain Adda group thought to be the key moiety on the NOD causing PP1 inhibition. Due to lack in information,

newly characterised nodulopeptin 901 was quantified by ANA and ANB. All diluted fractions also showed activity against PP1, including those fractions, which were not contained the NOD and nodulopeptin 901. It may be due to green colouration of fractions, which had an effect on the OD values of spectrophotometer or *N. spumigena* produces other PP1 inhibitory compounds, which can be extracted using different solvents and extraction methods.

Like other strains *N. spumigena* can easily be grown in temperature controlled culture rooms in closed glass flasks/columns and provide a low cost source of cell biomass to discover new and bioactive compounds.

The mass culturing of *N. spumigena* KAC 66 in different vessels also provided a comparative analysis of Chl-*a*, cell biomass and peptide production. In this experiment, 10 L glass flasks were found to be easy to handle while harvesting. In the glass flasks the highest biomass and peptide levels were recorded compared to Perspex columns. Even light distribution to culturing glass flasks provided suitable conditions to produce high cell biomass and peptides levels. The present study indicated that the end of log phase and beginning of the stationary phase was a suitable time to obtain highest biomass and intra and extracellular peptides levels, while grown in glass flasks.

Suitable environmental conditions support high production of biomass and peptides. Extreme low or high growth conditions (eg. light and temperature), inappropriate culturing vessels and shortage or excessive availability of nutrients can affect on growth, biomass and peptide production. After 2 weeks, 3 Perspex columns out of five demonstrated

poor growth/death of cells resulted in reduced production of cell biomass and toxins, while extracellular NOD concentrations continued to increase after cells death. The death of cells or poor growth of cultures may have occurred due to low irradiance or bacterial contamination or not properly cleaning of columns. Light intensity is a major component with an effect on growth and peptide production. In growth experiment the cultures carried out in glass flasks and Perspex columns were receiving 17.35-17.47 μmol/s/m^2 and 1.4 to 42.6 μmol/s/m^2 light intensity, respectively. The high biomass and peptide production in glass flasks reviled that light is also play an important role in healthy growth of the strain. Low irradiance could not support the growth in columns than glass flasks, which were receiving equal and high light intensity compared to columns.

It is interesting to know that under stressed conditions in columns nodulopeptin 901 was not detected in the surrounding medium and they retained within the cells or they may be degraded by bacterial contaminants present in the growth medium.

The growth and peptide production by *N. spumigena* was strongly environmental factor-dependant. This study proved that 22°C was the optimal temperature for the growth and toxin production by *N. spumigena*, while the highest temperature (30°C) was found to have significant effect on the production of extra and intracellular peptides production. In this experiment some interesting results were obtained that *N. spumigena* kept growing well in all salt concentrations. Furthermore, increasing salinity affected the intra and extracellular NOD, while nodulopeptin 901 showed positive correlation with increasing salinity. It seems that release and production of NOD is more sensitive to

the salinity changes than nodulopeptin 901. In general, different nitrate and phosphate conditions had similar effects on intra and extracellular peptide levels, they decreased with increasing time. The absence of nitrate in the medium had a significant negative effect on the cell biomass, Chl-*a* concentrations and total NOD production (intra and extracellular). However, intra and extracellular nodulopeptin 901 were high under this condition. In phosphate deficient medium *N. spumigena* maintained its growth at all concentrations but after 3 weeks, a decrease in cell biomass, Chl-*a* and total peptides was observed. It may be due to shortage of stored phosphate within the cells.

It is suggested that the alteration in temperature and concentrations of salinity, nitrate and phosphorus, can enhance the yield of intra and extracellular peptides and biomass (summarized in Table 3.17) in laboratories and can be helpful to control the bloom formation and toxin production in natural environments.

Determination of cell density or biomass can be best estimated by chlorophyll-*a* concentration as this was found coorelate well with growth. The dead cells in the experimental flasks or especially salt contents on filter discs (especially during salinity experiment) can effect on the cell biomass estimation.

The role of toxin production by cyanobacteria is still unclear. In the result of cell lysis/death/damage, the peptides release in the surrounding medium (Sivonen and Jones, 1999), maybe they are not essential for the growth and reproduction. These toxins maybe the product of cell biological processes or use as signalling compounds or for defence

against microorganisms that feed on cyanobacterial strains (Mundt *et al*., 2001).

There is some information available on the fate of toxins released in surrounding medium. Sivonen and Jones, (1999) studied degradation of NOD under different environmental conditions. They reported that under light and dark conditions NOD was photochemically degraded into small components. These small components recycled by bacterial communities or maybe reused by cyanobacterial cell themselves.

It is suggested that there is much work needed to know about the fate and use extracellular peptides.

Microscopic identification provided immediate information about identification of strains present in bloom and could be helpful to distinguish between prokaryotes and eukaryotes, potential toxic and non toxic strains and abundance of certain strains in natural samples. This basic information can be helpful to alarm presence of dangerous strains in developing blooms. For rapid identification of strains several methods are available but in this study three isolation techniques (serial dilution, capillary and streaking methods) were found to be very effective and successful to isolate filamentous and non filamentous strains. In the present study a total of 26 strains were isolated from bloom samples from of Dian Lake and natural samples from the Dead Sea. It is suggested that unicellular strains i.e. *Synechococcus* spp. and *Chlorella* spp. were easily isolated by serial dilution method. Streaking and capillary methods or combination of both methods were effective to isolate some green algae and mostly filamentous cyanobacteria.

Creeping/oscillatory movements of *Phormidium*, *Pseudoanabaena*, *Oscillatoria* and *Lyngbya* spp., on solid media, helped to isolate them from other strains. *Pseudoanabaena* and *Phormidium* spp. were very difficult to distinguish and can be distinguished on the basis of their motality and aggregation of trichomes.

Phormidium spp. showed clear motile movements compared to trembling *Pseudoanabaena* spp. This study supported the importance of microscopic identification that a total of 5 *Phormidium* spp. were isolated from Dian Lake and the Dead Sea samples, which showed the abundance and diversity of strain in two different water bodies. In this study species only identified up to only genera levels. Traditional microscopy could not provide satisfactory results to identify strains up to species levels. It is proposed that combination of modern technique DNA analysis, habitat, size of strain, bioassays and microscopy made it possible to identify strains correctly and up to species.

There are several reports are available on microoragnisms including cyanobacterial diversity in the Dead Sea. Out of 53 samples from different locations of the Dead Sea, 6 cyanobacterial strains belonging to *Pseudoanabaena* and *Phormidium* spp. were isolated by streaking and capillary methods. Only one strain *Pseudoanabaena* sp. (D15) showed peaks of unknown compound. It shows that cyanobacterial strains are rich in compounds that can be further investigated to discover novel compounds.

Twenty strains were isolated from the bloom samples of the Dian Lake and out of which 2 strains identified as *Microcystis* spp. Due to

eutrophication in the Dian Lake annual toxic blooms of *Microcystis* and *Aphanizomenon* spp. have already been reported. The UPLC-PDA-MS analysis of isolated strains, *Microcystis* spp. indicated the presence toxic MC-LR, cyanopeptolin A, aerucyclamides A, B, C and D. These peptides have also been reported from *Microcystis aeruginosa* PCC 7806 and the presence of same peptides in the Dian Lake, showed the geographical diversity of similar strain.

This study contributes to the information of the diversity of cyanobacteria in the Dead Sea and Dian Lake.

In conclusion, the present study is first time providing information on the effects of environmental factors on the production of intra and extracellular levels of newly discovered nodulopeptin 901. Additionally, this study also presents information about the lethality of nodulopeptin 901 against daphnids and inhibition of PP1.

The information from effects of environmental factors on the production of biomass and total peptides can be used as a base line to understand the occurrence of peptides levels in nature.

CHAPTER 6

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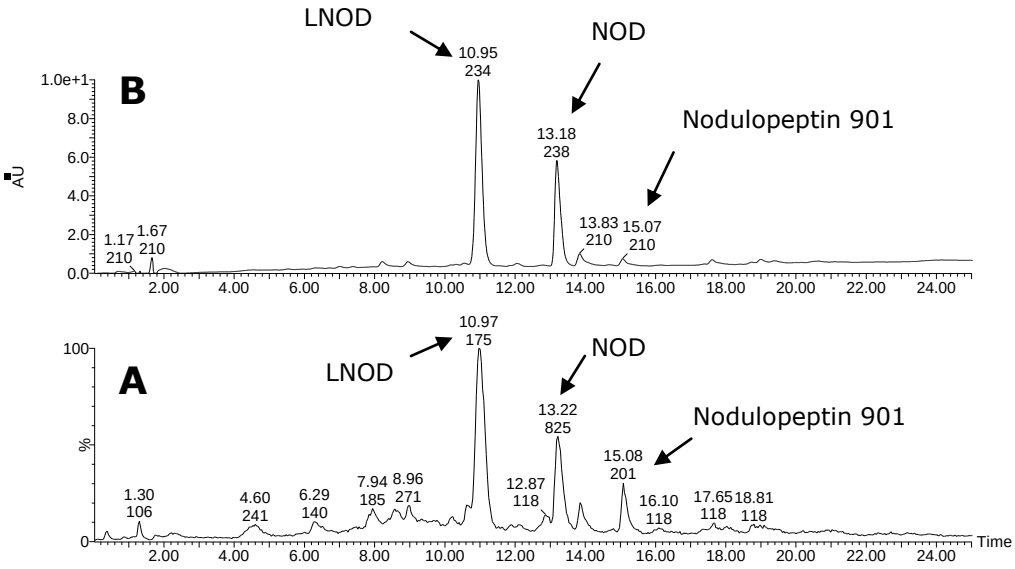
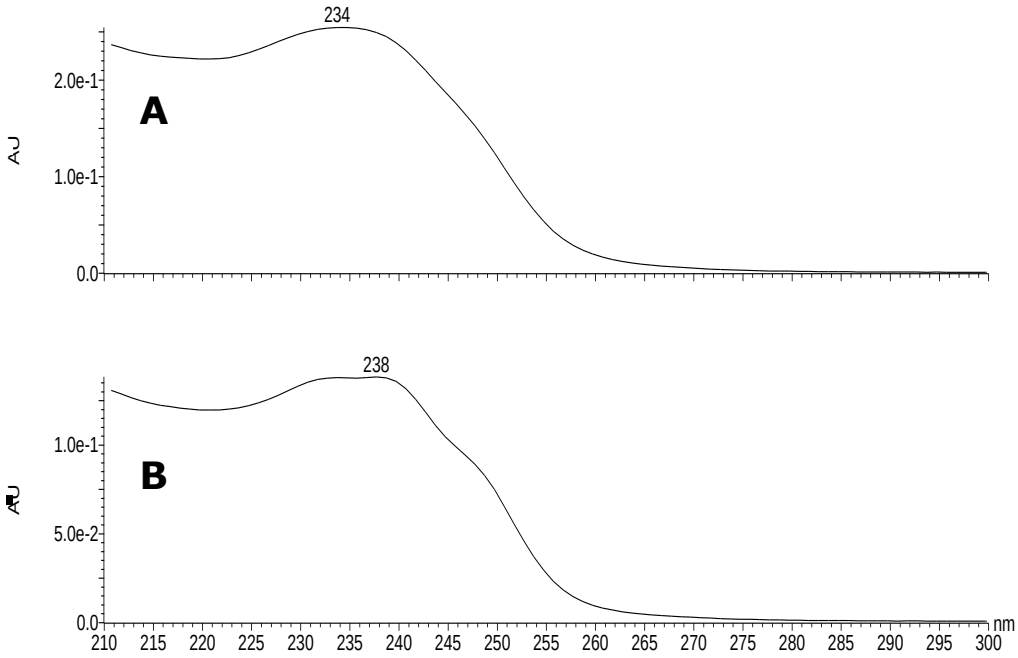
APPENDICES

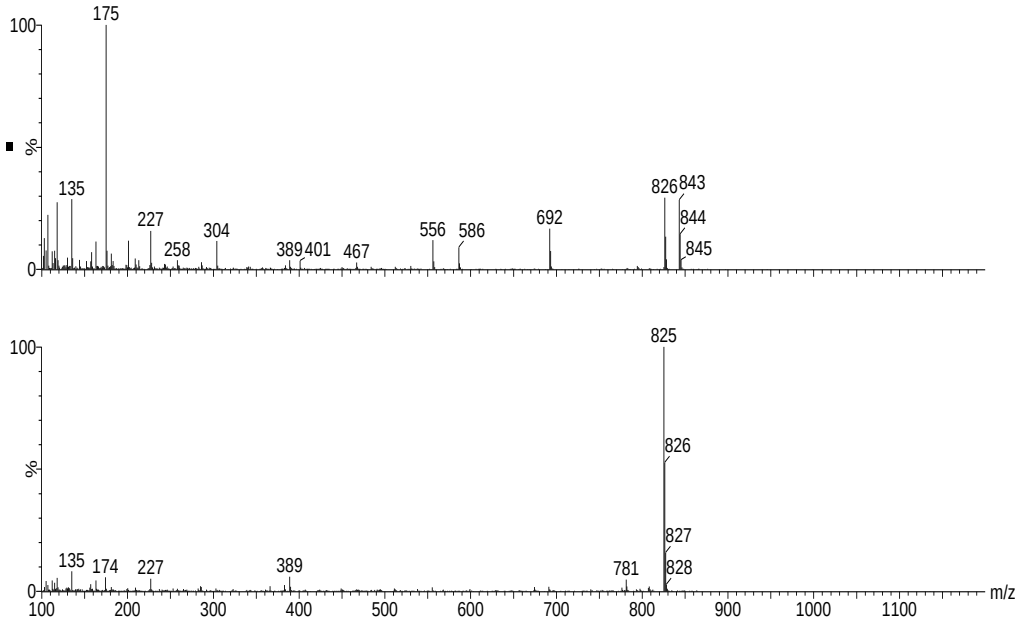
1. Full scanned HPLC-PDA-LCMS chromatograms of peptides (**A**) their absorbance (**B**).



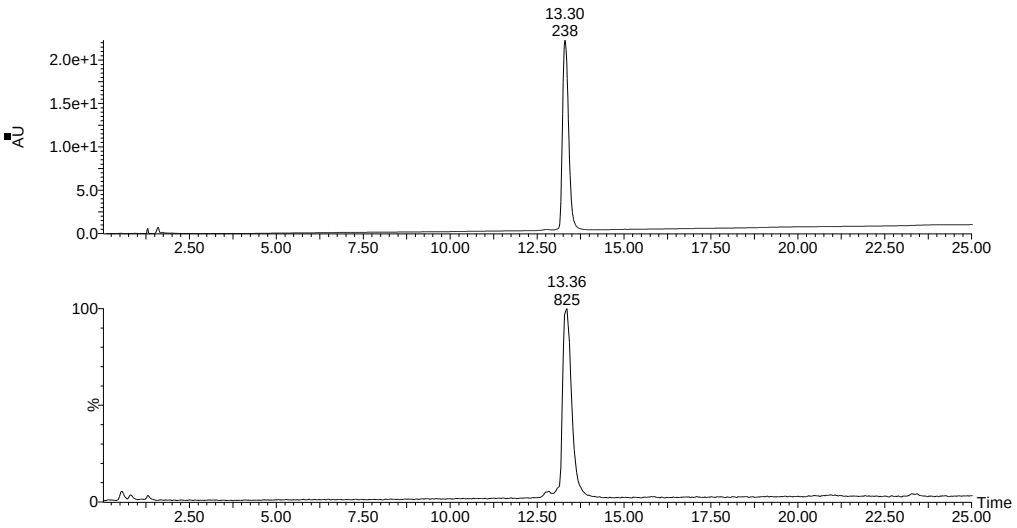
2. Typical absorbance spectra of LNOD (**A**) and NOD (**B**).



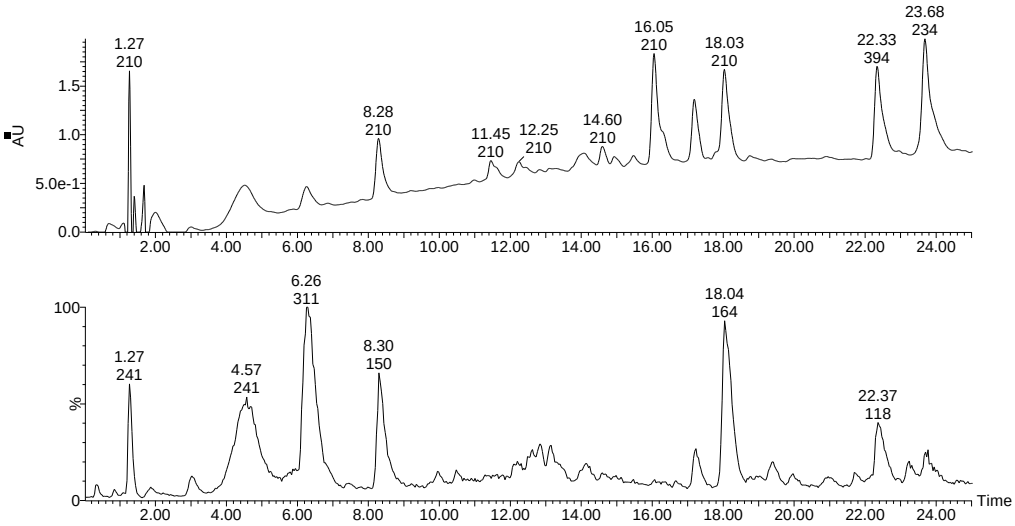
3. Typical absorption spectrum of LNOD with presence of peptide at 234 mass spectrum (*m/z*).



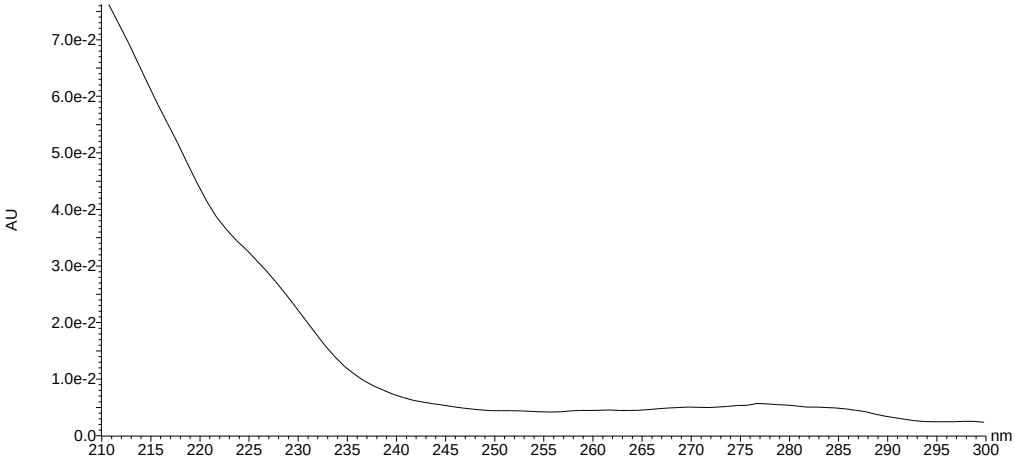
4. Typical absorption spectrum of NOD with presence of peptide at 238 mass spectrum (*m/z*) and 825 (MW).



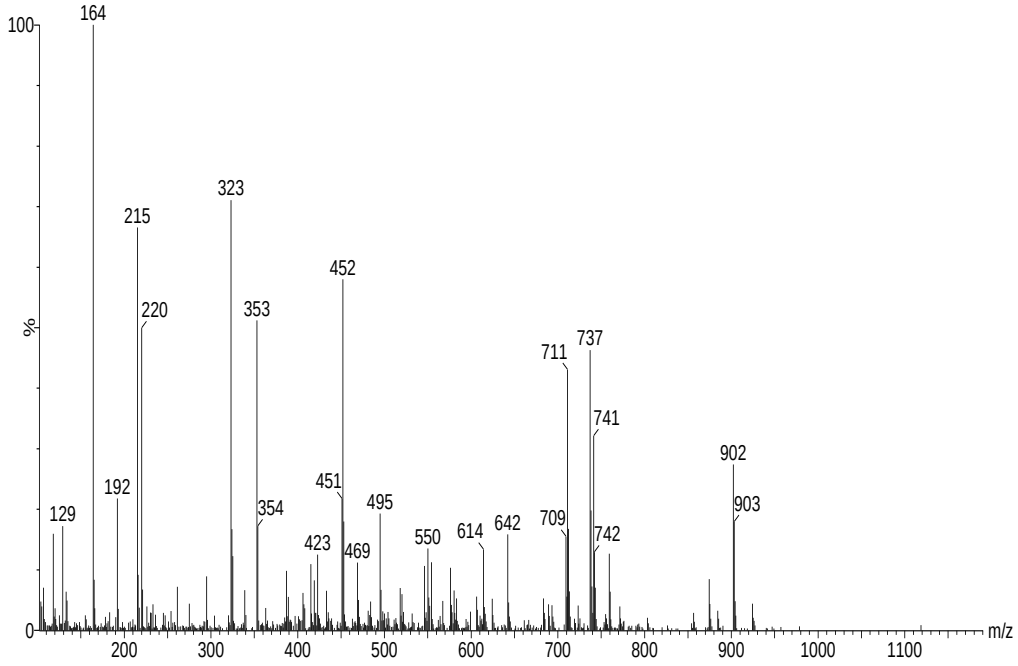
5. Typical absorption spectrum of nodulopeptin 901 with presence of peptide at 210 mass spectrum (*m/z*).



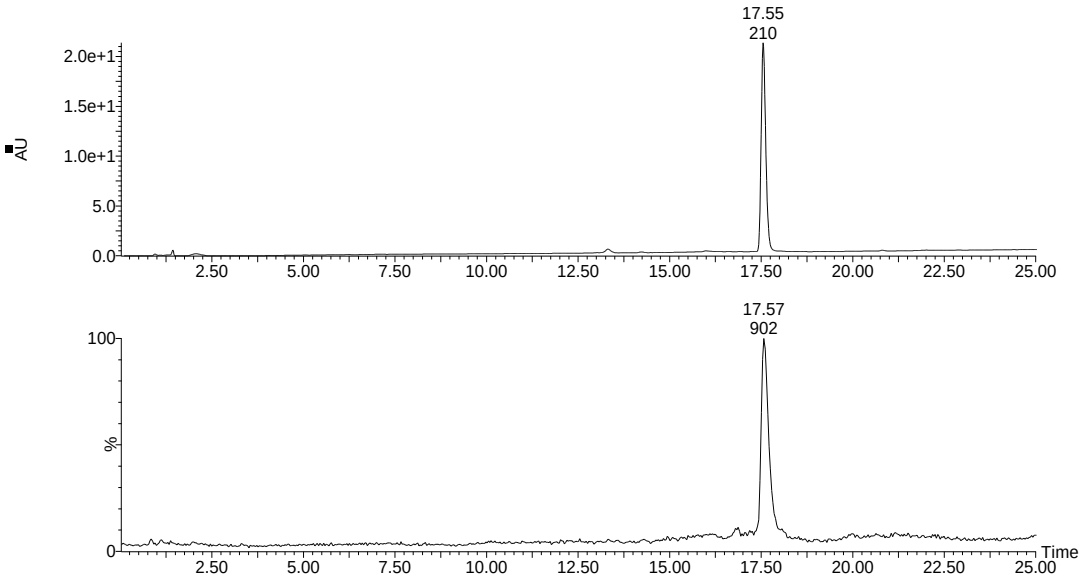
6. Typical absorbance spectrum of nodulopeptin 901 at 210 nm.



6. Typical absorption spectrum of nodulopeptin 901 with presence of peptide at 210 mass spectrum (m/z).



7. Typical absorption spectrum of nodulopeptin 901 with presence of peptide at 210 mass spectrum (*m/z*).



8. Calibration of standard NOD quantified at wavelength of 238 nm with diode array at 12:00 min retention time analysed on HPLC-PDA-MS (n=3). (±S.D = standard deviation).

Concentration ($\mu g\ mL^{-1}$)	**Peak area**	**mean (x)**	**STDev (σ_{n-1})**	**% error bar**
0.1	312 331 318	320	9.71	3.03
0.5	1677 1650 1669	1665	13.87	0.83
1.0	3115 3293 3292	3233	102.48	3.17
5.0	15224 15192 15588	15335	219.98	1.43
10	33212 33271 33185	33223	43.98	0.132
50	176315 175066 175040	175474	728.73	0.42
100	358840 355616 355805	356754	1809.29	0.51
200	699806 687737 698538	695360	6632.37	0.95

9. Calibration of standard nodulopeptin 901 based on the use of nodulopeptin 901 curve with diode array at 12:00 min retention time quantified at 210 nm analysed on HPLC-PDA-MS (±S.D = standard deviation), (n=3).

Concentration (µg mL^{-1})	**Peak area**	**Mean (x)**	**STDev (σ_{n-1})**	**% Error bar**
0.1	119 109 97	108	11.02	10.17
0.5	766 721 754	747	23.30	3.12
1.0	1754 1771 1706	1744	33.71	1.93
5.0	10378 9546 8979	9634	703.67	7.30
10	18181 18862 19377	18807	599.92	3.190
50	99656 97638 96414	97903	1637.12	1.67
100	168431 200898 193911	187747	17088.76	9.10

10. Calibration of standard NOD quantified at wavelength of 238 nm with diode array at 6.05-6.13 min retention time analysed on UPLC-PDA-MS (n=3). (±S.D = standard deviation).

