CYANOBACTERIA FROM THE BALTIC SEA AND FINNISH LAKES AS AN ENERGY SOURCE AND MODULATORS OF BIOENERGETIC PATHWAYS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals in the text.

I   Yagut Allahverdiyeva*, Hannu Leino*, Lyudmila Saari, David P.Fewer, Sumathy Shunmugam, Kaarina Sivonen, Eva-Mari Aro (2010) Screening for biohydrogen production by cyanobacteria isolated from the Baltic Sea and Finnish lakes. *Int. J. Hydrogen Energy* 35:1117–1127.

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ABBREVIATIONS

ADP	adenosine diphosphate
AOX	alternative oxidase
AsA	ascorbate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CAT	catalase
CET	cyclic electron transport
Chl	chlorophyll
CO_2	carbon dioxide
cyt	cytochrome
DCBQ	2,6-dichloro-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
DMSO	dimethyl sulphoxide
Fd	ferrodoxin
Fe	iron
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione S-transferase
H_2	molecular hydrogen
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
LET	linear electron transport
MC	microcystin
MDA	monodehydroascorbate
MMP	mitochondrial membrane potential
Mo	molybdenum
N_2	molecular nitrogen
NADH	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NH_3	ammonia
Ni	nickel
NTR	NAD(P)H-dependent thioredoxin reductase
O_2	molecular oxygen

PAGE	polyacrylamide gel electrophoresis
PC	plastocyanin
PCC	Pasteur Culture Collection
PHAs	polyhydroxyalkanoates
Pi	inorganic phosphate
POD	peroxidase
PP	protein phosphatase
PQ	plastoquinone
PRX	peroxiredoxin
PS	photosystem
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
S	sulphur
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SSC	Saline-Sodium Citrate
TCA	tricarboxylic acid
TRX	thioredoxin
UHCC	University of Helsinki Culture Collection
V	vanadium
WT	wild type

ABSTRACT

Cyanobacteria are a diverse group of oxygenic photosynthetic bacteria that inhabit in a wide range of environments. They are versatile and multifaceted organisms with great possibilities for different biotechnological applications. For example, cyanobacteria produce molecular hydrogen (H_2), which is one of the most important alternatives for clean and sustainable energy. Apart from being beneficial, cyanobacteria also possess harmful characteristics and may become a source of threat to human health and other living organisms, as they are able to form surface blooms that are producing a variety of toxic or bioactive compounds. The University of Helsinki Culture Collection (UHCC) maintains around 1,000 cyanobacterial strains representing a large number of genera and species isolated from the Baltic Sea and Finnish lakes. The culture collection covers different life forms such as unicellular and filamentous, N_2-fixing and non-N_2-fixing strains, and planktonic and benthic cyanobacteria.

In this thesis, the UHCC has been screened to identify potential strains for sustainable biohydrogen production and also for strains that produce compounds modifying the bioenergetic pathways of other cyanobacteria or terrestrial plants. Among the 400 cyanobacterial strains screened so far, ten were identified as high H_2-producing strains. The enzyme systems involved in H_2 metabolism of cyanobacteria were analyzed using the Southern hybridization approach. This revealed the presence of the enzyme nitrogenase in all strains tested, while none of them are likely to have contained alternative nitrogenases. All the strains tested, except for two *Calothrix* strains, XSPORK 36C and XSPORK 11A, were suggested to contain both uptake and bidirectional hydrogenases. Moreover, 55 methanol extracts of various cyanobacterial strains were screened to identify potent bioactive compounds affecting the photosynthetic apparatus of the model cyanobacterium, *Synechocystis* PCC 6803. The extract from *Nostoc* XPORK 14A was the only one that modified the photosynthetic machinery and dark respiration. The compound responsible for this effect was identified, purified, and named M22. M22 demonstrated a dual-action mechanism: production of reactive oxygen species (ROS) under illumination and an unknown mechanism that also prevailed in the dark.

During summer, the Baltic Sea is occupied by toxic blooms of *Nodularia spumigena* (hereafter referred to as *N. spumigena*), which produces a hepatotoxin called nodularin. Long-term exposure of the terrestrial plant spinach to nodularin was studied. Such treatment resulted in inhibition of growth and chlorosis of the leaves. Moreover, the activity and amount of mitochondrial electron transfer complexes increased in the leaves exposed to nodularin-containing extract, indicating upregulation of respiratory reactions, whereas no marked changes were detected in the structure or function of the photosynthetic machinery. Nodularin-exposed plants suffered from oxidative stress, evidenced by oxidative modifications of various proteins. Plants initiated strategies to combat the stress by increasing the levels of α-tocopherol, mitochondrial alternative oxidase (AOX), and mitochondrial ascorbate peroxidase (mAPX).

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1. INTRODUCTION

1.1. Ecology and diversity of cyanobacteria

Cyanobacteria are photosynthetic prokaryotes that possess chlorophyll (Chl) a and convert solar energy into chemical energy using water as an electron donor, and release oxygen (O_2) as a by-product during photosynthesis. The presence of phycocyanin in most cyanobacteria leads to the bluish colour of the organisms; thus, they are also known as blue-green algae. Cyanobacteria have a long evolutionary history. They are a morphologically diverse group of organisms and they exist as unicellular (e.g. *Chroococcus*), filamentous (e.g. *Anabaena*), or colonial forms (e.g. *Gloeothece*) (Whitton and Potts 2000). The early forms of unicellular and filamentous cyanobacteria formed 3.5 billion years ago, and the endolithic forms about 1.5 billion years ago (Wilmotte 1994). It is widely accepted that the cyanobacteria are responsible for the formation of atmospheric O_2 and that they have given rise to the present-day chloroplasts of algae and green plants (Miyagishima 2005; Mulkidjanian et al. 2006). Their long evolutionary history contributes to their successful adaptation to aquatic (e.g. freshwater, seawater, and brackish water), terrestrial and extreme environments (e.g. hot deserts, geothermal vents, hypersaline lakes, and polar regions with glaciers and ice-capped lakes) (Gademann and Portmann 2008). In aquatic environments, they are found both as floating planktonic and benthic forms in the sediment (Shilo 1989). For example, in the Baltic Sea, one of the largest brackish water basins in the world, several planktonic species such as *N. spumigena* and *Anabaena* spp. and also benthic forms of *Anabaena*, *Nostoc*, and *Calothrix* have been reported. About 1,000 different strains have been isolated from the Baltic Sea and Finnish lakes, and these strains are maintained in the UHCC.

1.2. Good and bad cyanobacteria

Our knowledge of the diversity and physiology of cyanobacteria from past research serves as an excellent platform for exploring their biotechnological applications for human welfare. They have been widely explored as an alternative energy source to replace the depleting fossil fuel resources (Asada and Miyake 1999). Cyanobacteria are also a promising and rich source of bioactive compounds (Borowitzka 1995) including antibacterial (Jaki et al. 2000), antifungal (Kajiyama et al. 1998), antiviral (Patterson et al. 1994), anticancer (Gerwick et al. 1994), and immunosuppressive agents (Koehn et al. 1992). *Spirulina plantensis* has been found to produce γ-linolenic acid, a medically important compound, which functions in lowering of blood pressure and lipid metabolism (Cohen 1999). Some cyanobacteria have the capacity to convert fixed carbon dioxide (CO_2) into biodegradable thermoplastic polyhydroxyalkanoates (PHAs) under nitrogen (N_2)-limiting conditions (Sudhesh et al. 2002). In addition to this, cyanobacteria can be used as biofertilizers because of their ability to fix atmospheric N_2, which improves the fertility of the soil in the tropical rice fields (Capone et al. 2005). Moreover, strains of *Spirulina*, *Anabaena*, and *Nostoc* are consumed as human

food in many countries, including Chile, Mexico, Peru, and the Philippines, as they are rich in proteins and β-carotene, thiamine, and riboflavin and are considered to be one of the richest sources of vitamin B12 (Abed et al. 2009).

At the same time, due to the growing human population and human activities, the ecosystem has been enriched with phosphorus and N_2 (Schindler 1977; Smith 1983). These nutrients trigger the formation of harmful algal blooms or toxic cyanobacteria, up to 1 m thick scum mats (Zohary and Roberts 1989; Dow and Swoboda 2000). There are two types of harmful algal blooms: toxin-producing, which cause human illness through drinking of contaminated water or consumption of seafoods exposed to toxins, and non-toxin-producing, which can cause harm through the development of scums, depletion of O_2 when blooms decay, or destruction of habitats for fish, shellfish, or sea animals (Anderson et al. 2002).

Toxic cyanobacteria produce a wide range of secondary metabolites with potent inhibitory effects on the growth, photosynthesis, respiration, carbon uptake, and enzyme activity of other algae or eukaryotic organisms growing in the same environment. Some of the secondary metabolites have generally been called cyanotoxins, which can cause acute and possibly chronic health problems in humans and fatal poisonings in mammals, fish, and birds (Carmichael 1992, 2001; Onodera et al. 1997; Liu et al. 2002). Cyanotoxins have been classified according to the symptoms they cause in humans and vertebrates: hepatotoxins (such as microcystin (MC), nodularin, and cylindrospermopsin), neurotoxins (such as anatoxin-a, anatoxin-a(S), and saxitoxins), and irritant-dermal toxins (Sivonen and Jones 1999; Carmichael 2001). Cyanobacterial toxins have, however, also been classified based on their chemical structures: cyclic peptides (MCs and nodularins), alkaloids (anatoxin-a, anatoxin-a(s), cylindrospermopsins, and saxitoxins), lipopolysaccharides, and polyunsaturated fatty acids (PUFAs) and their derivatives (2,4-heptadienal and 2,4-octadienal) (Leflaive and Ten-Hage 2007). Of all the toxins mentioned, cyclic hepatotoxic peptides such as MCs and nodularin are the most commonly found, and their toxicity to vertebrates, aquatic organisms, and higher plants etc. has been extensively reviewed (Wiegand and Pflugmacher 2005; van Apeldoorn et al. 2007).

1.3.   Cyanobacteria as an alternative fuel source

Due to the rapid depletion of fossil fuels and their associated problems such as environmental pollution, global warming, acid rain, and other multiplicative effects, more research is being focused on the development of clean and sustainable energy resources. Harvesting of solar energy to produce biological H_2 is considered to be one of the best approaches to developing clean and renewable energy resources. Photosynthetic microalgae convert light (solar) energy into chemical energy through photosynthesis. Under certain conditions, these photosynthetic microorganisms redirect the electrons originating from solar energy to the enzymes that mediate H_2 production. Cyanobacteria possess three enzymes that are directly involved in H_2 production such as nitrogenase, uptake hydrogenase, and bidirectional hydrogenase.

1.3.1 Nitrogenases

Nitrogenase catalyzes biological N_2 fixation, where the formation of H_2 accompanies ammonia production (Burris 1991) according to the following equation:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + 16ADP + 16Pi + \mathbf{H_2}$$
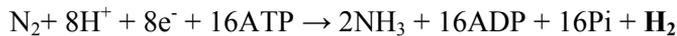
This reaction is highly endergonic, requiring metabolic energy in the form of ATP. The nitrogenase complex consists of two proteins: dinitrogenase (Mo-Fe protein or protein I) and dinitrogenase reductase (Fe protein or protein II). Dinitrogenase is a $\alpha_2\beta_2$ heterotetramer of about 240-kDa and encoded by the *nifD* and *nifK* genes (Bothe et al. 2010). Dinitrogenase reductase, encoded by *nifH*, is a homodimer of about 64-kDa and mediates electron transfer from either a ferredoxin or a flavodoxin to the dinitrogenase (Bothe et al. 2010). Nitrogenases are sensitive to O_2. Cyanobacteria have developed various mechanisms, such as temporal or spatial separation of N_2 fixation and O_2 evolution, to protect their N_2-fixing machinery not only from atmospheric O_2 but also from the intracellularly generated O_2 (Fay 1992; Wolk 1996; Mulholland and Capone 2000). Several filamentous cyanobacteria possess specialized cells called heterocysts for the spatial separation process (Wolk 1996; Thiel and Pratte 2001). Filamentous non-heterocystous (e.g. *Oscillatoria* and *Trichodesmium*) (Stal and Krumbein 1987) and non-filamentous cyanobacteria (*Cyanothece*) utilize a temporal separation mechanism, with photosynthesis performed in the light and N_2 fixation in the dark (Bergman et al. 1997; Misra and Tuli 2000). The heterocyst provides a microaerobic environment due to lack of active PSII, high respiration rate, and a thick envelope limiting the diffusion of O_2 through the cell wall (Fay 1992; Wolk et al. 1994). The heterocyst undergoes differention during the expression of nitrogenase genes, and the process has been well studied in *Anabaena variabilis* (hereafter referred to as *A. variabilis*) (Brusca et al. 1989) and *Anabaena* PCC 7120 (Carrasco et al. 2005).

In addition to the conventional nitrogenase (referred as *nifHDK1*), heterocystous cyanobacteria have been found to contain different alternative nitrogenases:

1. *Second molybdenum (Mo)-containing nitrogenase*: The conventional Mo-Fe nitrogenase occurs only in the heterocysts. The alternative Mo-containing enzyme is encoded by a separate *nifHDK* (referred as *nifHDK2*) genes and functions in vegetative cells. This enzyme has been described in *A. variabilis*, but strictly under N_2-fixing and anaerobic conditions (Thiel et al. 1995, 1997).

