The effects of low nitrate levels on the freshwater cyanobacterium *Synechocystis* sp. strain PCC 6803: Construction of a bioreporter assay and molecular characterization by transcriptome and proteome analysis

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vorgelegt von

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PLEDGE

I certify that the present thesis entitled:
"The effects of low nitrate levels on the freshwater cyanobacterium *Synechocystis* sp. strain PCC 6803: Construction of a bioreporter assay and molecular characterization by transcriptome and proteome analysis" was carried out without any unlawful devices. I did not use any other than the described literature sources or technical devices. This work has never been submitted before in this or similar form to any other university and has not been used for any examination.

Stuttgart, 17.09.2003

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LIST OF ABBREVIATIONS

µE	Micro Einstein
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
ACN	Acetonitrile
APS	Ammonium per sulfate
bp	Base pair
BSA	Bovine serumalbumine
cDNA	Complementary DNA
CHAPS	(3-[[3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate
dH_2O	Distilled water
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosid triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamin-N,N´,N´,N´ tetra acetic acid
Fig.	Figure
FluoroLink Cy3-dUTP	5-amino-propargryl-2´-deoxyuridine 5´-triphosphate coupled to Cy3 (cyanine) fluorescent dye
FluoroLink Cy5-dUTP	5-amino-propargryl-2´-deoxyuridine 5´-triphosphate coupled to Cy5 (cyanine) fluorescent dye
h	Hours
HEPES	*N*-2-hydroxyethylpiperazine –*N´*-2-ethane sulfonic acid
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IPG-Dalt	Two-dimensional polyacrylamide gel electrophoresis with immobilized gel electrophoresis
isi	Iron stress induced
kb	Kilobases
Km^R	Kanamycin resistance
LMW	Low molecular weight
M	Mole per liter
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight
min	Minutes
MOPS	3-(N-Morpholino)-propanesulfonic acid
mRNA	Messenger RNA
MS	Mass spectrometry
N	Nitrogen
nbl	Non bleaching
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	Potential hydrogen
PI	Point ioselectric
PVDF	Polyvinyledene difluoride
RNA	Ribonucleic acid
rpm	Round per minute
RT-PCR	Reverse transcription-polymerase chain reaction
s	Seconds

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp.	Species
SSC	Saline sodium citrate
SSPE	Saline sodium phosphate EDTA
TE	Tris-Cl+Na_2-EDTA
TEMED	N,N,N´,N´ Tetramethylendiamin
Tris	Tris (hydroxymethyl)-aminomethane
V	Volume
v/v	Volume per volume
w/v	Weight per volume

Units

°C	Degree Celsius	s	Second
Da	Dalton	M	Molar
g	Gram	V	Volt
h	Hour	l	Liter
min	Minute	m	Meter

Standard prefixes

Prefix	Symbol	Factor
mega	M	10^6
kilo	k	10^3
centi	c	10^{-2}
milli	m	10^{-3}
micro	µ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}

Symbols for amino acids

A	Ala	Alanine	M	Met	Methionine
B	Asx	Asparagine or aspartic acid	N	Asn	Asparagine
C	Cys	Cysteine	P	Pro	Proline
D	Asp	Aspartic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Cly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophan
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamine or glutamic acid

Nucleotide (base)

A	Adenine	G	Guanine
C	Cytosine	T	Thymine

ABSTRACT

Since a few decades the so-called blue algal blooms came to public awareness and their occurrence was more frequently reported. These blooms stem from the mass proliferation of some cyanobacterial species and they occur both in the sea as well as in fresh water. The exact reasons for this phenomenon have not been finally clarified, but nutrient availability, limitation and excess, has a proven influence. Some bioavailability patterns of P, N, S and Fe strongly promote cyanobacterial proliferation. Due to the negative effects of these blooms, *i.e.* severe neuro- and hepato-toxin release, a deeper understanding, prediction and monitoring are desirable. It was the aim of this work to develop and use biotechnological tools to fulfill this requirement. In order to avoid the problems associated with water blooms and to understand the behavior of these microorganisms at low nutrient concentration, an assay as an early warning system for monitoring of water blooms formation at low nitrate concentration was developed; and the analysis of the physiological change at the level of the transcriptome and proteome was performed.

Starting with a cyanobacterial reporter strain, *Synechocystis* sp. strain PCC 6803 harboring *PnblA::luxAB-km*^{R} in its genome, a luminescent reporter assay for the detection of nitrate bioavailability was constructed. In this construction, *luxAB* gene encoding the luciferase, from the luminescent bacterium *Vibrio harveyi* was fused with the kanamycin resistance gene (*km*^{R}), leading to a *luxAB-km*^{R} gene complex. This gene complex was then fused with the *nblA1* gene of *Synechocystis* and inserted in its chromosomal DNA. This reporter strain was designated N1LuxKm. The expression of the *luxAB* gene was induced by nitrate deficiency and was quantified by the bioluminescence emission. By means of immobilization of N1LuxKm in microtiter plates, the sensor was storable for about one month and showed a dose-dependent luminescence signal in a concentration range of 4-100 µM nitrate after a sample incubation time of 10 h under continuous illumination (50 $\mu E.m^{-2}.s^{-1}$ of white light). Combined with ecological and physiological data this sensor could be used as an early warning system for water blooms.

In order to further understand cellular processes resulting from nitrate starvation and their influence on cyanobacterial blooms, the proteome dynamics of *Synechocystis* sp. PCC 6803 was analyzed through 2D gel electrophoresis, MALDI-TOF/MS of trypsin-digested protein fragments and *N*-terminal amino acid sequencing. This simultaneous analysis of total gene

expression at the level of protein represents one of the premiere strategies for studying biological systems and understanding the relationship between various expressed genes and gene products. This approach allowed the identification of four proteins which were up-regulated under nitrate starvation conditions, namely two isoforms of "the nitrogen regulatory protein P-II" encoded by *glnB* gene; "the carbon dioxide concentrating mechanism protein" and the plastocyanin encoded by *ccmK* and *petE* genes respectively.

The information gained with proteomics was confirmed and extended by RNA expression analysis related to nitrate depletion using oligonucleotide sequences immobilized on microarrays. Total RNAs were reverse transcribed to fluorescent-labeled cDNAs, then hybridized to the immobilized probes. The difference in the abundance of the transcripts was recorded through the difference in the fluorescence emission. All the genes, which encoded the proteins, identified with proteomics were up-regulated. *nblA* gene used for the construction of the reporter strain and the *ntcA* gene (found in the literature to be induced under nitrate deficiency) were also up-regulated whereas those encoding some units of the phycobilisomes were constantly expressed.

ZUSAMMENFASSUNG

Seit mehreren Jahrzehnten wurde vermehrt vom Auftreten sogenannter Algenblüten berichtet. Diese Algenblüten deren Ursprung in der übermäßigen Vermehrung einiger Cyanobakterien liegt, können im Meer genauso auftreten wie in Süßwasser. Die genauen Hintergründe dieses Phänomens sind nicht vollständig geklärt; Nährstoffverfügbarkeit, Limitierungen und Überschüsse spielen jedoch eine wesentliche Rolle. Durch die Verfügbarkeit von Phosphor, Stickstoff, Schwefel und Eisen in bestimmten Verhältnissen wird das Algenwachstum stark gefördert. Aufgrund der verschiedenen negativen Auswirkungen der Algenblüten, u. a. die Freisetzung von Leber- und Neurotoxinen, ist ein tieferes Verständnis der Zusammenhänge notwendig um schließlich das Auftreten von Algenblüten bereits in ihrer Entstehung vorhersagen zu können.

Das Ziel dieser Arbeit war die Entwicklung und Validierung biotechnologischer Tools zur Beobachtung und Vorhersage von Algenblüten. Zur Vertiefung des Verständnisses des Verhaltens dieser Mikroorganismen bei niedrigen Nährstoffkonzentrationen, wird als Frühwarnsystem vor Algenblüten bei niedrigen Nitratkonzentrationen ein mikrobieller Test entwickelt und die Analyse der physiologische Änderung auf Basis von Transkriptom- und Proteomdaten wird aufgeführt.

Herstellung des Nitrat-Biosensors

Die Expression des *nblA* Gens wird in *Synechocystis* Stamm PCC 6803 und *Synechococcus* Stamm PCC 7942 bei Nitratmangel verstärkt induziert. Tatsächlich codiert *nblA* ein 59 Aminosäurepeptid (NblA), das essentiell für die Degradation von Phycobilin ist (Collier and Grossman, 1994, Richaud et al., 2001). Dieser Abbauprozess hat eine Veränderung der Zellfärbung von blau-grün zu gelb zur Folge (Bleichungsphänomen). Hauptgrund für die starke Verfärbung ist die eingestellte Synthese des Phycobilins (Collier and Grossman, 1992; Lau et al., 1977; Riethman and Sherman, 1988) in Verbindung mit dem schnellen Abbau des noch vorhandenen Phycobilins, das bis zu 50% des gesamten Zellproteins ausmachen kann.

Der im Rahmen dieser Arbeit zum Nachweis der Nitratverfügbarkeit in Wasser verwendete Reporter-Stamm wurde auf Basis des Cyanobakteriums *Synechocystis* sp. PCC 6803 hergestellt.

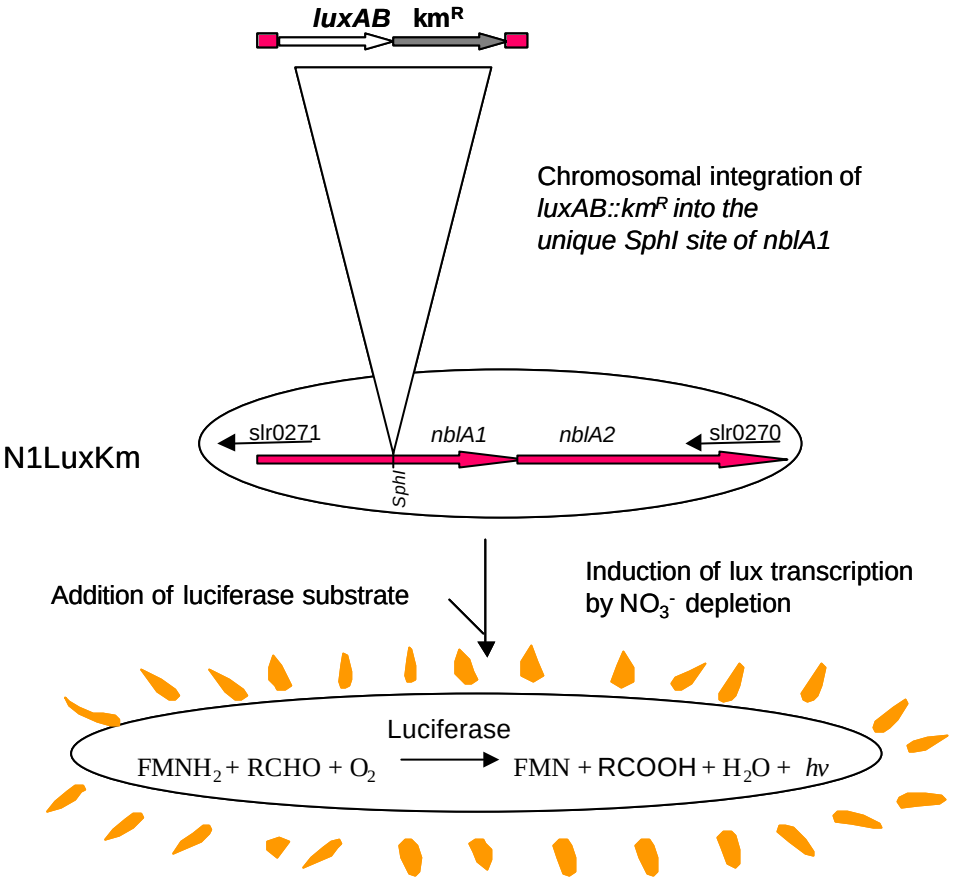


Abbildung 1: Herstellung des Nitrat-Reporter Stammes N1LuxKm

Durch Fusion des Gens dessen Expression durch Nitratlimitierung induziert wird, mit einem bakteriellen Biolumineszenz-Operon wurde ein Konstrukt erzeugt, das in Abhängigkeit von der Nitratkonzentration Licht emittiert: Hierzu wurde wie in Abb. 1 dargestellt, zunächst das Kanamycin Resistenzgen (km^R) mit dem Luciferase-Operon *luxAB* aus *Vibrio harveyi* fusioniert und der entstandene Komplex *luxAB-km*^{R} dann mit dem *nblA1* Gen von *Synechocystis* fusioniert und in dessen chromosomale DNA inseriert (*PnblA::luxABkm*^{R}). Der so entstandene Stamm N1LuxKm zeigte im Bereich von 4–100 µM Nitrat ein konzentrationsabhängiges Lumineszenzsignal (Abb. 2) und konnte nach Immobilisierung in Mikrotiterplatten als Biosensor für ca. einen Monat gelagert werden. Hierzu wurde N1LuxKm in „gewaschenem Agar“ immobilisiert und in Proben die unterschiedliche Nitratkonzentrationen enthielten für 10 Stunden bei kontinuierlicher Beleuchtung (50 $\mu E.m^{-2}.s^{-1}$) inkubiert. Die Messung der Biolumineszenz erfolgte dann im Luminometer. In Kombination mit ökologischen und physiologischen Daten kann dieser Sensor als Frühwarnsystem zur Vorhersage von Algenblüten eingesetzt werden.

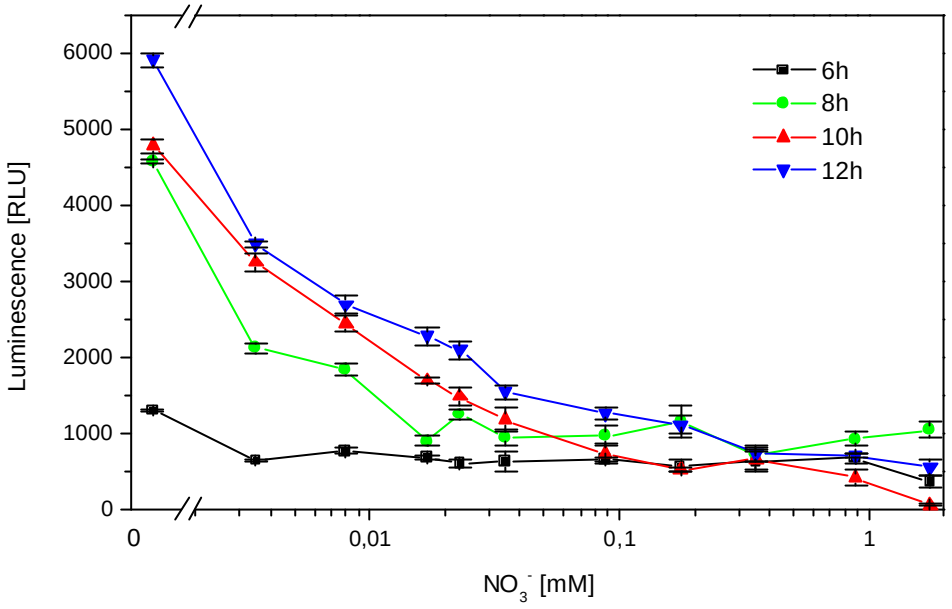


Abbildung 2: Induktion der Biolumineszenz im CyanoSensor in Abhängigkeit von der Probeninkubationszeit

Protein expression bei Nitratmangel

Die Analyse und Identifikation der während der Veränderung der Zellfärbung von blau-grün zu gelb des Cyanobakteriums bei Nitratmangel induzierten Proteine durch Proteomics könnte das Verständnis der Vorgänge vertiefen und gleichzeitig Zugang zu neuen Biosensoren ermöglichen. Hierfür wurden die löslichen Proteine von *Synechocystis* PCC 6803 aus Kulturen mit Nitratmangel und –überschuß extrahiert und durch zweidimensionale Gelelektrophorese aufgetrennt.

Die SDS-PAGE der zweiten Dimension erfolgte in einem selbst hergestellten Polyacrylamidgel (12% oder 15%, 200x250 mm^2, 1.5 mm) gemäß Görg et al. (1988). Nach dem Gellauf erfolgte ein Fixierungsschritt um die getrennten Proteine zu immobilisieren und Nicht-Protein-Bestandteile, die nach der Silberfärbung zu einen Hintergrund geführt hätten, zu entfernen.

Die gefärbten Gele wurden schließlich gescannt und die gefundenen Spots mit der Bildverarbeitung Phoretix 2D analysiert (Abb. 3). Zur Identifizierung wurden ausgewählte Proteine im Gel mit Trypsin geschnitten, anschließend die entsprechenden Spots aus den Coomassie-gefärbten Gelen ausgeschnitten und die Peptide durch HPLC und Massenspektrometrie analysiert.

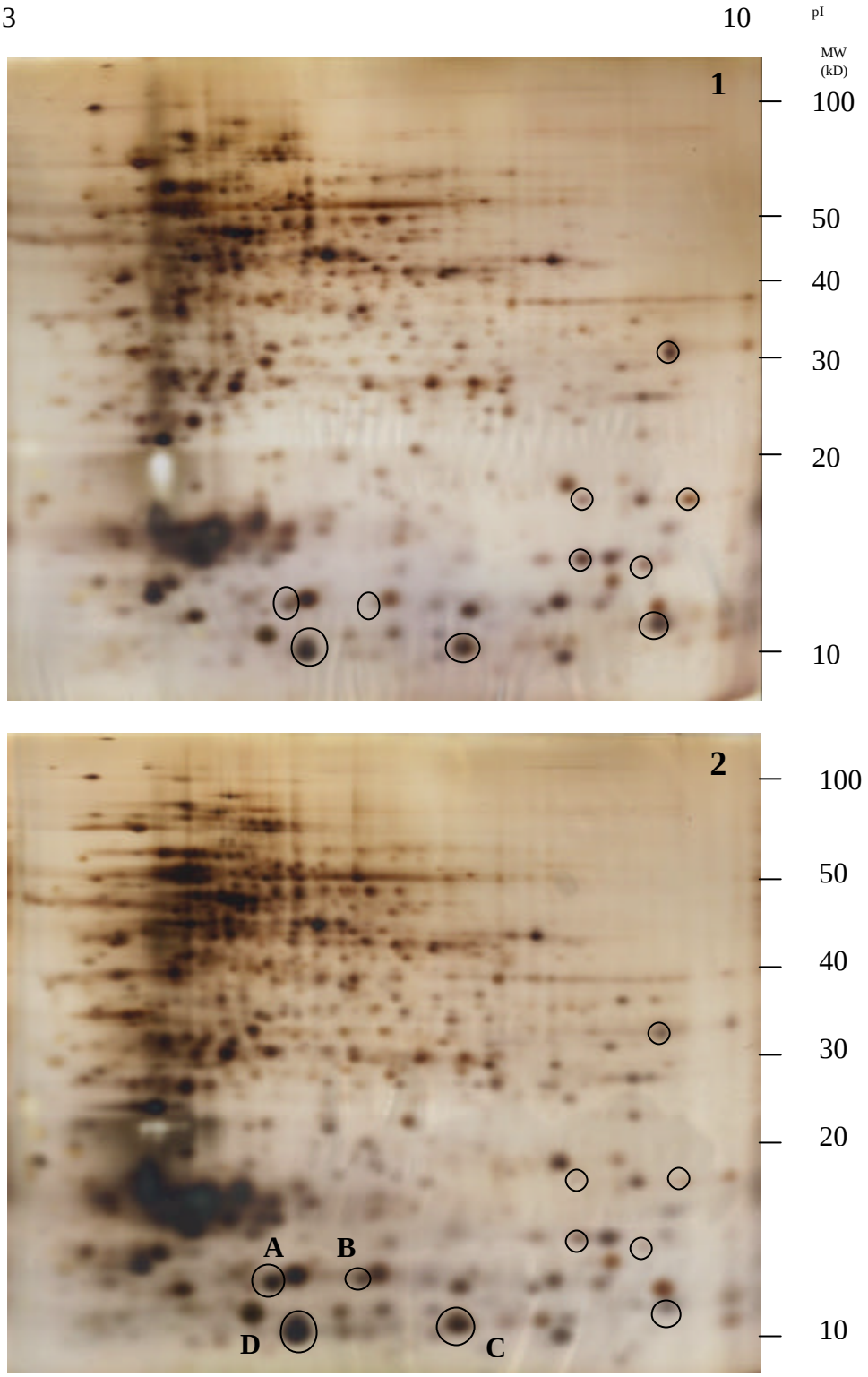


Abbildung 3: Proteinzusammensetzung der löslichen Fraktion von *Synechocystis* PCC 6803. Die Zellen wurden unter kontrollierten Bedingungen mit Nitratüberschuß (1, $+NO_3^-$) und unter Nitratmangelbedingungen kultiviert. Nach der 2D-PAGE erfolgte eine Silberfärbung

Alternativ wurden die Protein-Spots auf eine PVDF-Membran durch Elektro-Blotting übertragen und *N*-terminal ansequenziert. Vier Proteine die durch Nitratmangel induziert

wurden, konnten damit identifiziert werden: Bei den Spots A und B handelt es sich um zwei Isoformen des „Stickstoff-Regulator Proteins P-II“ welches durch das Gen *glnB* codiert wird. Bei Spot C handelt es sich um das “carbon dioxide concentrating mechanism protein”, das durch das *ccmK* Gen codiert wird und bei Spot D um das Plastocyanin welches durch das *petE* Gen codiert wird.
Aufgrund zu geringer Konzentrationen oder blockierter *N*-Termini konnten jedoch nicht alle interessierenden Proteinspots der 2D-Gele analysiert werden. Aufgrunddessen wurde im weiteren Verlauf des Projekts ein DNA-Microarray hergestellt, der eine Reihe von *Synechocystis*-Genen enthält, deren Regulation in Abhängigkeit der Nitratverfügbarkeit somit auf Transkriptionsebene parallel analysiert werden kann.

DNA Microarray, Analyse der Genexpression

Ein Microarray-Experiment besteht aus einem Array aus verschiedenen an eine Oberfläche immobilisierten DNA-Fragmenten, an die eine oder mehrere fluoreszenzmarkierte Proben hybridisiert werden. Der Hybridisierungsgrad wird dann über das Fluoreszenzsignal detektiert und gibt Aufschluß ob in der Probe die auf dem Träger immobilisierten DNA-Sequenzen vorhanden sind. Bei Genexpressionsanalysen werden zwei Proben miteinander verglichen wovon eine als Kontrolle dient. Um den Einluß des Nitrats auf das Genexpressionsverhalten von *Synechocystis* zu testen, wurden die Zellen in Nitrat-haltigen und Nitrat-freien Medien für 10 Stunden kultiviert. Von diesen Zellen wurde die RNA isoliert und daraus durch RT die cDNA synthetisiert. Die cDNAs der verschiedenen Proben wurden hierbei mit zwei unterschiedlichen Fluoreszenzfarbstoffen markiert. Anschließend wurden gleiche Mengen an cDNA der Proben die mit und ohne Nitrat kultiviert wurden auf den Arrays hybridisiert (Abb. 4). Anhand des Farbsignals (rot oder grün) ist zu erkennen ob die Expression der Gene durch Nitratmangel induziert oder reprimiert wurde.

Zur Quantifizierung der Spots wurde der Array mittels der Bildverarbeitungssoftware ImaGene ausgewertet. Für die Intensitäten jedes Gens wurde dabei der Mittelwert aus vier Spots gebildet (Abb. 5). Die Gene der durch Proteomics bereits identifizierten Proteine wurden wie erwartet durch Nitratmangel induziert. Das *ccmK* Gen wurde hierbei am stärksten induziert. Auch das *nblA* Gen, das im ersten Teil der Arbeit zur Herstellung des mikrobiellen CyanoSensors verwendet wurde, wurde exprimiert, stärker jedoch wurde das in der Literatur beschriebene *ntcA* Gen induziert, welches das „globale Stickstoff Regulationsprotein“ codiert.

Sämtliche Gene deren Produkte nach einiger Zeit des Nitratmangels degradierten, wurden wie in der Literatur beschrieben nicht exprimiert. Das *phoA* Gen, eines der Gene, dessen Verhalten unbekannt war wurde stark exprimiert, während das *isiA* Gen unter beiden Bedingungen konstant exprimiert wurde.

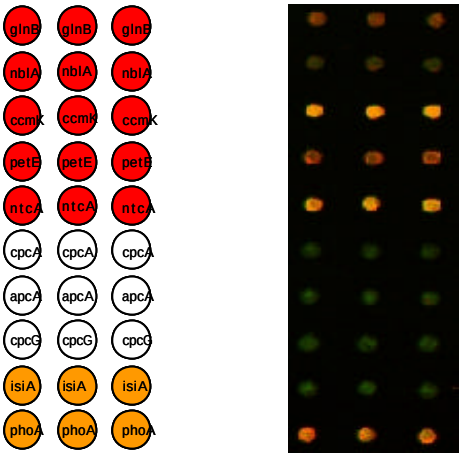


Abbildung 4: Zweifarben-Bild eines DNA-Microarrays von Genen aus Synechocystis PCC 6803 deren Expression durch Nitratmangel induziert oder reprimiert wurde. Die rechte Abbildung zeigt die Überlagerung zweier Hybridisierungen eines Microarrays in Falschfarbendarstellung, um die unterschiedliche Genexpression bei Wachstum mit Nitrat (Cy5, grün) bzw. bei Nitratmangel (Cy3, rot) zu visualisieren.

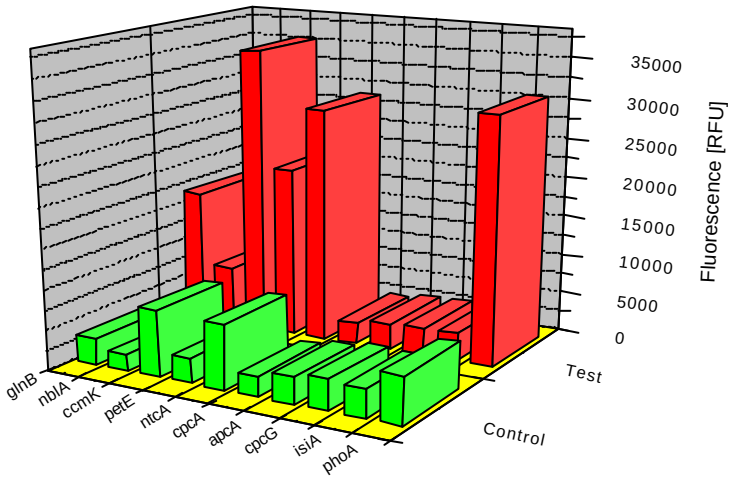


Abbildung 5: Fluoreszenzintensität von cDNA Proben aus Test- (Nitratmangel) und Kontrollbedingungen (mit Nitrat), die auf einem *Synechocystis* Microarray hybridisiert wurden.

Fazit

Der im Rahmen dieser Arbeit entwickelte Cyano-Sensor kann für den Nachweis der Bioverfügbarkeit von Nitrat in Wasser unterschiedlicher Herkunft eingesetzt werden. Die Immobilisierung der Cyanobakterien vereinfachte den Test und ermöglichte die Lagerung des Sensors über einen Zeitraum von einem Monat. In Kombination mit ökologischen und physiologischen Daten kann der Cyano-Sensor als Frühwarnsystem für Algenblüten eingesetzt werden.

Die physiologischen Veränderungen als Folge von Nitratmangel wurden mit Hilfe von Proteomics untersucht und durch Transkriptomics bestätigt. Dabei wurden neue Marker-Gene für niedrige Nitrat-Konzentrationen identifiziert, die für die Konstruktion neuer Bioreporter verwendet werden können.

1. INTRODUCTION

1.1. General introduction

An increasing awareness for environmental protection issues created a demand for progressively more sophisticated detection methods. In response, many chemical and physical methodologies were developed along with the required analytical equipment: gas or high-performance liquid chromatography, mass and atomic absorption spectrometry. The resulting techniques – accurate and sensitive – are costly and require specialized laboratories. In addition, they fail to provide data as to the bioavailability of pollutant, its effects on living systems, or its potential synergistic/antagonistic behavior in mixtures.

Thus, in parallel to the advances in the analytical chemistry, an increasing set of bioassays has been under continuous development for environmental monitoring purposes. A variety of organisms, cellular or subcellular systems has been employed for these purposes, from whole-organism assays such as fish toxicity testing to immunological determination of specific pesticides. Among the test organisms, a special position is held by bacteria: their large population sizes, rapid growth rates, low costs and easy maintenance often make them a more attractive option than other systems. Furthermore, as is becoming increasingly obvious, bacteria are endowed with an additional characteristic, which further augments their attractiveness: they are rapidly amenable to genetic manipulation. Thus, by relatively simple molecular biology techniques, bacterial strains can be “tailored” to emit a detectable signal upon a pre-specified change in environmental conditions.

In this work, a bioreporter assay employing a cyanobacterium was developed and its molecular characterization -grown in nitrate starvation conditions- at the levels of transcriptome and proteome was described.

1.2. Cyanobacteria

Like the discovery on the stromatolites from the Precambrian indicated, cyanobacteria belong to the oldest organisms on earth (Schopf and Packer, 1987). They are photosynthetic prokaryotes possessing the ability to synthesize chlorophyll *a*. Because of their superficial resemblance to eukaryotic green algae, the autotrophic cyanobacteria were once classified as "blue green algae". Although both groups are photosynthetic, they are only distantly related: cyanobacteria lack the histone proteins associated with eukaryotic chromosomes, a discrete

nucleus and internal organelles. According to this prokaryotic structure, cyanobacteria are classified as bacteria (Rippka et al., 1979). They are Gram-negative bacteria, but are, however, unique in having an internal membrane system, the photosynthetic thylakoid membrane.

The ecological diversity of cyanobacteria is noteworthy. They are found in almost every conceivable habitat, from oceans to fresh water to bare rock to soil. They are also found in extreme conditions of temperature, for example in the thermal springs or in the earth´s crust in the desert (Whitton and Potts, 2000). Some of the reasons for the success of cyanobacteria in modern habitats can be related to their long evolutionary history. Symbiotic interactions between cyanobacteria and other organisms are surprisingly diverse, including examples from many of the major phyla of plants and animals. Some cyanobacteria belong to the few groups of organisms that can convert inert atmospheric nitrogen into an organic form, such as nitrate ammonia or urea. Plants need for their growth this organic form of nitrogen, which must be obtained from soil. Therefore, cyanobacteria are very important organisms for the health and growth of many plants.

Cyanobacteria also provide an extraordinarily wide-ranging input to human daily life (Tiffany, 1958) and are of economic importance (Mann and Carr, 1992). Both the beneficial and detrimental features of the cyanobacteria are of considerable significance. Cyanobacteria are important primary producers and their general nutritive value is high. The nitrogen-fixing species contribute globally to soil and water fertility (Rai, 1990). Food production and solar energy conversion by cyanobacteria hold promising potential for the future (Skulberg, 1995). However, cyanobacteria may also be a source of nuisance in many situations. Proliferation of cyanobacteria in water reservoirs creates severe practical problems for water supplies. The development of strains containing toxins is a common experience in polluted inland water systems all over the world, as well as in some coastal waters.

1.2.1. Cyanobacterial blooms

The optimal growth of a cyanobacterial species depends on its ability to optimize resource capture, to utilize proficiently these resources and to minimize losses. It is improbable that any one organism will have the flexibility to excel under all conditions, but the appearance of a dominant suggests it has characteristics needed to maximize net growth under the prevailing environmental conditions. Nowadays cyanobacteria may come to dominate the phytoplankton of lakes, reservoirs and rivers. The term “bloom” describes generally a phytoplankton

biomass significantly higher than the lake's average. Surface blooms are comprised of cyanobacteria made buoyant by the presence of gas-filled cell inclusions (gas-vacuoles). It is these that have historically been referred to as "water blooms" (Reynolds and Walsby, 1975). The species containing gas-vacuoles are distributed across a number of genera and vary in form and size from small filaments to large globular colonies (Table 1.1).