Concentration ($\mu g\ mL^{-1}$)	Peak area	Mean ($\bar{x}$)	StDev (σ_{n-1})	% Error bar
0.1	72 68 64	68	4.00	5.88
0.5	319 344 337	333	12.90	3.87
1.0	689 690 637	672	30.32	4.51
5.0	3524 3527 3555	3535	17.10	0.48
10	7357 7342 7256	7318	54.50	0.745
50	38327 38302 38170	38266	84.36	0.22
100	72542 72501 72353	72465	99.42	0.14

11. Calibration of standard nodulopeptin 901 based on the use of nodulopeptin 901 curve quantified at 210 nm with diode array at 7.88-7.90 min retention time analysed on UPLC-PDA-MS (n=3). (±S.D = standard deviation) (n.d.=not detected).

Concentration (µg mL^{-1})	**Peak area**	**Mean ($\bar{x}$)**	**StDev (σ_{n-1})**	**% Error bar**
0.1	n.d n.d n.d	n.d	n.d	n.d
0.5	n.d n.d n.d	n.d	n.d	n.d
1.0	530 498 532	520	19.08	3.67
5.0	2566 2540 2317	2474	136.87	5.53
10	4200 4222 4239	4220	19.55	0.463
50	18332 18517 18260	18370	132.58	0.72
100	30735 34638 34598	33324	2241.94	6.73

12. Concentrations and lethality of standard toxin, NOD for *D. pulex* exposed to NOD for 24 h (n= 2).

NOD Concentrations (µg/ml)	Set number 1			Set number 2					
	Total no. of daphnids	No. of alive daphnids	No. of dead daphnids	Total no. of daphnids	No. of alive daphnids	No. of dead daphnids	Mean of total daphnids	Mean of dead daphnids	% of dead daphnids
100	10	0	10	10	0	10	10	10	100
50	10	0	10	11	0	11	11	11	100
10	11	5	6	11	5	6	11	6	55
5	10	6	4	10	6	4	10	4	40
1	11	8	3	10	9	1	11	2	18
0.5	11	11	0	10	10	0	11	0	0
0.1	10	10	0	11	11	0	11	0	0
-ve Control	15	15	0	13	13	0	14	0	0

13. *D. pulex* assay of positive, negative controls and undiluted 17 fractions (collected from the RPFC) of *N. spumigena* KAC 66.

Fractions of *N. spumigena* KAC 66	Total no. of neotales		No. of dead neotales		No. of alive neotales		Mean total no. of neotales	Mean alive neotales	Mean dead neotales	% of dead neotales
	1	2	1	2	1	2				
F1	13	10	1	1	12	9	12	11	1	8
F2	10	8	10	7	0	1	9	1	9	100
F3	10	9	10	9	0	0	10	0	10	100
F4	12	10	12	10	0	0	11	0	11	100
F5	9	10	9	10	0	0	10	0	10	100
F6	9	10	9	10	0	0	10	0	10	100
F7	15	10	5	5	10	5	13	8	5	38
F8	10	9	7	7	3	2	10	3	7	70
F9	10	10	10	10	0	0	10	0	10	100
F10	10	10	10	10	0	0	10	0	10	100
F11	10	10	10	10	0	0	10	0	10	100
F12	10	10	10	10	0	0	10	0	10	100
F13	10	10	10	10	0	0	10	0	10	100
F14	10	10	10	10	0	0	10	0	10	100
F15	10	10	10	10	0	0	10	0	10	100
F16	10	10	1	1	9	9	10	9	10	100
F17	13	11	0	1	13	10	12	12	1	8
-ve Control	11	12	2	0	9	12	12	11	1	8
+ ve Control	10	10	10	10	0	0	10	0	10	100

14. *D. pulex* assay of positive, negative controls and 17 fractions (collected from flash chromatography) of *N. spumigena* KAC 66 at x2 dilution.

Fractions of *N. spumigena* KAC 66	Total no. of neotales		No. of dead neotales		No. of alive neotales		Mean total no. of neotales	Mean alive neotales	Mean dead neotales	% of dead neotales
	1	2	1	2	1	2				
F1	14	9	0	1	14	8	12	11	1	8
F2	10	8	5	8	5	0	9	3	7	78
F3	10	10	10	10	0	0	10	0	10	100
F4	10	10	10	10	0	0	10	0	10	100
F5	10	10	8	7	2	3	10	3	8	80
F6	10	10	10	10	0	0	10	0	10	100
F7	10	10	3	5	7	5	10	6	4	40
F8	10	11	1	1	9	10	11	10	1	9
F9	11	11	8	7	3	4	11	4	8	73
F10	10	10	10	10	0	0	10	0	10	100
F11	10	10	10	10	0	0	10	0	10	100
F12	10	10	10	9	0	1	10	1	10	100
F13	10	10	10	10	0	0	10	0	10	100
F14	10	10	10	10	0	0	10	0	10	100
F15	10	12	5	3	5	9	11	7	4	36
F16	12	10	2	1	10	9	11	10	2	18
F17	10	9	0	1	10	8	10	9	1	10
-ve Control	11	12	2	0	9	12	12	11	1	8
+ ve Control	10	10	10	10	0	0	10	0	10	100

15. *D. pulex* assay of positive, negative controls and 17 fractions collected from flash chromatography) of *N. spumigena* KAC 66 at x4 dilution.

Fractions of *N. spumigena* KAC 66	Total no. of neotales		No. of dead neotales		No. of alive neotales		Mean total no. of neotales	Mean alive neotales	Mean dead neotales	% of dead neotales
	1	2	1	2	1	2				
F1	10	11	0	0	10	11	11	11	0	0
F2	10	10	0	0	10	10	10	10	0	0
F3	10	11	5	5	5	6	11	6	5	45
F4	10	10	1	4	9	6	10	8	3	30
F5	10	11	1	1	9	10	11	10	1	9
F6	9	10	1	4	8	6	10	7	3	30
F7	14	10	1	1	13	9	12	11	1	8
F8	10	10	0	0	10	10	10	10	0	0
F9	10	10	0	0	10	10	10	10	0	0
F10	10	10	0	0	10	10	10	10	0	0
F11	11	10	2	1	9	9	11	9	2	18
F12	9	10	2	0	7	10	10	9	1	10
F13	10	10	0	0	10	10	10	10	0	0
F14	10	10	0	0	10	10	10	10	0	0
F15	10	10	0	0	10	10	10	10	0	0
F16	10	10	0	0	10	10	10	10	0	0
F17	10	10	0	0	10	10	10	10	0	0
-ve Control	11	12	2	0	9	12	12	11	1	8
+ ve Control	10	10	10	10	0	0	10	0	10	100

16. Concentrations and lethality of standard toxin, NOD for *D. magna* (exposed to NOD for 24 h; n=3)

NOD Concentrations (µg/ml)	Set number 1			Set number 2					
	Total no. of neonates	No. of alive neonates	No. of dead neonates	Total no. of neonates	No. of alive neonates	No. of dead neonates	Mean of total neotales	Mean of dead neotales	% of dead neotales
100	12	0	12	11	0	11	12	12	100
50	12	0	12	11	0	11	12	12	100
10	9	4	5	10	5	5	10	5	50
5	10	6	4	10	7	3	10	4	40
1	9	5	4	11	9	2	10	3	30
0.5	10	7	3	11	9	2	11	3	27
0.1	9	8	1	10	7	3	10	2	20
-ve Control	10	9	1	9	9	0	10	1	10

17. Concentrations and lethality of standard toxin, nodulopeptin 901 for *D. magna*(the daphnids exposed for 24 h with nodulopeptin 901; n=3)

Nodulopeptin 901 Concentrations (µg/ml)	Set number 1			Set number 2			Set number 3						
	Daphnids												
	Total no.	Alive	Dead	Total no.	Alive	Dead	Total no.	Alive	Dead	Mean of total ($\bar{x}$)	Mean of dead ($\bar{x}$)	% of dead	StDev (σ_{n-1})
120	12	6	6	11	5	6	11	6	5	11	6	55	0.6
100	12	7	5	11	5	6	10	7	3	11	5	45	1.5
50	9	3	6	8	5	3	9	5	3	9	4	44	1.7
10	9	9	0	10	9	1	10	6	4	10	2	20	2.1
5	10	9	1	10	9	1	10	9	1	10	1	10	0.0
1	10	10	0	10	9	1	9	6	3	10	1	10	1.5
0.5	10	10	0	10	9	1	9	6	3	10	1	10	1.5
0.1	10	10	0	9	6	3	9	8	1	9	1	11	1.5
-ve Control	12	12	0	10	9	1	10	9	1	11	1	9	0.6
+ve Control	10	0	10	13	0	13	10	0	10	11	11	100	1.7

18. *D. magna* assay of positive, negative controls and undiluted 17 fractions (collected from RPFC) of *N. spumigena* KAC 66.

Fractions of *N. spumigena* KAC 66	Total no. of neonates		No. of dead neonates		No. of alive neonates		Mean total no. of neonates	Mean alive neonates	Mean dead neonates	% of dead neonates
	1	2	1	2	1	2				
F1	7	10	2	4	5	6	9	6	3	33
F2	10	10	3	4	7	6	10	7	4	40
F3	10	9	10	9	0	0	10	0	10	100
F4	9	10	4	6	5	4	10	5	5	50
F5	10	10	8	4	2	6	10	4	6	60
F6	10	10	7	5	3	5	10	4	6	60
F7	11	10	4	4	7	6	11	7	4	36
F8	9	10	3	4	6	7	10	7	4	70
F9	10	10	2	3	8	7	10	8	3	30
F10	10	10	2	4	8	6	10	7	3	30
F11	10	10	8	9	2	1	10	2	9	90
F12	10	10	6	5	4	5	10	5	6	60
F13	10	10	10	7	0	3	10	2	9	90
F14	10	10	8	10	2	0	10	1	9	90
F15	10	9	3	4	7	5	10	6	4	40
F16	9	10	3	3	6	7	10	7	10	100
F17	11	10	2	1	9	9	11	9	2	18
-ve Control	9	10	0	1	9	9	10	9	1	10
+ ve Control	10	10	10	10	0	0	10	0	10	100

19. *D. magna* assay of positive, negative controls and 17 fractions (collected from RPFC) of *N. spumigena* KAC 66 at x2 dilution.

Fractions of *N. spumigena* KAC 66	Total no. of neonates		No. of dead neonates		No. of alive neonates		Mean total no. of neonates	Mean alive neonates	Mean dead neonates	% of dead neonates
	1	2	1	2	1	2				
F1	10	10	2	1	8	9	10	9	2	20
F2	11	9	2	2	9	7	10	8	2	20
F3	10	11	10	8	0	3	11	2	9	82
F4	10	10	4	3	6	7	10	7	4	40
F5	10	10	1	2	9	8	10	9	2	20
F6	11	10	4	6	7	4	11	6	5	45
F7	11	11	3	3	8	7	11	8	3	27
F8	10	10	2	3	8	7	10	8	3	30
F9	8	10	1	2	7	8	9	8	2	22
F10	10	9	2	1	8	8	10	8	2	20
F11	9	10	4	4	5	6	10	6	4	40
F12	9	10	2	1	7	9	10	8	2	20
F13	9	10	1	8	8	2	10	5	5	50
F14	9	9	6	3	3	6	9	5	5	56
F15	10	11	2	4	8	7	11	8	3	27
F16	10	9	1	3	9	6	10	8	10	100
F17	9	10	0	2	9	8	10	9	1	10
-ve Control	9	10	0	1	9	9	10	9	1	10
+ ve Control	10	10	10	10	0	0	10	0	10	100

20. *D. magna* assay of positive, negative controls and 17 fractions (collected from RPFC) of *N. spumigena* KAC 66 at x4 dilution.

Fractions of *N. spumigena* KAC 66	Total no. of neonates		No. of dead neonates		No. of alive neonates		Mean total no. of neonates	Mean alive neonates	Mean dead neonates	% of dead neonates
	1	2	1	2	1	2				
F1	10	9	1	0	9	9	10	9	1	10
F2	8	10	0	0	8	10	9	9	0	0
F3	10	9	0	1	10	8	10	9	1	10
F4	10	10	1	3	9	7	10	8	2	20
F5	10	10	0	2	10	8	10	9	1	10
F6	10	9	1	4	9	5	10	7	3	30
F7	11	10	2	2	9	8	11	9	2	18
F8	8	10	2	1	6	9	9	8	2	22
F9	11	11	0	2	11	9	11	10	1	9
F10	10	9	2	2	8	7	10	8	2	20
F11	10	10	0	0	10	10	10	10	0	0
F12	9	9	1	3	8	6	9	7	2	20
F13	10	10	2	1	8	9	10	9	2	20
F14	9	10	1	1	8	9	10	9	1	10
F15	10	10	0	3	10	7	10	9	2	20
F16	11	10	0	3	11	7	11	9	10	91
F17	10	10	0	0	10	10	10	10	0	0
-ve Control	9	10	0	1	9	9	10	9	1	10
+ ve Control	10	10	10	10	0	0	10	0	10	100

21. Inhibitory activity of standard NOD after 14 h incubation at 37°C. The plate was read at 405 nm.

Conc. of NOD (µg/ml)	OD				Std Dev (σ_{n-1})
	1	2	3	Mean ($\bar{x}$)	
10.0	0.127	0.118	0.117	0.121	0.006
5.0	0.132	0.137	0.132	0.134	0.003
2.5	0.15	0.15	0.15	0.150	0.000
1.25	0.167	0.157	0.172	0.165	0.008
0.625	0.197	0.187	0.198	0.194	0.006
0.312	0.226	0.225	0.222	0.224	0.002
0.156	0.256	0.256	0.259	0.257	0.002
0.078	0.328	0.337	0.335	0.333	0.005
0.039	0.391	0.486	0.509	0.462	0.063
0.019	0.711	0.724	0.683	0.706	0.021
0.009	0.845	0.858	0.815	0.839	0.022
0	0.956	0.985	0.976	0.972	0.015
Blank	0.044	0.044	0.044	0.044	0.000
Control	0.06	0.061	0.06	0.060	0.001

22. Inhibitory activity of standard linear NOD after 14 h incubation at 37 °C. The plate was read at 405 nm.

Conc of linear NOD (µg/ml)	OD				Std Dev (σ_{n-1})
	1	2	3	Mean ($\bar{x}$)	
Linear NOD					
100.0	0.213	0.23	0.232	0.225	0.010
50.0	0.325	0.321	0.303	0.316	0.012
10.0	0.819	0.787	0.841	0.816	0.027
5.0	1.043	1.079	1.053	1.058	0.019
2.5	1.178	1.238	1.18	1.199	0.034
1.25	1.26	1.174	1.252	1.229	0.048
0.63	1.232	1.25	1.27	1.251	0.019
0.31	1.266	1.294	1.22	1.260	0.037
0.16	1.268	1.244	1.258	1.257	0.012
0.07	1.277	1.275	1.257	1.270	0.011
0.009	1.248	1.357	1.229	1.278	0.069
0	1.272	1.255	1.314	1.280	0.030
Blank	0.043	0.043	0.044	0.043	0.001
Control	0.074	0.076	0.086	0.079	0.006

23. Inhibitory activity of standard ANA and ANB after 14 h incubation at 37 °C. The plate was read at 405 nm.

Conc of peptides (µg/ml)	**OD**				
	1	**2**	**3**	**Mean ($\bar{x}$)**	**Std Dev (σ_{n-1})**
ANA					
100.0	0.299	0.244	0.29	0.278	0.030
50.0	0.501	0.378	0.559	0.479	0.092
10.0	0.572	0.568	0.548	0.563	0.013
5.0	0.528	0.531	0.573	0.544	0.025
2.5	0.541	0.57	0.522	0.544	0.024
1.25	0.621	0.537	0.436	0.531	0.093
0.63	0.517	0.591	0.517	0.542	0.043
0.31	0.641	0.592	0.625	0.619	0.025
0.16	0.619	0.561	0.718	0.633	0.079
0.07	0.738	0.553	0.651	0.647	0.093
0.009	0.739	0.719	0.712	0.723	0.014
0	0.649	0.702	0.829	0.727	0.093
ANB					
100.0	0.347	0.351	0.285	0.328	0.037
50.0	0.399	0.385	0.311	0.365	0.047
10.0	0.363	0.355	0.347	0.355	0.008
5.0	0.399	0.39	0.374	0.388	0.013
2.5	0.49	0.418	0.506	0.471	0.047
1.25	0.402	0.589	0.445	0.479	0.098
0.63	0.404	0.816	0.403	0.541	0.238
0.31	0.512	0.666	0.473	0.550	0.102
0.16	0.539	0.676	0.527	0.581	0.083
0.07	0.51	0.618	0.584	0.571	0.055
0.009	0.59	0.61	0.58	0.593	0.015
0	0.61	0.678	0.66	0.649	0.035
Blank	0.044	0.044	0.044	0.044	0.000
Control	0.063	0.064	0.065	0.064	0.001

24. Inhibitory activity of standard nodulopeptin 901 after 14 h incubation at 37 °C. The plate was read at 405 nm.

Conc of pep 901 (µg/ml)	OD (after 14 hrs incubation)			Mean ($\bar{x}$) OD	Std Dev (σ_{n-1})
	1	2	3		
100.0	0.318	0.452	0.423	0.398	0.071
50.0	0.484	0.658	0.545	0.562	0.088
10.0	0.946	0.674	0.924	0.848	0.151
5.0	1.865	0.054	0.977	0.965	0.906
2.5	1.006	0.681	1.238	0.975	0.280
1.25	0.934	1.092	1.069	1.032	0.085
0.63	0.979	0.963	1.186	1.043	0.124
0.31	1.06	1.049	1.148	1.086	0.054
0.16	1.184	0.983	1.124	1.097	0.103
0.07	0.583	1.735	0.998	1.105	0.583
0.009	1.556	0.984	1.282	1.274	0.286
0	1.122	1.732	1.373	1.409	0.307
Blank	0.044	0.044	0.043	0.044	0.001
Control	0.067	0.066	0.067	0.067	0.001

25. PP1 inhibitory activity of undiluted 17 fractions of *N. spumigena* KAC 66 collected from reversed phase flash chromatography. The plate incubated for 14 h at 37 °C and read at 405 nm.

Conc. of fractions (2 ml dried and dissolved in 200 µl MQ water)	OD				Inibition (%)	Std Dev (σ_{n-1})
	1	2	3	Mean ($\bar{x}$)		
F1	0.216	0.215	0.22	0.217	77.6	0.003
F2	0.332	0.345	0.394	0.357	63.2	0.033
F3	0.145	0.144	0.144	0.144	85.1	0.001
F4	0.127	0.128	0.129	0.128	86.8	0.001
F5	0.259	0.237	0.278	0.258	73.4	0.021
F6	0.152	0.157	0.158	0.156	83.9	0.003
F7	0.176	0.179	0.182	0.179	81.5	0.003
F8	0.168	0.17	0.169	0.169	82.6	0.001
F9	0.215	0.22	0.217	0.217	77.6	0.003
F10	0.202	0.211	0.206	0.206	78.7	0.005
F11	0.228	0.241	0.235	0.235	75.8	0.007
F12	0.2	0.201	0.204	0.202	79.2	0.002
F13	0.135	0.136	0.133	0.135	86.1	0.002
F14	0.111	0.114	0.11	0.112	88.5	0.002
F15	0.098	0.096	0.099	0.098	89.9	0.002
F16	0.134	0.133	0.135	0.134	86.2	0.001
F17	0.162	0.158	0.165	0.162	83.3	0.004
Blank	0.044	0.044	0.044	0.044		0.000
Control	0.06	0.061	0.06	0.060		0.001

26. PP1 inhibitory activity of diluted 17 fractions (x10) of *N. spumigena* KAC 66 collected from reversed phase flash chromatography. The plate incubated for 14 h at 37 °C and read at 405 nm.

Fractions	OD					
	1	2	3	Mean ($\bar{x}$)	Inhibition (%)	Std Dev (σ_{n-1})
F1	0.147	0.146	0.14	0.144	85.2	0.004
F2	0.117	0.108	0.111	0.112	88.5	0.005
F3	0.093	0.089	0.088	0.090	90.7	0.003
F4	0.094	0.092	0.092	0.093	90.5	0.001
F5	0.093	0.095	0.092	0.093	90.4	0.002
F6	0.099	0.089	0.093	0.094	90.4	0.005
F7	0.101	0.095	0.095	0.097	90.0	0.003
F8	0.099	0.095	0.081	0.092	90.6	0.009
F9	0.104	0.105	0.157	0.122	87.5	0.030
F10	0.126	0.115	0.109	0.117	88.0	0.009
F11	0.185	0.109	0.108	0.134	86.2	0.044
F12	0.103	0.102	0.104	0.103	89.4	0.001
F13	0.099	0.102	0.096	0.099	89.8	0.003
F14	0.103	0.12	0.103	0.109	88.8	0.010
F15	0.12	0.155	0.157	0.144	85.2	0.021
F16	0.529	0.524	0.527	0.527	45.8	0.003
F17	0.736	0.768	0.717	0.740	23.9	0.026
Blank	0.044	0.044	0.043	0.044		0.001
Control	0.071	0.068	0.068	0.069		0.002
0 µg/ml NOD (control)	0.956	0.985	0.976	0.972		0.015

27. PP1 inhibitory activity of diluted 17 fractions (x100) of *N. spumigena* KAC 66 collected from reversed phase flash chromatography. The plate incubated for 14 h at 37 °C and read at 405 nm.

Fractions	OD				Inhibition (%)	Std Dev (σ_{n-1})
	1	2	3	Mean ($\bar{x}$)		
F1	0.18	0.172	0.175	0.176	81.9	0.004
F2	0.128	0.208	0.142	0.159	83.6	0.043
F3	0.096	0.095	0.094	0.095	90.2	0.001
F4	0.119	0.117	0.116	0.117	87.9	0.002
F5	0.098	0.098	0.099	0.098	89.9	0.001
F6	0.094	0.092	0.092	0.093	90.5	0.001
F7	0.097	0.098	0.097	0.097	90.0	0.001
F8	0.106	0.107	0.106	0.106	89.1	0.001
F9	0.112	0.106	0.104	0.107	89.0	0.004
F10	0.153	0.152	0.153	0.153	84.3	0.001
F11	0.141	0.14	0.142	0.141	85.5	0.001
F12	0.133	0.135	0.137	0.135	86.1	0.002
F13	0.186	0.144	0.148	0.159	83.6	0.023
F14	0.201	0.191	0.19	0.194	80.0	0.006
F15	0.421	0.351	0.633	0.468	51.8	0.147
F16	0.59	0.599	0.504	0.564	42.0	0.052
F17	0.612	0.602	0.588	0.601	38.2	0.012
Blank	0.044	0.044	0.044	0.044		0.000
+ve control	0.646	0.61	0.606	0.621		0.022
Control 0 µg/ml NOD (control)	0.063 0.956	0.062 0.985	0.063 0.976	0.063 0.972		0.001 0.015

28. PP1 inhibitory activity of diluted 17 fractions (x1000) of *N. spumigena* KAC 66 collected from reversed phase flash chromatography. The plate incubated for 14 h at 37 °C and read at 405 nm.

Fractions	OD				StDev (σn_{-1})	Inhibition (%)
	1	2	3	Mean ($\bar{x}$)		
F1	0.953	0.968	0.956	0.959	0.01	1.4
F2	0.574	0.614	0.593	0.594	0.02	38.9
F3	0.238	0.219	0.23	0.229	0.01	76.4
F4	0.33	0.341	0.336	0.336	0.01	65.5
F5	0.251	0.223	0.228	0.234	0.01	75.9
F6	0.22	0.239	0.217	0.225	0.01	76.8
F7	0.395	0.392	0.36	0.382	0.02	60.7
F8	0.376	0.38	0.38	0.379	0.00	61.1
F9	0.399	0.468	0.484	0.450	0.05	53.7
F10	0.498	0.538	0.499	0.512	0.02	47.4
F11	0.344	0.526	0.481	0.450	0.09	53.7
F12	0.47	0.512	0.47	0.484	0.02	50.2
F13	0.476	0.466	0.408	0.450	0.04	53.7
F14	0.483	0.464	0.435	0.461	0.02	52.6
F15	0.47	0.447	0.409	0.442	0.03	54.5
F16	0.465	0.466	0.454	0.462	0.01	52.5
F17	0.407	0.457	0.362	0.409	0.05	58.0
Blank	0.046	0.049	0.049	0.048	0.00	
Control	0.065	0.064	0.063	0.064	0.00	
+ve control	0.416	0.445	0.468	0.443	0.03	
0 µg/ml NOD (control)	0.956	0.985	0.976	0.972	0.01	

29. PP1 inhibitory activities of 17 undiluted and dilution fractions of *N. spumigena* KAC 66 collected from reversed phase flash chromatography.

Fractions	Undiluted	Diluted		
		(x10)	(x100)	(x1000)
	2 ml dried fraction dissolved in 200 µl MQ water multiply by 10	100 µl of 1 + 900 µl MQ water divided by 10	100 µl of 2 + 900 µl MQ water divided by 100	10 µl of 3 + 990 µl MQ water divided by 1000
	1	2	3	4
	% inhibition			
F1	77.6	85.2	81.9	1.4
F2	63.2	88.5	83.6	38.9
F3	85.1	90.7	90.2	76.4
F4	86.8	90.5	87.9	65.5
F5	73.4	90.4	89.9	75.9
F6	83.9	90.4	90.5	76.8
F7	81.5	90.0	90.0	60.7
F8	82.6	90.6	89.1	61.1
F9	77.6	87.5	89.0	53.7
F10	78.7	88.0	84.3	47.4
F11	75.8	86.2	85.5	53.7
F12	79.2	89.4	86.1	50.2
F13	86.1	89.8	83.6	53.7
F14	88.5	88.8	80.0	52.6
F15	89.9	85.2	51.8	54.5
F16	86.2	45.8	42.0	52.5
F17	83.3	23.9	38.2	58.0

30. Weight of empty, freeze dried filter discs (with cells) and cell biomass for *N. spumigena* KAC 66 to determine relationship between cell biomass and chlorophyll-*a* (±S.D= standard deviation).

