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Physiological and Protein-Biochemical Analysis of UV-A and UV-B Tolerance of the Terrestrial Cyanobacterium *Nostoc commune*

Monika Ehling-Schulz

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"Und alle großen Wissenschaftler waren sich klar darüber, daß jede Lösung eines wissenschaftlichen Problems viele neue ungelöste Probleme aufwirft. Je mehr wir über die Welt lernen, um so bewußter, um so detaillierter und um so genauer wird unser Wissen von den noch ungelösten Problemen, unser sokratisches Wissen von unserem Nichtwissen. Die wissenschaftliche Forschung ist in der Tat die beste Methode, uns über uns selbst und über unser Nichtwissen aufzuklären."

Karl R. Popper

Abstract

The increase of UV-B radiation (280 nm – 320 nm) in the solar spectrum due to the depletion of the stratospheric ozone causes enhanced exposure to UV-B, which is dangerous for all living cells, but especially to photosynthetic organisms due to their light dependency. In search of the basis of UV tolerance in terrestrial cyanobacteria, liquid cultures *Nostoc commune* derived from field material were treated with artificial UV-B and UV-A irradiation. The induction of various pigments which are thought to provide protection against damaging UV-B irradiation were studied. First, UV-B irradiation induced a rapid increase in carotenoids, especially echinenone and myxoxanthophyll, but did not influence chlorophyll *a*. Second, an enormous increase of an extracellular, water-soluble UV-A/B-absorbing mycosporine occurred, which was associated with extracellular glycan synthesis. Finally, synthesis of scytonemin, a lipid-soluble, extracellular pigment known to function as UV-A sunscreen was observed. After longtime exposure the UV-B effect on carotenoid and scytonemin synthesis ceased while the mycosporine content remained constantly high. It is proposed that the outer membrane-bound carotenoids provide a fast, active SOS response to counteract acute cell damage whereas the glycan with its UV absorbing pigments is a passive UV screen against longtime exposure. The UV-B sunscreen mycosporine is exclusively induced by UV-B (< 315 nm). The UV-A sunscreen scytonemin is only slightly induced by UV-B (< 315 nm), very strongly by near UV-A (350 - 400 nm) and not at all by far UV-A (320 - 350 nm). These results may indicate that the synthesis of these UV sunscreens is triggered by different UV photoreceptors.

By applying two-dimensional (2D) gel electrophoresis coupled to computerized image analysis and database analysis the influence of UV was monitored on protein level. UV-A had only little influence on the protein pattern, nevertheless, it had remarkable influence on the pigment composition. In contrast, UV-B led to tremendous changes in the protein expression profile of *N. commune*. At least 493 proteins of 1350 protein spots analyzed displayed statistical significant changes in their relative rate of synthesis. A programmed acclimation to the new growing conditions was observed. In contrast to shock proteins , which are usually bulk proteins, the majority of stimulated proteins during UV-B acclimation were low abundant ’acclimation’ proteins. Cytosolic water-soluble proteins showed different kinetics in their response compared to membrane-associated and membrane-bound proteins. The cellular adjustment resulted in alternative metabolic fluxes under this stress conditions. Like the physiological reaction, the reaction on the protein level could be divided in two phases. Early acclimation response within

the first 24 hours, and late acclimation response which requires one up to three days. Most of the protein changes observed during early acclimation were transient. The importance of long time studies for a holistic understanding of UV tolerance in cyanobacteria is discussed.

The presented study is the first global study of UV-B effects on the proteome of cyanobacteria and demonstrates the complex physiology of UV-B adaptation.

Zusammenfassung

Durch die Abnahme der stratosphärischen Ozonschicht steigt der UV-B-Anteil (280 nm – 320 nm) im solaren Strahlungsspektrum. Diese erhöhte UV-B-Strahlung ist für alle lebenden Organismen schädlich, besonders gefährlich ist sie jedoch für photosynthetische Organismen aufgrund ihrer Lichtabhängigkeit. Um die Grundlage der UV-Toleranz von terrestrischen Cyanobakterien zu verstehen, wurde ein aus Freilandmaterial isolierter und in Flüssigmedium wachsender *Nostoc commune* Stamm mit künstlichem UV-A und UV-B bestrahlt. Es wurden Pigmente untersucht, die Schutz gegen die schädliche UV-Strahlung bieten können. Als erste Reaktion auf die UV-B-Bestrahlung wurde eine rasche Zunahme von Carotinoiden, insbesondere von Echinenon und Myxoxhanthophyll, beobachtet, während der Chlorophyll *a* Gehalt unverändert blieb. Dann wurde die starke Zunahme eines extrazellulären wasserlöslichen UV-A/B-absorbierenden Mycosporins festgestellt, welche mit der Synthese von extrazellulärem Polysacchariden und der Synthese des sauren Wasserstreßproteins Wsp verbunden war. Außerdem wurde Scytonemin synthetisiert, ein lipidlösliches extrazelluläres Pigment, das als UV-A-Schutzpigment bekannt ist. Nach Dauerbestrahlung nahm der UV-B-Einfluß auf die Carotinoid- und Scytonemin-Synthese ab, während der Mycosporingehalt gleichmäßig hoch blieb. Wahrscheinlich bieten Carotinoide, die an die äußere Membran gebunden sind, einen schnellen aktiven SOS-Schutz vor akuten Zellschädigungen, wohingegen die Schleimhüllen mit den eingelagerten UV-absorbierenden Pigmenten einen passiven Langzeitschutz darstellen. Das UV-B-Sonnenschutzmittel Mycosporin wird nur durch UV-B (< 315 nm) induziert. Das UV-A-Sonnenschutzmittel Scytonemin wird durch UV-B (< 315 nm) nur sehr schwach, durch langwelliges UV-A (350 – 400 nm) sehr stark und durch kurzwelliges UV-A (320 – 350 nm) gar nicht induziert. Diese Ergebnisse deuten darauf hin, daß die Synthese dieser UV-Sonnenschutzpigmente durch verschiedene Photorezeptoren reguliert wird.

Durch die Kombination von zweidimensionaler Gelelektrophorese, computergestützter Gelanalyse und Datenbankanwendung wurde der Einfluß von UV auf Proteinebene untersucht. UV-A hat nur einen geringen Einfluß auf die Proteinmuster von *N. commune*, obwohl es einen signifikanten Einfluß auf dessen Pigmentzusammensetzung hat. Im Gegensatz dazu beeinflußte UV-B die Proteinzusammensetzung des Organismus sehr stark. Mindestens 493 Proteine von insgesamt 1350 Proteinen, die analysiert wurden, zeigten signifikante Änderungen. Es wurde eine programmierte Anpassung an die neuen Wachstumsbedingungen beobachtet. Im Gegensatz zu Schockproteinen, die in der Regel stark expremiert werden, wurde die Mehrzahl der UV-B-

‚Adaptionsproteine' nur in geringer Konzentration in der Zelle gefunden. Die zelluläre Anpassung an den UV-B-Streß führte zu Veränderungen in den Stoffwechselwegen. Die Adaption von *N. commune* an UV-B kann in zwei Phasen geteilt werden: frühe Anpassung während der ersten 24 Stunden nach Beginn des UV-Stresses und Langzeitanpassung, die nach ein bis drei Tagen zu beobachten ist. Diese zwei Phasen waren sowohl auf physiologischer als auch auf Proteinebene zu beobachten. Die meisten Proteinänderungen, die während der frühen Anpassungsphase registriert wurden, waren nur vorübergehend. Die Bedeutung von Langzeitstudien für ein ganzheitliches Verständnis der UV-Toleranz von Cyanobakterien wird diskutiert.

Vorliegende Arbeit ist die erste globale Untersuchung des Einflusses von UV-B auf ein Proteome von Cyanobakterien und gibt einen Einblick in die komplexe Physiologie der UV-B-Anpassung von *N. commune*.

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General Introduction

The decrease of stratospheric ozone caused by anthropogenic inputs of chlorinated fluorocarbons has resulted in an increase in UV-B radiation reaching the Earth's surface (Fraser *et al.*, 1992). Due to the high molecular absorption coefficient of ozone in the UV-B region (280 – 320 nm), depletion of ozone increases the amount of UV-B reaching the Earth's surface and shifts the solar spectrum to shorter wavelengths (Fig. 1). Within the UV-B waveband (especially 290 – 315 nm) the solar irradiance decreases by more than four orders of magnitude due to ozone absorption. With ozone reduction, the enhancement of UV-B is also highly wavelength dependent (Fig. 1, inset). UV-A (320 – 390 nm) and PAR (photosynthetically active radiation, 400 – 700 nm) are not attenuated by ozone, so their fluence will be unaffected by the ozone layer reduction. UV-C (< 280 nm) is effectively absorbed by the stratospheric ozone layer (Caldwell *et al.*, 1989). UV-B is only a small proportion of the total solar radiation and less than 1 % of the total solar flux reaching the Earth's surface, but it is biologically highly active. The biological effectiveness of most reactions to UV-B increases considerably with decreasing wavelength (Caldwell *et al.*, 1986). Recent studies have shown, not only in Antarctica but also in the northern hemisphere, that UV-B radiation increases due to the depletion of the ozone layer (Stolarsky *et al.*, 1992; Madronich *et al.*, 1995). This enhanced exposure to UV-B is potentially detrimental to all living organisms, but especially to photosynthetic organisms due to their requirements for light. The UV-A proportion of solar radiation plays an important role in both the inhibition and the repair mechanisms of photosynthetic organisms (Cullen *et al.*, 1992; Queseda *et al.*, 1995).

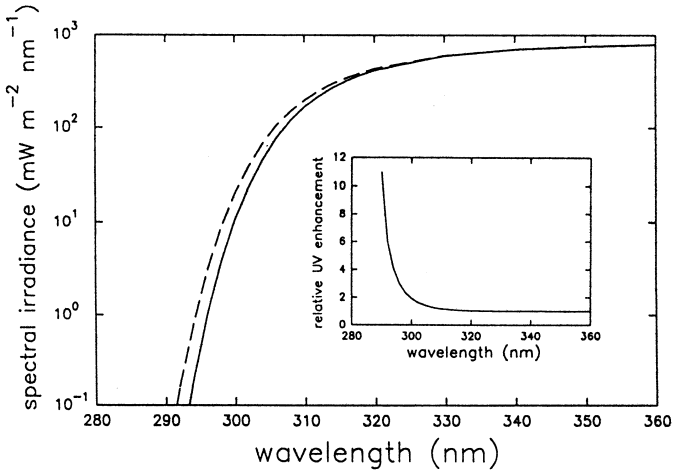
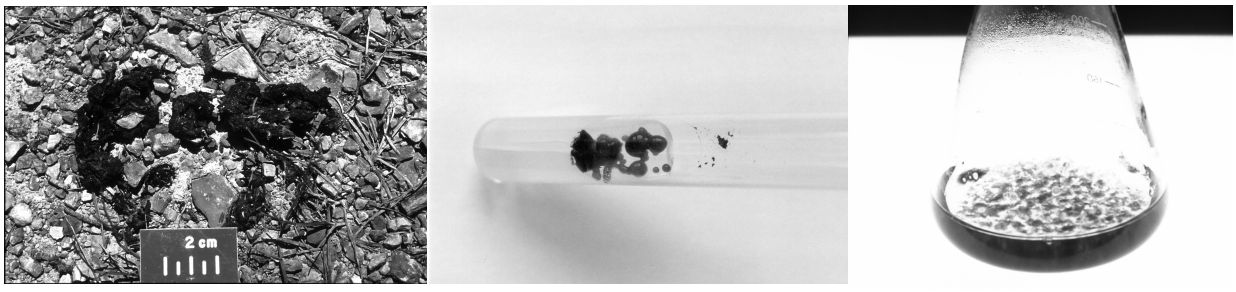


Fig. 1: Solar spectral irradiance. Solid line shows irradiance computed for normal ozone column thickness in the summer at temperate latitudes (midday). The dashed line shows irradiance for the same conditions, but with 20 % ozone column reduction. The inset shows the wavelength-dependent factor by which irradiance is increased due to ozone reduction (from Caldwell and Flint, 1994).

Cyanobacteria are oxygen-producing photosynthetic prokaryotes dominating the microbial communities of the most extreme environments on earth. They are present in habitats of great diversity, such as hot springs, antarctic ice shelves or deserts (e.g., Fogg and Steward, 1968; Potts and Friedmann, 1981). Cyanobacteria are abundant in marine and fresh water environments and are important contributors to global photosynthetic biomass production. Terrestrial cyanobacteria are predominant in habitats exposed to high solar irradiance; they prevent erosion and preserve water in soil (Booth, 1941). Due to the ability of many cyanobacteria to fix nitrogen, these organisms provide a substantial source of nitrogen to terrestrial ecosystems and they may play a central role in successional processes (Bliss *et al.*, 1990).

A terrestrial nitrogen-fixing cyanobacterium, which is well adapted to live under extraordinary environmental conditions is *Nostoc commune* VAUCHER. It flourishes in extreme cold and dry habitats which are characterized by intense solar radiation, extreme temperature differences, and regular periods of desiccation (Whitton *et al.* 1979; Scherer and Zhong, 1991). The morphology of *N. commune* depends on its growing conditions. In its natural habitat *N. commune* forms macroscopic colonies, which typically appear as brittle, dried and dark crusts and cover areas of several square centimeters. On semi-solid media *N. commune* forms little pearls and in liquid culture it shows diffuse growth (Fig. 2). Beside its extreme desiccation tolerance this organism shows a high UV tolerance (Scherer *et al.*, 1988; Whitton, 1992). In the past, most studies concentrated on the extraordinary drought resistance of *N. commune* (Scherer and Potts, 1989; for review see Potts, 1999), but only little is known about its UV tolerance.



A B C

Fig. 2: Different life forms of *N. commune*. **A:** *N. commune* in its natural habitat (Grainberg, Karlstadt am Main). **B:** *N. commune* grown on agar. **C:** *N. commune* grown in liquid media.

Nevertheless, UV irradiance might be a special stress factor for this cyanobacterium, since it is subject to regular cycles of desiccation and rewetting and often must survive long periods of quiescence, during which active repair mechanisms are inactive.

The present study documents a comprehensive physiological and protein-biochemical analysis of the UV-A and UV-B tolerance of *Nostoc commune*.

Due to the fact, that the chapters have been written for separate publication, minor overlaps of the introduction contents emerged.

Chapter 1
UV Protection in Cyanobacteria*

1 Targets and effects of UV on photosynthetic organisms

The negative effects of UV irradiation on photosynthetic organisms have been studied thoroughly. Growth, cell differentiation, motility and photoorientation are affected by UV irradiation (for review see Häder and Worrest, 1991; Tevini, 1994; Franklin and Forster, 1997). A UV-A- induced growth delay has been reported for cyanobacteria (Garcia-Pichel *et al.*, 1992). However, it is difficult to generalize concerning influences of UV irradiation on growth since cyanobacterial species, or even strains, show large differences in UV sensitivity and their physiological response to UV. Although UV-B- induced growth delay has been reported for the cyanobacterium *Gloeocapsa* (Garcia-Pichel *et al.*, 1993), UV-B exposure had negligible short term effects on the growth of *Nostoc commune*. Prolonged UV-B, but not UV-A, exposure of *N. commune* led to decreased cell number but enhanced dry weight in comparison to control cultures (Ehling-Schulz *et al.*, 1997). The decreased cell number was suggested to be the result of a slower cell replication caused by the metabolic cost of increased glycan production, and is not a consequence of photoinhibition of cell replication. UV- absorbing compounds may also be synthesized at a metabolic cost for growth (Raven, 1991).

Changes observed at the organismal and physiological level could result from a number of primary UV effects. To determine which factors are involved, action spectra and kinetics of the response must be established. Molecular targets of UV irradiation-induced damage include DNA (Caldwell, 1979), the D1 reaction centre protein of photosystem II (Barbato *et al.*, 1995), ribulose-1,5-biphosphate carboxylase (Jordan *et al.*, 1992), phycobiliproteins (Lao and Glazer, 1996), nitrogenase (Newton *et al.*, 1979) and membranes (Tuveson *et al.*, 1988).

DNA and proteins are primary targets of UV-B irradiation because they absorb UV-B. DNA lesions caused by UV-B irradiation include single-stranded and double-stranded breaks, DNA-protein cross-links and the predominant formation of cyclobutane dimers and pyrimidine - (6-4) – pyrimidone photoproducts (Peak and Peak, 1986; Mitchell and Nairn, 1989). On the other hand, DNA and proteins must be indirectly affected by UV-A irradiation because they absorb it

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very weakly if at all. UV-A damage to DNA occurs by energy transfer from UV-A stimulated chromophores to the DNA target or the photosensitized production of active oxygen species (Peak and Peak, 1986; Eisenstark, 1989).

2 UV protection mechanisms

Cyanobacteria display a variety of strategies for protection against the detrimental effects of UV. Three general types of stress responses are found among cyanobacteria: stress avoidance, stress defense, and repair mechanisms.

2.1 UV-stress avoidance

Motile cyanobacteria can escape from high solar radiation by downward migration into mat communities (Queseda and Vincent, 1997) or by sinking deeper into the water column (Reynolds *et al.*, 1987). Although most of the filamentous cyanobacteria are motile by gliding, information on the influence on UV on vertical migration of cyanobacteria is limited. Daily vertical migration to avoid periods of incident high solar irradiance has been reported for *Oscillatoria* sp., and *Spirulina cf. subsala* (Garcia-Pichel *et al.*, 1994). The vertical migration of *Microcoleus chthonoplastes* has been shown to be UV- and PAR- inducible (Bebout and Garcia-Pichel, 1995). Since UV-B was by far the most effective waveband to promote migration, it has been suggested that *M. chthonoplastes* can sense UV-B directly. UV-B- induced vertical migration may be an effective strategy to minimize UV- induced damage. On the other hand, migration led to a decreased overall productivity of the mat ecosystem (Bebout and Garcia-Pichel, 1995). Further investigations are needed to assess the effect of migration on net primary productivity of mats.

2.2 UV-stress defense

Synthesis of UV-absorbing compounds is an important mechanism preventing UV photodamage. Several studies provide evidence that mycosporine amino acids (MAA), which have absorption maxima between 310 and 360 nm, protect cyanobacteria and other lower organisms by absorbing harmful UV radiation (Scherer *et al.*, 1988; Karentz *et al.*, 1991; Ehling-Schulz *et al.*, 1997). Mycosporine amino acids are water-soluble, substituted cyclohexenones which are linked to amino acids and amino alcohols, and have absorption maxima between 310 nm and 360 nm (Fig. 3). Their synthesis probably originates from the first part of the shikimate

pathway (Favre Bonvin *et al.*, 1987). MAAs are widely distributed among cyanobacteria. However, the relative protection against UV-B-damage provided by MAAs depends on the species and the location of the pigments therein. Significant, but limited, protection has been reported for various cyanobacteria with MAAs located in the cytoplasm. In these cases, only 10 - 26 % of the photons are absorbed by the pigment (Garcia-Pichel and Castenholz, 1993). In *Nostoc commune*, MAAs are thought to play an important role in photoprotection because the MAAs are located in the extracellular glycan. Two out of three photons are absorbed by the pigment before cell membranes or targets within the cell are reached (Böhm *et al.*, 1995). Two UV-A/B-absorbing pigments of *N. commune* with absorption maxima at 312 nm and 335 nm were found in colonies exposed to high solar radiation (Scherer *et al.*, 1988). One of them was the first mycosporine reported to be covalently linked to oligosaccharides (Fig. 3) and shown to be located in the extracellular glycan (Hill *et al.*, 1994a; Böhm *et al.*, 1995). The pigment provides protection, mainly by absorbing the harmful radiation, but the 312 chromophore of one pigment, which is thought to be a MAA-Gly (Fig. 3), may provide additional protection by radical quenching (Dunlap and Yamomoto, 1995). Because *N. commune* is subject to regular cycles of desiccation and rewetting and has often to survive long times in quiescence during which repair mechanisms are ineffective, UV-absorbing compounds may play a key role in UV photoprotection of *N. commune* (Ehling-Schulz *et al.*, 1997).

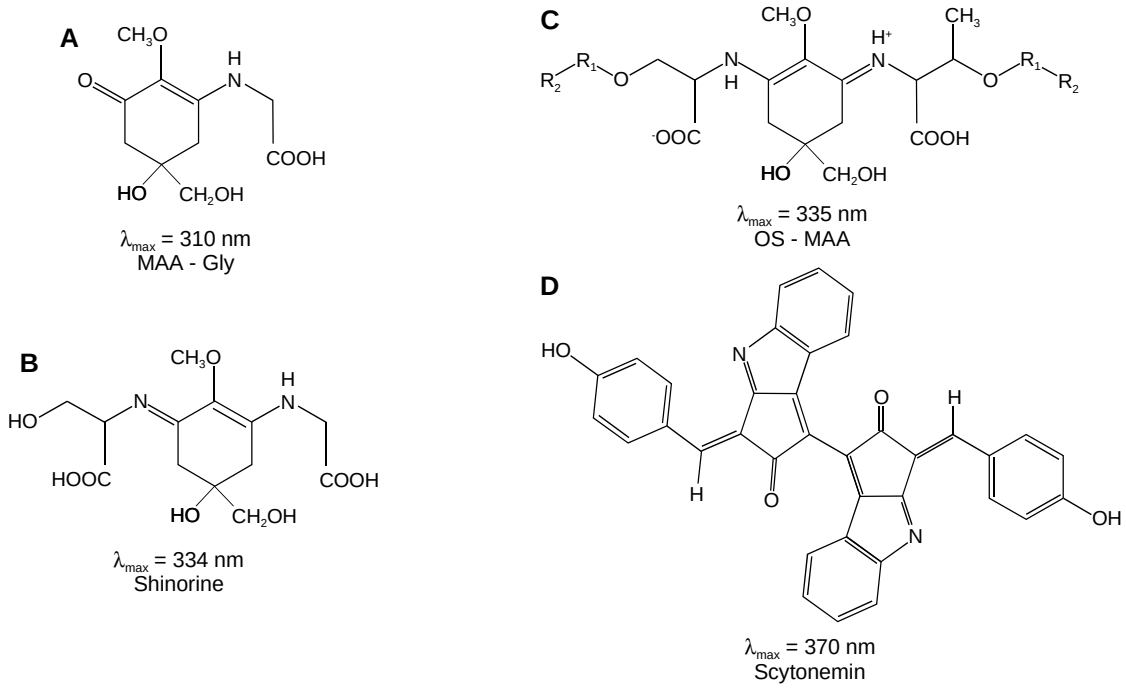


Fig. 3: Structures of UV sunscreen pigments. **A:** The monosubstituted mycosporine-like amino acid (MAA), MAA-Gly; **B:** The bisubstituted MAA, shinorine; **C:** The 335 chromophore of the oligosaccharide-MAA of *N. commune* (according to Böhm *et al.*, 1995). R1 (galactose, xylose, glucuronic acid), R2 (galactose, glucose, glucosamine); **D:** Scytonemin (according to Proteau *et al.*, 1993).

Scytonemin is another pigment with UV- shielding properties found in cyanobacteria. It has an *in vivo* absorption maximum at 370 nm (Fig. 4) and is located in the cyanobacterial sheath. Scytonemin is a yellow-brownish, lipid-soluble dimeric pigment with a molecular mass of 544 Da and a structure based on indolic and phenolic subunits (Fig. 3). It has been suggested that it is formed by condensation of tryptophan- and phenylpropanoid-derived subunits (Proteau *et al.*, 1993). Its synthesis is strongly inducible by UV-A irradiation, but only weakly by UV-B irradiation and it has been proposed to serve as UV-A sunscreen (Garcia-Pichel *et al.*, 1992; Ehling-Schulz *et al.*, 1997). Scytonemin plus extracellularly located mycosporines can provide an effective passive screen against harmful UV-irradiance (Fig. 4).

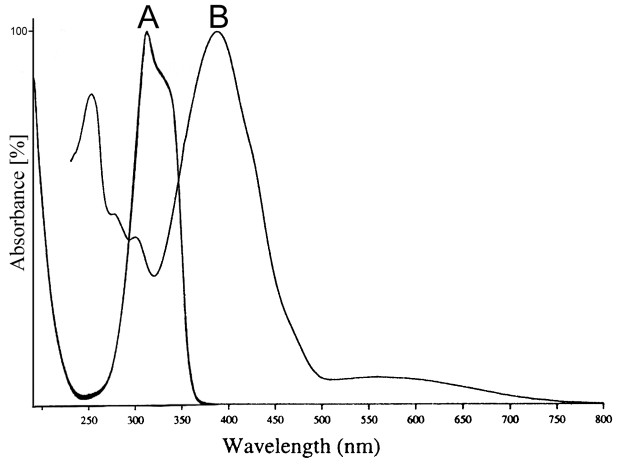


Fig. 4: UV screen. **A:** Absorption spectrum of oligosaccharide-MAA in H_2O (according to Böhm *et al.*, 1995). **B:** Absorption spectrum of scytonemin in tetrahydrofuran (according to Proteau *et al.*, 1993).

Removal of toxic oxygen species can be another defense strategy. Carotenoids are well known for their antioxidant activity. They remove singlet oxygen, triplet chlorophyll and inhibit lipid peroxidation (for review see Edge *et al.*, 1997). Their photoprotective role against high intensity of visible light is evident. UV-A and UV-B can cause oxidative stress by photodynamically generating reactive oxygen intermediates (Cunningham *et al.*, 1985; Shibata *et al.*, 1996). An increase in the carotenoid / Chl*a* ratio of cyanobacteria has been reported in response to UV-A and UV-B radiation (Paerl, 1984; Ehling-Schulz *et al.*, 1997; Quesada and Vincent, 1997), supporting the role of carotenoids as important reactive oxygen - quenching pigments. Recent studies indicated that carotenoids have a protective function against UV-B irradiation in plants (Middleton and Teramura, 1993). In the cyanobacterium *N. commune*, changes in the carotenoid pattern in response to UV-B irradiation have been observed and

myxoxanthophyll as well as echinenone were suggested to act as outer membrane-bound UV-B photoprotectors (Ehling-Schulz *et al.*, 1997).

Scavenging enzymes such as superoxide dismutase and peroxidases can help to decrease reactive oxygen species. In plants and microalgae synthesis of superoxide dismutase and ascorbate peroxidase have been shown to be inducible by UV-B irradiation (Lesser, 1996; Rao *et al.*, 1996). Cyanobacteria are known to synthesize scavenging enzymes such as ascorbate peroxidase and catalases (Miyake *et al.*, 1991). However, the importance of enzymes with radical scavenging properties for UV tolerance by cyanobacteria deserves further investigation.

Synthesis of extracellular polysaccharides may also help to limit UV-damage. Bacterial extracellular polysaccharides (EPS) have been reported to provide protection against desiccation, phagocytosis, antibody recognition and lysis by viruses (Dudman, 1977; Tease and Walker, 1987; Hill *et al.*, 1994b). The EPS - containing sheath of cyanobacteria forms a buffer zone between the environment and the cell. Recently, it has been reported that UV-B irradiation stimulates the extracellular glycan production of *N. commune*. The yield of EPS isolated from UV-B irradiated cultures was three times higher than that from control cultures (Ehling-Schulz *et al.*, 1997). Leaf thickening in higher plants has been reported as a response to UV-B (Balakumar *et al.*, 1993). With a thicker sheath, effective path lengths for the absorption of radiation are much longer and it has been suggested that EPS synthesis is stimulated to provide a matrix for the UV-A/B- absorbing oligosaccharides-mycosporines which are located in the sheath of *N. commune* (Ehling-Schulz *et al.*, 1997).

2.3 Active repair mechanisms

UV-damaged targets can be replaced by increased synthesis of the targets or by repair of damaged targets without *de novo* synthesis.

DNA repair mechanisms are universal for all types of cells and have been studied extensively in *Escherichia coli*. UV- induced photoproducts can be recognized and repaired by several mechanisms in *E. coli* including photoreactivation, excision repair and postreplication repair (SOS repair) (Walker, 1985). During photoreactivation cyclobutane-type pyrimidine dimers are monomerized by the enzyme DNA photolyase, which is activated by UV-A and blue light (Pang and Hays, 1991). Excision repair is light - independent and various enzymes are involved. First, damaged DNA is nicked, then short single-stranded segments spanning the base lesions are removed and the gaps are filled by resynthesis. Cyanobacteria have been found to exhibit both photoreactivation and excision repair (O'Brian and Houghton, 1982; Levine and

Thiel, 1987; Eker *et al.*, 1990). *RecA*- like genes from cyanobacteria have been shown to complement a *recA* deletion in *E. coli* (Geoghegan and Houghton, 1987; Owttrim and Coleman, 1987). The complemented *recA* strains showed an increased UV-C resistance. The activation of the *RecA* protein by DNA damage is the first step of the SOS repair mechanism. The RecA protein cleaves the LexA repressor and the SOS genes are expressed (SOS regulon) (Walker, 1985). In most studies related to DNA damage repair UV-C irradiation has been used, and the induction of the expression of *recA* by UV-A and UV-B irradiation has only recently been reported in *Pseudomonas aeruginosa* (Kidambi *et al.*, 1996).

Increased protein degradation and resynthesis to replace UV-sensitive proteins as fast as they are damaged may help to counteract UV-damage. Sass *et al.* (1997) reported an increased turnover of D1 and D2 proteins of the photosystem II reaction-centre in *Synechocystis* sp. PCC6803 in response to UV-B irradiation. They suggested that UV-damaged D1 and D2 proteins are removed from the thylakoid and are replaced by newly synthesized D1 and D2 molecules. A specific cleavage site has been reported to be involved in the degradation of UV-B-damaged D1 protein (Barbato *et al.*, 1995). During recent years the turnover of D1 protein has been studied in detail. It has been shown to be regulated by most stress conditions (Giardi *et al.*, 1997) and its turnover has been proposed to be a general adaptive response to environmental stresses.

2.4 Combinatory strategies

A combination of several strategies may be used by photosynthetic organisms to acclimate to UV irradiation. In *N. commune* a cascade of physiological reactions was observed in response to UV-B irradiation (Fig. 5): first a rapid increase in carotenoids, especially echinenone and myxoxanthophyll, and second, an increase of an extracellular UV-A/B-absorbing mycosporine, which was associated with extracellular glycan synthesis. Finally, scytonemin was

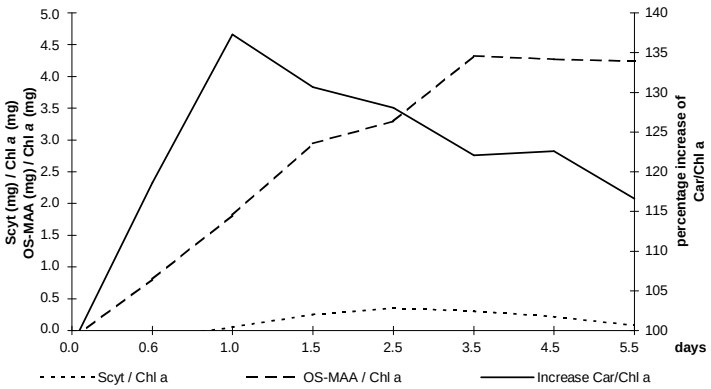


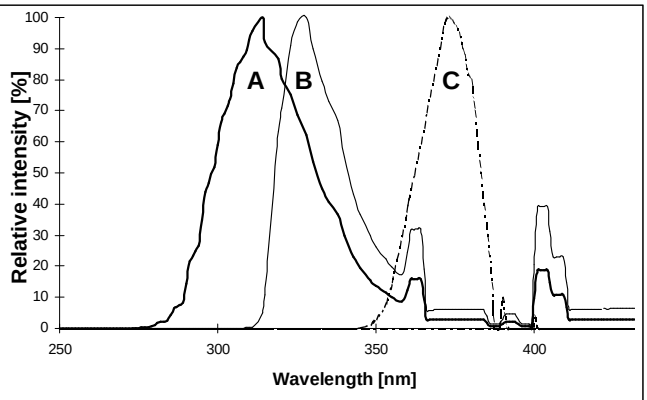
Fig. 5: Time course of the response of photosynthetic and UV-absorbing pigments to UV-B irradiation (1 W m^{-2}). (For details see Ehling-Schulz *et al.*, 1997). Abbreviations: scyt: scytonemin; OS-MAA: oligosaccharide-mycosporine; car: total carotenoid; chl*a*: chlorophyll *a*.

induced slightly by UV-B and very strongly by UV-A irradiation. It has been proposed that the outer membrane-bound carotenoids provide a fast, active SOS response to counteract acute cell damage whereas the glycan with its UV- absorbing compounds is a passive UV screen against long term exposure (Ehling-Schulz *et al.*, 1997).

3 UV photoreceptors

It is evident that several different photoreceptors mediate the response to UV and blue light in higher plants (Jenkins *et al.*, 1995). Phytochrome, a blue light photoreceptor and a UV-B photoreceptor have been shown to be involved in the induction of UV-absorbing flavonoids (Bruns *et al.*, 1986).

Information about photoreceptors in cyanobacteria responsible for photoresponses to UV irradiance is still lacking. A UV-B photoreceptor has been proposed to regulate the oligosaccharide-mycosporine in *N. commune* since its synthesis is only inducible by wavelengths below 315 nm and a UV-A photoreceptor might be involved in the regulation of scytonemin synthesis (Fig. 6).



	UV-B + farUV-A	farUV-A only	nearUV-A
	A	B	C
Car / Chl*a*	+	-	+
Scyt / Chl*a*	+	-	+++
OS-MAA / Chl*a*	+++	-	-

Fig. 6: Spectral characteristics of UV-treatments and wavelength dependence of pigment induction. **A:** λ_{max} = 315 nm; **B:** λ_{max} = 330 nm; **C:** λ_{max} = 370 nm. UV intensities applied were in the range of 0.5 W m^{-2} to 1.7 W m^{-2} (for details see Ehling-Schulz *et al.*, 1997). Abbreviations see Fig. 4.

A UV- A photoreceptor with a maximum at 370 nm, but no blue light stimulation, has been reported to be involved in the carotenoid synthesis of *Verticillium agaricinum* (Osman and Valadon, 1977). Campos *et al.* (1991) reported that UV-B and UV-C irradiation increased levels of 3-hydroxy-3-methylglutaryl CoA reductase RNA, which may induce carotenoid synthesis. Since carotenoids in cyanobacteria showed a different response to high intensity visible light, UV-A and UV-B (Leisner *et al.*, 1994; Ehling-Schulz *et al.*, 1997) it is possible that special UV-B photoreceptors are involved. However, for identification and functional analysis of photoreceptors and signal transduction pathways, the isolation of mutants will be necessary.

4 Effects of UV on protein composition

The knowledge about the effects of UV at the protein level is limited (Table 1). An induction of UV-shock proteins in response to high intensities of “near” UV (295 nm –390 nm) and in response to UV-C (265 nm) irradiation has been reported in cyanobacteria (Nicholson *et al.*, 1991; Shibata *et al.*, 1991). Some of the UV shock proteins were also inducible by heat shock (Shibata *et al.*, 1991) and heat shock like proteins have been found to be UV-B inducible in plant seedlings (Nedunchezhian *et al.*, 1992). Recently, it has been reported that UV-B induced the expression of *psbAII* and *psbAIII* genes in *Synechococcus* sp. PCC 7942 (Campbell *et al.*, 1998). *psbAII* and *psbAIII* encode for a second form (D1:2) of the photosystem II D1 protein (D1:1) in *Synechococcus* sp. PCC 7942. In response to UV-B D1:1 is transiently replaced by D1:2. After 2 hours of UV-B irradiation D1:2 is exchanged back for D1:1 (Porankiewicz *et al.*, 1998). In contrast to *Synechococcus*, *Synechocystis* PCC 6803 has only one form of the D1 protein which is encoded by *psbAII* and *psbAIII*. Under normal growth conditions, mainly *psbAII* is expressed. In response to UV-B stress the *psbAIII* gene is switched on and the pool of *psbA* mRNA for the production of new D1 protein increases. *psbAIII* has been considered to be a stress gene, which is regulated, at least partially, at the transcription level (Mate *et al.*, 1998). It has been suggested that a specific UV-B- related signal transduction pathway is involved in the induction of *psbAIII*. An ATP- dependent Clp protease (ClpP1) isolated from *Synechococcus* sp. PCC 7942 has been shown to be UV-B- and cold- inducible (Porankiewicz *et al.*, 1998).

Table 1: UV- inducible gene products in cyanobacteria.