Table 1.1 The major genera of gas-vacuolate, planktonic cyanobacteria (Oliver and Ganf, 2000)

Genus	N_2-fixer	Familly	Order
Anabaena	+	Nostocaceae	Nostocales
Anabaenopsis	+	Nostocaceae	Nostocales
Aphanizomenon	+	Nostocaceae	Nostocales
Nodularia	+	Nostocaceae	Nostocales
Cylindrospermopsis	+	Nostocaceae	Nostocales
Gloeotrichia	+	Rivulariaceae	Nostocales
Planktothrix	+/-	Oscillatoriaceae	Oscillatoriales
Spirulina	-	Oscillatoriaceae	Oscillatoriales
Microcystis	-	Chroococcaceae	Chroococcales
Gomphosphaeria	-	Chroococcaceae	Chroococcales
Coelosphaerium	-	Chroococcaceae	Chroococcales

Cyanobacterial blooms have been recorded from early history (Reynolds and Walsby, 1975) and for some decades those involved in water supply have been aware of their economic impacts due to impairment of water treatment processes including filter blockage, increased disinfection's costs and taste and odor problems. Cyanobacterial blooms also degrade the recreational value of surface waters, particularly where thick surface scums reduce the use of amenities for contact sports or large concentrations cause deoxygenation of the water leading to fish kills. Concern about the detrimental effects of freshwater cyanobacteria on water quality was heightened during the 1980s and 1990s as information accumulated on the potency of their toxins (Carmichael, 1994; Codd, 1994; Falconer, 1993; Gorham and Carmichael, 1988). It is during calm weather in summer and autumn that surface blooms of cyanobacteria frequently develop, often associated with minimum nutrient concentrations in the surface layer. In the tropics, cyanobacterial blooms and surface scums can be formed at almost any time of the year, as the annual solar input and air temperature are relatively constant. However, despite this relative constancy, there are major seasonal hydrographic and

meteorological changes that alter the phytoplankton community structure. Marked similarities occur between large tropical lakes such as Lake Victoria in East Africa (Talling, 1987) and Lake Lanao in the Philippines (Lewis, 1978).

1.2.2. Cyanotoxins

Toxic cyanobacterial blooms have been reported for over a century, one of the first being a *Nodularia* bloom described by Francis (Francis, 1878) in *Nature* (Carmichael, 1994; Luukkainen et al., 1994). The dense freshwater blooms not only affect the water's taste, odor or appearance, but more drastically, they are frequently highly toxic to wildlife, domestic livestock and humans, causing a range of allergic and gastroenteric responses. In the case of humans, the primary cyanobacterial toxicoses include acute liver diseases (hepatotoxicoses), peracute neurotoxicoses and gastrointestinal disturbances (Kuiper-Goodman et al., 1999).

Table 1.2 General features of the cyanotoxins (Sivonen and Jones, 1999)

Toxin group[1]	Primary target organ in mammals	Cyanobacterial genera[2]
Cyclic peptides Microcystins	Liver	*Microcystis, Anabaena, Planktopthrix, Nostoc, Hapalosiphon, Anabaenopsis*
Nodularin	Liver	*Nodularia*
Alkaloids, Anatoxin-a	Nerve synapse	*Anabaena, Planktothrix, Aphanizomenon*
Anatoxin-a(S), Aplysiatoxins	Nerve synapse Skin	*Anabaena* *Lyngbya, Schiszothrix, Planktothrix*
Cylindrosperopsins	Liver[3]	*Cylindrospermopsis, Aphanizomenon, Umezakia*
Lyngbyatoxin-a Saxitoxins	Skin, Gastrointestinal tract Nerve axons	*Lyngbya* *Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharides (LPS)	Potential irritant; affects any exposed tissue	All

[1]Many structural variants may be known for each toxin group.
[2]Not produced by all species of the particular genus.
[3]Whole cells of toxic species elicit widespread tissue damage, including damage to kidney and lymphoid tissue.

Despite their aquatic origin, most cyanotoxins appear to be less toxic to aquatic biota than to humans and other terrestrial mammals (Sivonen and Jones, 1999). Table 1.2 shows the different classes of cyanotoxins and their producers.

In recent years, the frequency of cyanobacterial blooms in amenity water bodies has greatly increased the interest shown by various groups, such as the water management organizations and environmental protection agencies (Codd and Beattie, 1991). This interest can be traced not only to illness and death of domestic animals and wildlife, as well as to human illnesses, which have been attributed to contact and/or ingestion of toxic cyanobacterial blooms, but to the potential of the microcystins as potent tumors promoters (Codd and Beattie, 1991; Falconer, 1991). There is still insufficient information to evaluate the extend of the problem caused by the latter, since it is not yet considered in most water quality guidelines, such as the World Health Organization's recommended limit of 1 µg microcystin LRL-1 drinking water (Falconer, 1991; Ito et al., 1997). Relatively little is known on the natural grazers of cyanobacteria and other aquatic biota. There is evidence to show that zooplankton may exhibit both physiological and behavioral adaptations, which enhance their ability to co-exist with toxic cyanobacteria (DeMott et al., 1991). Atoxigenic and toxigenic strains have been isolated from the same bloom, but there is little information as to the factors that regulate expression i.e. a toxic strain may become a non-toxic strain or *vice versa*. Toxicity not only varies between strains, but between clones of the same isolate (Carmichael, 1992; Lukac and Aegerter, 1993; Utkilen and Gjölme, 1992). In addition, some strains produce three or more toxins, with the relative proportions being influenced by environmental factors such as light, pH, nitrate, phosphate, metal ions and temperature (Carmichael, 1992; Keevil, 1991; Lukac and Aegerter, 1993; Utkilen and Gjölme, 1992; Watanabe and Oishi, 1985; Wicks and Thiel, 1990). Field data on the influence of environmental factors on toxin formation in particulars species have to be assessed with care, because any observed effect may be due to varying influences on different species. For instance, a comparison of 72 Finnish lakes (Vaitomaa et al., 1998) indicated that higher concentrations of aqueous phosphate (P) favoured hepatoxic *Microcystis* and anatoxin-a containing *Anabaena* blooms, whereas *Anabaena* blooms with unknown neurotoxicity were associated with low P and high nitrate.

1.3. Assimilatory nitrogen metabolism and its regulation

Nitrogen (N) can represent as much as about 11% of the dry weight of a cyanobacterial cell (Wolk, 1973). The cyanobacteria mainly use inorganic compound (dinitrogen) to fulfill their

N requirements, but urea and other organic sources of N, such as some amino acids, nitrate and ammonium, can also be assimilated by some cyanobacteria. A general scheme describing major routes in N-assimalatory metabolism is depicted in Fig. 1.1. The fact that urea and inorganic N sources other than ammonium are first metabolized to ammonium to allow assimilation of their N atoms is highlighted in the left part of Fig. 1.1. Some amino acids like arginine are assimilated by the production of ammonium among their metabolic products. The central position played in cyanobacterial N metabolism by the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle for ammonium assimilation is also highlighted in Fig. 1.1. Glutamate produced in this pathway is not only the major N-donor for the biosynthesis of other N-containing metabolites but is itself a precursor for some amino acids and 5-aminolevulinate, the immediate precursor for porphyrin, phycobilin and chlorophyll biosynthesis. Glutamine also donates N to some metabolites. Finally, Fig. 1.1 shows that the amino acids arginine and aspartate together make up cyanophycin, a unique storage reservoir of N (and carbon) found in many cyanobacteria.

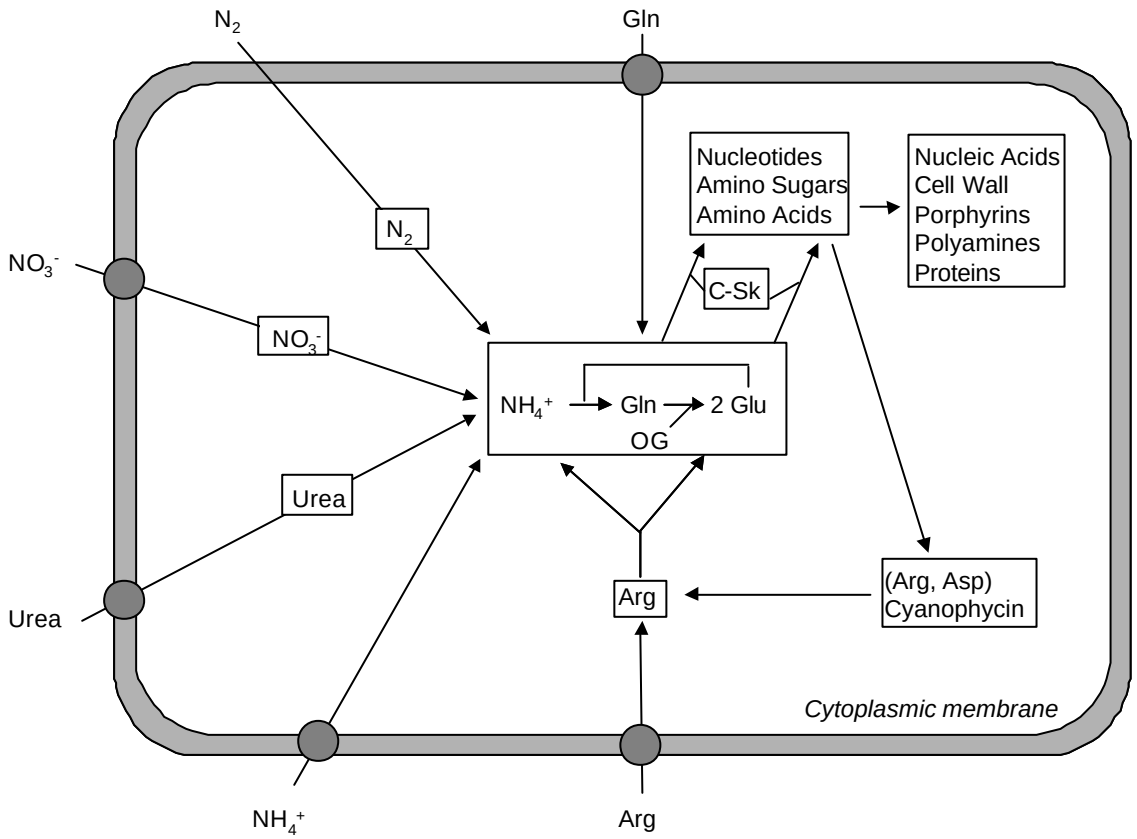


Fig. 1.1 Major routes in cyanobacterial nitrogen. Stoichiometries between substrates and products are not indicated. The participation of permease in the uptake of some nitrogen sources is illustrated. C-Sk, carbon skeletons; OG, 2-oxoglutarate.

1.3.1. Nitrogen fixation

Many cyanobacteria are able to grow at the expenditure of atmospheric N_2 under aerobic conditions and many more are able to perform N_2 fixation when anaerobic conditions are provided experimentally. The machinery for fixing N_2 has been shown to be present in all the currently recognized taxonomic groups of cyanobacteria. A phylogenetically coherent group of filamentous cyanobacteria, that includes genera such as *Anabaena*, *Nosto*c, and *Fischerella* of the taxonomic classification of Rippka (Rippka et al., 1979), has developed a complex and efficient mechanism for performing N_2 fixation under aerobic conditions. This consists of the development of heterocysts, specialized cells that, under conditions of aerobiosis and combined-N deprivation, differentiate from vegetative cells at semi-regular intervals in the filament and bear a series of modifications devoted to the protection of N_2-fixation apparatus from O_2. The enzyme complex nitrogenase catalyses the ATP-dependent reduction of N_2 to two molecules of ammonium during the N_2 fixation reaction. A region of the chromosome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 containing the *nifHDK* genes, encoding the three polypeptides of nitrogenase, was identified and cloned by means of hybridization with the *Klebsiella pneumoniae nif* genes (Mazur et al., 1980; Rice et al., 1982). In the same cyanobacterium, mRNAs hybridizing to *nifH, nifD, nifK* (Rice et al., 1982), *nifB* (Mulligan et al., 1988) have been found in filaments derepressed by incubation in N-free medium, but not in ammonium-grown cells. However, because heterocysts do not develop in the presence of ammonium, absence of *nif* gene expression in ammonium-supplemented cultures might be a consequence of inhibition by ammonium of heterocyst development, with ammonium not having a direct effect on *nif* gene expression. Therefore, ammonium seems negatively affect nitrogenase synthesis besides the effect it has on heterocyst development.

1.3.2. Nitrate assimilation

Nitrate is almost certainly the most abundant source of combined N for cyanobacterial nutrition. The assimilation of nitrate by cyanobacteria involves nitrate uptake and reduction of intracellular nitrate (via nitrite) to ammonium, which is the N form incorporated into organic compounds. Nitrite, which can also fulfill the N requirement of cyanobacteria, is taken up into the cell and then reduced to the level of ammonium. Intracellular nitrate is reduced to nitrite in a two-electron reaction catalyzed by nitrate reductase (Nar), the resulting nitrite being then reduced to ammonium in a six-electron reaction catalyzed by nitrite reductase (Nir). Both Nar

and Nir use reduced feredoxin as physiological electron donor (Arizmendi and Serra, 1990; Manzano et al., 1976; Méndez et al., 1981).

When reduced N in the form of ammonium is available, it is preferentially used over other good N sources such as nitrate and nitrite. The negative effect of ammonium on nitrate assimilation is exerted at two different levels: (1) ammonium addition provokes an almost immediate cessation of the uptake of nitrate by cyanobacterial cells that are actively assimilating this nutrient; this inhibitory effect operates at the level of nitrate transport (Flores et al., 1980; Lara et al., 1987; Ohmori et al., 1977); (2) exposure to ammonium results in repression of the synthesis of proteins involved in nitrate assimilation. The inhibition of nitrate uptake by ammonium requires that ammonium be incorporated into carbon skeletons through the GS/GOGAT cycle, as ammonium is unable to exert any negative effect on nitrate uptake in cells that bear an inactive GS or an inactive GOGAT (Flores et al., 1980). It seems more likely that ammonium inhibition of nitrate uptake operates through feedback inhibition exerted by product(s) of ammonium assimilation via GS (Flores et al., 1980). Nitrate uptake by *Synechocystis* sp. strain PCC 6801 exhibits a strict dependance upon the operation of CO_2 fixation, and it has been proposed that some CO_2-fixation product(s) may have a regulatory, positive action on nitrate uptake (Flores et al., 1983). When cells of a variety of cyanobacteria are transferred from ammonium-containing media to media containing either nitrate or nitrite as the sole of N source, an increase in the cellular levels of Nar (Herrero et al., 1981), Nir (Herrero and Guerrero, 1986) and the 48 kDa cytoplasmic membrane protein that is part of the nitrate transporter (Madueno et al., 1988; Sivak et al., 1989), is observed. Moreover, in *Synechococcus* sp. strain PCC 7942 (Luque et al., 1994; Suzuki et al., 1993), mRNA of nitrate assimilation genes is not detected in the presence of ammonium. Thus, ammonium promotes inhibition of the synthesis of nitrate-assimilation proteins.

1.4. Nitrate starvation in cyanobacteria

Nutrient-limited growth of non N_2-fixing cyanobacteria induces a set of general responses, which include cessation of cell division and important morphological and physiological alterations such as loss of photosynthetic membranes, increase of glycogen and inclusion bodies, and loss of pigments (chlorophyll, phycobiliproteins, and all carotenoids except zeaxanthin). Besides these general effects, certain nutrient-specific responses have been described, such as increased synthesis of high-affinity transport systems, synthesis of more

readily transported metabolites, and synthesis of new type of phycocyanin (Bhaya et al., 2000).

The effect of nitrogen (N) depletion on the abundance of pigment molecules in several cyanobacteria has been well documented, in *Anacystis nidulans* (Allen and Smith, 1969), *Synechococcus* sp. (Yamanaka et al., 1978), *Anabaena* (Foulds and Carr, 1977; Wood and Haselkorn, 1980), and *Synechocystis* strain PCC 6803 (Elmorjani and Herdman, 1987). The resulting decrease in chlorophyll and phycolbilisome (PBS) content leads to a dramatic change in cell color from normal blue-green to yellow-green, which is known as "bleaching" or "chlorosis". PBS, which can constitute up to 50% of the total cellular protein, is progressively, rapidly, and almost completely degraded; the chlorophyll content also declines. In this sense, phycocyanin (PC), the major constituent of PBS, acts as a nitrogen store. The material released by protein degradation may provide substrates for the synthesis of polypeptides required for acclimation to new N status (Allen and Smith, 1969). In some *Synechococcus* species, bleaching also occurs in response to nutrient starvation for sulfur (S) (Jensen et al., 1984; Schmidt et al., 1982) phosphorus (P) (Ihlenfeldt and Gibson, 1975), carbon (Miller and Holt, 1977), and iron (Fe) (Sherman and Sherman, 1983). For starvation under such nutrients conditions, as PBSs are poor source of S-containing amino acids and do not contain P and Fe, their degradation would be rather for minimizing the absorption of excess excitation energy under stress conditions (Schwarz and Grossman, 1998). Nutrient starvation in various bacteria has been shown to induce the synthesis of proteins that are involved in DNA protection (Pena et al., 1995), detoxification (Hummerjohann et al., 1998; Pena and Bullerjahn, 1995), or the formation of storage products (Lehmann and Wober, 1976). In cyanobacteria, the amino acids used for the synthesis of such proteins could be provided by proteolysis of the phycobiliproteins. In *Synechococcus* PCC 7942, three genes (*nblA*, *nblR*, and *nblB*) are essential for degradation of the phycobilisomes, and loss of their function leads to a non-bleaching phenotype during nitrogen and sulfur starvation (Collier and Grossman, 1994; Dolganov and Grossman, 1999; Schwarz and Grossman, 1998). The *nblB* gene is constitutively expressed, whereas transcription of the *nblA* gene (encoding a small polypeptide of 59 amino acids) initiates phycobilisome degradation during nutrient starvation (Dolganov and Grossman, 1999). Thus, expression of *nblA* is controlled by the *nblR* gene product, which exhibits strong similarity to the response regulator OmpR (Schwarz and Grossman, 1998). It has been suggested that NblR integrates diverse environmental signals that lead to the degradation of the phycobilisomes (Schwarz and Grossman, 1998). Various regulatory proteins exert acclimation to the availability of various nitrogen sources. NtcA was

originally identified in Synechococcus PCC 7942 as a global activator of ammonium–repressed genes (Luque et al., 1994; Vega-Palas et al., 1992). It has also been shown that NtcA is involved in the early steps of heterocyst differentiation (Frias et al., 1994; Wei et al., 1994) and the expression of genes that are not directly associated with nitrogen metabolism, e.g., rbcLS (carbon-fixation gene; (Ramasubramanian et al., 1994)) and *gor* (encoding the antioxidant protein gluthatione reductase; (Jiang et al., 1995)). In the genome of *Synechocystis* sp. strain PCC 6803, two nblA homologs, nblA1 and nblA2 are present (Baier et al., 2001; Kaneko et al., 1996). In the present work, *nblA* gene is involved in the construction of a bioreporter (CyanoSensor) for nitrate availability monitoring in water.

1.5. *Synechocystis* sp. strain PCC 6803

Synechocystis sp. PCC 6803 is an unicellular non-nitrogen-fixing cyanobacterium and an inhabitant of freshwater. This strain is naturally transformable by exogenous DNA, and has been one of the most popular organisms for genetic, biochemical and physiological studies particularly for the analysis of oxygenic photosynthesis because of the capability of growth photoheterotrophically (Grigorieva and Shestakov, 1982). The genome of *Synechocystis* sp. PCC 6803 is small (3.57 Mbp) compared to those of plants and has been completely sequenced (Kaneko et al., 1996). The *Synechocystis* genome contains 3168 genes. Genome sequencing has been followed by proteome analysis using 2D electrophoresis combined with microsequencing to characterize expressed proteins. With the use of these techniques, 234 protein spots have so far been identified (Sazuka et al., 1999).

1.6. Cyanobacterial reporter-assay

In order for a bacterial cell to function as a “microbial bioreporter” or a “microbial biosensor”, it has to contain two linked genetic elements: a sensing element and a reporter. The former senses the presence of the target molecule(s), and turns on the latter which emits a detectable signal. The reporter element is always one of a typical set of genes or groups of genes, coding for proteins with an easily detectable presence or activity, as will be described in more details below. The sensing element, in contrast is different in each bacterial sensor, and in its selection lies the distinctiveness and specificity of the final construct. In most cases, the sensing element is a promoter for a gene or a group of genes normally activated in response to a specific or general environmental change. Under normal conditions, this

activation would lead to the synthesis of proteins, the presence or activity of which would help the cell combat the sensed hazard or adapt to it. In the recombinant strain, in addition to this function, the selected promoter also drives the synthesis of the reporter protein(s).

Using this principle, a promoter sequence from one bacterial species can be genetically fused to a reporter gene from a second species and introduced into the cells of a microorganism. In practice, in order for the promoter sequence to sense its target chemicals, an additional element has often to be included: the regulatory mechanism mediating the signal from the sensed molecule to the promoter. This can easily be achieved if the host cell is also the origin of the promoter used. The fused promoter::reporter can be introduced into the host cell in one of two options: either as a plasmid, normally a multicopy one, or integrated into the bacterial chromosome, as depicted in Fig. 1.2. The latter option, which calls for a somewhat lenghtier molecular procedure, allows better stability of the system but may suffer from reduced signal strength.

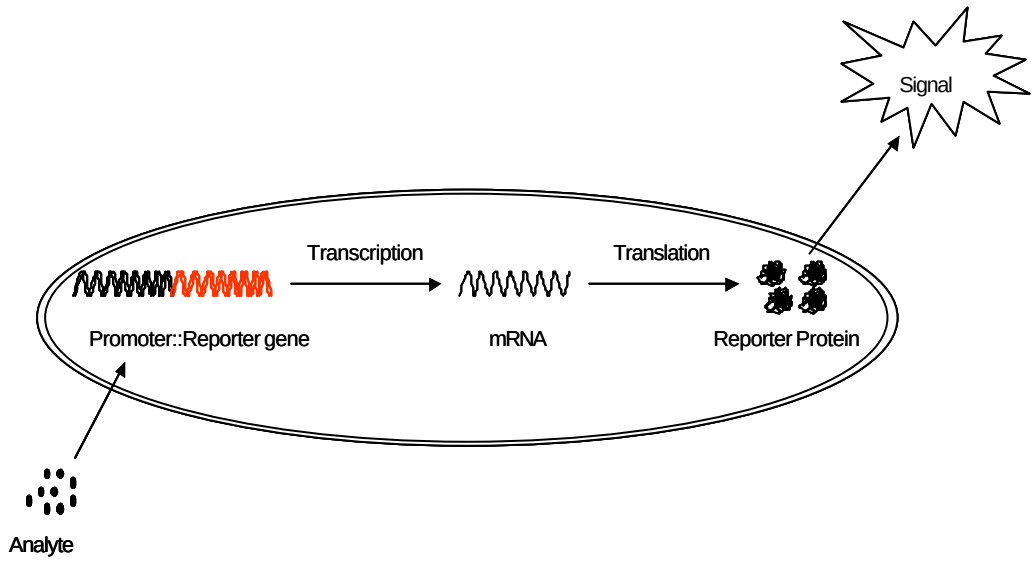


Fig. 1.2 Principe of a microbial reporter-assay

In environmental analytics many microbial reporter assays were developed using appropriate promoters. Their application lies mainly in the investigation of pollutants, like heavy metals and organic solvents, as well as stress factors and genotoxicity (Köhler et al., 2000). Some examples of the application of the microbial reporter assays are outlined in table 1.3. Beside ß-galactosidase, alkaline phosphatase, and green fluorescent protein are luciferases, which are

strongly applicated as reporter proteins in the reporter strains since their discovery. Bacterial luciferases catalyze the obligately aerobic oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde (RCHO) to flavin mononucleotide (FMN) and the corresponding carboxylic acid (RCOOH), with light emission at around 490 nm and a quantum yield of about 0.1.

$$FMNH_2 + RCHO + O_2 \xrightarrow[\text{luciferase}]{\text{Bacterial}} FMN + RCOOH + H_2O + h?$$

The luciferase is encoded by *luxA* and *luxB* of the *lux* operon, and the synthesis enzymes for the aldehyde substrate are coded by *luxCDE* (Meighen and Dunlap, 1993). In constructs where only luxAB is present, the aldehyde has to be added externally.

Table 1.3 Examples of the application of microbial reporter assays.

Analyte	Promoter	Reporter	Reporter strain	References
Heavy metals	*smt*	*lux*	*Synechococcus*	(Erbe et al., 1996)
Hg^{2+}	*mer*	*lux*	*E. coli*	(Selifonova et al., 1993)
Naphtalene	*nahG*	*lux*	*Pseudomonas*	(Heitzer et al., 1994)
Stress conditions	*grpE, dnaK, ion*	*lux*	*E. coli*	(Van Dyk et al., 1995)
	OxyR, micF, katG	*lux*	*E. coli*	(Belkin et al., 1996)
Genotoxicity	*rad54*	*gfp*	*Saccharomyces*	(Billinton et al., 1998)
	cda	*lux*	*E. coli*	(Ptitsyn et al., 1997)
	umu	*lacZ*	*Salmonella*	(Oda et al., 1985)
	Sfi	*lacZ*	*E. coli*	(Quillardet et al., 1982)

The firefly luciferase is encoded by the *luc* gene. The oygen-dependent bioluminescent reaction is based on energy transfer from ATP to the substrate, D-luciferin, to yield oxyluciferin, AMP, carbon dioxide and light emission at 560 nm with a quantum yield of 0.88 which is the highest known for bioluminescent reactions (Gould and Subramani, 1988). The substrate, luciferin, has always to be added externally because of its negative charge at the physiological pH, which makes its transfer difficult through the cellular membrane.

$$ATP + Luciferin + O_2 \xrightarrow[\text{luciferase}]{\text{Firefly}} AMP + PPi + Oxyluciferin + CO_2 + h?$$

Modern analytical methods, along with sophisticated instruments, which allow the rapid and sensitive detection of virtually any chemical in a high-throughput format, are developed; but these analytical techniques do not provide information about the potential biological damaging or the bioavailability of compounds under study. To overcome this problem, bioanalytical methods have been developed as a powerful supplement to chemicals analysis. Cyanobacteria were among the first organisms to be used for the construction of whole-cell biosensors for environmental monitoring. Because of the ecological importance of these phototrophic microorganisms, they are ideally suited for the monitoring of compounds that inhibit photosynthetic activity.

Cyanobacterial amperometric sensors employing shuttled (Rawson et al., 1989) or direct electron transfer (Croisetiere et al., 2001) have been used for the detection of phytotoxic pollutants, but these sensors were unstable and lacked robustness and specific technical requirements. The use of genetically modified microbial cells by introduction of a "reporter gene" to connect the initial biological interaction of the tested chemical or physical event to an easily recordable output signal (e.g. light) could overcome these shortcomings. The most commonly used reporter proteins are ß-galactosidase, green-fluorescent protein (GFP) and luciferase, from either bacteria (e.g. from *Vibrio fisheri*, *Vibrio harvei*, *Xenorhabdus luminescens*) or insects (e.g. *Photinus pyralis*). The advantages and disadvantages of reporter proteins used in cell-based biosensing systems are depicted in table 1.4. The use of the bacterial luciferase in cyanobacteria as a bioreporter for physiological studies has been reviewed by Fernandez-Pinas et al. (2000). The application of genetically-engineered bioluminescent cyanobacteria as whole cell biosensors in ecotoxicology and environmental monitoring (Bachmann, 2003) has recently flourished. A bioluminescent *Synechocystis* PCC 6803-derived reporter strain for monitoring the correlation between cyanobacterial activity and the presence of environmental toxicants was recently described by Shao et al. (Shao et al., 2002). Shao al. introduced a construction consisting of a constitutive *tac* promoter fused to *luc* (the gene encoding the firefly luciferase) and *luxAB* (encoding the bacterial luciferase) into the cyanobacterial chromosome, which ensured a genetically stable bioreporter. Finally, only the firefly luciferase was used because the substrate of the bacterial enzyme proved to be inhibitory in their assays. Furthermore, Firefly luciferase is especially well suited to the measurement of the energy status of an organism because it depends directly on ATP concentration. A bioluminescent reporter assay based on the fresh water cyanobacerium *Synechococcus* PCC 7942, for the detection of iron availability at trace levels was developed

(Durham et al., 2002) and was recently characterized fully and applied to further field samples (Durham et al., 2003).

Table 1.4 Advantages and disadvantages of reporter proteins used in cell-based biosensing systems (Daunert et al., 2000)

Reporter protein	Advantages	Disadvantages
Chloramphenicol acetyl transferase	No endogenous activity	Often employs radioisotopes. Requires addition of a substrate. Requires separation of substrate and product. Narrow linear range.
ß-galactosidase	Sensible and stable. Moderate linear range. Applicable in anaerobic environment	Endogenous activity. Requires addition of a substrate.
Bacterial luciferase	High sensitivity. Does not require addition of substrate. No endogenous activity in mammalian cells	Heat labile therefore limited use in mammalian cells. Narrow linear range.
Firefly luciferase	High sensitivity. Broad linear range. No endogenous activity in mammalian cells	Requires addition of a substrate. Requires an aerobic environment and ATP
Aequorin	High sensitivity. No endogenous activity in mammalian cells	Requires addition of a substrate and the presence of Ca^{2+}
Green fluorescent protein	Autofluorescent, therefore, does not require addition of a substrate or cofactors. Spectral variants. No endogenous homologues in most systems. Stable at biological pH	Moderate sensitivity. Requires posttranslationnal modification. Background fluorescence from biological system may interfere. Potential cytotoxicity in some cell types
Uroporphyrinogen III methyltransferase	Autofluorescent, therefore, does not require addition of a substrate or cofactors. May have better signal-to-noise ratio than green fluorescent protein. Does not require costly reagents or special host strains.	Endogenous activity.

Using *luxAB* gene, a variety of other cyanobacterial reporters for bioavailability determination have been described in *Synechocystis* PCC 7942 for heavy metal (Erbe et al., 1996) and phosphorus (Gillor et al., 2002), and in *Synechocystis* PCC 6803 for iron (Kunert et al., 2000) and nitrogen (Richaud et al., 2001). A bioreporter strain for the detection of chemicals as benzene, toluene, ethylbenzene, and xylene was constructed in *Pseudomonas putida* using the

lux cassette (*luxCDABE*) (Applegate et al., 1998), and in *Escherichia coli* using *luc* gene for firefly luciferase (Willardson et al., 1998). Cyanobacterial biosensors used in environmental monitoring offer a means for the biologically relevant estimation of chemical residues and nutrients that cannot be achieved by conventional analyses; therefore, genomics and proteomics methods will help to identify genes and proteins that respond to particular analytes.

1.7. Transcriptome and proteome analysis

Recently, biological vernacular has been expanded with a series of "omes": the genome (the complete genetic information of an organism), the transcriptome [all messenger RNAs (mRNA) expressed at a given time in a cell] and the proteome (the protein equivalent). The latest in the family has been dubbed the metabolome and is a term for all small molecules that are a product of enzymatic and chemically activity within the cell. In contrast to the genome, which is fairly inert, the latter three molecular groups are highly dynamic and vary greatly according to endogenous and exogenous conditions; they also vary throughout the life cycle of an organism. The dynamic expression of genes as mRNA (the transcriptome) can be followed in a quantitative and qualitative manner using binding assays based on DNA arrays (Schena et al., 1995). Alternatively, sequencing methods based on either differential-display polymerase chain reactions (PCRs) (Liang and Pardee, 1992) or tagged-DNA approach termed serial analysis of gene expression (Velculescu et al., 1995) can be used. However, there are several key objections to reducing biological studies to the monitoring of changes in mRNA: (1) the level of mRNA does not allow one to predict the level of protein expression (Anderson and Seilhamer, 1997; Gygi et al., 1999); (2) protein function is controlled by many post-translational modifications; and (3) protein maturation and degradation are dynamic processes that dramatically alter the final amount of active protein, independent of the mRNA level. In order to be able to correlate mRNA levels with protein expression, modification and activity, there should be a systematic method for separating and visualizing the proteins components of a cell that allows the: (1) extraction and high-resolution separation of protein components, including membrane, extreme-pI and low-copy-number proteins; (2) identification and quantification of each component; and (3) comparison, analysis and visualization of complex changes in expression patterns.

The technology of DNA-microarrays relies on the ability to decipher the genetic basis of various phenotypes. It is an emerging tool for analysing the biological function of the

information encoded in the genome of particular organisms and/or tissues focus on the monitoring of transcription (mRNA) and translation (proteins) processes. mRNA and protein expression patterns should be simultaneously considered to fully develop a conceptual understanding of the functional architecture of genomes and gene networks.

1.7.1. DNA-microarrays

DNA-microarray analysis has emerged in the last few years as a flexible method for analyzing large numbers of nucleic acid fragments in parallel. Its origins can be marked out to several different disciplines and techniques. Microarrays can be defined as a continued development of molecular biology hybridization methods, as an extension of the use of fluorescence microscopy in cell biology, as well as a diagnostic assay using capture to solid surface. The convergence of ideas and principles utilized in these fields, together with technological advancements in preparing miniaturized collections of nucleic acids on solid supports, have all contributed to the appearance of microarray and microchip technologies. Analysis of nucleic acids by hybridization is a universally adopted key method for analysis in molecular biology. Filter-based dot blot analysis has been used for a long time as a suitable method for analyzing multiple samples by hybridization. Classical gene expression analysis methods such as Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR) and nuclease protection assays, are best suitable for analyzing a limited number of genes and samples at a time. Filter-based gene expression analysis has enabled simultaneous determination of expression levels of thousands of genes in one experiment, by reversing the Northern blotting principle so that the labeled moiety is derived from the mRNA sample and the immobilized fractions are the known sequences traditionally used as probes. These macroarrays have been widely adopted for gene expression studies, because of the ease of use of these filter-based methods and their compatibility with general lab equipment (Baldwin et al., 1999). One disadvantage to using this method has been the relatively large size and the autofluorescence of the membrane, which prevents efficient use of multiplexed fluorescent probes and subsequently limits the number of samples that can be analyzed in each experiment.