Dilutions	Empty filter paper wts. (mg)						Freeze dried filter paper with cells (mg)							Biomass				
	1	2	3	4	5	6 (a)	1	2	3	4	5	6 (b)	Freeze dried cells (c) (b-a=c) (mg/20ml)	(mg/20ml)	(mg/ml)	(ug/ml)	Mean (x) (ug/ml)	Stdev (σ_{n-1})
100ml																		
1	118.80	118.80	118.73	118.90	118.90	118.90	141.25	141.02	141.05	141.03	141.02	141.01	22.12	22.11	1.106	1105.5	1108.0	2.29
2	119.50	119.22	119.00	119.06	119.00	119.00	141.38	141.20	141.31	141.18	141.17	141.17	22.17	22.17	1.109	1108.5		
3	115.70	116.55	116.53	116.57	116.54	116.53	139.02	138.70	139.09	138.75	138.73	138.73	22.19	22.20	1.110	1110.0		
50ml																		
1	117.20	117.40	117.50	117.36	117.36	117.40	129.09	129.07	129.10	128.97	128.74	128.73	11.38	11.33	0.566	566.5	597.0	26.47
2	116.26	116.64	116.74	116.74	116.71	116.72	129.33	129.13	129.11	129.15	128.96	129.00	12.25	12.28	0.614	614.0		
3	117.13	117.50	117.56	117.56	117.56	117.55	129.80	129.79	129.77	129.78	129.77	129.76	12.21	12.21	0.611	610.5		
25ml																		
1	117.44	117.44	117.50	117.53	117.52	117.52	125.43	125.40	125.32	125.25	125.03	125.01	7.51	7.49	0.375	374.5	367.0	16.12
2	117.21	118.84	118.80	118.80	118.80	118.80	125.94	125.68	125.76	125.92	125.76	125.77	6.96	6.97	0.349	348.5		
3	118.22	118.10	118.00	118.13	118.13	118.12	125.74	125.66	125.59	125.75	125.72	125.68	7.59	7.56	0.378	378.0		
10ml																		
1	116.84	117.02	117.20	117.07	117.08	117.00	121.06	121.06	121.08	121.12	121.02	120.95	3.94	3.95	0.198	197.5	214.0	22.79
2	118.52	118.46	118.40	118.28	118.27	118.27	122.65	122.33	122.47	122.45	122.38	122.36	4.11	4.09	0.205	204.5		
3	118.60	118.71	118.88	118.40	118.80	118.80	123.84	123.75	123.64	123.64	123.64	123.60	4.84	4.80	0.240	240.0		
5ml																		
1	116.14	116.00	116.09	116.10	116.10	116.10	120.40	120.30	120.34	120.32	120.29	120.30	4.19	4.20	0.210	210.0	171.3	40.07
2	119.05	119.60	119.41	119.32	119.30	119.30	123.01	123.00	123.03	123.01	122.77	122.78	3.47	3.48	0.174	174.0		
3	118.88	118.60	118.86	118.93	118.90	118.90	121.76	121.70	121.77	121.88	121.50	121.50	2.60	2.60	0.130	130.0		
1ml																		
1	117.00	116.90	117.19	117.22	117.23	117.23	120.82	120.72	120.80	120.89	120.83	120.80	3.60	3.57	0.179	178.5	163.2	44.05
2	118.06	117.80	118.09	118.07	118.00	118.03	120.35	120.34	120.30	120.29	120.29	120.30	2.29	2.27	0.114	113.5		
3	117.04	117.04	117.20	117.15	117.12	117.01	121.09	121.07	121.09	120.97	120.97	120.96	3.85	3.95	0.197	197.5		

31. Chl-*a* concentrations for *N. spumigena* KAC 66 to determine relationship between cell biomass and chlorophyll-*a* (±S.D = standard deviation).

Dilutions	Absorbance	Concentration of Chl-*a* (μg/20ml)	(μg/ml)	Mean (μg/ml)	±STDev (σ_{n-1})
100ml					
	2.322	7.55	7546.5	7554.1	10.45
	2.328	7.57	7566.0		
	2.323	7.55	7549.8		
50ml					
	1.800	5.85	5850.0	5796.9	78.29
	1.756	5.71	5707.0		
	1.795	5.83	5833.8		
25ml					
	0.886	2.88	2879.5	2785.3	84.31
	0.849	2.76	2759.3		
	0.836	2.72	2717.0		
10ml					
	0.386	1.25	1254.5	1206.8	50.59
	0.373	1.21	1212.3		
	0.355	1.15	1153.8		
5ml					
	0.185	0.60	601.3	619.7	34.75
	0.184	0.60	598.0		
	0.203	0.66	659.8		
1ml					
	0.042	0.14	136.5	133.3	5.63
	0.042	0.14	136.5		
	0.039	0.13	126.8		

32. Serial dilution for cell biomass and Chl-*a.*

Dilutions (ml)	**Cell biomass mean (x) (µg/ml)**	**Mean of Chl-*a* (µg/ml)**
100	1108	7554.1
50	597	5796.9
25	367	2785.3
10	214	1206.8
5	171.3	619.7
1	163.2	133.3

33. Absorption and standard deviation of concentrated sample.

Absorbance (100% extract)	**Mean absorbanc (x)**	**Stdev (σ)**	**Absorbance (50% extract)**	**Mean absorbanc (x)**	**Stdev (σ_{n-1})**
2.32	2.32	0.003	1.64	1.66	0.049
2.33			1.72		
2.32			1.63		

34. Cell biomass and Chl-*a* concentrations for *N. spumigena* KAC 66 grown in 10 L glass for 7 weeks at 20ºC (±S.D = standard deviation).

Time (weeks)	Freeze dried biomass of cells						Chlorophyll-a estimation			
	Freeze dried cells (mg/20ml)	mg/ml	ug/ml	mean (x) (ug/ml)	±STDev (σn-1)	Absorbance	Chlorophyll *a* (µg/20ml)	Chl-*a* (µg/ml)	Mean Chl-*a* (µg/ml)	±STDev (σn-1)
To										
1	129.0	6.45	6450.0	6150.0	396.86	0.113	0.37	0.018	0.018	0.001
2	126.0	6.30	6300.0			0.122	0.40	0.020		
3	114.0	5.70	5700.0			0.105	0.34	0.017		
T1										
1	131.9	6.60	6595.0	6386.7	220.93	0.276	0.90	0.045	0.048	0.003
2	128.2	6.41	6410.0			0.310	1.01	0.050		
3	123.1	6.16	6155.0			0.295	0.96	0.048		
T2										
1	135.0	6.75	6750.0	6715.0	44.44	0.460	1.50	0.075	0.083	0.008
2	134.6	6.73	6730.0			0.512	1.66	0.083		
3	133.3	6.67	6665.0			0.557	1.81	0.091		
T3										
1	126.8	6.34	6340.0	6641.7	261.36	0.818	2.66	0.133	0.130	0.008
2	136.0	6.80	6800.0			0.837	2.72	0.136		
3	135.7	6.79	6785.0			0.744	2.42	0.121		
T4										
1	137.0	6.85	6850.0	6735.0	103.32	0.878	2.85	0.143	0.134	0.008
2	134.1	6.71	6705.0			0.815	2.65	0.132		
3	133.8	6.65	6650.0			0.786	2.55	0.128		
T5										
1	137.0	6.85	6850.0	6688.3	207.02	1.062	3.45	0.173	0.153	0.017
2	135.2	6.76	6760.0			0.878	2.85	0.143		
3	129.1	6.46	6455.0			0.891	2.90	0.145		
T6										
1	139.0	6.95	6950.0	6678.3	237.50	1.395	4.53	0.227	0.199	0.024
2	131.5	6.58	6575.0			1.143	3.71	0.186		
3	130.2	6.51	6510.0			1.130	3.67	0.184		
T7										
1	141.5	7.08	7075.0	6916.7	137.33	1.255	4.08	0.204	0.193	0.013
2	136.9	6.85	6845.0			1.096	3.56	0.178		
3	136.6	6.83	6830.0			1.203	3.91	0.195		

35. Intra and extracellular levels of NOD and nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown in 10 L glass flasks for 7 weeks at 22°C during the analysis on LC-MS. (for NOD 238 nm wavelength and retention time 12.18 to 14.12 min, for nodulopeptin 210 nm wavelength and retention time 17.98 to 18.00 min; RT= retention time, PA= peak area, n.d= not detected)

Time (weeks)	Nodularin				Nodulopeptin 901			
	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev (σ_{n-1})
Extracellular								
To	-	n.d	n.d	n.d	-	n.d	n.d	n.d
	-	n.d			-	n.d		
	-	n.d			-	n.d		
T1	13.10	29	23	5.7	18.03	100	81	50.0
	13.10	23			18.02	119		
	13.12	18			18.03	24		
T2	13.13	30	29	7.0	18.07	138	177	57.5
	13.10	22			18.03	243		
	13.08	36			18.02	151		
T3	13.08	71	55	15.2	18.02	238	248	51.5
	13.08	51			18.02	304		
	13.08	41			18.02	203		
T4	13.07	45	130	148.4	18.00	258	319	77.8
	13.07	301			18.00	407		
	13.08	43			18.00	292		
T5	-	n.d	n.d	n.d	17.98	291	327	65.6
	-	n.d			17.98	402		
	-	n.d			17.98	287		
T6	-	n.d	n.d	n.d	17.98	486	467	28.4
	-	n.d			17.98	481		
	-	n.d			17.98	434		
T7	-	n.d	n.d	n.d	17.97	375	442	129.8
	-	n.d			17.98	592		
	-	n.d			18.00	360		

Time (weeks)	Nodularin				Nodulopeptin 901			
	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev (σ_{n-1})
Intracellular								
To	13.07	196	189	16.1	18.00	50	46	7.7
	13.05	200			17.98	50		
	13.08	170			17.98	37		
T1	13.08	430	489	62.7	18.03	131	179	44.5
	13.12	482			18.07	219		
	13.12	555			18.05	186		
T2	13.08	1100	1117	16.8	18.02	222	283	53.8
	13.08	1118			18.02	308		
	13.07	1133			18.00	321		
T3	13.08	1187	1192	45.7	18.02	291	288	47.0
	13.07	1241			18.00	332		
	13.08	1150			18.02	239		
T4	13.07	1465	1287	206.0	18.00	422	416	17.5
	13.07	1061			18.00	431		
	13.07	1336			17.98	397		
T5	13.07	1234	1346	110.4	17.98	422	484	113.1
	13.07	1455			17.98	614		
	13.07	1350			17.98	415		
T6	13.05	1438	1390	79.0	17.97	699	708	117.8
	13.05	1299			17.98	831		
	13.08	1434			18.00	596		
T7	13.08	825	1085	247.7	18.00	654	725	111.3
	13.08	1318			18.00	854		
	13.08	1113			18.00	669		

36. The amount (ng/ml) of intra and extracellular concentrations of NOD and nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown in 10 L glass flasks for 7 weeks at 22°C. (n.d= not detected)

Time	**Nodularin**			**Nodulopeptin 901**		
(weeks)	**Amount (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**STDev (σ_{n-1})**	**Amount (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**STDev (σ_{n-1})**
Extracellular						
To	n.d n.d n.d	n.d	n.d	n.d n.d n.d	n.d	n.d
T1	8.2 6.4 5.0	6.6	1.6	51.1 60.8 12.3	41.4	25.7
T2	8.4 6.2 10.1	8.2	2.0	70.5 124.1 77.1	90.6	29.2
T3	19.9 14.3 11.5	15.2	4.3	121.6 155.3 103.7	126.8	26.2
T4	12.6 84.4 12.1	36.4	41.6	131.8 207.9 149.1	162.9	39.9
T5	n.d n.d n.d	n.d	n.d	111.8 205.3 146.6	154.6	47.2
T6	n.d n.d n.d	n.d	n.d	146.1 143.5 221.7	170.4	44.4
T7	n.d n.d n.d	n.d	n.d	191.5 302.3 183.9	225.9	66.3

Time (weeks)	Nodularin			Nodulopeptin 901		
	Amount (ng/ml)	Mean ($\bar{x}$) (ng/ml)	STDev (σ_{n-1})	Amount (ng/ml)	Mean ($\bar{x}$) (ng/ml)	STDev (σ_{n-1})
Intracellular						
To	54.9 56.1 47.7	52.9	4.6	25.5 25.5 18.9	23.3	3.8
T1	120.5 135.1 155.6	137.1	17.6	66.9 111.8 95.0	91.2	22.7
T2	308.4 313.4 317.6	313.1	4.6	113.4 157.3 163.9	144.9	27.5
T3	332.8 347.9 322.4	334.4	12.8	148.6 169.6 122.1	146.7	23.8
T4	410.7 297.4 374.5	360.9	57.8	215.5 220.1 202.8	212.8	9.0
T5	345.9 407.9 378.5	377.4	31.0	215.5 313.6 212.0	247.0	57.7
T6	403.1 364.2 402.0	389.8	22.2	357.0 424.4 304.4	361.9	60.2
T7	231.3 369.5 312.0	304.3	69.4	334.0 436.2 341.7	370.6	56.9

37. Weight of empty, freeze dried filter discs (with cells) and cell biomass for *N. spumigena* KAC 66 grown in 8 L Perspex columns for 5 weeks at 20ºC (±S.D = standard deviation).

Columns	Empty filter paper weights (mg)			Freeze dried filter paper with cells (mg)			Freeze dried cells (c) (b-a=c) (mg/20ml)	(mg/ml)	Biomass (µg/ml)	Mean ($\bar{x}$) (µg/ml)	StDev (σ_{n-1})
	1	2	3 (a)	1	2	3 (b)					
To											
1	116.67	116.63	116.61	120.67	120.67	120.68	4.07	0.20	203.5	213.6	19.51
2	116.76	116.77	116.76	121.14	121.13	121.14	4.38	0.22	219.0		
3	118.50	118.51	118.50	123.45	123.41	123.41	4.91	0.25	245.5		
4	118.07	118.01	118.01	122.09	122.05	122.04	4.03	0.20	201.5		
5	116.34	116.32	116.32	120.31	120.30	120.29	3.97	0.20	198.5		
T1											
1	115.10	115.03	115.00	121.93	121.90	121.91	6.91	0.35	345.5	309.5	37.82
2	116.20	116.15	116.13	122.00	121.34	121.36	5.23	0.26	261.5		
3	116.00	115.96	115.95	122.89	122.85	122.82	6.87	0.34	343.5		
4	115.99	115.97	115.98	121.62	121.53	121.57	5.59	0.28	279.5		
5	115.79	115.76	115.75	122.12	122.09	122.1	6.35	0.32	317.5		
T2											
1	115.70	115.57	115.56	123.81	123.79	123.77	8.21	0.41	410.5	447.6	54.71
2	117.77	117.73	117.70	125.17	125.14	125.15	7.45	0.37	372.5		
3	116.60	116.59	116.54	126.65	126.63	126.64	10.10	0.51	505.0		
4	117.50	117.51	117.48	126.89	126.83	126.80	9.32	0.47	466.0		
5	118.90	118.90	118.90	128.59	128.59	128.58	9.68	0.48	484.0		
T3											
1	116.19	116.08	116.06	122.65	122.62	122.62	6.56	0.33	328.0	420.5	94.62
2	116.90	116.82	116.83	125.46	125.45	125.43	8.60	0.43	430.0		
3	117.90	117.69	117.70	126.71	126.70	126.69	8.99	0.45	449.5		
4	116.70	116.71	116.70	127.89	127.87	127.88	11.18	0.56	559.0		
5	116.40	116.40	116.38	123.11	123.11	123.10	6.72	0.34	336.0		
T4											
1	118.86	118.84	118.85	124.97	124.95	124.96	6.11	0.31	305.5	281.1	104.10
2	118.34	118.32	118.33	127.39	127.36	127.37	9.04	0.45	452.0		
3	118.33	118.31	118.32	122.45	122.43	122.44	4.12	0.21	206.0		
4	117.56	117.55	117.55	122.39	122.35	122.34	4.79	0.24	239.5		
5	118.78	118.72	118.73	122.79	122.78	122.78	4.05	0.20	202.5		
T5											
1	120.33	120.31	120.30	125.15	125.13	125.13	4.83	0.24	241.5	344.9	112.21
2	120.10	120.06	120.06	125.56	125.51	125.52	5.46	0.27	273.0		
3	117.06	117.01	117.00	127.14	127.13	127.12	10.12	0.51	506.0		
4	118.40	118.39	118.38	124.13	124.11	124.12	5.74	0.29	287.0		
5	117.66	117.64	117.62	125.98	125.95	125.96	8.34	0.42	417.0		

38. Chl-*a* concentrations for *N. spumigena* KAC 66 grown in 8 L Perspex columns 5 weeks at 22ºC (±S.D = standard deviation).

Columns	Absorbance	Concentration of Chl-*a*			±STDev (σ_{n-1})
		Chlorophyll-*a* (μg/20ml)	Chl-*a* (μg/ml)	Mean Chl-*a* (μg/ml)	
To					
1	0.358	1.16	0.058	0.057	0.005
2	0.384	1.25	0.062		
3	0.312	1.01	0.051		
4	0.334	1.09	0.054		
5	0.370	1.20	0.060		
T1					
1	0.550	1.79	0.089	0.066	0.031
2	0.662	2.15	0.108		
3	0.362	1.18	0.059		
4	0.246	0.80	0.040		
5	0.218	0.71	0.035		
T2					
1	0.698	2.27	0.113	0.207	0.058
2	1.238	4.02	0.201		
3	1.355	4.40	0.220		
4	1.414	4.60	0.230		
5	1.657	5.39	0.269		
T3					
1	0.786	2.55	0.128	0.125	0.058
2	0.760	2.47	0.124		
3	1.044	3.39	0.170		
4	1.079	3.51	0.175		
5	0.185	0.60	0.030		
T4					
1	0.680	2.21	0.111	0.062	0.037
2	0.571	1.86	0.093		
3	0.254	0.83	0.041		
4	0.216	0.70	0.035		
5	0.186	0.60	0.030		
T5					
1	0.269	0.87	0.044	0.028	0.011
2	0.119	0.39	0.019		
3	0.165	0.54	0.027		
4	0.104	0.34	0.017		
5	0.196	0.64	0.032		

39. Intra and extracellular concentrations of NOD and nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown in 8 L Perspex columns for 5 weeks at 22°C during the analysis on LC-MS. (for NOD 238 nm wavelength and retention time 14.18 to 14.12 min, for nodulopeptin 210 nm wavelength and retention time 17.98 to 18.00 min; RT= retention time, PA= peak area, n.d= not detected)

Time (weeks)	Nodularin				Nodulopeptin 901			
	RT (min)	PA	mean of PA ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	mean of PA ($\bar{x}$)	STDev (σ_{n-1})
Extracellular								
To								
1	-	n.d	n.d	n.d	-	n.d	n.d	n.d
2	-	n.d			-	n.d		
3	-	n.d			-	n.d		
4	-	n.d			-	n.d		
5	-	n.d			-	n.d		
T1								
1	-	n.d	n.d	n.d	-	n.d	n.d	n.d
2	-	n.d			-	n.d		
3	-	n.d			-	n.d		
4	-	n.d			-	n.d		
5	-	n.d			-	n.d		
T2								
1	14.28	1409	384	537.1	-	n.d	n.d	n.d
2	14.25	129			-	n.d		
3	14.27	166			-	n.d		
4	14.23	147			-	n.d		
5	14.27	67			-	n.d		
T3								
1	14.23	2319	718	866.1	-	n.d	n.d	n.d
2	14.22	454			-	n.d		
3	14.22	466			-	n.d		
4	14.22	310			-	n.d		
5	14.23	41			-	n.d		
T4								
1	14.20	4435	1006	1768.6	-	n.d	n.d	n.d
2	14.23	416			-	n.d		
3	14.23	82			-	n.d		
4	14.22	43			-	n.d		
5	14.22	53			-	n.d		
T5								
1	14.13	8562	1968	3399.9	-	n.d	n.d	n.d
2	14.22	705			-	n.d		
3	14.22	307			-	n.d		
4	14.23	96			-	n.d		
5	14.23	171			-	n.d		

Time (weeks)	Nodularin				Nodulopeptin 901			
	RT (min)	PA	mean of PA ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	mean of PA ($\bar{x}$)	STDev (σ_{n-1})
Intracellular								
To								
1	-	n.d	n.d	n.d	17.47	102	58	53.4
2	-	n.d			-	n.d		
3	-	n.d			17.43	92		
4	-	n.d			17.45	99		
5	-	n.d			-	n.d		
T1								
1	14.32	1716	1204	821.3	17.47	289	153	124.5
2	14.32	1793			17.45	261		
3	14.32	1481			17.45	125		
4	14.30	1032			17.57	92		
5	-	n.d			-	n.d		
T2								
1	14.27	2590	2438	1003.8	17.42	179	308	241.6
2	14.23	2435			17.43	92		
3	14.23	2284			17.40	171		
4	14.23	2297			17.40	462		
5	14.25	2581			17.42	635		
T3								
1	14.23	102	1731	2070.2	-	n.d	n.d	n.d
2	14.27	284			-	n.d		
3	14.20	126			-	n.d		
4	14.25	3507			-	n.d		
5	14.23	4638			-	n.d		
T4								
1	14.20	5199	1613	1925.9	-	n.d	16	23.8
2	14.20	1123			-	n.d		
3	14.25	582			17.50	23		
4	14.25	376			-	n.d		
5	14.23	786			17.84	58		
T5								
1	14.22	387	131	146.5	-	n.d	n.d	n.d
2	14.28	9			-	n.d		
3	14.22	146			-	n.d		
4	14.23	34			-	n.d		
5	14.22	78			-	n.d		

40. The amount (ng/ml) of intra and extracellular concentrations of NOD and nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown in 8 L Perspex columns for 5 weeks at 22°C (n.d= not detected).

Time (weeks)	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	STDev (σ_{n-1})
Extracellular						
To						
1	n.d	n.d	n.d	n.d	n.d	n.d
2	n.d			n.d		
3	n.d			n.d		
4	n.d			n.d		
5	n.d			n.d		
T1						
1	n.d	n.d	n.d	n.d	n.d	n.d
2	n.d			n.d		
3	n.d			n.d		
4	n.d			n.d		
5	n.d			n.d		
T2						
1	395	107.5	161.0	n.d	n.d	n.d
2	36			n.d		
3	47			n.d		
4	41			n.d		
5	19			n.d		
T3						
1	650	201.3	255.5	n.d	n.d	n.d
2	127			n.d		
3	131			n.d		
4	87			n.d		
5	11			n.d		
T4						
1	1244	282.0	539.3	n.d	n.d	n.d
2	117			n.d		
3	23			n.d		
4	12			n.d		
5	15			n.d		
T5						
1	673	206.3	269.0	n.d	n.d	n.d
2	198			n.d		
3	86			n.d		
4	27			n.d		
5	48			n.d		

Time (weeks)	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	STDev (σ_{n-1})
Intracellular						
To						
1	n.d	n.d	n.d	52	29.9	27.4
2	n.d			0		
3	n.d			47		
4	n.d			51		
5	n.d			0		
T1						
1	481	337.7	206.3	148	78.3	61.5
2	503			133		
3	415			64		
4	289			47		
5	0			0		
T2						
1	726	683.3	41.4	91	157.2	117.8
2	683			47		
3	640			87		
4	644			236		
5	724			324		
T3						
1	29	485.4	609.8	n.d	n.d	n.d
2	80			n.d		
3	35			n.d		
4	983			n.d		
5	1300			n.d		
T4						
1	1458	452.3	567.3	0	8.3	13.0
2	315			0		
3	163			12		
4	105			0		
5	220			30		
T5						
1	108	36.7	42.7	n.d	n.d	n.d
2	3			n.d		
3	41			n.d		
4	10			n.d		
5	22			n.d		

41. Chl-*a* concentrations for *N. spumigena* KAC 66 grown for 6 weeks at different temperatures (±S.D = standard deviation).

Time (Weeks)	Conditions and temperatures	Absorbance	Concentration of Chl-*a*			
			Chlorophyll-*a* (ug/20ml)	Chl-*a* (ug/ml)	Mean Chl-*a* (ug/ml)	±STDev (σn-1)
To	**22 °C**	0.211	0.686	0.034	0.033	0.003
		0.184	0.598	0.030		
		0.218	0.709	0.035		
	25 °C	0.214	0.696	0.035	0.034	0.005
		0.178	0.579	0.029		
		0.243	0.790	0.039		
	30 °C	0.204	0.663	0.033	0.036	0.002
		0.229	0.744	0.037		
		0.229	0.744	0.037		
T1	**22 °C**	0.516	1.677	0.084	0.085	0.001
		0.521	1.693	0.085		
		0.527	1.713	0.086		
	25 °C	0.593	1.927	0.096	0.098	0.003
		0.625	2.031	0.102		
		0.597	1.940	0.097		
	30 °C	0.643	2.090	0.104	0.111	0.010
		0.653	2.122	0.106		
		0.750	2.438	0.122		
T2	**22 °C**	0.761	2.473	0.124	0.127	0.007
		0.831	2.701	0.135		
		0.753	2.447	0.122		
	25 °C	0.842	2.737	0.137	0.142	0.008
		0.930	3.023	0.151		
		0.855	2.779	0.139		
	30 °C	1.025	3.331	0.167	0.168	0.010
		0.981	3.188	0.159		
		1.097	3.565	0.178		
T3	**22 °C**	0.899	2.922	0.146	0.149	0.008
		0.971	3.156	0.158		
		0.873	2.837	0.142		
	25 °C	1.022	3.322	0.166	0.169	0.003
		1.059	3.442	0.172		
		1.031	3.351	0.168		
	30 °C	1.093	3.552	0.178	0.177	0.002
		1.099	3.572	0.179		
		1.073	3.487	0.174		
T4	**22 °C**	1.152	3.744	0.187	0.189	0.002
		1.170	3.803	0.190		
		1.169	3.799	0.190		
	25 °C	1.072	3.484	0.174	0.165	0.010
		1.019	3.312	0.166		
		0.947	3.078	0.154		
	30 °C	1.308	4.251	0.213	0.213	0.000
		1.311	4.261	0.213		
		1.307	4.248	0.212		
T5	**22 °C**	1.122	3.647	0.182	0.194	0.010
		1.223	3.975	0.199		
		1.237	4.020	0.201		
	25 °C	1.065	3.461	0.173	0.154	0.017
		0.910	2.958	0.148		
		0.861	2.798	0.140		
	30 °C	1.248	4.056	0.203	0.202	0.000
		1.244	4.043	0.202		
		1.242	4.037	0.202		
T6	**22 °C**	1.153	3.747	0.187	0.193	0.006
		1.226	3.985	0.199		
		1.193	3.877	0.194		
	25 °C	0.888	2.886	0.144	0.126	0.016
		0.738	2.399	0.120		
		0.696	2.262	0.113		
	30 °C	1.024	3.328	0.166	0.171	0.016
		1.163	3.780	0.189		
		0.977	3.175	0.159		

42. Intra and extracellular levels of NOD for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different temperatures analysed on LC-MS. (NOD 238 nm wavelength and retention time 14.37 to 14.48 min; RT= retention time, PA= peak area, n.d= not detected).