Gene product	UV-source	organism	reference
RecA like protein	UV-C	*Anabaena variabilis*	Owttrim and Coleman 1987
	UV-C	*Gloeocapsa alpicola*	Geoghegan and Houghton 1987
UV shock proteins	UV-C	*Phormidium laminosum*	Nicholson *et al.* 1987, 1991
UV shock proteins	UV-B + UV-A	*Synechococcus* PCC7942	Shibata *et al.* 1991
D1:2 protein	UV-B	*Synechococcus* PCC7942	Campbell *et al.* 1998
psbAmRNA	UV-B	*Synechocystis* PCC6803	Mate *et al.* 1998
ClpP1 protein	UV-B	*Synechococcus* PCC7942	Porankiewicz *et al.* 1998

5 Proteome analysis

The term "proteome" refers to all proteins expressed by a genome at a given time point and was first mentioned in the literature four years ago (Wasinger *et al*., 1995). High-resolution two-dimensional (2D) gel electrophoresis is the basic technology of proteome analysis. In the first dimension proteins are separated by their surface charge (isoelectric focusing) in a pH gradient. In the second dimension proteins are separated, most commonly, by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) according to their molecular mass. The complex protein pattern are analyzed and compared by computer assisted image analysis. Often, 2D electrophoresis is combined with high throughput technology for amino acid analysis, peptide-mass fingerprinting and associated sequence tags in combination with nanoelectrospray tandem mass spectrometry and N-terminal protein sequencing (for review see Humphery-Smith *et al.*, 1997; Williams and Hochstrasser, 1997)

The influence of UV irradiation on the proteome of cyanobacteria is poorly understood and restricted to UV-shock response and early acclimation processes (Table 1). *Nostoc commune* DRH1 cultures were treated with UV-B irradiation as described previously (Ehling-Schulz *et al.*, 1997), cells were harvested and proteins were analyzed by SDS-PAGE after different exposure times (3 hours up to 5 days).

Beside some degradation of phycobiliproteins in response to UV-B, no major differences were observed between the protein pattern of UV-B- treated and untreated cells (Fig. 7). It has been reported that UV-B has no influence on the protein pattern on the basis of SDS-PAGE data (Gerber and Häder, 1995; Chauhan *et al.*, 1998). However, the resolution of SDS-PAGE is far too low to monitor the UV-acclimation process.

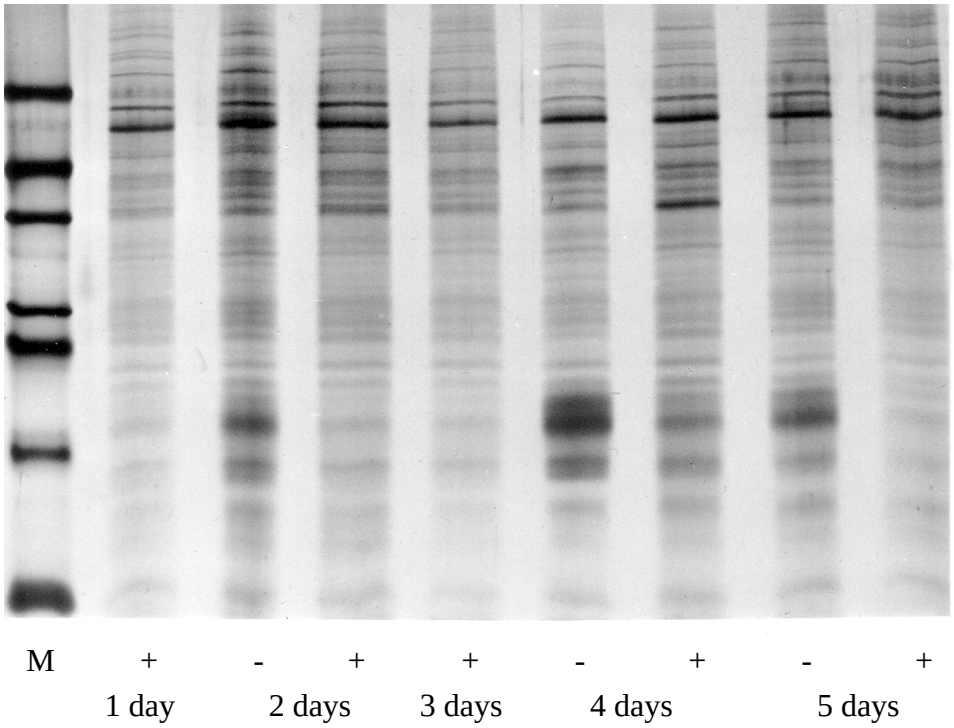


Fig. 7: SDS-PAGE of *N. commune* DRH1 was performed on 15 % (w/v) polyacrylamide gel in a discontinuous buffer system according to Laemmli (1970). Cellular proteins were analyzed after different periods of exposure to UV-B (1.0 W m^{-2}). For details of culture conditions see Ehling-Schulz *et al.* (1997). Gels were silver stained according to Blum *et al.* (1987). Abbreviations: + cultures exposed to UV-B; - cultures grown without UV-B; M protein marker (MW = 66, 45, 36, 29, 24, 20, 14 kDa).

In the past, limited resolution of proteins by 2D electrophoresis was often obtained for sheath- producing cyanobacteria since high concentrations of extracellular polysaccharides negatively influence isoelectric focusing (first dimension). With the improvement of 2D electrophoresis, especially by the IPG-Dalt system (Görg *et al.*, 1988; Weiss *et al.*, 1993) larger volumes can be loaded in the first dimension and polysaccharides are diluted to a degree where successful 2D-analysis can be performed. IPG-Dalt of *N. commune* showed that the UV-B response is extremely complex, involving the induction and the repression of a large number of proteins (Fig. 8).

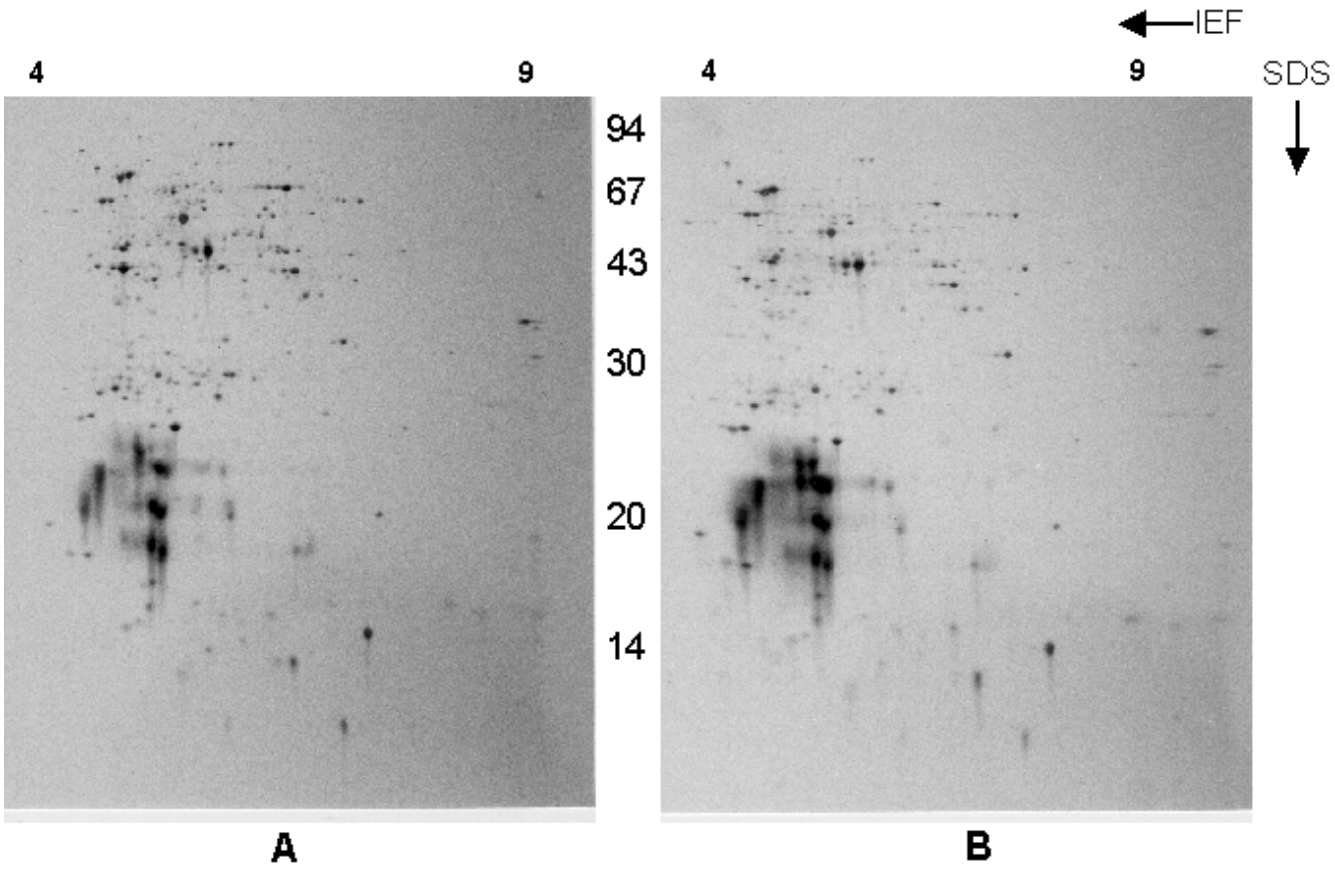


Fig. 8: IPG-Dalt of 60 µg of total cellular proteins from *N. commune* DRH1 performed with immobilized pH gradient 4-9 in the first dimension (according to Görg *et al.* 1988). Proteins were focused for 16 000 Vh at 20°C under oil. Second dimension SDS-PAGE was carried out at 20°C on 13 % (w/v) polyacrylamide gels, cast on GelBond PAGfilms (BioRad Laboratories). **A:** *N. commune* DRH1 cultures grown for three days with supplemented UV-B (1.0 W m^{-2}); **B:** Control cultures grown without UV-B. For details on culture conditions see Ehling-Schulz *et al.* (1997). Gels were silver stained according to Blum *et al.* (1987).

The proteins can be grouped into three categories by their response to UV-B irradiation. First, proteins whose synthesis is stimulated by UV-B; second, proteins which are repressed and /or degraded in response to UV-B; and third, proteins which are not affected by UV-B (Fig. 8). A detailed analysis of the UV influence on the proteome of *N. commune* is provided in chapter 3.

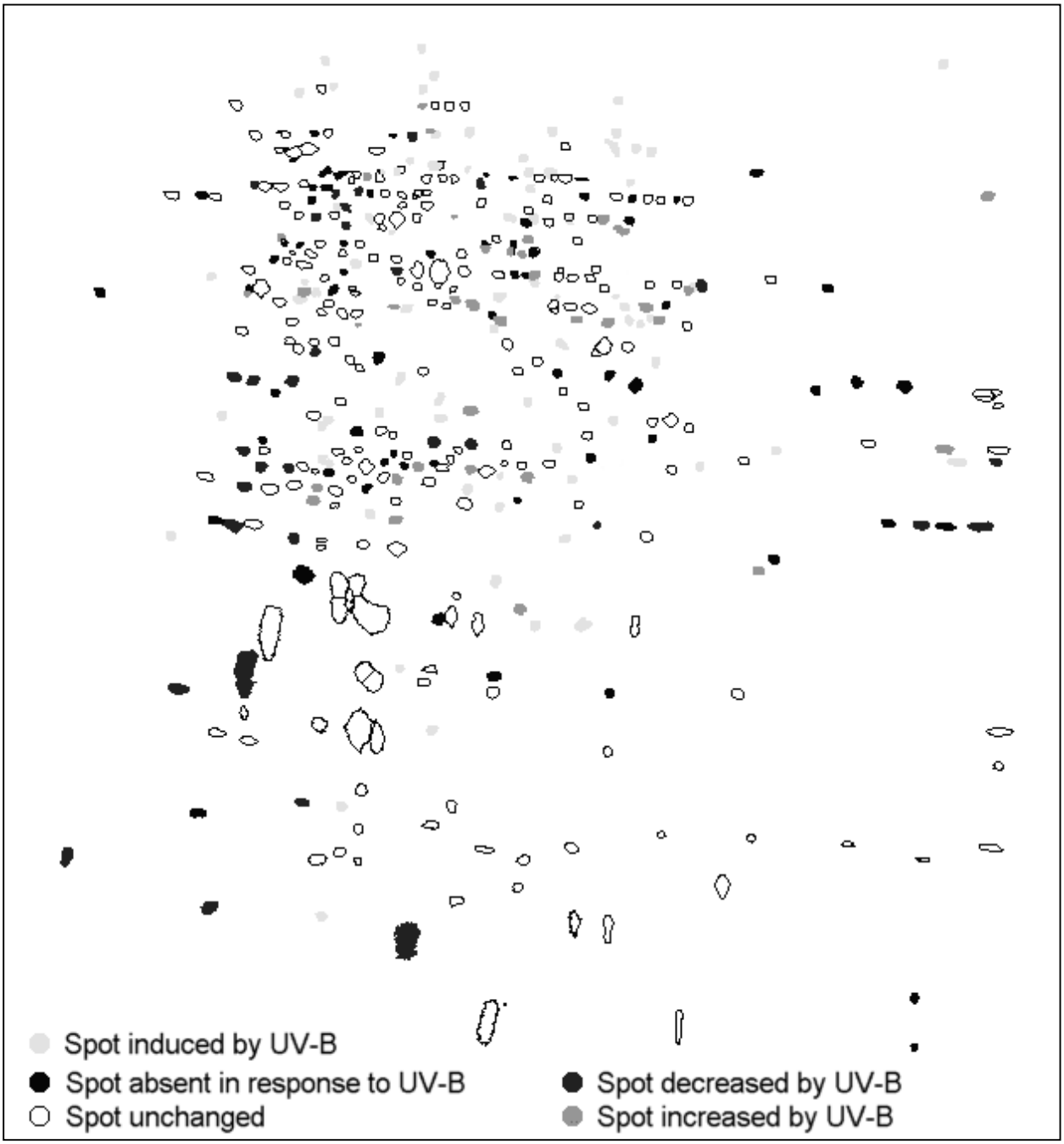


Fig. 9: Schematic map of *N. commune* DRH1. The protein profile of *N. commune* treated with UV-B (Fig. 7A) is compared to the protein profile of control cultures (Fig. 7B). The presented map is an overlay of five images which were generated with the Imagemaster 2D Elite software (Amersham Pharmacia Biotech).(i) spots, whose spot volumes are more than threefold the spot volumes of control spots, (ii) spots, whose spot volumes are less than one third of the spot volumes of control spots, (iii) spots, induced by UV-B, (iv) spots, absent in UV-B, (v) spots, unchanged in UV-B.

Chapter 2
UV-B-induced synthesis of photoprotective pigments and extracellular polysaccharides in the terrestrial cyanobacterium *Nostoc commune*[*]

1 Introduction

The terrestrial nitrogen fixing cyanobacterium *Nostoc commune* Vaucher flourishes in extremely cold and dry habitats which are characterized by intense solar radiation, extreme temperature differences and regular periods of desiccation (e.g., Whitton *et al.*, 1979, Scherer and Zhong, 1991, for review see Dodds *et al.*, 1995). *N. commune*, in its natural habitat, forms macroscopic colonies with filaments embedded in gelatinous glycan. In the past, most studies concentrated on the extraordinary drought resistance of *N. commune* (e.g., Scherer and Potts, 1989; for review see Potts, 1994), but only few investigated its UV tolerance (Scherer *et al.*, 1988; Whitton, 1992).

Mechanisms counteracting UV-B damage have been demonstrated in plants and cyanobacteria. Besides repair of UV-induced damages of DNA by excision repair and photoreactivation (e.g., O´Brian and Houghton, 1982; Eker *et al.*, 1990) and accumulation of detoxifying enzymes and carotenoids (e.g., Mittler and Tel-Or., 1991; Middleton and Teramura, 1993), an important mechanism to prevent UV photodamage is the synthesis of UV-absorbing compounds. Several studies provide evidence that epidermally located phenylpropanoids, especially flavonoid derivatives, protect higher plants by absorbing harmful UV radiation (Tevini *et al.*, 1991; Kootstra, 1994). Mycosporine amino acids (MAA) are thought to fulfill a comparable purpose in lower organisms (Karentz *et al.*, 1991; Garcia-Pichel *et al.*, 1993). Mycosporine amino acids are water-soluble, substituted cyclohexenes which are linked to amino acids and iminoalcohols and have absorption maxima between 310 nm and 360 nm. Scytonemin, with an *in vivo* absorption maximum at 370 nm and its location in the cyanobacterial sheath, has been proposed to serve as UV-A sunscreen (Garcia-Pichel *et al.*, 1992). It is a yellow-brown, lipid-soluble dimeric pigment of terrestrial cyanobacteria with a molecular mass of 544 D and a structure based on indolic and phenolic subunits (Proteau *et al.*, 1993).

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A UV-A/B-absorbing pigment with absorption maxima at 312 nm and 335 nm was found in *N. commune* colonies exposed to high solar radiation (Scherer *et al.*, 1988). Recently, its chemical structure has been shown to be a oligosaccharide mycosporine amino acid (OS-MAA) (Böhm *et al.*, 1995). It was the first mycosporine reported to be covalently linked to oligosaccharides and is located in the extracellular glycan where it forms high molecular weight complexes which are attached to the cyanobacterial sheath by non covalent interactions (Hill *et al.*, 1994; Böhm *et al.*, 1995). Because *N. commune* is subject to regular cycles of desiccation and rewetting and has often to survive long periods in quiescence in which repair mechanisms are ineffective, UV-absorbing compounds may play a key role in UV photoprotection of *N. commune*.

The aim of this work was to study, in a single organism, the sequence of UV-induced synthesis of carotenoids, scytonemin and a mycosporine, which are suggested to provide protection against UV damage.

2 Materials and Methods

2.1 Organism and growth conditions.

The cyanobacterium *Nostoc commune* Vaucher strain DRH 1 was derived from field material of *N. commune* collected in the Hunan province, People´s Republic of China (Hill *et al.*, 1994). The strain grows in liquid media under laboratory conditions without producing a visible glycan sheath surrounding single filaments. For UV induction experiments 50 ml *N. commune* DRH 1 liquid cultures were grown under nitrogen fixing conditions at 30° C in 200 ml flask in $BG11_o$ (Rippka *et al.*, 1979) medium with constant shaking (80 rpm) to avoid self shading. The flasks (Duran, Schott, Mainz, Germany) function as UV-C filter. The cultures were illuminated from above. Visible light was obtained from a cool white fluorescent tube (L 40 W / 25 S, Osram, Munich, Germany, ca. 2.4 W m^{-2}). Additional illumination was provided from a Philips TL 40 W / 12 lamp with an incident irradiance of 100 to 140 mW m^{-2} nm^{-1} at 310 nm and 50 to 70 mW m^{-2} nm^{-1} at 330 nm. UV-A control experiments were performed using a Philips TL 36 W / 08 lamp with an emission maximum centered at 375 nm. Foils with a cutoff at 315 nm were used as UV-B blocking filters. The spectral irradiance which was received by the cultures after passing the flask and filters is presented in Figure 10. Since no measurements are available the maximum values of incident solar UV-B radiation in the natural habitat of *N. commune* DRH1 were calculated according to Feister (Feister, 1995) to vary between 0.7 W m^{-2} (winter) and

2 W m^{-2} (summer), around noon and on cloudless days. The UV-B irradiation applied in our experimental setting was 1 W m^{-2}.

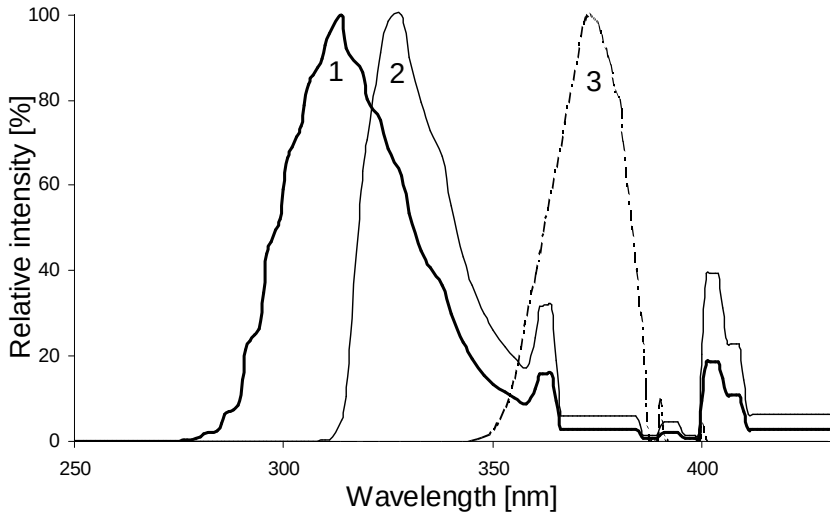

Fig. 10: Comparison of the spectral characteristics of UV light received by the cultures within the flasks from different light sources and filters. Curves: 1, lamp TL-12 (UV-B + far UV-A: λ_{max} 315 nm, approx. 1.0 W m^{-2} UV-B + 0.6 W m^{-2} UV-A); 2, lamp TL-12 plus 315 nm cutoff filter (far UV-A only: λ_{max} 330 nm, approx. 0.5 W m^{-2} or 1.0 W m^{-2} respectively UV-A); 3, lamp TL-08 (near UV-A: λ_{max} 375 nm, approx. 1.7 W m^{-2} UV-A).

Photon flux density in the visible spectral region was measured with a photodiode (G1118, Hamamatsu Photonics K. K., Hamamatsu, Japan), which was calibrated with an Osram L 40 W / 25 S lamp against a quantumsensor (LI-190B, Li-Cor, Nebraska, USA). UV radiation was measured with a UV-B sensor (UV-S-310-C, Scintec, Tübingen, Germany) calibrated by the manufacturers with a Philips TL 40 W / 12 lamp as light source.

2.2 Growth measurements.

Cultures were sampled under sterile conditions at indicated intervals and homogenized with a motor driven tissue-grinder (Glas-Col, Terra Haute, Indiana, USA) rotating at 500 rpm. Cell number was determined by cell counting of 1:2 dilution series using a hemocytometer (Neubauer Chamber, Brand, Germany). Dry weight was determined gravimetically after desiccation at 85° C overnight and room temperature equilibration under $CaSO_4$.

2.3 Extraction of pigments.

For extraction of carotenoids and scytonemin, cells were harvested by centrifugation and concentrated under vacuum to remove the remaining water. Samples were extracted with 100 % acetone by grinding the cells in the solvent with a motor driven tissue-grinder (Glas-Col, Terra Haute, Indiana, USA), rotating at about 1500 rpm, under nitrogen in darkness. Extracts were clarified by centrifugation. Samples for the carotenoid composition determination were stored under nitrogen at -70° C in darkness until HPLC analysis were performed. For extraction of OS-MAA, samples were extracted in 30 % methanol (30 min at 50° C) or in 100 % methanol (30 min at 60° C) in darkness according to Scherer *et al.* (Scherer *et al.*, 1988) and clarified by centrifugation.

2.4 Determination of pigment contents.

UV-visible spectra were obtained with a Pharmacia Ultraspec 2000 photometer (Pharmacia Biotech, Upsala, Sweden) immediately after extraction of the pigments. Scytonemin, chlorophyll *a* and total carotenoids were quantified from the recorded spectra of acetone extracts using a set of trichromatic equations (Garcia-Pichel and Castenholz, 1991). Specific extinction coefficients used were 92.60 L g^{-1} cm^{-1} at 663 nm for chlorophyll *a* (Vernon, 1960), 112.6 L g^{-1} cm^{-1} at 384 nm for scytonemin (Garcia-Pichel *et al.*, 1992), 250 L g^{-1} cm^{-1} at 490 nm for total carotenoids (Britton, 1985). The OS-MAA content was calculated from recorded spectra of the 30 % methanol extracts using a specific extinction coefficient of 17 L $g^{-1}cm^{-1}$ at 312 nm (Böhm *et al.*, 1995).

Carotenoid composition was analyzed using reversed - phase high pressure liquid chromatography (Hypersil ODS 5 µ column, 250 x 4.6 nm, Alltech Ass. Inc., Deerfield, IL, USA). For details of the system used see Leisner *et al.* (Leisner *et al.*, 1994). Before injection, the 100% acetone extracts were diluted with water to 80 % acetone. Solvent A consisted of 35 % acetone, 52 % methanol and 13 % water (v/v/v), solvent B consisted of 100 % acetone. Chromatography was started with 100 % solvent A for 8.5 min and changed in a linear gradient to 69:31 (v/v) solvent A : solvent B within 30 s. After running isocratically at this composition for 3.5 min, the composition was changed in a linear gradient to 30:70 (v/v) solvent A : solvent B within 14 min, followed by a linear change to 100 % solvent B within 30s. After 2 min at 100 % solvent B the system was returned to the initial conditions and was equilibrated for 9 min. Carotenoids were identified and calibrated using standards (zeaxanthin ((3R,3'R)-ß,ß-carotene-

3,3'-diol) and canthaxanthin (ß,ß-carotene-4,4'-dione) from Roth, Karlsruhe, Germany, ß-carotene from Sigma, Deisenhofen, Germany, echinenone (ß,ß-carotene-4-one) was an isolate donated by Dr. Czygan, Wuerzburg). Myxoxanthophyll (myxol-2'-rhamnoside or 2'-(ß-L-rhamnopyranosyloxy-)3',4'-didehydro-1',2'-dihydro-ß,ψ- carotene-3,1'-diol) was tentatively identified by comparing retention time and online absorbance spectra with published values (Davies, 1976). Other carotenoids were not detected. However, in *Nostoc sp*. Bu94.1, which was isolated by Dr. Büdel, Rostock from the lichen *Peltigera rufescens* (Weis) Humb., two further carotenoids could be separated which were tentatively identified as myxol-2'-o-methyl-methylpentoside and 4-keto-myxol-2'-rhamnoside using the same chromatography system (Woitke and Bilger, unpublished). Chlorophyll *a* was calibrated using an extract of *Synechococcus*.

All experiments described were conducted independently at least twice with three replicates each, the mean values given in the result section representing averages of six assays. Mean separation was based on the calculation of 95 % confidence limits from the appropriate experiment error mean square and tabulated t value.

3 Results

3.1 General growth response upon UV irradiation.

Short time exposure to UV-B for 1 or 1.5 days had negligible effects on the growth of *N. commune* DRH 1. No significant differences ($p < 0.05$) in cell number, dry weight or chlorophyll *a* content were observed (Table 2). Prolonged UV-B exposure led to a decrease of cell replication by about 60 % and, simultaneously, increased dry weight per cell two to three fold when compared to control cultures (Table 2). Light microscopic observation showed that UV-B irradiation induced synthesis of sheath material surrounding the filaments (Fig. 11). The sheath can be visualized by negative staining with 1 % nigrosin and was absent around single filaments in control cultures (data not shown). The yield of a large scale glycan isolated from UV-B-irradiated cultures was about three times higher than the one from control cultures (data not shown), indicating that the dry weight increase upon UV-B radiation can be essentially attributed to a stimulated extracellular glycan production.

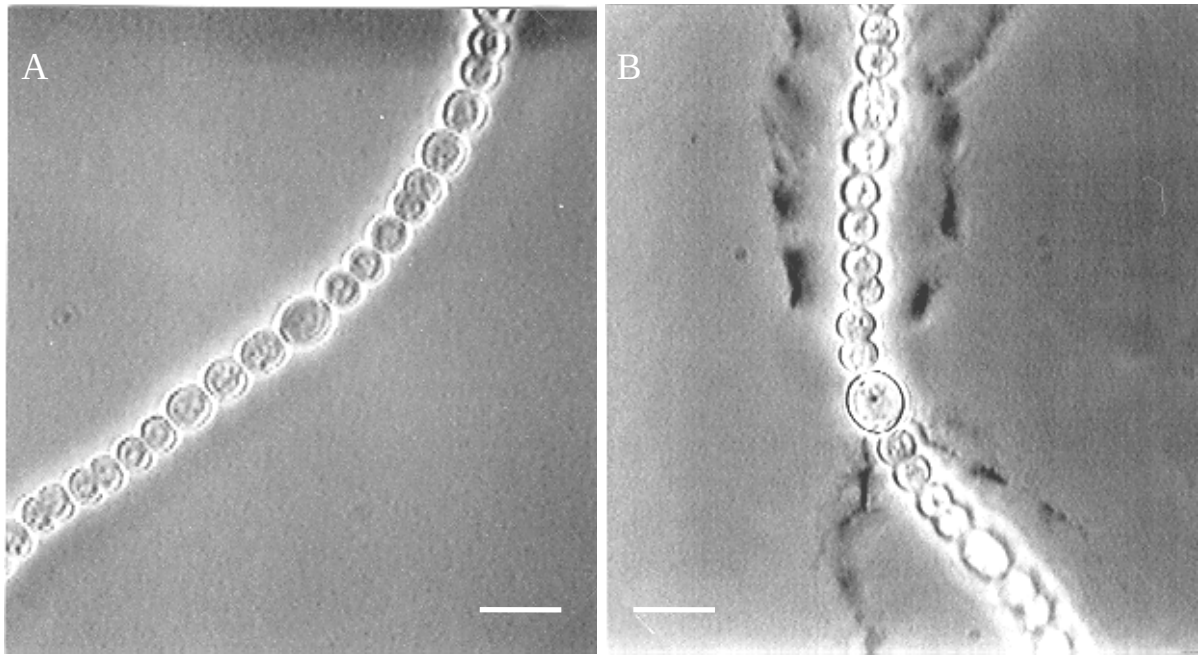

Fig. 11: *Nostoc commune* DRH1 filaments grown in liquid culture; unstained light microscopic picture. **A:** Typical appearance of *Nostoc commune* DRH1 grown in liquid culture without UV-B. **B:** DRH1 liquid culture after 72 hours of UV-B irradiation (1.0 W m^{-2}). Note that glycan is absent around heterocysts. Bars indicate 10 µm.

The UV-B dependent induction of pigments occurred in three phases, involving carotenoids, mycosporine and scytonemin. Because chlorophyll *a* content per cell remained unaffected even after prolonged exposure to UV-B, while total dry weight was rising (Table 2), all pigment contents are given in relation to chlorophyll *a*.

Table 2: Growth response of *Nostoc commune* DRH1 cultures upon UV-B irradiation.[a)]

Exposure time [days]	Mean ± SD					
	Cell number [10^7 cells / ml]		Chl *a* [µg / 10^7 cells] [b)]		Dry weight [µg / 10^7 cells]	
	Control	+ UV-B	Control	+ UV-B	Control	+ UV-B
1	2.2 ± 0.5	2.5 ± 0.5	1.7 ± 0.4	1.5 ± 0.4	n.d.[c)]	n.d. [c)]
6.5	9.3 ± 0.7	3.9 ± 0.4	1.1 ± 0.2	1.4 ± 0.2	150 ± 20	370 ± 50

a) DRH1 was cultivated at 30° C with cold fluorescent light of 2.4 W m^{-2} and artificial UV-B irradiation of 1.0 W m^{-2} (emission spectra 1, Fig. 10). The control culture received the same intensity of fluorescent light without UV-B.
b) no significant differences were detected ($p < 0.05$). Chl *a*: chlorophyll *a*.
c) n.d.: not determined

3.2 Induction of carotenoids.

After 5 hours of UV-B treatment with 1.0 W m^{-2}, a first significant increase of total carotenoids was observed (data not shown). The total carotenoid to chlorophyll *a* ratio after 1 day UV-B irradiation was 34 - 40 % higher than the control value (Fig. 12A). After 5.5 days of UV-B exposure the enhanced carotenoid to chlorophyll *a* ratio declined to about 115 % compared to controls.

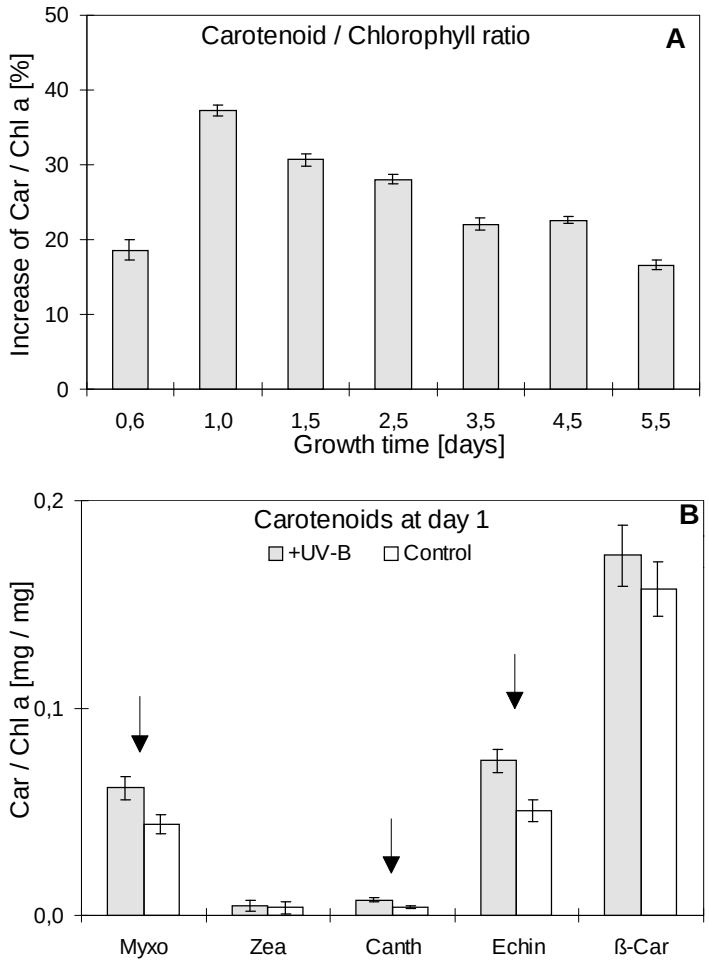

Fig. 12: Influence of UV-B irradiation on carotenoid synthesis. **A:** Changes in total carotenoid / chlorophyll ratios in response to UV-B irradiation of 1.0 W m-2. Values of UV exposed cells were calculated and compared with those of non-UV-exposed cells and presented as a percentage increase, ($p < 0.05$) ± se. **B:** Changes in carotenoid patterns after 1 day UV-B irradiation monitored by RP-HPLC. Abbreviations: myxoxanthophyll : myxo, zeaxanthin : zea, canthaxanthin : canth, echinenone : echin, ß-carotene : ß-car, chlorophyll a : chl a. ↓ denotes significantly different values compared to control ($p < 0.05$).

To analyze whether UV-B led to a general shift up in all carotenoids or whether specific carotenoids were induced, carotenoid patterns were analyzed by RP-HPLC. Figure 12B shows the carotenoid pattern of *N. commune* DRH 1 after 1 day of UV-B treatment and the corresponding pattern of the control culture. As reported for other cyanobacteria (Hertzberg and Liaaen-Jensen, 1971), the carotenoid composition of *N. commune* was dominated by ß-carotene, echinenone, and myxoxanthophyll, while canthaxanthin and zeaxanthin were only minor components. Specific contents (mg pigment / mg chlorophyll *a*) of echinenone, myxoxanthophyll and canthaxanthin were significantly increased ($P < 0.01$) while ß-carotene and zeaxanthin showed no significant differences in comparison to control cultures. No additional carotenoids were induced by UV-B irradiation. The carotenoids mainly affected by UV-B were echinenone and myxoxanthophyll. Their specific content increased about 40 % to 50 % in comparison to control cultures.

The induction of carotenoids was due to UV-B, since filters with a cutoff at 315 nm (Fig. 10) prevented an increase in carotenoids (Table 3). Cultures irradiated with near UV-A (λ_{max} 375 nm) also showed an increase in carotenoid to chlorophyll *a* ratios, but the induction followed completely different kinetics because carotenoids increased continuously over five days (Table 3).

Table 3: Wavelength dependence of pigment induction[a]

Time [days]	Car / Chl a [% increase]			OS-MAA / Chl a [mg / mg]			Scyt / Chl a [mg / mg]		
	farUV-A + UV-B [b]	farUV-A only [c]	nearUV-A [d]	farUV-A + UV-B [b]	farUV-A only [c]	nearUV-A [d]	farUV-A + UV-B [b]	farUV-A only [c]	nearUV-A [d]
1	37.3 ± 0.7	3.1 ± 0.5	26.2 ± 0.9	2.0 ± 0.3	N.D.	N.D.	0.05 ± 0.02	N.D.	n.d.
2.5	28.1 ± 0.7	5.2 ± 0.6 (10.9 ± 0.7)*	30.3 ± 0.7	3.6 ± 0.5	N.D. (N.D.)*	N.D.	0.38 ± 0.03	0.02 ± 0.01 (0.03)*± 0.01	1.1 ± 0.04
4.5	22.6 ± 0.4	0.2 ± 0.4	45.3 ± 0.5	4.6 ± 0.4	N.D.	N.D.	0.23 ± 0.05	N.D.	n.d.