Despite the variety of technical solutions that have been developed for performing microarray analysis, all are miniaturized hybridization assays for studying thousands of nucleic acid fragments at the same time. All microarray systems share the following key components: (1) the array, which contains immobilized nucleic acid sequences, or ‘probes’, (2) one or more

labeled samples or 'targets', that are hybridized with the microarray, and (3) a detection system that quantitates the hybridization signal.

DNA-Microarrays consist of a set of nucleic acid sequences immobilized onto a solid support so that each unique sequence forms a small feature, called a 'spot'. The size of these spots varies from one system to another, but it is usually less than two hundred micrometers in diameter. A glass slide or glass wafer acts as the solid support onto which up to tens of thousands of spots can be arrayed in a total area of a few square centimeters. The microarray sample that is being analyzed, whether it is mRNA for a gene expression study or DNA derived from genomic analysis, is transformed to a labeled population of nucleic acids, the target. Fluorescent dyes, and especially the cyanine dyes Cy3 and Cy5, have been adopted as the main label in microarray analysis. In microarray hybridization, the labeled fragments in the target are expected to form duplexes with their immobilized complementary probes. This requires that the nucleic acids are single-stranded and accessible to each other. The number of duplexes formed reflects the relative number of each specific fragment in the target, as long as the amount of immobilized target nucleic acid is in excess and not limiting the kinetics of hybridization.

Microarrays have not become a replacement to established techniques, but more a novel, high-power approach to perform analyses that were previously time consuming. Gene expression analysis, genome analysis, and drug discovery have been three of the main areas in which microarray analysis has been applied so far.

1.7.2. Gene expression analysis

Two or more RNA samples are compared in the gene expression analysis to identify differences in the abundance and identity of the transcripts they contain. The transcript populations need to be labeled in order to convert the information they contained into a form that can hybridize with microarrays and subsequently detected. The convertion of mRNA population into a labeled first-strand cDNA population is one of the simplest and most popular labeling strategies. This is achieved by copying the transcripts into cDNA molecules with a reverse transcriptase while incorporating a modified CyDye nucleotide (Fig. 1.3).

Usually, hybridization involves annealing a single-stranded nucleic acid to a target complementary strand. In the gene expression technique, nucleic acid sequences (samples) from the genomic DNA are attached to a membrane and incubated within a solution of fluorescently labeled cDNA -the target (Fig.1.3).

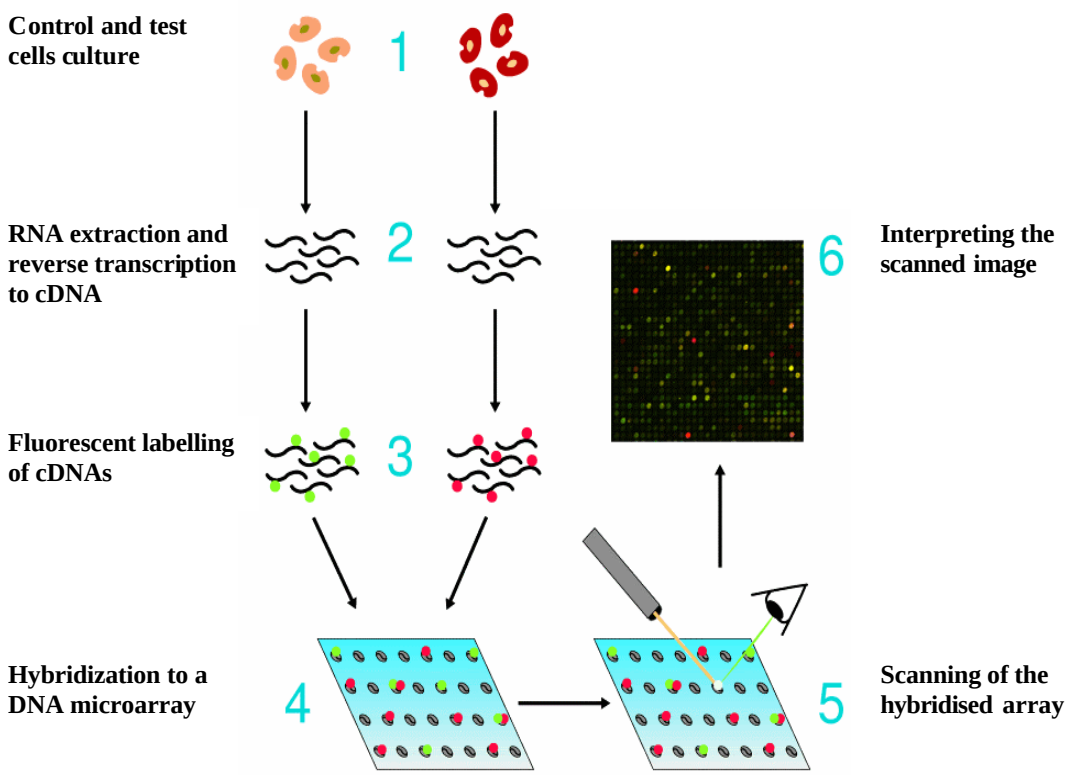


Fig. 1.3 Comparative hybridization experiment
[http://www.cs.wustl.edu/~jbuhler/research/array/array.png]

The binding of probe molecules to the sample, the target, highlights complementary sequences. The cDNA synthesis can be primed with a choice of primers including random primers, anchored oligo (dT) as well as gene specific primers. This allows the use of both mRNA and total RNA as sample. Messenger RNA molecules, otherwise called transcripts, carry the genetic information encoded in genes. These transcripts represent only a small proportion of the total RNA in most cells, whereas ribosomal and transfer RNA account for more than 98% of total RNA. In any cell type, the transcript population typically consists of thousands of distinct transcripts, most of which are transcribed from different genes (although splice variants of genes exist too). These transcripts can be present in widely varying amounts ranging from just a few copies per cell to thousands of copies. Furthermore, the relative levels of transcripts are constantly changing as the cell responds to different environmental signals. The amount of transcripts is estimated to follow a normal distribution in which a small number of genes are expressed at high or very low levels. The majority of the genes are expressed at intermediate levels. The identity of transcripts that make up the messenger RNA populations and their expression levels are informative of cell state and activity of genes and, as the precursors of translated proteins, changes in mRNA levels are related to changes in the

proteome. Gene expression using DNA microarray technique has been already used in the cyanobacterium *Synechocystis* sp. PCC 6803 for the identification of genes expressed in response to irradiation with intermediate-wavenlength UV light and white light (Huang et al., 2002b); the transcriptional profile of the light-to-dark transition (Gill et al., 2002); and the identification of genes expressed during acclimation to high light (Hihara et al., 2001).

1.7.3. Proteomic technologies

Genomics-based approaches to biomarker development include the measurements of expression of full sets of mRNA, such as differential display (Liang and Pardee, 1995; Zhang et al., 1997), serial analysis of gene expression (Datson et al., 1999; Velculescu et al., 1995), and large-scale gene expression arrays. However, interpreting the best data and adapting the results to a particular application remain challenging. Although studies of differential mRNA expression are informative, they do not always correlate with proteins concentrations (Anderson and Seilhamer, 1997; Gygi et al., 1999). The field of proteomics (the simultaneous analysis of total gene expression at the protein level) represents one of the premiere strategies for studying biological systems and understanding the relationship between various expressed genes and gene products. The great task lying ahead is to elucidate the functions of all proteins encoded in the genomes of sequenced model organisms. This process involves the collection of information about the temporal, spatial, and physiological regulation of proteins, their interaction partners, biochemical activities, posttranslational modifications, and the mutual influence of all these parameters on the physiology of the organism. Proteomics is a large-scale screening of the proteins of a cell, organism or biological fluid; a process, which requires stringently controlled steps of sample preparation, 2D electrophoresis, image detection and analysis, spot identification, and database searches. The core technology of proteomics is 2D electrophoresis. At present, there is no other technique, which is capable of simultaneously resolving thousands of proteins in one separation procedure.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O´Farrell, 1975), in which proteins are separated according to the isoelectric point (pI) by isoelectric focusing (IEF) in the first dimension and according to size (MW) by SDS-PAGE in the second dimension, has the unique capacity for the resolution of complex mixtures of proteins, permitting the simultaneous analysis of hundreds or even thousands of gene products. However, the exchange of 2D gel data between laboratories has been a major problem because of the spatial

irreproducibility of 2D gels generated by the conventional method of 2D-PAGE using carrier ampholite (CA) IEF. Equilibrium CA-IEF cannot be achieved because of pH gradient instability with prolonged focusing time, as the pH gradient move towards the cathode and flattens in the centre. Finally, the problems of pH gradient instability and irreproductibility were overcome by the introduction of immobilized pH gradients (IPG) for IEF (Fig. 1.4) (Bjellqvist et al., 1982). A basic protocol for horizontal as well as vertical two-dimensional electrophoresis with IPGs in the first dimension (IPG-Dalt) was established in 1988 (Görg et al., 1988). Since that time, the protocol has not been changed essentially but the employment of IPG-Dalt has produced significant improvements in 2D electrophoresis separation, permitting higher resolution, especially with narrow-range IPGs and reproducibility of 2D patterns both within a laboratory and, more important, between laboratories (Blomberg et al., 1995; Corbett et al., 1994). Two-dimensional electrophoresis is unique in its ability to detect post- and co-translational modifications, which cannot be predicted from the genome sequence. Application of 2D gel electrophoresis include proteome analysis, cell differentiation, detection of disease markers, monitoring therapies, drug discovery, cancer research, purity checks, and microscale protein purification.

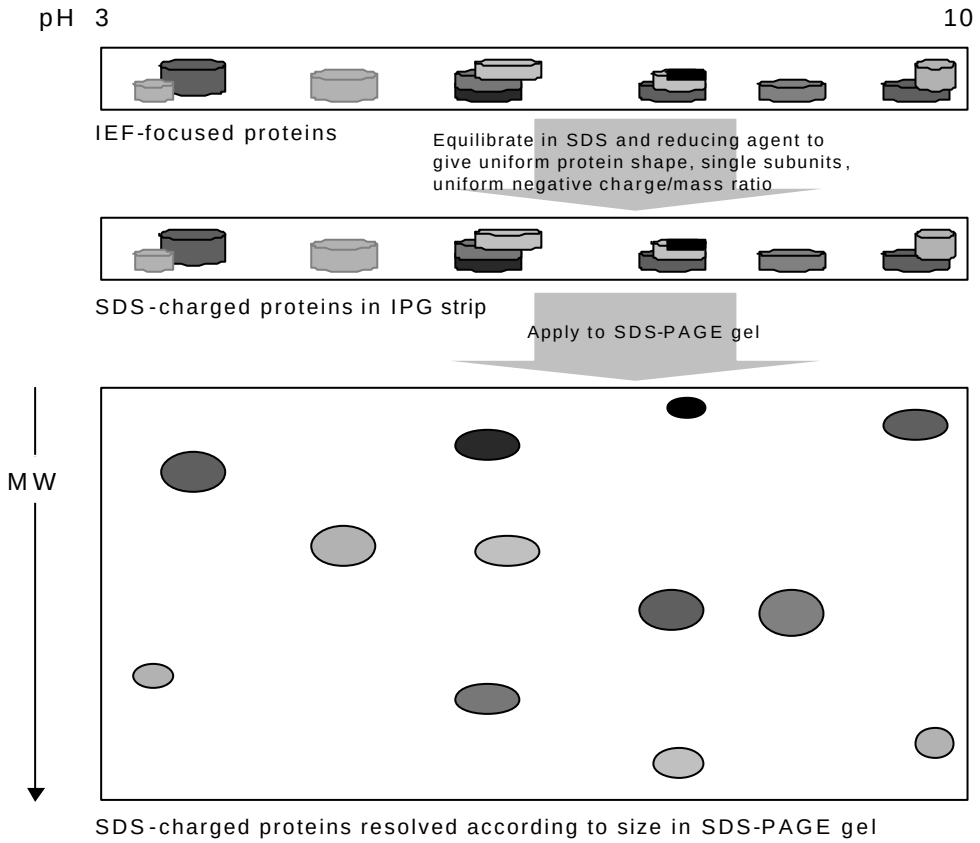


Fig. 1.3 Schematic diagram showing separation of proteins by SDS-PAGE after separation by IEF

The proteome of *Synechocystis* PCC 6803 has been studied by electrophoresis and *N*-terminal amino acid sequencing. In this study 234 protein spots could be identified (Sazuka et al., 1999). Plasma membrane protein (Huang et al., 2002a) and peripheral proteins from thylakoid membranes (Wang et al., 2000) were also identified in this cyanobacterium. The proteome analysis also represents a powerful tool for the study of acclimation to environmental stress conditions, because newly synthesized or enhanced expressed proteins, so-called stress proteins, can be identified. Salt-induced proteins (Fulda et al., 2000), and light-induced proteins (Merrick and Edwards, 1995) have been already identified in *Synechocystis* PCC 6803.

1.7.4. MALDI-TOF/Mass spectrometry analysis of proteins

Conventionally, Edman degradation has been used to obtain partial amino acid sequences for protein identification and the design of an oligonucleotide for gene cloning (Edman and Begg, 1967; Totty et al., 1992). This method requires substantial amounts of materials, and its low sensitivity makes it difficult to sequence the regulatory proteins, which are often present in low abundance. Therefore, the analysis of these proteins requires more sensible tools.

Mass spectrometry (MS) has changed its appearance in the scientific world considerably during recent years. It started out as a tool in atomic physics 100 years ago to a new ionization method, fast atom bombardment, which made MS a viable tool in the field of biochemistry 20 years ago (Barber et al., 1981). Approximately 13 years ago, with the invention of electrospray ionization [ESI; (Fenn et al., 1989)] and matrix-assisted laser desorption ionization [MALDI; (Karas and Hillenkamp, 1988)], MS became a major tool for biology, biomedicine and molecular medicine. MALDI has helped to establish the MS platform as an important tool in proteomics. This technique of ionization has been instrumental in bringing the mass spectrometer, which measures the mass of a molecule, to the forefront of proteomics research. MALDI enables conversion of biomolecules into a charged gaseous state that is essential for analysis by the mass spectrometer. Today, MALDI has successfully entered the field of medical research (for example, in cellular diagnostics, gene therapy and cancer research). Investigation of complex (multi-component) samples and the direct characterization of cellular material are two typical applications of MALDI. In proteome research, MALDI is routinely employed for protein characterization, typically after two-dimensional gel electrophiresis and enzymatic digestion. Although MALDI is limited to providing molecular

weight information only (rather than structural information), its spread in the proteome field is unprecedented.

MALDI was developed as an extension of the older technology of laser desorption ionization (LDI), which has been used extensively (and still being used) in the field of microprobe analysis (Van Vaeck and Gijbels, 1990a; Van Vaeck and Gijbels, 1990b). LDI of soluble compounds is based on the simple approach of air-drying analyte solutions on a metal target and forming ions [using an ultra-violet (UV) laser pulse] that can be detected by time-of-flight (TOF) mass analysis. The main limitation of this approach is that analyte substances usually have rather different spectral absorptions at the laser wavelength used and often are completely transparent. Only highly absorbing molecules are accessible by LDI, while non-absorbing molecules can only be ionized at the expense of extensive fragmentation. The useful mass range is rather low ($m < 1000$ u), because the thermal stress from absorption of the laser light does not allow the desorption of larger molecules as intact entities. Furthermore, even under ideal conditions, LDI has a rather low ionization yield (and, thus, analytical sensitivity) and mainly produces neutral desorbed material (Spengler et al., 1988). All these disadvantages are circumvented by detaching the energetic processes necessary for desorption and ionization from the analyte molecules; this is done by employing an intermediate matrix for energy transfer.

The mechanisms of ion formation from solid material by laser irradiation were found to consist of at least three different pathways; each contributes to the ionization of molecules to a variable degree, depending on the experimental conditions (Spengler et al., 1987). The pathways are: (1) photo-ionisation by individual photo-molecule interactions, as gas-phase photo-ionisation of single molecules; (2) ionization by protonation or de-protonation via interactions between excited, ionized and/or protonated (or de-protonated) molecules and neutral molecules, as in chemical ionization mechanisms; (3) cluster decay of small peptides formed by lattice disintegration, leading to charge inhomogeneities and charging of the remaining molecular entities, as liquid-spray methods. The introduction of the MALDI method has overcome the strong substance-specific variability that exists among the various ion-formation processes. The matrix in the MALDI approach allows the separation of the analytes molecules from each other and allows only small interactions between the analytes molecules and the target. Matrix substances are usually relatively volatile in comparison to the analyte molecules, leading to an efficient desorption ("evaporation") of material.

1.8. Aim of the work

There is a wealth of information on the biochemical and physiological responses of cyanobacteria to environmental stress. The nutrient status of the environment is critical in modulating the activity and biogenesis of the photosynthetic apparatus. In both terrestrial and aquatic environments cyanobacteria experience prolonged periods during which they are limited for one or more nutrients. Under conditions of limiting nutrient availability, most cyanobacteria increase the efficiency with which they scavenge nutrients from the environment, initiate the metabolization of internal nutrients reserves, and limit metabolic activities, which are not essential for survival. Some of the responses to nutrient limitation are specific to the deficiency of a particular nutrient and include elevated synthesis of transport systems and enzymes that may increase the availability of the limiting nutrient to the cell.

There is a lack of tools sensitive enough to study the effects of the minute nutrient concentrations on cyanobacterial physiology. The methods used till now to understand the nutritional status of natural cyanabacteria communities are mostly based on chemical determination of nutrient concentrations, assays of uptake rate, study of the effects of added nutrients on primary production. These techniques fail to provide data such as the bioavailability of a nutrient, its effects on living systems or its potential synergistic/antagonistic in mixtures. Moreover, it is unclear whether these techniques would function in natural environments with variable chemical composition. In this work, we attempted to overcome this problem with a novel approach: We monitored the responses of a cyanobacterium under nitrate limitation condition. For this purpose, a gene that is induced upon nitrate limitation was fused to a bacterial bioluminescence operon to yield a construct that emits light according to the ambiant concentration of nitrate. In order to increase the practicality of this concept, the sensor strain called CyanoSensor was immobilized into an easy-to-use format, which can be stored.

In order to understand cellular processes resulting from nitrate starvation and their influence on cyanobacterial blooms, the proteome dynamics of *Synechocystis* PCC 6803 was analyzed through 2D gel elctrophoresis, MALDI-TOF/MS of trypsin-digested protein fragments and N-terminal amino acid sequencing. The information gained with proteomics was confirmed and extended by RNA expression analysis related to nitrate depletion using DNA microarrays where some genes of the cyanobacterium *Synechocystis* PCC 6803 were spotted.

2. MATERIALS AND METHODS

In the present work, all the chemicals in pure degree were supplied by the firms Sigma, Fluka, Merck and Riedel de Haen. The cyanobacterium *Synechocystis* PCC 6803 WT and mutant used in this work were grown in BG-11 medium.

2.1. BG-11 medium for cyanobacteria

Component	Final concentration	Amount
$NaNO_3$	17.65 mM	1.5 g
$K_2HPO_4.3H_2O$	0.18 mM	0.04 g
$MgSO_4.7H_2O$	0.30 mM	0.075 g
$CaCl_2.2H_2O$	0.25 mM	0.036 g
Citric acid	0.03 mM	0.006 g
Ferric ammonium citrate	0.03 mM	0.006 g
EDTA (disodium salt)	0.003 mM	0.001 g
Na_2CO_3	0.19 mM	0.02 g
Trace metal mix	See below	1.0 ml
HEPES	200 mM, pH 7	10 ml
Distilled water		to 1.000 l

Trace metal mix

Component	Amount ($g.l^{-1}$)
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.222 g
$NaMoO_4.2H_2O$	0.390 g
$CuSO_4.5H_2O$	0.079 g
$Co(NO3)_2.6H_2O$	0.049 g
dH_2O	to 1.000 l

Synechocystis PCC 6803 WT and mutant were grown in BG-11 medium and the different tests were performed according to various nitrate concentrations in the medium. In nitrate free

BG-11 medium, $NaNO_3$ was replaced by equimolar amount of NaCl. For the culture of the mutant N1LuxKm, 25 µg/ml kanamycin were added to the medium.

The present work is divided into 3 sections. The list of specific materials used and the methodologies are described in each of the three sections. Table 2.1 and table 2.2 show the list of devices used in all the 3 sections and the list of software and online resources used.

Table 2.1 List of devices used and the corresponding suppliers

Purpose	Instrument	Company
Autoclave	Autoclav IP 44 Autoclav Pacs 2000	Certoclav, Traun, Austria Getringe, Rochester, USA
Balance	Basic, MC Research RC 210D Precision Advanced	Sartorius, Göttingen, Germany
Centrifugation	Centrifuge 5417R, Centrifuge 5810R Minispin	Eppendorf, Hambourg, Germany
Incubator	Certomat R with Certomat HK	B. Braun Biotech, Melsungen, Germany
pH-Meter	Digital pH-Meter pH500	WTW, Weilheim, Germany
Photometer	Ultrospec 3000 UV/Vis	Amersham Biosciences, Freiburg, Germany
Pipettes	Eppendorf Pipettes	Eppendorf, Hamburg, Germany
Vortex	Vortex Genie2	Scientific Industries, New York, USA
Water bath	DC10 + K20	Thermo Haake, Karlsruhe, Germany

Table 2.2 Software and online ressources

Products	Manufacturer or Website
Array designer 2.0	Bio.com, Emeryville, USA
Cyano2Dbase	http://www.kazusa.or.jp/tech/sazuka/cyano/proteome.html
ImaGene	BioDiscovery, Los Angeles, USA
Melanie Viewer	GENEBIO, Geneva, Switzerland
Phoretix 2D Software	Nonlinear Dynamics, Newcastle, UK
Protein Prospector	UCFS, San Francisco, USA

2.2. Development of a bioreporter assay for the detection of nitrate

2.2.1. Materials

The materials used in this section are described in table 2.3. Each item is described with the corresponding purpose and supplier.

Table 2.3 List of materials

Item	Purpose	Company
Spectroquant Nitrat-Test kit	Nitrate concentration determination	Merck, Darmstadt, Germany
Optiplate white, 24 wells	Immobilization of the bioreporter	Packard, Groninger, Nederland
Luminometer (LUMIStar)	Bioluminescence measurement	BMG, Offenburg, Germany

2.2.2. Microbiological methods

2.2.2.1. Strains and growth conditions

The recombinant bioluminescent reporter strain N1LuxKm, a nitrate reporter strain of *Synechocystis* PCC 6803, was constructed by the CNRS laboratory in Paris, France (Richaud et al., 2001). The culture medium BG-11 was prepared as described (Rippka, 1988) and modified by buffering to pH = 7.0 with 2 mM HEPES. To substitute nitrate in nitrate free medium, $NaNO_3$ was replaced by equimolar amount of NaCl. The medium was called: BG-$11_{(-N)}$ (nitrate free medium). N1LuxKm was grown photoautotrophycally in BG-11^{km} or 1/10BG-11^{km} in the same conditions as *S.* PCC 6803 is cultivated. *S.* PCC 6803 and N1LuxKm cells were incubated in 50 ml medium (OD_{720} = 0.2) in an Erlenmeyer flask (250 ml) at 29°C with orbital shaking (100 rpm) and continuous illumination (50 µE.m^{-2}.s^{-1} of white light) up to the optical density (OD_{720}) 1.0. 25 µg/ml kanamycin was added in the medium for N1LuxKm. Afterwards, the cells were newly incubated in fresh medium at OD_{720} = 0.2 for further experiments.

2.2.2.2. Storage of bacterial strains

For a short storage of the bacterial strain, cells were spreaded on a solid agar medium with a loop. Recovery and growth of the strains on agar surfaces was enhanced by autoclaving the agar (30 g) in 500 ml H_2O and 500 ml BG-11 medium separately and mixing the solutions at 45-50°C just before pouring into the plates. Cells on agar plates were incubated in the same conditions as liquid cultures for up to 6 weeks. For a long storage of the strains, 25 ml cells at the stationary phase were harvested and resuspended in 900 µl fresh medium. 100 µl of 50% sterile DMSO were added to the suspension and immersed immediately in liquid nitrogen, then stored at – 80°C. To make a new culture, 1 ml of the DMSO stored cells were slowly thawed on ice and resuspended in 50 ml fresh medium in a 250 ml Erlenmeyer at low light intensity (5 $\mu E.m^{-2}.s^{-1}$) without shaking for 3 days. Afterwards, cells were incubated in the standard conditions described above.

2.2.3. Preparative methods

2.2.3.1. Construction of the bioluminescent strain

The genetically engineered, bioluminescent reporter strain, used in this work for the detection of nitrate availability in water, was constructed from the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Richaud et al., 2001). This reporter strain was constructed by the fusion of a gene that is induced upon nitrate limitation to a bacterial bioluminescence operon to yield a construct that will emit light according to the ambient concentration of nitrate. The nitrate reporter strain was constructed by the fusion of a kanamycin resistant gene (km^R) to the luciferase operon *luxAB* from *Vibrio harveyi* leading to a *luxAB-km*^{R} gene complex. This gene complex was then fused with the *nblA1* gene of *Synechocystis* in its chromosomal DNA, leading to *PnblA::luxABkm*^{R}. This reporter strain was designated N1LuxKm. Because the *luxCDE* genes of the *lux* operon, which encoded the enzymes for the synthesis of the substrate for the luciferase enzyme was absent in the reporter strain N1LuxKm, the substrate solution (nonanal) was added externally. The resulted light emission (bioluminescence) level was expected to increase with decreasing the nitrate concentration in the medium.

2.2.3.2. Immobilization of the reporter strain in agar and agarose

The reporter strain N1LuxKm was immobilized in agar and agarose as follows: N1LuxKm cells from 1/10BG-11 were harvested by centrifugation, washed twice with $BG\text{-}11_{(-N)}$ medium

and resuspended in the same medium at the final cell density (OD_{720}) = 1.0. Cells were immobilized by pipetting 100 µl of cells suspension and 150 µl of matrix (agar or agarose, 1% in ddH_2O at 39°C) in each well of a 24-wells microtiter plate followed by mixing. After gelation, the sensors were either used immediately or stored at 4°C in the dark. To avoid drying during storage the sensor was covered with 1 ml 1/500 BG-11 medium per well. Prior to use, the stored sensor was preconditioned by adjusting to room temperature and washed with 1 ml $BG\text{-}11_{(-N)}$ per well.

2.2.3.3. Immobilization of the reporter strain in washed agar

Washed agar was prepared according to the method of Waterbury (Waterbury et al., 1987): 100 g of agar were washed by stirring with 3 liters of double-distilled water in a 4-liter beaker. After 30 min of stirring, the agar was allowed to settle, the water was siphoned off, and the agar was filtered onto whatman filter paper in a Büchner funnel. This procedure was repeated once more until the filtrate was clear. The agar was then washed with 3 liters of 95% ethanol followed by a final 3-liters wash with analytical grade acetone. The agar was then dried at 50°C in glass baking dishes for 2-3 days and stored in a tightly covered container.
Cells were immobilized by pipetting 100 µl of cells suspension and 150 µl of washed agar (0.8% in ddH_2O at 39°C) in each well of a 24-well microtiter plate followed by mixing. After gelation, the sensors were either used directly or stored as described with agarose or agar.

2.2.4. Analytical methods

2.2.4.1. Characterization of N1LuxKm in liquid medium

N1LuxKm and *S.* PCC 6803 cells from BG-11 medium cultures at the exponential growth phase were harvested by centrifugation (3220 x g, 10 min, 15°C), washed twice with $BG\text{-}11_{(-N)}$ medium and resuspended in different media containing various nitrate concentrations at a final OD_{720} = 0.12-0.2. During the growth and after different incubation times, N1LuxKm was characterized through 3 parameters: (1) the growth rate, (2) the bioluminescence of the cells and (3) the nitrate concentration residual in the medium.

Growth rate: The optical density was measured photometrically at 720 nm using a fresh medium as blank.

Bioluminescence of the cells: In order to have a linearity at high cell density, the cell culture was diluted with the supernatant to the range OD_{720} = 0.1–0.5 before bioluminescence

measurement. For the bioluminescence measurement, 250 µl of the cell suspension were pipetted in a well of 24-wells microtiter-plate and measured at 26°C with a luminometer. The substrate solution (table 2.4) for bioluminescence reaction catalyzed by the luciferase enzyme, added automatically by the luninometer, was prepared daily. The luminescence was measured in intervals 15 minutes after the addition of substrate (50 µl) and the maximal value attained was taken into account and expressed in relative light unit (RLU). The bioluminescence intensity was expressed in RLU/OD_{720}.

Nitrate concentration residual: After cells harvesting by centrifugation, the concentration of nitrate left in the supernatant was determined by the kit Spectroquant^{R} Nitrate-Test following manufacturer's instructions.

Table 2.4 Substrate solution for the luciferase enzyme

Component	Concentration	Amount
n-nonanal in DMSO	0.1%(V/V)	1 ml
Igepal in ddH_2O	0.1%(W/V)	1 ml
ddH_2O		to 8 ml

2.2.4.2. Spectrum of analytes

The reporter strain N1LuxKm and the WT of *Synechocystis* PCC 6803 were incubated in BG-11 medium lacking one essential nutrient each, such as nitrate, ammonium, phosphate and sulfate. The induction and the bioluminescence measurement were performed after different incubation times as described for the liquid culture (see section 2.2.4.1).

2.2.4.3. Induction and measurement of the bioluminescence in the immobilized reporter strain

The bioluminescence induction studies were performed by adding 1 ml of various media depending on NO_3^- concentration (0-17.6 mM) to freshly prepared sensors or preconditioned stored ones. After different incubation times of the immobilized sensor at 29°C and continuous illumination (50 $\mu E.m^{-2}.s^{-1}$ of white light), the luminescence measurement was performed as described for liquid culture (see section 2.2.4.1.). Either in liquid cultures or in the immobilized forms, the bioluminescence could be detected in each type of incubation; the time of incubation and the concentration of different nutrients were chosen accordingly to the goals of a given experiment.

2.3. Proteomics technologies

2.3.1. Materials

The materials used in this section are described in table 2.5 and 2.6

Table 2.5 List of some materials used in proteomics

Item	Supplier
10 kDa Protein ladder	Gibco BRL Life Technologies, Eggenstein, Germany
Acrylamide/Bis solution (30%)	Bio-Rad Laboratories, Richmond, USA Roth GmbH, Karlsruhe, Germany
BCA protein Assay reagent kit	Pierce, Rockford, Germany
Cocktail tablets Complete^{R} (protease inhibitor)	Boehringen, Mannheim, Germany
Glass beads	Helmut Clauss, Nidderau, Germany
IPG gel	Amersham Biosciences, Freiburg, Germany
Plain Glass Plates	Amersham Biosciences, Freiburg, Germany
SDS-PAGE Caster of 2D gel	Amersham Biosciences, Freiburg, Germany
SDS-PAGE standard (low molecular weight)	Bio-Rad Laboratories, Richmond, USA
Sequi-blot PVDF membrane	Bio-Rad, Munich, Germany

Table 2.6 List of devices used in proteomics

Instrument	Purpose	Company
Alpha I-5	Freeze-dryer	Christ medizi-nischer Apparatebau, Osterode, Germany
EPS 2A200	Power supply for 2D-PAGE	Amersham Biosciences, Freiburg, Germany
HP1100	HPLC	Agilent Technologies, Palo Alto, USA
HS 501 digital IKA-Horizontal shaker	Shaker	IKA Werke, Staufen, Germany
IPGphor	1. Dimension electrophoresis	Amersham Biosciences, Freiburg, Germany
ISO-DALT	2. Dimension electrophoresis	Amersham Biosciences, Freiburg, Germany

Minigel-Twin G42 Model 583 Gel Dryer Power Pac 3000, Power Pac 300, Model 200/2.0 Power Supply	PAGE-System	Biometra, Göttingen, Germany BioRad, Munich, Germany
Multitemp III	Thermostatic circulator	Amersham Biosciences, Freiburg, Germany
Precise Protein Sequencer	Protein Sequencing	Applied Biosystems, Weiterstadt, Germany
ScanJet 5p	2D gel scanning	Hewlett-Packard, Palo Alto, USA
Trans-blot SD	Protein Blotting	Bio-Rad, Munich, Germany
Voyager STR-DE	Mass Spectrometry	Applied Biosysttems, Weiterstadt, Germany

2.3.2. Preparation of the polyacrylamide gel for 2D-PAGE

Vertical SDS-PAGE was performed in the DALT apparatus originally described by Anderson and Anderson (1978). A stacking gel is usually not necessary in this system. SDS gels were casted in the polymerization cassettes (200 x 250 mm^2), which are made in the shape of books consisting of two glass plates connected by a hinge strip, and 1.5 mm thick spacer between them. The cassettes were stacked vertically into the gel casting box with the hinge strips to the right interspersed with plastic sheets. The front plate of the casting box was put in place and screwed on the nuts, then a polyethylene tube was connect to a funnel held in a ring-stand at a level of about 30 cm above the top of the casting box.