2. *Vanadium (V)-containing nitrogenase*: This enzyme is encoded by the *vnfDGK* cluster and expressed under conditions of Mo depletion, in the presence of V. This enzyme has been studied extensively in *A. variabilis* (Kentemich et al. 1991; Thiel 1993) and catalyzes the following reaction:

$$N_2 + 12H^+ + 12e^- + 24ATP \rightarrow 2NH_3 + 24ADP + 24Pi + \mathbf{3H_2}$$
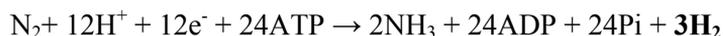
Importantly, the rate of H_2 evolution and acetylene reduction is higher when cyanobacteria are grown in the presence of V as compared to Mo-containing medium (Kentemich et al. 1988)

3.   *Iron (Fe)-containing nitrogenase*: This enzyme does not contain Mo nor V at the active site, and it is encoded by an *anfHDGK* cluster, which was cloned and sequenced from *Clostridium pausterianum* (Zinoni et al. 1993), *Rhodobacter capsulatus* (Schüddekopf et al. 1993), and *Azotobacter vinelandii* (Joerger et al. 1989). Although physiological evidence for the occurrence of this enzyme has been reported in *A. variabilis* (Kentemich et al. 1991), the complete genome sequence did not reveal the genes coding for Fe nitrogenase (Pratte et al. 2006). The overall reaction catalyzed by this enzyme is as follows:

$$N_2 + 21H^+ + 21e^- + 42ATP \rightarrow 2NH_3 + 42ADP + 42Pi + \mathbf{7.5H_2}$$
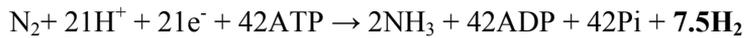
1.3.2. Uptake hydrogenases

The uptake hydrogenase catalyzes consumption of H_2 produced by nitrogenase, and has been found in almost all the N_2-fixing cyanobacteria analyzed so far (Lambert and Smith 1981; Houchins 1984; Tamagnini et al. 2000). Exceptionally, unicellular non-N_2-fixing *Anacystis nidulans* (*Synechococcus* strain PCC 6301) may also possess an uptake hydrogenase enzyme (Boison et al. 1996), but N_2-fixing *Synechococcus* does not (Ludwig et al. 2006). The uptake hydrogenase has various functions such as (1) O_2 removal from the heterocysts via the respiratory oxyhydrogen (Knallgas) reaction, (2) regaining of ATP used in H_2 production during nitrogenase reaction, and (3) supply of electrons to N_2 fixation and other metabolic processes of the cell (Bothe et al. 1977; Howarth and Codd 1985; Weisshaar and Böger 1985). Uptake hydrogenase is a nickel (Ni)-containing enzyme; it is encoded by the *hupSL* operon, where *hup*S codes for the small subunit of 35-kDa and *hupL* codes for the large subunit of 60-kDa (Carrasco et al. 1995; Happe et al. 2000; Lindberg et al. 2000). The localization of uptake hydrogenase still remains controversial. An immunological study revealed that it may be associated with the thylakoid membrane in N_2-fixing non-heterocystous *Lyngbya majuscula* CCAP 1446/4 and present in both heterocysts and vegetative cells in *Nostoc punctiforme* (hereafter referred to as *N. punctiforme*) (Seabra et al. 2009). Recently, Camsund et al. (2011) reported expression of the *hupSL* operon exclusively in the heterocysts of *N. punctiforme*. In most of the heterocystous cyanobacteria, the *hupL* gene undergoes genetic rearrangement during the late stage of heterocyst differentiation, whereby excision of a 9.5-kb element is catalyzed by the recombinase Xis C (Carrasco et al. 2005). The expression of the *hupSL* operon is regulated by the N_2 transcriptional regulator (NtcA) (Weyman et al. 2008), the availability of Ni (Axelsson and Lindblad 2002), and O_2 (Kovacs et al. 2005). In some microorganisms, a third *hup* gene, *hupC*, has been identified and found to be located downstream of the *hupSL* operon (Van Soom et al. 1993; Vignais and Toussaint 1994).

1.3.3. Bidirectional hydrogenases

Bidirectional or reversible hydrogenase catalyzes both H_2 uptake and H_2 evolution (Lambert and Smith 1981). This NAD(P)H-dependent enzyme (Schmitz et al. 1995) exists both in N_2-fixing and non-N_2 fixing cyanobacteria (Kentemich et al. 1989, 1991; Serebryakova et al. 2000; Hansel and Lindblad 1998; Tamagnini et al. 2002) and it

functions as a safety valve during the dark-light transition (Appel et al. 2000). The localization of the enzyme is still under debate. It possesses a pentameric structure and is encoded by the *hoxEFUYH* operon. The *hoxYH* genes code for the hydrogenase part, which contains the motif for binding to both Ni-Fe-S and Fe-S centres, while the *hoxFU* genes code for the diaphorase part that transfers the electrons to $NAD(P)^+$ and has binding sites for $NAD(P)^+$, flavin mononucleotide (FMN), and Fe-S centres (Schmitz et al. 1995; Boison et al. 1996, 1998; Appel and Schulz 1998). The *hoxE* gene codes for a subunit that co-purifies with the active bidirectional enzyme (Schmitz et al. 2002). Recently, the whole enzyme was purified from *Synechocystis* PCC 6803 under aerobic conditions, as a functional heteropentameric protein (Germer et al. 2009). In *Synechocystis* sp. PCC 6803, the *hoxEFUYH* genes are co-transcribed with the transcription start point located 168 bp upstream of the start codon (Gutekunst et al. 2005; Oliveira and Lindblad 2005). In recent years, several significant transcription factors such as LexA-related protein (Gutekunst et al. 2005; Oliveira and Lindblad 2005) and two members of the AbrB-like family (Oliveira and Lindblad 2008) were identified and found to be activators of bidirectional hydrogenase.

1.3.4. *Mutational approaches with hydrogenase and nitrogenase enzymes to improve cyanobacterial H_2 production*

The function of uptake hydrogenase during N_2 fixation and H_2 consumption activity of bidirectional hydrogenase are the major challenges when utilizing the cyanobacterial H_2 production system. In recent years, there have been several attempts by various laboratories to overcome these barriers, mainly using mutational approach. Various targeted mutants have been created with reduced or no uptake hydrogenase activity. Uptake hydrogenase-deficient mutants of *A. variabilis* (Happe et al. 2000), *N. punctiforme* (Lindberg et al. 2002), *Nostoc* sp. PCC 7120 (Lindblad et al. 2002; Masukawa et al. 2002a; Carrasco et al. 2005), and *Nostoc* sp. PCC 7422 (Yoshino et al. 2007) were generated and shown to produce H_2 at a higher rate than wild type (WT) strains. Except for *N. punctiforme*, other strains possess both bidirectional hydrogenase and H_2 uptake enzymes. Masukawa and co-workers produced a *hox*-defective mutant (Δ*hoxH*) and a mutant deficient in both hydrogenases (Δ*hupL*/Δ*hoxH*), and showed that the double mutant produced H_2 at a rate similar to other uptake hydrogenase-deficient mutants whereas the bidirectional hydrogenase-deficient mutant (Δ*hoxH*) produced less H_2 than the WT (Masukawa et al. 2002b). Very recently, an alternative nitrogenase (*nifD2*) was generated using chemical mutagenesis and by replacing valine with isoleucine at the NifD2 α-75 site. This resulted in four-fold higher H_2 production in a N_2 atmosphere than in the WT, and as much H_2 production as in an argon atmosphere (Weyman et al. 2010).

1.4. Effects of cyanobacterial secondary metabolites on photosynthetic organisms

The secondary metabolites produced by cyanobacteria have been shown to target bioenergetic pathways such as photosynthesis and respiration, leading to modification of the electron transport chain and generation of harmful ROS.

1.4.1. Photosynthesis

The photosynthetic reactions are divided into two steps: light reactions and fixation of CO_2. Light-driven photosynthetic electron transport converts solar energy into chemical energy of reducing equivalents in the form of NAD(P)H and energy equivalents in the form of ATP, using water as an electron donor. Next, the Calvin-Benson cycle enzymes use reducing and energy equivalents produced in the light reactions to assimilate CO_2 into triose phosphates (Bräutigam and Weber 2011). The schemes of photosynthetic electron transport reactions in cyanobacteria and higher plants are presented in Fig. 1. The light reactions of photosynthesis occur at the special internal membrane system called thylakoids, both in cyanobacteria and chloroplasts. Photosynthesis involves a flow of electrons through three major multisubunit protein complexes, namely photosystem (PS) II, cytochrome (cyt) b_6f, and PSI (Nelson and Ben-Shem 2004), all of which are embedded in the thylakoid membrane. The light reactions begin with absorption of light by the antenna pigments. The excitation energy is transferred to PSII and PSI reaction centres, where charge separation occurs and photosynthetic electron transport is initiated. The electrons extracted from water splitting in PSII are transferred via the PSII reaction centre, plastoquinone (PQ), cyt b_6f complex, and plastocyanin (PC) to PSI. Concomitantly, during this process a proton gradient (ΔpH) is formed, which is further used for ATP synthesis by ATP synthase. At the same time, electron transfer also results in the generation of NAD(P)H, the process termed linear electron transport (LET). Sometimes the electrons are recycled from NAD(P)H or ferredoxin (Fd) to PQ in a process called cyclic electron transport (CET), whereby ΔpH can be generated without any production of NAD(P)H.
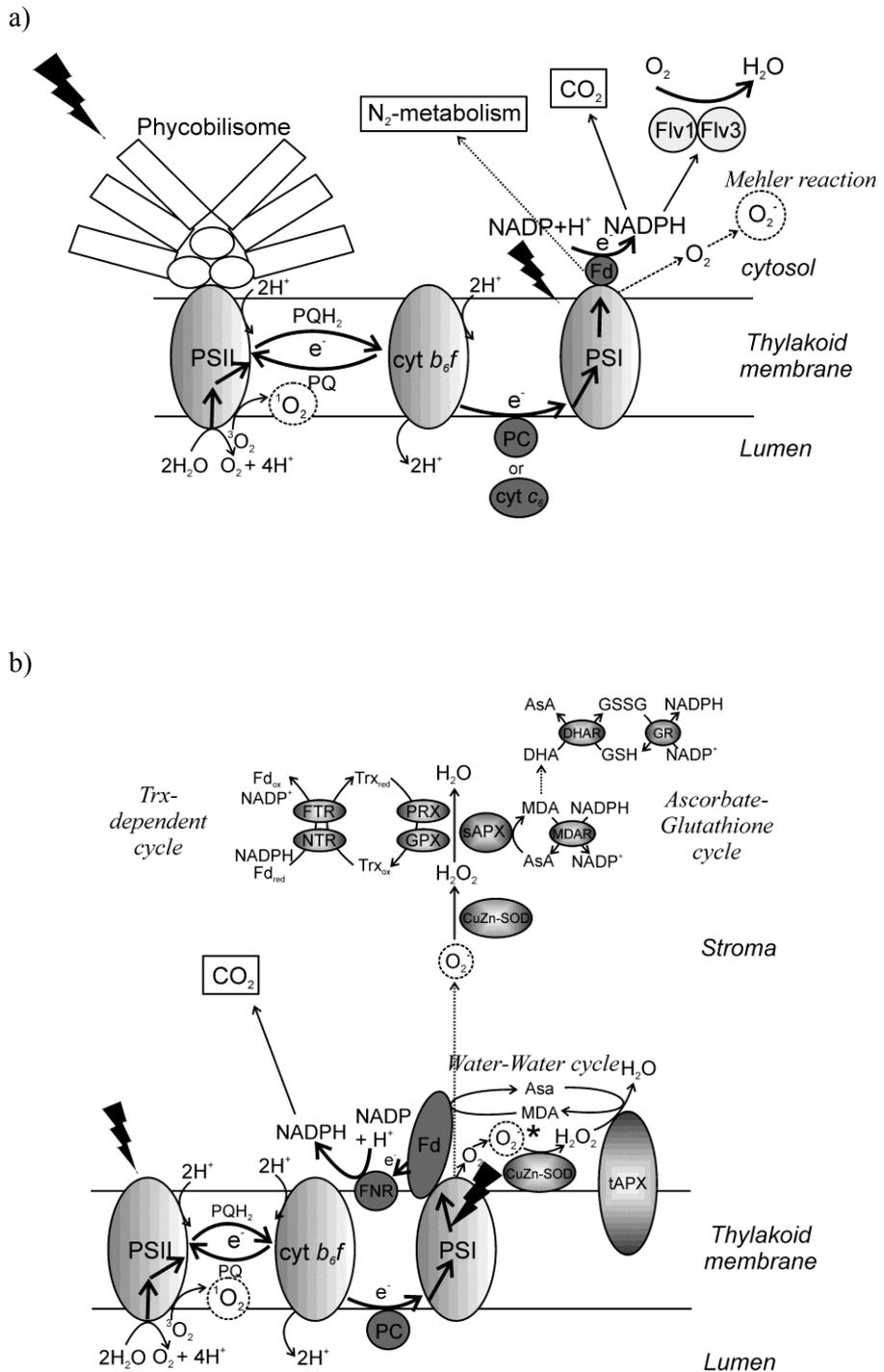
Fig. 1. Cyanobacterial (a) and plant (b) photosynthetic electron transport chain showing the linear electron transport (marked with heavy arrows). The asterisk denotes the superoxide radical produced by the Mehler reaction. The pathways for formation of ROS (marked with a dashed circle) and scavenging of ROS are also shown. *See text for details and abbreviations.*

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1.4.2. Photosynthesis-related ROS metabolism

Aerobic organisms such as cyanobacteria, green algae, and higher plants use O_2 as a terminal electron acceptor, capable of giving rise to harmful reactive free radicals and derivatives called ROS. An imbalance between the excessive formation of ROS and limited antioxidant defences may result in a condition called oxidative stress (Halliwal and Gutteridge 1999). Higher plants generate ROS, including singlet oxygen ($^1O_2$), superoxide ions (O_2^-), and hydrogen peroxide (H_2O_2) in chloroplasts and peroxisomes (Apel and Hirt 2004) (Fig. 1b). In both cyanobacteria and plants, $^1O_2$ is generated at PSII by interaction between the triplet state of P680 ($^3P680^*$) and O_2 (Telfer et al. 1994) (Fig. 1). O_2^- is produced at the acceptor side of PSI (Mehler 1951) and subsequently dismutated to H_2O_2 by superoxide dismutase (SOD) in higher plants (Asada et al. 1974). In the cyanobacteria, the heterodimer of A-type flavodiiron proteins, Flv1/Flv3, mediates the photo-reduction of O_2 directly to water without generating ROS downstream of PSI (Helman et al. 2003; Allahverdiyeva et al. 2011) (Fig. 1a). However, under certain conditions, the plant-like Mehler reaction may also occur in cyanobacteria (Latifi et al. 2009).