Temperature (°C)	To				T1				T2				T3			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Exrtracellular																
22	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d			n.d	n.d		
25	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d			n.d	n.d		
30	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d			n.d	n.d		
Intracellular																
22	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	14.42	1585	1684	93.2	13.98	2866	3037	148.8
	n.d	n.d			n.d	n.d			14.37	1770			14.43	3128		
	n.d	n.d			n.d	n.d			14.88	1698			14.40	3119		
25	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	14.40	1157	1156	81.5	14.43	1558	1688	128.8
	n.d	n.d			n.d	n.d			14.42	1075			14.43	1815		
	n.d	n.d			n.d	n.d			14.40	1238			14.45	1692		
30	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	14.43	183.0	61	105.7	14.45	87	167	69.3
	n.d	n.d			n.d	n.d			n.d	0			14.47	209		
	n.d	n.d			n.d	n.d			n.d	0			14.47	205		

Temperature (°C)	T4				T5				T6			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Exrtracellular												
22	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d		
25	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d		
30	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d		
Intracellular												
22	14.52	3903	3784	208.3	14.47	3812	3677	136.3	14.55	3459	3611	314.6
	14.48	3906			14.42	3680			14.52	3973		
	14.50	3543			14.45	3540			14.53	3402		
25	14.50	1994	1687	300.0	14.43	1806	1600	205.8	14.55	1339	1212	123.2
	14.50	1674			14.45	1394			14.07	1093		
	14.52	1395			14.48	1601			14.50	1204		
30	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	n.d	n.d			n.d	n.d			n.d	n.d		
	n.d	n.d			n.d	n.d			n.d	n.d		

43. Intra and extracellular levels of nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different temperatures analysed on LC-MS. (nodulopeptin 210 nm wavelength and retention time 17.98 to 18.00 min; RT= retention time, PA= peak area, n.d= not detected).

	To				T1				T2				T3			
Temperature (°C)	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**STDev (σ_{n-1})**	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**STDev (σ_{n-1})**	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**STDev (σ_{n-1})**	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**STDev (σ_{n-1})**
Exrtracellular																
22	17.50	76	99	25.1	18.02	83	93	8.8	17.50	126	162	32.1	17.55	147	218	62.3
	17.48	95			17.97	99			17.50	173			17.55	243		
	17.48	126			18.00	96			17.50	187			17.53	264		
25	17.50	92	105	12.2	18.02	82	69	17.9	17.52	136	146	9.4	17.52	307	270	52.9
	17.48	108			18.00	49			17.50	147			17.53	210		
	17.48	116			18.02	77			17.50	155			17.55	294		
30	17.50	104	113	10.4	18.00	79	86	8.1	17.53	100	119	17.5	17.53	131	142	9.4
	17.50	124			18.00	84			17.52	123			17.52	147		
	17.50	109			18.00	95			17.50	135			17.53	148		
Intracellular																
22	-	n.d	-	-	18.03	103	115	12.5	17.53	307	374	71.5	17.53	436	1156	632.0
	-	n.d			18.08	128			17.48	367			17.53	1414		
	-	n.d			18.00	113			17.50	450			17.50	1619		
25	-	n.d	-	-	18.02	93	77	14.0	17.52	203	228	35.7	17.53	572	617	138.6
	-	n.d			18.02	68			17.52	212			17.55	507		
	-	n.d			18.00	70			17.50	269			17.57	773		
30	-	n.d	-	-	18.03	58	62	11.7	17.52	128	145	16.5	17.53	135	159	26.7
	-	n.d			18.00	54			17.50	148			17.53	188		
	-	n.d			18.02	76			17.48	160			17.53	154		

Temperature	T4				T5				T6			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Exrtracellular												
22	17.53	317	450	117.5	17.58	404	595	166.6	17.55	618	771	133.3
	17.55	492			17.53	679			17.60	860		
	17.57	541			17.53	704			17.55	835		
25	17.53	580	631	68.3	17.55	639	681	58.4	17.55	649	613	53.9
	17.55	604			17.55	656			17.62	551		
	17.53	709			17.53	748			17.62	639		
30	17.55	263	261	15.6	17.53	253	233	17.3	17.58	197	185	16.1
	17.57	245			17.58	228			17.60	190		
	17.57	276			17.53	220			17.62	167		
Intracellular												
22	17.57	912	1111	176.2	17.55	1164	1301	125.4	17.63	1266	1922	855.4
	17.53	1247			17.50	1330			17.60	1611		
	17.57	1174			17.52	1410			17.60	2890		
25	17.53	876	838	34.0	17.50	979	956	101.4	17.60	1024	942	124.2
	17.53	809			17.52	845			17.58	799		
	17.53	830			17.53	1044			17.57	1002		
30	17.53	198	194	3.6	17.50	217	214	9.2	17.62	214	201	15.4
	17.52	191			17.53	203			17.57	204		
	17.53	194			17.50	221			17.58	184		

44. The amount (ng/ml) of intra and extracellular levels of NOD for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different temperatures (n.d= not detected).

Temperature (°C)	To			T1			T2			T3		
	Amount of NOD (ng/ml)	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**	**Amount of NOD (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**	**Amount of NOD (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**	**Amount of NOD (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**
Exrtracellular												
22	n.d	0	0	n.d	0	0	n.d	0	0	n.d	0	0
	n.d			n.d			n.d			n.d		
	n.d			n.d			n.d			n.d		
25	n.d	0	0	n.d	0	0	n.d	0	0	n.d	0	0
	n.d			n.d			n.d			n.d		
	n.d			n.d			n.d			n.d		
30	n.d	0	0	n.d	0	0	n.d	0	0	n.d	0	0
	n.d			n.d			n.d			n.d		
	n.d			n.d			n.d			n.d		
Intracellular												
22	n.d	0	0	n.d	0	0	444.4	472.21	26.13	803.5	851.60	41.70
	n.d			n.d			496.2			876.9		
	n.d			n.d			476.0			874.4		
25	n.d	0	0	n.d	0	0	324.4	324.27	22.85	436.8	473.32	36.04
	n.d			n.d			301.4			508.8		
	n.d			n.d			347.1			474.3		
30	n.d	0	0	n.d	0	0	51.3	17.10	29.62	24.4	46.78	19.40
	n.d			n.d			0			58.6		
	n.d			n.d			0			57.4		

Temperature (°C)	T4			T5			T6		
	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Exrtracellular									
22	n.d n.d n.d	0	0	n.d n.d n.d	0	0	n.d n.d n.d	0	0
25	n.d n.d n.d	0	0	n.d n.d n.d	0	0	n.d n.d n.d	0	0
30	n.d n.d n.d	0	0	n.d n.d n.d	0	0	n.d n.d n.d	0	0
Intracellular									
22	1094.2 1095.0 993.3	1060.84	58.51	1068.7 1031.7 992.4	1030.93	38.13	969.7 1113.8 953.7	1012.43	88.17
25	559.0 469.3 391.1	473.13	84.03	506.3 390.8 448.8	448.65	57.75	375.4 306.4 337.5	339.78	34.54
30	n.d n.d n.d	0	0	n.d n.d n.d	0	0	n.d n.d n.d	0	0

45. The amount (ng/ml) of intra and extracellular concentrations of nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different temperatures (n.d= not detected).

	To			T1			T2			T3		
Temperature (°C)	**Amount of nodulopeptin 901 (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**	**Amount of nodulopeptin 901 (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**	**Amount of nodulopeptin (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**	**Amount of nodulopeptin 901 (ng/ml)**	**Mean ($\bar{x}$) (ng/ml)**	**StDev (σ_{n-1})**
Exrtracellular												
22	38.9	50.6	12.8	42.2	47.3	4.5	64.2	82.8	16.4	75.2	111.3	31.8
	48.6			50.7			88.5			124.0		
	64.3			49.1			95.6			134.9		
25	64.3	59.6	4.6	41.9	35.4	9.1	69.4	74.4	4.8	156.9	138.0	27.0
	55.2			25.0			74.9			107.1		
	59.2			39.4			78.9			150.1		
30	53.2	57.5	5.3	40.5	43.9	4.1	51.3	61.0	8.9	67.0	72.5	4.8
	63.4			42.8			63.0			75.0		
	55.9			48.5			68.8			75.5		
Intracellular												
22	n.d	0	0	52.8	58.6	6.4	156.9	191.2	36.5	222.7	590.5	322.8
	n.d			65.4			187.3			721.9		
	n.d			57.5			229.6			826.8		
25	n.d	0	0	47.5	39.3	7.1	103.8	116.5	18.2	292.0	315.2	70.8
	n.d			34.7			108.3			259.0		
	n.d			35.6			137.4			394.7		
30	n.d	0	0	29.6	31.9	6.0	65.1	74.1	8.4	68.9	81.1	13.7
	n.d			27.3			75.5			95.9		
	n.d			38.6			81.8			78.7		

Temperature (°C)	T4			T5			T6		
	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Exrtracellular									
22	162.1	229.8	60.0	206.1	304.1	85.1	315.5	393.8	68.1
	251.2			346.8			439.4		
	276.3			359.4			426.5		
25	296.2	322.2	34.9	326.5	347.9	29.8	331.6	313.2	27.6
	308.5			335.1			281.6		
	361.9			382.0			326.6		
30	134.1	133.5	8.0	129.1	119.2	8.8	100.8	94.4	8.2
	125.2			116.2			97.2		
	141.2			112.2			85.1		
Intracellular									
22	465.7	567.4	90.0	594.6	664.7	64.1	646.6	981.6	436.9
	636.7			679.1			822.6		
	599.8			720.3			1475.7		
25	447.3	428.2	17.4	499.8	488.1	51.8	523.0	480.9	63.5
	413.4			431.5			407.9		
	424.0			533.0			511.7		
30	101.3	99.3	1.8	110.9	109.2	4.7	109.5	102.5	7.9
	97.8			103.9			104.1		
	98.8			112.8			94.0		

46. Weight of empty, freeze dried filter discs (with cells) and cell biomass for *N. spumigena* KAC 66 grown at different salinities for 6 weeks at 22ºC (±S.D = standard deviation).

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
To											
2	119.50	119.30	119.31	121.80	121.82	121.83	2.52	0.13	126.0	113.7	24.5
	120.32	120.30	120.31	122.01	122.02	122.02	1.71	0.09	85.5		
	118.80	118.76	118.76	121.36	121.35	121.35	2.59	0.13	129.5		
7	119.97	120.05	120.00	122.02	122.01	122.00	2.00	0.10	100.0	98.7	10.6
	120.00	119.75	119.73	121.92	121.91	121.90	2.17	0.11	108.5		
	120.83	120.77	120.76	122.56	122.50	122.51	1.75	0.09	87.5		
11	119.94	120.21	120.20	122.18	122.15	122.16	1.96	0.10	98.0	106.5	8.3
	117.14	116.85	116.84	119.15	119.15	119.13	2.29	0.11	114.5		
	117.02	117.00	117.01	119.20	119.14	119.15	2.14	0.11	107.0		
20	117.96	118.25	118.24	112.79	112.75	121.74	3.50	0.18	175.0	147.2	25.2
	119.40	119.20	119.19	121.74	121.73	121.71	2.52	0.13	126.0		
	118.00	116.96	116.97	119.80	119.78	119.78	2.81	0.14	140.5		
25	118.06	117.99	118.00	120.77	120.76	120.78	2.78	0.14	139.0	156.2	26.8
	115.84	115.87	115.83	119.61	119.54	119.57	3.74	0.19	187.0		
	118.05	118.08	118.06	120.95	120.93	120.91	2.85	0.14	142.5		

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T1											
2	117.11	117.03	117.04	123.52	123.50	123.49	6.45	0.32	322.5	270.3	45.3
	118.74	118.79	118.78	123.76	123.73	123.72	4.94	0.25	247.0		
	119.21	119.15	119.17	124.05	124.01	124.00	4.83	0.24	241.5		
7	120.07	120.01	120.03	125.14	125.12	125.11	5.08	0.25	254.0	305.0	57.0
	118.32	118.30	118.29	125.67	125.63	125.62	7.33	0.37	366.5		
	119.59	119.52	119.53	125.45	125.42	125.42	5.89	0.29	294.5		
11	117.39	117.38	117.40	124.94	124.93	124.90	7.50	0.38	375.0	324.8	44.4
	118.29	118.26	118.24	124.46	124.41	124.42	6.18	0.31	309.0		
	118.60	118.58	118.59	124.43	124.41	124.40	5.81	0.29	290.5		
20	115.72	115.60	115.62	124.07	124.05	124.04	8.42	0.42	421.0	410.3	31.4
	116.62	116.55	116.56	124.07	124.09	124.06	7.50	0.38	375.0		
	115.89	115.84	115.86	124.59	124.57	124.56	8.70	0.44	435.0		
25	109.00	108.95	108.99	119.10	119.07	119.05	10.06	0.50	503.0	408.2	84.7
	116.89	116.84	116.85	124.54	124.50	124.48	7.63	0.38	381.5		
	119.48	119.40	119.42	126.25	126.23	126.22	6.80	0.34	340.0		

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	StDev (σ_{n-1})
T2											
2	116.90	116.88	116.89	132.39	132.34	132.34	15.45	0.77	772.5	638.7	142.1
	116.40	116.39	116.38	129.49	129.47	129.46	13.08	0.65	654.0		
	119.10	119.06	119.04	128.83	128.81	128.83	9.79	0.49	489.5		
7	119.80	119.78	119.76	133.09	133.03	133.05	13.29	0.66	664.5	549.7	102.2
	117.06	117.02	116.99	126.39	126.37	126.36	9.37	0.47	468.5		
	118.45	118.43	118.43	128.78	128.76	128.75	10.32	0.52	516.0		
11	119.65	119.63	119.64	133.88	133.86	133.85	14.21	0.71	710.5	653.0	94.9
	118.10	118.09	118.08	132.21	132.20	132.18	14.10	0.71	705.0		
	118.95	118.93	118.91	129.81	129.79	129.78	10.87	0.54	543.5		
20	118.46	118.45	118.44	127.89	127.86	127.86	9.42	0.47	471.0	534.3	76.3
	117.25	117.23	117.23	127.51	127.50	127.49	10.26	0.51	513.0		
	119.65	119.63	119.64	132.05	132.03	132.02	12.38	0.62	619.0		
25	118.95	118.90	118.91	131.28	131.27	131.28	12.37	0.62	618.5	588.2	33.6
	119.95	119.93	119.94	131.01	130.99	130.98	11.04	0.55	552.0		
	118.26	118.24	118.25	130.19	130.15	130.13	11.88	0.59	594.0		

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	StDev (σ_{n-1})
T3											
2	118.05	118.03	118.04	132.68	132.62	132.63	14.59	0.73	729.5	706.5	19.9
	118.46	118.45	118.46	132.39	132.37	132.37	13.91	0.70	695.5		
	117.58	117.57	117.58	131.50	131.48	131.47	13.89	0.69	694.5		
7	115.69	115.66	115.67	132.45	132.42	132.41	16.74	0.84	837.0	801.5	45.7
	117.39	117.37	117.37	133.76	133.73	133.72	16.35	0.82	817.5		
	119.83	119.82	119.81	134.36	134.80	134.81	15.00	0.75	750.0		
11	119.34	119.31	119.31	136.14	136.12	136.11	16.80	0.84	840.0	827.0	14.7
	118.64	118.63	118.64	135.29	135.25	135.24	16.60	0.83	830.0		
	118.56	118.53	118.53	134.78	134.76	134.75	16.22	0.81	811.0		
20	118.33	118.30	118.29	131.83	131.80	131.79	13.50	0.67	675.0	672.2	3.3
	117.83	117.82	117.83	131.26	131.21	131.20	13.37	0.67	668.5		
	121.63	121.61	121.60	135.08	135.06	135.06	13.46	0.67	673.0		
25	118.79	118.77	118.78	138.69	138.65	138.65	19.87	0.99	993.5	972.7	24.6
	119.67	119.65	119.66	138.59	138.56	138.57	18.91	0.95	945.5		
	119.53	119.50	119.49	139.10	139.07	139.07	19.58	0.98	979.0		

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T4											
2	116.91	116.89	116.85	138.50	138.48	138.48	21.63	1.08	1081.5	940.2	126.5
	110.53	110.50	110.49	127.29	127.26	127.24	16.75	0.84	837.5		
	119.69	119.68	119.67	137.76	137.73	137.70	18.03	0.90	901.5		
7	119.09	119.05	119.03	139.38	139.36	139.37	20.34	1.02	1017.0	1116.3	142.8
	116.67	116.63	116.61	137.64	137.64	137.65	21.04	1.05	1052.0		
	112.82	112.80	112.79	138.42	138.40	138.39	25.60	1.28	1280.0		
11	119.29	119.26	119.25	138.09	138.10	136.08	16.83	0.84	841.5	879.2	55.2
	119.48	119.47	119.47	136.59	136.55	136.54	17.07	0.85	853.5		
	119.36	119.34	119.34	138.21	138.19	138.19	18.85	0.94	942.5		
20	119.10	119.08	119.08	134.83	134.80	134.80	15.72	0.79	786.0	784.3	8.1
	118.35	118.32	118.32	134.20	134.16	134.15	15.83	0.79	791.5		
	119.65	119.63	119.60	135.18	135.12	135.11	15.51	0.78	775.5		
25	117.89	117.87	117.85	128.71	128.69	128.67	10.82	0.54	541.0	549.0	8.0
	120.23	120.20	120.17	131.30	131.25	131.15	10.98	0.55	549.0		
	119.59	119.55	119.55	130.71	130.68	130.69	11.14	0.56	557.0		

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	StDev (σ_{n-1})
T5											
2	119.86	119.80	119.81	143.37	143.39	143.39	23.58	1.18	1179.0	1207.5	47.6
	120.61	120.58	120.56	144.15	144.15	144.18	23.62	1.18	1181.0		
	117.10	117.03	117.00	142.23	142.24	142.25	25.25	1.26	1262.5		
7	120.49	120.43	120.41	142.30	142.31	142.32	21.91	1.10	1095.5	1228.2	133.3
	117.47	117.45	117.43	144.63	144.66	144.67	27.24	1.36	1362.0		
	119.39	119.36	119.34	143.35	143.86	143.88	24.54	1.23	1227.0		
11	118.53	118.52	118.51	149.39	149.38	149.33	30.82	1.54	1541.0	1451.8	78.0
	119.67	119.65	119.65	148.05	148.03	148.02	28.37	1.42	1418.5		
	118.99	118.94	118.92	146.89	146.85	146.84	27.92	1.40	1396.0		
20	119.29	119.25	119.23	140.67	140.68	140.68	21.45	1.07	1072.5	1089.2	56.4
	118.17	118.16	118.15	138.96	139.03	139.01	20.86	1.04	1043.0		
	120.29	120.27	120.26	143.31	143.31	143.30	23.04	1.15	1152.0		
25	120.73	120.70	120.71	128.79	128.75	128.74	8.03	0.40	401.5	490.0	78.3
	118.80	118.79	118.79	129.85	129.82	129.80	11.01	0.55	550.5		
	118.58	118.56	118.55	128.94	128.93	128.91	10.36	0.52	518.0		

Conditions and salinity (‰)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	StDev (σ_{n-1})
T6											
2	117.80	117.74	117.73	140.93	140.91	140.91	23.18	1.16	1159.0	1222.2	71.0
	116.26	116.26	116.24	140.44	140.43	140.41	24.17	1.21	1208.5		
	116.91	116.87	116.86	142.86	142.85	142.84	25.98	1.30	1299.0		
7	116.99	116.97	116.95	139.27	139.25	139.23	22.28	1.11	1114.0	1156.7	75.6
	117.29	117.25	117.22	142.13	142.12	142.10	24.88	1.24	1244.0		
	115.79	115.78	115.76	138.06	138.03	138.00	22.24	1.11	1112.0		
11	115.90	115.89	115.87	145.88	145.87	145.85	29.98	1.50	1499.0	1496.7	75.0
	115.35	115.33	115.33	146.76	146.75	146.74	31.41	1.57	1570.5		
	118.46	118.41	118.39	146.85	146.82	146.80	28.41	1.42	1420.5		
20	118.53	118.51	118.49	153.24	153.25	153.27	34.78	1.74	1739.0	1739.5	9.8
	118.24	118.23	118.22	152.80	152.80	152.82	34.60	1.73	1730.0		
	116.29	116.23	116.21	151.18	151.20	151.20	34.99	1.75	1749.5		
25	118.55	118.52	118.50	126.47	126.41	126.39	7.89	0.39	394.5	428.2	48.7
	116.61	116.59	116.58	126.29	126.27	126.26	9.68	0.48	484.0		
	117.85	117.83	117.82	125.98	125.96	125.94	8.12	0.41	406.0		

47. Chl-*a* concentrations for *N. spumigena* KAC 66 grown at different salinities for 6 weeks at 22ºC (±S.D = standard deviation).

Conditions	Salinity (‰)	Absorbance (pure sample)	Absorbance (sample:MeOH) (1:1, v/v)	Diluted values multiplied by 2	Concentration of Chl-*a* Chlorophyll *a* (μg/ml) = (13.0xAxv)/(dxV)	Concentration of Chl-*a* Mean Chl-*a* (x) (μg/20ml)	Concentration of Chl-*a* Chl-*a* (μg/ml)	±STDev (σ_{n-1})
To	**2**	0.334	-	0.33	1.07	1.06	0.05	0.02
		0.319	-	0.32	1.04			
		0.328	-	0.33	1.07			
	7	0.349	-	0.35	1.13	1.09	0.05	0.03
		0.334	-	0.33	1.07			
		0.325	-	0.33	1.07			
	11	0.335	-	0.34	1.10	1.11	0.05	0.05
		0.360	-	0.36	1.17			
		0.334	-	0.33	1.07			
	20	0.336	-	0.34	1.10	1.08	0.05	0.03
		0.323	-	0.32	1.04			
		0.340	-	0.34	1.10			
	25	0.323	-	0.32	1.04	1.02	0.05	0.04
		0.320	-	0.32	1.04			
		0.302	-	0.30	0.97			
T1	**2**	0.951	-	0.95	3.08	2.95	0.14	0.17
		0.850	-	0.85	2.76			
		0.934	-	0.93	3.02			
	7	0.988	-	0.99	3.21	3.26	0.16	0.04
		1.007	-	1.01	3.28			
		1.006	-	1.01	3.28			
	11	0.956	-	0.96	3.12	3.29	0.16	0.15
		1.032	-	1.03	3.34			
		1.053	-	1.05	3.41			
	20	0.960	-	0.96	3.12	3.24	0.16	0.11
		0.995	-	1.00	3.25			
		1.031	-	1.03	3.34			
	25	0.953	-	0.95	3.08	3.03	0.15	0.05
		0.918	-	0.92	2.99			
		0.928	-	0.93	3.02			
T2	**2**	2.348	-	2.35	7.63	6.85	0.34	0.78
		2.108	-	2.11	6.85			
		1.873	-	1.87	6.07			
	7	2.044	-	2.04	6.63	5.75	0.28	1.38
		1.823	-	1.82	4.16			
		1.992	-	1.99	6.46			
	11	2.240	-	2.24	7.28	7.14	0.35	0.55
		2.335	-	2.34	7.60			
		2.014	-	2.01	6.53			
	20	1.584	-	1.58	5.13	5.53	0.27	0.53
		1.887	-	1.89	6.14			
		1.644	-	1.64	5.33			
	25	1.988	-	1.99	6.46	6.42	0.32	0.78
		1.727	-	1.73	5.62			
		2.208	-	2.21	7.18			
T3	**2**	2.274	-	2.27	7.37	7.26	0.36	0.14
		2.188	-	2.19	7.11			
		2.254	-	2.25	7.31			
	7	2.353	1.684	3.37	10.95	10.98	0.54	0.09
		2.352	1.704	3.41	11.080			
		2.351	1.682	3.36	10.920			
	11	2.342	1.670	3.34	10.85	9.72	0.48	2.56
		2.356	1.775	3.55	11.53			
		2.335	1.046	2.09	6.79			
	20	2.140	1.531	3.06	9.94	9.90	0.49	0.65
		2.151	1.418	2.84	9.23			
		2.096	1.620	3.24	10.53			
	25	2.404	1.853	3.71	12.05	10.60	0.53	2.51
		2.392	1.186	2.37	7.70			
		2.407	1.856	3.71	12.05			

Conditions	Salinity (‰)	Absorbance (pure sample)	Absorbance (sample:MeOH) (1:1, v/v)	Diluted values multiplied by 2	Concentration of Chl-*a* Chlorophyll *a* (µg/ml) = (13.0xAxv)/(dxV)	Mean Chl-*a* (x) (µg/20ml)	Chl-*a* (µg/ml)	±STDev (σ_{n-1})
T4	**2**	2.425	1.900	3.80	12.35	12.44	0.62	0.08
		2.417	1.921	3.84	12.48			
		2.410	1.919	3.84	12.48			
	7	2.446	1.213	2.43	7.89	7.86	0.39	0.22
		2.454	1.240	2.48	8.06			
		2.432	1.174	2.35	7.63			
	11	2.412	1.854	3.71	12.05	11.87	0.59	0.32
		2.408	1.857	3.71	12.05			
		2.409	1.770	3.54	11.50			
	20	2.372	1.700	3.40	11.05	11.24	0.56	0.48
		2.384	1.817	3.63	11.79			
		2.347	1.674	3.35	10.88			
	25	2.464	1.851	3.70	12.02	10.63	0.53	2.38
		2.483	1.843	3.69	11.99			
		2.461	1.213	2.43	7.89			
T5	**2**	2.443	1.265	2.53	8.22	7.61	0.38	0.78
		2.443	1.176	2.35	6.73			
		2.441	1.216	2.43	7.89			
	7	2.446	1.192	2.38	7.73	7.70	0.38	0.21
		2.386	1.217	2.43	7.89			
		2.368	1.150	2.30	7.47			
	11	2.427	1.325	2.65	8.61	8.65	0.43	0.33
		2.451	1.385	2.77	9.00			
		2.427	1.285	2.57	8.35			
	20	2.414	1.844	3.69	12.0	10.31	0.51	3.11
		2.415	1.879	3.76	12.22			
		2.436	1.036	2.07	6.72			
	25	2.480	1.433	2.87	9.32	9.06	0.45	0.51
		2.486	1.456	2.91	8.48			
		2.487	1.443	2.89	9.39			
T6	**2**	2.444	1.372	2.74	8.90	8.53	0.42	0.34
		2.448	1.267	2.53	8.22			
		2.438	1.303	2.61	8.48			
	7	2.488	1.463	2.93	9.52	9.41	0.47	0.13
		2.507	1.456	2.91	9.45			
		2.494	1.442	2.88	9.26			
	11	2.523	1.453	2.91	9.45	9.47	0.32	0.07
		2.512	1.450	2.90	9.42			
		2.507	1.471	2.94	9.55			
	20	2.427	1.158	2.32	7.54	7.48	0.37	0.93
		2.406	1.007	2.01	6.53			
		2.407	1.292	2.58	8.38			
	25	2.413	1.339	2.68	8.71	7.80	0.39	1.11
		2.386	1.252	2.50	8.12			
		2.387	1.011	2.02	6.56			

48. Intra and extracellular levels of NOD for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different salinities at 22ºC analysed on LC-MS. (NOD 238 nm wavelength and retention time 13.00 to 13.25 min; RT= retention time, PA= peak area, n.d= not detected).