[a] Car: total carotenoids, chl *a*: chlorophyll *a*, scyt: scytonemin, n.d.: not determined, N.D.: not detected. DRH1 cultures received about 2.4 W m^{-2} visible light supplemented with following UV irradiations as indicated in the footnotes *b* to *d*. For detailed spectra characteristics of UV treatments, see Fig. 10.
[b] λ_{max} 315 nm, approx. 1.6 W m^{-2},
[c] λ_{max} 330 nm, approx. 0.5 W m^{-2} or * 1.0 W m^{-2}
[d] λ_{max} 375 nm, approx. 1.7 W m^{-2}.

3.3 Induction of mycosporines.

UV-B exposure led to the production of UV-A/B-absorbing oligosaccharide mycosporine amino acid (OS-MAA) with absorption maxima at 312 nm and 335 nm (Fig. 13).

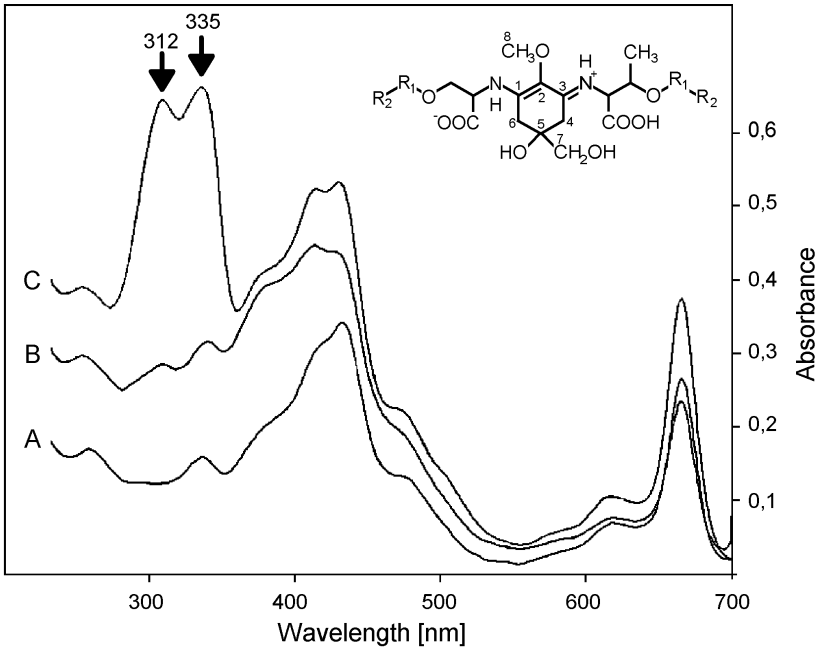

Fig. 13: Absorption spectra of DRH1 in 100 % methanol. **A:** before UV-B treatment, **B:** 1 day UV-B irradiation, **C:** 5.5 days UV-B irradiation. Inset: Structure of the 335-chromophore according to Böhm *et al.* (2). R1, galactose, xylose, glucuronic acid, R2, galactose, glucose, glucosamine.

The total amount of OS-MAA per ml culture increased during the entire UV-B exposure time, but the specific content rose to a maximum of about 4.5 mg per mg chlorophyll *a* at 3.5 days and remained at this high value (Fig. 14A). Synthesis of the pigment was neither promoted by supplemented UV-A irradiation (Table 3), nor by other stresses such as increased temperature, desiccation, or salt (data not shown). Traces of OS-MAA could be detected in old control cultures not subjected to UV stress.

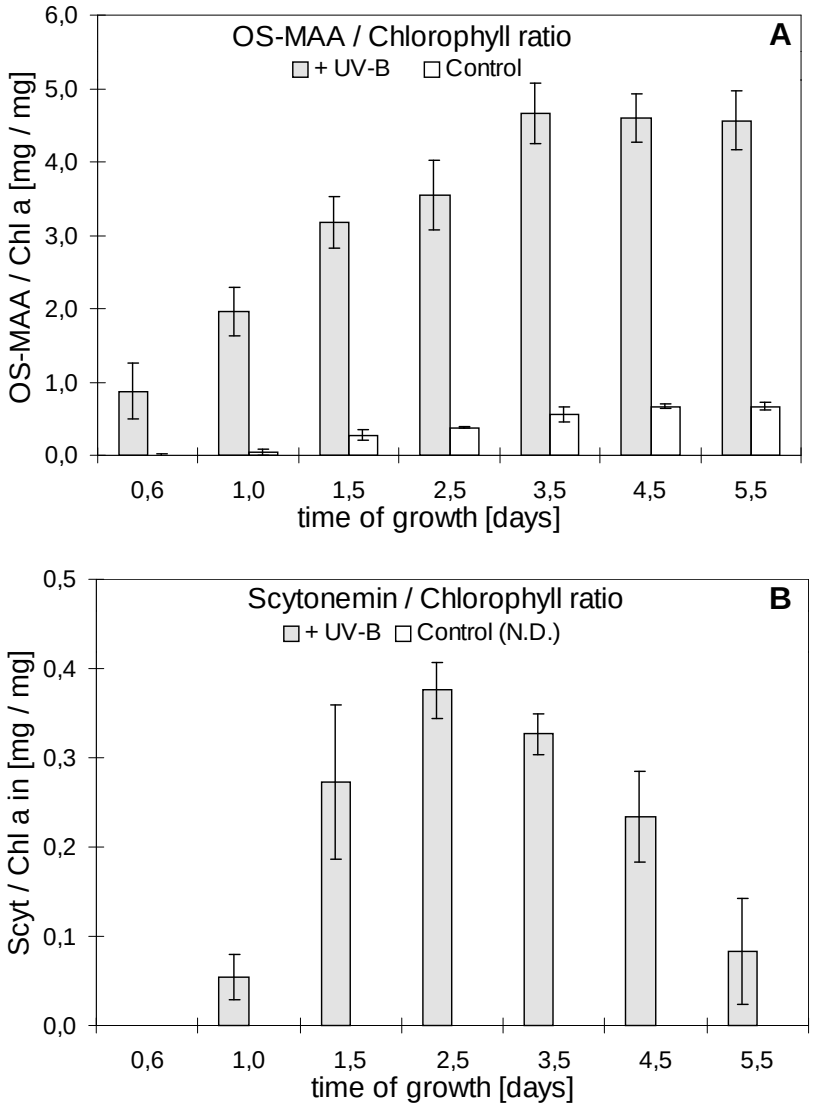

Fig. 14: UV-B induced synthesis of extracellular UV absorbing compounds in liquid cultures of DRH1. Time course of oligosaccharide-MAA **(**OS-MAA, **A)** and scytonemin **(**Scyt; **B)** content at 1.0 W m^{-2} UV-B. Bares denote standard derivations of means. N.D.: not detected. Chl a, chlorophyll *a*.

3.4 Induction of scytonemin.

No scytonemin was detected in control cultures grown without UV. UV-B irradiation induced scytonemin production after an initial lag of about 1 day. The specific content rose to a maximum of about 0.38 mg / mg chlorophyll *a* at 2.5 days and declined thereafter (Fig. 14B). The induction of scytonemin was mostly due to the UV-B part (λ_{max} 315 nm) emitted by the UV-B light source, because cultures protected by filters with a cutoff at 315 nm (far UV-A irradiation, λ_{max} 330 nm) showed only a very low scytonemin production of about 6 % compared to unfiltered cultures (Table 3). Even higher far UV-A irradiation only let to very low

scytonemin production, whereas near UV-A irradiation (λ_{max} 375 nm) induced a 2 to 3 fold scytonemin production in comparison to cultures treated with UV-B (Table 3).

4 Discussion

Synthesis of extracellular polysaccharides is induced by UV-B. Long-time UV-B, but not UV-A, exposure of *N. commune* DRH1 led to a decreased cell number but enhanced dry weight in comparison to control cultures, while short time UV-B exposure had negligible effects on the growth of *N. commune* (Table 2). We suggest that the decreased cell number observed after 6 days of UV-B exposure is due to a slower cell replication caused by the metabolic cost of increased glycan production and is not a consequence of inhibition of cell replication by UV-B. To our knowledge, the influence of UV-B on extracellular glycan production has not been studied so far. Since the UV-absorbing mycosporines induced simultaneously are located in the glycan sheath (Scherer *et al.*, 1988; Böhm *et al.*, 1995), it is likely that an increased glycan production serves to provide a matrix for the OS-MAA, which is closely attached to the glycan by noncovalent interactions (Hill *et al.*, 1994; Böhm *et al.*, 1995). A thicker sheath provides much longer effective path lengths for the absorption of radiation. Whether UV-B led to structural changes of exopolysaccharides is currently under investigation.

Myxoxanthophyll and echinenone could be envelope membrane-bound UV photoprotectors. For photosynthetic organisms, the protective role of carotenoids against high visible radiation is well known (Siefermann-Harms, 1987; for review see Demmig-Adams and Adams III, 1992) and a protective role of carotenoids in cyanobacteria against UV-A radiation was reported (Buckley and Houghton, 1976; Paerl, 1984). Only little is known about the role of carotenoids in photoprotection against UV-B radiation (Middleton and Teramura, 1993; Quesada *et al.*, 1995). Cyanobacteria produce some unique types of xanthophylls, such as ketocarotenoids and glycosides (Hirschberg and Chamovitz, 1994). Interestingly, in *N. commune* DRH 1 these unique types were induced by UV-B whereas ß-carotene and zeaxanthin showed no response (Fig. 12B). Analysis of cyanobacterial envelope membranes demonstrated that xanthophylls are the predominant carotenoids, whereas ß-carotene was found almost exclusively in the thylakoids (Jürgens and Weckesser, 1985; Karentz *et al.*, 1991). Myxoxanthophyll, a pigment induced by UV-B in *N. commune* DRH 1 (Fig. 14B), has been shown to be the predominant pigment in the outer membrane of *Synechocystis* (Jürgens and Mäntele, 1991). Echinenone, the other carotenoid strongly induced by UV-B in *N. commune* DRH 1 (Fig. 14B), has also been found in the outer membrane of *Synechocystis*, but only as a minor compound. The function of the carotenoids in

outer membranes of cyanobacteria is still not clear. Since it has been shown that heterologous expression of carotenoid genes in *Escherichia coli* led to an increased resistance to UV radiation (Tuveson *et al.*, 1988; Tuveson and Sandman**,** 1993), our results suggest that myxoxanthophyll and echinenone may, indeed, act as outer membrane-bound UV-B photoprotectors of *N. commune*. They may be induced as a fast, SOS-type response before extracellular UV sunscreens can be synthesized.

UV-B induced the synthesis of two extracellular sunscreen pigments. UV-B irradiation of *N. commune* DRH 1 led to the production of the water-soluble UV-A/B absorbing pigment, which belongs to the group of mycosporine like amino acids (MAAs), and the production of the lipid-soluble scytonemin (Fig. 14). However, the scytonemin content of UV-B treated cultures was one order of magnitude less than OS-MAA content. MAAs may play an important role in photoprotection of *N. commune* because the OS-MAA is located in the extracellular glycan. The pigment provides protection, mainly by absorbing the harmful radiation, but the 312 nm chromophore of the pigment, which is thought to be a MAA-Gly (Böhm *et al.*, 1995), may provide additional protection by radical quenching (Dunlap and Yamamoto, 1995). No photobleaching of chlorophyll *a* was observed in OS-MAA producing *N. commune* DRH1 upon UV-B irradiation (see Table 2), whereas *Nostoc* Bu 94.1, which produces scytonemin but no MAAs, completely bleached when it was treated with UV-B (Ehling-Schulz and Scherer, unpublished).

UV-B irradiated liquid cultures of *N. commune* contained about 2 % OS-MAA by dry weight (this study). Amounts of OS-MAA found in desiccated field material (Scherer *et al.*, 1988), estimated using the same extinction coefficient, correlate well with this value. As both OS-MAA and glycan synthesis increased due to UV-B, but not in response to UV-A, some correlation of extracellular polysaccharide and OS-MAA induction may exist. UV-A and UV-B induced synthesis of scytonemin in *N. commune* DRH 1 followed similar kinetics, but pigment concentrations in UV-B treated cultures were only about 30 % of those of UV-A treated cultures.

We propose that OS-MAA is the key pigment in UV-B protection while scytonemin is most effective in UV-A protection. However, the latter may have some special role as UV-B protectant immediately after rewetting of desiccated colonies. In contrast to OS-MAA, scytonemin is not lost upon rewetting. Since it has some absorption in the UV-B range it may provide some protection against UV-B in MAA-depleted field material.

Potential UV photoreceptors. Our results suggest that OS-MAA and scytonemin synthesis may be regulated by different photoreceptors. The synthesis of OS-MAA is induced by

a UV-B photoreceptor absorbing at wavelengths below 315 nm (Table 3). A separate UV-A photoreceptor probably regulates scytonemin because its synthesis is most pronounced at near UV-A (350 - 400 nm) irradiation, whereas far UV-A (320 - 350 nm) had only little effect. In addition to UV-A there is a slight induction of scytonemin by UV-B (Table 3). UV-B and UV-A induction of chalcone synthetase are regulated separately (Christie and Jenkins**,** 1996). Based on our data, however, it is not possible to assess the small effects of UV-B on scytonemin.

5 Conclusion

Photon fluence rates of UV-B which are within the magnitude of solar fluence rates induce a cascade of physiological reactions in *N. commune*. In its natural habitat, *Nostoc* has to cope with high solar radiation in its dry state, in which photodamage cannot be efficiently repaired. Therefore, passive photoprotective mechanisms are needed. The water-soluble OS-MAA provides passive protection against UV-B and far UV-A irradiation (Böhm *et al.*, 1995) whereas the lipid-soluble scytonemin, beside some absorption in the UV-B, absorbs mainly UV-A (Garcia-Pichel and Castenholz, 1991). Since carotenoid synthesis is induced very fast upon UV-B irradiation, outer membrane-bound carotenoids may play a role in photoprotection immediately after rewetting of desiccated colonies when the OS-MAA content is low. It is submitted that carotenoids, in UV protection, provide fast, active SOS response to counteract acute cell damage whereas the extracellular glycan with its UV absorbing pigments is a passive UV screen against long-time exposure.

Chapter 3

Semiquantitative, differential 2D Display of the Dynamics of UV-B triggered *versus* Growth-Cycle dependent Proteome Changes in the Terrestrial Cyanobacterium *Nostoc commune* [*]

1 Introduction

Cyanobacteria dominate the microbial communities of some of the most extreme environments on earth. They are often the primary colonizers of rock surfaces and soil (Whitton 1987) and play an important role in preventing erosion and preserving water in the soil (Booth 1941). It is, therefore, of considerable importance to understand their adaptive strategies towards changes of environmental conditions. One environmental factor which is receiving more and more attention is the increase of solar UV-B radiation reaching the earth's surface due to the depletion of the stratospheric ozone layer (Fraser *et al.*, 1992). DNA, proteins and membranes are likely to be important targets of detrimental UV-B radiation (Caldwell, 1979; Tuveson *et al.*, 1988; Barbato *et al.*, 1995). However, photosynthetic microorganisms display a variety of mechanisms to counteract UV damage which have been studied at the organismal and physiological level (for review see Ehling-Schulz and Scherer, 1999).

In the terrestrial, highly UV tolerant cyanobacterium *Nostoc commune* a cascade of physiological reactions was observed in response to UV-B: first, a rapid increase in carotenoids, especially echinenone and myxoxanthophyll; second, an enormous increase of an extracellular UVA-/B- absorbing pigment which was associated with extracellular glycan synthesis and, finally, scytonemin, was slightly induced by UV-B and very strongly by UV-A irradiation (Ehling-Schulz *et al.*, 1997). The UVA-/B- absorbing pigment has been shown to be an oligosaccharide MAA which is located in the extracellular glycan (Hill *et al.*, 1994; Böhm *et al.*, 1995). Several studies provide evidence that mycosporine amino acids (MAA) with absorption maxima between 310 and 360nm protect cyanobacteria and other lower organisms by absorbing harmful UV radiation (Scherer *et al.*, 1988; Karenz *et al.*, 1991; Ehling-Schulz *et al.*, 1997). Scytonemin, an extracellular pigment which has an in vivo absorption maximum at 370nm, is

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known to function as a UV-A sunscreen in terrestrial cyanobacteria. It has been shown that scytonemin synthesis requires protein *de novo* synthesis (Garcia-Pichel and Castenholz, 1991). The biochemical pathways which are involved in its synthesis are unknown. MAAs are thought to originate from the first part of the shikimate pathway, but this still requires evidence (Favre-Bonvin *et al.*, 1987).

The knowledge about the effects of UV at the biochemical and molecular level in cyanobacteria is limited. In the past, most studies have been focused on active repair mechanisms, like DNA repair and turnover of the UV sensitive D1 reaction center protein of photosystem II (O'Brien and Houghton, 1982; Eker *et al.*, 1990; Sass *et al.*, 1997; Cambell *et al.*, 1998; Mate *et al.*, 1998) but information about the global cellular response to long-time UV irradiation is lacking. Nevertheless, it has been claimed on the basis of SDS-PAGE data that UV-B has nearly no influence on the protein pattern of cyanobacteria (Gerber and Häder, 1995; Masi and Melis, 1997; Chauhan *et al.*, 1998). However, SDS-PAGE may not be suitable to monitor the UV acclimation process at the protein level since its resolution is far too low (Ehling-Schulz and Scherer, 1999). Proteome analysis, based on high-resolution two-dimensional (2D) gel electrophoresis has become a powerful tool for investigating global changes in the gene expression program of organisms (Antelmann *et al.*, 1997; Godon *et al.*, 1998; VanBogelen *et al.*, 1996, 1999). However, there is little information available on the cyanobacterial proteome. The only proteome which has been studied in some detail is the proteome of *Synechocystis* sp. PCC 6803 (Sazuka and Ohara, 1997; Sazuka *et al.*, 1999), but no stress response studies have been made.

In this study, we report on dramatic global changes of the *N. commune* proteome during UV acclimation, as is revealed by subtractive high-resolution two-dimensional gel electrophoresis (differential 2D display). Subtractive protein analysis allows the correlation of environmental effects with protein composition, but in long-time experiments growth dependent proteome changes have to be taken into account. In order to monitor the adaptation process over different growth stages of *N. commune*, a database application for Microsoft Access was developed.

2 Materials and Methods

2.1 Organism growth conditions and growth measurement.

The cyanobacterium *Nostoc commune* Vaucher strain DRH 1 was derived from field material of *N. commune* collected in the Hunan province, People's Republic of China (Hill *et al.*,

1994). For UV induction experiments, *N. commune* DRH 1 liquid cultures were grown under nitrogen fixing conditions at 30° C as described previously (Ehling-Schulz *et al.*, 1997). Visible light was obtained from a cool white fluorescent tube (L 40 W / 25 S, Osram, Munich, Germany, ca. 2.4 W m^{-2}). Additional UV illumination was provided by a Philips TL 40 W / 12 lamp with an incident irradiance of 100 to 140 mW m^{-2} nm^{-1} at 310 nm and 50 to 70 mW m^{-2} nm^{-1} at 330 nm. UV-A experiments were performed using a Philips TL 36 W / 08 lamp with an emission maximum centered at 375 nm. The spectral irradiance which was received by the cultures has been described in detail previously (Ehling-Schulz *et al.*, 1997). Because chlorophyll *a* content per cell remained unaffected even after prolonged exposure to UV-B, while total dry weight was rising, growth curves were calculated from chlorophyll *a* spectra (1.4 ± 0.2 µg chlorophyll a correspond to 10^7 cells). Chlorophyll *a* contents were determined as described previously (Ehling-Schulz *et al.*, 1997).

2.2 Preparation of protein fractions.

Cultures were precultivated for 2.5 days, thereafter UV irradiation was provided and cells were harvested after different incubation times (3 hours up to 3 days). Cultures were harvested by centrifugation and cells were washed three times with Tris-HCl pH 7.8 (10 mM, 30 °C) to remove extracellular polysaccharides and proteins. Supernatants were concentrated by ultrafiltration (Centricon, 10 kD cutoff, Amicon) and speedvac evaporation. This fraction contained the extracellular proteins. The extracellular protein fractions were tested to be free of intracellular proteins by spectrophotometric tests (excision spectra, excision 355 nm) to detect phycobiliproteins, which are the most abundant proteins in cyanobacterial cells. After the last washing step, cells were resuspended in Tris-HCl pH 7.8. Proteinase inhibitor Pefabloc (0.1 mM Boehringer Mannheim) was added and the suspension was passed two times through a French pressure cell (SLM AMINCO) at 140 MPa. Cell debris was removed by ultracentrifugation at 100 000 x g for 1 hour at 15°C. Protein content was determined by the Bradford test (Bradford 1976) with bovine serum albumin as a standard. The efficiency of the removal of extracellular proteins was tested by immunoblotting according to Hill *et al.* 1994. To the supernatant fraction, which contained the intracellular water-soluble proteins ("cytosolic protein fraction"), the following substances were added (final concentrations): 8M urea, 2% CHAPS, 1% DTT and 0.8% Pharmalyte 3-10 (Amersham-Pharmacia Biotech) and the samples were stored in aliquots at -70°C until analysis.

The 100 000 x g pellet was washed with Tris-HCl pH 7.8, (15 °C, 100 000g, 30 min), resuspended in a detergent buffer containing 8M urea, 2% CHAPS, 1% DTT and 0.8% Pharmalyte 3-10 (Amersham-Pharmacia Biotech) and sonicated 3 x for 10 seconds each in an ice-water bath using a Sonoplus microtip sonicator (Bandelin, Berlin, Germany). Samples were then centrifuged (15°C, 8 000 x g, 30 min). The supernatant fraction, containing membrane bound and membrane associated proteins ("membrane protein fraction"), was centrifuged again (15 °C, 8 000 x g, 15 min), recovered and stored at -70°C until analysis.

2.3 SDS-PAGE and western analysis.

SDS-PAGE was performed on 15 % (w/v) polyacrylamide gels in a discontinuous buffer system according to Laemmli (1970). Western blot analysis was performed using a Wsp antibody according to Hill *et al.* (1994). For detection, the BM chemiluminiscence western blotting kit from Boehringer Mannheim was used.

2.4 High resolution two-dimensional (2D) gel electrophoresis

High resolution two-dimensional gel electrophoresis was performed with immobilized pH gradients in the first dimension and discontinuous SDS-PAGE in the second dimension (IPG-Dalt) according to Görg *et al.* (1988). Both dimensions were run using a Multiphor II horizontal electrophoresis unit (Amersham -Pharmacia Biotech). Isoelectric focusing was carried out on IPG 4 - 9 gel strips, which had been rehydrated in 8M urea, 0.5 % CHAPS, 0.2 % DTT, 0.2 % Pharmalyte 3 - 10 over night. Each IPG strip was loaded with approx. 60 µg protein using the Immobiline Dry-Strip Kit (Amersham - Pharmacia Biotech) and focused for 16 000 Vh at 20 °C under oil. Prior to the second dimension, strips were equilibrated for 2 x 15 min in Tris-HCl, pH 8.8 (50mM), containing 6M urea, 30% glycerol, 1 % DTT was added to the first equilibration step and 4.8 % iodoacetamide was added to the second equilibration step. SDS-PAGE was carried out in 13% (w/v) polyacrylamide gels which were cast on GelBond PAGfilms (Biorad Laboratories). Resolved polypeptides were silver stained according to Blum *et al.* (1987). UV treated and corresponding control cultures were focused together, run on the same second dimension SDS-PAGE and stained together. Silver staining was used because a good linear relationship has been demonstrated between protein abundance and integrated optical density of protein spots, over 40-50 fold range from 0.04 ng/mm^2 to 2 ng/mm^2 (Hochstrasser, 1997). The intensity of a large majority of proteins is linearly related to the protein quantity (Damerval,

1994). Most proteins that do not have a linear response to silver stain show a plateau, for these proteins , the computed induction rate is a minimum value of induction (Burstin *et al.*, 1993).

2.5 Data analysis.

Computer assisted image analysis was used for spot detection and volume measurement (ImageMaster 2D Elite version 2, Amersham-Pharmacia Biotech). pIs were calculated from the pH gradient of the focused IPG strips and molecular weights were estimated from co-migrated standard proteins in the second dimension. Reproducibility of the 2D analysis was tested by running the same sample twice on independent gels and reproducibility of the growth experiments was tested by principal component analysis (PCA). A total of about 100 gels have been run during this approach, a subset of 46 gels have been analyzed by computer assisted image analysis. Subtractive gel comparison was performed at different time points. Gels were matched in a two step procedure. In the first step, gel images of stress treated and their corresponding control cells were matched. In a second step, gel images of the different timepoints were matched to provide the possibility to follow the changes of individual spots over time. All gels were matched to each other. A database application for Microsoft Access was developed to construct a three dimensional virtual master gel, which allowed to monitor the UV adaptation process over different growth stages of *N. commune*. The mean of the log-normal distribution of spot volume (ln mean) was used as normalization variable since it is independent of the number of spots in a gel and the stain variability from protein to protein (Garrels, 1989; Vohradsky *et al.*, 1997). Spots with a volume smaller than 0.15, or area smaller 200 pixel, or density (volume/area) lower 0.018 were discarded. Spots which were outside the 68% interval of ln mean were considered to be significantly changed (this correspond approximately to a factor of three). A cross-match algorithm was used to check all pairs of matched gels for consistency. It compared the spots matched directly between each gel pair to the spots matched indirectly between the same two gels. If the direct match disagreed with the majority of the indirect matches, the direct match was corrected. Statistical analysis was performed using the software package XLSTAT 3.5 (Thierry Fahmy). Two independent time-course growth experiments were completely analyzed.

3 Results

3.1 Subcellular protein fractions.

Total cellular protein was isolated from *N. commune* DRH1 cultures after different stress

induction and growth times. The time points of sampling are indicated in figure 15. The protein samples were fractionated prior to electrophoresis to get a better understanding of the location of proteins affected by UV-B. An immunoassay was used to test the cytosolic water-soluble protein fractions for contaminated extracellular proteins. Cytosolic protein fraction was tested by immunostaining for contamination of Wsp. The desiccation stress protein Wsp is an abundant

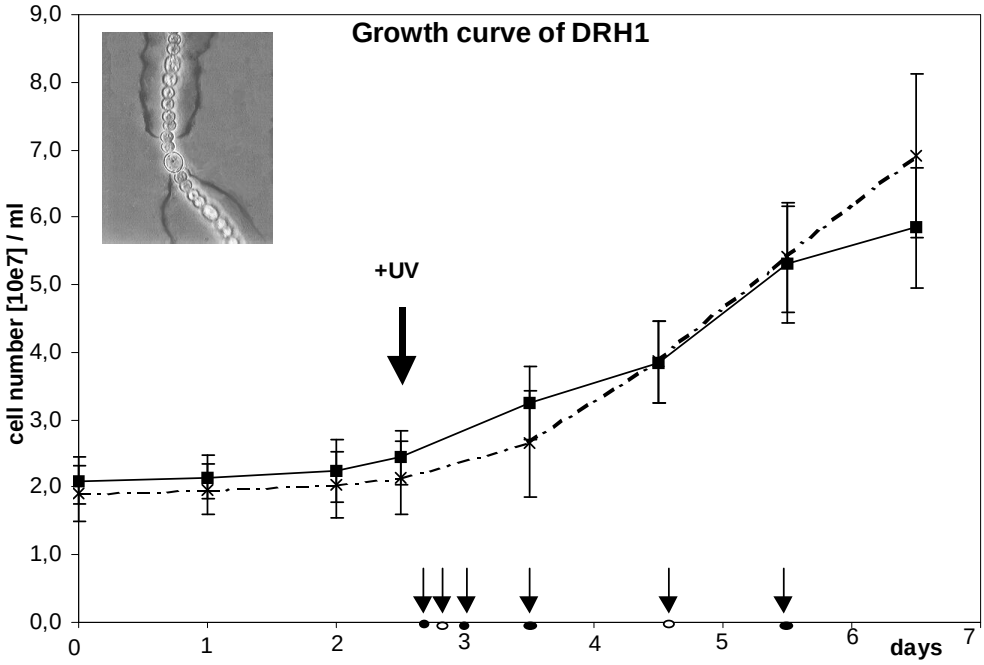

Fig. 15: Growth curve of *Nostoc commune* DRH1 liquid cultures grown under nitrogen-fixing conditions at 30°C receiving continuous light. For detail of culture conditions see Ehling-Schulz et al. (1997). Inset: *N. commune* DRH1 filament, grown 3 days with UV-B. Legend: solid line: cultures exposed to UV-B (1.0 Wm^{-2}); dashed line: cultures grown without UV-B; bares denote standard derivation of means; Thick arrow marks the beginning of UV-B irradiation; thin arrows mark time points at which samples for 2D electrophoresis were obtained. Time points which were sampled only in one experiment are indicated by white dots.

protein in the extracellular glycan. None of the cytosolic water-soluble protein fractions showed any signal in the immunoassay (data not shown). The membrane and cytosolic protein fractions showed completely different 2D patterns, the most abundant proteins of the cytosolic fraction are missing in the protein profile of the membrane fraction (Fig. 16). Because of the high extracellular polysaccharide content, which disturbs the first dimensional IEF, 2D turned out not to be a appropriate technique for analysis of the extracellular proteins of *N. commune* DRH1. Nevertheless, SDS-PAGE showed also some influence of UV-B irradiation, especially the induction of a band of approx. 22 kD. Immunodetection showed that UV-B induced the desiccation stress protein Wsp (Fig. 17).

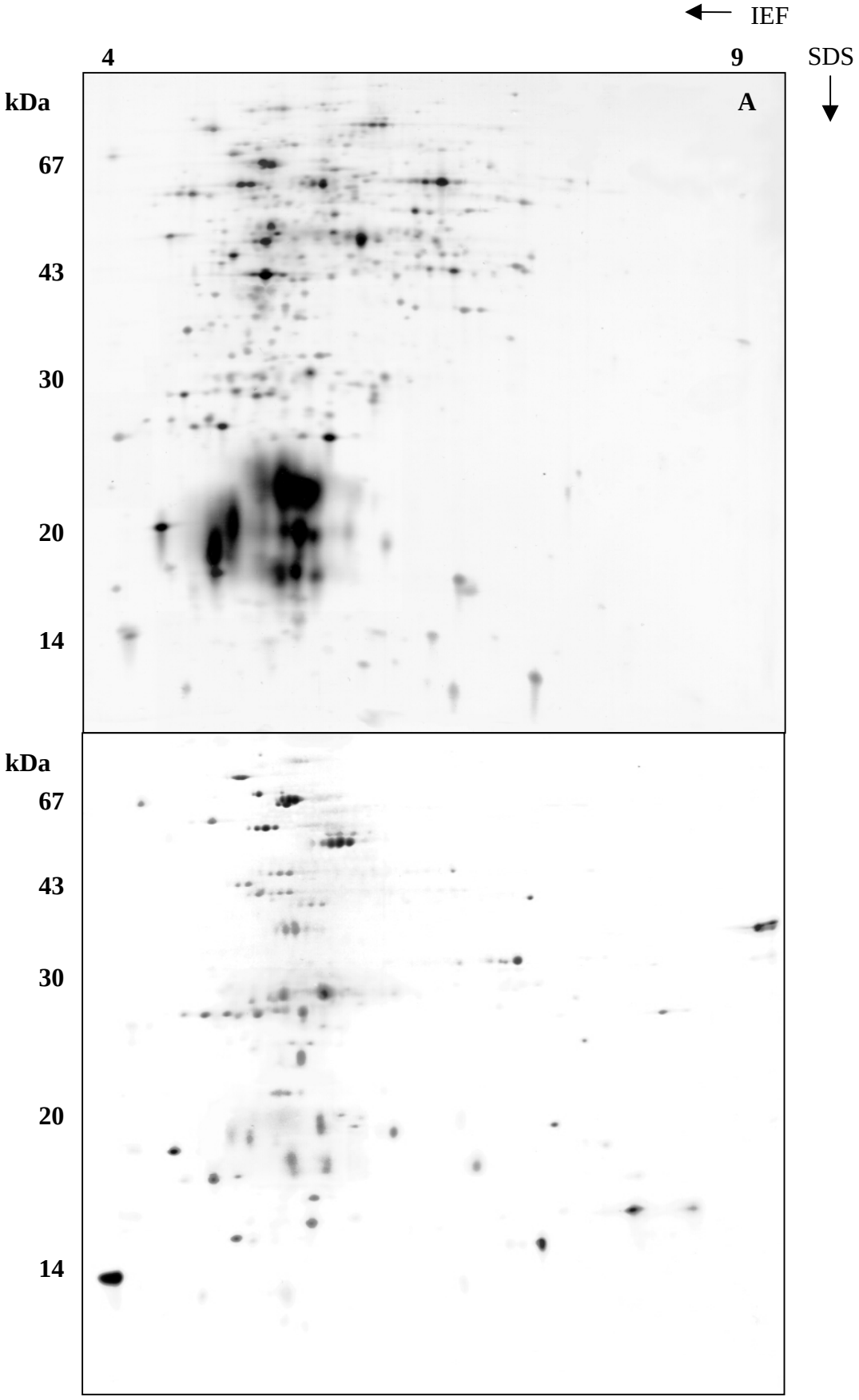

Fig. 16: Subcellular protein preparations of liquid cultures of *N. commune* DRH1 treated with UV-B. **A:** A representative 2D-electrophoresis gel of water-soluble cytosolic proteins of DRH1. **B:** A representative 2D-electrophoresis gel of membrane and membrane associated proteins of DRH1.

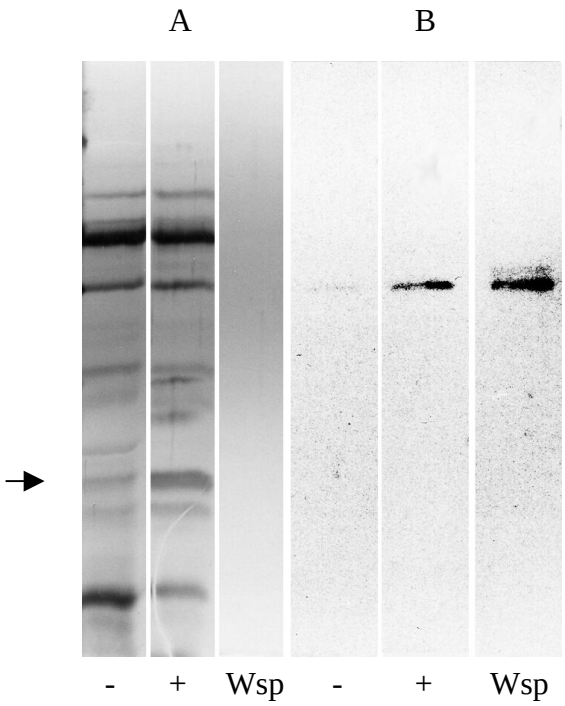

Fig. 17: SDS-PAGE and western analysis of *N. commune* DRH1. **A**: Extracellular proteins were analyzed by SDS-PAGE after 12 hours of UV-B irradiation (1.0 W/m²). Gel was silver stained. **B**: Western analysis of extracellular proteins, using the Wsp antibody to test the influence of UV-B on the synthesis of Wsp. Abbreviations: + cultures exposed to UV-B; - cultures grown without UV-B; Wsp purified fraction of the water stress protein; ➔ indicates the position of the predominant band induced by UV-B.

In the case of cytosolic and membrane fractions, highly reproducible protein pattern were obtained by 2D. Typical silver stained gels of the subcellular protein fractions are shown in figure 16. In the cytosolic fraction a total of about 750 protein spots, and in the membrane fraction a total of about 600 proteins could be detected in the molecular mass range of 10kDa to 110kDa and the pI range of pH4 to 9. A synthetically master gel was generated showing all proteins detected either in the cytosol or membrane fraction (Fig. 18). Cytosolic fraction and membrane fraction showed different frequency distributions of pIs and molecular weights of the detected protein spots (Fig. 19). The frequency distribution of pIs of membrane fraction proteins showed a clear center of gravity in the frequency class pH 5.5 to 6, whereas in the cytosolic protein fraction no clear center of gravity could be detected. Most proteins spread over five frequency classes, ranging from pH 4 to pH 7. Dependent on molecular weight frequency classes, the membrane fraction proteins were concentrated in the class 20 kDa to 30 kDa, whereas the cytosolic fraction proteins were concentrated in the 40 kDa to 50 kDa frequency class (Fig. 19).