ROS-scavenging mechanisms include non-enzymatic and enzymatic systems. The enzymatic system has been characterized best in higher plants. These include the water-water cycle, ascorbate (AsA)-glutathione (GSH) cycle, and thioredoxin (Trx)-dependent pathway (Asada 1999). The major non-enzymatic ROS-scavenging systems in cyanobacterial thylakoid membranes and plant chloroplasts include ascorbate (AsA), glutathione (GSH), β-carotene, and α-tocopherol. The $^1O_2$ generated at PSII is efficiently quenched by xanthophylls (Dall'Osto et al. 2007), β-carotene in both cyanobacteria and higher plants (Telfer 2005; Trebst 2003), and α-tocopherol (Krieger-Liszkay and Trebst 2006).

In higher plants, photo-reduction of O_2 at the acceptor side of PSI leads to the formation of O_2^-, which is called the Mehler reaction (Mehler 1951). Disproportionation of O_2^- to H_2O_2 is catalyzed by thylakoid-bound SOD, and the final disproportionation of H_2O_2 catalyzed by thylakoid-bound AsA-dependent ascorbate peroxidase (tAPX) leads to the formation of O_2 and water. This process is called the water-water cycle (Asada 2000). The O_2^- that escapes this cycle and diffuses into the stroma is scavenged by SOD and the AsA-GSH cycle, peroxiredoxin (PRX), and glutathione peroxidase (GPX) localized in the stroma. The AsA-GSH cycle generally utilizes AsA as a specific electron donor to reduce H_2O_2 to water, with the concomitant generation of monodehydroascorbate (MDA), a univalent oxidant of AsA. This reaction is catalyzed by APX. MDA is spontaneously converted to AsA and dehydroascorbate (DHA), and may be rapidly reduced to AsA by the action of an NAD(P)H-dependent MDA reductase. DHA reductase (DHAR) utilizes GSH to reduce DHA, and thereby regenerates AsA. GSH is regenerated from oxidized glutathione, also called glutathione disulphide (GSSG), by the action of glutathione reductase (GR) using NAD(P)H as a reducing power (Fig. 1b). Another important antioxidant system in the chloroplast is PRX and GPX, which function in cooperation with the Trx-dependent cycle. PRX and GPX utilize reduced Trx to reduce H_2O_2, and oxidized Trx

is regenerated by the action of either Ferredoxin-Trx reductase (FTR) or NAD(P)H-dependent Trx reductase (NTR). GPXs can use both GSH and Trx as reducing substrates, to reduce H_2O_2 (Herbette et al. 2002) (Fig. 1b).

1.4.3. Respiration

In both cyanobacteria and higher plants, the process of cellular respiration is essentially the reverse of photosynthesis. Respiration is the process in which the oxidation of carbohydrates (CH_2O) leads to the formation of CO_2 and water. This process releases a large amount of free energy, much of which is coupled to the conversion of ADP and Pi to ATP (Taiz and Zeiger 1991). Cyanobacterial respiration occurs in both plasma membrane and thylakoid membrane, the latter one sharing the components of oxygenic photosynthesis. Cyanobacteria possess complexes specific for respiration, e.g. bacterial-type complex I (NAD(P)H dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome c reductase), and the terminal oxidase (cytochrome oxidase), whereas PQ, cyt b_6f, and soluble electron carriers like PC and cyt c_6 are shared by both the respiratory and photosynthetic electron transport pathways (Cooley and Vermaas 2001; Peschek et al. 2004). In higher plants, respiration occurs in a special compartment at the inner membrane of mitochondria (Fig. 2).
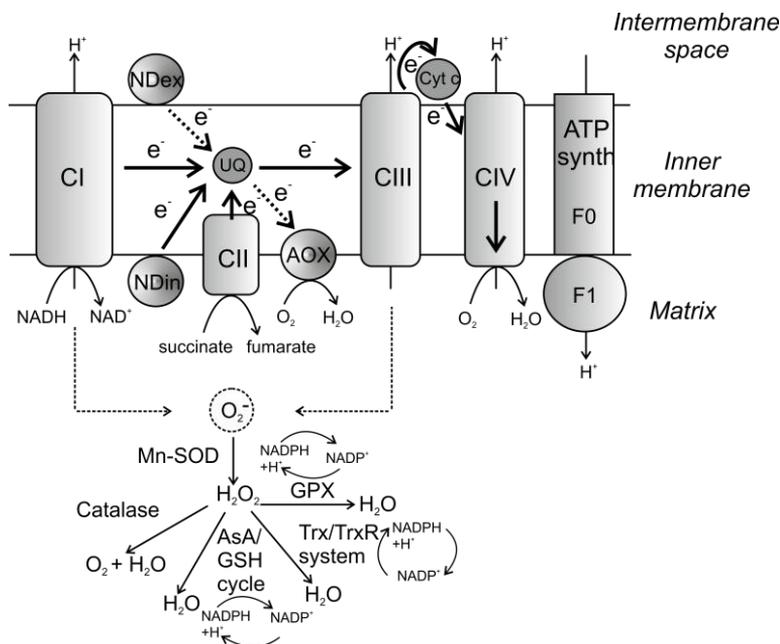
Fig. 2. Scheme of the respiratory oxidative pathway of higher plants showing the route of normal electron transport (marked with heavy arrows) and the alternative electron transport route (marked with dotted arrows). The pathways for formation of ROS (marked with a dashed circle) and removal of ROS in mitochondria are also shown. *See text for details and abbreviations.*

Plant mitochondrial respiration consists of four integral multiprotein complexes (complexes I to IV) (Nicholls and Ferguson 1992; Trumpower and Gennis 1994) (Fig. 2). Complex I (NADH ubiquinone oxidoreductase) is an NADH dehydrogenase, which oxidizes the NADH generated in the mitochondrial matrix by the tricarboxylic acid (TCA) cycle and transfers the electrons to ubiquinone. Complex II (succinate:ubiquinone oxidoreductase), the only TCA cycle enzyme (succinate dehydrogenase) present in the membrane, catalyzes the oxidation of succinate to fumarate in the TCA cycle, transferring the electrons to ubiquinone. Complex III (ubiquinone:cytochrome c oxidoreductase or bc1 complex) oxidizes the ubiquinone reduced by complexes I and II and transfers the electrons to cytochrome c. Reduced cytochrome c is oxidized by complex IV (cytochrome c oxidase), the terminal electron transfer complex in the series. At complexes I, III, and IV, protons are translocated across the inner membrane to generate the proton motive force that drives ATP synthesis. Although, the F_0F_1-ATP synthase is not part of the electron transfer chain because of its role in oxidative phosphorylation, it is referred as complex V (Hatefi 1985). Plant mitochondria differ from other eukaryotic mitochondria by the presence of four NAD(P)H dehydrogenases, two internal (facing the intermembrane space) (ND_{in}) and two external (facing the matrix) (ND_{ex}) in addition to complex I (Møller and Lin 1986; Douce and Neuburger 1989). It appears that the NAD(P)H dehydrogenase facing the matrix competes with complex I for oxidation of the NADH generated during the TCA cycle, and probably acts as an NAD(P)H dehydrogenase *in situ* (Rasmusson and Møller 1990, 1991). Moreover, plant mitochondria also contain an additional terminal oxidase, the AOX, which is insensitive to inhibitors of the bc_1 complex and cytochrome c oxidase such as myxothiazol, antimycin, and cyanide (Moore et al. 2002).

1.4.4. Production of ROS in plant mitochondria

As already mentioned, in green tissues, higher plants generate ROS in chloroplasts and peroxisomes (Apel and Hirt 2004) (Fig. 1b), whereas in the non-green tissues and in the dark, ROS are generated in the mitochondria (Navrot et al. 2007) (Fig. 2). In mitochondria, the major sites of ROS production are complex I (Chance et al. 1979; Turrens and Boveris 1980) and the ubisemiquinone in complex III (Turrens et al. 1985); this generates O_2^-, which is futher disproportionated to H_2O_2 by Mn-SOD (Fig. 2). The H_2O_2 is finally disproportioned to water and O_2 by the enzymatic system (water-water cycle, AsA-GSH cycle, and Trx-dependent pathway) and the non-enzymatic system (AsA and GSH) by a mechanism similar to the ROS-scavenging system in the chloroplast. The plant-specific AOX is also involved in controlling the production of ROS, mostly O_2^- and H_2O_2, by diverting the electron flow from the cytochrome oxidase pathway (Moore et al. 2002; Juszczuk and Rychter 2003; McDonald 2008; Noguchi and Yoshida 2008; Rasmusson et al. 2009). Recently, ROS have been shown to be signaling molecules involved in the activation of the stress response and defence pathways (Desikan et al. 2001; Knight and Knight 2001). Thus, the steady-state level of ROS used for the signal transduction has to be tightly controlled because increases in ROS, often as a result of environmental changes, may result in cell death through peroxidation of membrane lipids, protein oxidation, DNA

damage, etc. (Apel and Hirt 2004). This delicate balance is determined by the cooperation between the ROS-producing and ROS-scavenging mechanisms.

1.4.5. *Effect of cyanobacterial secondary metabolites on photosynthesis*

Several cyanobacterial secondary metabolites, including toxins, are known to interact with other prokaryotic cyanobacteria or eukaryotic algae, collectively called microalgae, eventually affecting their growth and metabolism (Leflaive and Ten-Hage 2007). These compounds are generally known as algicides or antialgal compounds. Most of the algicides that have been characterized are found to target photosynthesis, and are thus termed natural herbicides (Smith and Doan 1999). As photosynthesis is shared by both cyanobacteria and algae, it can be a reasonable target for the antialgal producer organism to compete out other such organisms in the same habitat (Smith and Doan 1999).

Cyanobacterin, a compound produced by *Scytonema hofmanni* (Mason et al. 1982), possess the specific site of action near PSII, on the oxidizing side of the Q_B site (Gleason and Paulson 1984). It has been reported that this site of action is different from the 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) - binding site (Gleason et al. 1986). Other compounds that have been reported to inhibit PSII are fischerellin A (Flores and Wolk 1986; Gross et al. 1991; Srivastava et al. 1998) and fischerellin B (Papke et al. 1997), both of which are produced by *Fischerella muscicola*, and cyanobacterin LU-1 and LU-2 (Gromov et al. 1991; Vepritskii et al. 1991), which are produced by *Nostoc linckia*.
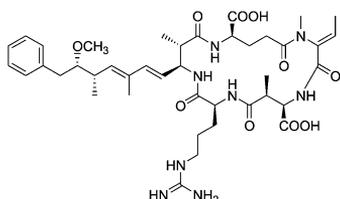
a)   b)
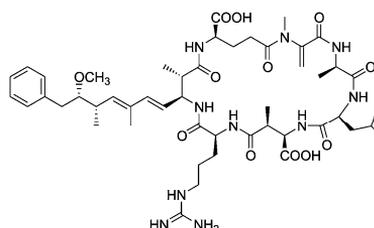
Fig. 3. The structure of nodularin (a) and microcystin (b) (Kurmayer and Christiansen 2009).

The action of toxic secondary metabolites has also been studied extensively in higher plants, either in terrestrial or aquatic plants depending on the habitat of the toxin producer. Terrestrial plants growing on the shore may be exposed to cyanotoxins during the formation of blooms, and crop plants can be exposed to toxins or toxin producers when the surface water used for irrigation is contaminated with toxic cyanobacteria (Abe et al. 1996; Codd et al. 1997). Nodularin inhibits serine-threonine type 1 (PP1) and 2A (PP2A) protein phosphatases in animals and plants (Honkanen et al. 1991; MacKintosh et al. 1990; Matsushima et al. 1990). Most research has focused on the effects of the well-studied cyanotoxin MC (Fig. 3) variants on terrestrial and aquatic plants.

Toxins such as MCs inhibit germination, growth, and photosynthesis of various plants, e.g. *Spinacia oleracea* (Siegl et al. 1990; Pflugmacher et al. 2007a), *Sinapsis alba* (Kós et al. 1995), *Oryza sativa*, *Brassica napus* (Chen et al. 2004), *Zea mays*, and *Pisum sativum* (Saqrane et al. 2008, 2009). Cylindrospermopsin inhibits protein synthesis and hinders germination of *Nicotiana tabacum* pollen (Metcalf et al. 2004), while anatoxin-a inhibits germination and root growth of *Medicago sativa* seedlings (Pflugmacher et al. 2006). At the same time, association of cyanotoxins with aquatic plants grown in the same locality has also been studied (Babica et al. 2006). Exposure of aquatic macrophytes such as *Ceratophyllum demersum*, *Myriophyllum spicatum*, *Lemna minor*, and *Wolffia arrhiza* (Pflugmacher et al. 1999; Weiss et al. 2000; Pflugmacher 2002; Mitrovic et al. 2005) to MC variants resulted in uptake of MC and subsequent growth inhibition, reduction in photosynthetic oxygen production, and changes in pigment patterns.