Table 2.7 Recipes for vertical SDS-PAGE gel casting

Component	Final acrylamide gel concentration	
	12%	15%
Acrylamide/Bisacrylamide (30.8% T, 2.6% C)	400 ml	500 ml
Gel buffer: 1.5 M Tris-HCl pH 8.8	250 ml	250 ml
dH_2O	329 ml	229 ml
SDS (10%)	10 ml	10 ml
Ammonium persulfate (10%)	10 ml	10 ml
TEMED (10%)	1.43 ml	1.14 ml
Final volume	1000 ml	1000 ml

The other end of the tube was placed in the grommet in the casting box side chamber. This side chamber was filled with heavy displacing solution (50% (v/v) glycerol in dH_2O and 0.01% (w/v) bromo-phenol blue) and immediately before gel casting TEMED and ammonium persulfate solutions were added to the gel solution (table 2.7) and poured into the funnel. When pouring was complete, the tube was removed from the side chamber grommet so that the level of the displacing solution in the side chamber fell. About 1 ml of overlay buffer (buffer-saturated 2-butanol) was pipetted very carefully onto the top of each gel in order to obtain a smooth, flat gel top surface. Gels were allowed to polymerize for about five hours at room temperature.

2.3.3. Molecular methods

2.3.3.1. Extraction of soluble proteins

Soluble proteins were extracted after different incubation times of the test (nitrate starved) and the control cells. 2D-PAGE sample preparation was carried out as described in Pena et al. (1995) with some modifications. *Synechocystis* sp. strain PCC 6803 cells (50 ml) were collected by low-speed centrifugation (3220 x g). The harvested cells were suspended in 1 ml buffer containing 10 mM Tris-HCl pH 7.6 and 50 µl 25x protease inhibitor cocktail tablets (Complete^{TM}, EDTA-free). The suspended cells were disrupted with glass beads (100-250 µm diameter) by vortexing 3 times for 2 min with 2 min intervals on ice. Subsequent centrifugation at 3220 x g for 30 min at 4°C to remove glass beads, unbroken cells and cellular debris, and centrifugation of the supernatant at 12000 x g for 30 min. Protein extract was concentrated by lyophylisation and the concentration was checked with the BCA protein Assay reagent. Samples suspended in lysis buffer (O´Farrell, 1975) were immediately used for 2D-PAGE or stored at –80°C.

2.3.3.2. Electrophoretical separation of proteins

To have an overview on the change in the protein composition of the extracts from nitrate starved cells and control, one-dimensional SDS-PAGE was performed in polyacrylamide gels (14 x 18 cm and 0.8 mm thick) as described by Laemmli (1970). Indeed, the resolving gel (table 2.8) was prepared and poured between the glass plates and allowed to polymerize after overlaying gently with iso-propanol. After polymerization, the overlay was decanted and the stacking gel was poured on the polymerized separating gel. The protein samples diluted in the

SDS loading-buffer and heat at 95°C for 5 min, were loaded together with the LMW standard in the wells of the stacking gel. The electrophoresis was carried out at 10 mA for 10 min and 25 mA for 50 min.

Table 2.8 Buffers and solutions for polyacrylamide gel electrophoresis

Stacking gel buffer

Tris/HCl pH 6.8	0.5 M
SDS	0.4% (M/V)

Resolving gel buffer

Tris/HCl pH 8.8	1.5 M
SDS	0.4% (M/V)

Stacking gel (4%)

Acrylamide solution 30%	0.52 ml
Stacking gel buffer (4x)	1 ml
dH_2O	2.47 ml
TEMED	4 µl
APS 10% (M/V)	40 µl

Resolving gel (12.5%)

Acrylamime solution 30%	3.33 ml
Resolving gel buffer (4x)	2 ml
dH_2O	2.67 ml
TEMED	4 µl
APS 10% (M/V)	40 µl

5x SDS loading buffer

Tris/HCl, pH 6.8	320 mM
Glycerin	50% (V/V)
SDS	10% (W/V)
ß-mercaptoethanol	25% (V/V)
Bromophenol blue	0.1% (M/V)

Electrophoresis buffer

Tris/HCl pH 8.3	3 g.l^{-1}
Glycin	14.4 g.l^{-1}
SDS	2 g.l^{-1}

Coomassie Brillant Blue staining solution

Coomassie Brillant Blue R-250	1 g.l^{-1}
Methanol	30% (V/V)
Acetic acid	10% (V/V)

Coomassie Brillant Blue destaining solution

Methanol	30% (V/V)
Acetic acid	10% (V/V)

After electrophoresis, proteins were visualized by Coomassie Brillant Blue staining. The excess of dye was then allowed to diffuse from the gel during a long period of destaining. Finally, the gel was dried at 80°C under vacuum between filter paper and cellophane foil. Two-dimensional polyacrylamide gel eclectrophoresis, in which proteins are separated according to charge (pI) by isolectric focusing in the first dimension and according to size by SDS-PAGE in the second dimension was performed as described (O´Farrell, 1975). Proteins on the 2D-PAGE were visualized either by Coomassie Brillant Blue staining or by ammoniacal silver staining.

2.3.3.3. First dimension: IEF in individual IPG strips

The first dimension of IPG-Dalt, isoelectric focusing (IEF), was performed in individual 3 mm wide IPG gel strips (ready-made Immobiline DryStrips). IPG-IEF was simplified by use of an integrated system, the IPGphor where rehydration with sample and IEF were performed automatically (Islam et al., 1998). The central part of this instrument are so-called strip holders made from an aluminium oxide ceramic, in which IPG strip rehydration with sample solution and IEF are performed without further handling after the strip is placed into the strip holder. Gel electrophoresis in the first dimension was carried out using an immobilized pH gradient gel (Immobilized Dry Strip Gel, pH 4-7/18 cm or pH 3-10/18 cm) with IPGphor

apparatus. Proteins solubilized with sample solubilization buffer (lysis buffer, table 2.9), were suspended in the rehydration buffer (table 2.9).

Table 2.9 Buffers and solutions for sample preparation and IEF

Lysis Buffer

Component	Final concentration	Amount
Urea	9.5 M	30.0 g
CHAPS	3% (W/V)	1.5 g
DTT	1% (W/V)	0.5 g
Double distilled water		to 50 ml

Rehydration solution with IPG buffer

Component	Final concentration	Amount
Urea	8 M	1.25 g
CHAPS	2% (W/V)	0.05 g
IPG buffer	0.5% (V/V)	12.5 µl
DTT	0.4%	0.01 g
Double distilled water		to 2.5 ml

Table 2.10 Guidelines for IPGphor with rehydration loading/IEF

Temperature	20°C
Current max.	0.05 mA per IPG strip
Sample volume	350 µl

Voltage	Time
50 V	12 hours (Rehydration)
500 V	1 hour (Isoelectric focusing)
1000 V	1 hour (Isoelectric focusing)
3500 V	2 hours (Isoelectric focusing)
5000 V	10 hours (Isoelectric focusing)

Voltage	Time
50 V	12 hours (Rehydration)
500 V	1 hour (Isoelectric focusing)
1000 V	1 hour (Isoelectric focusing)
3500 V	2 hours (Isoelectric focusing)
5000 V	8 hours (Isoelectric focusing)
8000 V	4 hours (Isoelectric focusing)

The first-dimension gels (Immobilized DryStrips) were rehydrated in 350 µl rehydration buffer containing the sample (Rabilloud et al., 1994) and the IPG-IEF was performed with voltage setting as described in table 2.10.

2.3.3.4. Equilibration of the IPG gel strips

After the isoelectric focusing, the IPG gel strip was equilibrated twice, each time for 15 min in 2x10 ml equilibration buffer (table 2.11). During the first equilibration step 100 mg DTT was dissolved in 10 ml equilibration buffer. In the second equilibration step, 400 mg iodoacetamide was added to the equilibration buffer in order to remove excess DTT (Görg et al., 1987). The equilibrated IPG gel strips were rinsed with deionized water for a second and placed on a piece of filter paper at one edge for a few minutes to drain off excess equilibration buffer.

Table 2.11 Equilibration solution for IPG gel strips

Component	Final concentration	Amount
Urea	6 M	180.0 g
Tris-HCl, pH 8.8, 1.5 M	50 mM	16.7 ml
Glycerol (87% V/V)	30% (V/V)	172.5 ml
SDS	2% (W/V)	10.0 g
Double distilled water		to 500 ml

2.3.3.5. Second dimensional gel electrophoresis: SDS-PAGE

The second dimension electrophoresis was run on ISO-DALT vertical electrophoresis system. It was carried out on a laboratory-made polyacrylamide gel (12% or 15% PAGE, 200x250

mm2, 1.5 mm thick) in the presence of SDS as described in Görg et al. (1988). The equilibrated IPG gel strips were placed on top of the SDS-PAGE gel and overlayed with 2 ml of hot (75°C) agarose solution. The SDS gel cassettes were inserted in the electrophoresis apparatus containing the electrode buffer (table 2.12) and run overnight (15 hours) with a voltage setting of 50 V for 1 hour and 100 V for 14 hours at 15°C. To estimate the molecular mass of each spot, marker proteins (Protein ladder 10 kD, 10 to 200 kD) were separated in the same way.

Table 2.12 Electrophoresis solution for 2D-PAGE

Component	Final concentration	Amount
Tris base	24 mM	58 g
Glycine	200 mM	300 g
SDS	0.1% (W/V)	20.0 g
Double distilled water		to 20 l

2.3.3.6. Visualization of proteins on acrylamide gels

After termination of the second dimension run, fixing is necessary to immobilize the separated proteins in the gel and to remove any non-protein components, which might interfere with subsequent staining. Here, protein spots were fixed and visualized according to the ammoniacal silver staining method shown in SWISS-2DPAGE (table 1.13). Indeed, all the steps were performed on an orbital shaker at 36 rpm. After removing gels from the glass plates and washing in dH_2O for 5 min, they were soaked in ethanol: acetic acid: water (40:10:50) for 1 hour, then in ethanol: acetic acid: water (5:5:90) for 2 hours or overnight. Afterwards, gels were washed in dH_2O for 5 min and soaked in a solution containing glutaraldehyde (1% V/V) and sodium acetate (0.5 M) for 30 min. After 3 times washing in dH_2O for 10 min, gels were soaked in a 2,7 naphtalene-disulfonic acid (0.05% W/V) for 1 h, in order to obtain homogenous dark brown staining of proteins. Gels were rinsed four times in dH_2O for 15 min, and then stained in a freshly made ammoniacal silver nitrate solution for 30 min. After 4 times washing in dH_2O for 4 min, the image were developed in a solution containing citric acid (0.01% W/V) and formaldehyde (0.1% V/V) for 5 to 10 min. When a slight background stain appeared, the development was stopped with a solution containing Tris (5% W/V) and acetic acid (2% v/v). Gels were finally scanned with ScanJet 5p scanner to obtain image for the analysis with Phoretix 2D Software. For the mass spectrometry and *N*-terminal amino acid sequencing of protein spots, gels were stained with Coomassie Brilliant

Blue as follow. All the steps were performed on an orbital shaker at 36 rpm. After electrophoresis, gels were soaked in the Coomassie Brillant Blue staining solution for 4-8 h. After staining, gels were washed in water and soaked repeatedly in the destaining solution until the background of the gels became clear.

Table 2.13 Ammoniacal silver staining solutions

Solutions	Composition	Concentration
1. Fixing solution	Ethanol:acetic acid:water	40:10:50 (% V/V)
	Ethanol:acetic acid:water	5:5:90 (% V/V)
2. Sensitizing solution	Glutaraldehyde Sodium acetate	1% (V/V) 0.5 M
	2,7 naphtalene disulfonic acid	0.05% (W/V)
3. Silver nitrate solution	Silver nitrate Ammonia Sodium acetate (10 N)	0.6% (W/V) 0.25% (V/V) 0.15% (V/V)
4. Developing solution	Citric acid Formaldehyde	0.01% (W/V) 0.1% (V/V)
5. Stopping solution	Tris Acetic acid	5% (W/V) 2% (V/V)

2.3.4. Protein analysis

2.3.4.1. In-gel digestion and mass spectrometry

In situ proteolytic digestion with endoproteinase trypsin sequencing grade was performed according to Rosenfeld et al. (1992) with some modifications. The gel pieces were excised with a scalpel from 2D-PAGE and washed twice with 50% acetonitrile in 100 mM ammonium hydrogen carbonate pH 8.5 for 20 min at 30°C, and left to semidry at room temperature. Trypsin sequencing grade was first dissolved in 1 mM HCl to prevent the auto-digestion, and then diluted in the digestion buffer (table 2.14). The dried gel pieces were partially rehydrated with 10 µl digestion buffer containing trypsin (250 µg/ml) at 4°C for 1 h. After absorption of the protease solution, 50 µl digestion buffer without trypsin were added to totally immerse the gels pieces and the digestion was carried out at 30°C for 18 h. The resulting peptides were recovered by two extractions of 20 min each, with 100 µl of the

extraction solution at 30°C with shaking in an Eppendorf thermomixer. After extraction, the eluted peptides were injected directly onto a reverse HPLC column or a mass spectrometer.

Table 2.14 Solutions for in-gel digestion of protein spots with trypsin

In-gel digestion buffer

NH_4HCO_3	100 mM
$CaCl_2$	1 mM

Extraction solution

Acetonitrile	60%
Trifluoroacetic	0.1%

2.3.4.2. *N*-terminal sequencing of amino acids

For microsequencing, the protein spots separated by 2D-PAGE were electroblotted onto a polyvinylidene difluoride membrane (Sequi-blot PVDF membrane) with a semi-dry transfer cell apparatus (Trans-blot SD). Three filter papers 1, 2, 3 at the same size as the PVDF membrane were incubated for 10 minutes in anode 1 buffer, anode 2 buffer and cathode buffer respectively (table 2.15). The gel was equilibrated for 15 minutes in the cathode buffer. The PVDF membrane was immersed for a few seconds in 100% methanol, and then incubated for three minutes in anode 2 buffer. Filter papers, the PVDF membrane and the gel were placed without air bubbles on the semi-dry transfer cell apparatus following this order from bottom to top: filter paper 1, filter paper 2, PVDF membrane, gel and filter paper 3. The blotting was run at 15 V for 1 h at room temperature. The membrane was stained with the PVDF membrane staining solution and destained with the respective destaining solution, and dried in air. Two to four excised spots were combined from multiple membranes by repetition of 2D-PAGE and sequenced by the Edman degradation method using an automated protein-sequencing machine (Precise Protein Sequencer).

Table 2.15 Buffers for protein spots electroblotting on the PVDF membrane

Anode 1 buffer

Tris/HCl pH 10.4	0.3 M
Methanol	10% (V/V)

Anode 2 buffer

Tris/HCl pH 10.4	25 mM
Methanol	10% (V/V)

Cathode buffer

Tris/HCl pH 9.4	25 mM
6-aminocaproic acid	40 mM
Methanol	10% (V/V)

PVDF membrane staining solution

Coomassie Brillant Blue R-250	0.025% (W/V)
Methanol	40% (V/V)

PVDF membrane destaining solution

Methanol	50%

2.3.5. Database searching of proteins

Proteins, corresponding to spots of interest during the 2D-PAGE, were identified by searching in the *Synechocystis* database using MS-Fit (Protein Prospector) or in the Cyano2Dbase. All searches were performed using a mass window between 1 and 100 kDa. The full pI range was considered. The search parameters allowed for oxidation of methionine and carbamidomethylation of cysteine.

2.4. Gene expression analysis

2.4.1. Materials

The materials used in this section are described in table 2.16 and 2.17

Table 2.16 List of some materials used in gene expression analysis

Item	Supplier
Bacterial mRNA purification kit	Ambion, Huntingdon, UK
Cy3-dUTP, Cy5-dUTP, Ultrapure dNTP-set	Amersham Biosciences, Freiburg, Germany

Gibco distilled water	Gibco BRL Life Technologies, Eggenstein, Germany
Oligonucleotides for DNA microarray	Metabion, Martinsried, Germany
RNA 6000 Assay kit	Agilent Technologies, Palo Alto, USA
RNase block ribonuclease inhibitor	Stratagene, La Jolla, USA
RNeasy Midi kit, RNeasy Mini kit	Qiagen, Hilden, Germany
SuperScript II enzyme, cDNA Cycle kit	Invitrogen life Technologies, Paisley, UK

Table 2.17 List of devices used in gene expression analysis

Instrument	Purpose	Company
Agilent 2100 Bioanalyzer	Bioanalysis	Agilent Technologies, Palo Alto, USA
BWM 9X Monitor	Agarose gel electrophoresis	Javelin Electronics, Schaumburg, USA
Centri-Sep column	cDNA purification	Princeton separations, Adelphia, USA
CreativeChip oligo slides	Microarray	Eppendorf, Hambourg, Germany
DNA Sub Cell GT system, Mini Sub DNA Cell, Mini Sub Cell GT	Agarose gel electrophoresis	BioRad, Munich, Germany
GMS 418 Array scanner	Scanner	Affymetrix, Santa Clara, USA
Microgrid II	Spotter	BioRobotics, Cambridge, UK
Thermomixer 5436	Thermomixer	Eppendorf, Hambourg, Germany
UV-Light	Agarose gel electrophoresis	MWG-Biotech, Ebersberg, Germany
Video Copy Processor P66E	Agarose gel electrophoresis	Mitsubishi, Cambridge, USA

2.4.2. Oligonucleotide sequences used in the construction of the oligo-microarray

For the DNA microaarray experiment, oligonucleotides (18-22 nucleotides length) with defined sequences from *Synechocystis* genes were spotted on CreativeChip oligo slides. The gene names, the sequences and the position in the genome are shown in table 2.18.

Table 2.18 Oligonucleotide sequences used in the construction of the microaarays

Genes	Sequence (5´ ? 3´)	Position
glnB	GCGTTACCGTGGCTCTGA	2152423 - 2152440
nblA	CGCCAACATCAGCCAGGA	1523865 - 1523882
ccmK	TGTTGTAGAAGCAGCCGACT	219251 - 219270
petE	CCTTCTGCTGGTGGTCTCC	2525860 - 2525878
ntcA	TTGAACAGGTGGAACAGGCT	1589704 - 1589723
cpcA	TACTACCTCCGCATCGTTACC	726617 - 726637
apcA	ATGTCCTCCGATGATGCC	1430840 - 1430857
cpcG	AACATCCTCTCCAGCAGCG	596601 - 596619
isiA	ACGCTCACTTCTTCCTGGC	1518490 - 1518508
phoA	CGGCGGTGATGCTCCATT	257566 - 257583

2.4.3. Construction of the microarray

Oligonucleotide microarrays (made *in house*) on which some gene sequences from *Synechocystis* PCC 6803 were immobilized, was used. Genes were selected according to their relevance during nitrate starvation condition (induction/repression). The probes were designed using Array Designer 2.0 and 18-22 nucleotides were selected in each gene sequence. To avoid cross homology, the oligonucleotide sequences were BLAST searched against the *Synechocystis* genome database at NCBI. The amino-modified oligonucleotides consisted of a terminal amino group coupled to a poly-dTTP sequence (12 nucleotides) spacer linked to the oligonucleotide sequence of the gene. The amino-modified oligonucleotide sequences were suspended in the Eppendorf spotting buffer to a concentration of 20 µM, then spotted on the CreativeChip oligo slide using MicroGrid II, and immobilized by incubation at 60°C for 30 min. Fig. 2.1 shows a schematic representation of a DNA-probe immobilized on the CreativeChip oligo slide in the same manner as the amino-modified oligonucleotides. After immobilization, the arrays could be used immediately or stored at RT in dark.

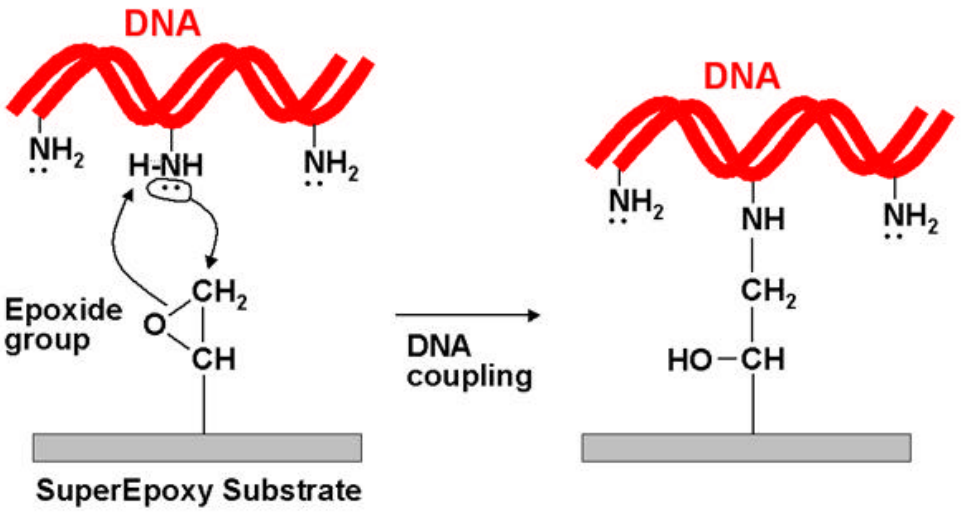


Fig. 2.1 Coupling of a DNA molecule to a slide surface activated with epoxide groups. The lone pair electrons on the amine group attack the electrophylic carbon on the epoxide group, forming a covalent bond between the DNA and the substrate. [TeleChem International]

2.4.4. Molecular methods

2.4.4.1. Total RNA extraction

The wild-type strain of *Synechocystis* PCC 6803 was grown photoautophycally in liquid BG-11 medium with or without nitrate at 30°C for 10 h under continuous illumination (50 µE.m^{-2}.s^{-1} of white light), then RNA was extracted. Total RNA was purified by using Qiagen Mini and Midi kits. Immediately after transfer from the growth culture into 50 ml polypropylene centrifuge tubes, the cells were placed into liquid nitrogen and chilled to a temperature below 5°C within 20 s. Chilled cells were immediately centrifuged at 3220 x g for 10 min in a precooled centrifuge (4°C), supernatant was discarded, and cell pellets were immediately frozen in liquid nitrogen prior to storage at –20°C. Cell pellets were resuspended in RLT buffer (RNA purification kit), an equal volume of 0.1-0.2 mm diameter glass beads was added and ground for four cycles of 1 min of vortexing and 1 min on ice. Lysed cells were then purified by following the exact protocols of the Qiagen RNA purification kit, in which RNA bound to an ion exchange column, and then eluted with RNase-free water.

After extraction, RNA was concentrated by adding 0.1 volume 3 M sodium acetate, 2.5 volumes 100% ethanol and mixing well. Then left to precipitate at – 80°C for 30 min and recovered by centrifugation at 12000 x g for 30 min at 4°C. The supernatant was discarded, 500 µl ice cold 70% ethanol was added and RNA was repelletet by centrifugation for 10 min

at 4°C. RNA was dissolved in TE buffer (table 2.19) at a concentration ≥ 2 µg/µl and stored at – 80°C. The concentration of RNA was determined by diluting an aliquot of the preparation in TE buffer and reading the absorbance with a Biophotometer at 260 nm (an absorption of 1.0 corresponds to the RNA concentration of 40 µg/ml) or by reading the absorbance of 1 µl of the preparation with the ND-1000 Spectrophotometer.

Table 2.19 TE buffer for RNA solution

Tris/HCl pH 8.0	10 mM
EDTA	1 mM

2.4.4.2. mRNA extraction from total RNA

mRNA was purified from total RNA by using the Ambion Bacterial mRNA purification kit. This kit is designed to rapidly enrich bacterial mRNA from purified total RNA by removing the 16S and 23S ribosomal RNAs. The kit employs a modified capture hybridization approach (Fig. 2.2), to remove these abundant rRNAs from the mixture.

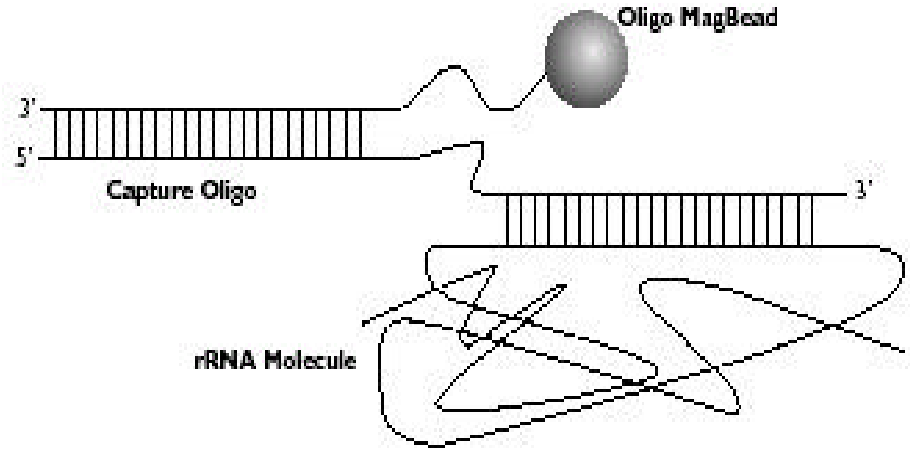


Fig. 2.2 Principle of bacterial mRNA purification from total RNA. One end of the capture oligo hybridizes to the rRNA molecule whereas the other end hybridizes to the oligo MagBead. [Ambion, The RNA Company]

The purified total RNA (10 µg) was incubated with the Capture Oligonucleotide Mix in binding buffer for 60 min. Magnetic beads, derivatized with an oligonucleotide that hybridizes to the capture oligonucleotide, were then added to the mixture and allowed to hybridize for 15 min. The magnetic beads, with 16S and 23S rRNAs attached were pulled to the side of the tube with a magnet. The enriched RNA in the supernatant was removed. The magnetic beads were briefly washed with the washing solution and the supernatant was once

more removed. The enriched mRNA containing mRNAs, tRNAs, 5S rRNA, and other small RNAs was concentrated and the concentration was checked as described above with total RNA.

2.4.4.3. Integrity and characterization of RNA

The integrity and size distribution of total RNA and mRNA were checked by agarose and denaturing-agarose gel electrophoresis and ethidium bromide staining. RNA samples were run on 1% agarose gel and 1.2% formaldehyde agarose gel electrophoresis for 30 min at a constant volt setting of 120 V and 100 V respectively. The RNA was visualized on the gel containing ethidium bromide, which binds to RNA and is fluorescent meaning that it absorbs invisible UV light.

For the agarose gel electrophoresis (table 20), 1% (M/V) agarose was suspended in 1x TAE buffer and heat to melt. After cooling in a water bath to 55°C, 0.5 µg.l^{-1} ethidium bromide was added, then mixed thouroughly and poured onto the gel support. For RNA sample preparation, 1 volume of 5x loading buffer and 4 volumes of RNA sample were mixed, incubated for 4 min at 65°C, chilled on ice and loaded onto the agarose gel. The electrophoresis was run for 30 min at 120 V.

Table 2.20 Recipes for agarose gel electrophoresis

50x TAE buffer

Tris	242 g.l^{-1}
Acetic acid	57.1 g.l^{-1}
EDTA, pH 8.0	0.5 M

5x RNA loading buffer

Glycerin	30% (M/V)
Bromophenol blue	0.2% (M/V)
EDTA, pH 7.5	25 mM

To prepare 100 ml of 1.2% formaldehyde agarose (FA) gel electrophoresis (table 2.21), 1.2 g agarose, 10 ml 10x FA gel buffer and water up to a total volume of 100 ml were mixed and heat to melt agarose. After cooling in a water bath to 65°C, 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide were added, then mixed thouroughly and poured onto the gel support. Prior to run the gel, it was equilibrated in 1x FA gel running

buffer for at least 30 min. For RNA sample preparation for FA gel electrophoresis, 1 volume of 5x loading buffer per 4 volumes of RNA sample were mixed, incubated for 4 min at 65°C, chilled on ice, and loaded onto the equilibrated FA gel. The gel was then run at 5-7 V/cm in 1x FA running buffer.

Table 2.21 Recipe for formaldehyde agarose gel electrophoresis

10x FA gel buffer

3-[N-morpholino] propanesulfonic acid (MOPS)	200 mM
Sodium acetate	50 mM
EDTA	10 mM
Ad just pH to 7.0 with NaOH	

1x FA Gel running buffer

10x FA gel buffer	100 ml
37% (12.3 M) formaldehyde	20 ml
RNase-free water	880 ml

5x RNA loading buffer

Saturated aqueous bromophenol blue solution	16 µl
500 mM EDTA, pH 8.0	80 µl
37% (12.3 M) formaldehyde	720 µl
100% glycerol	2 ml
Formamide	3084 µl
10x FA gel buffer	4 ml
RNase-free water	100 µl

The Agilent 2100 bioanalyzer with the RNA 6000 LabChip Kit was also used to check the size distribution of total RNA and to evaluate the rRNA removal after enrichment of mRNA with MICROB*Express* Kit. Each RNA sample (1 µl) was loaded in each well of the chip and the measurement was performed together with a RNA marker

2.4.4.4. Reverse transcription of RNA to cDNA and labeling

Fluorescently labeled cDNA probes were prepared from the total RNA or mRNA pool by direct incorporation of fluorescent nucleotide analogs during the first-strand reverse

transcriptase reaction (table 2.22). Each 14 µl of solution consisted of 50 µg of total RNA or 3 µg of mRNA, and 300 pmol of random hexamer was incubated at 70°C for 5 min to remove the secondary structure of RNA. After cooling on ice for about 5 min, the following reagents were added: 8 µl 5x reaction buffer provided with the Supersript II enzyme, 1 mM each of dATP, dCTP and dGTP, 0,5 mM dTTP, 50 units of RNase inhibitor and 3 mM of either cy3-dUTP or Cy5-dUTP to a final volume of 38 µl. The mixture was incubated at 42°C for 5 min and then 200 units (1 µl) of Superscript II were added, and the reverse transcription was performed at 42°C for 2 hours. Another 1 µl of Superscript II was added, 1 hour after the beginning of reverse transcription.

Table 2.22 Composition of the reverse transcription reaction of RNA to cDNA and labeling

Component	Amount
Total RNA /	20-40 µg /
mRNA	2-3 µg
Random primer (300 pmol)	1 µl
5x Superscript reaction buffer	8 µl
10x dNTP (A, C, G) mix	3 µl
1 mM dTTP	1µl
1 mM Cy3 or Cy5 dUTP	3 µl
RNase inhibitor (10 units/µl)	5 µl
RNase-free water	to 38 µl
SuperScript II (200 units/µl)	2 µl

After reverse transcription of RNA to cDNA, the remaining RNA was hydrolyzed by adding 5 µl of 0.5 M EDTA and well mixing. Then 10 µl of 1 N NaOH were added and incubated at 65°C for 30 min. Afterwards, the mixture was allowed to cool at room temperature and the reaction neutralized by the addition of 25 µl of 1 M Tris-Cl pH 7.5.

2.4.4.5. Purification of the labeled cDNA

The labeled cDNA was purified from the unincorporated fluorescent nucleotides and Cydye with Centri-Sep spin columns. The columns were hydrated with 0.8 ml RNase-free water after gently taping the columns to insure that the dry gel has settled in the bottom. The columns were hydrated at room temperature for at least 30 min and the interstitial fluid was removed by short spinning at 750 x g for 2 min. The sample (80 µl) was applied to the top of the gel

and the purified cDNA was collected by centrifugation of the columns at 750 x g for 2 min. The labeling efficiency was checked with N-1000 spectrophotometer. The labeled cDNA were purified from other contaminants by the addition of equal volume of rothiphenol (phenol/chloroform/isoamylalcohol 25/24/1) followed by well mixing and centrifugation at 12000 x g at RT for 5 min. The supernatant was removed and the cDNA was concentrated by ethanol precipitation as described above. The subsequent pellet was dissolved in 30 µl hybridization buffer (table 2.23).

Table 2.23 Hybridization solution for oligonucleotide microarrays (20x SSPE stock solution)

Component	Concentration	Amount
Sodium phosphate	0.2 M	23.99 g
NaCl	3.6 M	210.42 g
EDTA	20 mM	7.40 g
dH_2O		to 1000 ml

2.4.5. DNA microarrays

2.4.5.1. Hybridization of the target on the microarray

The spotted arrays were first blocked by 5 min incubation in rinsing solution 1, 4 min incubation in rinsing solution 2, 10 min incubation in rinsing solution 3 and 1 min incubation in ddH_2O at RT while constantly stirring (table 2.24). The arrays were then transferred into a heat-resistant petri dish, covered with blocking solution and incubated by slightly shaking for 15 min at 50°C.