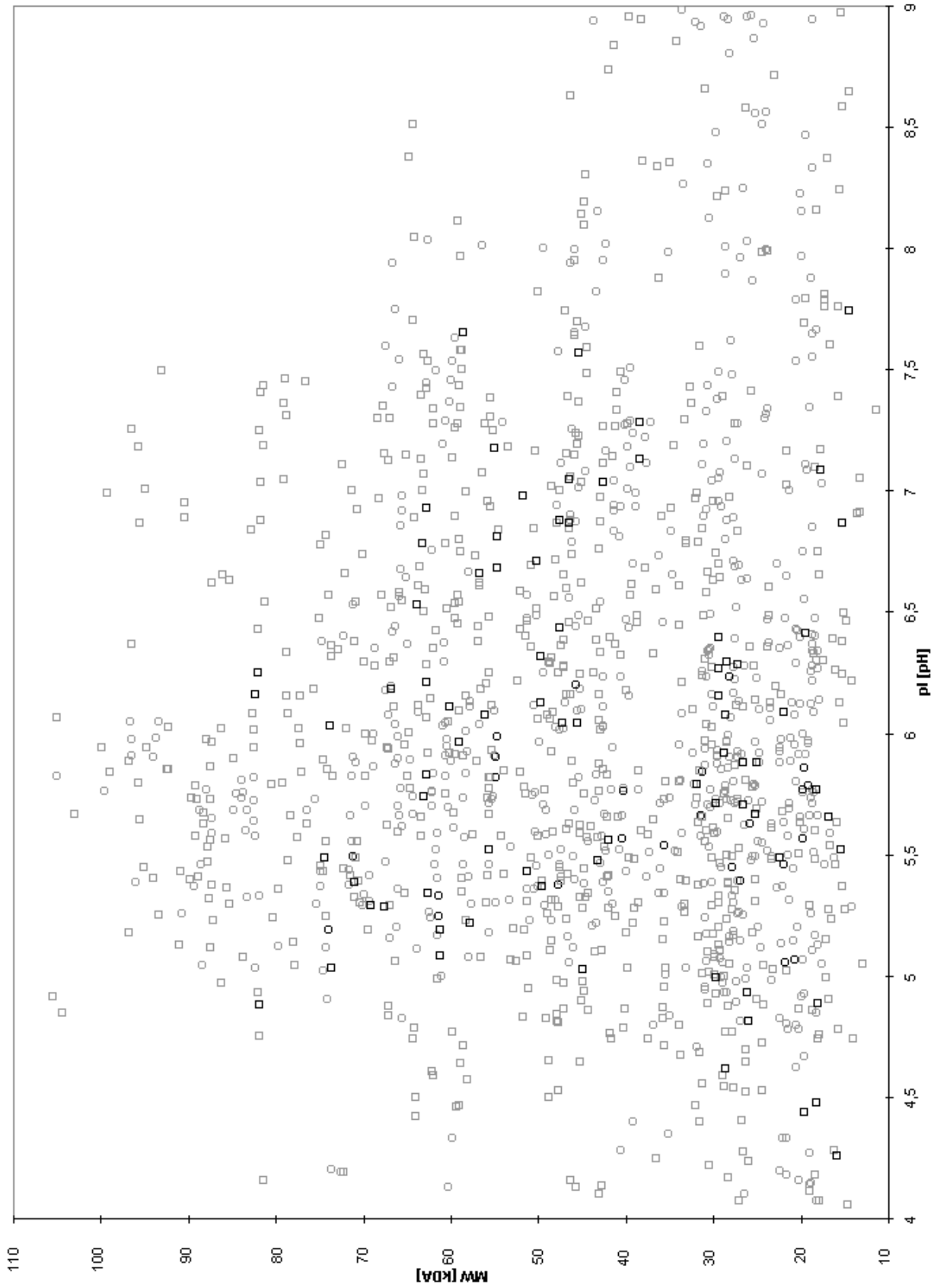

Fig. 18: Schematic reference map of *Nostoc commune* DRH1 proteome generated from the subcellular protein maps (see Fig. 22, 23). Squares indicate cytosolic protein spots, whereas circles refer to membrane and membrane associated protein spots. Spots which are not influenced by growth stage nor by UV-B or UV-A are highlighted.

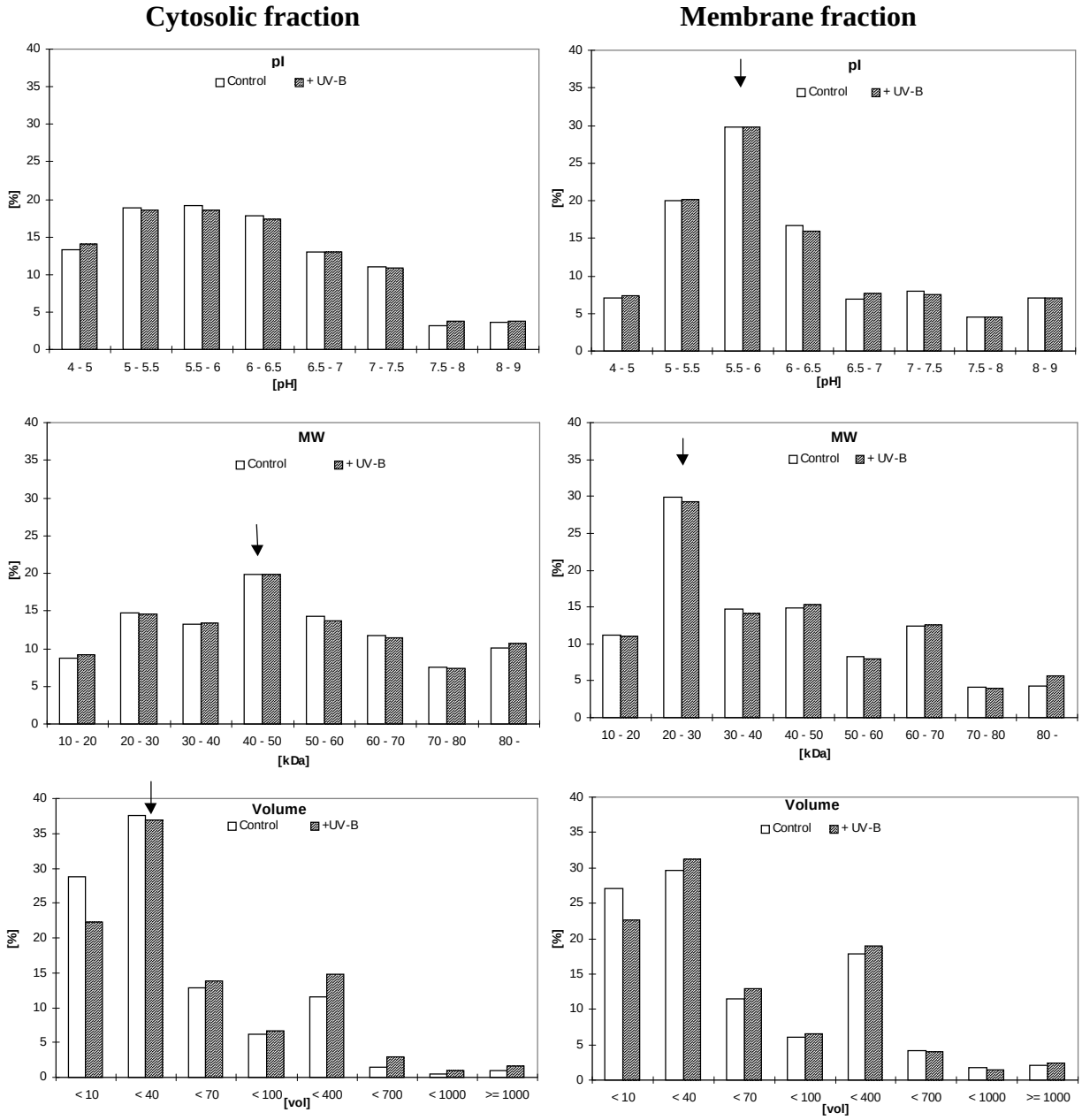

Fig. 19: Frequency distributions of all protein spots detected by 2D electrophoresis in dependence of isoelectric point (pI), molecular weight (MW) and volume (vol.).

3.2 Database application and statistical analysis.

The database application for Microsoft Access provided the possibility to analyze the two parameters, growth and stress, at the same time. A three dimensional virtual master gel allowed to monitor the UV-B influence over different growth stages of individual spots, as well as global changes in gel profiles. Principal component analysis (PCA) showed that the corresponding protein fractions from different experiments are strongly correlated, whereas UV treated gel profiles are clearly separated from their corresponding controls (Fig. 20).

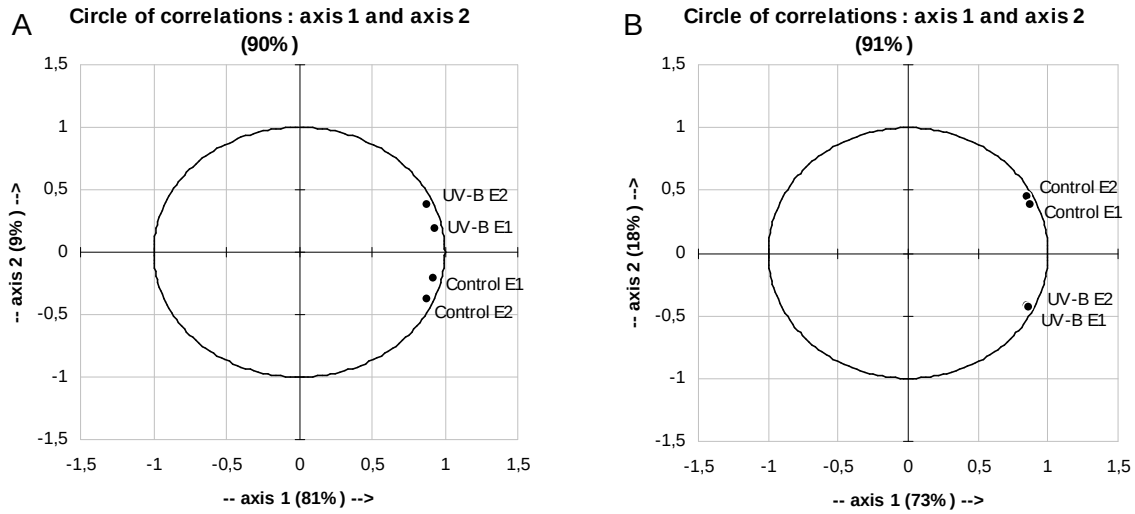

Fig. 20: Principal component analysis (PCA) of corresponding gel profiles from two independent experiments to test the reproducibility of the experimental procedure. Gel data are projected into a factorial subspace defined by the first two factors. The distance between gel profiles (indicated by points) within this space represents the degree of similarity between the gel profiles. The closer the gel profiles are located to the circle the more of the variability is explained. **A:** cytosolic fraction, **B:** membrane fraction. Abbreviations: UV-B: Gel profile of a culture treated with supplemented UV-B (1.0 Wm^{-2}); control: Gel profile of a corresponding control culture grown without UV-B. E1 refers to the first experiment, E2 refers to a second experiment.

To investigate the main sources of variation in the gel profiles, principal component analysis (PCA) was performed (Fig. 21). In the cytosolic fraction, the first two factors cumulated 86% of the variability and in the membrane fraction, the first two factors cumulated 88% of the variability (data not shown). In both fractions, the first factor is the same for all protein samples, whereas the second factor only affected UV-B treated samples. It was therefore concluded that, the growth dependent changes are reflected in the first factor and the UV-B dependent changes are reflected in the second factor.

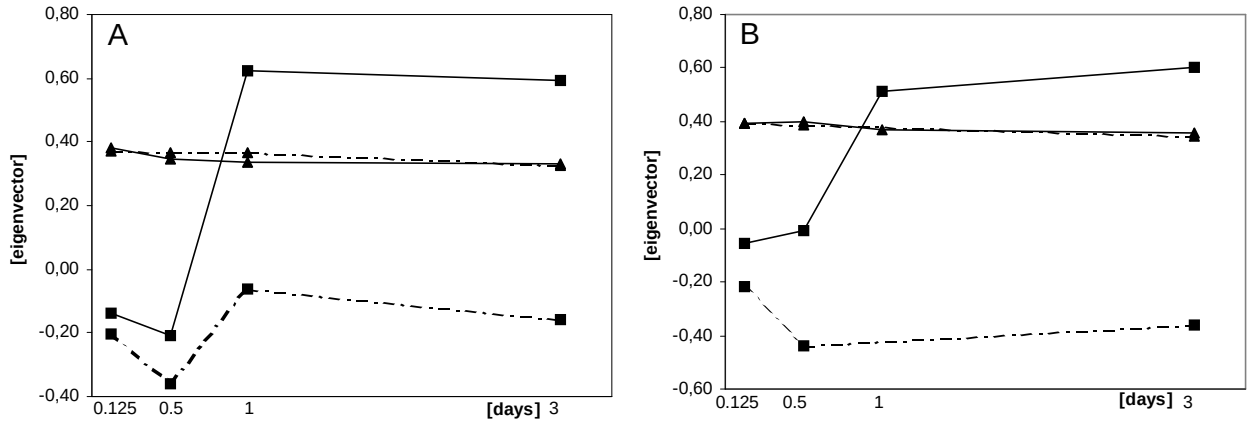

Fig. 21: Principal component analysis (PCA) was performed using gel profiles of cytosolic protein fractions **(A)** and of membrane protein fractions **(B)**. The first 2 factors cumulated 86 % of the variability in case of the cytosolic fractions (A) and cumulated 88 % of the variability in case of the membrane fractions (B). Triangles indicate the first factor of PCA and squares indicate the second factor of PCA. The solid lines indicate gel profiles of UV-B treated cultures and the dashed lines indicate gel profiles of control cultures grown without UV-B.

3.3 Growth state dependence of the proteome.

Long-time exposure experiments include growth of organism (Fig. 15). This resulted in significant changes of the proteome of the control cultures with time (Fig. 22). Changes in the protein profiles were monitored from the start of exponential growth to late exponential growth. A minimum of 214 protein spots in the cytosolic fraction and a minimum of 112 protein spots in the membrane fraction turned out to be affected by growth stage (Table 4). In both protein fractions spots were identified which were not influenced by growth state nor by UV-B or UV-A irradiation (Fig. 18). These proteins, 78 spots in the cytosolic fraction and 25 spots in the membrane fraction, which are most likely involved in the primary metabolic pathways and probably fulfill housekeeping functions, can be used as internal markers.

Table 4: Number of proteins from DRH1 influenced by growth phase or UV-B irradiation.

Growth

	Water-soluble cytosolic proteins [a]		**Membrane and membrane associated proteins** [a]	
	+ [b]	- [b]	+ [b]	- [b]
Start of exponential growth	39	24	25	3
Early exponential growth	29	21	20	7
Late exponential growth	60	30	48	9
Total	128	75	93	19

UV-B (1 Wm^{-2})

	Water-soluble cytosolic proteins [a]		**Membrane and membrane associated proteins** [a]	
	+ [b]	- [b]	+ [b]	- [b]
Early transient reaction	25	71	33	85
Early durable reaction	6	6	3	2
Late reaction	71	91	50	50
Total	102	168	86	137

[a] Proteins which are in the 68% interval of ln mean are omitted. Total number of proteins analyzed: cytosolic protein fraction about 750, membrane protein fraction about 600.

[b] +: new and increased proteins; -: absent and decreased proteins.

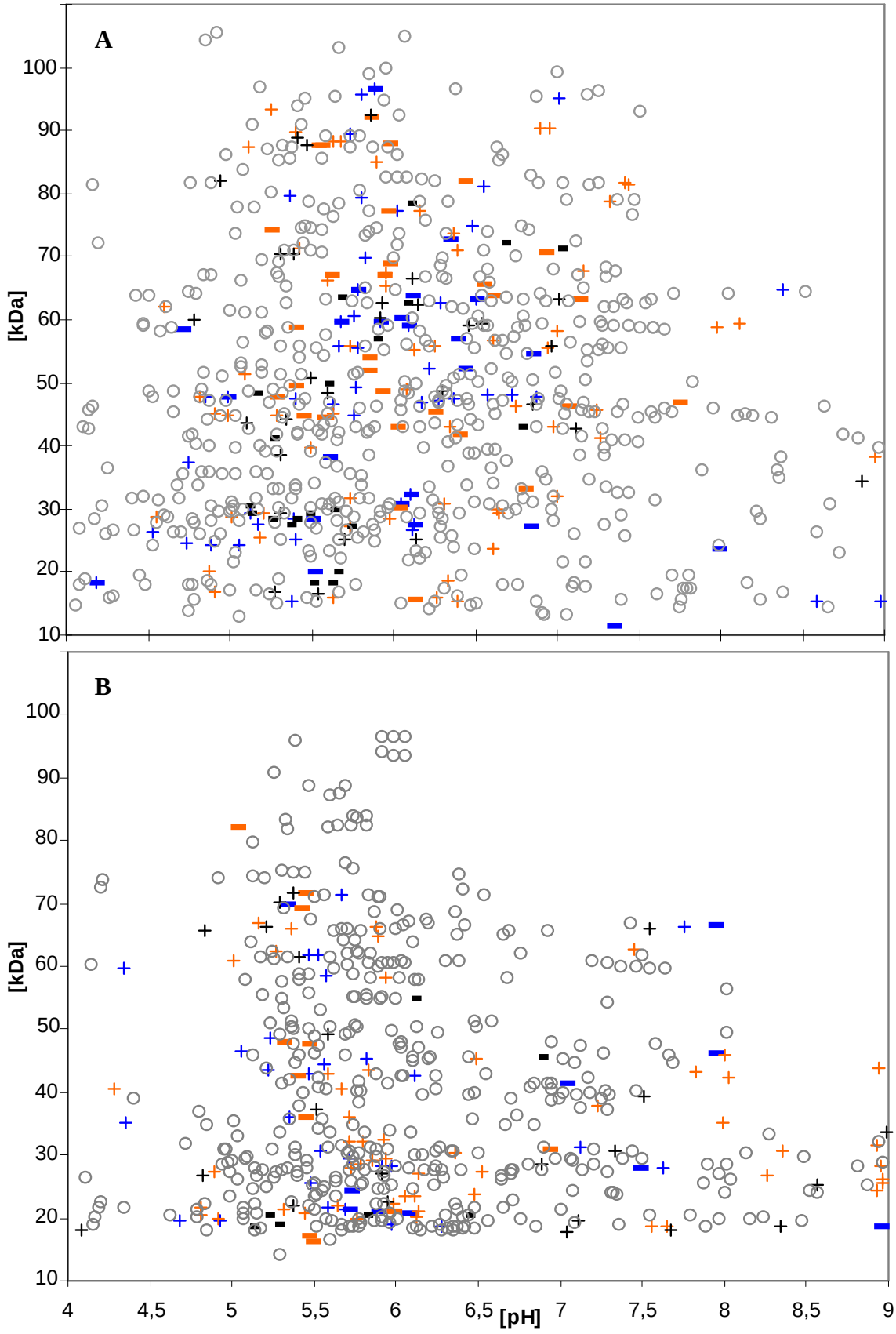

Fig. 22: Schematic 2D pattern of *N. commune* DRH1 presenting proteins which are growth cycle dependent. **A:** Cytosolic protein fraction. **B:** Membrane protein fraction. Blue: spots which are effected at the onset of exponential growth; black: spots which are effected during the early exponential growth phase; orange: spots which are effected during late exponential growth. Symbols: plus: spots which are positively effected (increased or induced); minus: spots which are negatively effected (decrease or absent); circles indicate all other spots detected.

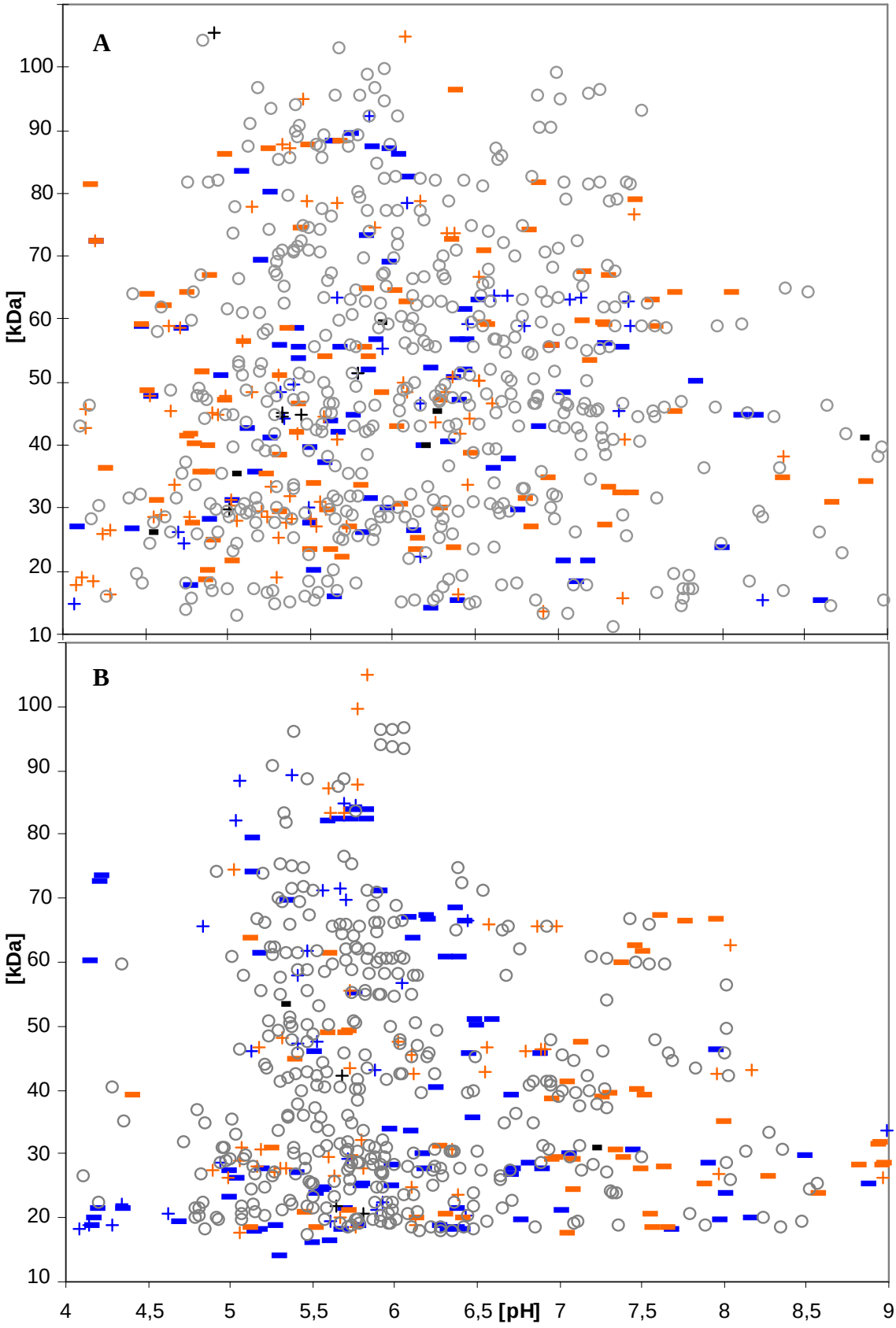

Fig. 23: Schematic 2D pattern of *N. commune* DRH1 presenting proteins which are influenced by UV-B. **A:** Cytosolic protein fraction. **B:** Membrane protein fraction. Blue: spots transiently influenced during early acclimation; black: spots durably influenced; orange: Spots influenced during long-time acclimation. Symbols as in Fig.8

3.4 UV-B stress influence on the proteome.

Around 40 % of all proteins detected by 2D-electrophoresis were significantly affected by UV-B (Fig. 23), whereas UV-A influenced less than 10 % of the proteins (data not shown). During the first 12 hours of UV-B treatment, 108 spots of the cytosolic protein fractions and 123 spots of the membrane fractions were influenced by UV-B. Most early reactions were transient, only 12 protein spots of the cytosolic fractions and 5 protein spots of the membrane fractions were permanently changed. After prolonged UV-B treatment for 3 days, 162 spots of the cytosolic fractions and 100 spots of the membrane fraction showed significant differences to the control factions (Table 4). The influence of UV-B on protein synthesis can be grouped in 4 categories: First, synthesis of novel proteins after short-term exposure, which diminished after prolonged exposure (Fig. 24, type 1). Second, transient increase or decrease of proteins which are present in the controls (Fig. 24, type 2). Third, synthesis of novel proteins after prolonged exposure (Fig. 24, type 3). Fourth, continuous increase or decrease of proteins which are present in the controls after prolonged exposure (Fig. 24, type 4). Cytosolic water-soluble proteins showed different kinetics in their response to UV-B irradiation compared to membrane-bound / -associated proteins. The protein response in the membrane fraction to UV-B was faster and

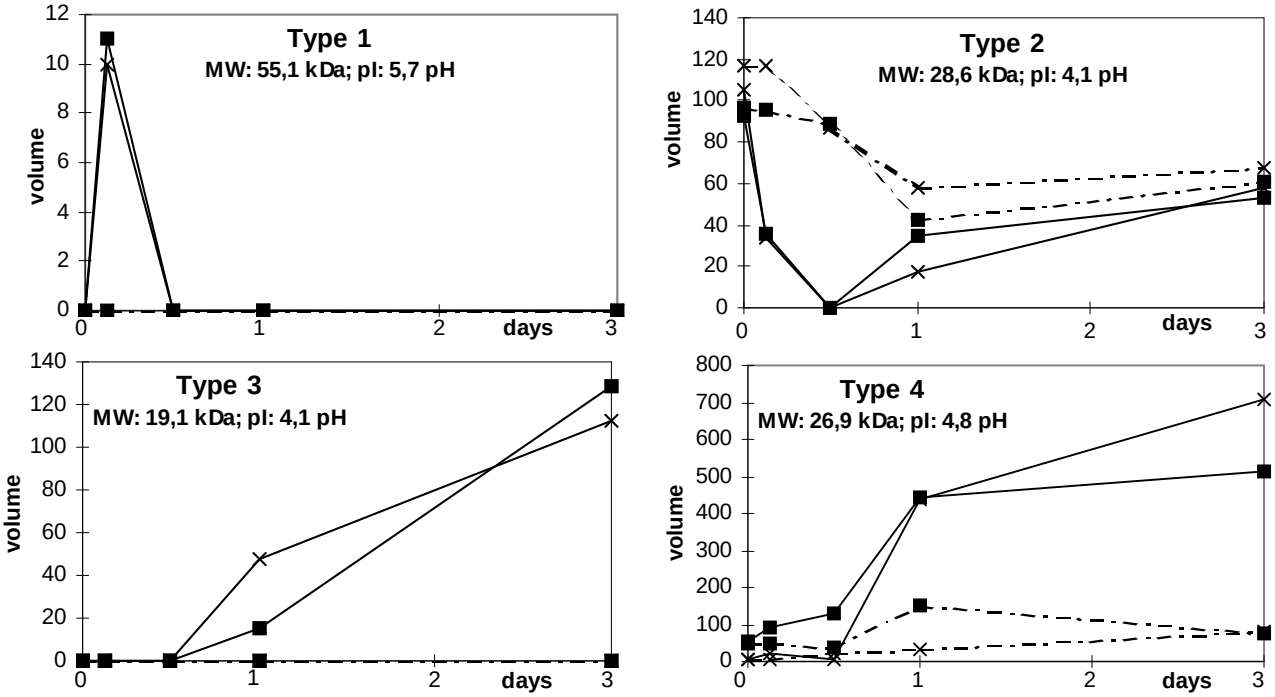

Fig. 24: Time course of protein changes in response to UV-B is shown for selected proteins of DRH1. Legend: solid lines: UV-B treated; dashed lines: control grown without UV-B; squares: first experiment; crosses: second experiment.

stronger during early acclimation than in the cytosolic fraction (Fig. 21, Fig. 23). During early acclimation 27% of the analyzed proteins in the membrane fraction changed significantly, while in the cytosolic fractions 18% of the analyzed spots significantly changed. Spots which contributed to the UV-B triggered changes where distributed over the entire proteome (Fig. 23). UV-B did not significantly change the frequency classes of pI or molecular weight distribution in the cytosolic or membrane protein profiles (Fig. 19). The majority of proteins (about 70%) influenced by UV-B were low abundant proteins , volumes <100, (data not shown).

4 Discussion

This proteomic approach showed the dynamic nature of the protein expression profile of *N. commune*. Programmed changes in the protein expression profile were observed during exponential growth of the organism. Which proteins were stimulated was strongly influenced by the specific growth stage. For each phase, onset of exponential growth, early exponential growth and late exponential growth a set of specific stimulated or specific expressed proteins was observed (Fig. 22). Growth cycle specific (expressed) proteins could be used as marker proteins to identify the growth stage of a culture. Proteome analysis of the microsymbiont *Sinorhizobium meliloti* revealed a minimum of 52 reproducible changes in expression profile of total cellular proteins when early exponential growing cells were compared to late exponential cells (Guerrero *et al.*, 1999). If proteins are fractionated before 2D electrophoresis a more detailed analysis is possible. Pre-fractionating of proteins provides information about the subcellular location of affected proteins and information concerning subcellular specific kinds of reaction (see Fig. 22), changes can be detected, which are not observed in whole cell extracts (Zischka *et al.*, 1999). Therefore whole cell extracts of *N. commune* were fractionated prior to analysis. In the membrane fraction, mostly positive stimulated proteins were detected, whereas nearly no negative stimulated proteins were found. This indicates that the membrane responses to exponential growth with the addition of novel metabolic features while keeping old ones intact. In the cytosol more proteins showed to be growth stage dependent than in membrane and more proteins were repressed (Table 4). Cytosol turned out to be more reactive to growth than the membrane.

In long-time stress experiments influence of growth on the protein expression profile has to be taken into account. The three dimensional virtual gel, generated with the database application, was used to investigate the UV-B triggered versus the growth stage dependent changes in the protein expression profile of *N. commune*. The UV-B response turned out to be

surprisingly complex. Semiquantitative analysis of about 1350 proteins revealed at least 493 proteins (37%) belonging to the UV-B stimulon. A minimum of 168 proteins were positive stimulated, whereas a minimum of 305 proteins were negative stimulated (Fig. 23). The relative decrease of proteins in stressed cells could be due to the repression of their synthesis, but could also be the result of their differential turnover. The term stimulon refers to a set of proteins whose amount or synthesis rate changes in respond to a single stimulus, a specific environmental condition (reviewed in VanBogelen *et al.*, 1999). The phosphate limitation (PL) stimulon in *Escherichia coli* included 413 proteins, half of this proteins were positive stimulated and half of them were negative stimulated (VanBogelen *et al.*, 1996). Another complex stimulon which has been described only recently is the H_2O_2 stimulon in *Sacharomyces cerevisiae*. The synthesis of 115 proteins was stimulated by H_2O_2, whereas 52 other proteins were repressed. Except for a few targets the H_2O_2 response was transient (Godon *et al.*, 1998). In *E. coli, Salmonella typhimurium* and *Bacillus subtilis* different types of stimulons have been investigated (Spector *et al.*, 1986; Antelmann *et al.*, 1997; VanBogelen *et al.*, 1997), while in cyanobacteria detailed global analysis of protein synthesis under specific environmental conditions to define stimulons are still missing.

The induction of UV-shock proteins has been reported in response to UV-C irradiation (265nm) and high intensities of “near” UV (295nm -390nm) (Nicholson *et al.*, 1991; Shibata *et al.* 1991) and an ATP dependent Clp protease (ClpP1) isolated from *Synechococcus* sp. PCC 7942 has been shown to be UV-B and cold inducible (Porankiewicz *et al.*, 1998). To our knowledge the influence of UV-B on total protein pattern of cyanobacteria had not previously been studied. The 37 % observed changes in the protein expression profile of *N. commune* reflect that the organism completely changed its physiology in response to UV-B. These changes are the result of true adaptation and not of damage, since no growth delay was observed in *N. commune* (Fig. 15). We showed that short-time (1 day) UV-B treatment had no effects on the growth whereas in long-time studies the cell number decreased at the (metabolic) cost of glycan and sunscreen production (Ehling-Schulz *et al.*, 1997). The cells adjusted their physiology to the new conditions. The regulation of gene expression underlying these adaptations is of considerable interest, since the response observed on the physiological level showed a stringent order of reactions. The control of these processes is likely to be complex. The sensing of UV-B signals by cyanobacteria is still cryptic. A UV-B photoreceptor has been proposed to regulate the oligosaccharide-mycosporine in *N. commune* and a UV-B special photoreceptor might be involved in the regulation of carotenoid synthesis (Ehling-Schulz *et al.*, 1997; Ehling-Schulz and Scherer, 1999). However, for identification and functional analysis of photoreceptors and signal

transduction pathways, the isolation of mutants will be necessary. Genes involved in signal transduction normally expressed in low copy number, which are unlikely to be identified from 2D electrophoresis because of the detection limit of 2D. Thus 2D differential display and molecular genetics have to complement each other to get a more holistic understanding of acclimation of cyanobacteria to UV-B.

70% of the stimulated proteins during UV-B acclimation were low abundant proteins, 'acclimation proteins', with volumes below 0.4% of total cellular proteins, whereas shock proteins are usually bulk proteins. E.g., the major cold shock protein CspA of *Escherichia coli* accounts for 13% of total cellular protein 1 hour after cold shock (Goldstein *et al.*, 1990). Our approach emphasized adaptations to stress rather than shock response, therefore silver staining was used for protein detection. In long-term studies the actual turnover of proteins, which can be measured by labeling experiments is not as interesting as the actual composition which is reflected in the amount of each protein presented in the cell. Pulse labeling provide an instantaneous image of the protein synthesis at the time point. However, in long-time experiments the amount of each protein available for metabolic, biochemical activity of the cells is more interesting than its actual synthesis. No differentiation was made between novel and increased proteins since proteins which where often stated to be novel may just be in the control protein patterns below the detection limit of the staining method used and not really novel. Recently, it has been stated that almost all proteins of a cell are expressed constitutionally in low copy number by replication induced protein synthesis (RIPS, for review see Humphery-Smith, 1999)

A programmed acclimation to the new growing conditions under UV-B was observed. The influence of UV-B on the proteome can be divided into early acclimation response, within the first 12 hours, and late acclimation response, which requires 1 up to 3 days. Most of the protein changes observed during early acclimation were transient (Table 4). This finding is consistent with the observations from physiological investigations. UV-B irradiation of *N. commune* led to a rapid, but transient increase of outer membrane bound carotenoids, and a slower, but constant production of extracellular UV-A/B absorbing mycosporines and extracellular glycan. It has been proposed that the outer membrane-bound carotenoids provide a fast, active response to counteract acute cell damage whereas the glycan with its UV absorbing compounds is a passive UV screen against long-time exposure (Ehling-Schulz *et al.*, 1997). The response to UV-B turned out to be subcellular specific. Influence of UV-B on the protein expression profile of the membrane was faster than on the cytosolic protein expression profile

(Fig. 21, factor 2). The protein composition of the membrane fraction was more strongly influenced during early acclimation, while the cytosolic fraction was more strongly influenced after long-time UV-B (Fig. 23). Membranes are known to be primary targets for UV-B induced damage by reactive oxygen intermediates and free radicals (Tuveson *et al.*, 1988; Hideeg and Vass, 1996). The transient stimulated proteins, especially in the membrane, may be important for early adaptation after UV-B shock, whereas the majority of the stimulated proteins are need for continuous growth under UV-B light.

Our results clearly showed the importance of long-time experiments, which can provide valuable information on the mechanisms and adaptation to UV radiation in cyanobacteria. The cellular adjustment to UV-B results in alternative metabolic fluxes. After prolonged UV-B irradiation, the cell number decrease due to a slower cell replication caused by the metabolic cost of MAA and glycan synthesis (Ehling-Schulz *et al.*, 1997). The induction of MAA synthesis is associated with the induction of a water stress protein (Wsp) (Fig. 17) and acidification of the glycan (Ehling-Schulz and Scherer, unpublished results). The induction of Wsp by UV-B support its role in the synthesis of the oligosaccharide MAA which has been proposed by Hill *et al.* 1994. However, further studies are necessary to unravel its specific role, if it is directly involved in the synthesis of oligosaccharide-MAA or via modification of the glycan to provide a matrix for oligosaccharide-MAA. UV-B is switching the cellular activity from biosynthetic towards protective functions.