1.4.6. Effect of cyanotoxins on respiration

Unlike photosynthesis, there have been very few studies on the mechanism(s) of action of cyanotoxins on the respiratory electron transport pathway. In particular, there have been no studies on the effect of nodularin on the plant respiration. Recently, it has been shown that MC-LR acts as an uncoupler, affecting the function of complex I and enhancing mitochondrial O_2 consumption in isolated rat liver mitochondria (Jasionek et al. 2010). Zhao and co-workers reported that exposure of crucian carp liver to MCs significantly impaired the activities of mitochondrial complexes I, II, III, IV, and V and the expression of genes coding for complexes IV and V (Zhao et al. 2011). Furthermore, MC-LR strongly reduced the transmembrane potential as a consequence of inhibition of redox complexes in isolated mitochondria of kidney cells (La-Salete et al. 2008). MCs have been shown to induce apoptosis in mitochondria and subsequent cell death through loss of the mitochondrial membrane potential (MMP) in primary cultured rat hepatocytes (Ding et al. 1998, 2000), in rabbit liver and heart (Zhao et al. 2008), in mice (Weng et al. 2007) and also in suspension cells of tobacco BY-2 (Huang et al. 2008). Exposure to MCs also resulted in morphological and ultrastructural changes such as necrosis, swelling of mitochondria, and loss of cristae in tobacco BY-2 cells (Huang et al. 2009).

1.4.7. Induction of ROS-scavenging systems by cyanotoxins

It has been shown that cyanotoxins induce oxidative stress in terrestrial and aquatic plants as well as in cyanobacteria, which might cause serious cellular damage such as inhibition of protein synthesis, lipid peroxidation (LPO), genotoxicity, and modulation of apoptosis (Ding and Ong 2003; Esterbauer et al. 1991; Pflugmacher 2004). The uptake of MC-LR by an aquatic plant, *Ceratophyllum demersum*, increased the activity of microsomal and soluble glutathione S-transferase (GST) above a threshold concentration, whereas the activity of the same enzymes was inhibited at lower concentrations of MC (Pflugmacher et al. 1999). Similarly, the exposure of yet another aquatic plant, *Lemna minor*, to MC-LR increased the peroxidase (POD) activity after 5 days of exposure (Mitrovic et al. 2005). MC-LR decreased the activity of SOD and

induced POD activity in *Brassica napus* whereas in *Oryza sativa*, it increased the activity of SOD, leaving the POD activity unaffected (Chen et al. 2004). MC-LR induced an oxidative stress response in *Lepidium sativum* through elevated activities of GPX, GST, and GR (Stüven and Pflugmacher 2007). Mitrovic and co-workers reported that anatoxin-a increased the activities of POD and GST after four days of exposure in the free-floating aquatic plant *Lemna minor* (Mitrovic et al. 2004). Exposure of *Scenedesmus armatus* to extract from a natural cyanobacterial bloom, composed of *Microcystis* and *Aphanizomenon*, elevated the POD activity and inhibited the activity of soluble GST (Pietsch et al. 2001). Moreover, MC-RR has been shown to increase ROS production by altering the levels of glutathione and GST activity in *Synechococcus elongatus* (Hu et al. 2005), and of many more antioxidant enzymes such as SOD, GPX, etc. in *Synechocystis* PCC 6803 (Li et al. 2009).

1.4.8. N. spumigena, *a toxin producer in the Baltic Sea*

N. spumigena was the first toxic cyanobacterium to be reported to cause animal poisoning in an Australian lake in 1878 (Francis 1878). It is a filamentous, N_2-fixing heterocystous cyanobacterium occuring mainly in brackish and saline water around the world (Sivonen et al. 1989; Harding et al. 1995; Bolch et al. 1999). *Nodularia* are divided into benthic form lacking gas vacuoles and planktic community possessing gas vacuoles. Benthic cyanobacteria consist of non-toxic strains only whereas planktonic forms include both nodularin-producing *N. spumigena* and non-toxic strains (Laamanen et al. 2001). *Nodularia* blooms have been reported in coastal areas of Baltic Sea (Lindstrøm 1976; Persson et al. 1984; Edler et al. 1985; Gussmann et al. 1985), where they appear regularly during summer (Sivonen et al. 1989; Kononen et al. 1993). A hepatotoxin produced by *N. spumigena*, known as nodularin, was first isolated from New Zealand bloom material (Carmichael et al. 1988). Unlike MC, which is produced in various cyanobacterial species, nodularin has been shown to be produced by *N. spumigena* alone (van Apeldoorn et al. 2007). Nodularin is a non-ribosomal, cyclic pentapeptide (MW 824) with a structure cyclo-(D-Masp-L-Arginine-Adda-D-Glutamic acid-Mdhb), where Masp denotes D-*erythro*-β-methylaspartic acid, an unusual amino acid, Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdhb is N-methyl-dehydrobutyrine (Rinehart et al. 1988) (Fig. 3). In contrast to the MCs with more than sixty-five variants, only seven structural variants of nodularins have been reported to date (Namikoshi et al. 1994; Sivonen and Jones 1999; Beattie et al. 2000; de Silva et al. 1992).

The function and action mechanism of nodularin has been studied extensively in mammalian systems (van Apeldoorn et al. 2007). It inhibits two key regulatory enzymes, PP1 and PP2A (Honkanen et al. 1991), in a manner similar to the diarrhoetic shellfish toxin, okadaic acid (Gehringer 2004). Nodularin binds to phosphatases by strong, non-covalent interaction, in contrast to MCs, which bind covalently to the target (Yoshizawa et al. 1990; Ohta et al. 1994; Annila et al. 1996). Phosphatases catalyze the reversibility of phosphorylation, which regulates various signaling processes from light responses in plants to muscle contraction in animals (Cohen 1989; MacKintosh and MacKintosh 1994). Nodularin has also been introduced as an

apoptogen (Fladmark et al. 1998) and as a direct carcinogen because of its ability to inhibit these phosphatases (Ohta et al. 1994). Nodularin-producing *N.spumigena* water blooms have also caused several episodes of animal poisoning in the Baltic Sea area (Edler et al. 1985; Nehring 1993).

The impact of transport of nodularin through the food web has been of great interest in the last decade. The bioaccumulation of nodularin and subsequent induction of oxidative stress have also been reported in important food sources for humans or aquatic species such as mussels (Sipiä et al. 2002; Davies et al. 2005), flounder (Sipiä et al. 2001, 2002; Persson et al. 2009), Atlantic cod (Sipiä et al. 2001), three-spined stickleback (Kankaanpää et al. 2001), prawns (Van Buynder et al. 2001), mysid shrimps (Engström-Öst et al. 2002), and clams (Lehtonen et al. 2003; Sipiä et al. 2002). Few studies have been done on the accumulation of nodularin and induction of oxidative stress in algae (Pflugmacher et al. 2007b, 2010).

2. AIMS OF THE STUDY

I am interested in using cyanobacteria for the biotechnological production of H_2, a potential source of energy in the future. For H_2 production, the biodiversity of cyanobacteria has not been well studied. The Baltic Sea and Finnish lakes comprise of morphologically and physiologically diverse set of cyanobacterial strains. More than 1,000 different strains have been isolated and are maintained in UHCC. This gave me an excellent opportunity to investigate the potential diversity in cyanobacteria for H_2 production. As a biologist, I have also paid attention to the growing cyanobacterial blooms in the Baltic Sea and Finnish lakes every summer and decided to study the effects of cyanobacterial secondary metabolites on the bioenergetic systems of other cyanobacteria and terrestrial plants.

The specific aims of my study are:

(1)   To explore superior H_2 producing cyanobacterial strains from natural diversity in the Baltic Sea and Finnish lakes and to further characterize the best H_2 producers at the molecular level.

(2)   To identify novel bioactive compounds and reveal their mechanism of action on the bioenergetic pathways of the model cyanobacterium, *Synechocystis* PCC 6803.

(3)   To study the bioenergetic responses of terrestrial plants to nodularin-producing *Nodularia* blooms.

3.   MATERIALS AND METHODS

3.1.   Cyanobacterial and plant material

3.1.1. Cyanobacterial strains

In my PhD study, several cyanobacterial strains from PCC, ATCC, and UHCC were used. The strains and growth conditions of the strains are listed in the following table.

Strains	Growth conditions	Strain characteristics	Publications
400 strains from UHCC	7 µmol photons m^{-2} s^{-1}, Z8 or Z8x or Z8xS, 22ºC	Unicellular, non-N_2-fixing; filamentous, N_2-fixing, heterocystous; and filamentous, non-N_2-fixing, non-heterocystous	Paper I
Calothrix 336/3 *Calothrix* XPORK 5E *Nostoc* XHIID A6	7 µmol photons m^{-2} s^{-1}, Z8x, 22ºC or 20 µmol photons m^{-2} s^{-1}, Z8x, 25ºC	N_2-fixing and heterocystous	Papers I & II
Calothrix BECID 33 *Nodularia* TRO31 *Nodularia* AV33 *Nostoc* BECID 19 *Anabaena* XSPORK 7B *Calothrix* XSPORK 36C *Calothrix* XSPORK 11A	7 µmol photons m^{-2} s^{-1}, Z8xS, 22ºC or 20 µmol photons m^{-2} s^{-1}, Z8xS, 25ºC	N_2-fixing and heterocystous	Papers I & II
<u>*Anabaena* PCC 7120</u> WT, Δ*hup*L, Δ*hup*L/ Δ*hox*H and Δ*hox*H	40 µmol photons m^{-2} s^{-1}, BG-11_o, 25ºC	N_2-fixing and heterocystous	Papers I & II
<u>*N. punctiforme* PCC 73102 (ATCC 29133)</u> WT and Δ*hup*L	40 µmol photons m^{-2} s^{-1}, BG-11 or BG-11_o, 25ºC	N_2-fixing and heterocystous	Papers I & II
A. variabilis ATCC 29413	50 µmol photons m^{-2} s^{-1}, BG-11, 30ºC	N_2-fixing and heterocystous	Paper II
Nodularin-producing *N. spumigena* AV1 non-nodularin-producing *Nodularia* sp. HKVV	20 µmol photons m^{-2} s^{-1}, Z8xS, 25ºC	N_2-fixing and heterocystous	Papers IV & V
Synechocystis sp. PCC 6803	50 µmol photons m^{-2} s^{-1}, BG-11, 30ºC	unicellular, non-N_2-fixing	Papers II & III

Z8x: Z8 medium (Kotai 1972) without added nitrogen; Z8xS: Z8 saline medium without added nitrogen; and BG-11_o: BG-11 medium (Rippka et al. 1979) without added nitrogen

3.1.2. *Plant material*

Spinach (*Spinacia oleracea* L.) plants were grown under 250 µmol photons m^{-2} s^{-1}, 8 h photoperiod, and 23ºC. The seeds were sown on vermiculite, and the seedlings were transferred to soil after two weeks of germination (Papers IV & V).

3.2. Screening of UHCC for H_2 production (Paper I)

For preliminary screening, 400 cyanobacterial strains from UHCC were grown in Helsinki for two weeks in appropriate liquid Z8 medium (pH 7.5) at 22ºC under approximately 7 µmol photons m^{-2} s^{-1}. The cells were harvested and resuspended in 50 mL of fresh growth medium, transported to Turku, and H_2 production assay was carried out on the next day. The strains selected for further study were cultivated in their respective medium with continuous bubbling of air under 40 µmol photons m^{-2} s^{-1}.

3.2.1. *H_2 production assay and determination of H_2 by gas chromatography*

The cyanobacterial strains harvested and resuspended in 5 mL of fresh medium were transferred to 20 mL gas chromatography (GC) vials with butyl-rubber septa. The vials were incubated for 24 h at 23°C under four different conditions: aerobic/light (70 µmol photons m^{-2} s^{-1}), aerobic/dark, microaerobic/light (70 µmol photons m^{-2} s^{-1}), and anaerobic/dark. The gas phase of the vial was changed to argon for anaerobic conditions. The aerobic and anaerobic treatments were performed in parallel. The vials were kept under continuous shaking.

Samples (150 µL) withdrawn from the headspace of the H_2 assay vials using a gas-tight syringe (Hamilton Co., USA) were injected into a Perkin Elmer Clarus 500 GC equipped with a thermal conductivity detector and a Molecular Sieve 5A column (60/80 mesh). Calibration was done with 0.5% H_2 (AGA, Finland). The rate of H_2 production was calculated on the basis of the Chl content of the cells. Argon was used as a carrier gas.

3.3. Preparation of cyanobacterial extracts

3.3.1. *Nodularia cell extract (Papers IV & V)*

Nodularin-producing *N. spumigena* strain AV1 and non-nodularin-producing *Nodularia* sp. HKVV were harvested after four weeks of growth and washed once with tap water. The pellets were weighed and stored at -20ºC. The frozen pellets were repeatedly thawed and re-frozen several times to break up the cells in order to release the toxin. Just before use, the pellets were suspended in tap water.

3.3.2. **Nostoc** *XP14A extract and M22 (Paper III)*

Nostoc XPORK 14A cells were harvested after 80 days of growth, freeze dried, and extracted with methanol. Equal volumes of dichloromethane and water were added to

the extract, followed by manual shaking for 5 seconds to form an emulsion. The emulsion was centrifuged and the upper phase (water/methanol) was collected and evaporated. The final residue was dissolved in high-performance liquid chromatography (HPLC) eluent for the purification of M22 or in methanol to obtain crude methanol extract. To avoid the hazardous effect of methanol on *Synechocystis*, the methanol was replaced with dimethyl sulfoxide (DMSO) for further physiological experiments.

Next, the crude methanol extract was injected into an Agilent 1100 Series LC/MSD Trap System HPLC (Agilent Technologies, USA) with an XCT Plus model ion trap as a mass detector and diode array UV detector. The column used was Phenomenex Luna C18 (2) (4.6 x 150 mm, particle size 5 μm), which was eluted isocratically at 1 mL min^{-1} with 45% ACN in water at ambient temperature. The M22 peak, eluting from 10 to 11.75 min, was collected and evaporated to dryness. This part of the work was done at the University of Helsinki.