Salinity (‰)	To				T1				T2				T3			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular																
2	13.32	37	36	11.3	13.10	26	29	2.9	13.08	133	108	21.7	13.05	105	95	8.6
	13.29	47			13.08	32			13.08	93			13.05	92		
	13.33	24			13.08	28			13.08	98			13.03	88		
7	13.27	22	22	0.9	13.10	34	34	0.3	13.07	82	85	10.8	13.02	107	112	5.8
	13.30	22			13.10	34			13.08	97			13.00	118		
	13.35	23			13.08	34			13.10	76			13.02	110		
11	13.30	75	58	15.1	13.08	29	30	2.9	13.10	242	163	75.8	13.07	151	138	12.0
	13.25	46			13.08	28			13.07	91			13.00	134		
	13.33	52			13.10	34			13.05	156			13.00	128		
20	13.33	65	104	34.9	13.10	41	40	1.8	13.05	133	134	2.1	13.00	160	178	38.5
	13.35	132			13.08	38			13.10	133			13.00	222		
	13.35	115			13.10	39			13.10	136			13.00	152		
25	13.31	53	75	18.9	13.10	22	22	3.4	13.07	108	102	22.9	12.98	120	109	17.9
	13.33	85			13.10	26			13.07	77			12.97	119		
	13.35	86			13.12	19			13.07	122			13.00	89		
Intracellular																
2	n.d	n.d	n.d	n.d	13.05	1610	1534	150.1	13.12	1349	1579	393.8	12.97	4684	4689	30.9
	n.d	n.d			13.03	1631			13.10	1354			12.98	4661		
	n.d	n.d			13.12	1361			13.13	2033			13.00	4722		
7	n.d	n.d	n.d	n.d	13.07	1118	1310	240.6	13.13	1642	1581	161.7	12.98	4438	4302	134.6
	n.d	n.d			13.1	1580			13.12	1703			12.98	4168		
	n.d	n.d			13.1	1232			13.12	1398			13.00	4301		
11	n.d	n.d	n.d	n.d	13.08	1121	1267	200.2	13.12	1346	1312	78.4	13.00	4315	4341	76.7
	n.d	n.d			13.03	1496			13.10	1367			13.00	4427		
	n.d	n.d			13.1	1186			13.08	1222			13.00	4280		
20	n.d	n.d	n.d	n.d	13.12	831	763	141.7	13.13	1227	1338	96.4	13.00	3026	2859	175.3
	n.d	n.d			13.12	857			13.10	1397			13.00	2676		
	n.d	n.d			13.13	600			13.07	1391			13.00	2874		
25	n.d	n.d	n.d	n.d	13.15	800	1062	493.3	13.10	1190	1118	78.4	13.00	2846	2851	5.1
	n.d	n.d			13.51	756			13.10	1131			13.02	2855		
	n.d	n.d			13.05	1632			13.08	1035			13.02	2854		

Salinity (‰)	T4				T5				T6			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
2	13.03	69	66	3.2	13.07	44	47	2.5	n.d	n.d	n.d	n.d
	13.02	63			13.05	48			n.d	n.d		
	13.05	66			13.03	49			n.d	n.d		
7	13.05	52	44	7.2	13.03	37	31	5.5	n.d	n.d	n.d	n.d
	13.05	44			13.03	28			n.d	n.d		
	13.05	37			13.02	27			n.d	n.d		
11	13.03	50	58	13.2	13.03	65	67	12.7				
	12.98	73			13.02	81			13.08	131	165	29.6
	13.10	51			13.03	56			13.07	181		
									1305	183		
20	13.05	77	94	15.6	13.02	89	91	5.2				
	13.05	106			13.03	87			13.08	85	88	5.0
	13.10	100			13.05	97			13.07	85		
									13.07	94		
25	13.05	83	92	10.4	13.05	204	217	17.6				
	13.05	104			13.05	237			13.10	89	73	14.7
	13.05	91			13.05	208			13.05	70		
									13.07	61		
Intracellular												
2	13.07	5225	5388	312.98	13.07	5770	5943	190.2	13.05	3515	3529	126.3
	13.07	5189			13.05	5912			13.05	3410		
	13.03	5748			13.05	6147			13.05	3662		
7	13.00	4331	4203	180.50	13.07	5232	5154	77.7	13.12	4109	3749	341.0
	13.03	4282			13.07	5155			13.05	3431		
	13.05	3997			13.07	5077			13.08	3707		
11	13.07	3509	3222	322.63	13.05	5345	5482	184.8	13.07	3833	3507	312.6
	13.05	3284			13.07	5693			13.1	3209		
	13.08	2873			13.00	5410			13.07	3480		
20	13.05	2837	2513	281.42	13.05	2816	2837	68.4	13.05	934	1295	325.9
	13.03	2368			13.05	2913			13.07	1383		
	13.03	2333			13.08	2780			13.05	1567		
25	13.05	3707	4102	342.92	13.07	5729	5756	312.4	13.08	973	732	214.8
	13.07	4281			13.10	6081			13.10	560		
	13.03	4318			13.08	5458			13.13	664		

49. Intra and extracellular levels of nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different salinities at 22ºC analysed on LC-MS. (nodulopeptin 901, 210 nm wavelength and retention time 18.07 to 18.25 min; RT= retention time, PA= peak area, n.d= not detected).

	To				T1				T2				T3			
Salinity (‰)	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**StDev (σ_{n-1})**	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**StDev (σ_{n-1})**	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**StDev (σ_{n-1})**	**RT (min)**	**PA**	**Mean ($\bar{x}$)**	**StDev (σ_{n-1})**
Exrtracellular																
2	18.10	19	18	0.5	18.10	311	302	12.5	18.07	835	599	221.3	18.15	457	466	13.5
	18.10	18			18.07	308			18.08	563			18.12	460		
	18.12	18			18.07	288			18.07	397			18.12	482		
7	18.82	14	16	2.5	18.10	268	273	7.6	18.05	500	445	90.8	18.10	644	650	13.0
	18.08	19			18.12	270			18.05	494			18.08	641		
	18.13	17			18.08	282			18.07	340			18.10	665		
11	18.13	19	20	2.1	18.08	277	271	5.8	18.07	517	479	32.9	18.12	608	640	34.6
	18.13	22			18.10	266			18.07	455			18.10	677		
	18.13	19			18.08	271			18.03	465			18.08	636		
20	18.13	19	21	2.8	18.08	217	225	6.4	18.02	345	350	19.3	18.08	438	426	45.6
	18.13	24			18.08	228			18.08	371			18.08	464		
	18.13	19			18.10	229			18.08	333			18.07	375		
25	18.12	26	22	3.0	18.10	131	126	4.6	18.05	191	227	38.7	18.07	241	224	28.7
	18.13	20			18.10	125			18.05	223			18.07	239		
	18.15	21			18.13	122			18.07	268			18.05	191		
Intracellular																
2	n.d	n.d	n.d	n.d	18.03	368	306	89.4	18.13	584	671	138.3	18.07	789	796	15.4
	n.d	n.d			18.03	346			18.12	598			18.07	785		
	n.d	n.d			18.10	204			18.13	830			18.08	813		
7	n.d	n.d	n.d	n.d	18.05	203	216	16.3	18.13	538	533	36.3	18.07	1007	937	61.6
	n.d	n.d			18.10	235			18.13	566			18.07	891		
	n.d	n.d			18.12	211			18.13	494			18.08	913		
11	n.d	n.d	n.d	n.d	18.08	203	192	16.3	18.12	498	503	26.3	18.08	815	817	28.2
	n.d	n.d			18.02	200			18.12	532			18.08	846		
	n.d	n.d			18.10	173			18.10	480			18.08	790		
20	n.d	n.d	n.d	n.d	18.12	80	107	27.4	18.13	436	438	9.2	18.07	580	563	31.7
	n.d	n.d			18.12	134			18.10	431			18.07	527		
	n.d	n.d			18.13	106			18.07	449			18.07	583		
25	n.d	n.d	n.d	n.d	18.15	81	113	40.9	18.08	236	231	4.6	18.07	316	309	8.1
	n.d	n.d			18.12	99			18.08	231			18.07	300		
	n.d	n.d			18.05	159			18.07	227			18.08	311		

Salinity (‰)	T4				T5				T6			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
2	18.12	595	614	26.1	18.13	796	775	18.1	18.15	897	930	28.3
	18.10	603			18.13	766			18.13	945		
	18.13	643			18.10	763			18.13	947		
7	18.13	755	763	13.6	18.08	987	1090	90.4	18.15	1369	1475	126.5
	18.10	755			18.17	1128			18.15	1442		
	18.12	779			18.10	1155			18.15	1615		
11	18.10	774	771	7.0	18.1	1109	1189	90.1	18.12	1468	1519	44.6
	18.08	776			18.12	1287			18.12	1538		
	18.17	763			18.10	1171			18.12	1550		
20	18.12	507	519	35.9	18.08	577	534	44.8	18.165	808	757	55.5
	18.13	560			18.12	487			18.12	766		
	18.17	491			18.12	537			18.13	698		
25	18.15	255	270	13.7	18.15	295	306	16.7	18.12	550	428	105.7
	18.13	274			18.13	325			18.10	369		
	18.13	281			18.12	297			18.12	364		
Intracellular												
2	18.15	1014	1042	58.2	18.13	1235	1294	50.6	18.08	1323	1291	74.9
	18.13	1003			18.10	1328			18.08	1205		
	18.12	1109			18.10	1317			18.08	1343		
7	18.08	1296	1260	59.2	18.12	1736	1712	32.1	18.12	2115	2110	219.3
	18.12	1292			18.10	1724			18.07	1889		
	18.12	1191			18.12	1676			18.10	2327		
11	18.13	1173	1030	144.9	18.12	1627	1691	82.0	18.10	2690	2260	373.8
	18.12	1033			18.10	1783			18.08	2009		
	18.15	884			18.13	1663			18.10	2081		
20	18.12	779	809	25.9	18.08	795	791	17.5	18.08	868	916	41.3
	18.10	819			18.08	807			18.08	940		
	18.08	828			18.12	773			18.10	939		
25	18.12	518	563	39.5	18.12	871	875	53.5	18.12	735	674	55.1
	18.12	591			18.12	930			18.13	628		
	18.10	580			18.13	823			18.13	658		

50. The amount (ng/ml) of intra and extracellular levels of NOD for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different salinities at 22ºC (n.d= not detected).

Salinity (‰)	To			T1			T2			T3		
	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular												
2	10.4	10.1	3.2	7.3	8.04	0.86	37.3	30.3	6.1	29.4	26.6	2.5
	13.2			9.0			26.1			25.8		
	6.7			7.8			27.5			24.7		
7	6.2	6.3	0.2	9.5	9.53	0.00	23.0	23.8	3.0	30.0	31.3	1.6
	6.2			9.5			27.2			33.1		
	6.4			9.5			21.3			30.8		
11	21.0	16.2	4.3	8.1	8.50	0.90	67.8	45.7	21.2	42.3	38.6	3.3
	12.9			7.8			25.5			37.6		
	14.6			9.5			43.7			35.9		
20	18.2	29.2	9.8	11.5	11.03	0.43	37.3	37.6	0.5	44.9	49.9	10.7
	37.0			10.7			37.3			62.2		
	32.2			10.9			38.1			42.6		
25	14.9	20.9	5.3	6.2	6.26	0.98	30.3	28.7	6.5	33.6	30.7	4.9
	23.8			7.3			21.6			33.4		
	24.1			5.3			34.2			25.0		
Intracellular												
2	0	0.0	0.0	451.4	430.05	42.11	378.2	442.6	110.3	1313.1	1314.6	8.6
	0			457.2			379.6			1306.7		
	0			381.6			569.9			1323.8		
7	0	0.0	0.0	313.4	367.26	67.47	460.3	443.2	45.2	1244.2	1206.1	37.8
	0			442.9			477.4			1168.5		
	0			345.4			391.9			1205.8		
11	0	0.0	0.0	314.3	355.39	56.18	377.3	367.7	22.0	1209.7	1216.9	21.5
	0			419.4			383.2			1241.1		
	0			332.5			342.6			1199.9		
20	0	0.0	0.0	233.0	213.81	39.66	344.0	375.2	27.0	848.3	801.4	49.2
	0			240.3			391.6			750.2		
	0			168.2			390.0			805.7		
25	0	0.0	0.0	224.3	297.92	138.36	333.6	313.2	22.5	797.9	799.5	1.4
	0			211.9			317.1			800.4		
	0			457.5			289.0			800.1		

Salinity (‰)	T4			T5			T6		
	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular									
2	19.3	18.5	0.8	12.3	13.2	0.7	0.0	0.0	0.0
	17.7			13.5			0.0		
	18.5			13.7			0.0		
7	14.6	12.4	2.1	10.4	8.6	1.5	0.0	0.0	0.0
	12.3			7.8			0.0		
	10.4			7.6			0.0		
11	14.0	16.3	3.6	18.2	18.9	3.5	36.7	46.3	8.3
	20.5			22.7			50.7		
	14.3			15.7			51.3		
20	21.6	26.4	4.3	25.0	25.5	1.5	23.8	24.7	1.5
	29.7			24.4			23.8		
	28.0			27.2			26.4		
25	23.3	26.0	3.0	57.2	60.6	5.0	25.0	20.6	4.0
	29.2			66.4			19.6		
	25.5			58.3			17.1		
Intracellular									
2	1464.8	1510.3	87.7	1617.6	1666.1	53.4	985.4	989.3	35.5
	1454.7			1657.4			956.0		
	1611.4			1723.3			1026.6		
7	1214.2	1178.4	50.6	1466.8	1445.1	21.7	1151.9	1051.0	95.6
	1200.4			1445.2			961.9		
	1120.5			1423.3			1039.2		
11	983.7	903.3	90.4	1498.5	1537.1	51.9	1074.6	983.3	87.7
	920.7			1596.0			899.6		
	805.4			1516.7			975.6		
20	805.4	707.8	84.7	789.5	795.2	19.3	261.8	363.0	91.3
	663.9			816.7			387.7		
	654.1			779.4			439.3		
25	1039.2	1150.0	96.0	1606.1	1613.7	87.6	272.8	205.3	60.2
	1200.2			1704.8			157.0		
	1210.5			1530.1			186.2		

51. The amount (ng/ml) of intra and extracellular concentrations of nodulopeptin 901 for cultures of *N. spumigena* KAC 66 grown for 6 weeks at different salinities at 22ºC (n.d= not detected).

Salinity (‰)	To			T1			T2			T3		
	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular												
2	9.7	9.4	0.3	158.8	154.4	6.4	426.5	305.6	112.9	233.4	238.2	7.0
	9.2			157.3			287.5			234.9		
	9.2			147.1			202.8			246.2		
7	7.2	8.5	1.3	136.9	139.6	3.9	255.4	227.1	46.3	328.9	332.0	6.7
	9.7			137.9			252.3			327.4		
	8.7			144.0			173.6			339.6		
11	9.7	10.2	0.9	141.5	138.6	2.8	264.0	244.6	17.0	310.5	327.0	17.7
	11.2			135.9			232.4			345.8		
	9.7			138.4			237.5			324.8		
20	9.7	10.6	1.5	110.8	114.7	3.4	176.2	178.6	9.9	223.7	217.4	23.4
	12.3			116.4			189.5			237.0		
	9.7			117.0			170.1			191.5		
25	13.3	11.4	1.6	66.9	64.4	2.3	191.0	147.3	39.6	123.1	114.2	14.5
	10.2			63.8			113.9			122.1		
	10.7			62.3			136.9			97.5		
Intracellular												
2	0	0	0	187.9	156.3	45.5	298.3	342.5	70.6	403.0	406.4	7.7
	0			176.7			305.4			400.9		
	0			104.2			423.9			415.2		
7	0	0	0	103.7	110.5	8.5	274.8	272.0	18.5	514.3	478.5	31.5
	0			120.0			289.1			455.1		
	0			107.8			252.3			466.3		
11	0	0	0	103.7	98.1	8.4	254.3	257.1	13.5	416.2	417.3	14.3
	0			102.1			271.7			432.1		
	0			88.4			245.1			403.5		
20	0	0	0	40.9	54.5	13.8	222.7	224.0	4.7	296.2	287.7	16.1
	0			68.4			220.1			269.2		
	0			54.1			229.3			297.8		
25	0	0	0	41.4	57.7	20.9	120.5	118.1	2.3	161.4	157.8	4.2
	0			50.6			118.0			153.2		
	0			81.2			115.9			158.8		

Salinity (‰)	T4			T5			T6		
	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular									
2	303.9	313.4	13.1	406.5	395.8	9.3	458.1	474.8	14.5
	308.0			391.2			482.6		
	328.4			389.7			483.7		
7	385.6	389.7	7.1	504.1	556.7	46.1	699.2	753.5	64.5
	385.6			576.1			736.5		
	397.9			589.9			824.8		
11	395.3	393.8	3.6	566.4	607.3	46.1	749.7	775.6	22.6
	396.3			657.3			785.5		
	389.7			598.1			791.6		
20	258.9	265.2	18.4	294.7	272.6	23.0	412.7	386.8	28.4
	286.0			248.7			391.2		
	250.8			274.3			356.5		
25	130.2	137.9	6.9	150.7	156.1	8.6	280.9	218.4	54.1
	139.9			166.0			188.5		
	143.5			151.7			185.9		
Intracellular									
2	187.9	422.2	204.7	630.7	660.5	26.0	675.7	659.0	38.1
	512.3			678.2			615.4		
	566.4			672.6			685.9		
7	661.9	643.3	30.4	886.6	874.4	16.2	1080.2	1077.8	111.9
	659.9			880.5			964.8		
	608.3			856.0			1188.5		
11	599.1	526.0	73.8	830.9	863.6	41.7	1373.9	1154.2	191.1
	527.6			910.6			1026.0		
	451.5			849.3			1062.8		
20	397.9	413.0	13.3	406.0	404.3	8.8	443.3	467.7	21.1
	418.3			412.2			480.1		
	422.9			394.8			479.6		
25	264.6	287.5	20.1	444.8	446.7	27.4	375.4	344.1	28.2
	301.8			475.0			320.7		
	296.2			420.3			336.1		

52. Weight of empty, freeze dried filter discs (with cells) and cell biomass for *N. spumigena* KAC 66 grown at different $NaNO_3$ concentrations for 5 weeks at 22ºC (±S.D = standard deviation).

Conditions (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
To											
0	128.5	128.5	128.1	131.8	131.7	131.5	3.4	0.17	170.0	183.3	11.5
	129.9	129.7	129.5	133.7	133.5	133.3	3.8	0.19	190.0		
	128.3	128.2	128.1	132.3	132.0	131.9	3.8	0.19	190.0		
3.5	129.1	129.0	128.9	133.0	132.9	132.7	3.8	0.19	190.0	215.0	21.8
	127.8	127.7	127.5	132.5	132.2	132.1	4.6	0.23	230.0		
	126.9	126.9	126.8	131.5	131.4	131.3	4.5	0.23	225.0		
6.5	126.7	126.6	126.6	131.5	131.3	131.2	4.6	0.23	230.0	261.7	38.8
	126.8	126.7	126.6	131.9	131.8	131.6	5.0	0.25	250.0		
	130.3	130.2	130.1	136.5	136.3	136.2	6.1	0.31	305.0		
7.5	128.9	128.7	128.7	133.2	133.1	132.9	4.2	0.21	210.0	206.7	10.4
	129.6	129.5	129.3	133.9	133.7	133.6	4.3	0.21	215.0		
	127.9	127.7	127.6	131.7	131.5	131.5	3.9	0.20	195.0		
8.5	126.9	126.9	126.7	131.9	131.8	131.7	5.0	0.25	250.0	240.0	17.3
	126.9	126.8	126.6	131.5	131.3	131.0	4.4	0.22	220.0		
	124.6	124.5	124.4	129.7	129.5	129.4	5.0	0.25	250.0		
9.5	126.8	126.7	126.5	133.4	133.2	133.0	6.5	0.33	325.0	250.0	65.4
	127.9	127.7	127.5	131.9	131.8	131.9	4.4	0.22	220.0		
	129.5	129.2	129.1	133.5	133.3	133.2	4.1	0.21	205.0		

Conditions (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T1											
0	125.5	15.3	125.0	130.5	130.4	130.2	5.2	0.26	260.0	261.7	7.6
	129.6	129.3	129.0	134.6	134.3	134.1	5.1	0.26	255.0		
	127.5	127.4	127.3	132.9	132.9	132.7	5.4	0.27	270.0		
3.5	127.9	16.9	126.8	132.0	131.9	131.8	5.0	0.25	250.0	270.0	43.6
	123.7	123.8	124.0	130.7	130.5	130.4	6.4	0.32	320.0		
	126.5	126.3	126.2	131.6	131.5	131.0	4.8	0.24	240.0		
6.5	127.9	127.7	127.6	132.9	132.8	132.7	5.1	0.26	255.0	278.3	32.1
	125.9	125.9	125.8	132.4	132.3	132.1	6.3	0.32	315.0		
	129.6	129.5	129.3	134.9	134.7	134.6	5.3	0.26	265.0		
7.5	124.6	124.5	124.3	130.6	130.5	130.5	6.2	0.31	310.0	275.0	31.2
	130.3	130.2	130.0	135.5	135.2	135.0	5.0	0.25	250.0		
	125.5	125.4	125.3	130.8	130.7	130.6	5.3	0.27	265.0		
8.5	126.3	126.2	126.0	132.2	131.9	131.9	5.9	0.30	295.0	275.0	17.3
	127.2	127.1	127.0	132.5	132.4	132.3	5.3	0.27	265.0		
	129.8	129.7	129.6	135.1	135.0	134.9	5.3	0.27	265.0		
9.5	129.4	129.3	129.2	134.8	134.7	134.6	5.4	0.27	270.0	253.3	20.8
	128.2	128.1	128.0	132.9	132.7	132.6	4.6	0.23	230.0		
	127.2	127.1	126.9	132.5	132.2	132.1	5.2	0.26	260.0		