Table 2.24 Blocking solutions for oligonucleotide microarrays

Solution	Components	Concentration
Rinsing solution 1	Triton X100	0.1%
Rinsing solution 2	HCl	0.05% (V/V)
Rinsing solution 3	KCl	100 mM
Blocking solution	Succinic anhydride	176 mM
	1-methyl 2-pyrrolidinone	95% (V/V)
	Sodium borate, pH 8.0	1 M

The arrays were subsequently washed at RT for 1 min in ddH_2O and dried with a flow of nitrogen. The target solution (containing target and hybridization buffer) was denatured at 95°C for 3 min following by brief centrifugation and subsequently applied to the microarray and the hybridization was carried out at 45°C for 12 hours.

2.4.5.2. Washing of the microarray and detection of the hybridization

The hybridized oligonucleotide arrays were washed in washing buffer 1, in washing buffer 2 and in washing buffer 3 (table 2.25) each for 10 min at RT with constantly stirring. Subsequently, the arrays were dried with a flow of nitrogen. Microarrays were scanned with two wavelengths either Cy3 (570 nm) or Cy5 (660 nm) by using a laser fluorescent scanner. Data analysis was performed using ImaGene version 3.0 software.

Table 2.25 Washing solutions for oligonucleotide arrays

Washing buffer 1	2x SSC / 0.2 SDS
Washing buffer 2	2x SSC
Washing buffer 3	0.2x SSC

20x SSC stock solution

Component	Concentration	Amount
Sodium citrate	0.3 M	88.3 g
Sodium chloride	3 M	175.38 g
dH_2O		to 1000 ml
pH 7.0 with 1 M HCl		

3. RESULTS

3.1. Development of a reporter assay in an immobilized format for the detection of nitrate

The recombinant bioluminescent cyanobacterial reporter strain *Synechocystis* sp. PCC 6803 was constructed by Richaud et al. (2001). This reporter strain harbors a kanamycin resistant gene (km^R) through the insertion of *luxAB-km*^{R} fusion with *nblA1* gene in its chromosomal DNA, leading to *PnblA::luxAB-km*^{R}. This reporter strain was designated N1LuxKm and showed a dose-dependent response to nitrate starvation. The construction of the reporter strain is shown in Fig. 3.1.

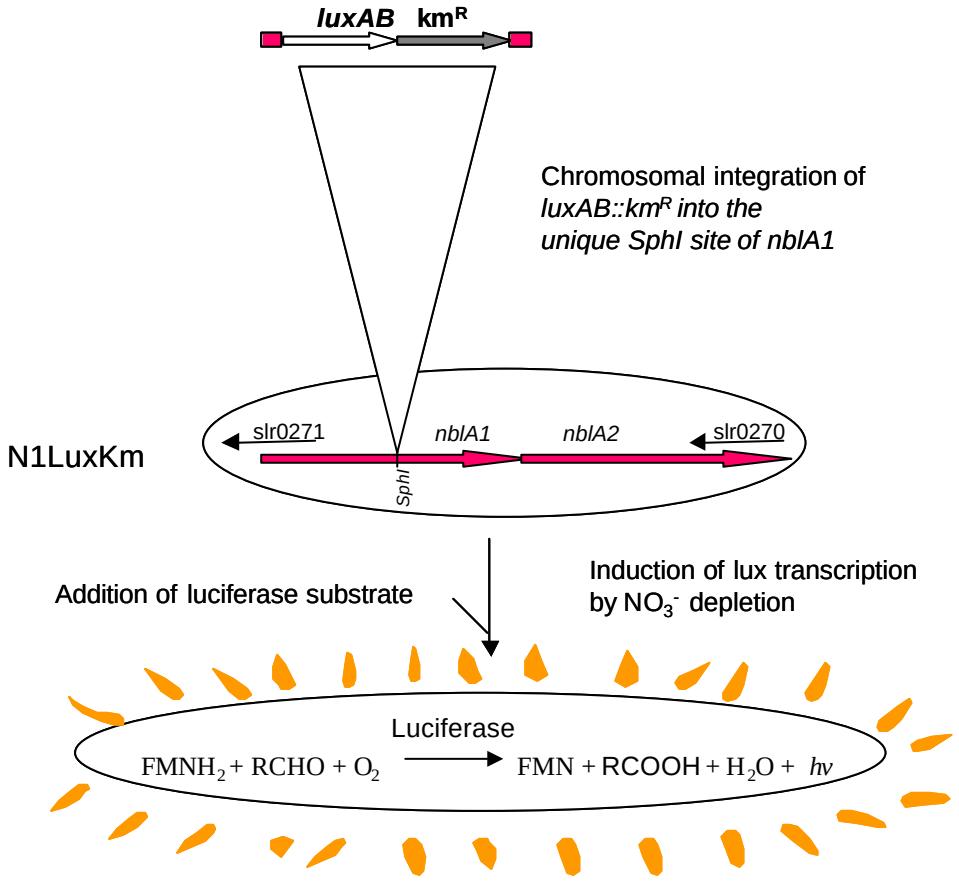


Fig. 3.1 Construction of the nitrate reporter strain N1LuxKm. The *luxAB-km*^{R} fusion is integrated into the chromosomal DNA of the cyanobacterium *Synechocystis* PCC 6083, leading to *PnblA::luxABkm*^{R} (Richaud et al., 2001).

3.1.1. Characterization of N1LuxKm reporter strain in liquid mediun

The *Synechocystis* PCC 6803 wild-type (WT) and the reporter strain N1LuxKm were grown in BG-11 medium and then transferred into media containing various nitrate concentrations. The time-courses of growth are shown in Fig. 3.2. To show the behaviors of the WT and the mutant, they have been monitored in the exponential phase of growth because of the objective of the experiment, which was to test the behaviors of the WT and mutant in different culture conditions. It appeared that after 15 hours of incubation in BG-$11_{(-N)}$ the growth rate of both WT and N1LuxKm was reduced. Moreover, WT cells appearance changed from blue-green to yellow while the color of the mutant remained unchanged. Expression of the *lux* genes from the *nblA* promoter resulted in constitutive bioluminescence emission by the reporter strain N1LuxKm. No bioluminescence was noticed in the WT even when cultivated in medium lacking nitrate.

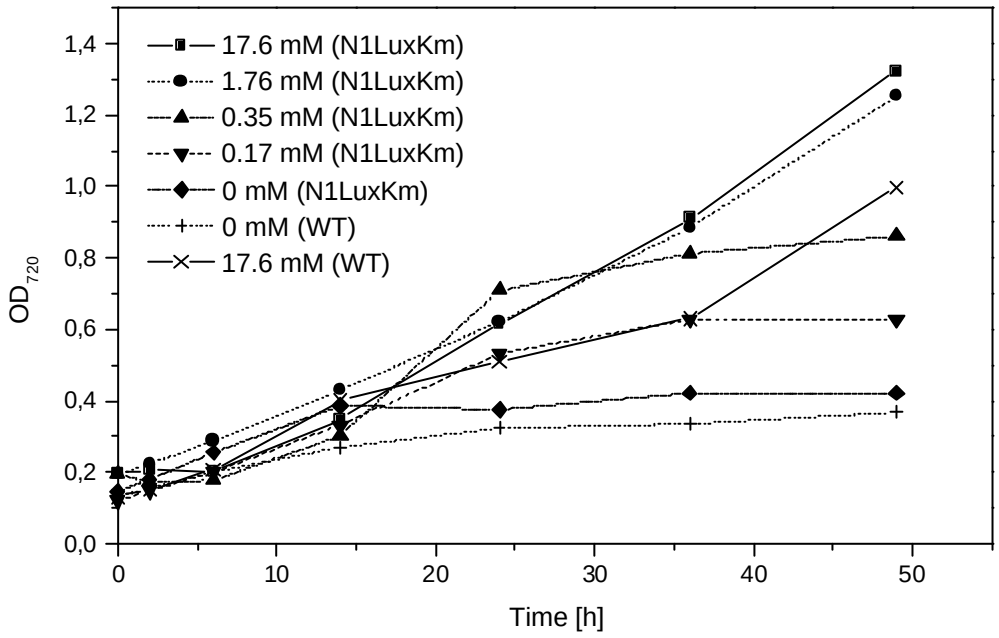


Fig. 3.2 Effect of nitrate concentration on the growth behavior of *Synechocystis* PCC 6803 N1LuxKm and wild type. N1LuxKm cells were incubated in media containing various nitrate concentrations (0-17.6 mM) and WT cells in media containing 0 mM and 17.6 mM nitrate after a pre-culture in BG-11 medium.

To test the effectiveness of the reporter strain under nitrate starvation, N1LuxKm was incubated in media containing various nitrate concentration and the bioluminescence response

was recorded. This assay is shown in Fig. 3.3. The bioluminescence response, whose intensity is correlated to the nitrate concentration in the medium, decreased whereas the nitrate concentration in the medium increased. Up to 7 hours of induction, the bioluminescence was inversely proportional to the nitrate concentration in a range 4–100 µM in the medium. After this time, the relationship was no longer linear.

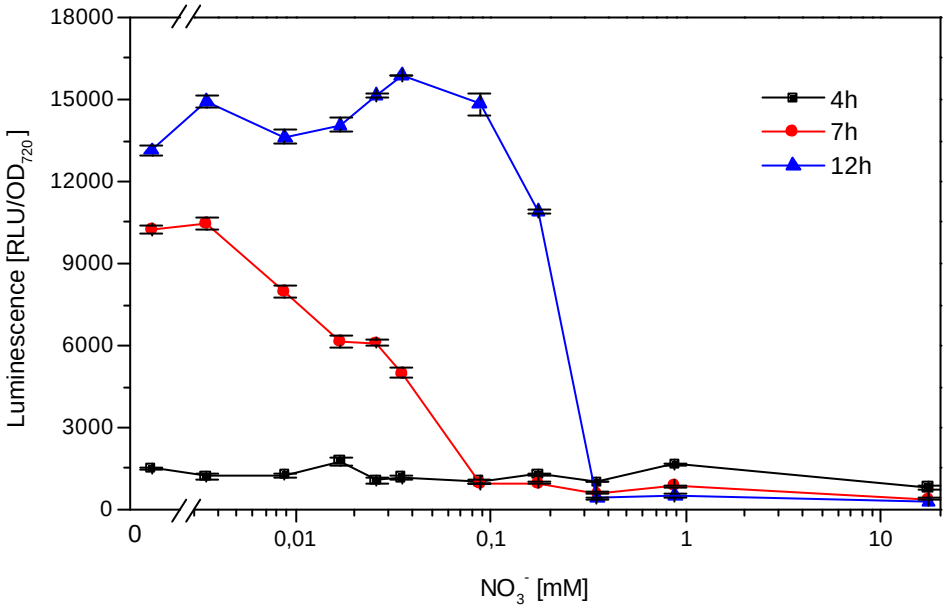


Fig. 3.3 Bioluminescence response of the N1LuxKm cells grown in liquid culture. N1LuxKm cells were incubated in media containing various nitrate concentrations and bioluminescence was measured after different incubation durations. In liquid culture, the relative light unit (RLU) is divided by the optical density of the cells at 720 nm.

In order to improve the bioluminescence response and to determine the optimal conditions for the immobilization, N1LuxKm was cultivated in BG-11 and 1/10-BG-11 media and three parameters were investigated: (1) the time-course of growth, (2) the bioluminescence activity and (3) the nitrate concentration left in the medium (Fig. 3.4). After 70 hours, a rapid increase in bioluminescence was noticed in 1/10-BG-11 medium (Fig. 3.4.b). This corresponded exactly to the time of complete depletion of nitrate in the medium. According to this result, cells from 1/10BG-11 at the optical density OD_{720} = 0.8-1.0 (after about 60 hours incubation) were used for immobilization. As expected, no luminescence was observed in BG-11 medium and the nitrate concentration decreased slowly during 90 hours incubation (Fig. 3.4.a).

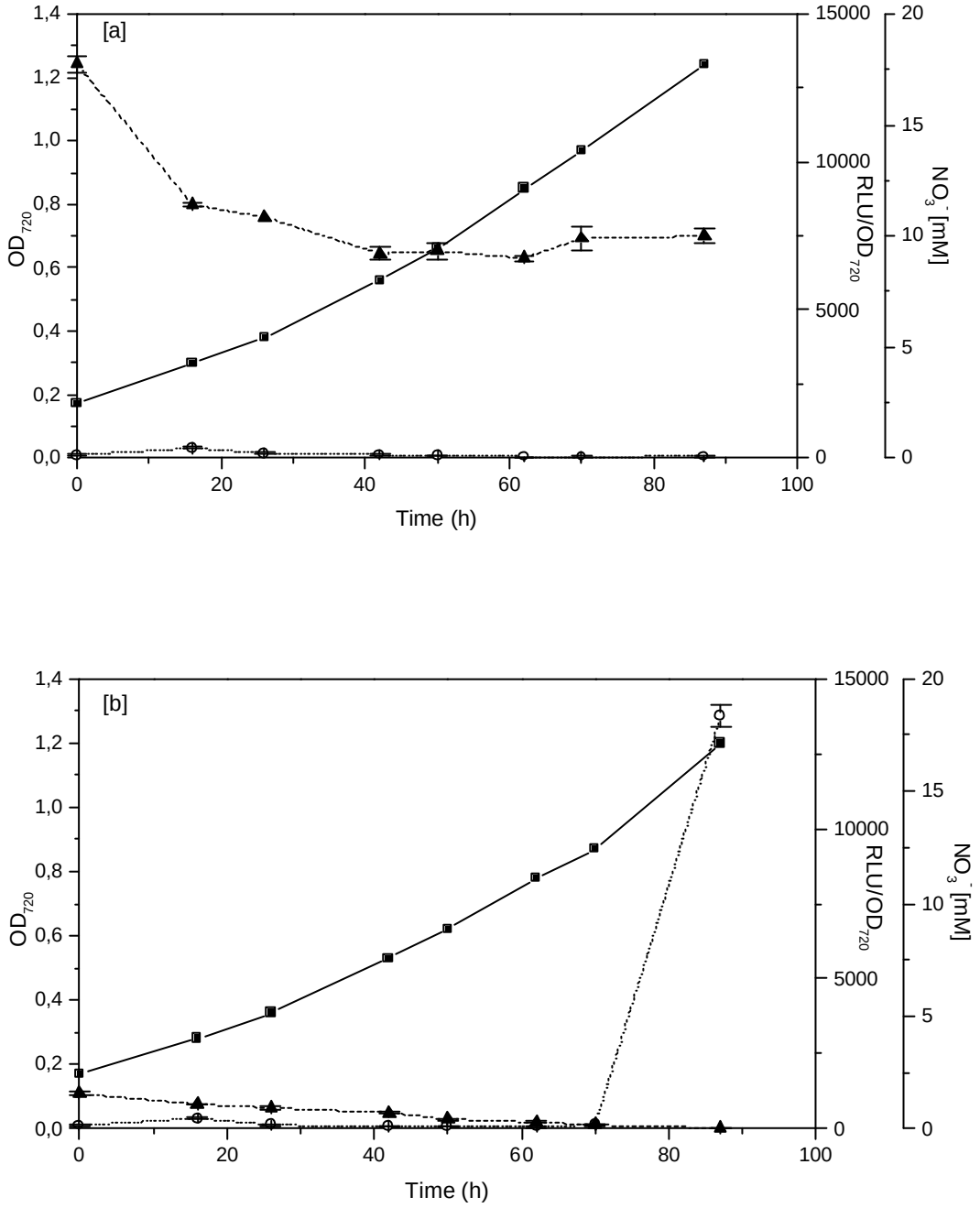


Fig. 3.4 Monitoring of 3 parameters for N1LuxKm cells grown in bath culture: Time-courses of growth (filled squares), nitrate concentration left in the medium (filled triangles) and bioluminescence response (open circles) in media containing [a] 17.6 mM and [b] 1.76 mM nitrate (1/10-BG-11).

3.1.2. Immobilization of the reporter strain N1LuxKm

3.1.2.1 Immobilization in agar and agarose

N1LuxKm from 1/10BG-11 was immobilized in agar, incubated in media containing various nitrate concentrations and the bioluminescence was measured after different incubation times (Fig. 3.5). The time needed to induce a detectable signal was longer for the immobilized cells (18 h) compared to free cells (4 h) (see Fig. 3.3). The results obtained with the chemical method used to test the presence of nitrate in agar led to the suggestion that this matrix contained some nitrate derivatives.

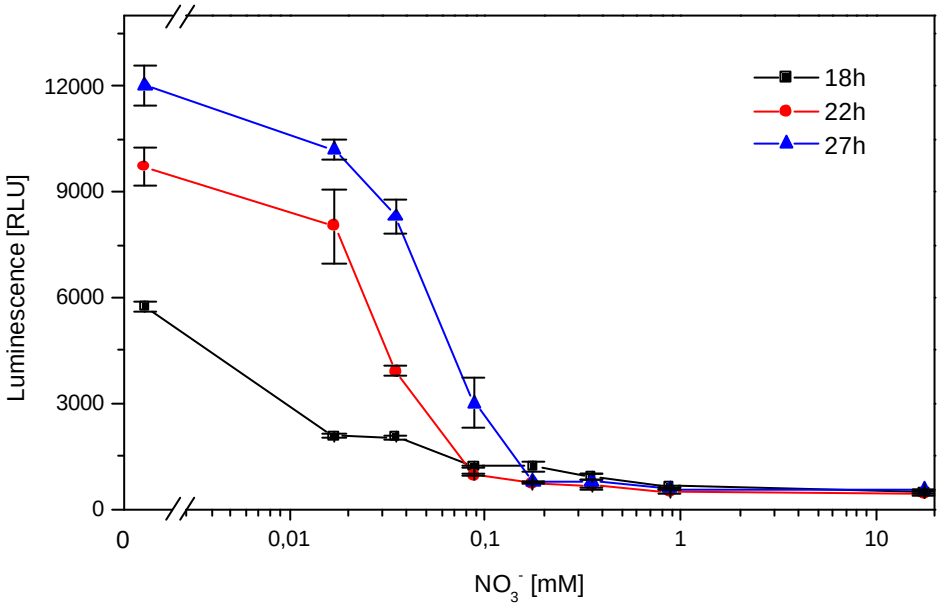


Fig. 3.5 Bioluminescence response of N1LuxKm mutant immobilized in agar. N1LuxKm cells from 1/10BG-11 (1.76 mM NO_3^-) were immobilized in agar and incubated in media containing various nitrate concentrations. The bioluminescence response was measured after different incubation durations (18h, 22h, 27h).

To improve the immobilized sensor response, two other matrices were tested: agarose and "washed agar". The agar washing procedure removed not only nitrate but also some other impurities that could affect the sensor response. In both matrices, the induction time was reduced (5 h) and the intensity of bioluminescence in "washed agar" was higher than in agarose (Fig. 3.6). Consequently "washed agar" was selected as the immobilization matrix for the CyanoSensor.

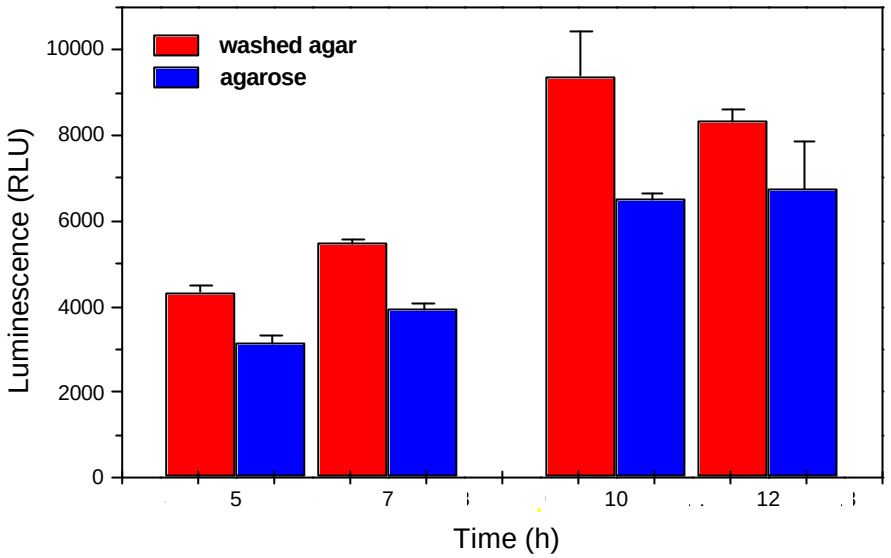


Fig. 3.6 Comparison of the matrices for immobilization. N1LuxKm was immobilized in agarose and washed agar, and the bioluminescence response was measured after different incubation times.

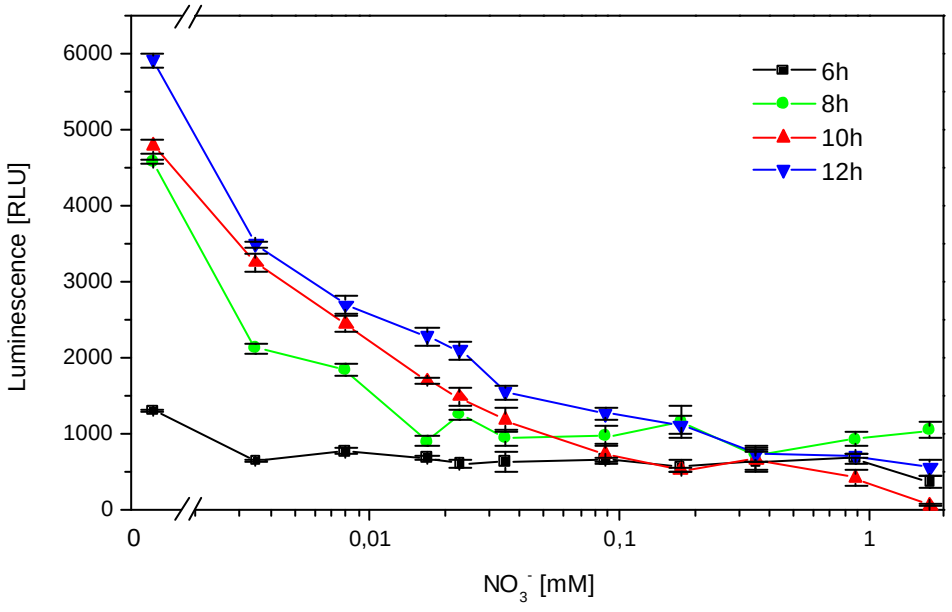


Fig. 3.7 Bioluminescence response of N1LuxKm immobilized in "washed agar". N1LuxKm cells from 1/10 BG-11 were immobilized in washed agar and incubated in media containing various nitrate concentrations. The bioluminescence response was measured after different incubation times.

3.1.2.2. Immobilization in washed agar

Fig. 3.7 shows the nitrate calibration curve obtained with N1LuxKm, immobilized in washed agar and pre-cultivated in 1/10BG-11 medium. The bioluminescence was measured after different times of incubation: 10 hours appeared optimal because of the best linearity of the curve compared to other times. A good linearity of sensor response related to nitrate concentration was found in the range of 4-100 µM nitrate, proving the possibility for a quantitative detection of nitrate by use of this CyanoSensor.

3.1.3. Optimization of the sensor

3.1.3.1. Effect of the amount of substrate on the bioluminescence response

N1LuxKm was incubated in BG-$11_{(-N)}$ medium for 10 h and the bioluminescence activity was measured after addition of different amount of the substrate solution. This experiment was to check the optimal amount of substrate to be added in the bioluminescence reaction, for a good sensor response (Fig. 3.8). It appeared that a substrate volume in the range 100-150 µl is optimal for the bioluminescence activity measurement.

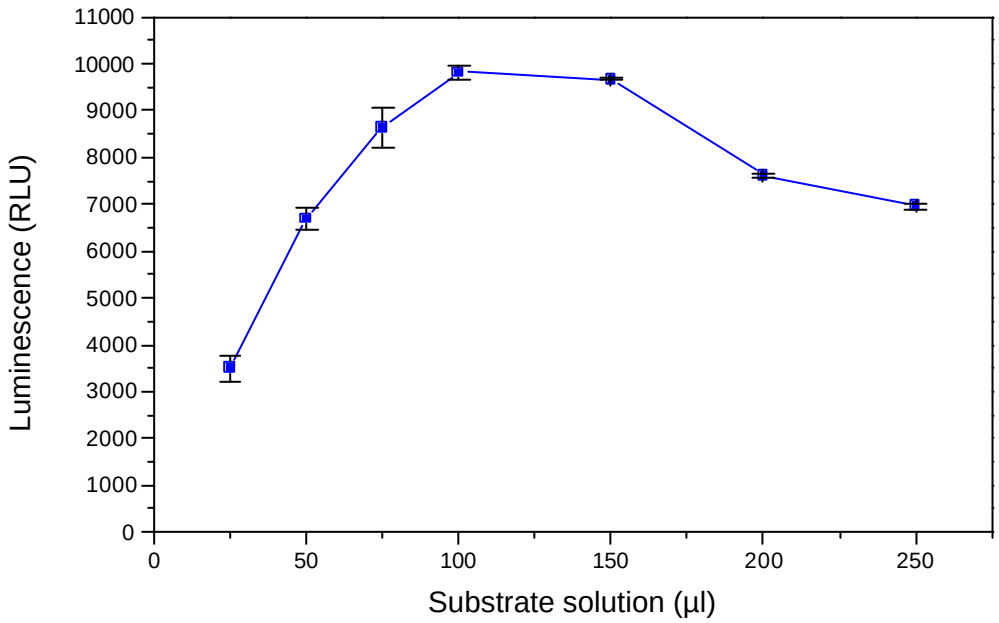


Fig. 3.8 Effect of the substrate volume on the bioluminescence activity. The bioluminescence activity of N1LuxKm incubated in BG-$11_{(-N)}$, was measured after addition of different substrate volumes (25-250 µl) containing 0.1 mg/ml nonanal

3.1.3.2. Storage stability

Several coverage media containing different nitrate concentrations were tested to store the immobilized sensor. The coverage medium containing 35 µM nitrate was found to be the optimal because it maintained good stability of the sensor after different durations of storage. Fig. 3.9 shows nitrate calibration curves after different storage times. The bioluminescence response upon sample addition decreased with the duration of storage but the sensitivity remained similar to freshly prepared sensors. Accordingly, N1LuxKm CyanoSensor could be stored up to one month.

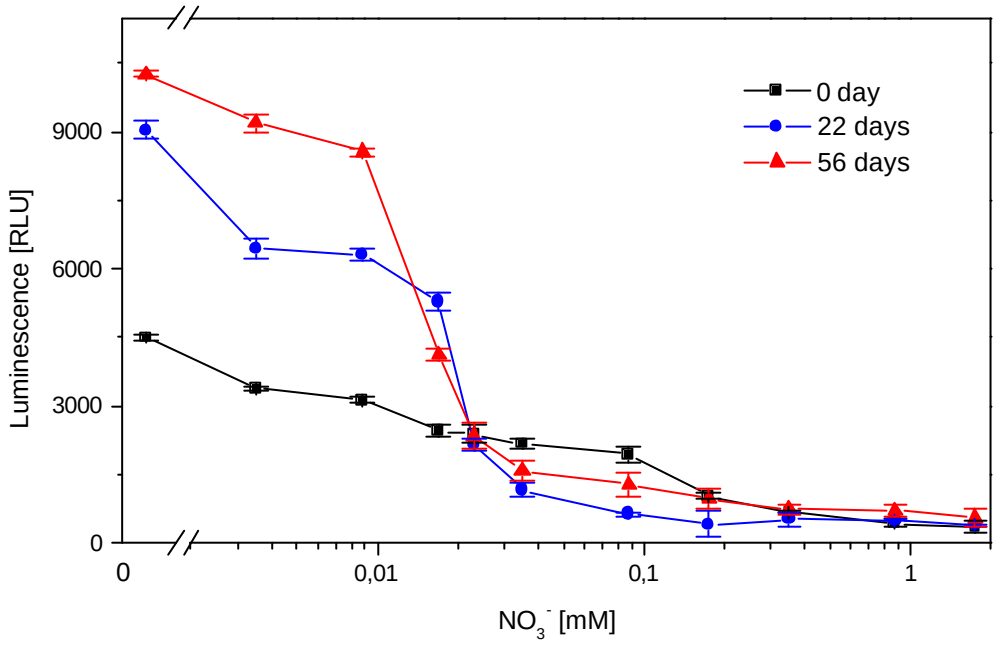


Fig. 3.9 Effect of storage on the response of the biosensor. N1LuxKm cells from 1/10 BG-11 and immobilized in "washed agar" were stored at 4°C in dark. For each storage duration, the bioluminescence response was measured after an incubation of the immobilized cells for 10h in media containing various nitrate concentrations.

3.1.3.3. Effect of ammonium and other nutrient limitations on the sensor

In order to characterize bioluminescence response on the absence of ammonium, free cells of N1LuxKm were incubated in BG-11 medium lacking nitrate and in BG-11 medium lacking nitrate and ammonium (Fig. 3.10). The bioluminescence, measured after different times of incubation, in medium lacking nitrate and ammonium, was higher than in medium lacking only nitrate. BG-11 media lacking phosphate, sulfate or nitrate (Fig. 3.11) were used to prove

N1LuxKm specificity to nitrogen-containing nutrients. Bioluminescence was found only in medium lacking nitrate. The results demonstrated the specificity of the reporter strain to nutrients bearing nitrogen such as nitrate and ammonium.

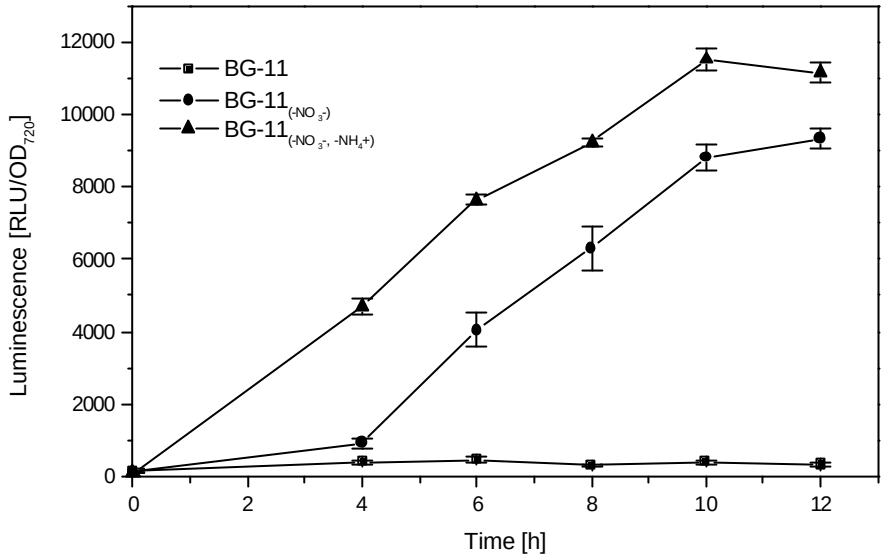


Fig. 3.10 Effect of ammonium-depletion on the biosensor response. N1LuxKm cells were incubated in liquid culture containing nitrate and ammonium (BG-11), lacking nitrate [BG-$11_{(-N)}$] and in medium lacking nitrate and ammonium [BG-11 ($-NO_3^-$, $-NH_4^+$)].

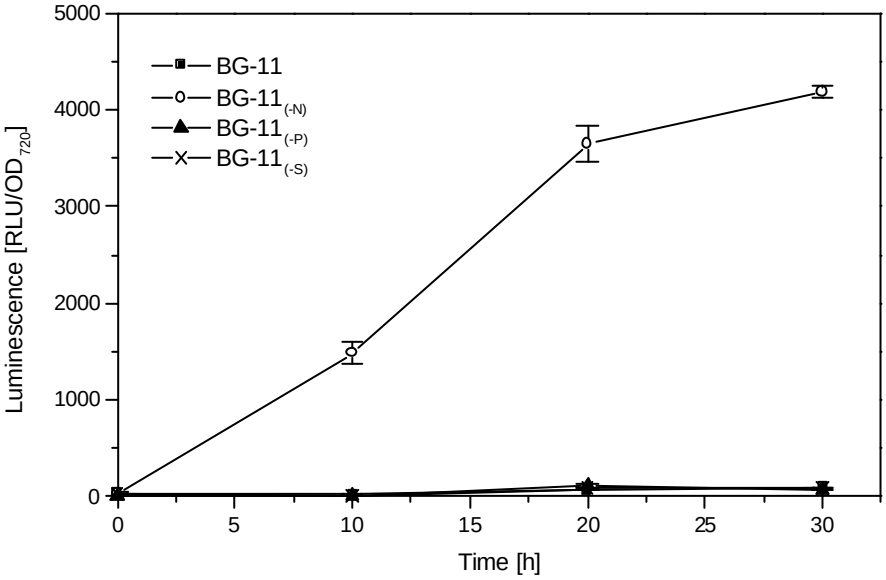


Fig. 3.11 Effect of depletion of other nutrients on the biosensor response. N1LuxKm cells were incubated in media lacking nitrate [BG-$11_{(-N)}$], phosphate [BG-11 (-P)], sulfate [BG-11 (-S)] and in BG-11 medium.