3.4. Treatments with cyanobacterial extracts

3.4.1. Treatment of Synechocystis *PCC 6803 with* Nostoc *XPORK 14A methanol extract (Paper III)*

The *Synechocystis* PCC 6803 (hereafter referred to as *Synechocystis*) cells at mid-logarithmic phase were diluted with fresh BG-11 medium to a final optical density of 0.1 or 0.4 at 750 nm (OD_{750}) for the treatments. For short-term treatment, the *Synechocystis* cells diluted to an OD_{750} of 0.4 were incubated with the *Nostoc* XPORK 14A methanol extract (hereafter referred to as XPORK 14A extract), or with purified M22 (hereafter referred to as M22) solution for two days under standard growth conditions with air-level CO_2 both in the light and in the dark. For long-term treatment, the *Synechocystis* cells diluted to an OD_{750} of 0.1 were grown in the presence of XPORK 14A extract for seven to eight days under standard growth conditions with air-level CO_2.

3.4.2. Treatment of spinach with Nodularia *cell extract (Papers IV & V)*

The spinach plants were divided into three groups and each group consisted of two pots, each containing four seedlings: (1) control plants, watered with tap water, (2) plants watered with tap water supplemented with *Nodularia* HKVV cell extract, and (3) plants watered with tap water supplemented with *Nodularia* AV1 cell extract containing 0.34 μM nodularin per plant. All three groups of plants were allowed to grow for five weeks. Watering with or without cell extracts were performed twice a week until the fifth week of growth or until generation of severe symptoms. Control plants were watered with tap water alone.

3.5.   Isolation of mitochondria and preparation of protein samples. Detection and analysis of proteins

The chloroplast-free mitochondria from spinach leaves were isolated as described in Paper IV. Total leaf extract, thylakoid membranes, and soluble proteins were isolated from spinach leaves after frozen in liquid N_2 as described in Papers IV & V. Thylakoid membrane proteins and crude cell extracts from *Synechocystis* cells were isolated as described in Paper III. The proteins were denatured and separated by one-dimensional SDS-PAGE. The western blotting was carried out by electroblotting the proteins onto polyvinylidene fluoride (PVDF) (Millipore, USA) or nitrocellulose membrane (Perkin Elmer, USA). The proteins of interest were detected by immunoblotting with appropriate antibodies. Oxidative modifications of proteins were studied using the OxyBlot[TM] Protein Oxidation Detection Kit (Chemicon International, USA).

3.6.   Determination of Chl and protein amount

The Chl content of the thylakoid membranes of spinach leaves was determined according to Porra et al. (1989) (Paper IV), and that of cyanobacterial cells was measured spectrophotometrically at 665 nm using 90% methanol (Meeks and Castenholz 1974) (Paper I & III). Protein content was measured as described by Bradford (1976) (Paper IV) and Lowry et al. (1951) (Paper III).

3.7.   Measurement of photosynthetic electron transport properties

The thylakoid membranes isolated from spinach leaf samples were used for the *in vitro* measurements (Paper IV). Leaf discs from spinach leaf samples (Paper IV) and *Synechocystis* cells harvested and resuspended in fresh BG-11 medium (Paper III) were used for the *in vivo* measurements. An appropriate volume of extracts was added to the culture in order to maintain the same growth environment for the cells as before the measurements (Paper III).

3.7.1. Chl a fluorescence

The single flash-induced increase in Chl a fluorescence yield and its subsequent relaxation (FF-relaxation) in darkness (Paper III) were determined with the FL3300 dual-modulation fluorometer (Photon System Instruments, Czech Republic) after 3 min of dark adaptation of the *Synechocystis* cells, either in the presence or absence of DCMU (Allahverdiyeva et al. 2004).

3.7.2. P700[+] oxido-reduction

The changes in the redox state of P700 in *Synechocystis* cells (Paper III) were measured by using DUAL-PAM-100 (Heinz Walz GmbH, Germany). P700 was oxidized in the presence of DCMU using red light applied from the actinic LED with intensity of 70 μmol photons m^{-2} s^{-1}. Subsequent re-reduction of P700[+] was recorded in

darkness. Redox changes in P700 in leaf discs from spinach plants exposed to nodularin-containing extract was monitored as described in Paper IV.

3.7.3. Oxygen evolution measurements

Steady-state rates of oxygen evolution were measured using a Hansatech DW1 oxygen electrode under saturating light intensity. The net photosynthesis and PSII capacity of the *Synechocystis* cells were determined in the presence of 10 mM $NaHCO_3$ and 2 mM 2,6-dichloro-p-benzoquinone (DCBQ), respectively, at 30°C (Paper III), whereas the PSII capacity of the thylakoid membranes isolated from spinach plants was measured in the presence of 1 mM DCBQ at 23°C (Paper IV).

3.8. Measurements of respiratory reactions

3.8.1. Dark respiration measurement (Paper III)

The respiratory activity of *Synechocystis* cells in the dark was measured with a Hansatech DW1 oxygen electrode at 30°C.

3.8.2. Spectroscopic assay of respiratory chain enzyme activities (Paper V)

The respiratory chain activities of isolated mitochondria were measured spectrophotometrically using an Aminco DW2 spectrophotometer (American Instrument Co., USA) as described earlier (Hinttala et al., 2005) with minor modifications (Vigani et al. 2009). Complex I activity was assayed by measuring rotenone-sensitive oxidation of NADH in the presence of decyl ubiquinone at 340/385 nm. Activities of complex II+III were determined by measuring the succinate-dependent reduction of cytochrome *c* at 550/540 nm. Activity of complex IV was assayed as an oxidation rate of reduced cytochrome *c* at 550/540 nm. This activity was monitored in the presence and absence of octylglucoside. The activity of citrate synthase was measured according to Tonkonogi and Sahlin (1997). The respiratory chain enzyme activities were then expressed relative to citrate synthase activity. These experiments were carried out at the University of Oulu, Finland.

3.9. Detection of ROS (Paper III)

Production of ROS was monitored as light-induced oxygen uptake by chemical-trapping technique in the presence of 5 mM histidine in BG-11 medium using a Hansatech oxygen electrode. This experiment was performed at the Laboratory of Molecular Stress and Photobiology, Szeged, Hungary.

3.10. Pigment and α-tocopherol analysis (Paper IV)

Pigments (Chl a and b, neoxanthin, violaxanthin, lutein, and β-carotene) and α-tocopherol were extracted from leaf discs of spinach with 100% methanol. The

photosynthetic pigments were separated by HPLC according to Gilmore and Yamamoto (1991) with a LiChroCART125-4 reverse-phase C18 column (Hewlett Packard, USA), and a series 1100 HPLC device with diode array and fluorescence detector (Agilent Technologies, USA).

3.11. APX activity and determination of ascorbate (Paper IV)

APX activity from fresh leaves was determined according to Foyer et al. (1989) using the modifications of Pätsikkä et al. (2002). Ascorbate was extracted from the leaves and was assayed according to Foyer et al. (1983).

3.12. DNA analysis (Paper II)

Genomic DNA was extracted according to Neilan et al. (1995) (Paper II). The quality of DNA was verified by agarose gel electrophoresis according to standard protocols (Sambrook et al. 1989). For Southern hybridization, 1-2 µg of genomic DNA was digested with *Hind*III and *EcoR*I. The restriction fragments were separated on 0.7% agarose gel. After denaturation and neutralization, restricted DNA was blotted onto Hybond-N nylon membranes (Amersham Bioscience, UK). The membranes were pre-hybridized, exposed to denatured probes (*hup*, *hox*, and *nif*) and hybridized overnight at 45ºC. After hybridization, the membranes were washed with 2x SSC, 0.1% SDS.

4.   RESULTS

4.1.   Identification and characterization of superior H_2 producers from the Baltic Sea and Finnish lakes (Papers I & II)

4.1.1   Screening of UHCC (Paper I)

In order to identify potential H_2 producers from UHCC, a preliminary screening process was carried out with 400 cyanobacterial species using four different conditions: aerobic/light, aerobic/dark, microaerobic/light, and anaerobic/dark. Two hundred strains produced detectable amounts of H_2. The list of 100 strains with an H_2 production rate of 0.1 µmol H_2 mg Chl^{-1} h^{-1} or higher is shown in Table 1 of Paper I. The highest H_2 production rate was obtained under microaerobic/light conditions and the strains producing H_2 at a rate higher than 0.15 µmol H_2 mg Chl^{-1} h^{-1} were selected for the second screening. The H_2 production rate of UHCC strains was compared to the H_2 production rate of reference strains *N. punctiforme* PCC 73102 (WT and Δ*hupL* deletion mutant) and *Anabaena* PCC 7120 (WT and Δ*hupL*, Δ*hoxH*, and Δ*hupL*/Δ*hoxH* deletion mutants). The screening revealed top ten strains with H_2-producing capacity similar to or up to 4 times higher than in the Δ*hup*L and Δ*hup*L/Δ*hox*H mutants (Fig. 1 of Paper I).

4.1.2. Identification of genes encoding the enzymes involved in H_2 metabolism in the top ten superior strains (Paper II)

The distribution of the genes encoding the enzymes involved in H_2 metabolism in the top ten strains was studied by Southern hybridization. The PCR product of *hoxY* from *Calothrix* XPORK 5E was used as probe for the detection of the *hoxY* gene. Similarly, the PCR products of *hupL*, *hupS*, *nif*1, *nif*2, and *vnf* genes from *A. variabilis* were used as probes for the detection of the *hup*, *nif*, and *vnf* genes. The genomic DNA of eight of the strains, with *Calothrix* XSPORK 36C and *Calothrix* XSPORK 11A as exceptions, showed hybridization signals in the *hupL*, *hupS*, and *hoxY* blots. As the strains tested are heterocystous, N_2-fixing cyanobacteria, they showed hybridization signals in the *nifH1*, *nifD1*, and *nifK1* blots, the subunits of conventional nitrogenase. However, none of the strains tested showed any hybridization signal in *nif*2 and *vnf* blots for the presence of these genes. The blots showing the hybridization signals for *nif1*, *nif2*, *vnf*, *hup*, and *hox* are shown as supplementary figures (Pages 57-58).

4.1.3. Effect of changing culture conditions on the rate of H_2 production (Paper I)

Differences in the cell density had a considerable effect on the H_2-producing capacity of the top ten H_2 producers. Increase in light intensity from 100 to 250 µmol photons m^{-2} s^{-1} enhanced the rate of H_2 production in top two H_2 producers, *Calothrix* 336/3 and *Calothrix* XPORK 5E. Further increase in light intensity to 350 and 500 µmol photons m^{-2} s^{-1} gradually reduced the capacity of H_2 production in *Calothrix* XPORK 5E, whereas high light still enhanced the H_2 production rate in *Calothrix* 336/3. The increase in

temperature in the H_2 production assay from 23ºC to 30ºC stimulated the H_2 production rate two-fold in *Calothrix* 336/3. Further increase was obtained at the same temperature when the position of the vials during incubation was shifted from vertical to horizontal position so that the light came from the sides (150 µmol photons $m^{-2} s^{-1}$). In this way, the surface area of the vials exposed to light was increased, thereby enhancing the H_2 production rate ca. 5 times relative to the standard vertical incubation position.

Next, the effect of pH on the H_2 production rate of two *Calothrix* strains was studied using Z8x medium at either pH 6.8 or pH 8.2, in addition to standard medium at pH 7.5. Interestingly, *Calothrix* 336/3 was not able to grow at pH 6.8 or pH 8.2. *Calothrix* XPORK 5E strain did not grow at pH 8.2 and grew relatively slowly at pH 6.8. At pH 6.8, *Calothrix* XPORK 5E produced much less H_2 than at the optimal pH (7.5). On the other hand, *Calothrix* XPORK 5E produced H_2 at a similar rate in both BG-11$_0$ and Z8x medium, whereas *Calothrix* 336/3, despite similar growth in both media, produced less H_2 in BG-11$_0$ compared to in Z8x medium. The third highest H_2 producer, *Nodularia* AV33, which is normally grown in Z8xS medium at pH 7.5, could not survive in BG-11$_0$ supplemented with salt. The H_2 production rate of most of the strains examined increased with decreasing cell density of the cultures. Nevertheless, some planktonic strains, such as *Nodularia* AV33 and *Nodularia* TRO31, did not show any difference in H_2 production rate upon lowering of the cell density. The H_2 production rate of *Calothrix* 336/3 and *Calothrix* XPORK 5E was compared on the basis of Chl and dry biomass. When compared to *Calothrix* XPORK 5E, *Calothrix* 336/3 produced 30% more H_2 on the basis of Chl whereas two-fold higher H_2 production was recorded on the basis of dry weight.

4.2. Screening of the UHCC for compounds with the capacity to modulate the photosynthetic apparatus of *Synechocystis* PCC 6803 (Paper III)

In preliminary screening assays, 55 methanol extracts of various cyanobacterial strains isolated from the Baltic Sea and Finnish lakes were monitored for their possible effects on the photosynthetic apparatus of *Synechocystis*, making use of Chl a fluorescence induction kinetics. The methanol extract from *Nostoc* XPORK 14A, the only one showing a dramatic effect on the kinetics and amplitude of Chl fluorescence induction, was selected for more detailed studies.

4.2.1. *Phenotype, pigment content, and oxidative status of* Synechocystis *exposed to XPORK 14A extract*

The short-term exposure of *Synechocystis* cells to the XPORK 14A extract resulted in significantly retarded growth rate and reduced Chl content under illumination as compared to the control cells after two days of incubation. Incubation of *Synechocystis* cells with XPORK 14A extract in darkness did not have any noticeable effect on the OD$_{750}$ or the Chl content when compared to the control cells. On the other hand, during long-term exposure to XPORK 14A extract, the growth of *Synechocystis* cells was inhibited for four days but then the cells slowly resumed their growth. The OD$_{750}$ of one-week-old treated cultures was equivalent to the OD$_{750}$ of three-day-old control

cultures. Interestingly, the *Synechocystis* cells incubated with XPORK 14A extract under standard growth conditions in the light or in darkness for two days demonstrated significant oxidative modifications (i.e. carbonylation) in the total protein fraction and the membrane protein fraction similar to those in spinach leaves exposed to nodularin-containing AV1 extract (Paper IV).