Conditions (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T2											
0	124.3	124.0	124.0	136.8	136.7	136.5	12.5	0.63	625.0	510.0	112.6
	123.3	123.1	122.9	133.5	133.2	133.0	10.1	0.51	505.0		
	125.3	125.2	125.0	133.3	133.1	133.0	8.0	0.40	400.0		
3.5	121.7	121.5	121.4	127.5	127.2	127.0	5.6	0.28	280.0	401.7	106.8
	122.6	122.4	122.2	131.9	131.8	131.8	9.6	0.48	480.0		
	120.9	120.8	120.6	129.8	129.6	129.5	8.9	0.45	445.0		
6.5	123.6	123.6	123.5	135.1	134.9	134.8	11.3	0.57	565.0	500.0	62.6
	123.0	122.9	122.8	132.9	132.8	132.7	9.9	0.50	495.0		
	124.8	124.6	124.5	133.7	133.5	133.3	8.8	0.44	440.0		
7.5	122.6	122.6	122.4	133.2	132.9	132.9	10.5	0.53	525.0	528.3	10.4
	124.8	124.7	124.5	135.6	135.5	135.3	10.8	0.54	540.0		
	123.5	123.3	123.2	133.9	133.7	133.6	10.4	0.52	520.0		
8.5	124.1	123.9	123.9	133.4	133.2	133.0	9.1	0.46	455.0	466.7	16.1
	124.9	124.8	124.7	134.6	134.5	134.4	9.7	0.49	485.0		
	120.3	120.2	120.1	129.6	129.5	129.3	9.2	0.46	460.0		
9.5	121.5	121.3	121.2	133.9	133.7	133.7	12.5	0.62	625.0	500.0	110.6
	123.2	122.9	122.8	131.5	131.2	131.1	8.3	0.42	415.0		
	129.2	128.9	120.9	130.4	130.2	130.1	9.2	0.46	460.0		

Conditions (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T3											
0	122.4	122.3	122.1	135.9	135.9	135.8	13.7	0.69	685.0	720.0	60.6
	122.7	122.8	122.6	138.7	138.5	138.4	15.8	0.79	790.0		
	123.2	123.1	122.9	136.9	136.7	136.6	13.7	0.68	685.0		
3.5	122.3	122.1	122.0	136.4	136.3	136.1	14.1	0.71	705.0	673.3	54.8
	124.5	124.2	124.0	136.7	136.4	136.2	12.2	0.61	610.0		
	120.9	120.8	120.8	135.3	135.1	134.9	14.1	0.71	705.0		
6.5	123.2	123.2	123.0	137.3	137.2	137.1	14.1	0.71	705.0	693.3	63.3
	122.8	122.7	122.6	135.5	135.3	135.1	12.5	0.63	625.0		
	119.4	119.3	119.2	134.4	134.3	134.2	15.0	0.75	750.0		
7.5	125.5	125.4	125.3	140.3	140.1	139.0	13.7	0.69	685.0	693.3	14.4
	122.7	122.7	122.5	136.9	136.8	136.7	14.2	0.71	710.0		
	125.7	125.6	125.5	139.5	139.3	139.2	13.7	0.68	685.0		
8.5	120.9	120.9	120.8	138.4	138.2	138.1	17.3	0.87	865.0	766.7	88.1
	123.8	123.6	123.5	138.6	138.4	138.3	14.8	0.74	740.0		
	124.7	124.5	124.5	138.7	138.5	138.4	13.9	0.70	695.0		
9.5	121.3	121.2	120.9	134.5	134.5	134.4	13.5	0.68	675.0	655.0	48.2
	123.6	123.5	123.3	135.7	135.4	135.3	12.0	0.60	600.0		
	124.5	124.4	124.3	138.4	138.3	138.1	13.8	0.69	690.0		

Conditions (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T4											
0	123.7	123.5	123.3	143.8	143.7	143.5	20.2	1.01	1010.0	941.7	67.5
	124.9	124.8	124.8	142.7	142.5	142.3	17.5	0.88	875.0		
	126.6	126.5	126.3	145.4	145.2	145.1	18.8	0.94	940.0		
3.5	123.8	123.6	123.5	141.9	141.8	141.7	18.2	0.91	910.0	898.3	38.8
	122.7	122.6	122.4	141.3	141.2	141.0	18.6	0.93	930.0		
	122.6	122.4	122.2	139.5	139.4	139.3	17.1	0.86	855.0		
6.5	125.5	125.2	125.0	145.8	145.7	145.6	20.6	1.03	1030.0	1075.0	39.1
	121.7	121.5	121.4	143.6	143.5	143.3	21.9	1.10	1095.0		
	122.9	122.8	122.7	144.9	144.8	144.7	22.0	1.10	1100.0		
7.5	124.7	124.5	124.4	140.1	139.9	139.9	15.5	0.78	775.0	801.7	50.6
	126.5	126.3	126.3	143.6	143.5	143.5	17.2	0.86	860.0		
	124.3	124.2	124.1	139.8	139.7	139.5	15.4	0.77	770.0		
8.5	122.6	122.5	122.4	145.6	145.5	145.3	22.9	1.15	1145.0	1106.7	50.1
	124.9	124.8	124.7	145.9	145.8	145.7	21.0	1.05	1050.0		
	124.8	124.7	124.6	147.3	147.2	147.1	22.5	1.13	1125.0		
9.5	125.6	125.3	125.2	144.1	143.9	143.8	18.6	0.93	930.0	1056.7	120.6
	122.4	122.3	122.0	143.7	143.5	143.4	21.4	1.07	1070.0		
	123.8	123.8	123.7	148.2	147.9	147.1	23.4	1.17	1170.0		

Conditions (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
	1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T5											
0	125.9	125.8	125.8	146.9	146.9	146.7	20.9	1.05	1045.0	1068.3	32.1
	121.5	121.4	121.3	142.7	142.6	142.4	21.1	1.06	1055.0		
	121.5	121.3	121.1	143.5	143.3	143.2	22.1	1.11	1105.0		
3.5	124.8	124.7	124.5	146.5	146.2	146.0	21.5	1.08	1075.0	1141.7	65.1
	127.5	127.3	127.2	150.3	150.2	150.1	22.9	1.15	1145.0		
	125.9	125.9	125.7	150.1	149.9	149.8	24.1	1.21	1205.0		
6.5	122.8	122.6	122.5	159.2	158.9	158.8	36.3	1.82	1815.0	1826.7	98.0
	123.7	123.6	123.4	162.3	162.3	162.0	38.6	1.93	1930.0		
	122.9	122.7	122.6	157.5	157.4	157.3	34.7	1.74	1735.0		
7.5	124.5	124.3	124.1	146.9	146.8	146.7	22.6	1.13	1130.0	1106.7	22.5
	124.7	124.6	124.5	146.8	146.7	146.6	22.1	1.11	1105.0		
	121.8	121.6	121.5	143.4	143.3	143.2	21.7	1.09	1085.0		
8.5	125.3	125.1	125.0	165.7	165.5	165.5	40.5	2.03	2025.0	1973.3	217.2
	122.9	122.8	122.8	157.8	157.6	157.5	34.7	1.74	1735.0		
	121.9	121.6	121.5	164.9	164.8	164.7	43.2	2.16	2160.0		
9.5	121.6	121.5	121.4	171.4	171.2	171.0	49.6	2.48	2480.0	2223.3	226.8
	123.6	123.4	123.3	164.6	164.5	164.3	41.0	2.05	2050.0		
	122.1	121.9	121.9	164.9	164.8	164.7	42.8	2.14	2140.0		

53. Chl-*a* concentrations for *N. spumigena* KAC 66 grown at different $NaNO_3$ concentrations for 5 weeks at 22ºC (±S.D = standard

Conditions	Concentrations of $NaNO_3$ (mg/L)	Absorbance (pure sample) (100% MeOH)	Absorbance (sample:MeOH) (1:1, v/v)	Diluted values multiplied by 2	Concentration of Chl-*a* Chlorophyll *a* (ug/20ml) = (13.0xAxv)/(dxV)	Chl-*a* (µg/ml)	Mean (x) (µg/ml)	±STDev (σ_{n-1})
To	**0**	0.07			0.23	0.01	0.01	0.00
		0.06			0.19	0.01		
		0.06			0.20	0.01		
	3.5	0.06			0.20	0.01	0.01	0.00
		0.05			0.18	0.01		
		0.06			0.19	0.01		
	6.5	0.03			0.11	0.01	0.01	0.00
		0.04			0.13	0.01		
		0.05			0.15	0.01		
	7.5	0.05			0.16	0.01	0.01	0.01
		0.03			0.08	0.00		
		0.10			0.32	0.02		
	8.5	0.03			0.09	0.00	0.01	0.00
		0.06			0.19	0.01		
		0.05			0.17	0.01		
	9.5	0.03			0.11	0.01	0.01	0.00
		0.05			0.17	0.01		
		0.05			0.17	0.01		
T1	**0**	0.31			1.01	0.05	0.05	#REF!
		0.34			1.10	0.06		
		0.26			0.85	0.04		
	3.5	0.26			0.84	0.04	0.05	0.01
		0.32			1.04	0.05		
		0.32			1.03	0.05		
	6.5	0.27			0.87	0.04	0.04	0.00
		0.25			0.80	0.04		
		0.28			0.92	0.05		
	7.5	0.29			0.94	0.05	0.04	0.00
		0.25			0.81	0.04		
		0.26			0.83	0.04		
	8.5	0.27			0.86	0.04	0.05	0.01
		0.26			0.85	0.04		
		0.33			1.08	0.05		
	9.5	0.21			0.67	0.03	0.03	0.00
		0.22			0.70	0.04		
		0.22			0.70	0.04		
T2	**0**	0.61			1.97	0.10	0.09	0.01
		0.49			1.59	0.08		
		0.65			2.12	0.11		
	3.5	0.77			2.51	0.13	0.12	0.00
		0.73			2.38	0.12		
		0.78			2.54	0.13		
	6.5	0.77			2.49	0.12	0.12	0.00
		0.72			2.33	0.12		
		0.74			2.42	0.12		
	7.5	0.69			2.23	0.11	0.11	0.02
		0.56			1.81	0.09		
		0.80			2.59	0.13		
	8.5	0.75			2.44	0.12	0.12	0.00
		0.70			2.27	0.11		
		0.73			2.37	0.12		
	9.5	0.49			1.58	0.08	0.09	0.01
		0.60			1.93	0.10		
		0.64			2.08	0.10		

Conditions	Concentrations of $NaNO_3$ (mg/L)	Absorbance (pure sample) (100% MeOH)	Absorbance (sample:MeOH) (1:1, v/v)	Absorbance Diluted values multiplied by 2	Concentration of Chl-*a* Chlorophyll *a* (ug/20ml) = (13.0xAxv)/(dxV)	Concentration of Chl-*a* Chl-*a* (µg/ml)	Concentration of Chl-*a* Mean (x) (µg/ml)	Concentration of Chl-*a* ±STDev (σ_{n-1})
T3	**0**	0.78			2.53	0.13	0.12	0.00
		0.76			2.45	0.12		
		0.73			2.39	0.12		
	3.5	1.18			3.83	0.19	0.20	0.01
		1.27			4.14	0.21		
		1.29			4.19	0.21		
	6.5	1.19			3.88	0.19	0.18	0.02
		0.98			3.19	0.16		
		1.08			3.50	0.17		
	7.5	1.19			3.86	0.19	0.19	0.01
		1.27			4.12	0.21		
		1.14			3.69	0.18		
	8.5	1.13			3.67	0.18	0.19	0.01
		1.27			4.11	0.21		
		1.13			3.67	0.18		
	9.5	0.97			3.14	0.16	0.17	0.01
		1.05			3.43	0.17		
		1.03			3.35	0.17		
T4	**0**	0.92			2.99	0.15	0.15	0.01
		0.97			3.15	0.16		
		0.83			2.70	0.13		
	3.5	1.39			4.53	0.23	0.23	0.01
		1.46			4.76	0.24		
		1.46			4.75	0.24		
	6.5	1.66			5.38	0.27	0.27	0.01
		1.72			5.60	0.28		
		1.55			5.02	0.25		
	7.5	1.33			4.32	0.22	0.22	0.00
		1.38			4.49	0.22		
		1.33			4.31	0.22		
	8.5	1.60			5.20	0.26	0.26	0.02
		1.74			5.66	0.28		
		1.53			4.96	0.25		
	9.5	1.39			4.50	0.23	0.25	0.02
		1.60			5.18	0.26		
		1.66			5.41	0.27		
T5	**0**	1.14			3.70	0.18	0.19	0.01
		1.19			3.87	0.19		
		1.13			3.68	0.18		
	3.5	1.69			5.48	0.27	0.27	0.01
		1.59			5.18	0.26		
		1.71			5.57	0.28		
	6.5	2.21	1.47	2.94	9.56	0.48	0.48	0.03
		2.16	1.39	2.78	9.04	0.45		
		2.26	1.55	3.10	10.08	0.50		
	7.5	1.89			6.13	0.31	0.30	0.00
		1.87			6.07	0.30		
		1.86			6.04	0.30		
	8.5	2.23	1.46	2.92	9.49	0.47	0.44	0.04
		2.07	1.20	2.40	7.80	0.39		
		2.19	1.39	2.78	9.04	0.45		
	9.5	2.05	1.21	2.42	7.87	0.39	0.39	0.09
		1.82			5.91	0.30		
		2.21	1.44	2.88	9.36	0.47		

54. Intra and extracellular levels of NOD during the analysis on LC-MS at different nitrate concentrations for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22°C. (for NOD 238 nm wavelength and retention time 13.10 to 13.52 min, RT= retention time, PA= peak area, n.d= not detected)

Nitrate concentrations (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Exrtracellular												
0	-	-	-	-	13.13	26	27	5.0	13.13	19	19	1.9
	-	-			13.12	32			13.12	17		
	-	-			13.12	22			13.08	21		
3.5	-	-	-	-	13.12	27	24	3.9	13.12	35	33	10.4
	-	-			13.12	19			13.10	42		
	-	-			13.12	25			13.12	22		
6.5	-	-	-	-	13.12	24	24	2.8	13.12	34	30	3.8
	-	-			13.10	26			13.12	28		
	-	-			13.10	21			13.08	28		
7.5	-	-	-	-	13.10	32	27	3.8	13.12	66	60	8.6
	-	-			13.10	25			13.10	63		
	-	-			13.08	26			13.10	50		
8.5	-	-	-	-	13.08	35	27	8.1	13.12	31	24	6.1
	-	-			13.10	19			13.12	21		
	-	-			13.10	27			13.12	20		
9.5	-	-	-	-	13.12	29	29	9.1	13.33	44	39	4.1
	-	-			13.12	39			13.12	36		
	-	-			13.10	20			13.13	39		

Nitrate concentrations (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Exrtracellular												
0	13.12	10	10	1.4	12.70	12	11	0.4	-	-	-	-
	13.15	9			12.67	11			-	-		
	13.17	12			12.70	11			-	-		
3.5	13.17	19	32	11.1	-	-	-	-	-	-	-	-
	13.17	38			-	-			-	-		
	13.17	38			-	-			-	-		
6.5	13.15	39	33	14.0	-	-	-	-	-	-	-	-
	13.15	17			-	-			-	-		
	13.15	43			-	-			-	-		
7.5	13.15	17	18	2.7	-	-	-	-	-	-	-	-
	13.15	16			-	-			-	-		
	13.15	21			-	-			-	-		
8.5	13.13	33	29	8.2	-	-	-	-	-	-	-	-
	13.15	36			-	-			-	-		
	13.15	20			-	-			-	-		
9.5	13.17	26	30	2.9	-	-	-	-	-	-	-	-
	13.17	32			-	-			-	-		
	13.17	31			-	-			-	-		

Nitrate concentrations (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Intracellular												
0	13.05	99.3	98	1.5	13.13	571	568	33.6	13.17	2348	2361	60.9
	13.07	99.0			13.12	600			13.18	2307		
	13.05	96.5			13.13	533			13.17	2427		
3.5	13.05	102.9	95	11.5	13.13	780	652	126.6	13.15	2222	2113	224.1
	13.05	101.0			13.12	650			13.15	2261		
	13.05	82.1			13.13	527			13.15	1855		
6.5	13.05	97.7	94	9.0	13.13	775	827	87.5	13.15	2152	2327	278.9
	13.05	83.5			13.13	779			13.17	2649		
	13.07	100.2			13.13	928			13.15	2181		
7.5	13.37	89.3	89	3.1	13.12	516	525	45.7	13.13	2485	2408	110.7
	13.03	92.2			13.15	485			13.15	2459		
	13.05	86.0			13.15	575			13.15	2281		
8.5	13.12	91.8	101	8.8	13.13	679	727	83.3	13.17	2614	2270	303.8
	13.13	109.4			13.13	823			13.13	2157		
	13.12	101.0			13.13	678			13.13	2039		
9.5	13.13	66.8	67	4.4	13.12	541	560	30.3	13.15	2022	2036	36.8
	13.15	71.1			13.15	544			13.13	2077		
	13.13	62.3			13.12	595			13.13	2008		

Nitrate concentrations (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	STDev (σ_{n-1})
Intracellular												
0	13.17	2908	2529	429.9	13.20	1982	2089	108.5	13.23	1977	2002	31.6
	13.18	2062			13.20	2199			13.27	2038		
	13.17	2616			13.20	2086			13.20	1992		
3.5	13.15	3450	3808	317.2	13.20	3981	3841	124.6	13.25	2339	2637	403.7
	13.13	4053			13.22	3742			13.23	3096		
	13.15	3921			13.22	3800			13.23	2475		
6.5	13.13	4268	4383	100.2	13.23	6619	6540	148.4	13.25	3513	3514	76.1
	13.17	4439			13.23	6369			13.23	3590		
	13.15	4443			13.25	6633			13.25	3438		
7.5	13.15	3706	3840	248.5	13.25	3448	3620	219.6	13.25	1929	1971	54.4
	13.17	4127			13.25	3545			13.25	1952		
	13.17	3688			13.25	3867			13.25	2033		
8.5	13.17	3441	3447	31.2	13.25	3413	3221	184.2	13.27	3030	3129	147.7
	13.15	3419			13.27	3204			13.25	3057		
	13.17	3480			13.28	3046			13.25	3298		
9.5	13.13	2789	2946	145.0	13.25	2798	2886	79.1	13.32	2429	2540	126.1
	13.15	2974			13.28	2912			13.32	2677		
	13.17	3075			13.25	2949			13.30	2514		

55. The amount (ng/ml) of intra and extracellular NOD at different concentrations of nitrate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 ºC (n.d= not detected).

Nitrate concentrations (mg/L)	To			T1			T2		
	Amount of NOD (ng/ml)	**Mean (x) (ng/ml)**	**STDev (σ_{n-1})**	**Amount of NOD (ng/ml)**	**Mean (x) (ng/ml)**	**STDev (σ_{n-1})**	**Amount of NOD (ng/ml)**	**Mean (x) (ng/ml)**	**STDev (σ_{n-1})**
Exrtracellular									
0	- - -	-	-	7.2 8.9 6.2	7.4	1.4	5.3 4.9 5.9	5.4	0.5
3.5	- - -	-	-	7.6 5.4 6.9	6.6	1.1	9.7 11.9 6.1	9.2	2.9
6.5	- - -	-	-	6.6 7.4 5.8	6.6	0.8	9.6 7.9 7.7	8.4	1.1
7.5	- - -	-	-	8.9 7.0 7.3	7.7	1.1	18.4 17.8 14.0	16.7	2.4
8.5	- - -	-	-	9.7 5.2 7.5	7.5	2.3	8.7 6.0 5.6	6.8	1.7
9.5	- - -	-	-	8.1 10.8 5.7	8.2	2.6	12.3 10.0 10.9	11.1	1.2

Nitrate concentrations (mg/L)	T3			T4			T5		
	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular									
0	2.9	3	0.4	3.3	3.2	0.1	-	-	-
	2.6			3.2			-	-	
	3.3			3.1			-	-	
3.5	5.4	9	3.1	-	-	-	-	-	-
	10.8			-			-	-	
	10.8			-			-	-	
6.5	11.0	9	3.9	-	-	-	-	-	-
	4.8			-			-	-	
	12.1			-			-	-	
7.5	4.8	5	0.8	-	-	-	-	-	-
	4.5			-			-	-	
	6.0			-			-	-	
8.5	9.2	8	2.3	-	-	-	-	-	-
	10.0			-			-	-	
	5.6			-			-	-	
9.5	7.4	8	0.8	-	-	-	-	-	-
	8.9			-			-	-	
	8.7			-			-	-	

Nitrate concentrations (mg/L)	To			T1			T2		
	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Intracellular									
0	27.8 27.8 27.1	27.5	0.4	159.9 168.3 149.5	159.2	9.4	658.2 646.8 680.3	661.8	17.1
3.5	28.8 28.3 23.0	26.7	3.2	218.6 176.6 147.7	181.0	35.7	623.0 633.8 520.0	592.2	62.8
6.5	27.4 23.4 28.1	26.3	2.5	217.2 218.4 260.3	232.0	24.5	603.3 742.6 611.4	652.4	78.2
7.5	25.0 25.8 24.1	25.0	0.9	144.7 135.8 161.1	147.2	12.8	696.6 689.3 639.6	675.2	31.0
8.5	25.7 30.7 28.3	28.2	2.5	190.4 230.7 190.1	203.8	23.4	732.9 604.6 571.7	636.4	85.2
9.5	18.7 19.9 17.5	18.7	1.2	151.8 152.5 166.8	157.0	8.5	566.8 582.4 562.9	570.7	10.3

Nitrate concentrations (mg/L)	T3			T4			T5		
	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Intracellular									
0	815.3 578.1 733.4	708.9	120.5	555.7 616.5 584.8	585.7	30.4	554.4 571.3 558.4	561.4	8.9
3.5	967.1 1136.2 1099.3	1067.5	88.9	1116.0 1049.0 1065.3	1076.8	34.9	655.6 868.0 693.9	739.2	113.2
6.5	1196.4 1244.5 1245.7	1228.9	28.1	1855.5 1785.5 1859.5	1833.5	41.6	984.9 1006.4 963.8	985.0	21.3
7.5	1038.9 1156.9 1033.8	1076.6	69.7	966.5 993.8 1084.2	1014.8	61.6	540.9 547.3 569.9	552.7	15.2
8.5	964.6 958.4 975.6	966.2	8.7	956.7 898.3 853.8	902.9	51.6	849.5 857.0 924.7	877.1	41.4
9.5	781.9 833.7 862.1	825.9	40.6	784.3 816.3 826.9	809.1	22.2	681.1 750.6 704.8	712.2	35.3

56. Intra and extracellular levels of nodulopeptin 901 during the analysis on LC-MS at different nitrate concentrations for of *N. spumigena* KAC 66 grown for 5 weeks at 22°C. (for nodulopeptin 901, 210 nm wavelength and retention time 18.12 to 18.42 min; RT= retention time, PA= peak area, n.d= not detected)

Nitrate conditions (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
0	-	-	-	-	18.18	128	142	12.3	18.18	379	363	20.5
	-	-			18.15	152			18.18	340		
	-	-			18.17	145			18.15	369		
3.5	18.38	9	15	9.3	18.18	122	116	5.1	18.15	318	322	9.2
	18.22	10			18.17	112			18.17	332		
	18.23	26			18.17	115			18.17	316		
6.5	18.22	10	10	0.4	18.17	94	88	4.8	18.18	296	269	23.8
	18.20	9			18.15	85			18.18	254		
	18.22	10			18.15	86			18.17	256		
7.5	18.23	9	6	4.9	18.15	88	83	3.8	18.17	226	218	7.4
	-	0			18.15	81			18.17	212		
	18.20	8			18.15	82			18.17	215		
8.5	18.20	9	12	5.5	18.13	97	90	7.2	18.18	228	214	17.3
	18.20	9			18.15	82			18.18	195		
	18.93	18			18.17	91			18.18	218		
9.5	-	-	-	-	18.22	47	43	6.0	18.22	86	85	1.0
	-	-			18.18	47			18.20	84		
	-	-			18.17	36			18.20	84		

Nitrate conditions (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
0	18.17	571	625	48.6	18.20	677	704	24.8	18.12	257	276	16.1
	18.18	665			18.20	726			18.10	287		
	18.18	638			18.20	710			18.10	283		
3.5	18.22	263	255	15.5	18.20	494	494	0.3	18.10	22	21	2.0
	18.20	237			18.17	493			18.17	18		
	18.20	265			18.18	494			18.20	22		
6.5	18.18	242	248	11.1	18.18	961	563	344.7	18.13	103	123	17.9
	18.18	261			18.18	361			18.13	135		
	18.22	241			18.17	368			18.13	132		
7.5	18.17	315	342	24.1	18.18	249	262	11.0	18.12	99	99	4.5
	18.17	354			18.17	266			18.13	94		
	18.17	358			18.20	270			18.15	103		
8.5	18.15	279	280	1.3	18.15	496	516	17.8	18.12	182	195	10.7
	18.17	281			18.17	523			18.13	201		
	18.17	281			18.17	530			18.15	200		
9.5	18.18	131	129	5.9	18.17	304	348	53.7	18.12	131	133	13.0
	18.18	135			18.15	332			18.12	147		
	18.20	123			18.17	408			18.13	122		

Nitrate conditions (mg/L)	To				T1				T2			
	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev of PA (σ_{n-1})	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev of PA (σ_{n-1})	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev of PA (σ_{n-1})
Intracellular												
0	18.22	40	40	1.8	18.20	402	438	43.0	18.20	1305	1320	21.1
	18.23	42			18.17	486			18.20	1312		
	18.22	39			18.18	427			18.18	1344		
3.5	18.2	39	37	3.2	18.20	489	443	55.0	18.17	1071	1049	55.0
	18.22	38			18.18	458			18.18	1091		
	18.22	33			18.18	382			18.18	987		
6.5	18.22	38	36	1.9	18.20	445	508	64.7	18.17	1208	1240	60.5
	18.22	35			18.18	504			18.18	1310		
	18.23	35			18.20	574			18.18	1202		
7.5	18.25	26	28	3.4	18.18	308	310	22.3	18.17	983	987	53.8
	18.2	32			18.22	290			18.17	1043		
	18.2	25			18.20	334			18.18	936		
8.5	18.18	35	39	3.0	18.2	337	359	39.9	18.17	874	774	100.0
	18.18	40			18.20	405			18.15	773		
	18.18	40			18.20	336			18.15	674		
9.5	-	-	-	-	18.18	223	234	24.2	18.15	647	650	8.8
	-	-			18.18	218			18.15	659		
	-	-			18.18	262			18.15	642		

Nitrate conditions (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev of PA (σ_{n-1})	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev of PA (σ_{n-1})	RT (min)	PA	Mean of PA ($\bar{x}$)	STDev of PA (σ_{n-1})
Intracellular												
0	18.2	2080	1931	196.3	18.17	2116	2369	274.8	18.13	1204	1209	8.5
	18.18	1708			18.17	2329			18.13	1219		
	18.18	2005			18.17	2661			18.12	1204		
3.5	18.18	1141	1253	97.4	18.17	1606	1551	51.0	18.15	645	733	113.3
	18.15	1302			18.18	1504			18.15	861		
	18.17	1317			18.18	1544			18.15	694		
6.5	18.15	1534	1586	45.2	18.20	2736	2412	281.8	18.15	1099	1097	10.7
	18.18	1612			18.18	2223			18.13	1107		
	18.18	1613			18.18	2278			18.13	1085		
7.5	18.17	1356	1359	71.2	18.20	997	1087	102.4	18.13	549	553	10.4
	18.18	1432			18.18	1067			18.15	546		
	18.17	1290			18.20	1199			18.13	565		
8.5	18.18	1090	1090	20.5	18.20	1568	1565	55.8	18.15	1192	1228	46.1
	18.17	1070			18.20	1620			18.15	1212		
	18.17	1111			18.22	1508			18.15	1280		
9.5	18.15	765	782	20.6	18.18	1069	1095	27.7	18.22	785	810	35.5
	18.15	776			18.22	1124			18.20	851		
	18.17	805			18,20	1092			18.17	795		

57. The amount (ng/ml) of intra and extracellular nodulopeptin 901 at different concentrations of nitrate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 ºC (for nodulopeptin 901, 210 nm wavelength and retention time 18.12 to 18.42 min; RT= retention time, PA= peak area, n.d= not detected).