UV-A had only little influence on the protein pattern of *N. commune*, nevertheless continuous growth under supplemented UV-A had remarkable effects on its pigment composition (Ehling-Schulz *et al.*, 1997). This confirms that UV-B response is highly complex and underlines the high biological effectiveness of short wavelength UV.

The number of proteins directly contributing to UV-B tolerance is currently unknown since an alteration in the level of expression in response to UV-B treatment does not necessarily imply a role in UV-B tolerance. Some of the proteins induced by UV-B are also salt inducible, but the response to salt stress is not as complex as the response to UV-B (Ehling-Schulz and Scherer, unpublished). To get a holistic understanding of cell response further studies at the biochemical level, especially combination of 2D with high throughput amino acid analysis, peptidemass fingerprinting and associated sequence tags in combination with nanoelectrospray tandem mass spectrometry will be necessary to unravel the global cell response.

5 Conclusion

Overall, these results clearly showed that the response to UV-B irradiation involves complex changes in gene expression. The complex proteome response observed during the adaptation period can not be explained by an adjustment of only a few cellular components. Therefore, it might not be sufficient to study only individual genes. 2D electrophoresis leads into a promising land of novel discoveries in cyanobacterial stress physiology. We monitored the UV induced changes in the proteome of *N. commune* as a first step toward the understanding of the molecular basis of the high UV tolerance of *N. commune*. To get more specific information on the proteins affected by UV-B protein biochemical techniques have to be combined with molecular techniques. Global analysis on change in protein expression profiles can yield important general information about the response of cellular systems to stress. However, the more specific information will be obtained by genetic analysis. It is suggested that the true level of complexity of the regulation of the UV response can only be addressed by the combination of mutants, signal transduction analysis and the complete analysis of the cellular response offered by high resolution 2D electrophoresis.

Chapter 4

Annual timecourse of the contents of carotenoids and UV-protective pigments in the cyanobacterium *Nostoc commune*[*]

1 Introduction

High irradiation potentially threatens all terrestrial plants. Solar radiation in the waveband between 400 and 700 nm (Photosynthetically Active Radiation, PAR) is necessary for photosynthetic carbon fixation. However, whenever the light absorbed by the photosynthetic apparatus exceeds the amount necessary to saturate light utilization by photosynthesis, damage to photosystem II (PS II) will result (Aro *et al.,* 1994). Although being only a minor portion of the total solar spectrum, ultraviolet radiation at wavelengths between 280 and 320 nm (UV-B) is highly damaging to living organisms. In contrast to long wavelength ultraviolet radiation (UV-A, 320-400 nm), UV-B radiation is absorbed by DNA and proteins and causes damage to these molecules due to its high energy (Strid *et al.,* 1994).

To protect themselves against the damaging effects of exessive radiation, plants have developed a variety of strategies. Higher plants can effectively dissipate excess absorbed PAR as heat (Demmig-Adams and Adams, 1996). There is much evidence that in this process the carotenoid zeaxanthin ((3R,3'R)-β,β-carotene-3,3'diol) is involved, which is formed in the so called xanthophyll cycle (Pfündel and Bilger, 1994; Demmig-Adams and Adams, 1996). However, cyanobacteria lack the xanthophyll cycle. This has been interpreted as one of the potential causes for their comparatively high sensitivity to high irradiation (Demmig-Adams *et al.,* 1990). Although cyanobacteria generally contain zeaxanthin, it apparently does not contribute to photoprotection as its contents did not correlate with light exposure of a variety of cyanobacterial lichens (Leisner *et al.,* 1994). In contrast, Leisner and coworkers found that another carotenoid, canthaxanthin (β,β-carotene-4,4'-dione), increased with increasing exposure in the cyanobacterial lichen *Peltigera rufescens* under natural conditions (Leisner *et al.,* 1994; Leisner, 1995). Antiparallel to the rise of canthaxanthin β-carotene contents declined. In terrestrial cyanobacteria and in all cyanobacterial lichens investigated so far high

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canthaxanthin/β-carotene ratios were consistently found under high irradiance (Leisner *et al.,* 1994; Vincent *et al.,* 1994). However, a photoprotective function of canthaxanthin has not yet been proven.

Cyanobacteria apparently protect themselves by synthesis of UV screening pigments against harmful effects of ultraviolet radiation. In the glycan sheath of terrestrial cyanobacteria the UV-A absorbing pigment scytonemin is located (Garcia-Pichel and Castenholz, 1991, 1993). Cyanobacteria also contain mycosporine like amino acid (MAA) pigments which have absorbance maxima in the UV-B and far UV-A waveband (Scherer *et al.,* 1988; Garcia-Pichel and Castenholz, 1993; Böhm *et al.,* 1995). The MAA found in *Nostoc commune* has been shown to be covalently linked to oligosaccharides and to be located in the glycan sheath (*Böhm et al*., 1995). Evidence has been presented that both MAA and scytonemin can screen the cell against ultraviolet radiation (Garcia-Pichel *et al.,* 1992, 1993).

In *N. commune* the synthesis of MAA is induced by UV-B radiation, while UV-A causes scytonemin formation (Scherer *et al.,* 1988; Ehling-Schulz *et al.,* 1997). Under natural conditions the UV radiation climate may vary largely. During the course of a year UV radiation is low during winter and high during summer (Dehne, 1989, Blumthaler *et al.,* 1985). Therefore, one would expect that synthesis of UV protective pigments is preferentially induced in the summer. In this study we explore if the natural variation of isolations of *N. commune* during the course of a year will result in parallel changes in the UV-protective pigment contents along with changes in the carotenoid composition. Since data on the variability of the contents of the hydrophilous carotenoid myxoxanthophyll under natural conditions were missing so far we determined the contents of this pigment as well.

2 Materials and Methods

2.1 Organism and collection sides

Specimens of *Nostoc commune* Vaucher were collected from August 1995 until November 1996 in monthly to three-monthly intervals at two different places, Reichenberg and Lindflur, 8 and 11 km, respectively, south of Würzburg, Germany. In most cases, samples were collected during humid weather since they could then be better recognized. At Reichenberg, the cyanobacteria were growing on a NNE-SSW directed field-path on a south facing slope. The inclination of the slope was between 7 and 11%. 6 sampling sites were distributed along the path

over a distance of about 130 m. At each site 2 specimens of *N. commune* were collected every time. While the path was open to the west, at its eastern side several buildings were located which shaded the path until between 9:00 AM to 12:00 AM. The sampling site at Lindflur was a horizontal area of about 10 m² on a field-path covered with limestone gravel. Here, irradiation of the site was not hindered throughout the day. At Lindflur each time 5 samples were collected from December 1995 until November 1996. After collection the samples were wetted and cleaned before they were frozen, freeze-dried and stored at -20°C until extraction.

2.2 Extraction and determination of pigments

Before extraction each sample was gently broken into small pieces. An aliquot with a mass of about 4 mg was extracted with aqueous methanol (30%, v/v) at 50°C for 30 min. From this extract MAA contents were determined spectrophotometrically (UVIKON 930, Kontron, Eching, Germany) in the supernatant after centrifugation. An extinction coefficient of 17 L g^{-1} cm^{-1} at 312 nm was used (Böhm *et al.*, 1995).

About 12 mg of the remaining sample were rewetted with 120 µl distilled water. After 15 min the sample was ground at 77K in a mortar and 1mL 100% acetone added together with about 0.5 µmol chlorophyll (chl) *b* as internal standard. After decanting and rinsing the mortar with 0.2 mL 100% acetone the mixture was gently sonicated for 5 min (Sonic Power Model S 125, Branson, Danbury, CT, USA). Subsequently, it was stored for 2h at 4°C in darkness. After centrifugation the pellet was extracted with 200 µL acetone for another 30 min. This extract was adjusted to 80% acetone by adding distilled water and was used for determination of carotenoids, chl *a* and scytonemin using HPLC (Ehling-Schulz *et al.*, 1997). Pigments were identified according to their absorbance spectra, retention times and by comparison with pigment standards. These were either purchased (β-carotene, chl *a*, chl *b*, Sigma, Deisenhofen, Germany; canthaxanthin, zeaxanthin, Roth, Karlsruhe) or prepared by TLC (myxoxanthophyll, scytonemin) according to the method of Garcia-Pichel and Castenholz (1991). Echinenone was a gift of F.-C. Czygan, Würzburg. These standards were used also for calibration using extinction coefficients from Davies (1976). Pigment contents were calculated after correction for the recovery of the internal standard.

Daily sums of global radiation measured at Würzburg were kindly provided by the Deutsche Wetterdienst, Potsdam.

3 Results

For the comparison of pigment contents with radiation climate the period before sampling is of interest. Therefore, daily sums of global radiation were averaged over the preceding 5 days for each day during the annual time course (Fig. 25). The data are corrected for the varying day length over the year and represent hourly averages. The irradiance at the sampling dates covered a range between 20 and 170 J m^{-2} h^{-1}. As expected, large fluctuations occurred during summer. For example, during the end of April there was a period of bright weather, whereas in the begin of May, rainy weather predominated. After both periods samples were collected.

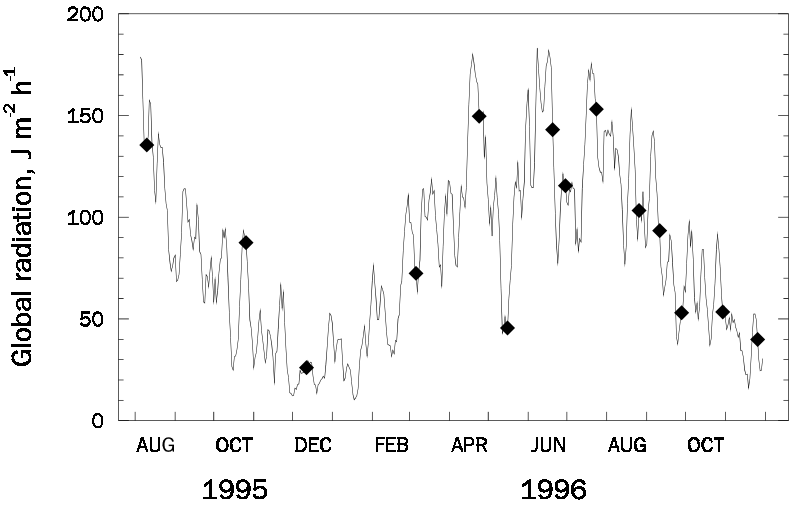

Fig. 25: Time course of global radiation from August 1995 until November 1996 measured at Würzburg, Germany. Daily sums of global radiation were averaged over 5 days preceding to the actual date and divided by day length to correct for seasonal variation in day length. Diamonds represent global radiation at the dates of sample collection.

At Reichenberg, no significant differences in pigment content were found between the 6 different sampling sites. The site at Lindflur was considerably more open than the site at Reichenberg where the samples partially grew between grass tufts or were shaded by adjacent houses for several hours in the morning. Nevertheless, no obvious differences between both places were found for the contents of all pigments with two exceptions, scytonemin and the ratio between canthaxanthin and β-carotene (see below).

N. commune collected during winter time was looking rather yellowish, whereas it had dark-green color during summer. These changes are also apparent in the chl *a* contents (Fig. 26). They showed a continuous decline during winter reaching minimal values in April. In May chl *a*

contents increased again, most probably because of humid weather and warmer temperatures. At that time also a resumption of growth was apparent.

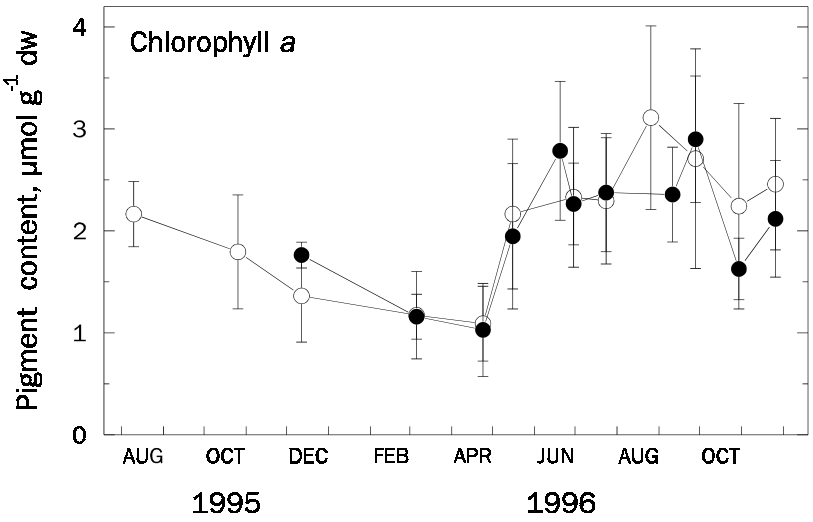

Fig. 26: Time course of chl a contents of N. commune from August 1995 until November 1996. Open circles denote samples collected at Reichenberg (n = 10 to 12), closed circles denote samples collected at Lindflur (n = 4 to 5). Error bars indicate standard deviation.

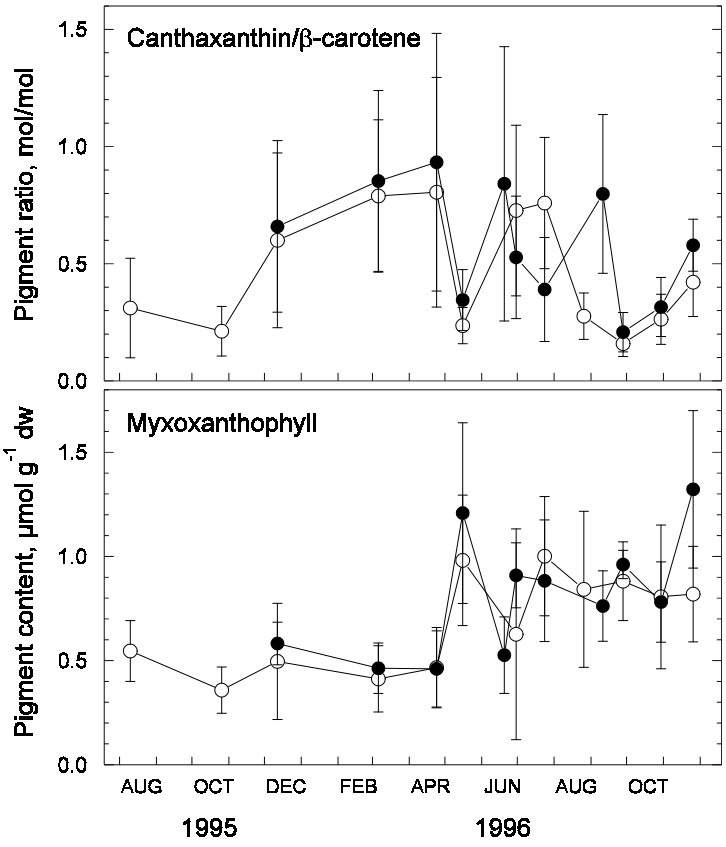

Fig. 27: Time course of the ratio between the carotenoids canthaxanthin and β-carotene (upper panel) and myxoxanthophyll contents (lower panel) in *N. commune* from August 1995 until November 1996. Symbols as in Fig. 26.

Total carotenoid content followed that of chl *a*, leading to an almost constant ratio between both parameters (data not shown). Similar results were obtained for myxoxanthophyll (Fig. 27, lower panel). About twice as much myxoxanthophyll was found in summer as compared to the winter. Myxoxanthophyll was tentatively identified as myxol-2'-rhamnoside from online absorbance spectra. In the cyanobionts from the lichen *P. rufescens* we had detected also myxol 2'-O-methyl-methylpentoside (M. Woitke and W. Bilger, unpublished). During summer an additional pigment appeared which was tentatively identified as 2'-O-Rhamnosyl-4-ketomyxol. However, its contents were too low for reproducible quantification.

β-Carotene contents were low during winter and did not increase significantly during summer, while canthaxanthin contents stayed roughly constant during the whole time (data not shown). In the Reichenberg material, the ratio between both pigments followed roughly irradiation (Fig. 27, upper panel). During the period of rainy weather in early May (compare Fig. 25) the ratio declined considerably. The same occurred in fall 1995 and 1996. However, in November '96 and December '95 the ratio increased again although radiation was low. This lack of correlation with PAR might have been caused by an interference with temperature. Also Leisner (1995) had found two different correlations between the canthaxanthin/β-carotene ratio for the cold and the warm season, with a tendency to higher values of the ratio during winter. While during the summer months canthaxanthin/β-carotene was high in the Reichenberg material, it was rather low in the samples from Lindflur. The low values of the latter might be caused by extended periods of drought during which *N. commune* was metabolically inactive. The slightly more favorable microclimatic conditions in Reichenberg may have allowed limited activity.

The contents of the UV-B protective pigment, MAA, represented a considerable fraction of dry weight and were highest during the winter time (Fig. 28, upper panel). Although there was variability within the different samples on a given date, it could be shown that the MAA contents in December 1995 and November 1996 were significantly higher than the summer values (Mann and Whitney's U-test, $p \leq 0.05$). For each sampling date complete UV-absorption spectra of the extract were recorded from 2 to 5 samples. Besides the main absorption band at 312 nm a shoulder around 335 nm was present. From the shape of the spectra no change in the composition of MAA during the observation period was obvious. Also other extracted components which have a relative absorbance maximum at 260 nm contributed equally over the year by 6.5% (stdev. 1.8%) to the absorbance at 312 nm when calculated according to the formula given by Garcia-Pichel *et al.* (1993). Therefore, the observed increase in MAA contents

during winter was not due to unspecific absorption changes at 312 nm or due to changes in the composition of MAA.

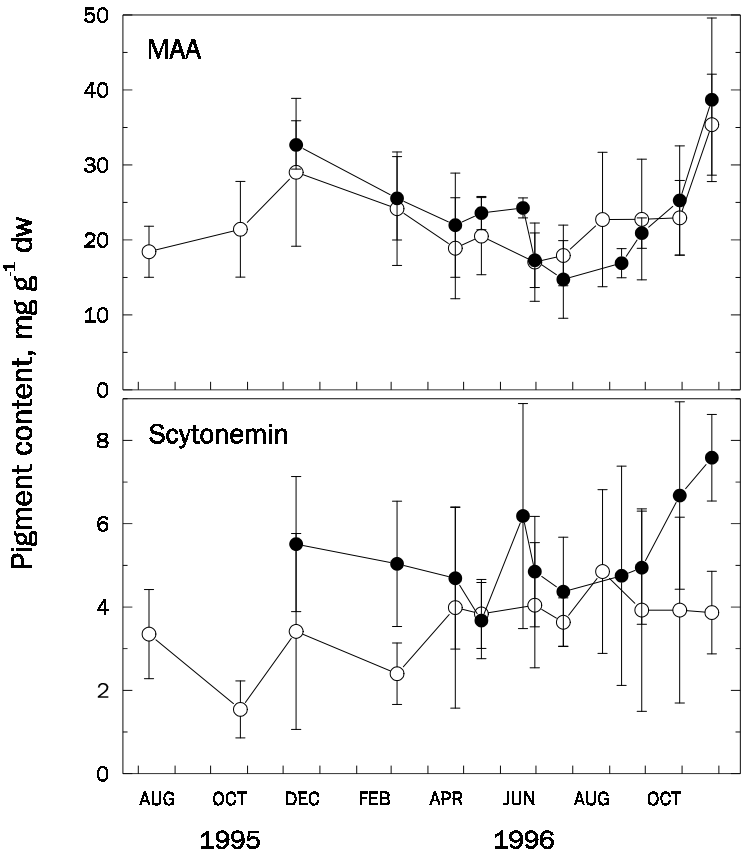

Fig. 28: Time course of the contents of MAA (upper panel) and of scytonemin (lower panel) in *N. commune* from August 1995 until November 1996. Symbols as in Fig. 26.

Scytonemin is a pigment which protects cyanobacteria against UV-A radiation. The annual time course of its contents is shown in Fig. 28, lower panel. Only statistically insignificant trends over the year are apparent. Furthermore, while scytonemin contents seem to rise slightly in the samples from Reichenberg during summer, it reached minimal values at the same time in the Lindflur material. Scytonemin often displayed the highest variability of all pigments within a given set of samples.

4 Discussion

The contents of most pigments of *N. commune* showed pronounced changes over the course of the year. Photosynthetic pigments and other carotenoids were low during winter and rose strongly in early May (Figs. 26 and 27). Opposite changes were found for MAA while

scytonemin was the only pigment for which no obvious trends were detected. A more detailed study of the climate related variation of pigment contents was not possible due to a large variability within each sample. *N. commune* thalli are sheath like, but often curled or folded into several layers. This causes a high degree of self-shading and concomitant variability of pigment contents which is sometimes visible to the bare eye when upper layers of a thallus piece are looking more bleached than the lower ones. The heterogeneity within a single thallus may not be the only reason for variability since thalli from Lindflur, which were considerably flatter than those from Reichenberg, displayed a similar degree of pigment variability.

Since it was not possible to relate pigment contents to cell number which would have been desirable, it was necessary to use dry weight as a basis. Therefore, changes in dry weight could cause apparent changes in pigment contents. The glycan sheath represents a considerable fraction of the dry weight of *N. commune*. An increased exopolysaccharide production in response to desiccation was reported from cyanobacteria (Grilli Caiola *et al.*, 1996) and other terrestrial bacteria (Roberson and Firestone, 1992). UV-B-induced exopolysaccharide production resulting in a two- to threefold increased dry weight per cell was observed in *N. commune* DRH1 (Ehling-Schulz *et al.*, 1997). While drought is a stress which is present throughout the year for a poikilohydrous organism, UV-B radiation is enhanced during summer. Increases in cell related dry weight during summer could have concealed increases in MAA or scytonemin at that time. On the other hand, the minimum of chl *a* content in winter would then be even more pronounced.

Our data suggest that winter may be considered as the most stressful time of the year for *N. commune*. Low chl *a* contents in late winter might have resulted from the concomitant exposure to increasing radiation at low temperatures. The latter may largely reduce metabolic reactions including photosynthetic CO_2 fixation which would cause increased photoinhibition with simultaneous inhibition of repair processes resulting in photobleaching. For the lichens *P. rufescens* and *P. praetextata* from different habitats minimal chl contents were observed for the months March and April (Leisner, 1995). March was also the time when PS II quantum yield of *P. rufescens* was severely reduced, indicating photoinhibition (Leisner *et al.*, 1996). In a study on *Aphanocapsa* spec. grown in liquid culture, Nonnengiesser *et al.* (1996) observed a strong reduction of chl *a* contents with increasing PAR. In contrast, under field conditions irradiance is not the only factor influencing chl content.

Also with regard to the contents of the UV-protective pigments scytonemin and MAA, irradiance seemed to have little influence. These pigments showed either no changes or varied

antiparallel to the radiation climate over the course of a year. This seems to be even more surprising, as both pigments are strongly induced by UV-A or UV-B radiation, respectively (Scherer *et al.,* 1988; Garcia-Pichel and Castenholz, 1991, 1993; Ehling-Schulz *et al.,* 1997).

Radiation in the UV-A waveband is a relatively constant fraction of total global radiation. Its proportion does not vary much between the different seasons of the year. For equal aerosol and water contents of the atmosphere and equal ozone column, the part of solar radiation at wavelengths below 400 nm, which is almost completely dominated by UV-A, varies from 5.0% during January to 5.6% during July at a latitude of 48°N (Dehne, 1989). At shorter wavelengths, especially in the UV-B waveband, the proportional decline of the UV-fraction at low solar zenith angle is even more pronounced, since then the radiation has to pass a larger ozone column which selectively reduces UV-B (Dehne, 1989). Measurements of Blumthaler *et al.* (1985) at the Jungfraujoch (3576 m) in the Alps are consistent with this notion. However, the relationship between UV-B and global radiation shows more scatter since it is strongly influenced by atmospheric factors such as clouds. Nevertheless, it is a safe assumption that during winter time the UV-B radiation reaching the earth is greatly reduced. Hence, the relatively high contents of UV-absorbing pigments in *N. commune* during the winter must be caused by other factors than exposure to high UV radiation.

Scytonemin and MAA are located in the glycan sheath of *N. commune* and, hence, are largely inaccessible for metabolism. Scytonemin is extremely stable and should not be degraded by physical factors (Garcia-Pichel and Castenholz, 1991). One could envisage that scytonemin synthesized in summer in response to high UV exposure would not disappear when the exposure is decreasing which would explain the relative constancy of its contents. However, MAA may be less stable *in vivo*. In extracts (30% methanol) this pigment had a half life time of about 3 months at 4°C in darkness (M. Ehling-Schulz, unpublished). After induction of MAA synthesis in liquid cultures of *N. commune* DRH1, MAA synthesis continued even in the absence of UV-B radiation when the cultures were in the exponential growth phase. However, in late stationary phase, MAA contents decreased under the same conditions (M. Ehling-Schulz, unpublished). This observation could indicate either spontaneous disintegration or bacterial metabolization of MAA. An involvement of bacteria in MAA decomposition has been described for the marine alga *Phaeocystis* (Marchant *et al.*, 1991). *N. commune* under natural conditions may well be comparable to a culture in the stationary phase and bacteria were probably also present under field conditions. Therefore, a decay of MAA is not improbable and high concentrations of MAA may indicate its continued synthesis.

N. commune is a poikilohydric organism and is frequently desiccated under natural conditions. This is occurring especially under high irradiation. For the lichen *P. rufescens* it was shown that on about 90% of the occasions when the lichen was exposed to a PAR above 1000 µmol m^{-2} s^{-1} it was in the desiccated state (Leisner, 1995). In this condition metabolism is arrested and exposure to high irradiation cannot have any influence on pigment synthesis. It is obvious that desiccation at high irradiances would level out seasonal differences in irradiance. The ratio of the carotenoids canthaxanthin/β-carotene has been suggested as an indicator for irradiance experienced in the metabolically active state (Leisner *et al.,* 1994). Our data show for the place Lindflur that indeed there was no large variation, whereas in Reichenberg, the ratio was higher in the summer and a response to the pronounced weather change in April/May 1996 was also obvious. In addition, we found wet thalli during summer around noontime which received much higher irradiation at this time as compared to the winter time. Even if cyanobacterial activity would have been restricted to days with humid weather conditions and complete cloud cover, there would have been substantial differences in UV-B exposure between the seasons (Blumthaler *et al.,* 1985). Therefore, although desiccation certainly may be a means for the cyanobacteria to avoid radiation stress, we consider this mechanism not sufficient to eliminate the large differences in UV radiation between summer and winter. Since UV-B radiation is probably detrimental also to dry cyanobacteria, they should be able to adapt the protective pigments even under weather conditions when desiccation occurs frequently.

If dry weight of the thalli did not vary to a considerable extent, our data would indicate that MAA synthesis was induced in late fall in spite of decreasing UV-B exposure. In view of this conclusion additional factors influencing MAA synthesis should be considered. An obvious factor could be low temperature. Short term treatment of liquid cultures of *N. commune* DRH1 at 7°C did not induce MAA synthesis in the absence of UV-B irradiation (M. Ehling-Schulz, unpublished). However, such results do not exclude a possible influence of long-term growth at low or freezing temperatures or the occurrence of synergistic effects between temperature and UV-B irradiance under natural conditions. This should be tested in future experiments.

At low temperatures enzymatic repair of UV-B-induced lesions is partially inhibited. This means that under such conditions avoidance of UV-B exposure by screening pigments is even more important than at elevated temperatures. Also in higher plants increased synthesis of photoprotective pigments at low temperatures was demonstrated (Christie *et al.,* 1994).

General Discussion

UV-protection mechanisms

Growing laboratory cultures of the highly UV tolerant terrestrial cyanobacterium *Nostoc commune* were treated with artificial UV-B and UV-A irradiation. Photon fluence rates of UV-B which are comparable to solar fluence rates induced a cascade of physiological reactions.

First, synthesis of carotenoids was rapidly induced by UV-B irradiation and changes in the carotenoid pattern of *N. commune* were observed (Fig. 12). Carotenoids are well known for their antioxidant activity. Their photoprotective role against high intensity visible light is evident (for review see Demming-Adams and Adams, 1992) and a protective role of carotenoids in cyanobacteria against UV-A radiation was reported (Buckley and Houghton,1976; Paerl, 1984). Cyanobacteria produce some unique types of xanthophylls, such as ketocarotenoids and glycosides (Hirschberg and Chamovitz, 1994). Interestingly, in *N. commune* these unique types were induced by UV-B whereas ß-carotene and zeaxanthin showed no response. This is in contrast to results observed in response to UV-A or high visible light. UV-A exposure of different cyanobacteria led to an increase of all carotenoids (Paerl, 1984) while high visible light exposure of *Nostoc* Bu 94.1 led to a sharp decline of ß-carotene and a 5 to 6 fold increase of canthaxanthin (Bilger, unpublished data). Xanthophylls are the predominant carotenoids in cyanobacterial envelope membranes, whereas ß-carotene was found almost exclusively in the thylakoids (Jürgens and Weckesser, 1985; Omata and Murata, 1984). Myxoxanthophyll and echinenone, pigments strongly induced by UV-B in *N. commune*, have been shown to be located in the outer membrane of *Synechocystis* sp PCC 6714 (Jürgens and Mäntele, 1991). The function of carotenoids in the outer membrane of cyanobacteria is still not clear. Since it has been shown that heterologous expression of carotenoid genes in *Escherichia coli* led to an increased resistance to UV radiation (Tuveson and Sandmann, 1993; Tuveson et al., 1988) it is suggested that myxoxanthophyll and echinenone act as outer membrane-bound UV-B photoprotectors of *N. commune*.

Second, UV-B induced the synthesis of two extracellular sunscreen pigments. UV-B irradiation of *N. commune* led to the production of the water-soluble UV-A/B absorbing pigment, which belongs to the group of mycosporine like amino acids (MAAs), and the production of the lipid-soluble scytonemin (Fig. 14). However, the scytonemin content of UV-B treated cultures was one order of magnitude less than MAA content. In *N. commune*, MAAs are thought to play an important role in photoprotection because the MAAs are located in the extracellular glycan.

Two out of three photons are absorbed by the pigment before cell membranes or targets within the cell are reached (Böhm *et al.*, 1995). The UV-A/B-absorbing pigment of *N. commune* with absorption maxima at 312 nm and 335 nm was the first mycosporine reported to be covalently linked to oligosaccharides (OS-MAA) and shown to be located in the extracellular glycan (Hill *et al.*, 1994a; Böhm *et al.*, 1995). The pigment provides protection, mainly by absorbing the harmful radiation, but the 312 chromophore of the pigment, which is thought to be a MAA-Gly, may provide additional protection by radical quenching (Dunlap and Yamomoto, 1995). No photobleaching of chlorophyll *a* was observed in MAA producing *N. commune* upon UV-B irradiation (Table 2), whereas *Nostoc* Bu 94.1, which produces scytonemin but no MAAs, completely bleached when it was treated with UV-B. Because *N. commune* is subject to regular cycles of desiccation and rewetting and has often to survive long times in quiescence during which repair mechanisms are ineffective, UV-absorbing compounds may play a key role in UV photoprotection of *N. commune*. UV-A and UV-B induced synthesis of scytonemin in *N. commune* followed similar kinetics, but pigment concentrations in UV-B treated cultures were only about 30 % of those of UV-A treated cultures. The content of about 12 to 16 µg scytonemin per mg dry weight observed in UV-A treated cultures is in good agreement with concentrations found in UV-A treated cultures of *Chlorogloeopsis* (Garcia-Pichel et al., 1992).

The OS-MAA is proposed to be the key pigment in UV-B protection while scytonemin is most effective in UV-A protection. However, the latter may have some special role as UV-B protectant immediately after rewetting of desiccated colonies. In contrast to OS-MAA, scytonemin is not lost upon rewetting.

Third, UV-B irradiation stimulated the extracellular glycan production of N. commune. Synthesis of extracellular polysaccharides may also help to limit UV-damage. Bacterial extracellular polysaccharides (EPS) have been reported to provide protection against desiccation, phagocytosis, antibody recognition and lysis by viruses (Dudman, 1977; Tease and Walker, 1987; Hill *et al.*, 1994b). The EPS - containing sheath of cyanobacteria forms a buffer zone between the environment and the cell. The yield of EPS isolated from UV-B irradiated cultures was three times higher than that from control cultures. Leaf thickening in higher plants has been reported as a response to UV-B (Balakumar *et al.*, 1993). With a thicker sheath, effective path lengths for the absorption of radiation are much longer. As both OS-MAA and glycan synthesis increased due to UV-B, but not in response to UV-A, some correlation of EPS and OS-MAA induction may exist. It is suggested that EPS synthesis is stimulated to provide a matrix for the OS-MAAs which are located in the sheath of *N. commune*.

Potential UV photoreceptors. The regulation of gene expression underlying these adaptations is of considerable interest, since the response observed on the physiological level showed a stringent order of reactions. The control of these processes is likely to be complex. It is evident that several different photoreceptors mediate the response to UV and blue light in higher plants (Jenkins *et al.,* 1995). Phytochrome, a blue light photoreceptor and a UV-B photoreceptor have been shown to be involved in the induction of UV-absorbing flavonoids (Bruns *et al.*, 1986) but information about photoreceptors in cyanobacteria responsible for photoresponses to UV irradiance is still lacking. A UV-B photoreceptor is proposed to regulate the oligosaccharide-mycosporine in *N. commune* since its synthesis is only inducible by wavelengths below 315 nm and a separate UV-A photoreceptor probably regulates scytonemin because its synthesis is most pronounced at near UV-A (350 - 400 nm) irradiation, whereas far UV-A (320 - 350 nm) had only little effect (Table 3). A UV- A photoreceptor with a maximum at 370 nm, but no blue light stimulation, has been reported to be involved in the carotenoid synthesis of *Verticillium agaricinum* (Osman and Valadon, 1977). Campos *et al.* (1991) reported that UV-B and UV-C irradiation increased levels of 3-hydroxy-3-methylglutaryl CoA reductase RNA, which may induce carotenoid synthesis. Since carotenoids in cyanobacteria showed a different response to high intensity visible light (Leisner *et al.*, 1994), UV-A and UV-B (Fig.6) it is possible that special UV-B photoreceptors are involved

In its natural habitat, *Nostoc* has to cope with high solar radiation in its dry state, in which photodamage cannot be efficiently repaired. Therefore, passive photoprotective mechanisms are needed. The water-soluble MAA provides passive protection against UV-B and far UV-A irradiation (Böhm *et al.*, 1995) whereas the lipid-soluble scytonemin, beside some absorption in the UV-B, absorbs mainly UV-A (Garcia-Pichel and Castenholz, 1991). Since carotenoid synthesis is induced very fast upon UV-B irradiation, outer membrane-bound carotenoids may play a role in photoprotection immediately after rewetting of desiccated colonies when the OS-MAA content is low. It is submitted that carotenoids, in UV protection, provide fast, active SOS response to counteract acute cell damage whereas the extracellular glycan with its UV absorbing pigments is a passive UV screen against long-time exposure.

Proteome Analysis

Proteome analysis, based on 2D electrophoresis has developed into a powerful tool to investigate global changes in the gene expression profile of organisms (Antelmann et al., 1997; Godon et al., 1998; VanBogelen et al., 1999). However, there is only few information available

on cyanobacterial proteome. The only cyanobacterial proteome which has bee studied in some detail is that of Synechocystis sp. PCC 6803 (Sazuka and Ohara, 1997; Sazuka et al., 1999), but no stress response studies have been performed. The proteomic approach presented in this work showed the dynamic nature of the protein expression profile of *N. commune*.

The proteome of N. commune was strongly influenced by the specific growth stage. For each phase, growth cycle specific expressed proteins were observed, which could be used as marker proteins to identify the growth stage of a culture (Fig. 22). Whole cell extracts of *N. commune* were fractionated prior to analysis to provide information about the subcellular location of affected proteins and information concerning subcellular specific kinds of reaction. In the membrane fraction, mostly positive stimulated proteins were detected, whereas nearly no negative stimulated proteins were found. This indicates that the membrane responses to exponential growth with the addition of novel metabolic features while keeping old ones intact. In the cytosol more proteins showed to be growth stage dependent than in membrane and more proteins were repressed (Table 4). Cytosol turned out to be more reactive to growth than the membrane. Influence of growth on the protein expression profile has to be taken into account when long-time stress experiments are performed and analyzed.