4.2.2. Action of XPORK 14A extract on bioenergetic pathways of Synechocystis

4.2.2.1. Short-term treatment in the light

Under growth light, the net photosynthetic activity of *Synechocystis* cells exposed to the XPORK 14A extract was drastically reduced. At the same time, the cells showed a significant decrease in the oxygen evolution capacity of PSII measured with DCBQ as an artificial electron acceptor. The treatment also demonstrated significant modifications in the shape of the FF-relaxation curve in the absence of DCMU, indicating that there were changes on the acceptor side of PSII. In the presence of DCMU, the FF-relaxation-curve showed a new fast phase, indicating serious modifications at the donor side of PSII. In addition to the effect on PSII, the extract also affected P700 oxido-reduction by slowing down the kinetics. Furthermore, the treated cells demonstrated a drastically reduced dark respiration rate compared to control cells.

4.2.2.2. Short-term treatment in darkness

Similarly to the experiment performed in the light, the XPORK 14A treatment in the dark reduced the net photosynthetic rate and the PSII activity of the *Synechocystis* cells relative to the control cells. The overall FF-relaxation kinetics in the absence of DCMU was slower compared to that of the control cells. In the presence of DCMU, no significant difference in the relaxation kinetics was detected. On the other hand, no difference in the $P700^{+}$ oxidation and re-reduction rates was recorded in darkness. Interestingly, the treatment increased the dark respiration rate of the *Synechocystis* cells nearly two-fold compared to the control.

4.2.2.3. Modification of the protein composition of the photosynthetic machinery by the XPORK 14A extract

In cells treated with XPORK 14A extract, under illumination, the amount of PsaB, one of the reaction centre proteins of PSI, was at an elevated level whereas the contents of D1, the PSII reaction centre protein, and cyt*f*, the subunit of the cyt b_6f complex, were clearly reduced compared to those in control cells. In cells treated with the extract in darkness, the amounts of both the D1 protein and PsaB were found to be significantly reduced, and the cyt*f* level increased, in comparison with the corresponding controls. Interestingly, the expression of the NdhJ subunit of the NDH-1 complex was downregulated in the cells treated with XPORK 14A extract, both in light and in darkness.

4.2.3. Characterization of the XPORK 14A extract and purification of M22

LC-MS analysis of the methanol extract of the *Nostoc* XPORK 14A strain revealed a prominent peak of an unknown secondary metabolite at the late growth phase. This

metabolite was isolated, purified, and named M22. The purity of M22 was confirmed by using HPLC. The molecular mass of M22 is 1,626.472 Da and the compound most likely possesses non-peptidic structure. Purified M22 absorbs within the UV-range and, interestingly, also in the blue region of visible light. The absorption properties of M22 suggested that it may be involved in photo-induced formation of ROS, and later on, M22 was indeed confirmed to produce ROS in a light-intensity-dependent manner.

4.2.4. M22 retards the growth and photosynthetic performance of Synechocystis

M22 treatment retarded the growth of *Synechocystis* cells in a similar way as XPORK 14A extract. The OD_{750} of *Synechocystis* cells incubated with purified M22 for two days were significantly reduced, and the pigmentation of *Synechocystis* cells was affected in similar way to that induced by the XPORK 14A extract. During long-term treatment with purified M22, the growth of *Synechocystis* cells was inhibited for four days, similar to the situation with XPORK 14A extract. The cells treated with M22 showed severe modifications in the overall FF-relaxation kinetics. These results indicate that the M22 may be responsible for major modifications in the growth and photosynthesis of *Synechocystis* cells reported in the presence of XPORK 14A extract. However, we cannot exclude the possibility that the *Nostoc* XPORK 14A extract also has other biologically active secondary metabolites.

4.2.5. Fate of M22 in the culture medium during long-term treatment and light exposure studies on XPORK 14A extract

M22 in *Synechocystis* culture medium was quantified every day in order to find any correlation between the inhibition of growth occurring during the first four days of long-term exposure of *Synechocystis* cells to XPORK 14A extract on the one hand and and the amount of M22 on the other. On second day, the concentration of M22 in the culture medium decreased to nearly half, and finally no M22 was detected after four days of incubation. To determine whether the loss of M22 in the culture medium was due to light-induced self-destruction, BG-11 medium containing only XPORK 14A extract was illuminated under standard growth conditions for four days. Quantification by HPLC revealed only a trace amount of M22 (5 nM) in illuminated BG-11 medium. Importantly, when *Synechocystis* cells were inoculated into BG-11 medium containing pre-illuminated XPORK 14A extract, a lag phase in the growth was still observed similar to that in cells exposed to XPORK 14A extract that had not been pre-illuminated.

4.3.   Nodularin modifies the bioenergetic pathways of *Spinacia oleracea* (Papers IV & V)

4.3.1. Phenotype and pigment changes in spinach plants exposed to nodularin extract (Paper IV)

Prolonged exposure of spinach seedlings (five weeks of growth) to nodularin-producing *Nodularia* AV1 extract resulted in significant accumulation of nodularin in

the leaves and in the roots. As a result, the spinach plants exposed to *Nodularia* AV1 extract had a distinct phenotype with the leaves suffering from severe chlorosis, which ultimately led to the death of the leaves. The most deleterious symptoms were detected in the oldest leaves, which were partly colourless and yellowish, while the youngest leaves were dark green and looked quite healthy. After six weeks of growth, the oldest leaves died. In contrast, treatment of plants with water extract from non-nodularin-producing *Nodularia* HKVV did not result in chlorosis and death of the leaves during the time course of the experiment. The control plants remained healthy and the leaves were dark green and exuberant (see Fig. 1 in Paper IV).

In line with the phenotypic features, the yellow part of the leaves of nodularin-exposed plants possessed ca. 50% less Chl than those of the control plants. However, the Chl content of the green parts of the leaves from nodularin-exposed plants and HKVV-exposed plants did not differ significantly from that of the control leaves. No distinct differences in the content of neoxanthin, lutein, β-carotene, and violaxanthin could be detected between the green parts of the leaves, but the yellow parts of the leaves from the nodularin-exposed plants always contained markedly less carotenoid pigments than the control or HKVV-exposed plants.

4.3.2. Effect of nodularin extract on the photosynthetic machinery of spinach plants (Paper IV)

As the plants treated with nodularin suffered from severe chlorosis and reduced pigment content, the possible effects of nodularin on the photosynthetic machinery of spinach leaves were studied. Immunoblotting of key photosynthetic proteins showed no marked difference in the level of PSII (D1, CP43, and CP47), PSI (PsaB) and ATPase proteins between the control plants and the plants treated with cyanobacterial extracts including nodularin. Likewise, the PSII function of the nodularin-exposed plants was not affected when oxygen evolution activity of the thylakoids was measured either from the young, healthy leaves or from old, yellowish leaves using DCBQ as an artificial electron acceptor. Nevertheless, the rate of re-reduction of P700$^+$, the primary donor of PSI, in the dark was slightly faster in the nodularin-exposed plants than in the control or HKVV-exposed plants. Nodularin exposure did not result in visible changes in the chloroplast ultrastructure, as the thylakoid organization remained intact in the chloroplasts of nodularin-treated plants (Paper V).

4.3.3. Upregulation of the mitochondrial electron transport chain of spinach plants by nodularin extract (Paper V)

In experiments with the crude membrane fraction from spinach, I noticed that the amount of mitochondrial COXII, a subunit of complex IV was significantly increased (Paper IV). To investigate the detailed effect of nodularin on mitochondrial respiration, the function of the respiratory enzyme complexes in mitochondria isolated from nodularin-exposed plants was determined using a spectrophotometry-based *in organello* assay. Nodularin exposure markedly increased the enzyme activities of complex I, complex II, complex III, and complex IV, as well as the activity of citrate

synthase, a marker enzyme of the citric acid cycle. Western blotting demonstrated the enhanced accumulation of the 39-kDa subunit and ND4 subunit of complex I, whereas no difference in the level of the 30-kDa subunit of complex I could be detected. Also, the amount of COXII from pure mitochondria was increased, which is in line with the upregulation of COXII when analyzed using crude membrane protein extract. Interestingly, the amounts of stress-induced AOX and NAD^+-dependent isocitrate dehydrogenase (Idh), an enzyme of TCA cycle, were significantly upregulated in the plants exposed to nodularin-containing extract.

SDS-PAGE analysis revealed the upregulation of expression of 30- and 20-kDa proteins and downregulation of the prominent protein band of approximately 55-kDa in mitochondria isolated from nodularin-exposed plants. Although the general morphology of the leaves did not differ between the control and nodularin-exposed plants, the defined structure of the cristae typical of the control mitochondria was more obscure in the mitochondria of the nodularin-treated plants. Moreover, at least part of the leaf mitochondria of nodularin-exposed plants were swollen compared to those of the control plants.

4.3.4. Organellar stress response of spinach plants exposed to nodularin extract (Papers IV & V)

The phenotype of the plants exposed to nodularin-containing extract indicated that the plants suffered from oxidative stress. As already mentioned, both chloroplasts and mitochondria are major sources and targets of ROS (Apel and Hirt 2004), and the oxidative stress response from both chloroplasts and mitochondria was therefore studied. Oxyblot analysis has been used to study the oxidative modifications, i.e. carbonylation of the proteins susceptible to oxidative damage. Chloroplast membrane and soluble proteins in the nodularin-exposed plants showed markedly more oxidative damage than those of the control or HKVV-exposed plants, whereas no such changes could be detected in the mitochondrial proteins. Also, the mAPX was upregulated during exposure to nodularin-containing extract, as a protective mechanism against the oxidative damage to mitochondria. No significant differences in the amounts of chloroplast PRXQ, SOD, or cytoplasmic APX (cAPX) were observed. However, the contents of tAPX and stromal APX (sAPX) were markedly reduced. Furthermore, α-tocopherol content both in green and yellow leaves of the plants treated with nodularin-containing extract was ca. 1.3 fold higher than in the control plants. Despite the obvious changes in oxidative status, no significant difference in the ratio of reduced to oxidized ascorbate could be detected in the differently treated spinach plants.

5. DISCUSSION

5.1. The Finnish cyanobacterial culture collection (UHCC) has several good H$_2$ producers (Papers I & II)

5.1.1. The top ten strains of UHCC are efficient H$_2$ producers

Cyanobacteria have the ability to produce H$_2$, a potential future source of energy, using the simplest raw materials such as water, mineral salts, and light. Although the potential light conversion efficiency to H$_2$ by cyanobacteria is theoretically high, the system is restricted by biochemical and metabolic pathways. Cyanobacterial H$_2$ production also faces hurdles, such as the sensitivity of nitrogenases and hydrogenases to O$_2$ and the consumption of H$_2$ by uptake hydrogenase (Rao and Hall 1996). A broad range of approaches to developing suitable and sustainable biohydrogen production methods using cyanobacteria have been utilized over the past three decades (Pinto et al. 2002). Importantly, in most cases, scientific and commercial interests have focused on the model and sequenced cyanobacterial strains. Biodiversity among cyanobacteria regarding H$_2$ production has not been well studied, which is the reason why cyanobacteria have a poor reputation regarding H$_2$ production. Several WT strains from natural environment, which could be potential H$_2$ producers as well as strains from the culture collections should be surveyed and exploited as the future energy resources.

To meet this requirement, we screened 400 out of 1,000 strains from the UHCC, which is a culture collection of cyanobacterial strains from the Finnish lakes and the Baltic Sea (Paper I). The ten cyanobacterial strains that produced the highest amounts of H$_2$ were selected from this screening. The rate of H$_2$ production of the top ten strains was similar or higher than the rate shown by the best H$_2$ producers and specifically generated mutant strains in other laboratories (Lindberg et al. 2002; Masukawa et al. 2002a, 2002b). Although *Nodularia* TRO31 and AV33 strains were among the best H$_2$-producing strains, they were excluded from further studies due to their toxic nature and light sensitivity. So, *Calothrix* 336/3 and *Calothrix* XPORK 5E were considered to be the two best H$_2$-producing strains.

5.1.2. Superior H$_2$-producing strains possess conventional nitrogenase enzymes

The molecular approach provides a means of identifying promising organisms that have the potential to produce H$_2$, characterizing the H$_2$-evolving machinery, and generating mutant strains. The molecular machinery for H$_2$ metabolism varies significantly between cyanobacterial strains. For example, strains such as *A. variabilis* and *Anabaena* PCC 7120 contain both uptake and bidirectional hydrogenases (Tamagnini et al. 2000), whereas *N. punctiforme* PCC 73102 contain only the uptake hydrogenase and lacks the bidirectional hydrogenase (Tamagnini et al. 1997). In an

attempt to characterize the top ten strains identified in the screening process at the molecular level, the Southern hybridization approach was employed (Paper II).

All the top superior strains were heterocyst-forming, N_2-fixing cyanobacteria and, as one would expect, the eight strains tested were found to contain all three subunits of *nif* genes, including *nif*H, D and K. N_2-fixing microorganisms may contain either one type of nitrogenase or a combination of two or three nitrogenase types (Kentemich et al. 1988; Bishop and Premakur 1992; Chakraborty and Samaddar 1995; Loveless and Bishop 1999; Oda et al. 2005; Betancourt et al. 2008). Cyanobacteria that harbor the Mo-based nitrogenase can produce only one molecule of H_2 at the expense of 16 molecules of ATP, but cyanobacteria that express V- and Fe-based nitrogenases, although not very common, can theoretically produce higher amounts of H_2 than those with only conventional Mo-nitrogenase. Thus, more attention should be paid to characterizing and utilizing alternative nitrogenase-based cyanobacterial H_2 production. Recently, Weyman and co-workers reported the amino acid substitution in *nifD2* as a first step towards the development of nitrogenase mutants in *A. variabilis*, which produce large amounts of H_2 in a N_2 atmosphere (Weyman et al. 2010). However, none of the N_2-fixing UHCC cyanobacterial strains tested contained the alternative nitrogenases *nif*2 or *vnf*. Alternatively, bidirectional hydrogenase-based H_2 production can be employed, which may produce H_2 under anaerobic conditions. This reaction is not driven by ATP, and it is energetically more favourable than nitrogenase-based H_2 production. In spite of being advantageous, this enzyme needs an O_2-protected environment, and the reaction can be reversed (with H_2 uptake) above certain partial pressure of H_2.