3.1.4 Conclusion

Comprising the preceding results, it can be stated, that the CyanoSensor could be used for the detection of nitrogen bioavailability from different sources of water. Immobilization simplified and minimized sensor operation, and rendered the sensor storable.
However, the change of the phenotype in the cyanobacterium *Synechocystis*, from blue-green to yellow, when grown at low nitrate concentration could be probably explained better at the molecular level. For this reason, and in order to find stronger induced genes under nitrate depletion, investigations to monitor the proteome of *Synechocystis* were performed and are described in the following chapter.

3.2. Protein expression under nitrate starvation

In this study, the proteome analysis in *Synechocystis* consisted of the separation of protein samples from different sources with 2D-PAGE; comparison of the 2D gels with an adequate software, and the identification of protein spots either by MALDI-TOF/MS or *N*-terminal amino acid sequencing.

3.2.1. Electrophoretical separation of soluble proteins

Proteins of *Synechocystis* cells were concentrated and resolved in SDS-PAGE (Fig. 3.12). Applying identical amounts of protein from the control and nitrate starved conditions, four major bands were observed on the gel, that showed the difference between the two sources of proteins. Three protein bands were down-regulated under nitrate starvation whereas only one was up-regulated. Because some proteins with the same molecular weight are different in their isoelectric point, isoelectric focusing and SDS electrophoresis were combined in a 2 dimensional electrophoresis system to improve the separation.

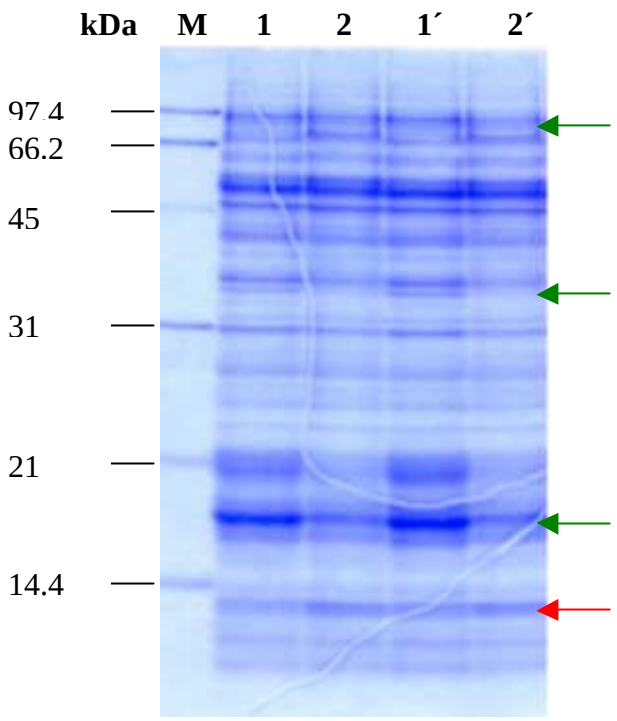


Fig. 3.12 Protein composition of soluble extracts obtained from control cells (lane 1, 1´) or nitrate starved cells (lane 2, 2´) of *Synechocystis* sp. PCC 6803 analyzed through SDS-PAGE and Coomassie Brillant Blue staining. 20 µg protein was loaded on each lane. Up-regulated protein band (red arrow), down-regulated protein bands (green arrow) under nitrate starvation.

3.2.2. Two -dimensional gel electrophoresis

2D-PAGE separates proteins both in terms of their isoelectric point (pI) and molecular weight (MW), and accordingly, its resolving power is unsurpassed when compared to one dimensional gel separation techniques (Celis and Bravo, 1984; Wilkins et al., 1987). A useful first-dimension separation requires selecting a first-dimension pH range appropriate for the sample. A pH 3-10 IPG strip displays the widest range of proteins on a single 2D gel. A silver-stained *Synechocystis* PCC 6803 proteome separated on a pH 3-10 linear gradient and 12%T SDS-PAGE is shown in Fig. 3.13.

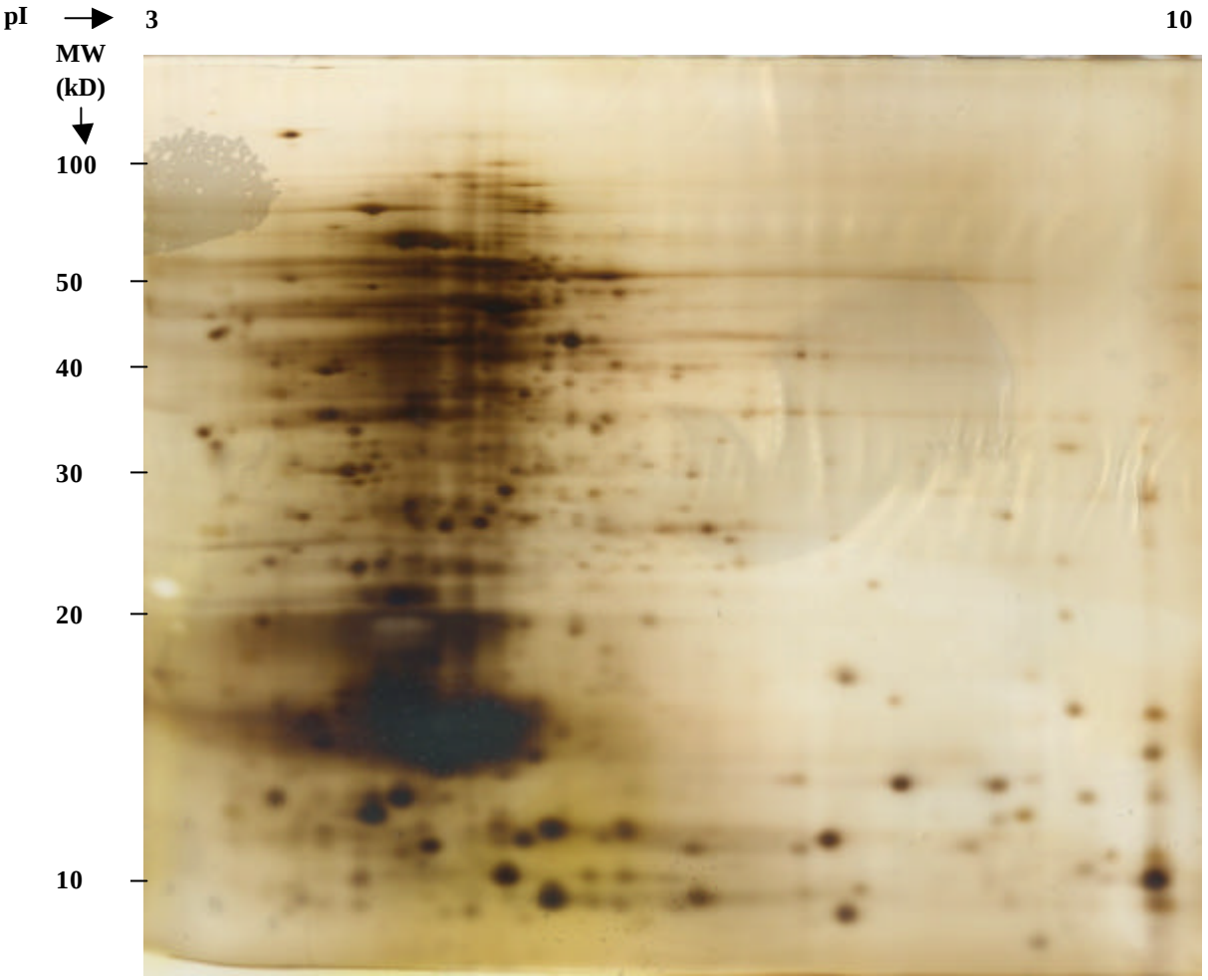


Fig. 3.13 Protein composition of soluble fraction from *Synechocystis* PCC 6803. Cells were grown under nitrate starvation conditions and the extracted proteins were separated using 2D-PAGE and stained with silver. 350 µl rehydration buffer containing 100 µg protein were applied in each IPG strip holder and the IEF was run with the Immobiline DryStrip gel pH 3-10 L/18 cm. After equilibration, the IPG gel was sealed on the top of a 12%T SDS-PAGE.

This figure shows that, proteins in *Synechocystis* PCC 6803 are more concentrated in the pH range 4-7. The resolution in this pH range is not effective and the pH 3-10 IPG non-linear gradient was used in the following experiments to improve the resolution.

3.2.2.1. 2D gel electrophoresis using IPG strips pH 3-10 NL and pH 4-7 L.

The pH 3-10 NL IPG strips have a roughly sigmoidal gradient that gives improved resolution between pH 5 and pH 7. The proteome of *Synechocystis* PCC 6803 separated on a pH 3-10 non-linear gradient and 12%T SDS-PAGE, and stained with silver is shown in Fig. 3.14. The resolution in the pH range 5-7 is improved compared to Fig. 3.13.

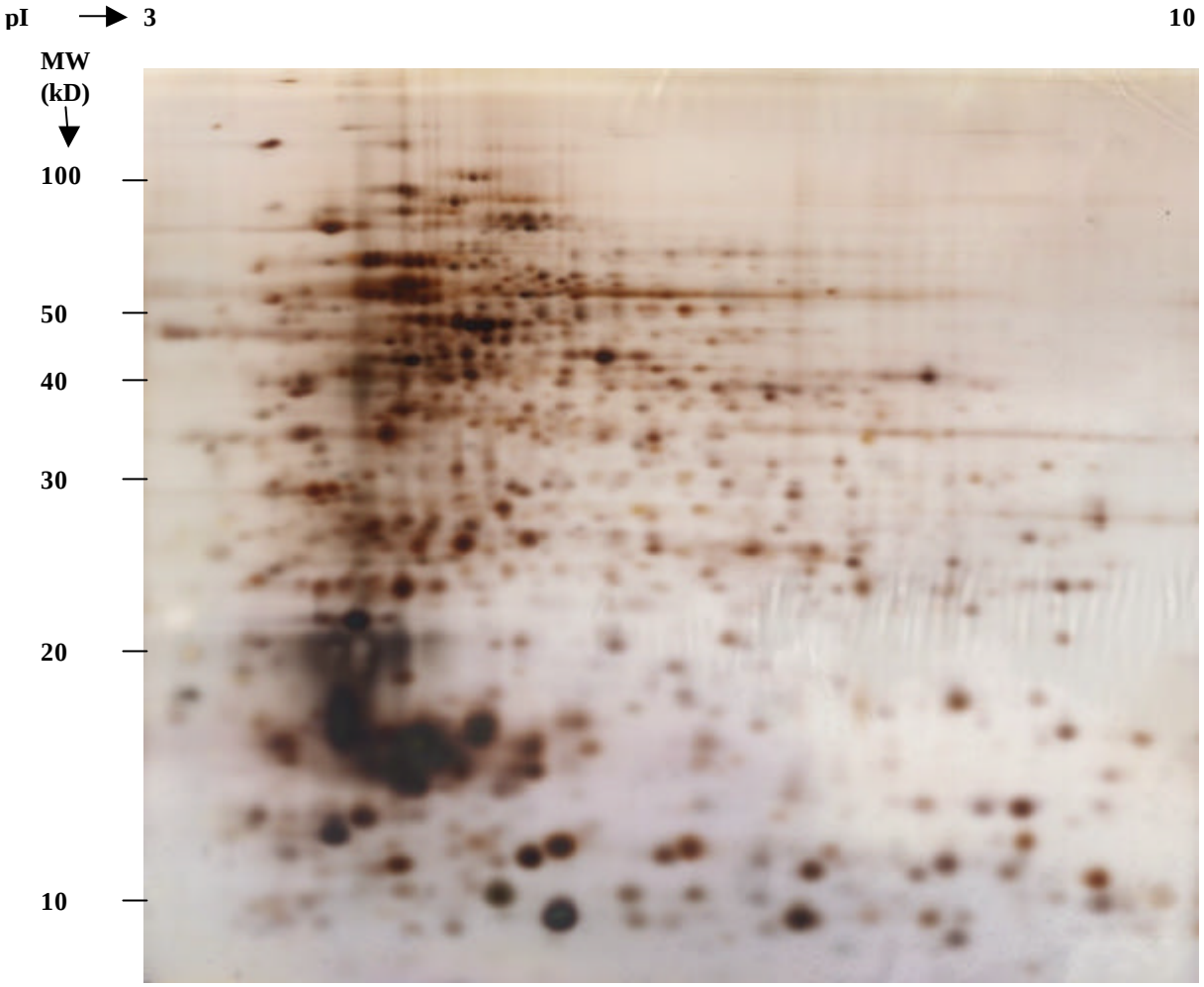


Fig. 3.14 Protein composition of soluble fraction from *Synechocystis* PCC 6803. Cells were grown under nitrate starvation conditions and the extracted proteins were separated using 2D-PAGE and stained with silver. The IPG gel pH 3-10 NL/18 cm and 12%T SDS-PAGE were used.

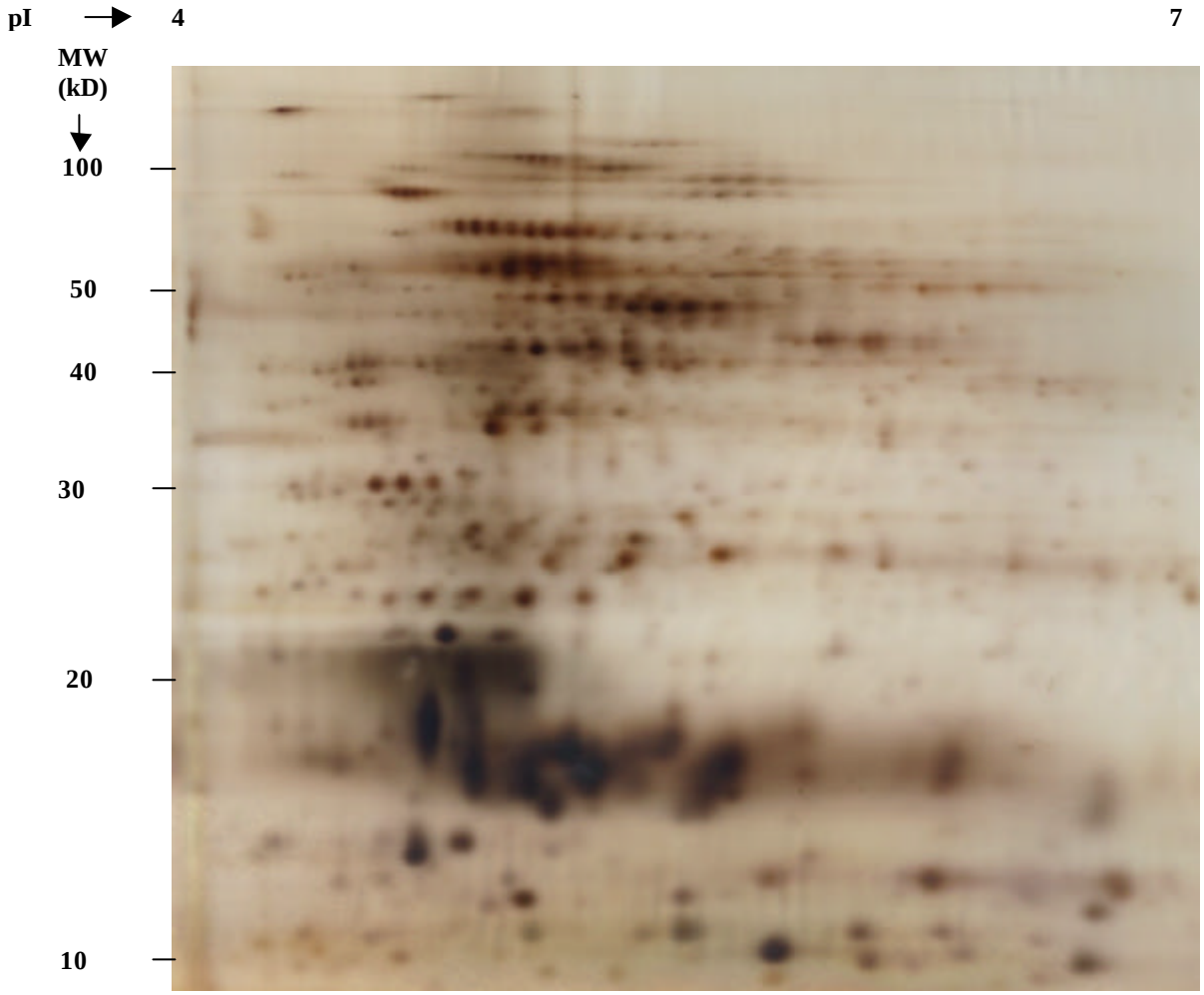


Fig. 3.15 Protein composition of soluble fraction from *Synechocystis* PCC 6803. Cells were grown under control conditions and the extracted proteins were separated using 2D-PAGE and stained with silver. The IPG gel pH 4-7 L/18 cm and 12%T SDS-PAGE were used.

Because of the high concentration of proteins in the pH range 4-7, a pH 4-7 IPG linear was used to improve the resolution and gels comparison. Fig. 3.15 shows a 2D-PAGE image of *Synechocystis* PCC 6803 separated on a pH 4-7 linear gradient and 12%T SDS-PAGE, stained with silver. The resolution in this pH range was improved but the difference with the pH 3-10 IPG non-linear gradient resolution was not significant. For 2D gels comparison, pH 3-10 IPG non-linear gradient and 12%T SDS-PAGE were used because of the high resolution and the wide pH range.

3.2.2.2. 2D-PAGE comparison using IPG strips pH 3-10 NL

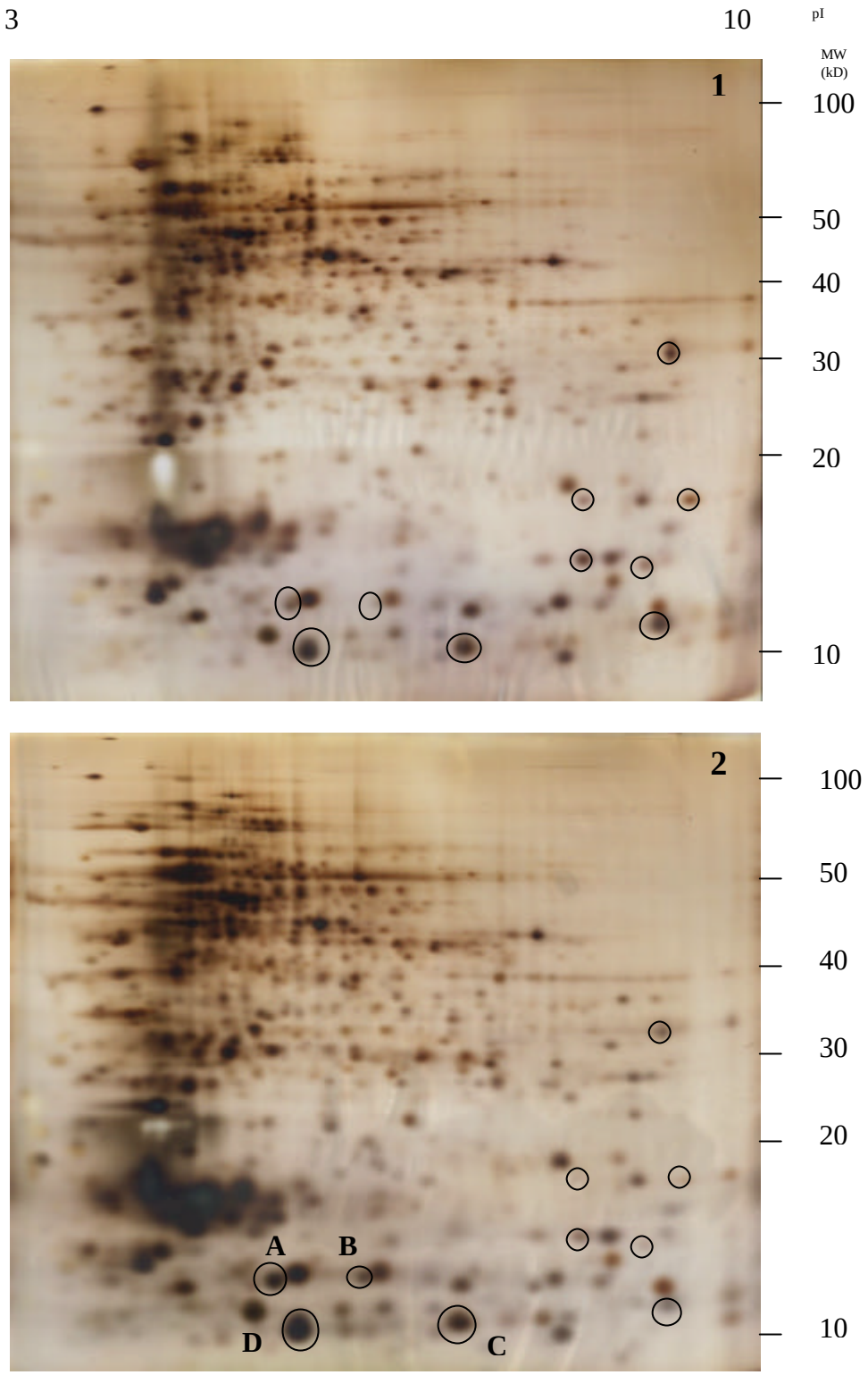


Fig. 3.16 Protein composition of soluble fraction from *Synechocystis* PCC 6803. Cells were grown under control conditions (1, $+NO_3^-$) and nitrate starved conditions (2, $-NO_3^-$); proteins were separated using 2D-PAGE and stained with silver. Encircled spots are those submitted to the analysis and the identified ones are indicated with a letter.

Cyanobacterial proteins in the soluble cell extract were resolved by 2D-PAGE using IPG strips pH 3-10 NL. About 160 protein spots became visible and were clearly resolved (Fig. 3.16). Reproducible results were obtained by extracting water-soluble proteins in low concentrated Tris-HCl buffer. The protein spots were evently distributed over the whole gel but more concentrated in the pH range 4-7. A similar general pattern of proteins spots was also observed after separation of proteins from nitrate-starved cells, although the intensity of the silver staining of some spots was increased, while others decreased or disappeared (Fig. 3.16(2)). The separation of the proteins was better at low molecular weight than high molecular weight range. Because of the concentration of high molecular weight protein in the pH range 4-7, the immobilized dry strip gel pH 4-7/18 cm was used (Fig. 3.15), but the great difference after comparison of the 2 gels from different extracts source remained at the low molecular weight range.

3.2.3. Differential expression of proteins in nitrate-starved cells

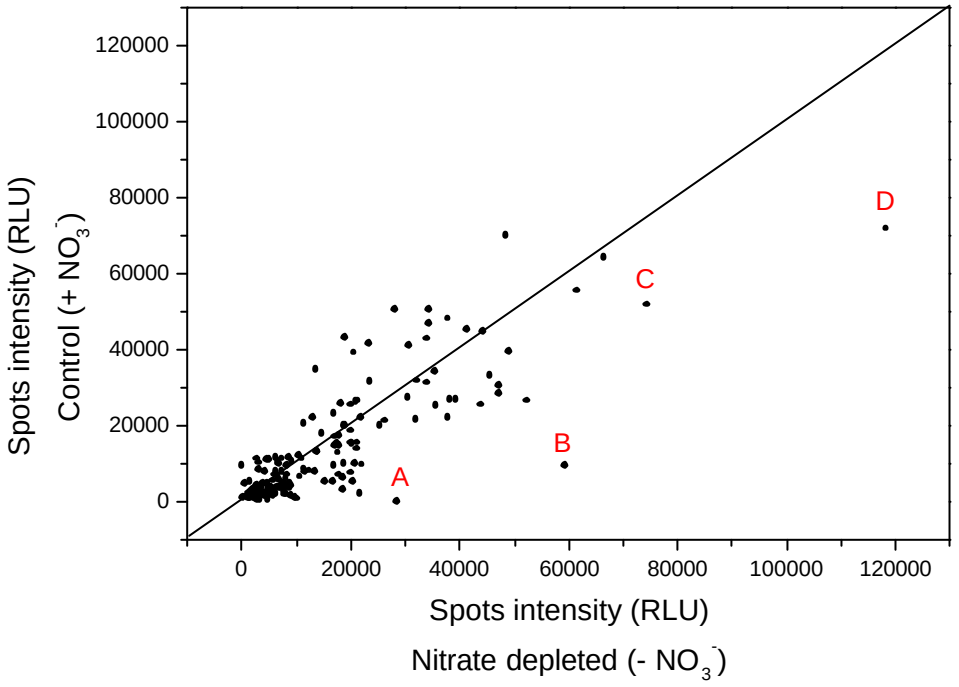


Fig. 3.17 Representative protein expression data in control versus nitrate starved cells. The 2D gels electrophoresis stained with silver were analyzed with Phoretix 2D software. 160 spots are represented on this graph and the intensity value represents the mean from 4 different gels. The analyzed and identified spots are indicated with a letter.

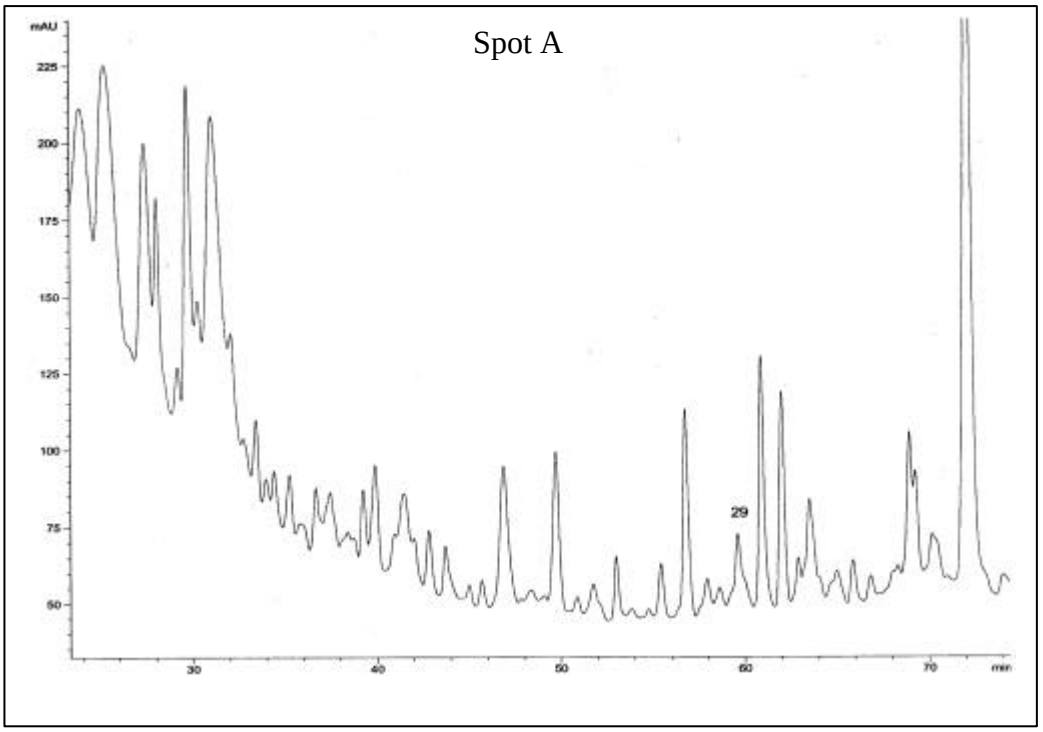
The intensity of each spot found on 2D-PAGE gel was analyzed with the Phoretix 2D Software. The differential expression of each spot from control and nitrate-starved cells is shown in Fig. 3.17. The spot intensities were normalized after background subtraction by single spot normalization method, in which the intensity of each spot is linearly scaled according to a constant value for a single spot. The intensity of the majority of spots did not change but some spots were found to be up or down-regulated under nitrate starvation and were subjected to further analysis. As it can be seen from Fig. 3.17, due to the variation between the gels/experiments it was difficult to conclude about the statistically significant behavior of low concentrated proteins with low spot intensities. We considered only the protein spots that were visualized on Coomassie Brillant Blue stained gel for the analysis because silver staining is incompatible with analysis.

3.2.4. Proteins analysis

The analysis of 2D–PAGE with Phoretix 2D software showed a number of protein spots, which were found to be interesting for further investigations. Therefore, the 2D-PAGE were stained with Coomassie Brillant Blue and the number of spots visualized on the gel decreased, due to the less sensitivity of CBB staining compared to silver staining. MALDI mass spectrometry and/or *N*-terminal amino acid sequencing were used to analyze protein spots excised from repetitive 2D-PAGE.

3.2.4.1 Identification of proteins with MALDI-TOF/MS

Spots A and B were in-gel digested with trypsin followed by the separation of the peptide mixture by RP-HPLC (Fig. 3.18). The peptide fractions were collected manually and some fractions (T17, T19 and T29) were submitted to the mass spectrometry analysis in order to define their mass. Fig. 3.19 shows the MALDI mass spectrum of peptide T17, T19 and T29. To confirm the result obtained with the mass spectrometry analysis, the same peptides were sequenced by Edman degradation. The sequencing of the peptide T29 of spot A with the monoisotopic protonated mass MH^+ of 1699 (Fig. 4) gave the sequence YRG(S)EYTVEFLQK. Table 3.1 shows the MS-Edman search results of the peptide T29. The database search with sequence identified the protein as nitrogen regulatory protein P-II from *Synechococcus* sp. (position 46 of the protein sequence). Serine at position 4 of the peptide could not be identified by Edman degradation, but the mass difference of 79 Da corresponded to the known phosphorylation site at position 49 of the protein sequence.



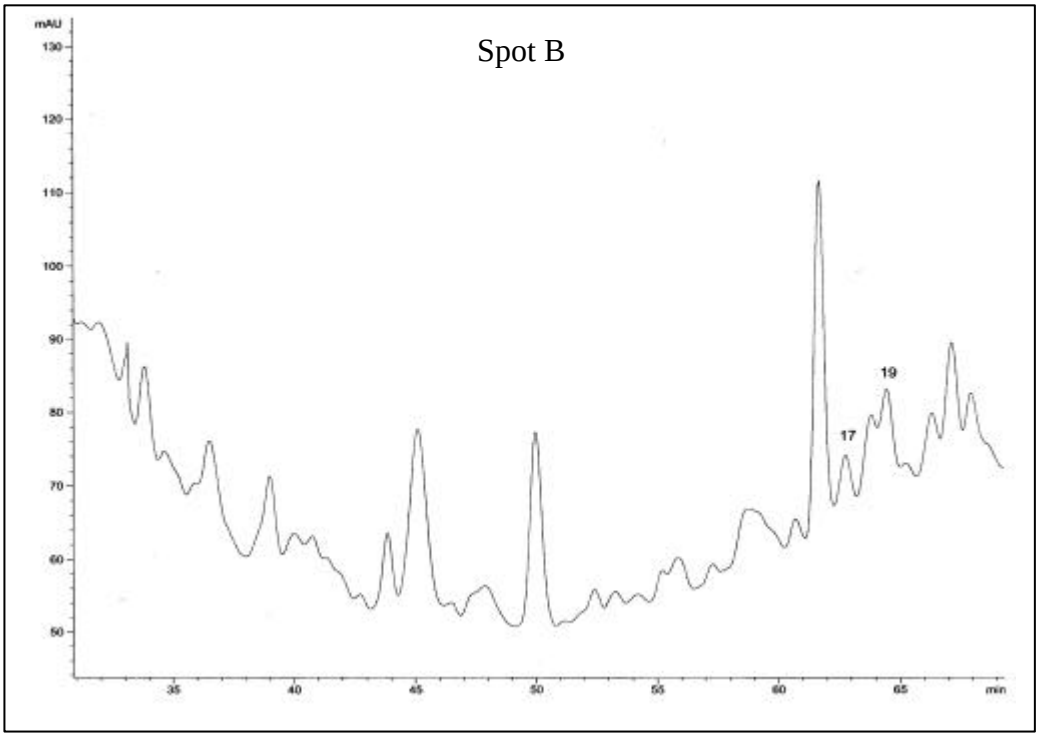


Fig. 3.18 RP-HPLC spectra of spots A and B. 300-400 µg proteins were separated by 2D-PAGE. The spots were excised from Coomassie Brillant Blue stained gels and digested with trypsin for 18 h at 30°C. The resulting peptides mixture were separated on a RP-HPLC column. Peptide fractions were collected manually and those labeled with a number were submitted to Edman degradation and MS analysis.