Influence of UV on the proteome. The three dimensional virtual gel, generated with the database application, was used to investigate the UV-B triggered versus the growth stage dependent changes in the protein expression profile of *N. commune*. The UV-B response turned out to be surprisingly complex. Semiquantitative analysis of about 1350 proteins revealed that at least 493 proteins (37%) belong to the UV-B stimulon. A minimum of 168 proteins were positive stimulated, whereas a minimum of 305 proteins were negative stimulated (Fig. 23). The term stimulon refers to a set of proteins whose amount or synthesis rate changes in respond to a single stimulus, a specific environmental condition (reviewed in VanBogelen *et al.*, 1999). The phosphate limitation (PL) stimulon in *Escherichia coli* included 413 proteins, half of this proteins were positive stimulated and half of them were negative stimulated (VanBogelen *et al.*, 1996). Another complex stimulon which has been described only recently is the H_2O_2 stimulon in *Sacharomyces cerevisiae*. The synthesis of 115 proteins was stimulated by H_2O_2, whereas 52 other proteins were repressed. Except for a few targets the H_2O_2 response was transient (Godon *et al.*, 1998). In *E. coli*, *Salmonella typhimurium* and *Bacillus subtilis* different types of stimulons have been investigated (Spector *et al.*, 1986; Antelmann *et al.*, 1997; VanBogelen *et al.*, 1997), while in cyanobacteria detailed global analysis of protein synthesis under specific environmental conditions to define stimulons are still missing.

The 37 % observed changes in the protein expression profile of *N. commune* reflect that the organism completely changed its physiology in response to UV-B. These changes are the result of true adaptation and not of damage, since no growth delay was observed in *N. commune*. The cells adjusted their physiology to the new conditions. 70% of the stimulated proteins during UV-B acclimation were low abundant proteins, 'acclimation proteins', with volumes below 0.4% of total cellular proteins, whereas shock proteins are usually bulk proteins. E.g., the major cold shock protein CspA of *Escherichia coli* accounts for 13% of total cellular protein 1 hour after cold shock (Goldstein *et al.*, 1990). The influence of UV-B on the proteome can be divided into early acclimation response, within the first 12 hours, and late acclimation response, which requires 1 up to 3 days. Most of the protein changes observed during early acclimation were transient (Table 4). This finding is consistent with the observations from physiological investigations. UV-B irradiation of *N. commune* led to a rapid, but transient increase of outer membrane bound carotenoids, and a slower, but constant production of extracellular UV-A/B absorbing mycosporines and extracellular glycan. The response to UV-B turned out to be subcellular specific. Influence of UV-B on the protein expression profile of the membrane was faster than on the cytosolic protein expression profile (Fig. 21, factor 2). The protein composition of the membrane fraction was more strongly influenced during early acclimation, while the cytosolic fraction was more strongly influenced after long-time UV-B. Membranes are known to be primary targets for UV-B induced damage by reactive oxygen intermediates and free radicals (Tuveson *et al.*, 1988; Hideeg and Vass, 1996). The transient stimulated proteins, especially in the membrane, may be important for early adaptation after UV-B shock, whereas the majority of the stimulated proteins are need for continuous growth under UV-B light.

These results clearly showed the importance of long-time experiments, which can provide valuable information on the mechanisms and adaptation to UV radiation in cyanobacteria. The cellular adjustment to UV-B results in alternative metabolic fluxes. After prolonged UV-B irradiation, the cell number decrease due to a slower cell replication caused by the metabolic cost of MAA and glycan synthesis (Table 2). The induction of MAA synthesis is associated with the induction of a water stress protein (Wsp) (Fig. 17) and acidification of the glycan. The induction of Wsp by UV-B support its role in the synthesis of the oligosaccharide MAA (OS-MAA) which has been proposed by Hill *et al.* 1994. However, further studies are necessary to unravel its specific role, if it is directly involved in the synthesis of OS-MAA or via modification of the glycan to provide a matrix for OS-MAA.

UV-A had only little influence on the protein pattern of *N. commune*, nevertheless continuous growth under supplemented UV-A had remarkable effects on its pigment composition (Table 3). This confirms that UV-B response is highly complex and underlines the high biological effectiveness of short wavelength UV.

The sensing of UV-B signals by cyanobacteria is still cryptic. A UV-B photoreceptor is proposed to regulate the oligosaccharide-mycosporine production in *N. commune* and a UV-B special photoreceptor might be involved in the regulation of carotenoid synthesis. However, for identification and functional analysis of photoreceptors and signal transduction pathways, the isolation of mutants will be necessary. Genes involved in signal transduction normally expressed in low copy number, which are unlikely to be identified from 2D electrophoresis because of the detection limit of 2D. Thus 2D differential display and molecular genetics have to complement each other to get a more holistic understanding of acclimation of cyanobacteria to UV-B.

Experiments under environmental conditions

Morphology of field material. In contrast to laboratory grown liquid cultures, which showed diffuse growth, *N. commune* forms in its natural habitat sheath like thalli, which are often curled or folded into several layers. This causes a high degree of self-shading and concomitant variability of pigment contents, even in a single thallus, which made a detailed study of climate related variations in pigment contents difficult. Since it was not possible to relate pigment contents to cell number which would have been desirable, it was necessary to use dry weight as a basis. The glycan sheath represents a considerable fraction of the dry weight of *N. commune*. An increased exopolysaccharide production in response to desiccation was reported from cyanobacteria (Grilli Caiola *et al.,* 1996) and other terrestrial bacteria (Roberson and Firestone, 1992). UV-B-induced exopolysaccharide production resulting in a two- to threefold increased dry weight per cell was observed in laboratory grown *N. commune* . While drought is a stress which is present throughout the year for a poikilohydrous organism, UV-B radiation is enhanced during summer. Increases in cell related dry weight during summer could have concealed increases in MAA or scytonemin at that time.

Annual time course of pigments. Photosynthetic pigments and other carotenoids were low during winter and rose strongly in early May (Fig. 26 and 27). In regard to the contents of the UV-protective pigments scytonemin and MAA, irradiance seemed to have little influence. These pigments showed either no changes or varied antiparallel to the radiation climate over the course

of a year (Fig. 28). This seems to be even more surprising, as both pigments are strongly induced by UV-A or UV-B radiation, respectively. The amount of MAA found in the field material correlated well with the amount of MAA found in of UV-B irradiated liquid cultures of *N. commune*. The MAA content was order of magnitude higher than the scytonemin content. Scytonemin and MAA are located in the glycan sheath of *N. commune* and, hence, are largely inaccessible for metabolism. Scytonemin is extremely stable and should not be degraded by physical factors (Garcia-Pichel and Castenholz, 1991). After induction of MAA synthesis in liquid cultures of *N. commune*, MAA synthesis continued even in the absence of UV-B radiation when the cultures were in the exponential growth phase. However, in late stationary phase, MAA contents decreased under the same conditions. This observation could indicate either spontaneous disintegration or bacterial metabolization of MAA. An involvement of bacteria in MAA decomposition has been described for the marine alga *Phaeocystis* (Marchant *et al.,* 1991). *N. commune* under natural conditions may well be comparable to a culture in the stationary phase and bacteria were probably also present under field conditions. Therefore, a decay of MAA is not improbable and high concentrations of MAA may indicate its continued synthesis.

Winter is considered to be the most stressful time of the year for N. commune. Low chlorophyll *a* contents in late winter might have resulted from the concomitant exposure to increasing radiation at low temperatures. The latter may largely reduce metabolic reactions including photosynthetic CO_2 fixation which would cause increased photoinhibition with simultaneous inhibition of repair processes resulting in photobleaching. If dry weight of the thalli did not vary to a considerable extent, the presented data would indicate that MAA synthesis was induced in late fall in spite of decreasing UV-B exposure. In view of this conclusion additional factors influencing MAA synthesis should be considered. An obvious factor could be low temperature. Treatment of liquid cultures of *N. commune* at 7°C did not induce MAA synthesis in the absence of UV-B irradiation. However, such results do not exclude a possible influence of long-term growth at low or freezing temperatures or the occurrence of synergistic effects between temperature and UV-B irradiance under natural conditions. This should be tested in future experiments. At low temperatures enzymatic repair of UV-B-induced lesions is partially inhibited. This means that under such conditions avoidance of UV-B exposure by screening pigments is even more important than at elevated temperatures. Also in higher plants increased synthesis of photoprotective pigments at low temperatures was demonstrated (Christie *et al.*, 1994).

General Conclusion

The acclimation response of cyanobacteria to UV stress appears to be rather complex. Photon fluence rates which are in the magnitude of solar fluence rates induce a cascade of physiological reactions in the terrestrial cyanobacterium *Nostoc commune*. The data presented in this work reflect the complex interactions at the physiological and molecular levels, and suggest that the study of individual genes may not provide sufficient information to allow holistic understanding of UV stress adaptation. High resolution 2D electrophoresis coupled to computerized image analysis and database analysis turned out to be a useful technique for studying the complex proteome changes during the UV-B acclimation process of *N. commune*. The results presented in this work revealed the importance of long-time experiments and emphasize further research to unravel the molecular basis of UV tolerance.

The combination of 2D electrophoresis with high throughput amino acid analysis, peptidemass fingerprinting and associated sequence tags in combination with nanoelectrospray tandem mass spectrometry can lead into novel discoveries in cyanobacterial UV stress physiology. Genetic approaches can also make significant contributions to the understanding of the UV acclimation processes, as has already been shown in studies of UV tolerance in higher plants (Jenkins *et al.,* 1995). The isolation and characterization of mutants will be an important step in the investigation of signal transduction pathways, which are still cryptic. For such studies, it will be advisable to use cyanobacterial strains, which are growing as single colonies rather than to use filamentous strains, like the *N. commune* strain used in this work.

Since the glycan of *N. commune* appeared to be play a central role not only in desiccation tolerance, but also in UV tolerance it would be an promising approach to study synergistic effects of UV-B and desiccation stress. The UV-B induced synthesis and secretion of UV-A/B-absorbing mycosporines, extracellular glycan and the water stress protein Wsp, which is involved in the desiccation tolerance of N. commune (Scherer and Potts, 1989), represent a considerable metabolic investment by *N. commune*, but it still remains to be determined whether and how this processes are related and coordinated. For such studies, defined laboratory conditions are necessary. On the other hand, this work also showed that it is of great importance to obtain more data under environmental conditions, especially concerning synergistic effects and defense mechanisms.

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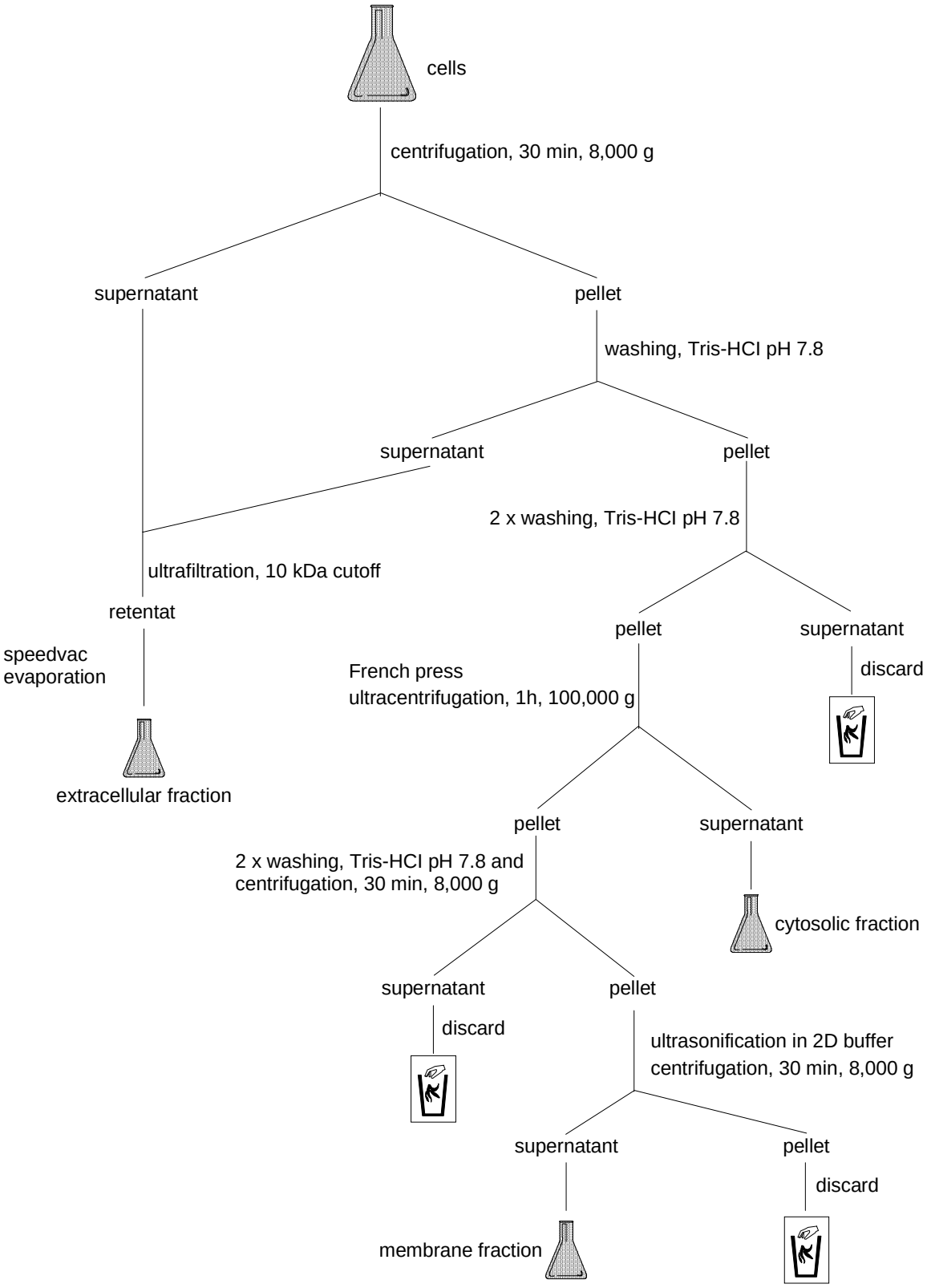
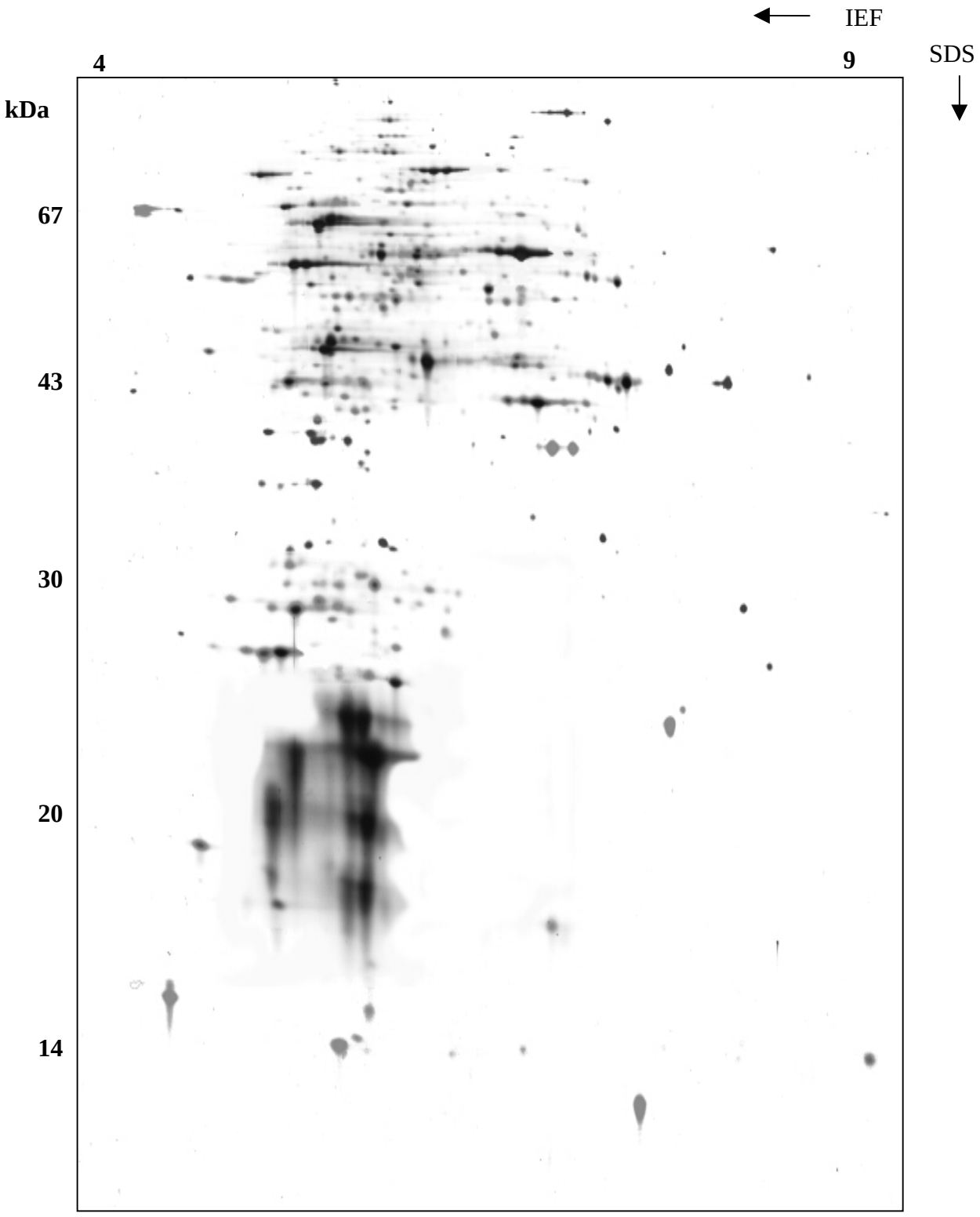
Appendix

Appendix A: Strategy of protein fraction preparations.



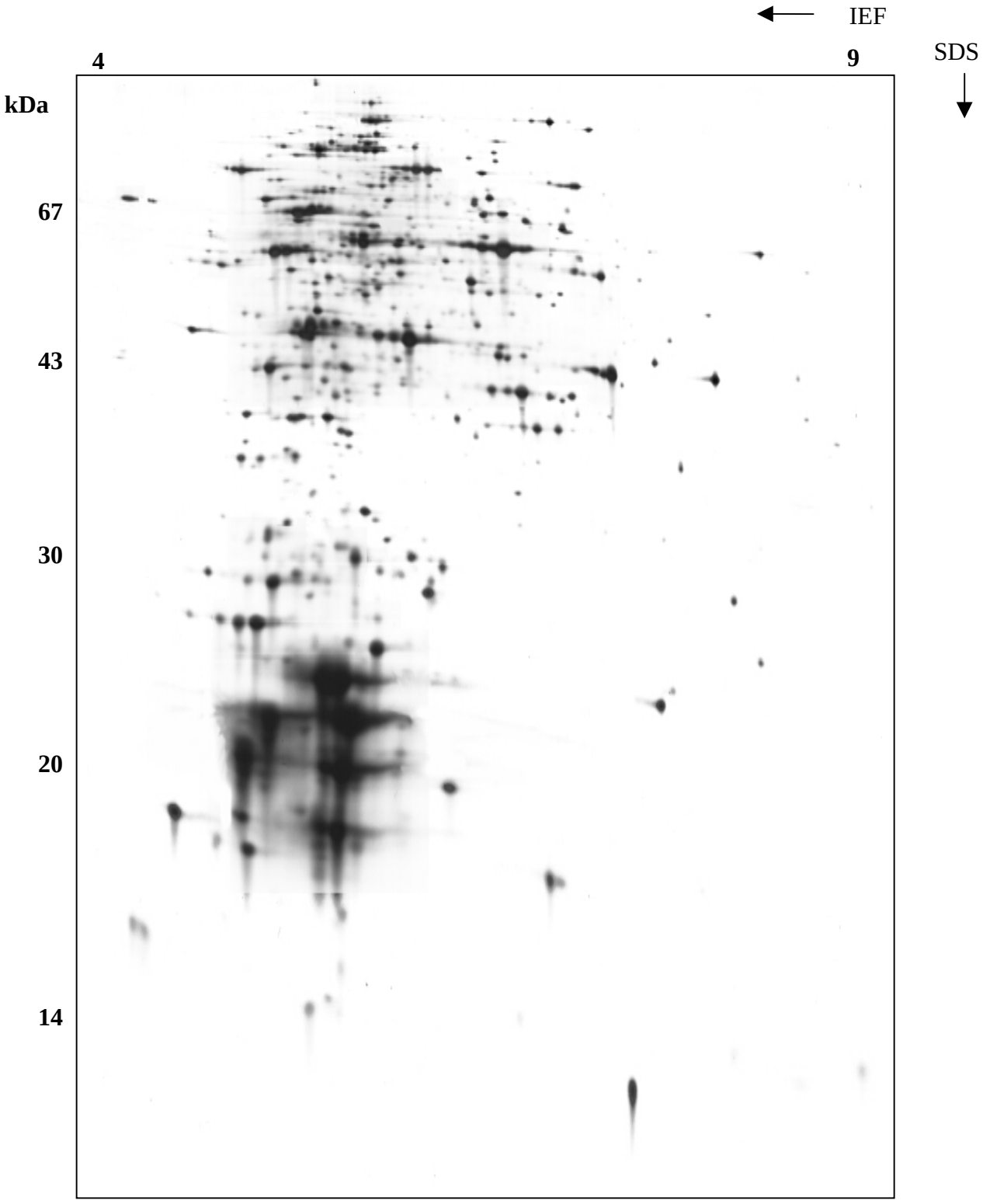
Appendix B: IPG-Dalt of subcellular protein fractions from *N. commune* DRH1.

B1: UV-B influence on the cytosolic protein fraction



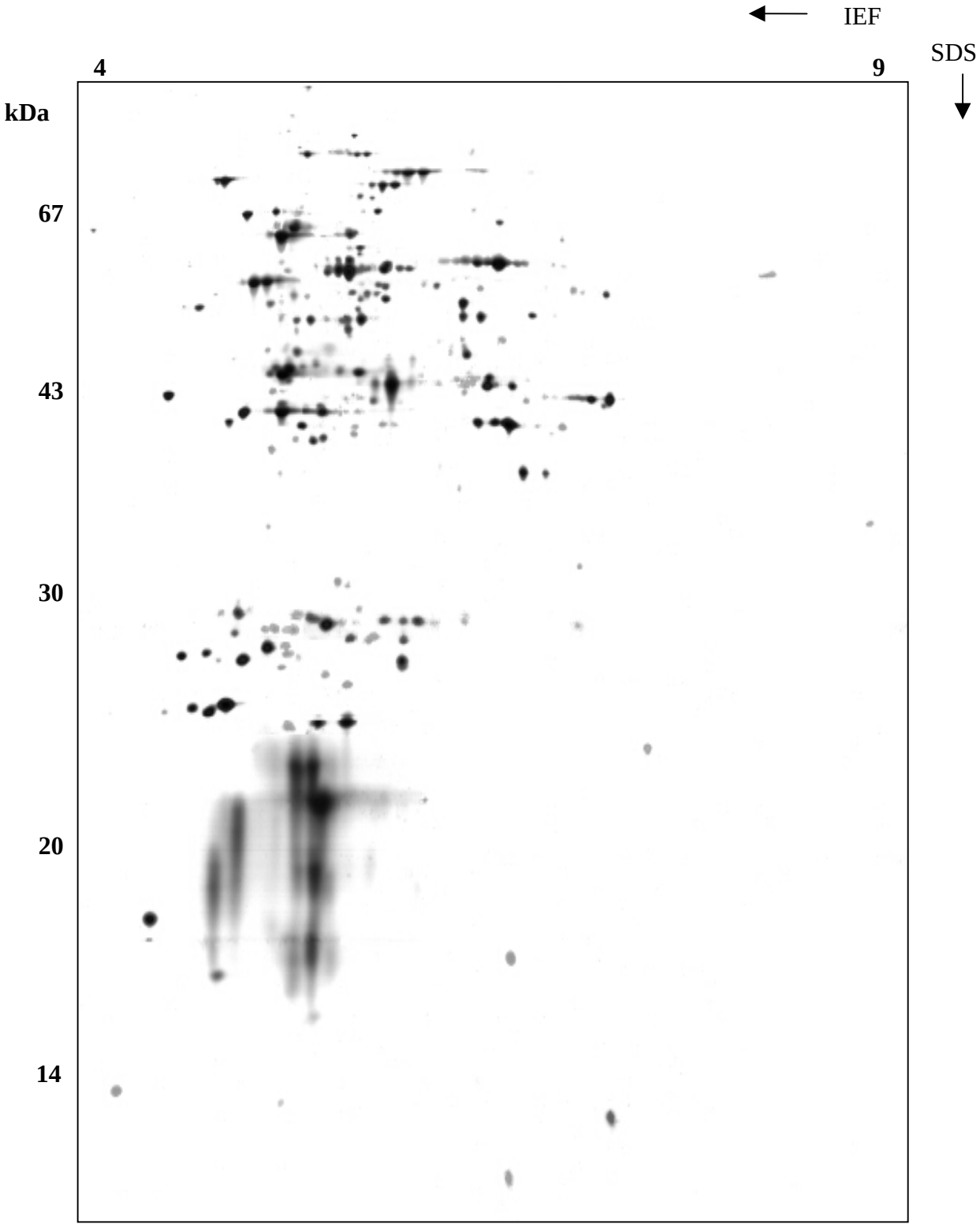
3 hours UV-B (1 Wm^{-2})

B1: UV-B influence on the cytosolic protein fraction (continued).



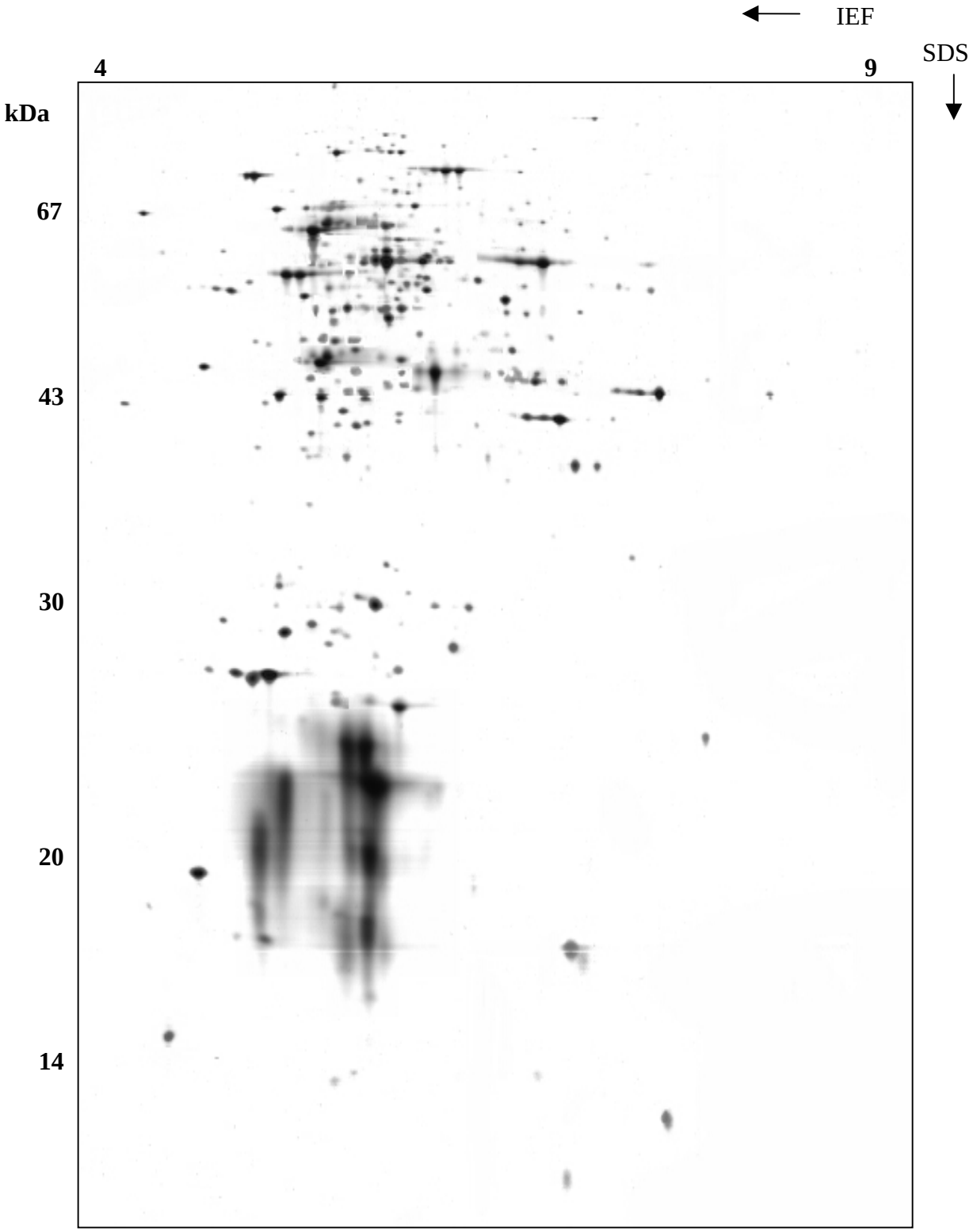
3 hours control

B1: UV-B influence on the cytosolic protein fraction (continued).



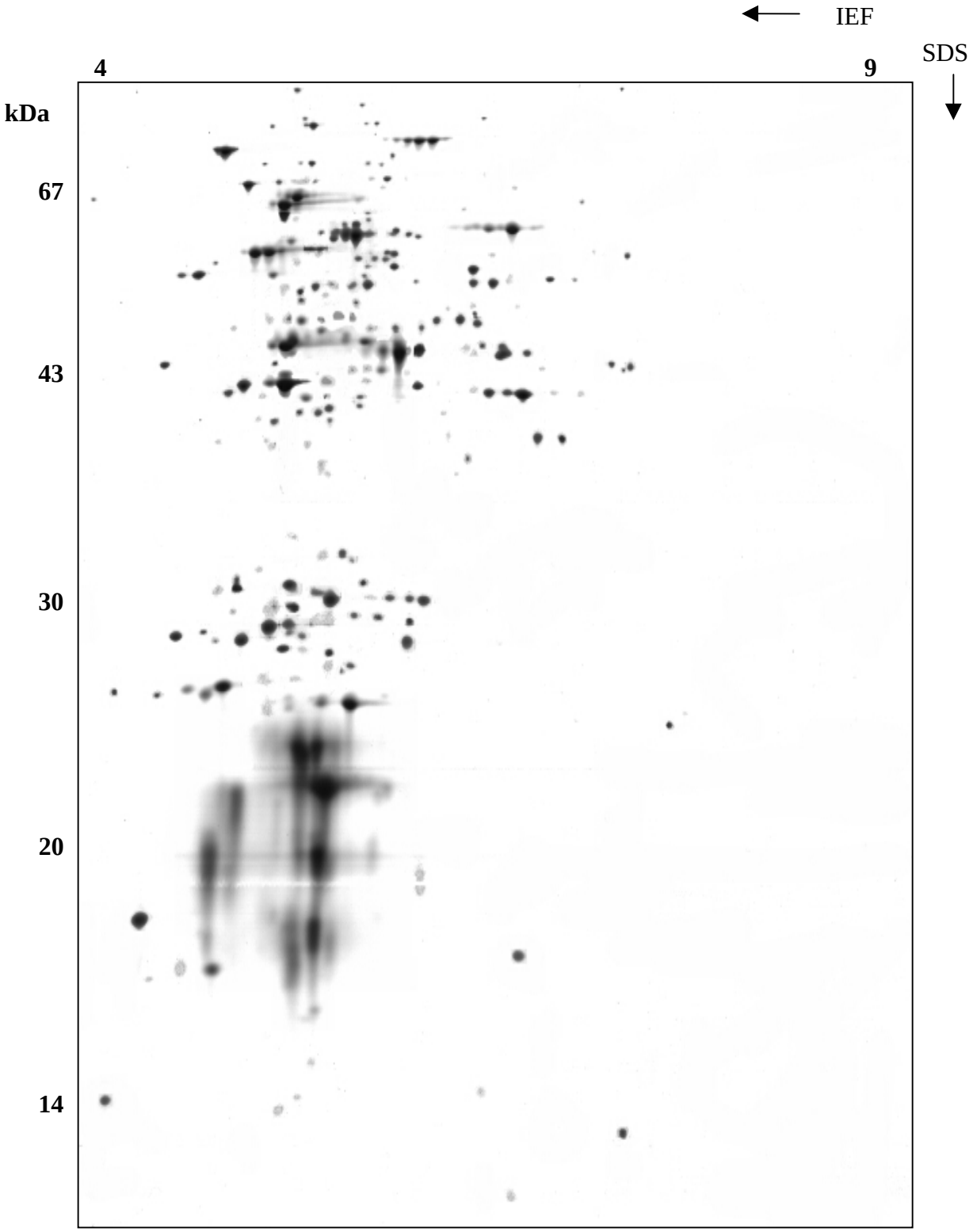
12 hours UV-B (1 Wm^{-2})

B1: UV-B influence on the cytosolic protein fraction (continued).



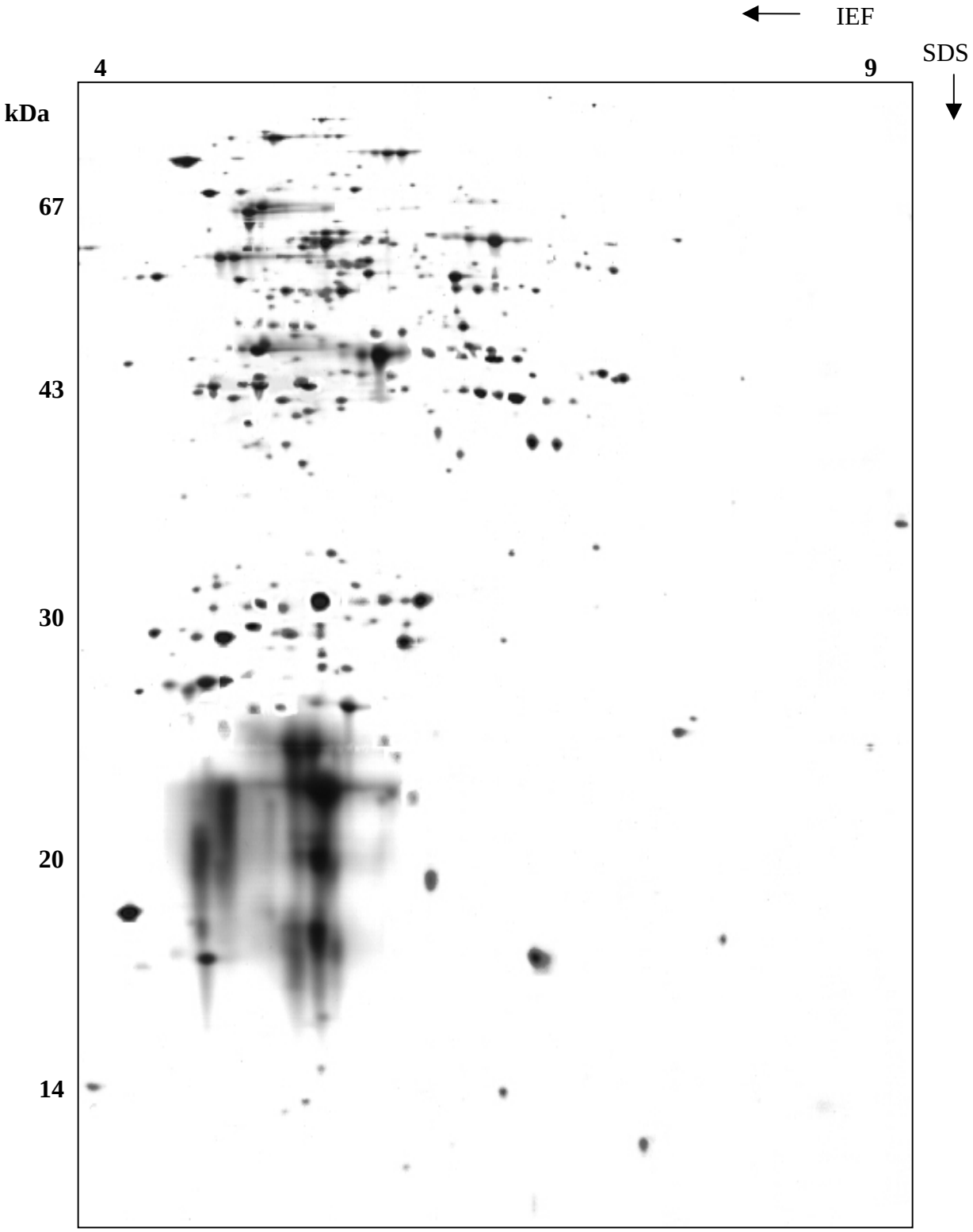
12 hours control

B1: UV-B influence on the cytosolic protein fraction (continued).