As the uptake hydrogenase can be an impediment to nitrogenase-based H_2 production systems (Tamagnini et al. 2002, 2007; Schütz et al. 2004; Sakurai and Masukawa 2007), targeted disruption of *hup* genes has been undertaken in several strains of *Anabaena* and *Nostoc* as a means of improving the efficiency of H_2 production (Happe et al. 2000; Lindberg et al. 2002; Masukawa et al. 2002a, 2002b; Schütz et al. 2004; Carrasco et al. 2005; Yoshino et al. 2007). These strains were used as reference strains in our research work (Paper I). Screening for *hup* genes in our top ten H_2-producing strains revealed the presence of *hupLS* genes in all *Calothrix* strains except two, XSPORK 36C and 11A. It is worth mentioning that Southern blot may yield ambiguous output and lead to negative results if the probe used and the target gene do not share high enough homology or it may result in cross-hybridization, when the probe shares homology with several target genes. Therefore, it may be highly possible that the two *Calothrix* strains contain an uptake hydrogenase but that the *hupSL* genes of the two strains are substantially different from those of the other eight strains. Although some N_2-fixing unicellular *Synechococcus* strains lack *hup* genes (Ludwig et al. 2006), the absence of those genes in our N_2-fixing, filamentous *Calothrix* strains needs further confirmation through direct assay of uptake hydrogenase activity, or by sequencing the genome before making the conclusion. The bidirectional hydrogenase catalyzes both uptake and evolution of H_2. Interestingly, the eight strains containing the *hupSL* genes also contain the *hox*Y genes.

5.1.3. *Growth conditions affect H$_2$ production*

It is well known that modification of the growth conditions may substantially enhance the photobiological production of H$_2$. For example, in *Chlamydomonos reinhardtii*, sulphur (S) deprivation became the most successful method and the one of choice for H$_2$ production (Melis et al. 2000). Two good H$_2$ producers, *Calothrix* 336/3 and *Calothrix* XPORK 5E, were used to optimize the culture conditions for improved H$_2$ production. The two *Calothrix* strains require higher light intensity (250 µmol photons m^{-2} s^{-1}) than the reference strains, such as Δ*hupL* mutants of *Anabaena* PCC 7120 and *N. punctiforme* PCC 73102, for maximal H$_2$ production. The decrease in cell density gradually improved the H$_2$ production rate in the H$_2$ assay, which may have been related to the light requirement of these planktonic strains in the Baltic Sea. The combination of larger surface area for light capture and lower cell density had a dramatic effect on the H$_2$ production rate of *Calothrix* 336/3 and the Δ*hupL* mutant of *Anabaena* PCC 7120. This set-up resulted in an improved rate of H$_2$ production in *Calothrix* 336/3 by up to 9 times. The optimal pH for our Finnish strains, *Calothrix* 336/3 and XPORK 5E, was pH 7.5 whereas the optimal pH for the Δ*hup*L/Δ*hox*H mutant of *Anabaena* PCC 7120 was 8.2. The optimal temperature for H$_2$ production varies between different cyanobacterial species (Dutta et al. 2005). Maximum H$_2$ production in *A. variabilis* SPU 003 (Shah et al. 2001) and *Nostoc muscorum* SPU 004 (Shah et al. 2003) has been achieved at 30ºC and 40ºC, respectively. In our studies, the H$_2$ production rate of *Calothrix* 336/3 increased two-fold on shifting from 23ºC to 30ºC. Above all, the *Calothrix* 336/3 strain had better H$_2$ production in the Z8x growth medium than in BG-11$_0$ medium, although the growth rates were similar in both media. On the other hand, *Calothrix* XPORK 5E showed similar growth rates and H$_2$ production rates in both BG-11$_0$ and Z8x. It has also been reported that *A. variabilis* ATCC 29413 has higher H$_2$ production in Allen-Arnon medium than in BG-11 and BG-11$_0$ media (Berberoğlu et al. 2008).

5.2. A novel bioactive compound, M22, from *Nostoc* XPORK 14A (Paper III)

Cyanobacteria produce many bioactive compounds that target photosynthesis in both eukaryotic algae and cyanobacteria. These compounds have mostly been reported to be algicides, as they kill other algae or cyanobacteria competing in the same habitat where the bioactive producer lives (Mason et al. 1982; Flores and Wolk 1986).

5.2.1. *M22 in* Nostoc *XPORK 14A attacks the bioenergetic pathways of* Synechocystis

The XPORK 14A extract affected the growth and pigment content of *Synechocystis* in the presence of light, thus revealing a preliminary clue about its significant effect on the photosynthesis. This was supported by the reduced steady-state oxygen evolution activity of PSII and the modified acceptor and donor sides of the PSII complex. In addition, XPORK 14A extract increased the proportion of PSII centres with an empty Q$_B$ pocket, most likely due to over-reduction of the PQ pool. Also, there was an increase in the fraction of PSII centres where re-oxidation of Q$_A^-$ occurs through back

reactions due to the impaired forward electron transfer from Q_A^-. Additionally, the appearance of a new fast phase of FF-relaxation kinetics in the presence of DCMU suggests the accumulation of PSII centres with modifications on the donor side of the PSII complex. A similar fast phase is characteristic of *Synechocystis* cells exposed to UV-B light, impairing the electron transfer step from Tyr_Z to $P680^+$ (Vass and Aro 2008). Furthermore, exposure of the cells to the XPORK 14A extract markedly reduced the cyclic electron flow around PSI, indicating the malfunctioning of PSI. These effects on PSII and PSI were evident from the increased PSI/PSII ratio, supported by upregulation of PsaB and downregulation of D1 at the protein level. Interestingly, dark respiration that shares components of the photosynthetic machinery (Peschek et al. 2004), was also significantly affected in the light treatment. This is in line with the downregulation of the cyt$f$ subunit of the cyt b_6f complex at the protein level.

5.2.2. M22 produces ROS in the light and is subject to degradation

The next task was to identify the compound responsible for the harmful effects of *Nostoc* XPORK 14A on *Synechocystis* culture. *Nostoc* XPORK 14A synthesized a specific compound during the late growth phase. This compound was purified from *Nostoc* XPORK 14A extract and named M22. M22 was identified as an active compound that modifies the bioenergetic pathways of *Synechocystis*. This was evident from reduction in the growth and pigment content of *Synechocystis* cells by M22 on treatment with light for two days, similar to that seen from XPORK 14A extract. Purified M22 absorbs light in the blue region, which suggested that it might behave as photo-induced ROS generator. A similar kind of photosensitizing compound, cercosporin, a fungal toxin, also kills plant cells rapidly in the light (Daub and Hangarter 1983). Indeed, M22 was found to generate ROS, and the production of ROS was linearly dependent on the light intensity. Also, the linearity was disturbed above a certain light intensity, suggesting photo-induced degradation. Degradation of M22 was evidenced by a gradual decrease in the amount of M22 during the first four days of incubation under standard growth light, and no M22 could be detected from four days onwards. During the long-term incubation, M22 inhibited the growth of *Synechocystis* for four days, and surprisingly, the cells started to grow like the control cells after four days. This behaviour contrasts with that of well-known algicidal compounds such as cyanobacterin (Mason et al. 1982; Gromov et al. 1991) and fischerellin (Gross et al. 1991; Srivastava et al. 1998), which completely kill the cyanobacteria or algae being tested. Besides the photo-generation of ROS, M22 can also undergo light-dependent destruction in its oxidative state. Degradation of M22 is a probable reason for the initiation of growth after four days in growth medium. In line with this, the photo-degradation of nodularin under visible and UV-light has also been reported (Twist and Codd 1997). Similarly, if M22 undergoes photo-degradation, one would expect normal growth of cells in the medium containing M22, pre-illuminated for four days without *Synechocystis* cells. However, the growth of the cells inoculated on the fourth day was still arrested, which could be due to a residual amount of M22 in the medium on the fourth day of the pre-light treatment. The reason for the absence of M22 in the medium including *Synechocystis* cells from the fourth day may have been due to degradation,

either by enzymatic means by *Synechocystis* or by physical means including prolonged exposure to unfavourable pH, light, or temperature. Another possible explanation for the faster destruction of M22 in the presence of *Synechocystis* cells might be the evolution of O_2 during photosynthesis, which might result in an oxidized state of M22 and therefore enhanced degradation.

5.2.3. M22 has a dual-action mechanism

M22 might induce oxidative stress via photo-generation of ROS, which was supported by the accumulation of oxidatively modified proteins in the presence of light. Surprisingly, the XPORK 14A extract caused upregulation of oxidative modification of proteins even in darkness. In addition, the treatment with XPORK 14A extract in darkness revealed significant effects on net photosynthetic rate, and the oxygen evolution capacity of PSII was similar to that under light treatment even though the cells do not grow in the dark. In contrast to light treatment, the dark treatment revealed significant effects that indicated that M22 has a dual-action mechanism. Dark-treated cells (1) lacked the prominent very fast phase in the FF-relaxation curves, which is specific to light-induced damage (Vass and Aro 2008), (2) showed no Chl destruction, (3) upregulated cellular respiration, which was also evidenced by increased amount of the cyt*f* protein, and (4) showed no difference in the oxido-reduction of P700 in the control cells and the cells treated with XPORK 14A extract. Thus, M22 apparently affects the photosynthetic and respiratory machineries in the light through photo-induced generation of ROS and in darkness by a still unknown mechanism.

5.3. Harmful nodularin-producing *N. spumigena* strain AV1 disturbs the growth and metabolism of plants (Papers IV & V)

5.3.1. Nodularin accumulates and affects the growth of spinach

Long-term exposure of spinach plants to nodularin-containing extract resulted in the uptake of nodularin in the roots and leaves of the plants, which is likely to be the reason for severe chlorosis in the leaves and reduction in the growth of the plants (Papers IV & V). High concentrations of nodularin, beyond ecologically relevant levels, were used to impose severe effects on metabolism in order to reveal the targets of nodularin. Nevertheless, it is highly possible that plants near the shore are not exposed to uniform concentrations of nodularin during the formation of thick blooms. In addition to its harmful effects on growth of the plants as such, accumulation of cyanotoxins in plants might lead to potential health risks for animals and humans consuming the contaminated plants. Interestingly, the nodularin that accumulated in the roots and leaves was stable and did not undergo degradation, as seen from analyzing the quantity of nodularin by HPLC. In contrast, the Baltic Sea trout accumulated nodularin-like compounds as degradative products in muscle, albeit in lower quantities, when exposed to *N. spumigena* AV1 (Kankaanpää et al. 2002), same strain as employed in our study. In our work, bleaching of leaves was observed in the older

leaves and the older leaves accumulated more nodularin than the young leaves (Papers IV & V). Similar leaf chlorosis has been detected in different variants of spinach plants treated with MC, which possess similar toxicity as nodularin (Pflugmacher et al. 2007a).

The hepatotoxin nodularin is produced by *N. spumigena*, a common harmful cyanobacterial species found in the Baltic Sea during the summer (Sivonen et al. 1989). Nodularin is known to accumulate in shell fish, mussels, prawns, flounder, etc. to a level high enough to cause hepatotoxicity in animals consuming them (Falconer et al. 1992; Van Buynder et al. 2001; Mazur-Marzec et al. 2007). Indeed Van Buynder et al. (2001) reported that nodularin was taken up by mussels and prawn at toxicity levels and that boiling of these seafoods redistributed the toxin between viscera and flesh. Therefore, they recommended restricting the use of seafoods harvested from the Sea contaminated with *Nodularia* blooms. Although there are enough studies demonstrating the effect of nodularin on sea animals, there has been a complete lack of studies concerning the toxicity, physiological and ecological consequences of nodularin on any kinds of plants, particularly terrestrial plants growing on the shores of the Baltic Sea (Fig. 4). It is also possible that agricultural crops are exposed to cyanotoxins if irrigated with surface waters containing bloom material.

Fig. 4. Blue-green algal bloom in the Baltic Sea (courtesy: www.yle.fi, published on 07.02.2009)

5.3.2. *The photosynthetic machinery is not the primary target of nodularin*

Severe chlorosis of older leaves of nodularin-exposed plants was evident from less Chl and carotenoids accumulating in the yellow parts of the leaves. However, the photosynthetic performance of nodularin-exposed plants was unaffected by the presence of nodularin (Paper IV). No differences were detected in the rate of PSII oxygen evolution in old and young leaves, and the levels of photosynthetic proteins in the thylakoid membranes of plants exposed to nodularin-containing extract remained unchanged. These results were supported by the intact chloroplast ultrastructure visualized by the electron microscopy (Paper IV). Therefore, it is possible to assume that although the amount of functional photosystems in the chloroplasts of yellow leaves is very low, the function of the remaining photosystems has not been disturbed by nodularin. The remarkable feature of the photosynthetic apparatus is its ability to adjust rapidly to changes in environmental and metabolic conditions (Rochaix 2011). Many studies reported that MC inhibits the photosynthetic activity of higher plants

(Babica et al. 2006), whereas long-term treatment of *Brassica oleracea* and *Sinapis alba* with MC did not affect the PSII capacity (Järvenpää et al. 2007). Moreover, Suikkanen and co-workers reported that nodularin has no role in allelopathy, and it has not been reported as an allelochemical (Suikkanen et al. 2006). An allelochemical is a compound that inhibits or benefits the growth of another organism in the same or a neighbouring habitat (Muller 1969). Intriguingly, the only photosynthetic parameter found to be affected by the presence of nodularin was the rate of P700 re-reduction, which reflects the status of cyclic electron transfer around PSI (Paper IV). The cyclic electron transfer was activated, which usually happens under stress conditions, for example, under low ambient CO_2 concentration (Rumeau et al. 2007) or drought (Golding et al. 2004).