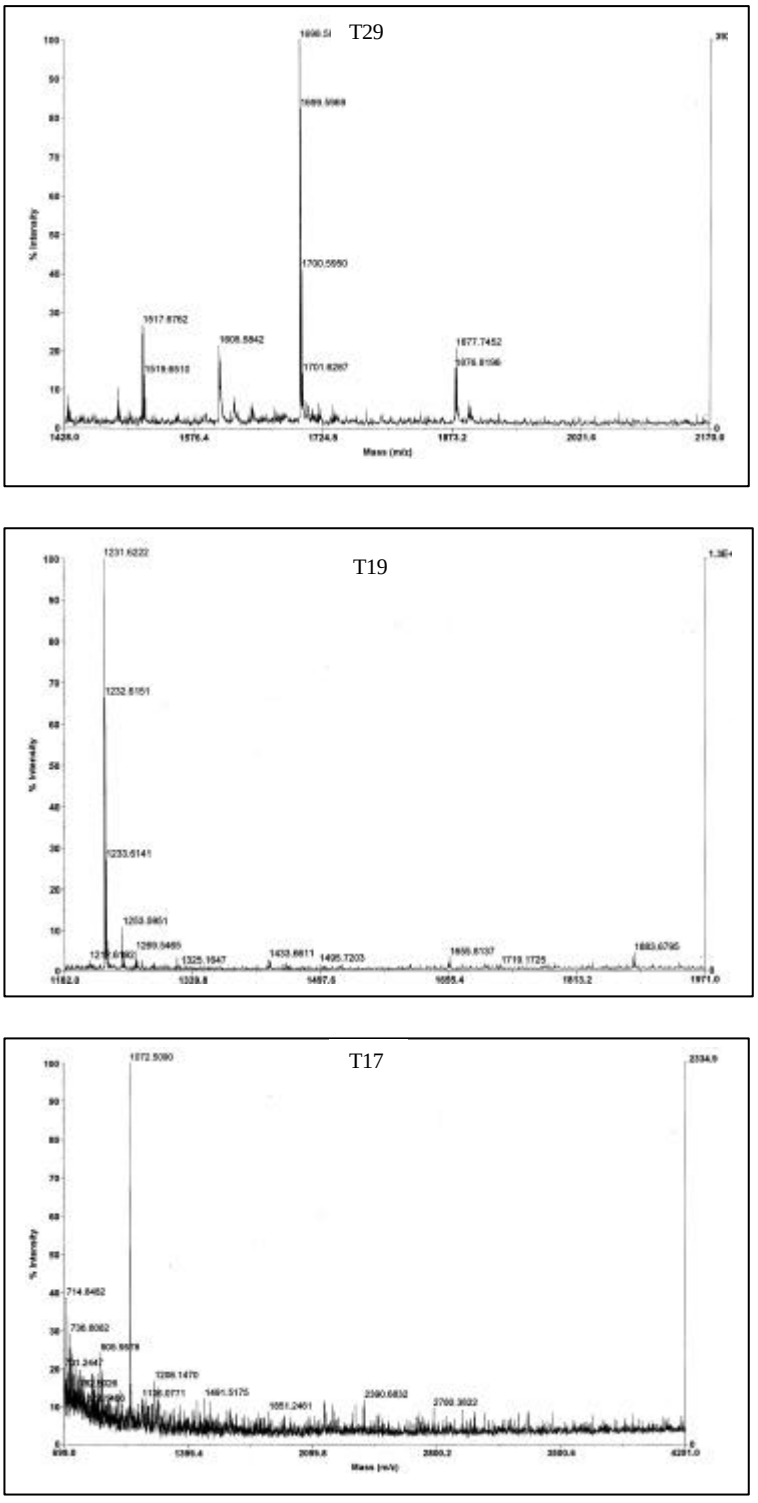


Fig. 3.19 MALDI mass spectra of different peptide fragments separated by RP-HPLC after in-gel digestion of spots A (T29) and B (T17 and T19) by tryptin. The monoisotopic protonated mass MH^+ of each peptide is indicated. The peptides were sequenced and used for MS-Edman search.

Table 3.1 Protein spots from 2D-PAGE identified with MALDI-TOF/MS

Spot	Peptide	*N*-terminal amino acid sequence	Position	Protein name	Gene
A	T29	YRG(S)EYTVEFLQK	46	P-II protein	*glnB*
B	T19	IFISPVDSVVR	91	P-II protein	*glnB*
	T17	KVEAII	3	P-II protein	*glnB*

Table 3.2 MS-Edman search results of the peptide T29 of spot A. 10 entries were selected and the regulation expression sequence is YRGSEYTVEFLQK.

Number of Substitutions	Matching Sequence	Protein MW (Da)/pI	Species	Protein name
0	(R)YRGSEYTVEFLQK(L)	12387.4/7.95	*Synechococcus* sp.	Photosystem II Signal transducing protein
0	(R)YRGSEYTVEFLQK(L)	12391.4/7.95	*Synechococcus* sp PCC 6301	Nitrogen regulatory protein P-II
0	(R)YRGSEYTVEFLQK(L)	12478.6/7.95	*Fremyella diplosiphon*	GlnB protein
0	(R)YRGSEYTVEFLQK(L)	12397.4/6.33	*Synechocystis* sp.	P-II protein
0	(R)YRGSEYTVEFLQK(L)	9462.8/5.14	*Synechocystis* sp. PCC 6803	Nitrogen regulatory protein P-II
0	(R)YRGSEYTVEFLQK(L)	12478.6/7.95	*Nostoc punctiforme*	P-II
0	(R)YRGSEYTVEFLQK(L)	12492.6/7.95	*Nostoc* sp. PCC 7120	P-II protein
0	(R)YRGSEYTVEFLQK(L)	12432.5/5.54	*Synechococcus* sp. PCC 7002	Nitrogen metabolism regulatory protein
0	(R)YRGSEYTVEFLQK(L)	10009.6/8.20	*Synechocystis* sp. PCC 9413	P-II protein
1	(R)YRGSEFTVEFLQK(L)	12314.2/5.28	*Prochlorococcus marinus*	P-II protein GlnB protein

Two peptides were sequenced from spot B; T19 with MH^+ 1231.7 (Fig. 3.19) had the sequence IFISPVDSVVR, similar to position 91 of the nitrogen regulatory protein P-II, and peptide T17 with MH^+ 1072.5 (Fig. 3.19) had the sequence KVEAII, and was similar to position 3 of the same protein sequence. Spots A and B were identified as isoforms of the signal transducing protein, "Nitrogen Regulatory Protein P-II" encoded by *glnB* gene.

3.2.4.2. Identification of proteins by *N*-terminal amino acid sequencing

Some proteins spots were electroblotted on PVDF membrane for *N*-terminal amino acid sequencing. The protein spots were excised from PVDF membrane after repetitive 2D-PAGE-blotting, and submitted to Edman degradation. Spots C and D were analyzed by amino-terminal sequencing (table 3.3). With this method, three parameters were involved in the identification of the protein: The apparent molecular weight (MW), the apparent isoelectric point (pI) and the *N*-terminal sequence.

Table 3.3. Protein spots from 2D-PAGE identified by *N*-terminal amino acid sequencing

Spot	*N*-terminal sequence	Apparent MW (kD)	Apparent pI	Gene product	Gene
C	SIAVGM	12.3	5.5	Carbon dioxide concentrating mechanism protein	*ccmK*
D	ANATVKM	11.8	4.9	Plastocyanin	*petE*

Spot C showed the *N*-terminal sequence SIAVGM. The apparent MW and the pI were 12.3 kDa and 5.5 respectively. Spot D showed the *N*-terminal sequence ANATVKM and the apparent MW of 11.8 kDa and pI of 4.9. These data were used to identify the protein in the Cyano2Dbase. Spot C was found to match with the "Carbon Dioxide Concentrating Mechanism Protein" encoded by *ccmK* gene and spot D with "Plastocyanin" encoded by *petE* gene.

3.2.5. Time-courses monitoring of the identified proteins

The expression of the identified proteins was monitored by incubation of the cells in nitrate-depleted medium for different times. Fig. 3.20 shows the time-course expression of the 2 isoforms of nitrogen regulatory protein P-II, the plastocyanin and the carbon dioxide concentrating mechanism protein. The intensity of each spot was measured with Phoretix 2D

software. The relative intensity is the difference between the intensity in the test and the control conditions. The nitrate-depleted induced proteins analyzed, were expressed during the first 8 h starvation and particularly between 6 and 8 h. After this time, the expression remained roughly stable or decreased.

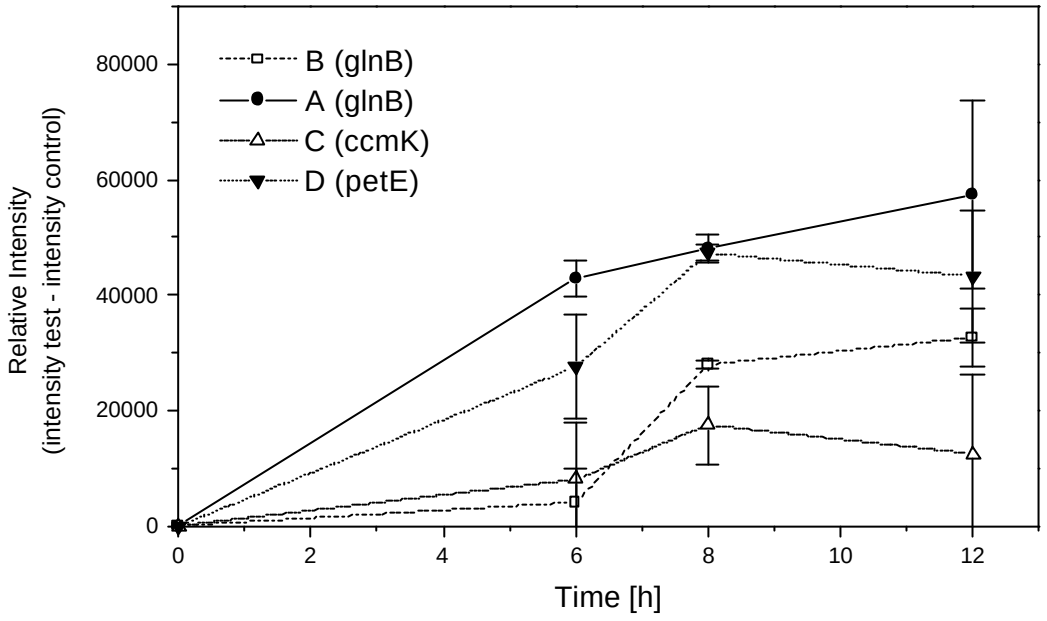


Fig. 3.20 Expression monitoring of the identified protein spots after different starvation times. The intensity value measured with the Phoretix 2D software is the difference between the intensity in the test and the control conditions.

The proteome analysis in *Synechocystis* sp. strain PCC 6803 provided new proteins, which are expressed under nitrate starvation. It is found that these proteins are not only expressed in nitrate starvation conditions. However, the genes that encode the nitrogen regulatory protein P-II or the carbon dioxide concentrating mechanism protein could be used for the construction of a biosensor both for nitrate and carbon.

3.2.6 Conclusion

In the proteome analysis of this study, three proteins were shown to be related to nitrate depletion. However, not all the proteins on the 2D gel could be analyzed because of the low concentration of most of them and the blocking of the *N*-terminal end during Edman degradation. The use of a microarray on which some genes in *Synechocystis* are spotted, could

be a means to confirm and extend the result gained with proteomics at the level of the transcriptome. For this reason, and in order to better quantify the expression of genes induced under nitrate depletion, investigations to monitor the gene expression in *Synechocystis* were performed and described in the following chapter.

3.3. DNA Microarray analysis of gene expression

A microarray experiment consists of an array, which contains immobilized nucleic sequences, or "probes", one or more labeled samples or "targets" that are hybridized with the microarray and a detection system that quantifies the hybridization signal. Gene expression analysis experiment compares the relative expression levels of specific transcripts in two samples. One of these samples is a control and the other is derived from cells whose response or status is being investigated. To elucidate the effect of nitrate starvation on the global gene transcription profile of *Synechocystis*, cells were grown in nitrate-depleted and nitrate-repleted media for 10 h and RNA was extracted. This incubation time was sufficient to induce large changes in the gene expression profile. In the present experiment, each of these RNA samples was reverse transcribed to cDNA, labeled with different fluorescent dyes, and equal amounts of the labeled cDNA were combined and hybridized with a microarray.

3.3.1. Integrity and size distribution of total RNA

Total RNA extracted from *Synechocystis* PCC 6803, were loaded on agarose and denaturing-agarose gel electrophoresis. Fig. 3.21 shows the size distribution of total RNA on agarose gel electrophoresis. The ribosomal RNA (16S and 23S) appeared as sharp bands on the ethidium bromide stained gel. The ribosomal RNA (23S) band should be present at approximately twice the size of 16S rRNA, but two 23S rRNA bands were found instead of one. After repetitive RNA extractions and agarose gel electrophoresis, the two 23S rRNA bands remained. The size distribution of 23S rRNA and 16S rRNA are 2.9 kb and 1.5 kb respectively. The sizes of these ribosomal RNAs on the agarose gel did not correspond to the expected ones compared to the marker RNA. To confirm the size of the rRNA, total RNA extracts were loaded on denaturing formaldehyde agarose gel electrophoresis (Fig. 3.22). The size of each rRNA was as expected according to the marker RNA.

The confirmation of the integrity and purity of total RNA is necessary before a microarray experiment. Therefore, the size distribution and the purity of the RNA sample was checked using an Agilent 2100 Bioanalyzer, which separates nucleic acids according to their size. Fig.3.23 shows the electrophoregrams of total RNA. No unknown peak was detected. Four peaks were found on the electrophoregram which corresponded to 23S rRNA (2 peaks), 16S rRNA (1 peak) and 5S rRNA (1 peak). Neither contamination nor degradation of total RNA was detected on the electrophoregram.

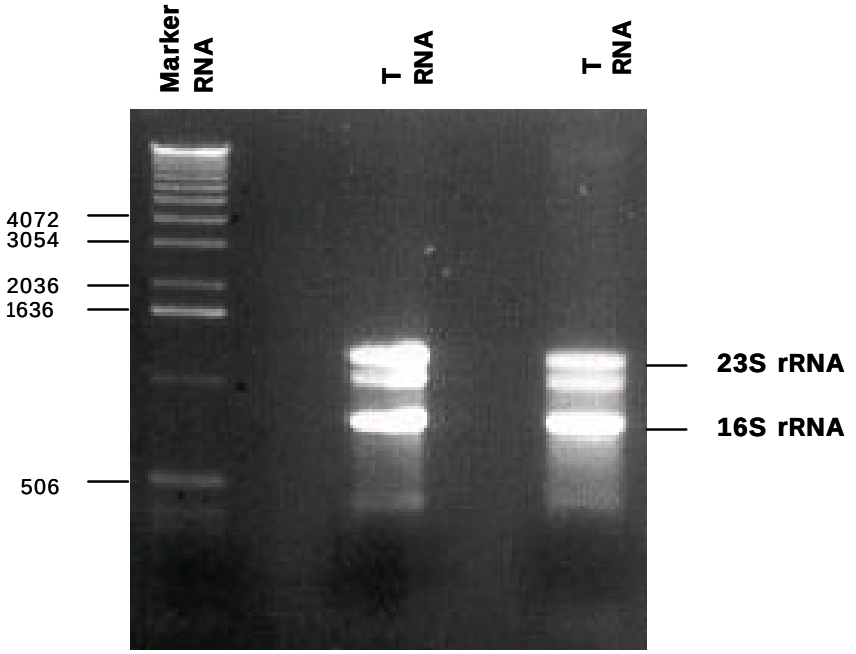


Fig.3.21 Gel electrophoresis of total RNA sample after extraction using RNeasy kits. 4 µg total RNA were loaded on an agarose gel electrophoresis. The gel was run together with a maker RNA.

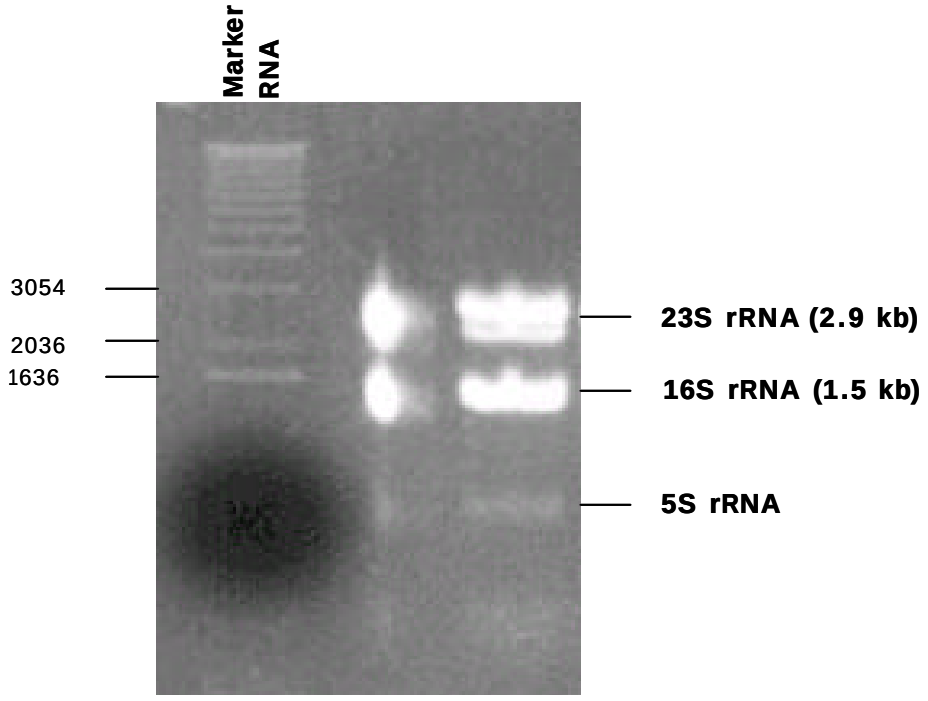


Fig.3.22 Gel electrophoresis of total RNA samples after extraction using RNeasy kits. 4 µg total RNA were loaded on a denaturing formaldehyde gel electrophoresis. The gel was run together with a maker RNA.

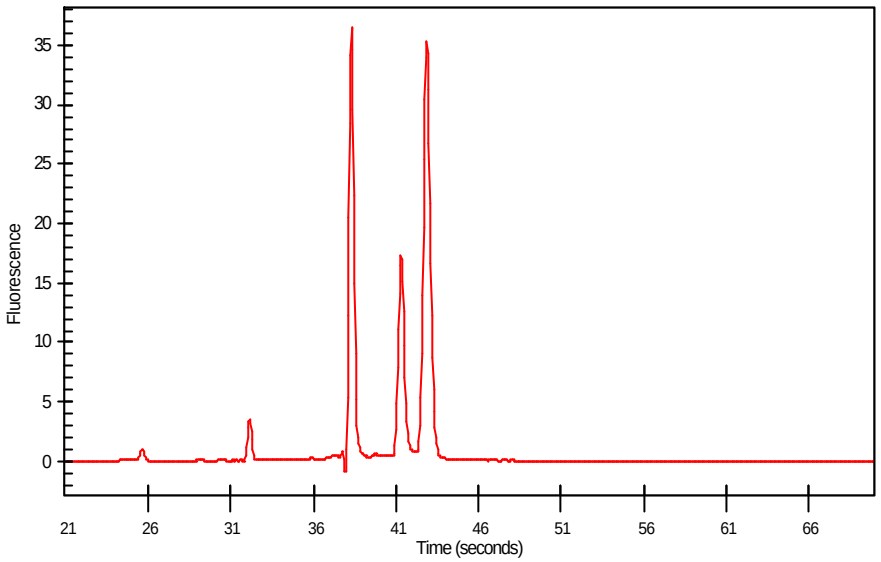


Fig.3.23 *Synechocystis* total RNA before mRNA enrichment. The total RNA was isolated using RNeasy kits and the electrophoregrams were generated by running 2.2 µg total RNA on an Agilent 2100 Bioanalyzer using RNA LabChip kit.

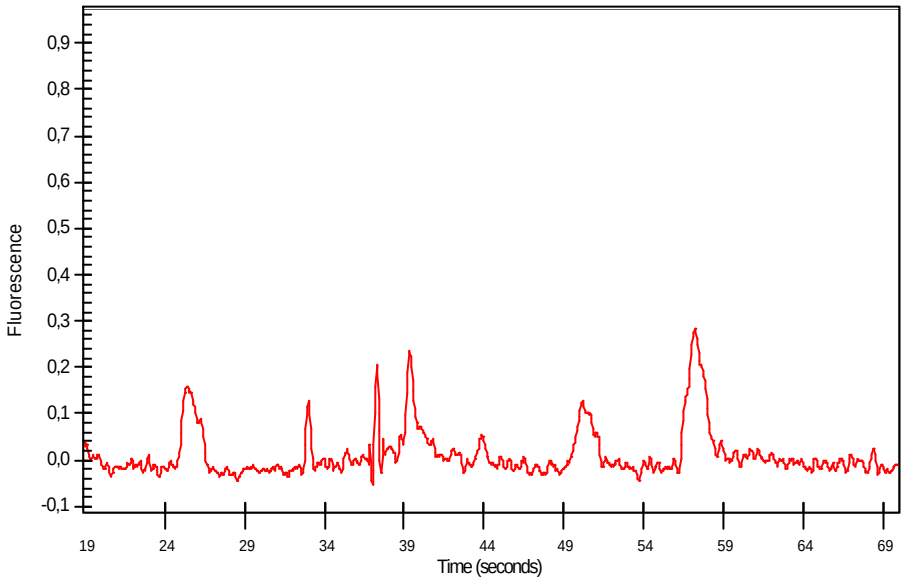


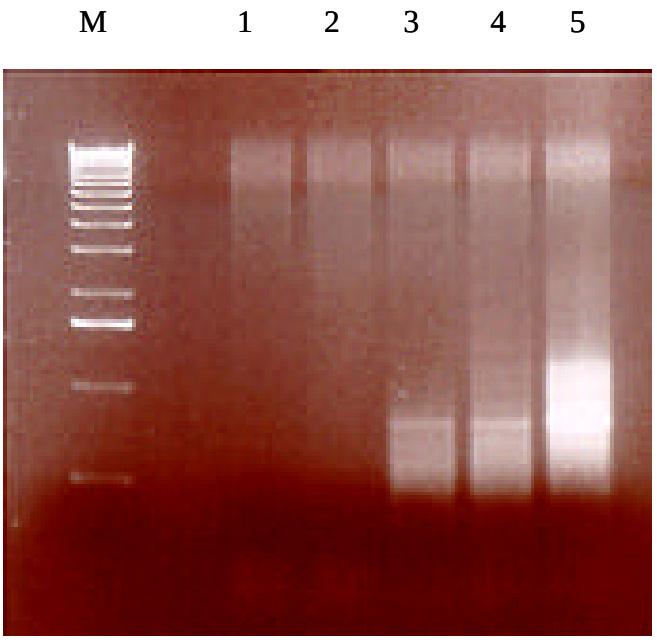
Fig.3.24 *Synechocystis* RNA after mRNA enrichment with a MicrobExpress Kit. The total RNA was isolated using RNeasy kits and mRNA was enriched from 10 µg total RNA using the MicrobExpress kit. The electrophoregrams were generated by running 0.14 µg enriched mRNA on an Agilent 2100 bioanalyzer using RNA LabChip kit.

3.3.2. Purification of mRNA from total RNA

mRNA was extracted from this pure total RNA and the purity and concentration were checked using Agilent 2100 Bioanalyzer (Fig. 3.24). In spite of the great difference in the concentrations of total RNA (2.2 µg) and mRNA (0.14 µg) on the electrophoregrams, almost all the high size ribosomal RNAs were removed. The enriched mRNA contained mRNA, 5S rRNA, tRNA and other small RNAs.

3.3.3. Reverse transcription and cDNA labeling

After purification of RNA, the sample was reverse transcribed to cDNA and labeled with Cy3-dUTP or Cy5-dUTP fluorescent dyes. The cDNA was then purified after degradation of the remaining RNA and the labeling efficiency was checked. Due to an assumed low incorporation rate of the Cydyes in the cDNA sequence, the optimal amount of dTTP (1 mM) to be added to the reverse transcription reaction was checked. The same amount of total RNA were reverse transcribed to cDNA and labeled with Cy3-dUTP. To the reverse transcription reaction mixture, different amount of dTTP were added. Afterwards, the resulting cDNA samples were loaded on an agarose gel electrophoresis (Fig. 3.25).



Incorporation rate

1 : 190

2 : 210

3 : 145

4 : 150

5 : 200

Fig. 3.25 Labeled cDNA on agarose gel electrophoresis. 20 µg total RNA were reverse transcribed to cDNA with different amount of 1 mM dTTP: 1 (0 µl), 2 (0.05 µl), 3 (0.1 µl) 4 (0.5 µl) and 5 (1 µl). M = RNA marker.

The incorporation rate was calculated according to the following equation:

Nucleotides/Cydye = [cDNA] / [[Cydye] x 330]

[cDNA] : cDNA concentration (pg/µl)

[Cydye] : Cydye concentration (pmol/µl)

330 : average nucleotide molecular weight

According to the concentration of cDNA yielded and the labeling efficiency, 0.5 µl and 1 µl dTTP (1 mM) were the best amounts for an optimal reverse transcription-labeling condition.

3.3.4. Design of the oligonucleotide sequences

Table 3.4 Selected genes for the *in-house* made chip

Gene categorie	Gene name	Gene product
Up-regulated genes	*glnB*	Nitrogen regulatory protein p-II
	nblA	Phycobilisome degradation protein
	ccmK	Carbon dioxide concentrating mechanism protein
	petE	Plastocyanin
	ntcA	Global nitrogen regulator
Down-regulated genes	*cpcA*	Phycocyanin alpha subunit
	apcA	Allophycocyanin alpha subunit
	cpcG	Phycobilisome rod-core linker polypetide
Unknown behavior	*isiA*	Ion-stress chlorophyll-binding protein
	phoA	Putative purple acid phosphatase

To confirm and extend the result gained with proteomics, three categories of genes (table 3.4) were selected according to their expected behavior (expressed, repressed, not known) under nitrate starvation. All the genes that encoded the proteins analyzed through proteomics and some others found in the literature, which are expected to be down-regulated, up-regulated or unknown (behavior not defined) under nitrate depletion, were used for the oligonucleotide microarray experiment. The amino-modified oligonucleotides were spotted on CreativeChip oligo slides.

3.3.5. Hybridization of the labeled cDNA on the oligo-slide

To characterize the microarray hybridization and to check the behavior of the olgonucletides immobilized on the slides, cDNA from one source labeled with Cy3-dUTP was hybridized on the oligonucleotide array. cDNA from total RNA extracted from test cells, labeled with cy3-dUTP were hybridized on the oligonucleotide slide and scanned. Each oligonucleotide spot on

the array was labeled with the corresponding gene's name. Fig. 3.27 shows the DNA microarray of cDNA hybridized on the oligonucleotide slide. All the up-regulated genes found in proteomics analysis and those found in the literature were highly expressed whereas the down-regulated ones were almost repressed or constant. The *phoA* gene that, the behavior was unknown was also highly expressed on the DNA microarray. The *isiA* gene, the other unknown behavior gene, which is expressed under iron depletion, was not induced in these conditions. *ApcA*, *cpcA* and *cpcG* genes encode protein units of the complex phicobiliproteins, and as expected, these genes were not expressed under nitrate starvation.

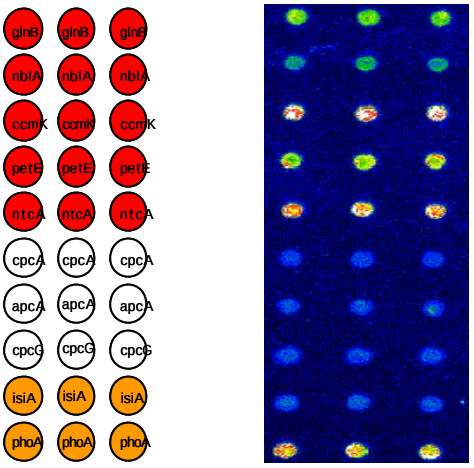


Fig. 3.26 One-color fluorescent image of a DNA microarray of nitrate depletion induced genes in *Syechocystis* sp. PCC 6803. The left image shows the array layout where spots are labeled with the corresponding gene's name.

3.3.6. Hybridization of test and control cDNAs on the oligo-array

For gene expression analysis, cDNAs from cells grown in control and test conditions were hybridized on the oligonucleotide microarray. cDNAs from total RNA (6 µg) labeled with Cy5-dUTP (control: green) and Cy3-dUTP (test: red) were mixed and hybridized on the oligonucleotide microarray (Fig. 3.27). Because of the absence of the internal control spot on the array, the spot intensities were normalized with a constantly expressed gene, which in this case was *isiA* gene.

To elucidate the intensity of each spot, the microarray was analyzed with ImaGene Software and the expression level of each gene, which represents the mean from 3 spots is depicted in Fig. 3.28. The genes, which encode the proteins identified with proteomics were up-regulated as the products found on 2D-PAGE.

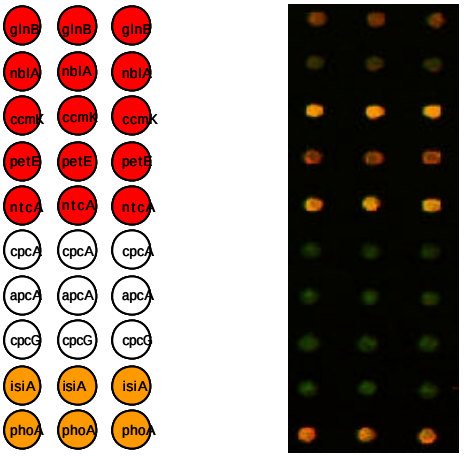


Fig. 3.27 Two-color overlaid fluorescent image of a DNA microarray of nitrate depletion induced and nitrate depletion-repressed gene in *Syechocystis* sp. PCC 6803. On the right image, two hybridization data sets from the same microarray were converted into pseudocolor images and superimposed to visualize differential gene expression between nitrate depleted (Cy3, red) and nitrate repleted (Cy5, green).

Among these genes, *ccmK* gene that encodes for the "carbon concentrating mechanism protein" was the most expressed one. The *nblA* gene, used in the first section of this work for the construction of the CyanoSensor for nitrate was also expressed but the *ntcA* gene, found in the literature, which encodes for the global nitrogen regulatory protein was more expressed. All the genes which are repressed and whose products are degraded after a long nitrate starvation time, according to the literature, were not expressed. The intensities remained almost constant in both conditions. The *phoA* gene, one of the genes, whose behavior was not known, was greatly expressed whereas the other one, *isiA* gene remained constant in both conditions.

The intensity of each gene was expressed as the percentage of test versus control, to highlight their relative expression (Fig. 3.29). According to this representation *petE* gene which encodes for the plastocyanin was the most expressed. In the same way, *nblA* gene which seemed weakly expressed on Fig. 3.28 was more expressed.

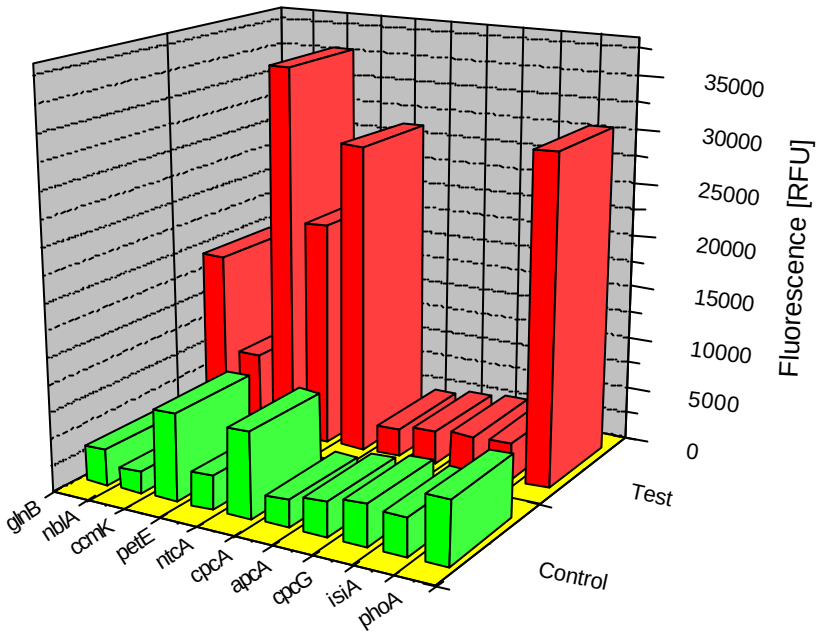


Fig. 3.28 Effect of nitrate depletion on the gene expression. Fluorescent cDNA from test and control conditions were hybridized on the oligonucleotide microarray.