Nitrate concentrations (mg/L)	To			T1			T2		
	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular									
0	-	-	-	65.6	72.5	6.3	193.8	185.2	10.5
	-			77.9			173.5		
	-			74.1			188.3		
3.5	4.7	7.7	4.8	62.3	59.4	2.6	162.2	164.4	4.7
	5.2			57.3			169.8		
	13.2			58.6			161.2		
6.5	5.2	4.9	0.2	47.9	45.1	2.4	151.3	137.3	12.2
	4.8			43.7			129.7		
	4.8			43.7			130.8		
7.5	4.5	2.9	2.5	44.8	42.6	1.9	115.4	111.1	3.8
	0.0			41.2			108.3		
	4.1			41.8			109.7		
8.5	4.6	6.2	2.8	49.3	45.9	3.7	116.6	109.2	8.9
	4.4			42.0			99.4		
	9.4			46.4			111.4		
9.5	-	-	-	23.8	22.1	3.1	43.8	43.2	0.5
	-			24.0			43.0		
	-			18.6			42.9		

Nitrate concentrations (mg/L)	T3			T4			T5		
	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Exrtracellular									
0	291.5 339.6 326.0	319.1	24.8	345.8 370.6 362.7	359.7	12.7	131.3 146.6 144.5	140.8	8.3
3.5	134.1 121.1 135.4	130.2	7.9	252.3 252.0 252.1	252.1	0.1	11.2 9.2 11.2	10.6	1.2
6.5	123.5 133.2 123.3	126.7	5.7	490.9 184.2 187.8	287.6	176.1	52.6 68.9 67.4	63.0	9.0
7.5	160.9 180.8 182.9	174.9	12.2	127.4 135.7 138.0	133.7	5.6	50.6 48.0 52.6	50.4	2.3
8.5	142.5 143.6 143.6	143.2	0.6	253.3 267.2 270.5	263.7	9.1	93.0 102.7 102.1	99.3	5.5
9.5	66.8 68.8 62.8	66.1	3.0	155.3 169.7 208.3	177.7	27.4	66.9 75.1 62.3	68.1	6.5

Nitrate concentrations (mg/L)	To			T1			T2		
	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Intracellular									
0	20.3 21.5 19.7	20.5	0.9	205.4 248.1 217.9	223.8	21.9	666.4 670.0 686.6	674.3	10.8
3.5	19.8 19.6 16.9	18.8	1.6	249.9 233.8 195.2	226.3	28.1	546.8 557.0 504.0	535.9	28.1
6.5	19.4 18.0 17.6	18.3	0.9	227.1 257.4 293.1	259.2	33.0	617.1 668.9 613.9	633.3	30.9
7.5	13.5 16.1 12.8	14.1	1.8	157.1 147.9 170.5	158.5	11.4	501.8 532.6 477.8	504.1	27.5
8.5	17.9 20.6 20.5	19.7	1.5	171.9 207.0 171.5	183.5	20.4	446.3 394.9 344.2	395.1	51.0
9.5	- - -	-	-	113.8 111.1 133.7	119.5	12.3	330.6 336.7 327.9	331.8	4.5

Nitrate concentrations (mg/L)	T3			T4			T5		
	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})	Amount of nodulopeptin (ng/ml)	Mean (x) (ng/ml)	STDev (σ_{n-1})
Intracellular									
0	1062.1 872.5 1024.0	986.2	100.3	1080.8 1189.6 848.5	1039.6	174.2	614.9 622.6 614.9	617.5	4.4
3.5	582.8 665.0 672.5	640.1	49.7	820.0 768.3 788.4	792.2	26.0	329.4 439.7 354.4	374.5	57.8
6.5	783.5 823.0 823.9	810.1	23.1	1397.3 1134.8 1163.3	1231.8	144.1	561.3 565.4 554.1	560.3	5.7
7.5	692.7 731.3 658.6	694.2	36.4	509.1 544.8 612.1	555.3	52.3	280.4 278.9 288.6	282.6	5.2
8.5	556.5 546.4 567.3	556.7	10.4	800.8 827.1 770.2	799.4	28.5	608.8 619.0 653.7	627.2	23.6
9.5	390.9 396.2 411.1	399.4	10.5	546.1 574.2 557.7	559.3	14.1	400.9 434.6 406.0	413.9	18.2

58. Weight of empty, freeze dried filter discs (with cells) and cell biomass for *N. spumigena* KAC 66 grown at different K_2HPO_4 concentrations for 5 weeks at 22ºC (±S.D = standard deviation).

Conditions	Amount of K_2HPO_4 (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
		1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
To	**0**	121.3	121.3	121.3	124.8	124.7	124.7	3.4	0.17	170.0	235.0	67.6
		119.7	119.7	119.6	124.3	124.3	124.2	4.6	0.23	230.0		
		118.9	118.8	118.7	125.0	125.0	124.8	6.1	0.31	305.0		
	0.1	121.2	121.2	121.1	124.9	124.8	124.7	3.6	0.18	180.0	243.3	77.7
		121.7	121.7	121.6	126.0	126.0	126.0	4.4	0.22	220.0		
		121.8	121.8	121.7	128.4	128.3	128.3	6.6	0.33	330.0		
	10	120.9	120.9	120.8	124.1	124.0	124.0	3.2	0.16	160.0	175.0	15.0
		121.4	121.3	121.3	124.9	124.9	124.8	3.5	0.18	175.0		
		122.4	122.4	122.4	126.3	126.2	126.2	3.8	0.19	190.0		
	40	124.9	124.8	124.8	129.7	129.6	129.6	4.8	0.24	240.0	260.0	52.9
		121.3	121.2	121.2	127.7	127.7	127.6	6.4	0.32	320.0		
		117.3	117.3	117.2	121.7	121.6	121.6	4.4	0.22	220.0		
	70	122.7	122.6	122.6	127.0	127.0	127.0	4.4	0.22	220.0	228.3	14.4
		118.2	118.2	118.1	122.7	122.6	122.5	4.4	0.22	220.0		
		118.0	118.0	117.9	122.9	122.8	122.8	4.9	0.25	245.0		
	100	122.0	122.0	122.0	127.2	127.2	127.1	5.1	0.26	255.0	263.3	7.6
		121.8	121.8	121.7	127.1	127.0	127.0	5.3	0.27	265.0		
		117.8	117.8	117.6	123.1	123.1	123.0	5.4	0.27	270.0		
	120	120.0	120.0	119.8	125.9	126.9	125.8	6.0	0.30	300.0	275.0	25.0
		118.7	118.6	118.6	123.7	123.7	123.6	5.0	0.25	250.0		
		122.7	122.6	122.6	128.3	128.2	128.1	5.5	0.28	275.0		

Conditions	Amount of K_2HPO_4 (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
		1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T1	**0**	117.9	117.8	117.8	123.9	123.8	123.8	6.0	0.30	300.0	320.0	18.0
		121.8	121.7	121.7	128.3	128.2	128.2	6.5	0.32	325.0		
		123.5	123.4	123.4	130.2	130.1	130.1	6.7	0.33	335.0		
	0.1	121.6	121.5	121.5	130.6	130.6	130.5	9.0	0.45	450.0	398.3	45.4
		121.2	121.2	121.1	128.9	128.8	128.7	7.6	0.38	380.0		
		119.9	119.8	119.8	127.3	127.2	127.1	7.3	0.37	365.0		
	10	120.7	120.6	120.6	128.5	128.4	128.3	7.7	0.39	385.0	358.3	25.2
		121.5	121.4	121.4	128.1	128.0	128.1	6.7	0.33	335.0		
		120.4	120.4	120.3	127.5	127.5	127.4	7.1	0.36	355.0		
	40	121.7	121.6	121.6	128.6	128.5	128.5	6.9	0.35	345.0	380.0	69.5
		119.6	119.5	119.5	128.8	128.8	128.7	9.2	0.46	460.0		
		118.7	118.6	118.6	125.5	125.4	125.3	6.7	0.34	335.0		
	70	123.3	123.3	123.2	131.5	131.4	131.4	8.2	0.41	410.0	391.7	16.1
		120.4	120.3	120.3	128.1	128.1	128.0	7.7	0.39	385.0		
		121.0	121.0	120.9	128.7	128.6	128.5	7.6	0.38	380.0		
	100	119.0	119.0	118.9	125.6	126.6	126.5	7.6	0.38	380.0	360.0	30.4
		118.0	117.8	117.9	124.5	124.4	124.4	6.5	0.33	325.0		
		121.4	121.3	121.2	128.8	128.8	128.7	7.5	0.37	375.0		
	120	121.8	121.7	121.7	130.2	130.1	130.1	8.4	0.42	420.0	395.0	52.2
		119.1	119.0	119.1	127.8	127.8	127.7	8.6	0.43	430.0		
		116.5	116.4	116.4	123.2	123.1	123.1	6.7	0.33	335.0		

Conditions	Amount of K_2HPO_4 (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
		1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T3	**0**	121.2	121.2	121.1	127.7	127.6	127.6	6.5	0.33	325.0	340.0	13.2
		119.2	119.1	119.1	126.1	126.0	126.0	6.9	0.35	345.0		
		118.5	118.4	118.4	125.4	125.3	125.4	7.0	0.35	350.0		
	0.1	122.3	122.2	122.2	134.7	134.7	134.6	12.4	0.62	620.0	586.7	38.2
		119.5	119.5	119.4	130.2	130.2	130.3	10.9	0.55	545.0		
		119.1	119.1	119.0	131.1	130.9	130.9	11.9	0.60	595.0		
	10	123.4	123.3	123.3	134.0	133.9	133.9	10.6	0.53	530.0	518.3	24.7
		122.2	122.1	122.1	132.9	132.9	132.8	10.7	0.54	535.0		
		121.9	121.9	121.8	131.7	131.7	131.6	9.8	0.49	490.0		
	40	121.9	121.8	121.9	133.5	133.4	133.3	11.4	0.57	570.0	590.0	20.0
		124.1	124.1	124.0	135.9	135.9	135.8	11.8	0.59	590.0		
		122.2	122.1	122.2	134.3	134.3	134.4	12.2	0.61	610.0		
	70	121.8	121.7	121.7	134.7	134.7	134.6	12.9	0.65	645.0	720.0	83.5
		121.5	121.4	121.4	137.5	137.5	137.6	16.2	0.81	810.0		
		124.3	124.2	123.2	137.4	137.3	137.3	14.1	0.71	705.0		
	100	122.1	122.1	122.0	133.8	133.8	137.7	15.7	0.78	785.0	691.7	144.7
		124.4	124.3	124.4	134.9	134.8	134.9	10.5	0.53	525.0		
		122.8	122.7	122.8	138.2	138.2	138.1	15.3	0.77	765.0		
	120	122.0	122.1	122.0	135.7	135.6	135.7	13.7	0.68	685.0	678.3	50.3
		123.5	123.5	123.4	138.1	137.9	137.9	14.5	0.73	725.0		
		123.1	123.0	123.0	135.6	135.6	135.5	12.5	0.63	625.0		

Conditions	Amount of K_2HPO_4 (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
		1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T4	**0**	121.3	121.3	121.2	127.5	127.4	127.3	6.1	0.31	305.0	250.0	49.2
		125.4	125.3	125.2	129.8	129.6	129.4	4.2	0.21	210.0		
		121.4	121.4	121.3	126.0	125.9	126.0	4.7	0.24	235.0		
	0.1	123.8	123.8	123.7	132.4	132.3	132.2	8.5	0.42	425.0	433.3	10.4
		122.3	122.3	122.2	130.9	130.9	130.8	8.6	0.43	430.0		
		122.4	122.4	122.3	131.5	131.3	131.2	8.9	0.45	445.0		
	10	122.9	122.9	122.8	132.8	132.7	132.7	9.9	0.50	495.0	560.0	91.8
		121.6	121.5	121.6	132.1	132.1	132.0	10.4	0.52	520.0		
		123.5	123.4	123.3	136.7	136.6	136.6	13.3	0.67	665.0		
	40	121.9	121.9	121.8	132.7	132.7	132.6	10.8	0.54	540.0	551.7	34.0
		121.8	121.8	121.7	132.2	132.3	132.2	10.5	0.52	525.0		
		121.9	121.9	121.8	133.7	133.5	133.6	11.8	0.59	590.0		
	70	122.9	122.9	122.8	134.8	134.7	134.6	11.8	0.59	590.0	623.3	80.4
		120.9	120.9	120.8	132.2	132.2	132.1	11.3	0.57	565.0		
		119.7	119.7	119.6	133.9	133.8	133.9	14.3	0.72	715.0		
	100	122.2	122.1	122.0	138.2	138.1	138.0	16.0	0.80	800.0	681.7	109.1
		122.5	122.4	122.3	134.2	134.1	134.0	11.7	0.59	585.0		
		121.0	120.9	120.9	134.2	134.2	134.1	13.2	0.66	660.0		
	120	124.7	124.7	124.6	137.4	137.4	137.4	12.8	0.64	640.0	740.0	88.9
		121.7	121.6	121.5	137.9	136.0	136.9	15.4	0.77	770.0		
		122.8	122.7	122.7	139.1	139.0	138.9	16.2	0.81	810.0		

Conditions	Amount of K_2HPO_4 (mg/L)	Empty filter papers (a) (mg)			Filter papers+freeze dried cells (b) (mg)			Cell biomass				StDev (σ_{n-1})
		1	2	3	1	2	3	(b-a=c) (mg/20ml)	(mg/ml)	(µg/ml)	Mean ($\bar{x}$) (µg/ml)	
T5	**0**	1213.5	123.5	123.4	124.9	124.8	124.8	1.4	0.07	70.0	200.0	113.6
		123.2	123.2	123.1	128.2	128.1	128.1	5.0	0.25	250.0		
		124.3	124.2	124.1	129.4	129.5	129.7	5.6	0.28	280.0		
	0.1	124.5	124.5	124.4	133.9	133.9	131.8	7.4	0.37	370.0	403.3	108.9
		121.3	121.3	121.2	127.6	127.6	127.5	6.3	0.32	315.0		
		120.9	120.8	120.7	131.3	131.2	131.2	10.5	0.52	525.0		
	10	119.8	119.8	119.7	129.7	129.7	129.6	9.9	0.50	495.0	571.7	104.0
		119.3	119.3	119.2	133.2	133.1	133.0	13.8	0.69	690.0		
		121.1	121.1	121.0	131.7	131.7	131.6	10.6	0.53	530.0		
	40	123.6	123.5	123.6	133.4	133.4	133.3	9.7	0.49	485.0	508.3	32.1
		122.5	122.5	122.4	133.5	133.4	133.3	10.9	0.55	545.0		
		121.2	121.2	121.1	131.2	131.1	131.0	9.9	0.50	495.0		
	70	122.2	122.2	122.1	135.3	135.2	135.0	12.9	0.65	645.0	603.3	36.9
		122.2	122.2	122.1	133.8	133.7	133.6	11.5	0.58	575.0		
		121.4	121.4	121.3	133.2	133.2	133.1	11.8	0.59	590.0		
	100	121.2	121.2	121.1	134.0	133.9	133.9	12.8	0.64	640.0	618.3	33.3
		122.3	122.2	122.2	135.2	135.1	134.9	12.7	0.64	635.0		
		122.4	122.3	122.2	133.9	133.8	133.8	11.6	0.58	580.0		
	120	121.9	121.9	121.8	139.5	139.4	139.4	17.6	0.88	880.0	931.7	65.3
		121.2	121.2	121.1	141.3	141.3	141.2	20.1	1.01	1005.0		
		122.0	121.9	121.9	140.2	140.2	140.1	18.2	0.91	910.0		

59. Chl-*a* concentrations for *N. spumigena* KAC 66 grown at different K_2HPO_4 concentrations for 5 weeks at 22ºC (±S.D = standard deviation).

Conditions	Amount of K_2HPO_4 (mg/L)	Absorbance	Concentration of Chl-*a*			±STDev (σ^{n-1})
			Chlorophyll *a* (ug/20ml) .= (13.0xAxv)/(dxV)	Chl-*a* (ug/ml)	Mean (x) (ug/ml)	
To	**0**	0.41	1.35	0.07	0.053	0.013
		0.26	0.84	0.04		
		0.30	0.99	0.05		
	0.1	0.41	1.34	0.07	0.060	0.014
		0.27	0.87	0.04		
		0.43	1.40	0.07		
	10	0.32	1.05	0.05	0.063	0.010
		0.43	1.39	0.07		
		0.42	1.36	0.07		
	40	0.44	1.43	0.07	0.061	0.009
		0.36	1.17	0.06		
		0.33	1.07	0.05		
	70	0.37	1.20	0.06	0.046	0.020
		0.14	0.46	0.02		
		0.33	1.08	0.05		
	100	0.18	0.60	0.03	0.046	0.022
		0.23	0.76	0.04		
		0.44	1.42	0.07		
	120	0.36	1.17	0.06	0.045	0.014
		0.18	0.59	0.03		
		0.28	0.92	0.05		
T1	**0**	0.61	1.98	0.10	0.104	0.009
		0.71	1.98	0.10		
		0.35	2.30	0.12		
	0.1	0.18	0.58	0.03	0.044	0.026
		0.46	0.58	0.03		
		0.37	1.49	0.07		
	10	0.76	2.48	0.12	0.117	0.011
		0.64	2.48	0.12		
		0.73	2.08	0.10		
	40	0.82	2.68	0.13	0.109	0.043
		0.36	2.68	0.13		
		0.24	1.18	0.06		
	70	0.48	0.00	0.00	0.073	0.071
		0.88	1.56	0.08		
		0.78	2.85	0.14		
	100	0.38	1.24	0.06	0.085	0.039
		0.80	1.24	0.06		
		0.67	2.60	0.13		
	120	0.49	1.58	0.08	0.082	0.018
		0.65	2.11	0.11		
		0.38	1.24	0.06		
T2	**0**	0.78	2.54	0.13	0.115	0.021
		0.55	2.54	0.13		
		0.36	1.80	0.09		
	0.1	0.98	3.19	0.16	0.165	0.009
		1.08	3.19	0.16		
		0.79	3.51	0.18		
	10	0.72	2.35	0.12	0.184	0.116
		1.96	2.35	0.12		
		1.10	6.37	0.32		
	40	0.77	2.50	0.12	0.144	0.033
		1.12	2.50	0.12		
		1.33	3.65	0.18		
	70	0.66	2.15	0.11	0.143	0.061
		1.31	2.15	0.11		
		0.54	4.27	0.21		
	100	0.77	2.52	0.13	0.121	0.007
		0.70	2.52	0.13		
		0.88	2.26	0.11		
	120	0.55	1.79	0.09	0.122	0.040
		0.90	2.92	0.15		
		0.80	2.58	0.13		

Conditions	Amount of K_2HPO_4 (mg/L)	Absorbance	Concentration of Chl-*a*			±STDev (σ^{n-1})
			Chlorophyll *a* (ug/20ml) .= (13.0xAxv)/(dxV)	Chl-*a* (ug/ml)	Mean (x) (ug/ml)	
T3	**0**	0.23	0.75	0.04	0.046	0.014
		0.23	0.75	0.04		
		0.38	1.23	0.06		
	0.1	0.69	2.25	0.11	0.094	0.028
		0.38	1.23	0.06		
		0.66	2.15	0.11		
	10	1.25	4.06	0.20	0.181	0.035
		1.23	4.01	0.20		
		0.87	2.81	0.14		
	40	0.67	2.17	0.11	0.114	0.012
		0.65	2.12	0.11		
		0.79	2.57	0.13		
	70	0.65	2.11	0.11	0.153	0.064
		1.39	4.51	0.23		
		0.78	2.54	0.13		
	100	0.35	1.14	0.06	0.095	0.070
		0.32	1.02	0.05		
		1.08	3.51	0.18		
	120	1.08	3.51	0.18	0.164	0.068
		0.49	1.58	0.08		
		1.46	4.74	0.24		
T4	**0**	0.02	0.07	0.003	0.003	0.001
		0.02	0.07	0.003		
		0.01	0.05	0.002		
	0.1	0.59	1.91	0.10	0.091	0.004
		0.55	1.78	0.09		
		0.55	1.78	0.09		
	10	0.64	2.06	0.10	0.104	0.038
		0.87	2.83	0.14		
		0.41	1.32	0.07		
	40	1.18	3.82	0.19	0.133	0.051
		0.59	1.93	0.10		
		0.68	2.21	0.11		
	70	1.20	3.90	0.20	0.121	0.066
		0.42	1.38	0.07		
		0.60	1.96	0.10		
	100	0.04	0.12	0.01	0.063	0.050
		0.58	1.88	0.09		
		0.55	1.80	0.09		
	120	0.64	2.09	0.10	0.076	0.024
		0.44	1.42	0.07		
		0.33	1.08	0.05		
T5	**0**	0.02	0.07	0.003	0.003	0.001
		0.02	0.06	0.003		
		0.01	0.04	0.002		
	0.1	0.07	0.24	0.01	0.015	0.003
		0.09	0.30	0.02		
		0.12	0.38	0.02		
	10	0.34	1.09	0.05	0.043	0.010
		0.21	0.68	0.03		
		0.25	0.81	0.04		
	40	0.40	1.30	0.07	0.092	0.031
		0.52	1.70	0.08		
		0.77	2.50	0.13		
	70	0.30	0.98	0.05	0.034	0.014
		0.13	0.41	0.02		
		0.20	0.64	0.03		
	100	0.22	0.71	0.04	0.050	0.013
		0.38	1.24	0.06		
		0.32	1.02	0.05		
	120	0.11	0.36	0.02	0.025	0.004
		0.08	0.25	0.01		
		0.27	0.89	0.04		

60**.** Intra and extracellular levels of NOD during the analysis on LC-MS at different phosphate concentrations for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22°C. (for NOD 238 nm wavelength and retention time 5.11-5.33 min, RT= retention time, PA= peak area, n.d= not detected).