5.3.3. Nodularin upregulates the activity of mitochondrial enzyme complexes

The mitochondrial electron transport chain and ATP synthases are very sensitive to inhibition and modification of the components by various stress conditions (Noctor et al. 2007). It has also been reported by several authors that mitochondria may be vulnerable targets of various cyanotoxins, e.g. MC-LR induced apoptosis, ultrastructural changes through the loss of MMP, formation of ROS, and cytochrome *c* release in primary cultured rat hepatocytes (Ding et al. 1998, 2000). However, very few studies have been done with plant mitochondria. It has been shown that MC-RR induces apoptosis in a dose-dependent manner by opening the mitochondrial permeability transition pores in tobacco BY-2 cell cultures (Huang et al. 2008, 2009). My study involving mitochondrial metabolism initiated when a high content of COXII, a marker for respiratory protein complexes, and increased oxygen consumption rate had been evidenced (Paper IV). Later on, I found that nodularin increased the specific activity of complex I and citrate synthase (Paper V). The increased rate of mitochondrial electron transport has already been reported in *Arabidopsis thaliana* exposed to oxidative stress (Tiwari et al. 2002) and *Chlamydomonas reinhardtii* cw92 exposed to anthracene (Aksmann and Tukaj 2008). Interestingly, the amounts of 39-kDa and ND4 subunits of complex I, COXII subunit of complex IV, and ATP synthase were also upregulated at the protein level in the plants exposed to nodularin-containing extract. It is important to note that the level of porin, the voltage-dependent anion channel on the outer mitochondrial membrane (Clausen et al. 2004), was unaffected by the exposure of plants to nodularin-containing extract in spite of the activated respiration rate. This clearly shows that the mitochondrial mass (Noguchi et al. 2005) was unaltered by the exposure to nodularin.

5.3.4. Nodularin induces oxidative stress and disturbs the balance of ROS production and scavenging

Nodularin-exposed plants suffered from oxidative stress, which was clearly revealed by the reduction in pigment content and carbonylation of oxidized proteins (Paper IV). Induction of oxidative stress is a natural phenomenon during cyanotoxin exposure, and nodularin has already been implicated in induction of oxidative stress in various organisms (Davies et al. 2005; Kankaanpää et al. 2007; Persson et al. 2009).

Nodularin exposure of the brown alga *Fucus vesiculosus* increased the lipid peroxidation activity and many ROS-scavenging enzymes (Pflugmacher et al. 2007b). In our case, nodularin-containing extract affected the chloroplast and mitochondria differently, as evidenced by the accumulation of oxidatively modified proteins in chloroplasts only. Moreover, the amount of α-tocopherol, an antioxidant molecule, was upregulated in plants treated with nodularin-containing extract (Paper IV). In line with our results, it has been shown that even a low concentration of cyanotoxins in the water has a strong influence on tocopherols in *Medicago sativa* seedlings (Peuthert and Pflugmacher 2010). However, the amounts of ROS-scavenging enzymes such as PRXQ, SOD, and cAPX were unaltered, whereas the contents of tAPX and sAPX were markedly reduced in chloroplasts (Paper IV). On the other hand, nodularin-containing extract upregulated the expression of stress-induced AOX and mAPX, indicating the induction of ROS scavenging enzymes in the mitochondria, which apparently protect the mitochondrial proteins from oxidative damage (Paper V). Moreover, enhanced expression of AOX, which controls the levels of ROS in the mitochondria (Moore et al. 2002) may protect mitochondria from the deleterious effects of nodularin. On the whole, nodularin exposure disturbs the balance of ROS formation and scavenging in chloroplasts and mitochondria, and consequently the network needed for maintenance of the redox balance is induced, which would help the organelles to function in a normal state.

6. CONCLUDING REMARKS

❖ UHCC strains possess tremendous potential to produce biohydrogen as a future alternative energy source. The H_2 production rate of UHCC strains was higher than that of the highest H_2 producers reported so far. The ten best H_2 producers were selected and all the strains tested were found to contain conventional nitrogenase enzyme. Moreover, eight strains contained uptake and bidirectional hydrogenase enzymes and two strains probably did not contain both the uptake and bidirectional hydrogenases. A detailed knowledge of the genomic background will be needed for genetic modification of the potential strains through a systems biology approach.

❖ A novel bioactive compound was identified from *Nostoc* XPORK 14A, and named M22. M22 affected growth and harmed the bioenergetic pathways, the photosynthetic and respiratory electron transport chains in the model cyanobacterium, *Synechocystis* PCC 6803. M22 possess a dual-action mechanism on bioenergetic pathways: light-induced production of ROS and a distinct, as yet unknown mechanism in the darkness.

❖ Exposure of the terrestrial model plant, *Spinacia oleracea* (spinach) to nodularin resulted in nodularin uptake to the tissues, growth retardation, chlorosis, and upregulation of respiratory complexes. Also, nodularin-exposed plants suffered from oxidative stress. Plants growing on the shores of the Baltic Sea that are contaminated with algal blooms may be subjected to oxidative stress and metabolic fluctuations. More extensive analysis, including proteomic and transcriptomic approaches, will be required to characterize the toxin import, the onset of the metabolic changes, and the mode of nodularin action in terrestrial plants.

FUTURE PERSPECTIVES

Today, when the world's energy demands are rising, one of the important goals is to produce clean and sustainable energy. Knowing that the cyanobacteria make use of solar energy, water, and simple nutrients for their growth, an exploration of morphologically and physiologically diverse cyanobacterial forms is required. Although the cyanobacterial H_2 production strategies have been studied extensively over the last three decades, screening for H_2 producers with flexible metabolism from culture collections and natural water bodies is still incomplete. Our laboratory is keen on this aspect of screening cyanobacterial forms from the UHCC to identify novel strains with better H_2-producing capacity than the standard strains reported so far. Moreover, a deeper knowledge of the genomic machinery of the best H_2 producers will be gained by sequencing of whole-genomes. This would provide an opportunity to either upgrade the H_2 production capacity of WT strains or to generate mutants on enzymes that are involved in H_2 metabolism. The generation of genetically modified strains, e.g. strains with modified hydrogenases showing O_2 insensitivity or with active hydrogenases or nitrogenases from other organisms, would pave the way either to combating the sensitivity of cyanobacterial H_2 metabolism to O_2 or to increasing the turnover of H_2 production through nitrogenase-based systems.

Although many compounds directly affecting the bioenergetics of other cyanobacteria have been discovered during the past 20-30 years, more recent activity in identification and characterization of such compounds has been very low. We have identified a novel compound, M22, from *Nostoc* XPORK 14A strain that affects bioenergetic pathways, both the photosynthetic and respiratory electron transport chains of *Synechocystis* PCC 6803. This kind of study could possibly also identify novel compounds with herbicidal activity, which might be helpful in reducing the formation of algal blooms. At the same time, the formation of harmful algal blooms in the Baltic Sea every summer is a well-known phenomenon. However, the condition of terrestrial plants after exposure to growing algal blooms by accidental contamination through irrigation is not well studied. It is also important to mention that the impact of cyanobacterial blooms on the plants growing on the shore has not been investigated at all. In my PhD study, I have found out that nodularin from *N. spumigena*, a prominent producer of toxin in the Baltic Sea, retards the growth and also affects the bioenergetic pathways of a terrestrial plant, spinach (*Spinacia oleracea*). Detailed biochemical, proteomic, and transcriptomic approaches could be taken to investigate the target pathways of the toxic bloom, the mode of import of toxins, and the onset of metabolic fluctuations. This research could also lead to prevention of the entry of toxins into the food web, thereby protecting the mankind from the consequences of hepatotoxicity.

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SUPPLEMENT
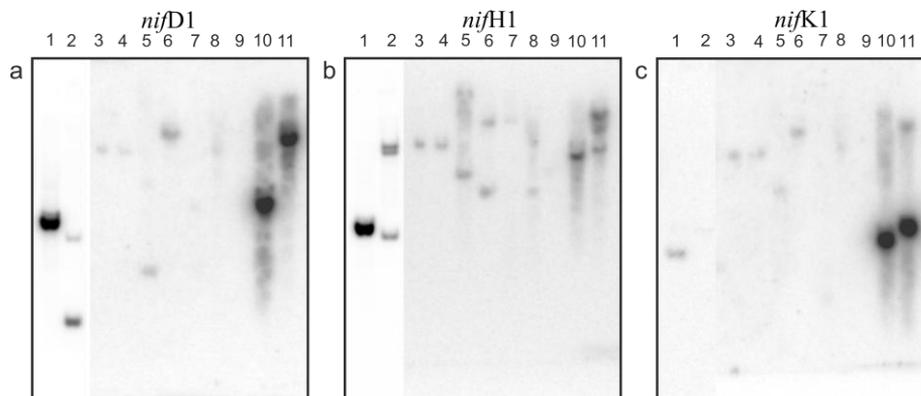
Supplementary Figure 1. Southern hybridization with *nifD1*, *nifH1*, and *nifK1*. The genomic DNA of cyanobacterial strains (including positive and negative controls) was digested with *Hind*III or *EcoR*I, electrophoresed in agarose gels and blotted onto membranes. 1-*Calothrix* 336/3, 2-*Calothrix* XPORK 5E, 3-*Nostoc* Becid 19, 4-*Calothrix* Becid 33, 5-*Nostoc* XHIID A6, 6-*Nodularia* AV33, 7-*Calothrix* XSPORK 11A, 8-*Nodularia* TRO31, 9-*Synechocystis* PCC 6803 (- control), 10-*Anabaena* PCC 7120 (+ control), 11-*A. variabilis* ATCC 29413 (+ control).
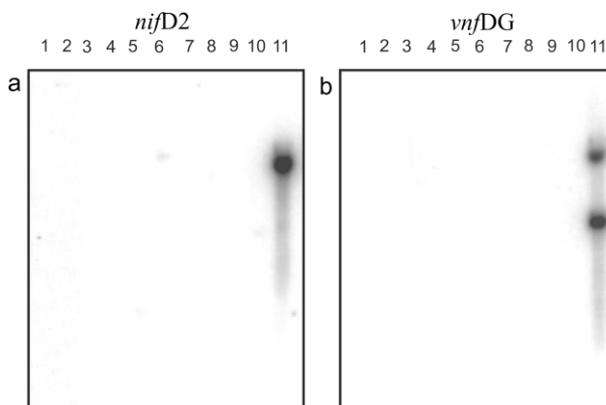
Supplementary Figure 2. Southern hybridization with *nifD2 and vnfDG*. The genomic DNA of cyanobacterial strains (including positive and negative controls) was digested with *Hind*III or *EcoR*I, electrophoresed in agarose gels and blotted onto membranes. 1-*Calothrix* 336/3, 2-*Calothrix* XPORK 5E, 3-*Nostoc* Becid 19, 4-*Calothrix* Becid 33, 5-*Nostoc* XHIID A6, 6-*Nodularia* AV33, 7-*Calothrix* XSPORK 11A, 8-*Nodularia* TRO31, 9-*Synechocystis* PCC 6803 (- control), 10-*Anabaena* PCC 7120 (- control), 11-*A. variabilis* ATCC 29413 (+ control).
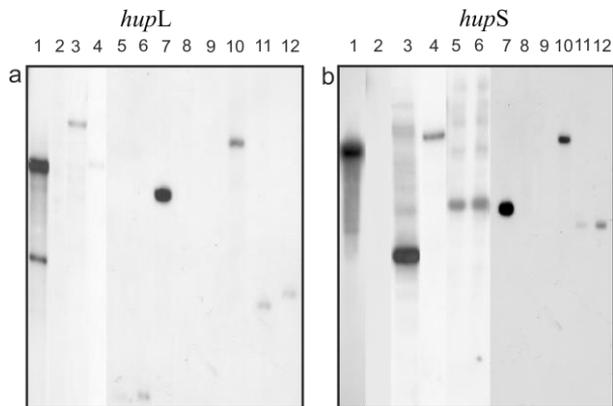
Supplementary Figure 3. Southern hybridization with *hup*L and *hup*S. The genomic DNA from top ten cyanobacterial strains (including positive and negative strains) was digested with *Hind*III or *EcoR*I, electrophoresed in agarose gels and blotted onto membranes. 1-*A. variabilis* ATCC 29413 (+ control), 2-*Synechocystis* PCC 6803 (- control), 3-*Calothrix* 336/3, 4-*Calothrix* XPORK 5E, 5-*Calothrix* Becid 33, 6-*Nostoc* Becid 19, 7-*Anabaena* XSPORK 7B, 8-*Calothrix* XSPORK 36C, 9-*Calothrix* XSPORK 11A, 10-*Nostoc* XHIID A6, 11-*Nodularia* AV33, 12-*Nodularia* TRO31.
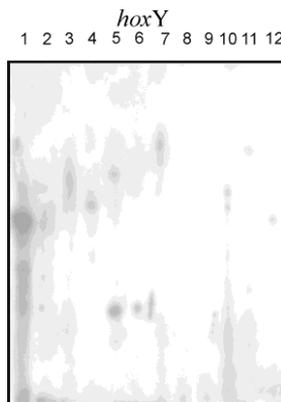
Supplementary Figure 4. Southern hybridization with *hoxY*. The genomic DNA from top ten cyanobacterial strains (including positive and negative strains) was digested with *Hind*III or *EcoR*I, electrophoresed in agarose gels and blotted onto membranes.1-*A. variabilis* ATCC 29413 (+ control), 2- *N. punctiforme* PCC 73102 (- control), 3-*Calothrix* 336/3, 4-*Calothrix* XPORK 5E, 5-*Calothrix* Becid 33, 6-*Nostoc* Becid 19, 7-*Anabaena* XSPORK 7B, 8-*Calothrix* XSPORK 36C, 9-*Calothrix* XSPORK 11A, 10-*Nostoc* XHIID A6, 11-*Nodularia* AV33, 12-*Nodularia* TRO31.