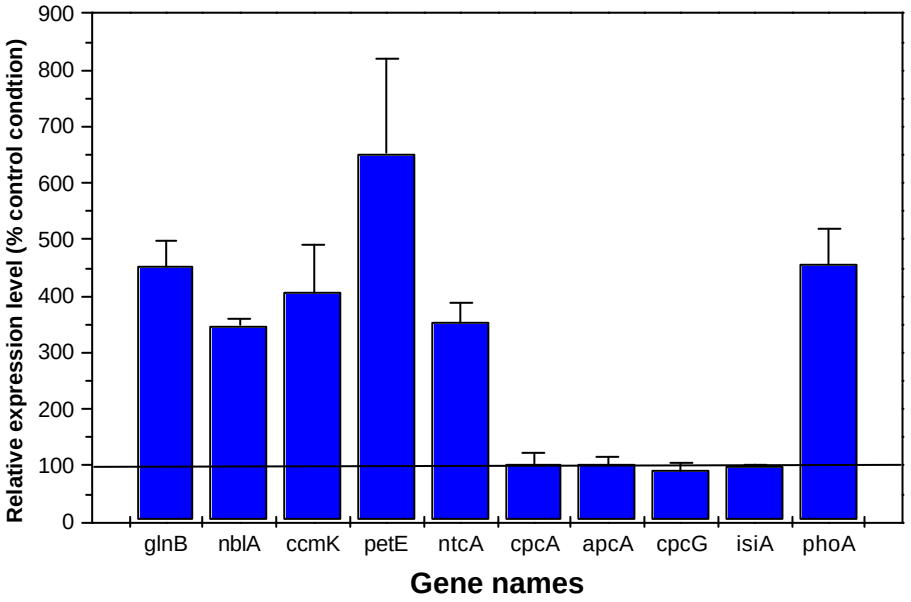


Fig. 3.29 Effect of nitrate depletion on the gene expression. The fluorescence intensity of each gene is expressed in percentage of the intensity in control condition.

3.3.7. Conclusion

This experiment revealed the behavior of each gene under nitrate depletion condition. It confirms not only the result gained with proteomics, but validates also the statement on some genes found in the literature. Furthermore, the differential expression of each gene was more emphasized compared to the differential expression of proteins. This experiment allowed the detection of the change in the behavior of less expressed genes, and therefore illustrated a good quantification of the expression.

4. DISCUSSION

4.1. Reporter assay

4.1.1. Characterization of the reporter strain N1LuxKm in liquid medium

In accordance to previous reports (Collier and Grossman, 1994; Sauer et al., 1999), results indicated that nitrate is essential for the growth of cyanobacteria: the reduction of the growth rates of N1LuxKm and WT cells cultivated in BG-$11_{(-N)}$. The nitrate calibration curve obtained with free N1LuxKm cultures showed that above 7 hours of incubation, the relationship between bioluminescence and nitrate concentration was no longer linear. A number of factors could contribute to the decrease of the bioluminescence response: (1) a reduced luciferase synthesis rate, (2) limited availability of reaction substrates such as O_2, aldehyde, reduced flavine mononucleotide, NADPH, and ATP, (3) intracellular dilution by cellular growth, and (4) intrinsic stability of the luciferase enzyme (Heitzer et al., 1994). The length of time required for the bioluminescence induction could be explained by the consumption of available internal nitrate by the reporter strain. The state of this sensor in liquid medium is not appropriate for field application. Therefore it was packed into an easy-to-use format (immobilized), which simplified and minimized its operation, and made it storable.

4.1.2. Matrix for immobilization

The immobilization of microbial cells in different carriers leads to change in their microenvironment (Shreve and Vogel, 1993). Because of these changes, immobilized cells show various modifications in physiology and biochemical composition when compared to suspended cells (Hilge-Rotmann and Rehm, 1990). Therefore the choice of an optimal matrix of immobilization is significant. This matrix did not interfere with the luminescence measurement and therefore was ideal for immobilization. It showed a good compromise between the stability and the sensor response.

4.1.3. CyanoSensor characterization

The biosensor immobilized in agar showed a longer bioluminescence induction time compared to free cells. This was probably due to the presence of some nitrogen derivatives in

this matrix: the cells could be using the nitrogen derivatives from the matrix, thus delaying the sensor response. In order to remove these derivatives, we tested the use of "washed agar". The washing steps of agar removed these derivatives; consequently the bioluminescence induction time for the immobilized cells in washed agar was reduced and similar to that of free cells. Due to these results and the simple immobilization procedure, agar was an ideal matrix for immobilization. The nitrate concentration in an environmental sample depends on the source. For instance, in rain-water, it varies between 20 and 78 µM and in freshwater between 214 and 885 µM (BCR reference materials, 2000). Our sensor could thus be directly used to detect nitrate in sample from rain-water but sample from freshwater should be diluted. The high detection range and the stability of this nitrate bioreporter make it a useful tool for examining bioavailable nitrate in freshwater environments.

4.1.4. Sensor optimization

Phosphorus and sulfur are also essential nutrients for cyanobacterial blooms (Collier and Grossman, 1994). Nevertheless, the lack of these nutrients in the medium did not induce any bioluminescence in our experiments, showing the specificity of the reporter strain response for nitrogen compounds. The *nblA* gene used in the construction of the reporter strain, is specific for nitrate (nitrogen compounds) deprivation in *Synechocystis* PCC 6803. The specificity of *nblA* gene for nitrate in *Synechocystis* PCC 6803 was previously demonstrated in Richaud et al. (2001).

Synechocystis PCC 6803 is not able to fix molecular nitrogen, a biosensor for nitrate instead of nitrogen was thus built. The utilization of nitrate in cyanobacteria is a genuine photosynthetic process directly driven by the assimilation power in the light. This process includes (1) the entrance of nitrate into the cell, apparently mediated by an active transport system, (2) the two-step reduction of nitrate to ammonium catalyzed by the ferredoxin-dependent enzymes nitrate and nitrite reductases and (3) the incorporation of ammonium to carbon skeletons via the ATP-dependent glutamine syntethase system (Flores et al., 1983). The effect of illumination is one of the most important factors for the CyanoSensor characterization. Schreiter et al. (2001) demonstrated that 50 $\mu E.m^{-2}.s^{-1}$ was the optimal light intensity for the response of the CyanoSensor APL of *Synechococcus* PCC 7942 immobilized in agar. Thus, this light intensity was used for the characterization of the CyanoSensor N1LuxKm. The optimal sample incubation time of the CyanoSensor N1LuxKm was 10 h. Considering that cyanobacteria exhibit relatively slow growth and metabolic rates (Paerl, 1996), this sensor's response was acceptable and in the range of naturally occuring changes.

The high sensitivity of this biosensor to ammonium and nitrate demonstrated that it could be used to monitor the bioavailability of nitrogen in environmental samples. Nitrite is an intermediate in nitrate utilization and the main sources of nitrogen in *Synechocystis* PCC 6803 are nitrate and ammonium. The lack of only ammonium could show a low sensor response comparing to the lack of only nitrate. The abundance of ammonium could inhibit the uptake of nitrate, thus leading to almost no sensor response (Flores et al., 1983). More detailed and intensive studies, especially physiological and long-term ecological studies among cyanobacteria and correlation between this reporter strain N1LuxKm and other bloom-forming cyanobacteria, are required in order to use this sensor as a tool for prediction of algal bloom formation.

We stored the immobilized cells in the refrigerator at 4°C in dark, thus preventing the growth of the cells. Nevertheless, the biosensor needed an energy source in which cells survive during the storage. The coverage media used for storage showed the best compromise between survival and bioluminescence response. The sensor proved sufficient stability after storage. The sensitivity of stored sensors was higher than that of freshly prepared ones at low nitrate concentration range. This effect can probably be attributed to the consumption of available nitrate resources during storage. This assumption remains to be verified by determination of cellular nitrate content before and after storage.

In this study, a novel *lux*-marked cyanobacterial biosensor was developed for the detection of nitrate in environmental samples (rain-water, freshwater, seawater). The biosensor described in this study could be stored for at least one month compared to the microscale NO_3^- biosensor developed by Larsen et al. (1997), which could be stored only for 2-4 days. Moreover, this mocroscale NO_3^- biosensor suffers from interferences from other substances like HCO_3^-, NH_4^- and acetate despite its high sensitivity (1 µM NO_3^-) and its short response time (30-60 s). The CyanoSensor N1LuxKm overcomes these interferences. An essay period of 10 h was suitable for the detection of nitrate in a wide concentration range (4-100 µM). Whole living-cell biosensors have been reported for a number of compounds (Scheller and Schubert, 1992). The advantages of whole-cell biosensors over enzyme biosensors are high stability and the information on physiological and biological parameters that can be obtained. Disadvantages include the longer response times due to the complex biochemical processes that are involved, as well as the higher diffusion resistances of cellular membranes and immobilization matrices. In addition, whole-cell systems exhibit reduced substrate specificities compared with enzymatic biosensors (Scheller and Schubert, 1992). However,

the use of genetically engineered bacteria with *lux* fusions to the regulatory elements of inducible genes or operons largely overcomes the specificity problem. The presence and concentration of nitrate can be determined with greater accuracy by chemical analytical methods rather than with a bioluminescent reporter bacterium. However, the biosensor reported here provides additional unique information on bioavailability and the specific catabolic/anabolic activity of a non-nitrogen fixing cyanobacterium.

The method used in this work for nitrate bioavailability monitoring in water, is simple and selective and the small size of the biosensor kit, in which the bioluminescent reporter strain N1LuxKm was immobilized, was found to be very advantageous. In order to confirm the CyanoSensor contribution to monitoring of algal blooms, the system will be evaluated using real samples from the environment. Some investigations will be made to improve the sensor response, by reducing the induction time and extending the storage duration. Because cyanobacterial occurrence and abundance depends on a complex interplay of factors, and not only on one environmental parameter like nitrate availability, the engineering of further reporter strains responsive to other factors is considered to be useful for the monitoring of algal bloom and cyanotoxin formation. In the present case, the change of the phenotype in the cyanobacterium *Synechocystis*, from blue-green to yellow, when grown under nitrate depletion conditions could be probably explained better at the molecular level. For this reason, and in order to find stronger induced genes under nitrate depletion, investigations to monitor the proteome of *Synechocystis* were performed.

4.2. Protein expression analysis

A proteome represents a protein pattern of an organism, a cell, an organelle, or even a body fluid determined quantitatively at a certain moment and under precisely defined limiting conditions. Unlike the genome, the proteome is thus a highly dynamic system, which is characteristically altered by changes in environmental conditions. Proteome analysis is only meaningful in combination with a subtractive procedure in which two or more well-defined states can be compared. Two-dimensional electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. An appropriate sample preparation is absolutely essential for good two-dimensional electrophoresis results.

4.2.1. Sample preparation

Synechocystis cells were disrupted at cold temperature with glass beads, which by vortexing broke cells walls, thus liberating the cellular contents (Blomberg et al., 1995; Cull and McHenry, 1990; Jazwinski, 1990). This vigorous lysis method was used instead of the gentle enzymatic lysis method because *Synechocystis* cells with tough cell walls are less easily disrupted. When cells are lysed, proteases are often liberated or activated. Degradation of proteins through protease action greatly complicates the analysis of 2D-PAGE results, so protease inhibitor was added to the sample preparation buffer. Because proteases are less active at lower temperatures and preparing the sample in the presence of Tris base, sodium carbonate, or basic carrier ampholyte can often inhibit proteolysis, the cell disruption process in Tris buffer at cold temperature greatly prevents the degradation of proteins. Non-protein impurities in the sample can interfere with separation and subsequent visualization of the 2D-PAGE result, so sample preparation can include steps to rid the sample of theses substances. Salt contamination is the most frequent cause of insufficient focusing of protein spots. The use of low concentrated Tris buffer minimized the salt contamination.

In order to achieve a well-focused first-dimension separation, sample proteins must be completely disaggregated and fully solubilized. The sample solutions must contain certain components to ensure complete solubilization and denaturing prior to first-dimension IEF. Complete denaturation ensures that each protein is present in only one configuration, and that aggregation and intermolecular interaction is avoided (table 4.1).

Table 4.1 Forces implied in protein cohesion and interactions with other molecules (Rabilloud, 1996).

Nature of the interactions	Energy of interaction (kCal/mole of bond)	Disruption method(s) or agent(s)
Disulfide bond	40	Reduction (facultative alkylation)
Hydrogen bond	3-8	Chaotropes
Electrostatic interactions	2-5	Salts, charged detergents, chaotropes, strong dipolar molecules (less efficient)
Charge-dipole	1	Salts, dipolar molecules, chaotropes
Dipole-dipole	0.3	Salts, dipolar molecules, chaotropes
Van der Waals	0.3	
Hydrophobic interactions		Chaotropes, detergents

Urea, a neutral chaotrope, was used as denaturant in the first-dimension of 2D-PAGE. It solubilizes and unfolds most proteins to their fully random conformation, with all ionisable groups exposed to solution. The non-ionic or zwitterionic detergent CHAPS was included in the sample solution to ensure complete sample solubilization and to prevent aggregation through hydrophobic interactions. Foregoing studies have demonstrated that this detergent is often more effective than NP-40 or Triton X-100 (Perdew et al., 1983). SDS is a very effective protein solubilizer, but because it is charged and forms complexes with proteins, it cannot be used as the sole detergent for solubilizing samples for 2D-PAGE.

4.2.2. 2D-PAGE using immobilized pH gradients

In the first-dimension isoelectric focusing, longer strips (18 cm) were used to maximize the resolution and the loading capacity; and the pH interval of 3-10 was used for an overview of total distribution. Sample was applied to the IPG strip holders, included in the rehydration solution for some reasons: (1) It allows larger quantities of proteins to be loaded and separated, (2) it eliminates the formation of precipitates at the application point that often occur when loading with sample cups, (3) the rehydration loading method is technically simpler, avoiding problems of leakage that can occur when using sample cups. DTT was added as reductant to the rehydration solution. It cleaves disulfide bonds to allow proteins to unfold completely. 2-Mercaptoethanol is not recommended, because higher concentrations are required, and impurities may result in artefacts (Marshall and Williams, 1984). Adding IPG buffer (carrier ampholytes mixtures) to the rehydration solution enhances protein solubility and produces more uniform conductivity across the pH gradient without disturbing IEF or affecting the shape of the gradient.

After IEF, the equilibration step saturates the IPG strip with the SDS buffer system required for the second dimension separation. Urea and glycerol present in the equilibration buffer reduce electroendosmosis and improve transfer of protein from the first to the second dimension (Görg et al., 1995) whereas DTT preserves the fully reduced of denatured, unalkylated proteins.

The equilibrated IPG gels were applied to the second-dimension gels, on which protein spots could be visualized with different detection methods. These features are desired for an effective detection method: High sensitivity, wide linear range for quantification, compatibility with mass spectrometry, low toxicity and environmental safe, and environmental friendly. Among the common detection methods are: (1) Autoradiography and fluorography which are the most sensitive detection methods, but sample must consist of

protein radiolabeled in vivo; (2) silver staining which is the most sensitive non-radioactive method. By omitting glutaraldehyde from the sensitizer and formaldehyde from the developing solution, this method becomes compatible with mass spectrometry analysis (Shevchenko et al., 1996), however at the expense of sensitivity; (3) Coomassie staining, although 50- to 100-fold less sensitive than silver staining, is a relatively simple method and more quantitative than silver staining. It is preferable when relative amounts of protein are to be determined by densitometry; (4) negative Zinc-Imidazole staining is well compatible with mass spectrometry, but it is a poor quantification technique; and (5) fluorescent labeling (Ünlü et al., 1997) and fluorescent staining (Patton, 2001; Steinberg et al., 2000; Yan et al., 2000), whose sensitivity is between colloidal Coomassie staining and silver staining. It requires fluorescence scanners, but it is compatible with mass spectrometry and shows a wide dynamic range for quantification.

On the 2D-PAGE stained with silver, 160 protein spots were clearly resolved after visualization with silver. There were a group of proteins with isoelectric point beyond the range of commercially available IPG strip, and in this gel system these proteins were either lost or remained at the top of the isoelectric focusing gel. Artifactual charge heterogeneity of a single protein produces a distinctive pattern, which can be recognized even in a complex mixture of proteins. The spots produced by a protein possessing charge heterogeneity form a series of spots with the same molecular weight; the spacing of the spots is consistent with single charge differences between consecutives spots. We have succeeded to obtain a good resolution of proteins and reproducible results on 2D-PAGE using the Immobilized Dry Strip Gel, pH 3-10 L/18cm, which cover almost the whole pI range of proteins. Because of the high concentration of proteins in the pH range 4-7 in *Synechocystis* sp. PCC 6803, the Immobilized Dry Strip Gel pH 3-10 NL/18 cm, in which the pH range 5-7 is larger was used. The 160 spots found on the 2D-PAGE represented 66% of the 244 spots from the whole cell extracts of *Synechocystis* sp. PCC 6803 described by Sazuka (Sazuka et al., 1999). This percentage of soluble proteins on 2D-PAGE represented almost the same found by Sazuka et al. (1999) in the whole cell extracts.

4.2.3. Protein identification

From Coomassie stained 2D-PAGE, 10 protein spots were excised and analyzed. From this analysis 4 protein spots were identified either by MALDI-TOF/MS or by *N*-terminal amino acid sequencing. The basic requirements of proteome analysis are: A wide dynamic-detection range, high-throughput and high-confidence protein identification, protein quantification, the

ability to deal with multiple proteins in a single spot and the ability to identify post-translational modifications. Mass spectrometry (MS) can fulfil all of these requirements. The direct digestion of CBB-stained proteins in the gel matrix is an attractive option to overcome the problem of producing sequence data from *N*-terminal blocked proteins. In-gel digestion circumvents many of the problems associated with electrotransfer of protein prior to digestion. However, only a limited number of protein spots were submitted during this study to this analysis, due to its cost.

The signal transducer "Nitrogen regulatory protein P-II" which was found in our study to be highly induced under nitrate starvation is known to be involved in the coordination of carbon and nitrogen metabilism through the control of both nitrate and bicarbonate uptake processes in *Synechocystis* PCC 6803 (Hisbergues et al., 1999). Huang et al. (2002a) found that the P-II protein, present as two spots –one phosphorylated and the other unphosphorylated- on the gel, was bound to the plasma membrane of *Synechocystis*. The mode of action of protein P-II, also known as GlnB, has been extensively studied in *Escherichia coli* (Merrick and Edwards, 1995). In this organism, the protein is covalently modified by uridylation of a conserved tyrosyl residue (Tyr-51). The modification takes place via phosphorylation of the Ser-49 residue of the corresponding protein in *Synechococcus* PCC 7942, although the protein also carries a conserved tyrosyl residue at position 51 (Forchhammer and Tandeau, 1994; Forchhammer and Tandeau, 1995). In *in-vitro* experiments, it was shown that the P-II proteins from *Escherichia coli* and from *Synechococcus* PCC 7942, in spite of their different modification types, share the property of binding two small molecule effectors, ATP and 2-oxoglutarate, in a positive cooperative manner (Forchhammer and Hedler, 1997; Jiang and Ninfa, 1999; Kamberov et al., 1995). The cellular concentration of the later metabolite has been postulated to be a crucial factor for the control of the modification level of P-II in both organisms. Since cyanobacteria lack 2-oxogluatarate dehydrogenase, 2-oxoglutarate is used solely as carbon skeleton for the incorporation of nitrogen into organic molecules. It is thus critical to coordinate synthesis of 2-oxoglutarate with nitrogen availability. This interdependence raised the possibility that P-II could play a role as a coordinator between 2-oxoglutarate production, inorganic carbon assimilation, and nitrogen metabolism. P-II protein indirectly controls the transcription of the glutamine synthetase gene (*glnA*). Nitrogen starvation provoked a strong increase in *Synecocystis glnB* mRNA levels that was also reflected in P-II protein levels (Garcia-Dominguez and Florencio, 1997). Under nitrogen deprivation, this might be a stand-by mechanism to assimilate nitrogen rapidly and efficiently when it becomes available. Among the proteins expressed, found up to date under nitrate

depletion condition, the signal transducing protein P-II is an interesting one for more study, because of its high expression, which could be used to sense nitrogen or carbon in water.

Plastocyanin, as second protein analyzed, and cytochrome C6 are soluble metalloproteins, which are very well characterized redox carriers located inside the thylacoidal lumen. They transfer electrons between the membrane complexes cytochrome b6-f and photosystem I (PSI). These two proteins possess similar physico-chemical and surface structural properties, which make them capable of replacing each other (De la Cerda et al., 1997; Grossman et al., 2001). In fact, their relative synthesis is regulated by copper availability in such a way that the cells produce either cytochrome C6 or plastocyanin in the absence or presence of copper, respectively (Ho and Krogmann, 1984). During nutrient limitation, when the anabolism of the cell is slowed down or completely arrested, $NADP^+$, the final electron acceptor of the photosynthetic electron transport chain, is not recycled as fast as under nutrient-repleted conditions and the electron carriers are maintained in a relatively reduced state (Grossman et al., 2001). In this case of nitrate limitation, the electron carrier plastocyanin is maintained in a reduced state and consequently the concentration in the cell increases. Nitrate limitation affects the photosynthesis process in cyanobacteia, thus modifiying the expression of some proteins involved in this event.

Carbon dioxide concentrating mechanism protein, the third protein analyzed is well characterized in some microorganisms. Huang et al. (2002a) found this protein as two copies of the *ccmK* gene in the plasma membrane of *Synechocystis*. Indeed, a number of aquatic photosynthetic microorganisms are able to concentrate dissolved inorganic carbon (DIC) intracellularly, allowing rapid growth despite low-CO_2 availability externally (Kaplan and Reinhold, 1999). This carbon concentrating mechanism (CCM) shows acclimation to external DIC to optimize CO_2 fixation efficiency (Badger et al., 1980).During acclimation these organisms induce the expression of a set of genes required for various aspects of the CCM. The dependence of nitrate utilization upon active CO_2 fixation was demonstrated in *Anacystis nidulans*, (Romero et al., 1985). The utilization of nitrate in cyanobacteria is a genuine photosynthetic process directly driven by the assimilatory power of the light. The nitrate uptake involves the participation of both ammonium assimilation products and CO_2 fixation products (Flores et al., 1983). Nitrate utilization and carbon assimilation in cyanobacteria are two processes, which are related and the depletion of one of these nutrients induces the change in the composition of the metabolites involved in theses processes.

Among the protein spots analyzed, some proteins like NblA (triggers phycobilisome degradation) and NtcA (global nitrogen assimilation protein), which were expected to be found on the 2D-PAGE of the nitrate-depleted extract were not. This could be related to the low concentration of these proteins in the soluble fractions (Corthals et al., 1997). To improve the visualization of the low concentrated proteins on the 2D-PAGE, one approach could be the elimination of the phycobiliproteins, which constitute 50% of the total proteins, from the soluble fraction before 2D-PAGE. In addition, all the proteins on the 2D gel could not been analyzed because of the low concentration of most of them and the blocking of the *N*-terminal end during Edman degradation. The use of a microarray on which some genes in *Synechocystis* are spotted, could be a means to confirm and extend these results at the level of the transcriptome. For this reason, and in order to better quantify the expression of genes induced under nitrate depletion, investigations to monitor the gene expression in *Synechocystis* were performed.

4.3. Expression analysis of RNA

The objectives of many microarray projects is to identify genes expression at different abundances in complex samples of RNA extracted from different types of cells or from the same cells growing under different conditions. In this work, gene expression analysis was used to compare the relative expression levels of specific transcripts in two samples of *Synechocystis*. One of these sample was control RNA from *Synechocystis* grown in nitrate-repleted condition and the other was derived from cells whose response or status was being investigated (nitrate-depleted condition). Each of these RNA samples were reverse-transcribed to cDNA, labeled with different fluorescent dyes, and equal amounts of the labeled samples were combined and hybridized with the microarray. Subsequently, RNA isolation, reverse transcription-labeling to cDNA, hybridization and scanning of the microarray were the major steps in this experiment.

4.3.1. Isolating RNA

The quality of information obtained from microarray experiments is primarily dependent upon the quality of RNA analyzed (Schena and Davis, 2000). The RNA sample should be devoid of DNA, protein, carbohydrates, lipids, and other compounds. The presence of these substances will not only make it difficult to correctly estimate the amount of RNA present in the sample, but can contribute to fluorescent background signals in the array hybridization.

Degradation of RNA, whether by enzymatic or chemical means, results in the loss of gene expression information from the labeled samples. Furthermore, if the quantity or quality of the two samples being compared differ, misleading conclusions can be made. Compared with DNA, RNA is relatively unstable and can be degraded either enzymatically, chemically or physically. The reactive hydroxyl groups on the 2´ and 3´ positions of ribose residues open RNA to an easy attack by RNases. Ubiquitous and stable, these enzymes are released from cells upon lysis and are present on hands, skin, and hair; and can be derived from bacterial or viral contamination of solutions or remnants of previous reagents in lab glassware.

After electrophoresis of RNA in the presence of ethidium bromide, the 23S and 16S species ribosomal RNA (rRNA) were clearly visible under UV illumination. The 23S specie rRNA, in two close bands, were stained at approximately twice the intensity of the 16S band. The absence of material stained with ethidium bromide close to the loading well on the electrophoresis gel, is a sure sign that genomic DNA was totally removed. Cells disruption with glass beads on ice followed by RNA extraction using RNeasy kits gave a pure non-degraded RNA, and consequently this procedure is a method of choice for RNA extraction in cyanobacteria (Hihara et al., 2001).

4.3.2. Sample labeling

In differential gene expression analysis two or more RNA samples are compared to identify differences in the abundance and identity of the transcripts they contain. In order to convert the information contained in the transcript populations into a form that can be hybridized with microarrays and subsequently detected, the transcript populations need to be labeled. Messenger RNA molecules, otherwise called transcripts, carry the genetic information encoded in genes. In most cells these transcripts constitute only a small proportion of the total RNA, whereas ribosomal and transfer RNA account for more than 98%. One of the simplest and most popular labeling strategies is to convert mRNA population into a labeled first strand cDNA population (Yu et al., 1994; Zhu et al., 1994; Zhu and Waggoner, 1997). This is achieved by copying the transcripts into cDNA molecules with a reverse transcriptase while incorporating a modified CyDye nucleotide.

The direct synthesis of fluorochrome-labeled first-strand cDNA was carried out in this study in a reaction catalyzed by reverse transcriptase that contained mRNA or total RNA as template, deoxynucleotide triphosphates and a dye-conjugated deoxynucleotide triphosphate as substrates, and random hexamer as primer. In general, dye-conjugated nucleotides are incorporated less efficiently than unmodified dNTPs into cDNA in reactions catalyzed by

reverse transcriptase. Because incorporation of a cyanine-labeled nucleotide often leads to chain termination at a nearby nucleotide (Zhu and Waggoner, 1997), the median length of the fluorescent cDNAs synthesized is inversely proportional to the concentration of dye-conjugated nucleotide in the reaction mixture. Lowering the nucleotide ratio (Cy-labeled dUTP/dTTP) can increase the yield of cDNA, but this will compromise labeling density and the brightness of the probe. A balance between these two factors and the consequences of quenching at high labeling densities, were performed by the investigation of the optimal amount of dTTP in the reaction mixture for optimal results.

Degradation of the RNA template after cDNA synthesis is necessary to prevent the labeled probe from hybridizing with the original template in solution instead of the microarray targets during microarray hybridization. The total removal of RNA template was effective at 65°C in the presence of EDTA and NaOH. Regardless of the labeling strategy, it is necessary to purify the labeled nucleic acid after labeling, as the amount of incorporated fluorescent dye is typically only a small fraction of all the dye present in the sample. The recovery of labeled nucleic acid from purification is a major limiting factor for most labeling methods. Centrifugal ultrafiltration through Centri-sep columns was used to purify fluorescently labeled cDNAs whose size was greater than 250-300 nucleotides and concentrate by ethanol precipitation. Targets shorter than 300 nucleotides have been reported to produce unreliable data in microarray experiments (Yang et al., 1999).

4.3.3. Microaray hybridization

The process of hybridization is typically performed in order to identify and quantitate nucleic acids within a larger sample. Generally, it involves annealing a single-stranded nucleic acid to a probe complementary strand. Binding of target molecules to the sample on the glass slide highlights complementary sequences, and the intensity of signal is proportional to the amount of the labeled sample.

The design of oligonucleotide probes to print on the oligonucleotide array took into account factors that influence the specificity and strength of hybridization with labeled targets. The specificity was estimated by comparing the oligonucleotide sequence with known gene sequences. The length of oligonucleotides spotted on the slide varied between 18-22 nucleotides. In gene expression analysis, systematic variation can arise, for example, as a result of differences in labeling efficiencies between the two fluorescent dyes. Yang et al. described three types of genes that can be used for the normalization: (1) DNAs included in the arrayed set of genes, (2) spiked controls, and (3) constantly expressed genes (Yang et al.,

2002). The first type was used to normalize the array. Indeed, in many type of microarray experiments, only a small number of genes are expected to be differentially expressed in the sample under comparison. The remaining genes, whose levels of expression are not expected to change, can be used to normalize the intensities of the signals from the two dyes. An advantage of using endogenous DNAs as normalization controls is that averaging the red and green signals across a large number of elements flattens nonsystematic differences between the expression levels of the arrayed genes. Short oligonucleotides (20 nucleotides) were successfully hybridized to the oligonucleotide microarray. Up to date, the optimal oligonucleotide length for microarray varied between 50-70 nucleotides (Kane et al., 2000; Li and Stormo, 2001).

In this study, all the genes which encoded the proteins up-regulated under nitrate starvation, gained with proteomics were also expressed on the microarray. There are several key objections to reducing biological studies to the monitoring of change in mRNA: (1) The level of mRNA does not allow one to predict the level of protein expression (Anderson and Seihamer, 1997; Gygi et al., 1999); (2) protein function is controlled by many post-translational modifications; and (3) protein maturation and degradation are dynamic processes that dramatically alter the final amount of protein, independent of the mRNA level. Nevertheless, the expression of the gene products in the proteome analysis correlated well with the corresponding gene on the microarray. The three genes spotted on the array as down-regulated genes under nitrate starvation encode for subunits of the phicobiliproteins. Indeed, when cyanobacteria are growing under nitrate depletion conditions, the cell appearance changes dramatically from blue-green to yellow chlorosis, which is largely a consequence of the cessation of new phicobiliproteins synthesis (Collier and Grossman, 1992; Gasparich et al., 1987; Lau et al., 1977) coupled with the rapid degradation of the pre-existing phycobiliproteins, which can constitute half of the cellular protein (Boyer et al., 1987; Riethman and Sherman, 1988; Yamanaka et al., 1982). The expression of these genes was almost constant in both conditions on the microarray. The inhibition of phycobiliproteins synthesis occurs after a long nitrate starvation duration. Cells were starved only for 10 hours and were still at this time at the adaptation step.

The nitrate depletion treatment led to the induction of several genes that, apparently, are not directly involved in the nitrate assimilation activity such as *ccmK*, *petE* and *phoA* genes. These observations may indicate that different environmental changes may lead to the

induction of similar sets of genes via common signaling pathway(s). In fact, it may enable one to distinguish between a specific response to a certain environmental response stimuli and general responses to a number of stressors (Schwarz and Grossman, 1998), possibly mediated by the redox state of component(s) on the photosynthetic electron transport chain. Further application of DNA microarray methodology on RNA isolated after exposure to various environmental conditions may help to clarify stress-sensing mechanisms and networks of gene expression for acclimation responses.

4.4. Conclusions

The primary objective of this study was the construction of a cyanobacterial reporter assay for the nitrate availability monitoring in water, and subsequently monitoring of the cyanobacterial blooms. A cyanobacterial biosensor called CyanoSensor was successfully constructed and packed in an easy-to-use format suitable for field application. It is storable and could be used to detect nitrate availability in the environmental samples (rain-water, freshwater and seawater). To better understanding the effect of nitrate depletion on the freshwater cyanobacterium *Synechocystis* at the molecular level, investigations to monitor the proteome of *Synechocystis* were performed. This approach was to find other nitrate depletion markers that could be used to construct novel bioreporter for nitrate, in order to fully controlling cyanobacterial proliferation. From this study some nitrate-regulated proteins were identified and the results were confirmed and extended at the level of transcriptome using DNA microarray method.

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- Licence (Bachelor´s degree) in Biochemistry (1992)
- Maîtrise (Postgraduate Diploma) in Biochemistry (1994)
- D.E.A. (M.Sc.) in Biochemistry (1995)

1995 – 1999 Doctorate research and Graduate assistant at the department of Biochemistry, University of Yaounde I, Cameroon

10/1999 – 03/2000 German language training, Goethe Institut Freiburg, Germany

04/2000 – 09/2003 Ph.D work at the Institute of Technical Biochemistry (University of Stuttgart) under the supervision of Professor Dr. Rolf D. Schmid

Scientific papers published

1. Flaubert Mbeunkui, Catherine Richaud, Anne-Lise Etienne, Rolf D. Schmid and Till T. Bachmann (2002) Bioavailable nitrate detection in water by an immobilized luminescent cyanobacterial reporter strain. Appl Microbiol Biotechnol **60**: 306-312.

2. Flaubert Mbeunkui, Rolf D. Schmid and Till T. Bachmann. Proteome analysis of nitrate-regulated proteins in *Synechocystis* PCC 6803: Identification of proteins separated by 2D-PAGE using MALDI-TOF/MS and *N*-terminal sequencing. (accepted).