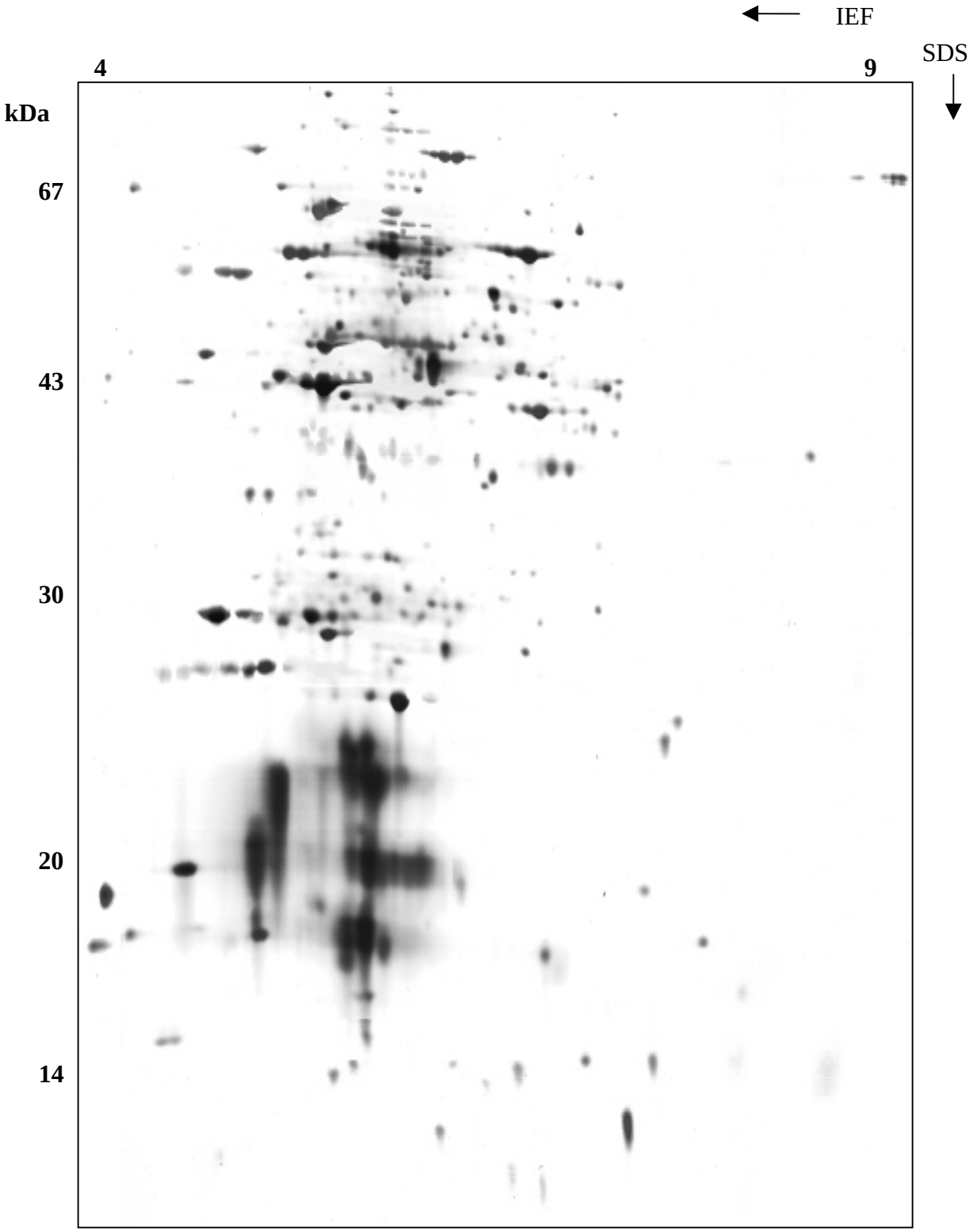
1 day UV-B (1 Wm^{-2})

B1: UV-B influence on the cytosolic protein fraction (continued).



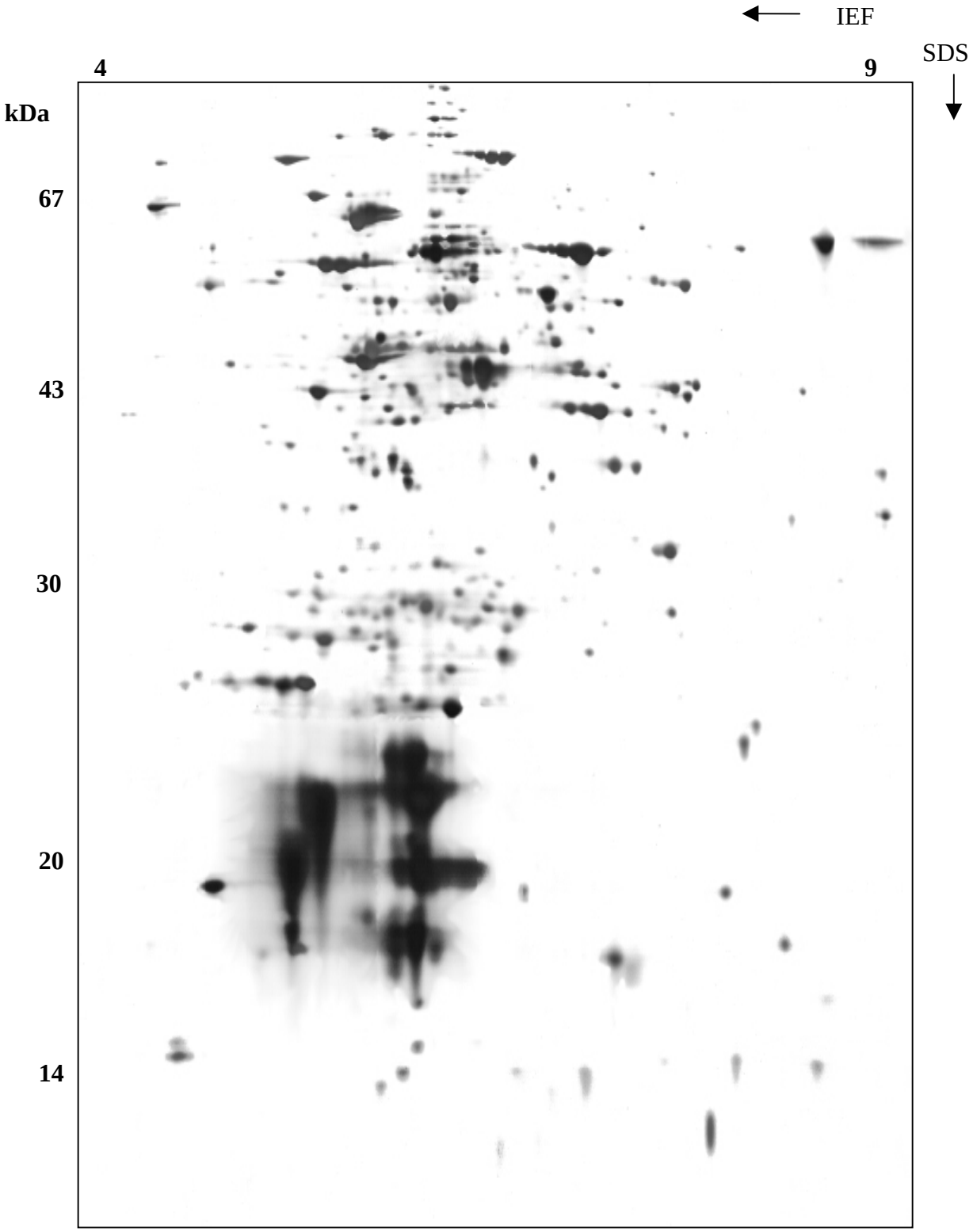
1 day control

B1: UV-B influence on the cytosolic protein fraction (continued).



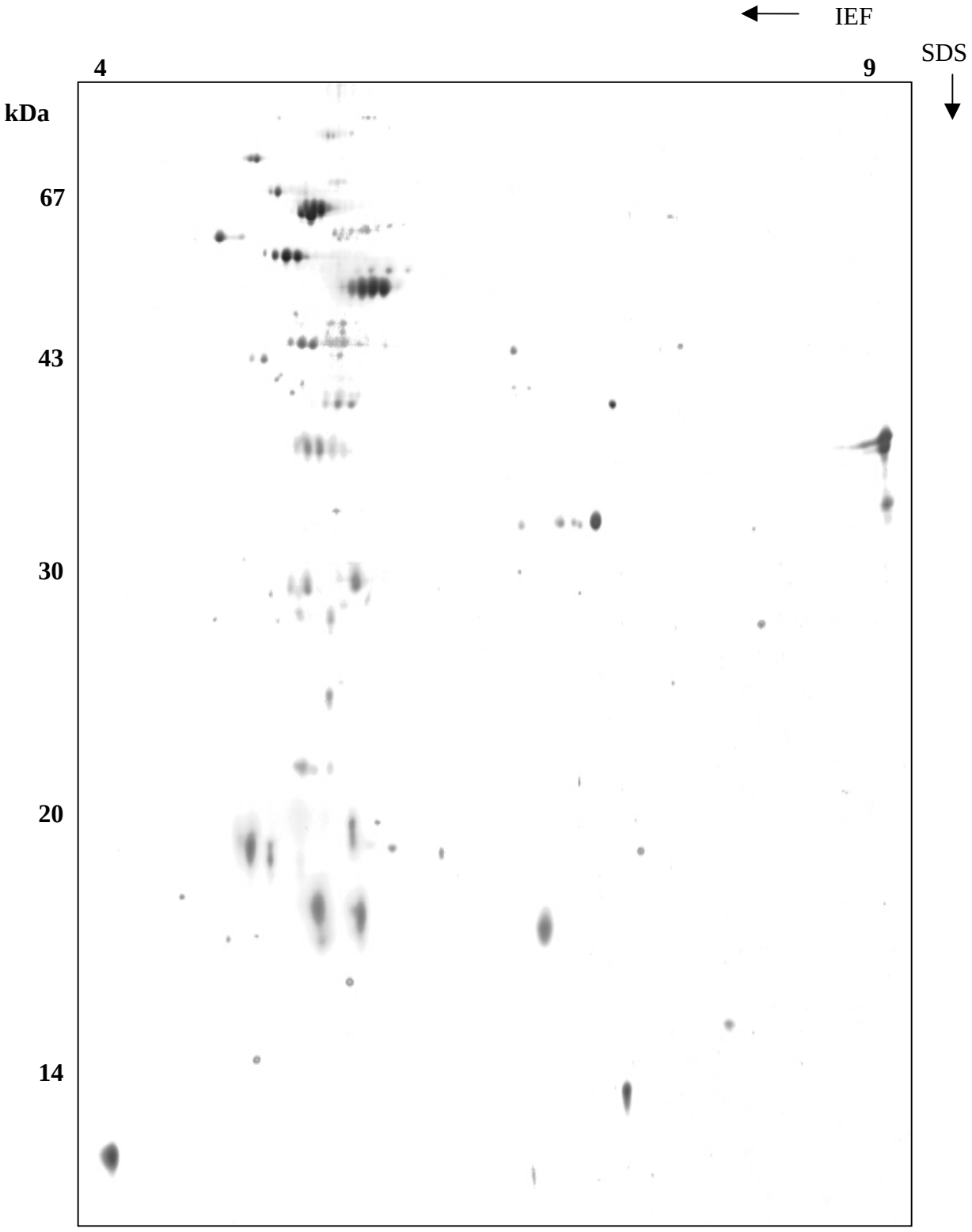
3 days UV-B (1 Wm^{-2})

B1: UV-B influence on the cytosolic protein fraction (continued).



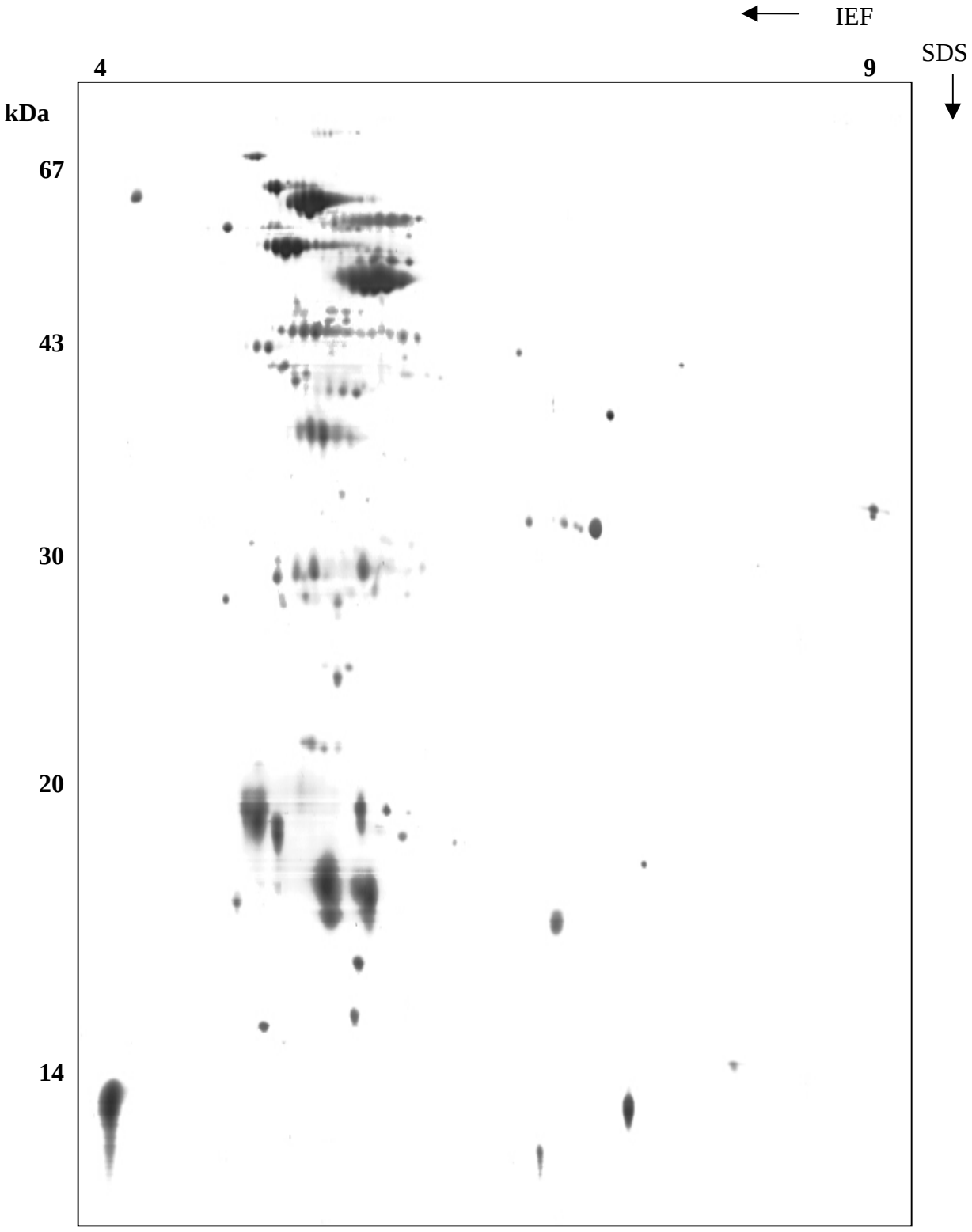
3 days control

B2: UV-B influence on the membrane protein fraction



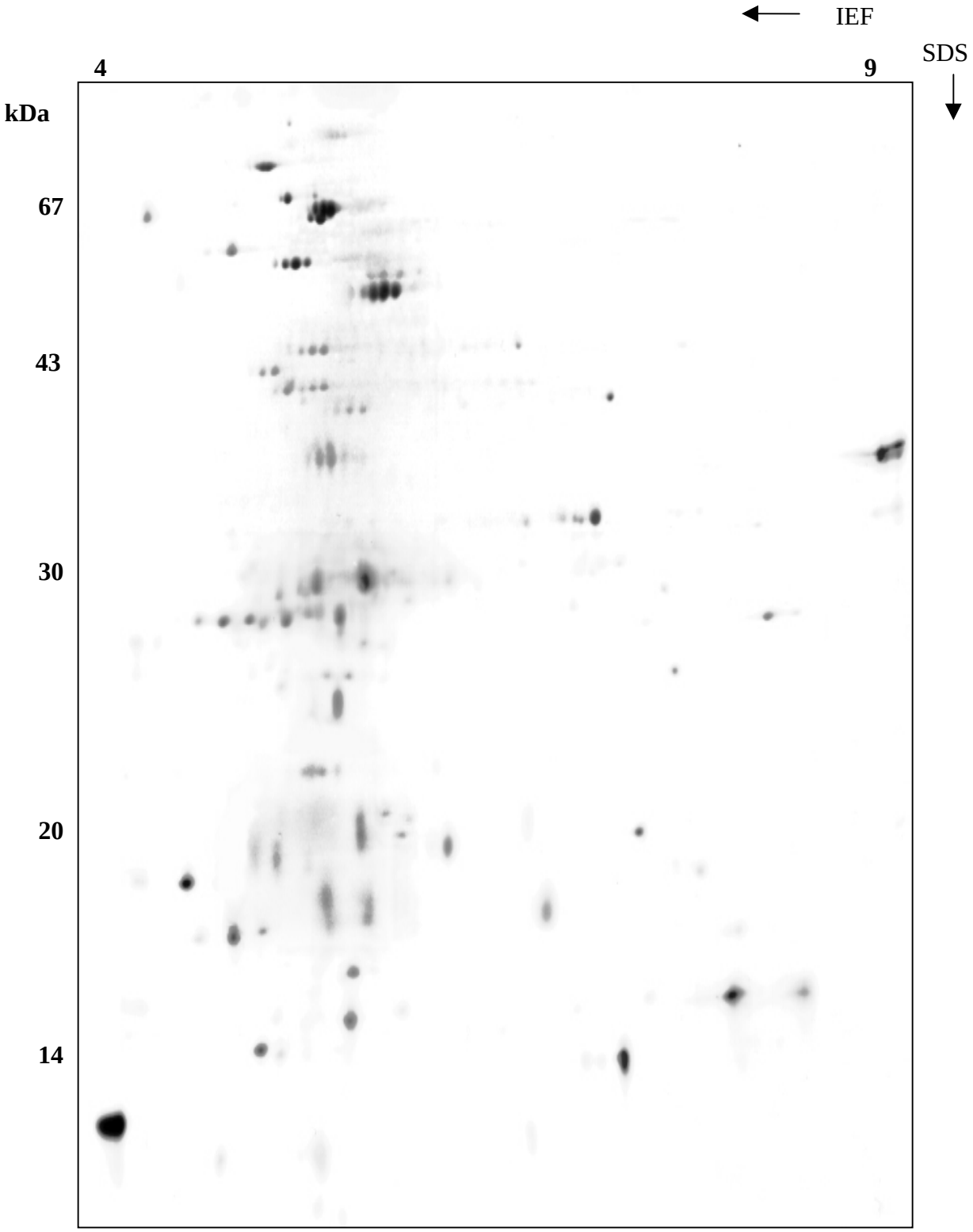
3 hours UV-B (1 Wm^{-2})

B2: UV-B influence on the membrane protein fraction (continued).



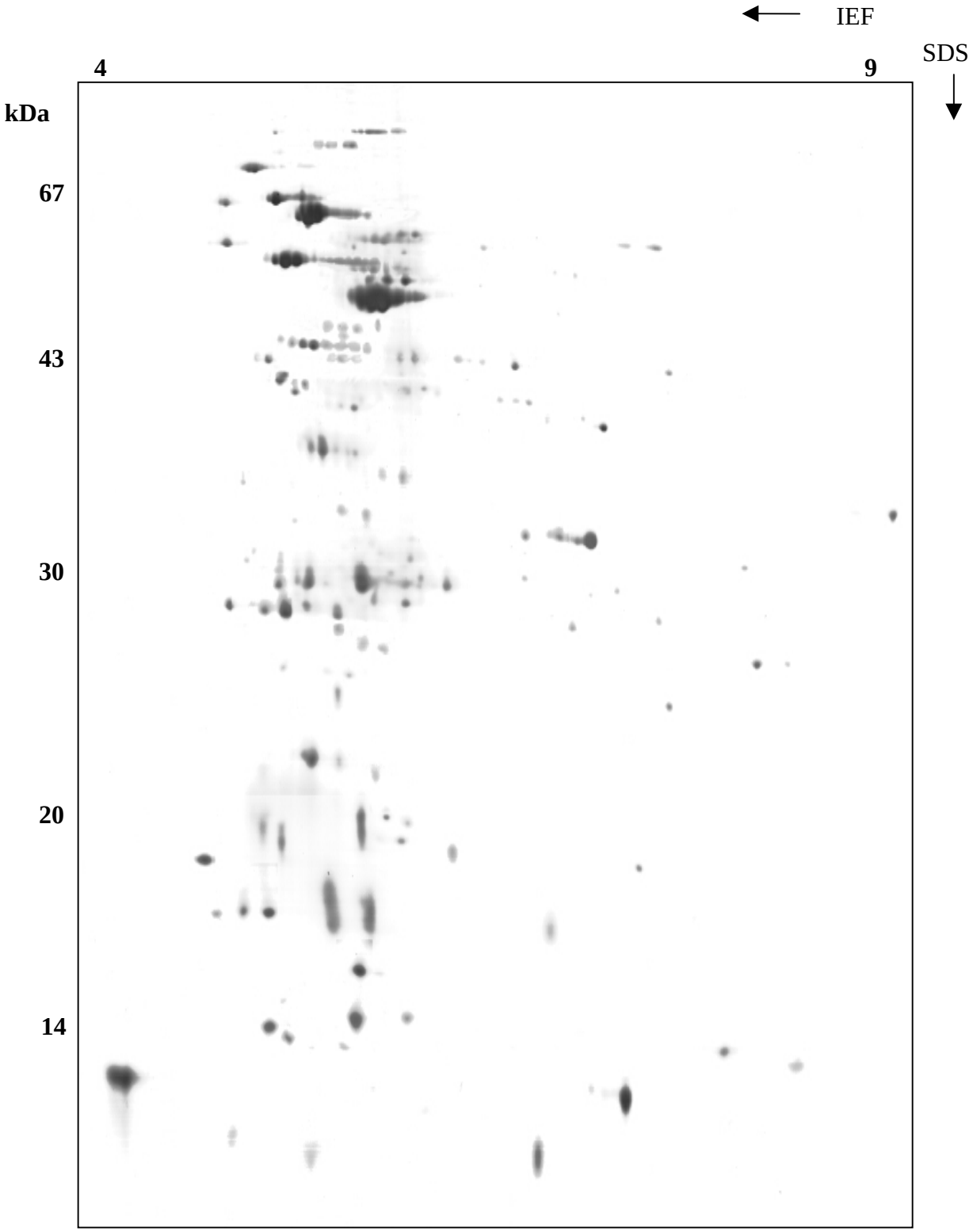
3 hours control

B2: UV-B influence on the membrane protein fraction (continued).



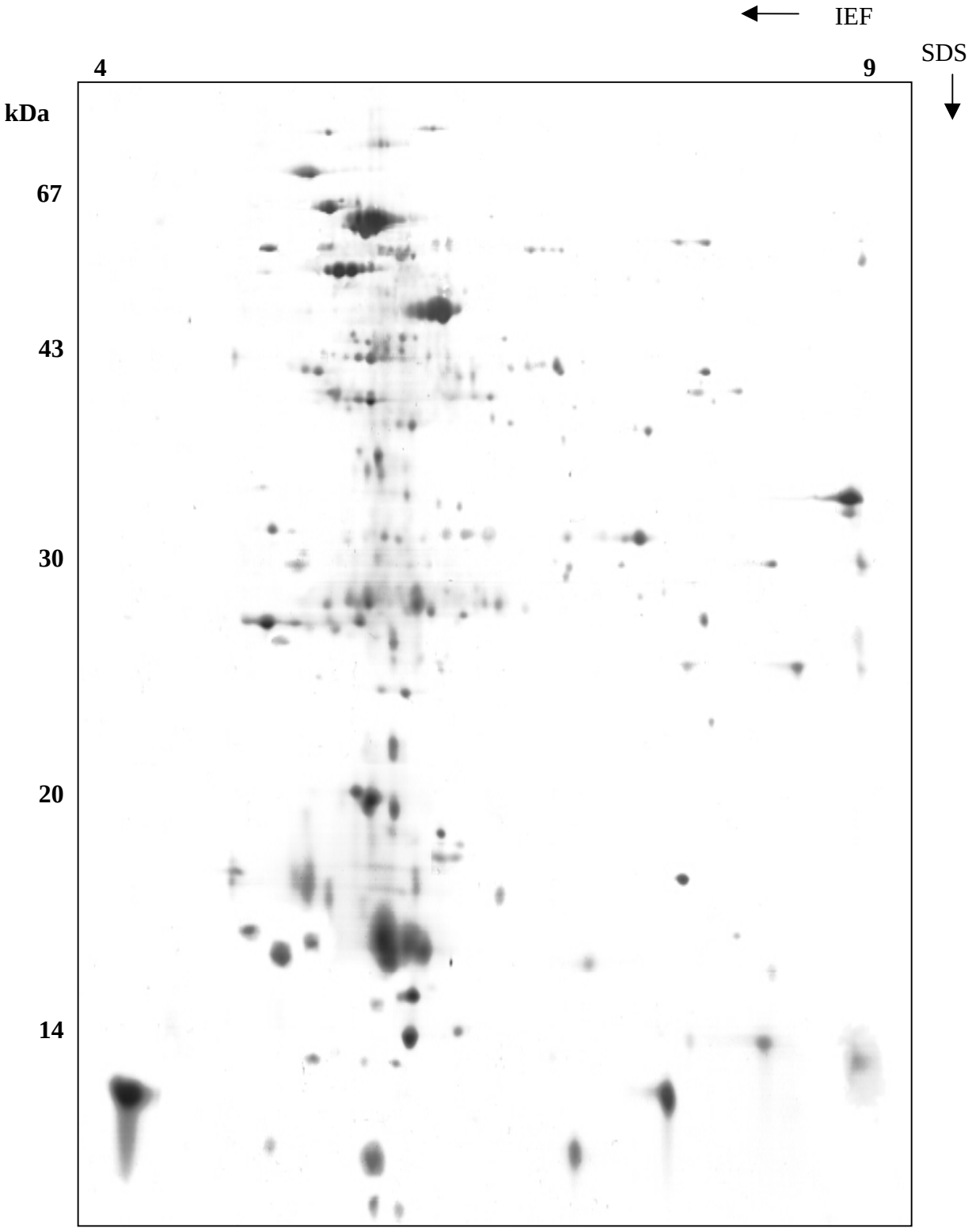
12 hours UV-B (1 Wm^{-2})

B2: UV-B influence on the membrane protein fraction (continued).



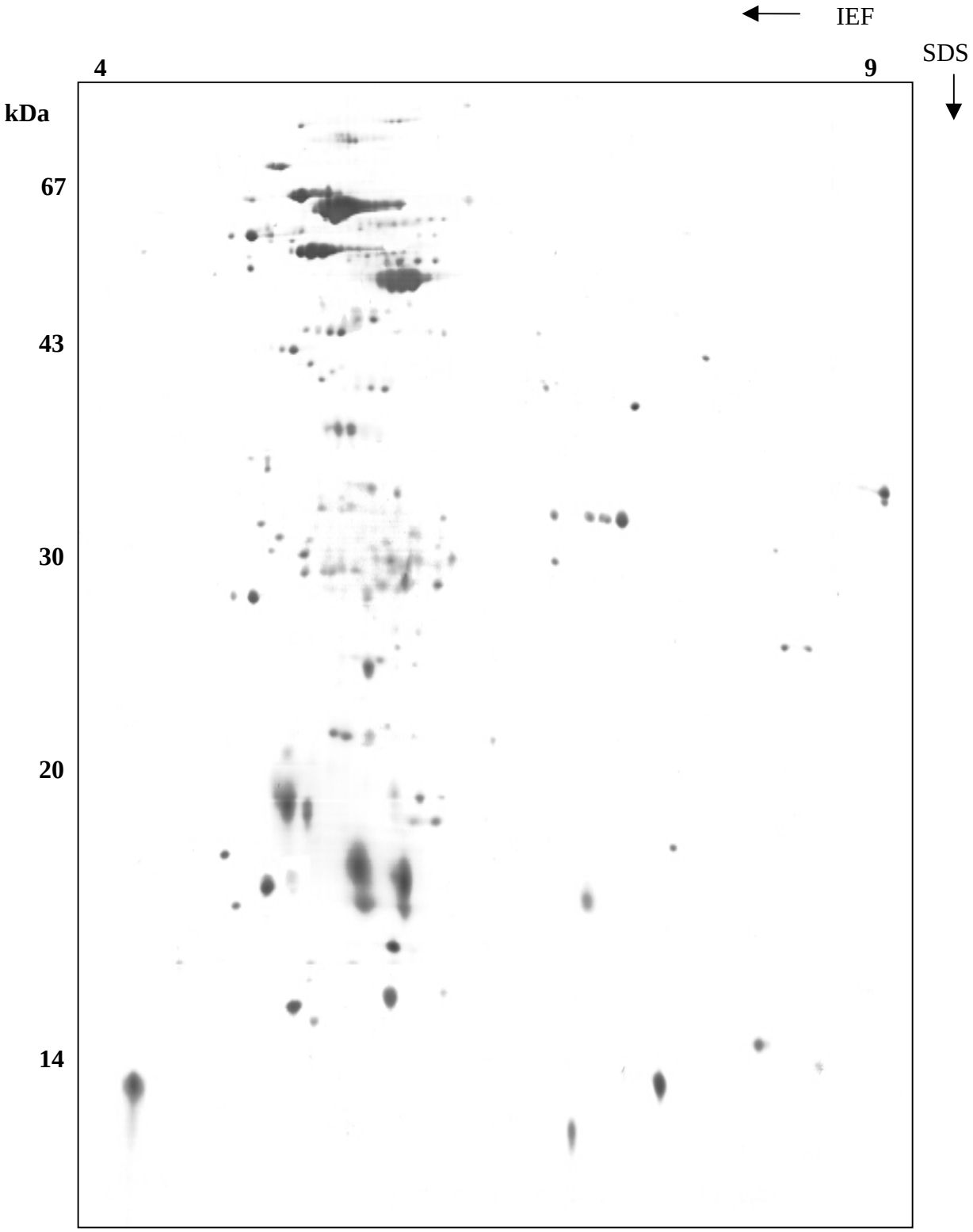
12 hours control

B2: UV-B influence on the membrane protein fraction (continued).



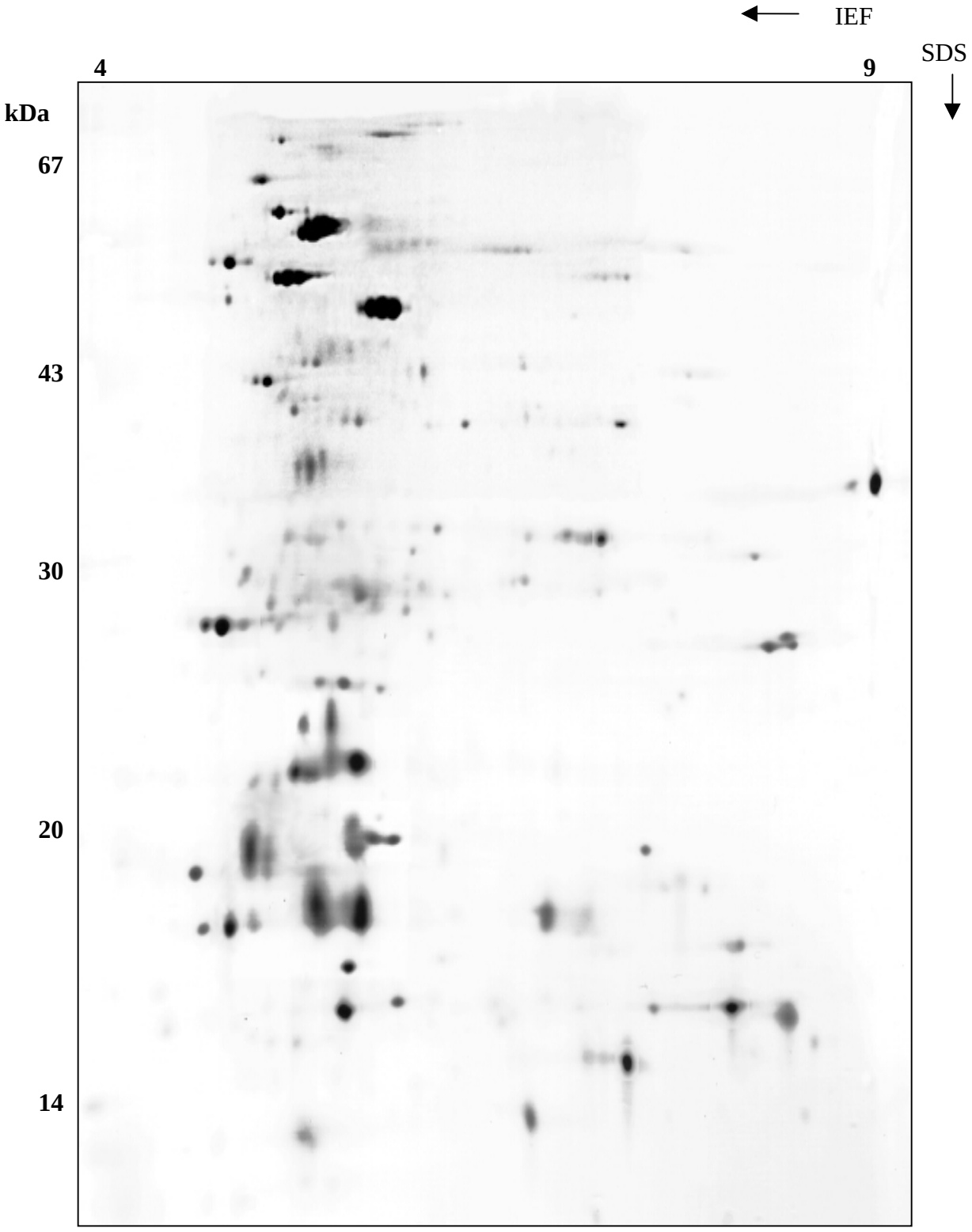
1 days UV-B (1 Wm^{-2})

B2: UV-B influence on the membrane protein fraction (continued).