Phosphate conditions (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
0	5.23	4	5	0.74	5.25	12	13	2.79	5.31	11	12	1.19
	5.22	5			5.25	11			5.31	11		
	5.23	5			5.25	16			5.3	13		
0.1	5.23	5	4	0.68	5.26	9	9	0.54	5.30	20	18	2.87
	5.22	4			5.25	10			5.30	20		
	5.23	3			5.25	9			5.30	15		
10	5.23	6	6	0.16	5.25	9	10	2.03	5.30	10	12	1.60
	5.23	5			5.25	13			5.30	13		
	5.22	6			5.25	10			5.30	13		
40	5.23	6	5	0.51	5.25	11	12	0.84	5.30	12	12	0.18
	5.22	6			5.25	11			5.29	12		
	5.23	5			5.25	13			2.30	12		
70	5.22	5	5	0.33	5.25	10	12	1.60	5.30	14	14	0.32
	5.23	5			5.25	11			5.30	14		
	5.22	5			5.25	13			5.29	15		
100	5.23	6	6	0.06	2.25	17	17	0.16	5.29	9	12	2.83
	5.23	6			5.24	17			5.29	12		
	5.22	6			5.24	17			5.29	14		
120	5.23	7	7	0.35	5.24	20	23	2.14	5.30	14	15	0.96
	5.22	8			5.25	24			5.29	15		
	5.22	7			5.24	24			5.30	15		

Phosphate conditions (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
0	5.32	3	3	0.28	5.33	1	1	0.05	5.22	3	3	1.90
	5.33	3			5.33	1			5.23	5		
	5.32	3			5.34	1			5.23	2		
0.1	5.32	27	18	7.62	5.33	2	2	0.69	5.23	1	1	0.23
	5.32	13			5.33	3			5.23	1		
	5.32	14			5.33	2			5.23	1		
10	5.32	1	1	0.33	5.33	1	1	0.33	5.23	1	1	0.08
	5.32	1			5.34	1			5.23	1		
	5.31	1			5.34	1			5.24	1		
40	5.32	5	6	4.81	5.33	1	1	0.14	5.23	3	3	2.08
	5.31	2			5.33	1			5.24	1		
	5.32	12			5.33	1			5.24	5		
70	5.31	5	5	1.18	5.34	2	11	10.85	5.24	6	3	3.08
	5.32	4			5.33	23			5.24	2		
	5.31	6			5.34	9			5.24	0		
100	5.31	8	9	2.05	5.33	26	22	17.89	5.24	1	1	0.72
	5.32	7			5.33	37			5.24	2		
	5.32	11			5.34	2			5.25	0		
120	5.32	7	5	2.11	5.34	2	2	0.20	5.24	4	4	3.95
	5.32	3			5.34	2			5.25	8		
	5.32	6			5.34	2			0	0		

Phosphate conditions (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Intracellular												
0	5.21	121	156	31.14	5.21	651	627	21.91	5.26	1452	1445	33.11
	5.21	179			5.21	610			5.28	1475		
	5.22	169			5.21	619			5.26	1410		
0.1	5.21	174	161	11.69	5.21	530	557	24.39	5.27	1478	1512	41.76
	5.21	160			5.21	561			5.27	1499		
	5.21	150			5.22	578			5.27	1558		
10	5.22	166	162	3.75	5.21	566	499	59.43	5.27	1432	1399	45.93
	5.22	160			5.22	481			5.27	1419		
	5.22	160			5.22	451			5.28	1347		
40	5.22	173	180	21.46	5.22	678	707	26.02	5.27	1767	1740	31.27
	5.22	204			5.23	729			5.28	1706		
	5.22	163			5.22	714			5.28	1748		
70	5.22	174	162	23.00	5.22	740	750	15.00	5.28	1902	1889	38.16
	5.22	177			5.22	742			5.29	1919		
	5.22	136			5.23	767			5.29	1846		
100	5.22	171	169	2.86	5.23	799	769	27.64	5.29	1735	1774	45.67
	5.22	166			5.23	745			5.29	1762		
	5.22	170			5.23	764			5.29	1824		
120	5.22	168	160	7.96	5.23	777	773	32.06	5.30	1757	1775	18.21
	5.22	152			5.23	740			5.30	1794		
	5.22	161			5.23	804			5.29	1773		

Phosphate conditions (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Intracellular												
0	5.30	287	277	12.78	5.33	19	18	1.03	5.18	23	22	1.72
	5.29	263			5.33	18			5.19	20		
	5.29	281			5.33	17			5.19	22		
0.1	5.28	1006	1047	116.10	5.32	271	274	7.57	5.19	35	35	1.13
	5.28	957			5.31	269			5.19	33		
	5.28	1178			5.31	283			5.19	35		
10	5.29	797	783	34.82	5.31	412	411	4.08	5.22	306	279	49.40
	5.29	809			5.31	414			5.21	308		
	5.29	744			5.31	406			5.22	222		
40	5.29	873	969	100.86	5.31	484	489	13.23	5.21	296	280	29.50
	5.29	959			5.31	504			5.22	246		
	5.29	1074			5.32	479			5.21	298		
70	5.30	998	1009	12.40	5.32	451	447	20.92	5.22	195	200	26.06
	5.30	1023			5.32	424			5.22	228		
	5.30	1006			5.32	466			5.22	177		
100	5.30	893	972	134.76	5.32	596	583	18.98	5.22	245	287	49.55
	5.31	895			5.32	562			5.22	342		
	5.31	1128			5.32	593			5.22	274		
120	5.31	845	851	38.84	5.33	262	265	18.60	5.23	47	45	3.95
	5.32	893			5.33	285			5.23	41		
	5.31	816			5.33	248			5.22	49		

61. The amount (ng/ml) of intra and extracellular NOD at different concentrations of phosphate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 ºC (for NOD 238 nm wavelength and retention time 5.11-5.33 min, RT= retention time, PA= peak area, n.d= not detected).

Phosphate concentrations (mg/L)	To			T1			T2		
	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Exrtracellular									
0	5.2 6.5 6.5	6.1	0.8	15.7 14.4 20.9	17.0	3.5	14.4 14.4 17.0	15.3	1.5
0.1	6.5 5.2 3.9	5.2	1.3	11.8 13.1 11.8	12.2	0.8	26.1 26.1 19.6	24.0	3.8
10	7.8 6.5 7.8	7.4	0.8	11.8 17.0 13.1	13.9	2.7	13.1 17.0 17.0	15.7	2.3
40	7.8 7.8 6.5	7.4	0.8	14.4 14.4 17.0	15.3	1.5	15.7 15.7 15.7	15.7	0.0
70	6.5 6.5 6.5	6.5	0.0	13.1 14.4 17.0	14.8	2.0	18.3 18.3 19.6	18.7	0.8
100	7.8 7.8 7.8	7.8	0.0	22.2 22.2 22.2	22.2	0.0	11.8 15.7 18.3	15.3	3.3
120	9.2 10.5 9.2	9.6	0.8	26.1 31.4 31.4	29.6	3.0	18.3 19.6 19.6	19.2	0.8

Phosphate concentrations (mg/L)	T3			T4			T5		
	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Exrtracellular									
0	3.9 3.9 3.9	3.9	0.0	1.3 1.3 1.3	1.3	0.0	3.9 6.5 2.6	4.4	2.0
0.1	35.3 17.0 18.3	23.5	10.2	2.6 3.9 2.6	3.1	0.8	1.3 1.3 1.3	1.3	0.0
10	1.3 1.3 1.3	1.3	0.0	1.3 1.3 1.3	1.3	0.0	1.3 1.3 1.3	1.3	0.0
40	6.5 2.6 15.7	8.3	6.7	1.3 1.3 1.3	1.3	0.0	3.9 1.3 6.5	3.9	2.6
70	6.5 5.2 7.8	6.5	1.3	2.6 30.1 11.8	14.8	14.0	7.8 2.6 0.0	3.5	4.0
100	10.5 9.2 14.4	11.3	2.7	34.0 48.4 2.6	28.3	23.4	1.3 2.6 0	1.3	1.3
120	9.2 3.9 0	4.4	4.6	2.6 2.6 2.6	2.6	0.0	5.2 10.5 0	5.2	5.2

Phosphate concentrations (mg/L)	To			T1			T2		
	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Intracellular									
0	158.2 234.0 220.9	204.4	40.5	851.0 797.4 809.2	819.2	28.2	1898.0 1928.1 1843.1	1889.8	43.1
0.1	227.5 209.2 196.1	210.9	15.8	692.8 733.3 755.6	727.2	31.8	1932.0 1959.5 2036.6	1976.0	54.2
10	217.0 209.2 209.2	211.8	4.5	739.9 628.8 589.5	652.7	78.0	1871.9 1854.9 1760.8	1829.2	59.9
40	226.1 266.7 213.1	235.3	27.9	886.3 952.9 933.3	924.2	34.3	2309.8 2230.1 2285.0	2274.9	40.8
70	227.5 231.4 177.8	212.2	29.9	967.3 967.3 1002.6	979.1	20.4	2486.3 2508.5 2413.1	2469.3	49.9
100	223.5 217.0 222.2	220.9	3.5	1044.4 973.9 998.7	1005.7	35.8	2268.0 2303.3 2384.3	2318.5	59.7
120	350.3 198.7 210.5	253.2	84.4	1015.7 967.3 1051.0	1011.3	42.0	2296.7 2286.3 2317.6	2300.2	16.0

Phosphate concentrations (mg/L)	T3			T4			T5		
	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of NOD (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Intracellular									
0	375.2 343.8 367.3	362.1	16.3	24.8 23.5 22.2	23.5	1.3	30.1 26.1 28.8	28.3	2.0
0.1	1315.0 1251.0 1539.9	1368.6	151.7	354.2 351.6 369.9	358.6	9.9	45.8 43.1 45.8	44.9	1.5
10	1041.8 1057.5 972.5	1024.0	45.2	538.6 541.2 530.7	536.8	5.4	400.0 402.6 290.2	364.3	64.2
40	1141.2 1253.6 1403.9	1266.2	131.8	632.7 658.8 626.1	639.2	17.3	386.9 321.6 389.5	366.0	38.5
70	1304.6 1337.3 1315.0	1319.0	16.7	589.5 554.2 609.2	584.3	27.8	254.9 298.0 231.4	261.4	33.8
100	1167.3 1169.9 1474.5	1270.6	176.6	779.1 734.6 775.2	763.0	24.6	320.3 447.1 358.2	375.2	65.1
120	1104.6 1167.3 1066.7	1112.9	50.8	342.5 372.5 324.2	346.4	24.4	61.4 53.6 64.1	59.7	5.4

62. Intra and extracellular levels of nodulopeptin 901 during the analysis on LC-MS at different phosphate concentrations for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22°C. (for nodulopeptin901, 210 nm wavelength and retention time 6.90-7.00 min; RT= retention time, PA= peak area, n.d= not detected)

Phosphate conditions (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
0												
	6.98	7	25	15.9	6.99	50	50	4.1	6.99	68	75	6.6
	6.98	32			6.98	45			6.99	81		
	6.97	36			6.99	54			6.99	77		
0.1												
	6.98	49	41	7.4	6.99	42	43	2.6	6.99	38	37	23.6
	6.98	34			6.98	46			6.99	61		
	6.98	41			6.98	40			6.99	14		
10												
	6.98	34	35	2.8	6.98	43	43	0.6	6.99	55	58	4.4
	6.98	38			6.99	44			6.99	55		
	6.99	33			6.99	43			6.99	63		
40												
	6.98	40	38	2.7	6.99	41	44	2.4	6.99	64	62	2.3
	6.97	35			6.99	45			6.99	60		
	6.980	37			6.99	45			6.99	60		
70												
	6.98	35	38	4.3	6.99	45	43	2.4	6.99	54	55	5.8
	6.98	43			6.99	41			6.99	61		
	6.98	36			7.00	42			6.99	50		
100												
	6.98	39	40	1.3	6.99	20	34	12.3	6.99	52	47	8.5
	6.98	40			6.99	37			6.99	38		
	6.97	41			6.98	44			6.99	53		
120												
	6.98	40	39	2.3	6.99	44	43	1.9	6.99	52	54	1.9
	6.97	41			7.00	41			6.99	55		
	6.97	36			6.99	43			6.99	56		

Phosphate conditions (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Exrtracellular												
0												
	6.99	117	120	3.3	7.00	32	45	11.9	6.90	11	12	3.0
	7.00	123			7.00	48			6.89	16		
	7.00	122			7.00	55			6.89	11		
0.1												
	6.99	85	91	6.2	7.00	82	109	36.7	6.90	24	24	2.7
	7.00	97			7.00	151			6.89	22		
	6.99	90			7.00	94			6.89	27		
10												
	6.99	64	67	3.1	6.99	69	70	7.9	6.90	55	55	4.0
	6.99	67			6.99	79			6.90	50		
	6.99	70			6.99	63			6.90	58		
40												
	7.00	78	67	12.2	7.00	75	76	8.7	6.90	79	84	8.9
	6.99	54			6.99	85			6.90	94		
	7.00	70			6.99	68			6.90	79		
70												
	6.99	63	71	8.6	7.00	78	88	8.5	6.90	62	59	6.1
	7.00	70			7.00	94			6.89	52		
	6.99	80			7.00	92			6.89	63		
100												
	6.99	79	71	6.8	7.00	63	74	10.0	6.89	51	46	4.5
	7.00	69			7.00	82			6.90	43		
	6.99	66			7.00	78			6.90	44		
120												
	7.00	74	70	4.3	7.00	81	85	3.9	6.89	25	26	3.4
	7.00	71			7.00	87			6.90	24		
	6.99	66			7.00	88			6.89	30		

Phosphate conditions (mg/L)	To				T1				T2			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Intracellular												
0	9.98	52	77	24.9	6.98	187	179	13.2	6.99	406	387	17.0
	6.98	102			6.98	187			6.99	383		
	6.98	76			6.98	164			6.99	373		
0.1	6.98	83	69	11.5	6.98	133	135	5.4	6.99	313	307	16.5
	6.98	63			6.97	130			6.98	288		
	6.98	62			6.98	141			6.98	319		
10	6.98	85	71	12.4	6.98	129	125	19.1	6.99	245	234	42.4
	6.98	63			6.98	105			6.98	270		
	6.97	64			6.98	142			6.99	187		
40	6.98	80	82	6.6	6.98	144	166	19.1	6.98	314	300	12.5
	6.98	89			6.98	177			6.98	295		
	6.98	76			6.98	177			6.98	290		
70	6.98	72	67	4.1	6.98	201	174	23.8	6.98	301	300	26.0
	6.98	67			6.98	157			6.99	326		
	6.98	63			6.98	164			6.98	274		
100	6.97	67	66	1.9	6.98	153	165	17.7	6.98	284	295	16.4
	6.98	68			6.98	157			6.99	314		
	6.98	64			6.98	186			6.99	288		
120	6.97	74	66	7.4	6.98	163	154	24.8	6.99	253	287	31.0
	6.98	60			6.98	126			6.99	314		
	6.98	64			6.98	173			6.99	295		

Phosphate conditions (mg/L)	T3				T4				T5			
	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})	RT (min)	PA	Mean ($\bar{x}$)	StDev (σ_{n-1})
Intracellular												
0	7.00	159	152	7.1	6.99	43	47	3.5	6.89	25	22	2.3
	6.99	145			7.00	48			6.90	21		
	6.99	152			7.00	50			6.90	22		
0.1	6.99	214	241	39.3	7.00	119	116	4.9	6.90	34	37	2.6
	6.99	224			6.99	110			6.89	39		
	7.00	286			7.00	119			6.90	38		
10	6.99	111	124	12.2	6.99	110	108	4.2	6.89	119	109	14.6
	6.99	128			7.00	110			6.89	115		
	6.99	134			7.00	103			6.90	92		
40	6.99	150	146	29.7	6.99	100	103	3.2	6.89	128	134	9.9
	6.99	115			6.99	106			6.90	129		
	6.99	174			7.00	103			6.89	145		
70	6.99	157	165	11.7	7.00	99	105	6.0	6.90	63	89	22.8
	6.99	160			7.00	106			6.90	107		
	6.99	179			7.00	111			6.89	95		
100	6.99	142	147	8.2	7.00	134	125	13.5	6.89	91	95	4.9
	6.99	142			7.00	131			6.90	100		
	7.00	156			6.99	110			6.89	95		
120	6.99	152	142	9.7	7.00	104	97	15.1	6.90	45	45	2.1
	7.00	132			7.00	79			6.89	43		
	6.99	143			7.00	107			6.89	47		

63. The amount (ng/ml) of intra and extracellular nodulopeptin 901 at different concentrations of phosphate for cultures of *N. spumigena* KAC 66 grown for 5 weeks at 22 ºC (for nodulopeptin901, 210 nm wavelength and retention time 6.90-7.00 min; RT= retention time, PA= peak area, n.d= not detected)

Phosphate concentrations (mg/L)	To			T1			T2		
	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Exrtracellular									
0	9.1 41.5 46.7	32.4	20.4	64.9 58.4 70.0	64.4	5.8	88.2 105.1 99.9	97.7	8.6
0.1	63.6 44.1 53.2	53.6	9.7	54.5 59.7 51.9	55.3	4.0	49.3 79.1 18.2	48.9	30.5
10	44.1 49.3 42.8	45.4	3.4	55.8 57.1 55.8	56.2	0.7	71.3 71.3 81.7	74.8	6.0
40	51.9 45.4 48.0	48.4	3.3	53.2 58.4 58.4	56.6	3.0	83.0 77.8 77.8	79.6	3.0
70	45.4 2.6 46.7	31.6	25.1	58.4 53.2 54.5	55.3	2.7	70.0 79.1 64.9	71.3	7.2
100	2.6 2.6 53.2	19.5	29.2	25.9 48.0 57.1	43.7	16.0	67.4 49.3 68.7	61.8	10.9
120	51.9 53.2 46.7	50.6	3.4	57.1 53.2 55.8	55.3	2.0	67.4 71.3 72.6	70.5	2.7

Phosphate concentrations (mg/L)	T3			T4			T5		
	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Exrtracellular									
0	151.8 159.5 158.2	156.5	4.2	41.5 62.3 71.3	58.4	15.3	14.3 20.8 14.3	16.4	3.7
0.1	110.2 125.8 116.7	117.6	7.8	106.4 195.8 121.9	141.4	47.8	31.1 28.5 35.0	31.6	3.3
10	83.0 86.9 90.8	86.9	3.9	89.5 102.5 81.7	91.2	10.5	71.3 64.9 75.2	70.5	5.2
40	101.2 70.0 90.8	87.3	15.8	97.3 110.2 88.2	98.6	11.1	102.5 121.9 102.5	108.9	11.2
70	81.7 90.8 103.8	92.1	11.1	101.2 121.9 119.3	114.1	11.3	80.4 67.4 81.7	76.5	7.9
100	102.5 89.5 85.6	92.5	8.8	81.7 106.4 101.2	96.4	13.0	66.1 55.8 57.1	59.7	5.7
120	96.0 92.1 85.6	91.2	5.2	105.1 112.8 114.1	110.7	4.9	32.4 31.1 38.9	34.2	4.2

Phosphate concentrations (mg/L)	To			T1			T2		
	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 901 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Intracellular									
0	67.4 132.3 98.6	99.4	32.4	242.5 242.5 212.7	232.6	17.2	526.6 496.8 483.8	502.4	21.9
0.1	107.7 81.7 80.4	89.9	15.4	172.5 168.6 182.9	174.7	7.4	406.0 373.5 413.7	397.8	21.3
10	110.2 81.7 83.0	91.7	16.1	167.3 136.2 184.2	162.6	24.3	317.8 350.2 242.5	303.5	55.2
40	103.8 115.4 98.6	105.9	8.6	186.8 229.6 229.6	215.3	24.7	407.3 382.6 376.1	388.7	16.4
70	93.4 86.9 81.7	87.3	5.8	260.7 203.6 212.7	225.7	30.7	390.4 422.8 355.4	389.5	33.7
100	86.9 88.2 83.0	86.0	2.7	198.4 203.6 241.2	214.4	23.4	368.4 407.3 373.5	383.1	21.1
120	96.0 77.8 83.0	85.6	9.4	211.4 163.4 224.4	199.7	32.1	328.1 407.3 382.6	372.7	40.5

Phosphate concentrations (mg/L)	T3			T4			T5		
	Amount of nodulopeptin 902 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 902 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})	Amount of nodulopeptin 902 (ng/ml)	Mean ($\bar{x}$) (ng/ml)	StDev (σ_{n-1})
Intracellular									
0	206.2 188.1 197.1	197.1	9.1	55.8 62.3 64.9	61.0	4.7	32.4 27.2 28.5	29.4	2.7
0.1	277.6 290.5 370.9	313.0	50.6	142.7 154.3 154.3	150.5	6.7	44.1 50.6 49.3	48.0	3.4
10	144.0 166.0 173.8	161.3	15.5	142.7 142.7 133.6	139.6	5.2	154.3 149.2 119.3	140.9	18.9
40	0.0 149.2 225.7	124.9	114.8	129.7 137.5 133.6	133.6	3.9	166.0 167.3 188.1	173.8	12.4
70	203.6 207.5 232.2	214.4	15.5	128.4 137.5 144.0	136.6	7.8	81.7 138.8 123.2	114.6	29.5
100	184.2 184.2 202.3	190.2	10.5	173.8 169.9 142.7	162.1	17.0	118.0 129.7 123.2	123.6	5.8
120	197.1 171.2 185.5	184.6	13.0	134.9 102.5 138.8	125.4	19.9	58.4 55.8 61.0	58.4	2.6

64. Details and salinity values of samples collected from the various habitats of the Dead Sea.

Original sample code	New codes	Salinity (‰)	Salinity groups
Shore sample 6	D39	4.0	4
Shore sample 7	D40	1.4	4
Shoreline 10a	D49	5.4	4
Sample 1b	D2	17.0	17
Spring spring 11a	D35	16.0	17
Sample 1a	D1	19.0	20
Sample 9a	D11	18.6	20
Shoreline 4	D46	18.8	20
Shoreline 10b	D50	19.6	20
Sample 2a	D3	22.4	22
Sample 2b	D4	22.4	22
Sample 4a	D5	23.0	22
Sample 4b	D6	22.4	22
Sample 6a	D7	22.4	22
Sample 6b	D8	22.4	22
Sample 9b	D12	22.4	22
Sample 12b	D16	23.2	22
Shore sample 2	D37	21.4	22
Spring spring 11b	D36	21.0	22
Sample 8a	D9	25.0	25
Sample 8b	D10	26.6	25
Sample 11a	D13	26.2	25
Sample 11b	D14	26.2	25
Sample 12a	D15	24.0	25
Spring sample 10	D31	26.4	25
Spring sample13	D34	25.0	25
Shore sample 5	D38	25.2	25
White hard a	D51	>32.0	32
White hard b	D52	32.0	32
Hot spring 1	D18	>32.0	32
Hot spring 2	D19	>32.0	32
Hot spring 3	D20	>32.0	32
Hot spring 4	D21	>32.0	32
Hot spring 7	D22	>32.0	32
Hot spring 9	D23	>32.0	32
Hot spring 11a	D24	>32.0	32
Hot spring 11b	D25	>32.0	32
Hot spring 12	D26	>32.0	32
Hot spring 17	D27	>32.0	32
Hot spring 18	D28	>32.0	32
Hot spring 19	D29	>32.0	32
Spring sample 6	D30	>32.0	32
Spring sample12a	D32	>32.0	32
Spring sample12b	D33	>32.0	32
Southern spring	D43	>32.0	32
Southern system a	D44	>32.0	32
Southern system b	D45	>32.0	32
Shore sample 12	D42	29.0	28
Shoreline 6	D47	28.6	28
Shoreline 9	D48	27.2	28
Sample 14	D17	29.8	28
Shore sample 11	D41	dry	dry
SHO shore	D53	19.4	20

CONFERENCE PRESENTATIONS

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Production of bioactive peptides by *Nodularia spumigena* KAC 66

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Under favourable conditions several freshwater and marine cyanobacterial strains are capable of forming blooms which may be toxic and thus hazardous to animals and humans. The growth and toxin production of cyanobacteria are affected by abiotic and biotic factors, but why blooms form is still largely a mystery. The nitrogen-fixing cyanobacterium, *Nodularia spumigena* frequently forms blooms in the Baltic Sea. In addition to producing peptide hepatotoxins, nodularins, this species produces many other bioactive compounds such as spumigins, nodulopeptins and recently three new peptides have been characterised (A, B & C). In order to unravel the roles of these compounds, intracellular-and extracellular levels have been monitored during growth. This study reports the production of nodularin and the recently characterised peptide "C", major secondary metabolites produced by *N. spumigena* KAC 66.

Cultures of *N. spumigena* were grown for 7 weeks with weekly sampling. Growth was measured by dry weight. The production of NOD and peptide C in cells and media were monitored by HPLC-PDA-MS.

In common with many studies, the majority of the NOD was in the cells throughout the seven week growth experiment, with maximum levels

detected at 6 weeks. In contrast, as much as 40% of peptide C was detected in the media thought the growth cycle.

HPLC-PDA-MS revealed that *N. spumigena* produces many characterised and uncharacterised compounds. Week 6 (T6) is the optimum time to get the highest amount of NOD and peptide C.

Keywords: extracellular, intracellular, biomass, HPLC-PDA-MS

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EFFECTS OF ENVIRONMENTAL FACTORS ON PRODUCTION OF BIOACTIVE PEPTIDES IN *NODULARIA SPUMIGENA* KAC 66

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Abstract

Due to heavy nutrient load, the Baltic Sea is *Nodularia spumigena* under the influence of eutrophication, which has resulted in the occurrence of heavy toxic algal blooms. In late summer the dominant and toxic strain *N. spumigena* along with the non-toxic *Aphanizomenon flos-aquae* and *Anabaena* spp., produces massive and lethal blooms in many areas of the Baltic Sea. *N. spumigena* has also been reported to have lethal blooms in Lake Alexandrina, Australia. As well as producing nodularins, *Nodularia* sp. also produces a range of other bioactive peptides such as spumigins and nodulopeptins, all of which have unclear function.

We recently characterised three new nodulopeptins (899, 901, 917) from *N. spumigena* KAC 66. Nodulopeptin 901 demonstrated weak inhibition of protein phosphatase 1 (IC_{50} 25 µg/mL). To gain further insight on the effects of environmental stress on growth and production of bioactive metabolites in *N. spumigena* KAC 66, a range of parameters were investigated which included; temperature, salinity, nitrate and phosphorus. Growth was monitored by cell biomass and chlorophyll-*a*. Intracellular and extracellular peptides were monitored by high

performance liquid chromatography with photodiode array and mass spectrometry (HPLC-PDA-MS).

In common with many studies, the maximum amount of nodularin was retained within the cells during the seven week growth experiment. In contrast, as much as 40% of nodulopeptin 901 was detected in the growth media throughout the duration of experiments.

Temperature had the greatest effect on peptide production. Whilst growth was similar at 22°C, 25°C and 30°C, increase in temperature had a profound effect on nodularin production in that an increase from 22°C to 25°C resulted in a 50% decrease in intracellular nodularin levels. At 30°C little or no nodularin was detected. In contrast, whilst concentrations of nodulopeptin 901 decreased with increasing temperature, they were still detected at consistent levels suggesting they play an important role.

This is the first study to evaluate the effects of selected environmental parameters on nodularin/nodulopeptin production which ultimately may be helpful to explain the distribution, control of natural blooms and toxin levels of *N. spumigena* in the Baltic Sea and as well as laboratory based experiments.