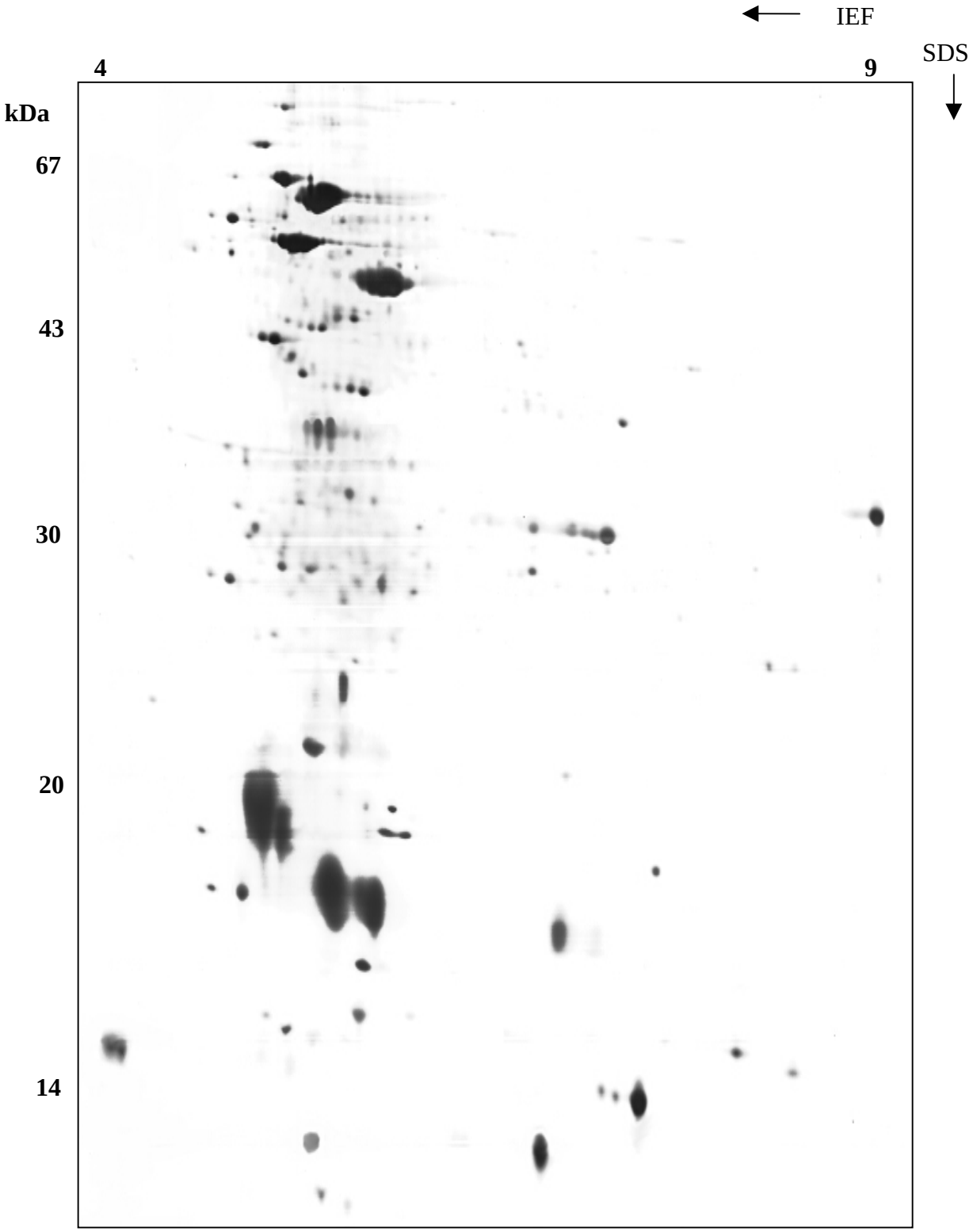
1 day control

B2: UV-B influence on the membrane protein fraction (continued).



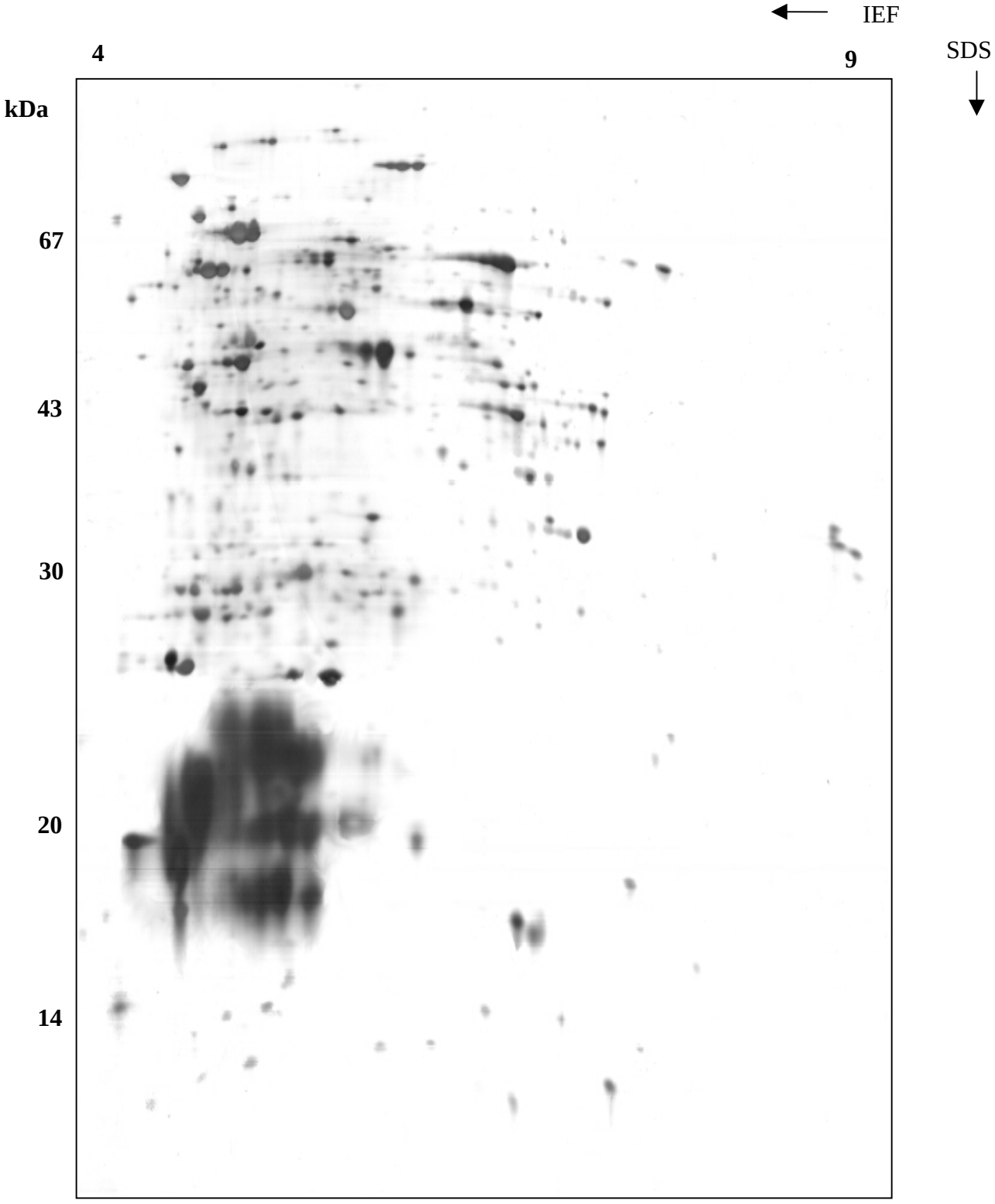
3 days UV-B (1 Wm^{-2})

B2: UV-B influence on the membrane protein fraction (continued).



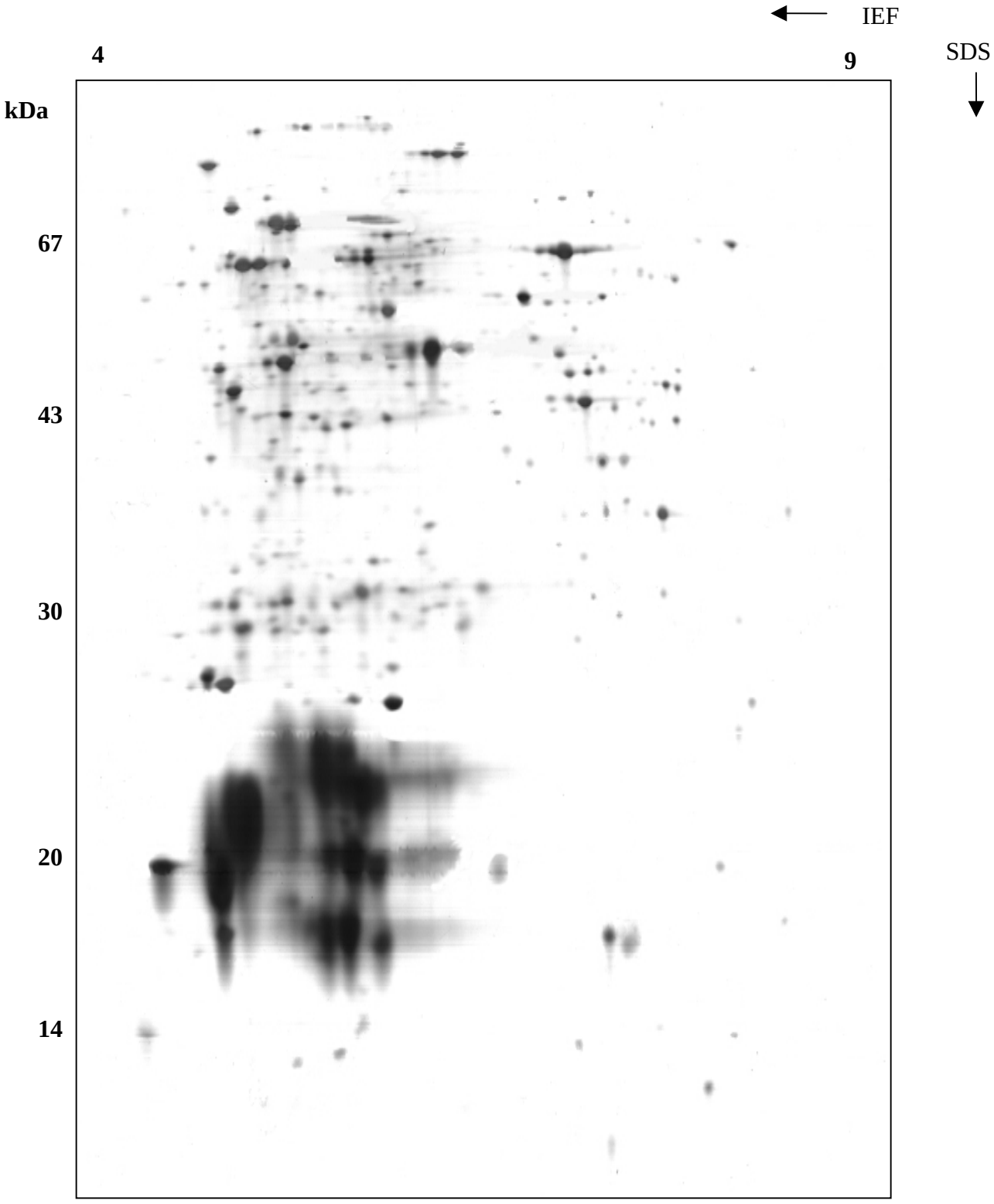
3 days control

B3: UV-A influence on the cytosolic protein fraction



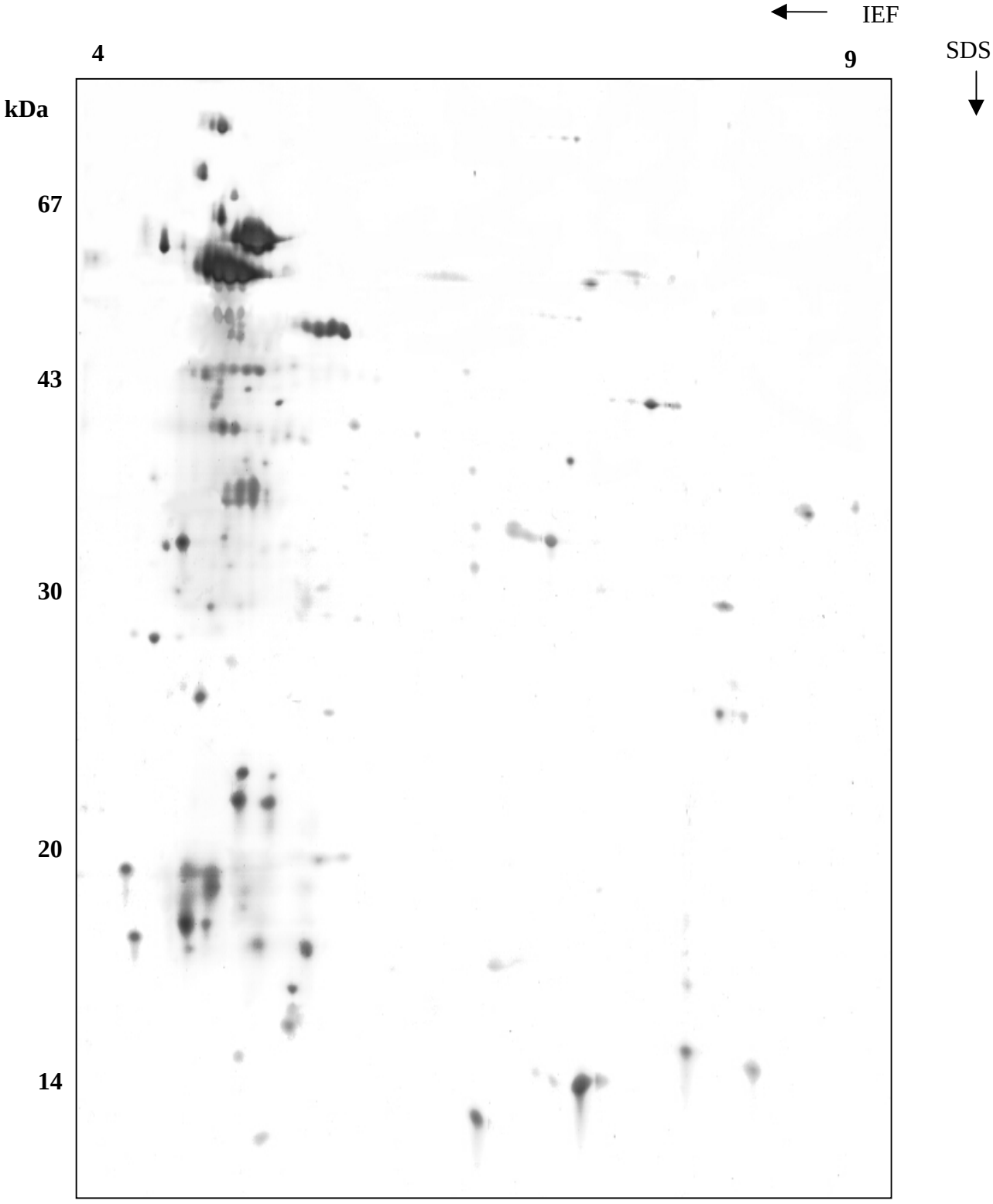
3 days UV-A (1,7 Wm^{-2})

B3: UV-A influence on the cytosolic protein fraction (continued).



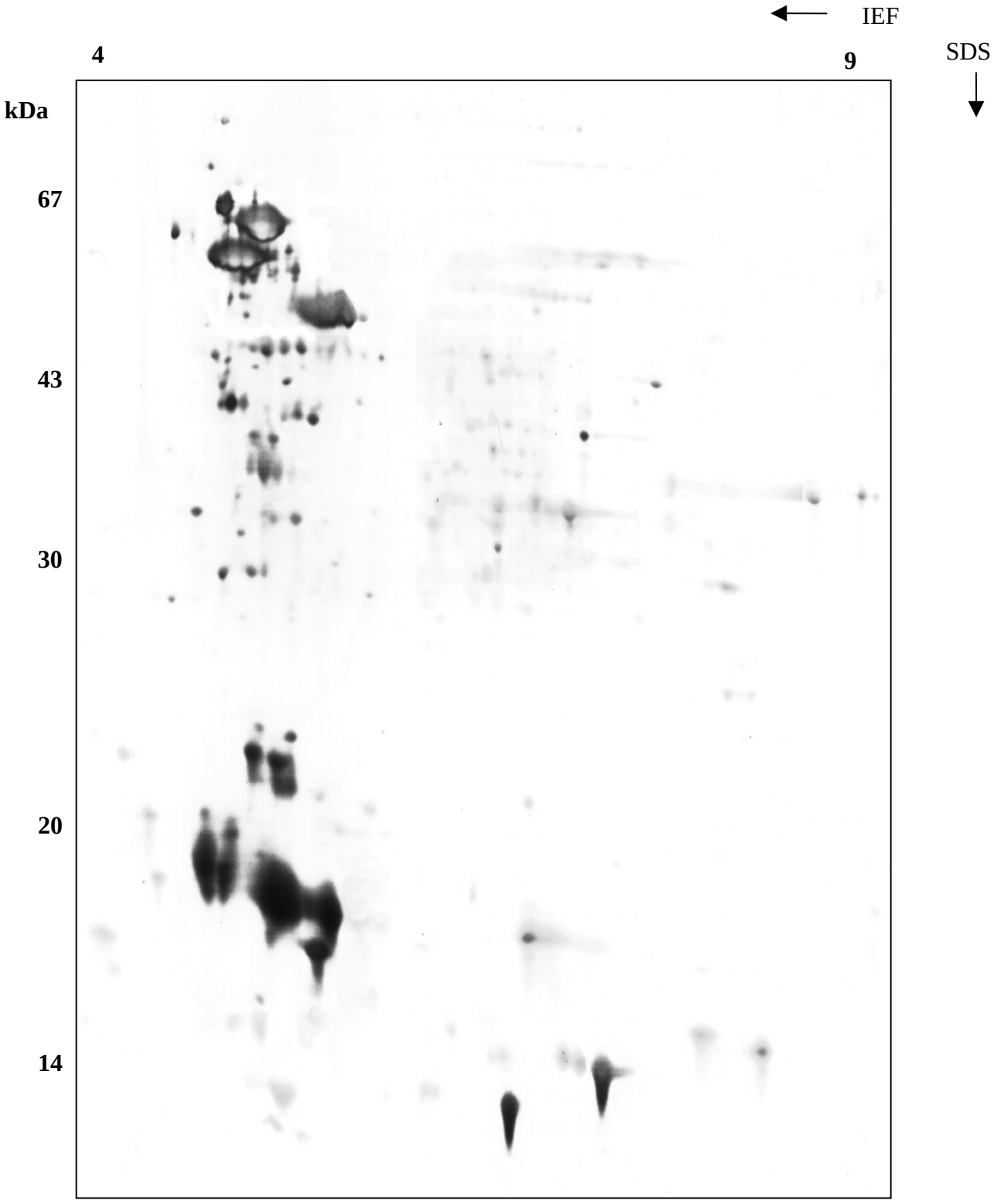
3 days control

B4: UV-A influence on the membrane protein fraction



3 days UV-A (1,7 Wm^{-2})

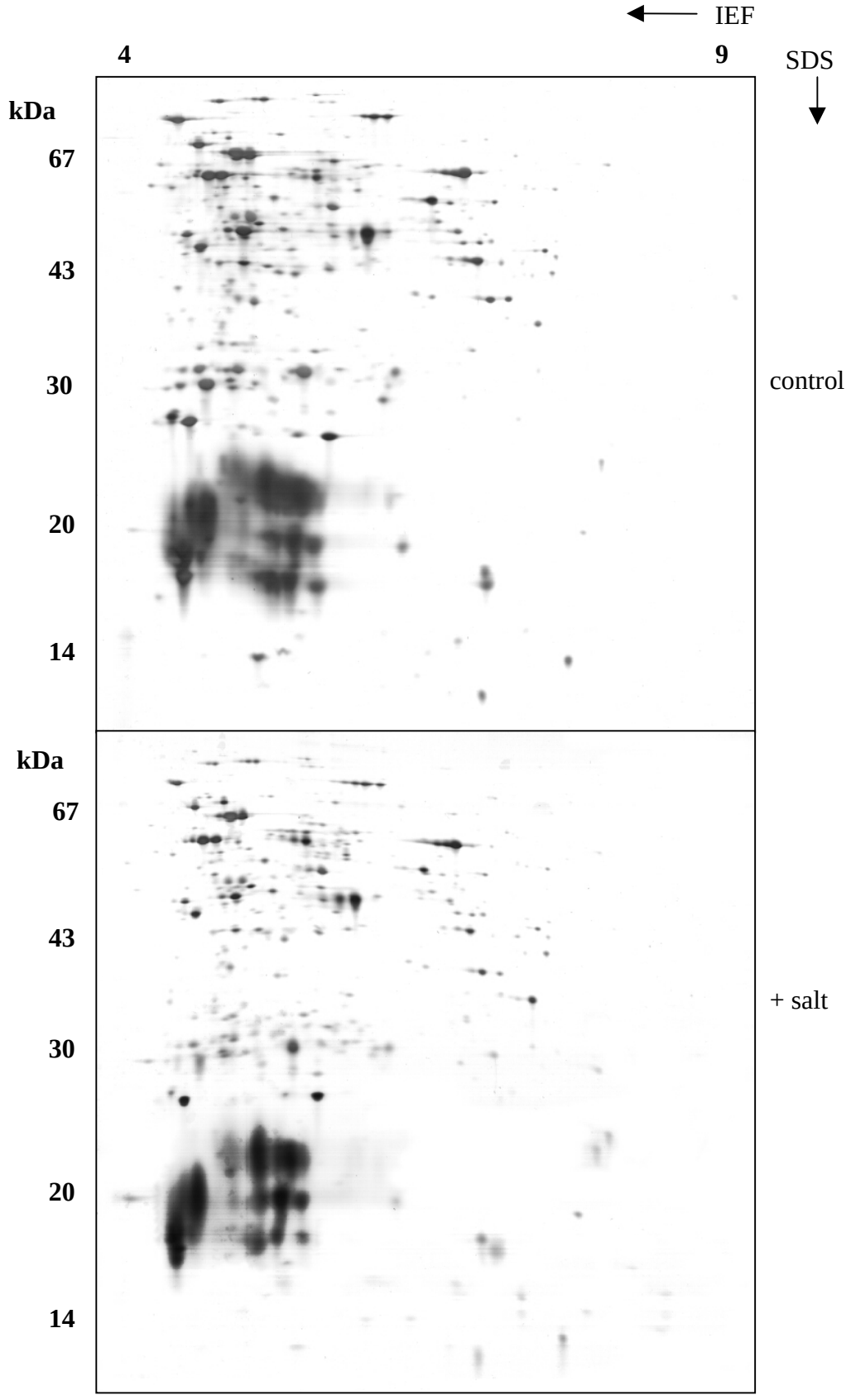
B4: UV-A influence on the membrane protein fraction (continued).



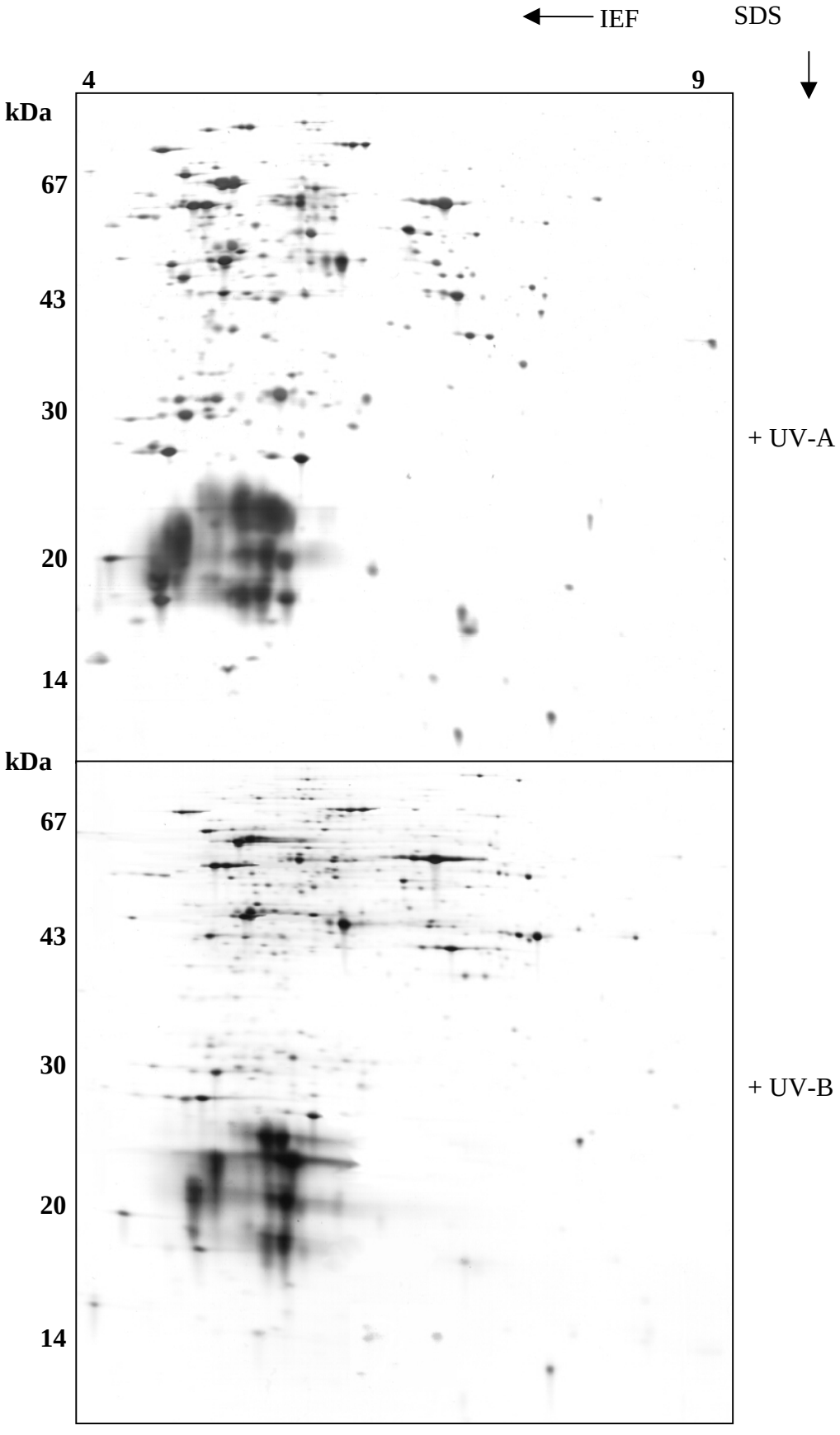
3 days control

Appendix C: Stress stimulons of *N. commune* DRH1.

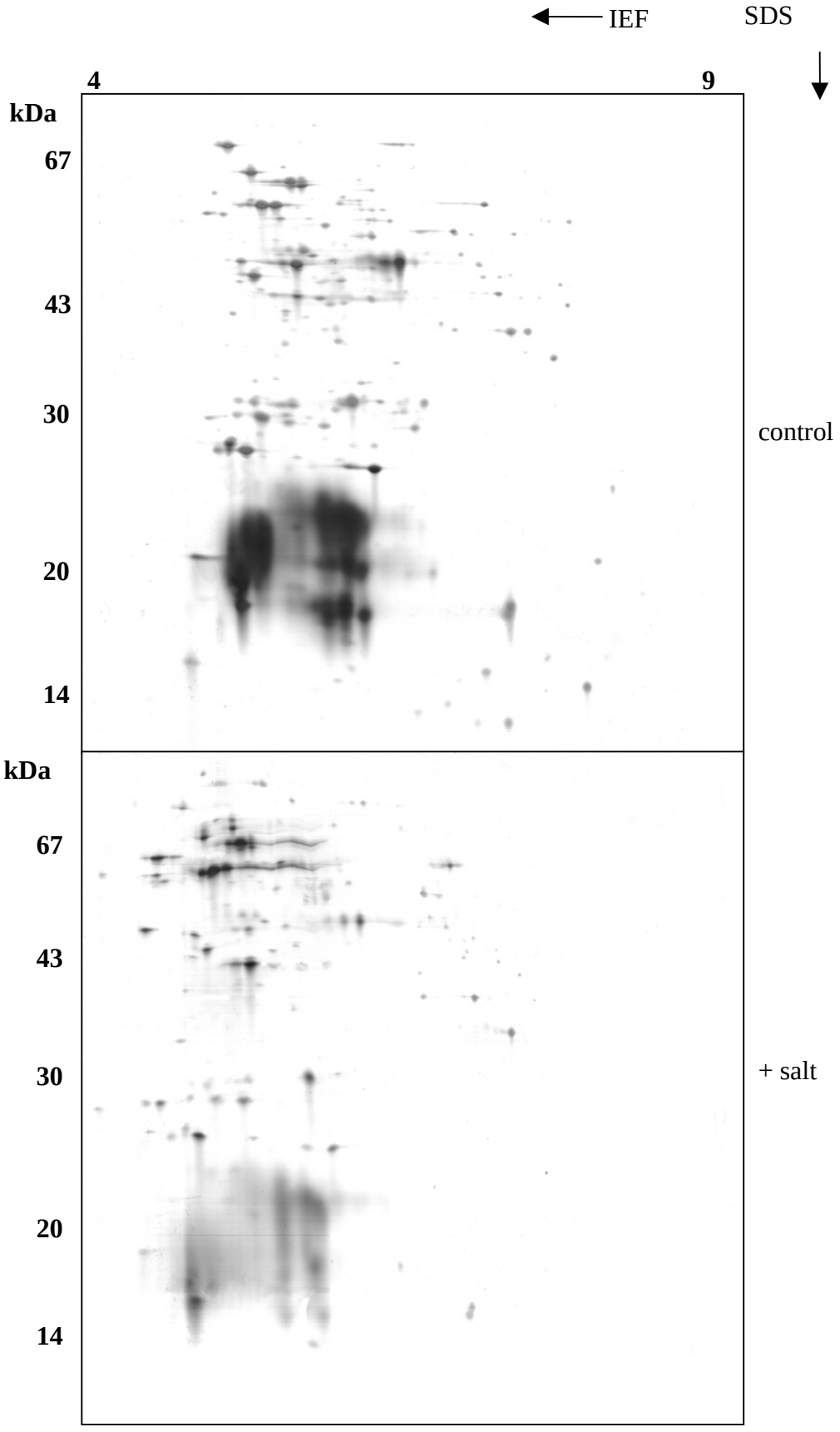
C1: Response of *N. commune* DRH1 protein expression profile to different kind of stress. - Early response (3 hours of particular stress). For details on stress conditions see chapter 2.



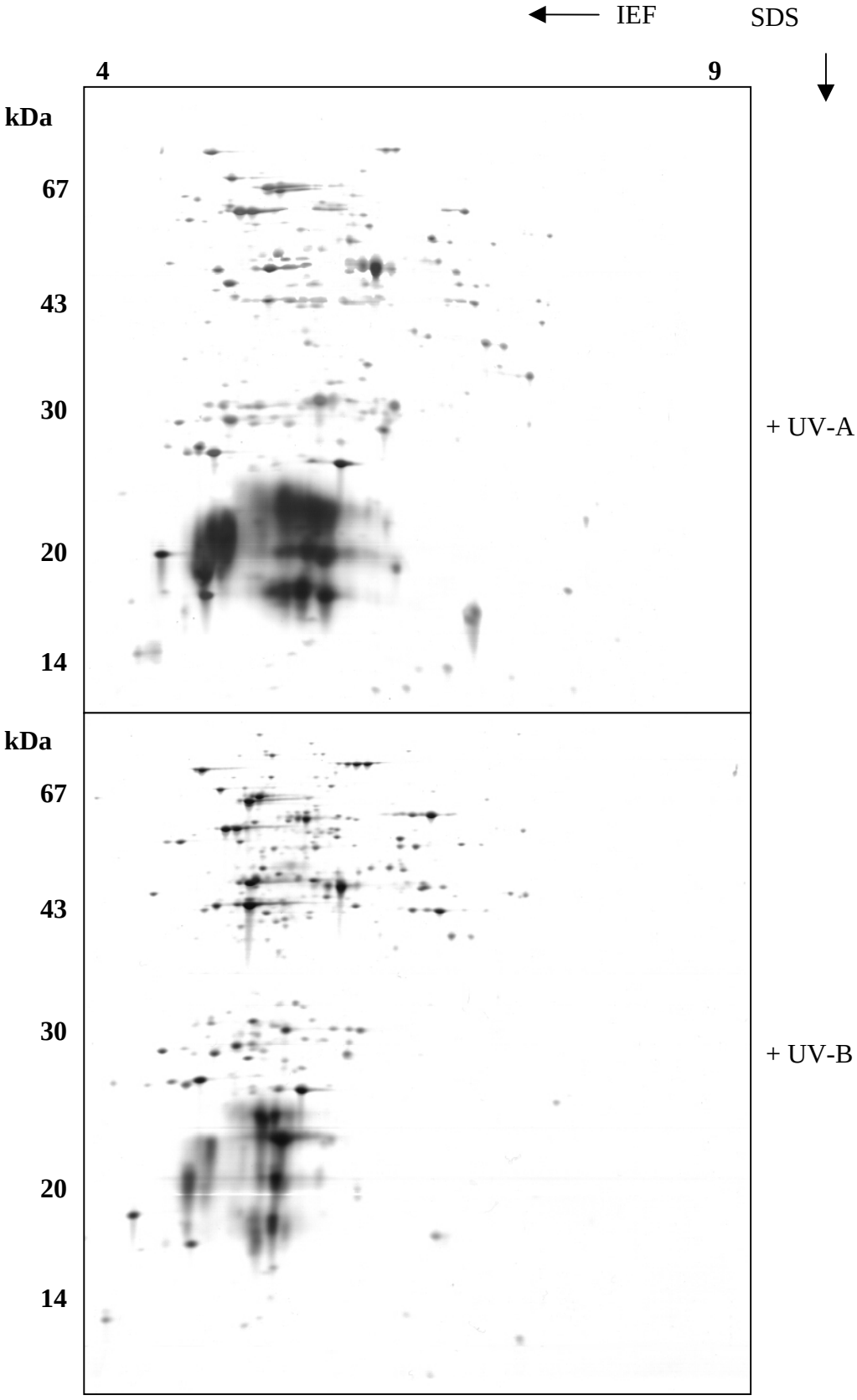
C1: Response of *N. commune* DRH1 protein expression profile to different kind of stress. - Early response (3 hours of particular stress) (continued).



C2: Response of *N. commune* DRH1 protein expression profile to different kind of stress. - Late response (1 day of particular stress).



C2: *Response* of *N. commune* DRH1 protein expression profile to different kind of stress. - Late response (1 day of particular stress) (continued).



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Ehling-Schulz, M., and S. Scherer. 1999. UV Protection in Cyanobacteria. Eur. J. Phycol. **34**: 329 – 338.

Ehling-Schulz, M., W. Bilger, and S. Scherer. 1997. UV-B-induced synthesis of photoprotective pigments and extracellular polysaccharides in the terrestrial cyanobacterium *Nostoc commune*. J. Bacteriol. **179**: 1940 - 1945.

Ehling-Schulz, M., S. Schulz, A. Görg, and S. Scherer. 2000. Semiquantitative, differential 2D Display of the dynamics of UV-B triggered *versus* growth-cycle dependent proteome changes in the terrestrial cyanobacterium *Nostoc commune*. submitted

Bilger, W., M. Bohuschke, and M. Ehling-Schulz. 1997. Annual time courses of the contents of carotenoids and UV-protective pigments in the cyanobacterium. *Nostoc commune*. Bibl. Lichenol. **67**: 223 – 234.

Joardar, V., M. Ehling-Schulz, S.C. Smith, S. Scherer, and M. Potts. Isolation, characterization and expression of a class II fructose-1,6-biphosphat aldolase gene from *Nostoc commune* UTEX 584 (Cyanobacteria), in preparation.

Erklärung

Ehling-Schulz, M., and S. Scherer (1999) UV Protection in Cyanobacteria. Eur. J. Phycol. 34: 329 - 338.

Die experimentellen Arbeiten wurden von Frau Monika Ehling-Schulz durchgeführt. Die Veröffentlichung wurde hauptsächlich von ihr geschrieben.

Ehling-Schulz, M., W. Bilger, and S. Scherer (1997) UV-B-induced synthesis of photoprotective pigments and extracellular polysaccharides in the terrestrial cyanobacterium *Nostoc commune*. J. Bacteriol. 179: 1940 - 1945.

Die experimentellen Arbeiten wurden von Frau Monika Ehling-Schulz durchgeführt. Nur die HPLC Analyse der Carotinoide wurde von Dr. Wolfgang Bilger, Department of Biology and Natural Conservation, Agricultural University of Norway durchgeführt. Die Veröffentlichung wurde hauptsächlich von Frau Monika Ehling-Schulz geschrieben.

Ehling-Schulz, M., S. Schulz, A. Görg, and S. Scherer (Submitted) Semiquantitative, differential 2D Display of the dynamics of UV-B triggered versus growth-cycle dependent proteome changes in the terrestrial cyanobacterium *Nostoc commune*.

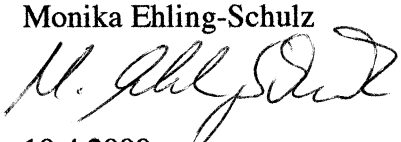
Dipl.-Inf. Stefan Schulz, München, entwickelte eine Database Application für Microsoft Access, welche für die Analyse der 2D Experimente benutzt wurde. Die Proteom-Gruppe von Prof. Angelika Görg, Lehrstuhl für Allgemeine Lebensmitteltechnologie, führte Monika Ehling-Schulz in das IPG Dalt System ein und begleitete die 2D Experiments mit durchgehenden technical support. Alle experimentellen Arbeiten wurden von Frau Monika Ehling-Schulz durchgeführt. Die Veröffentlichung wurde hauptsächlich von ihr geschrieben.

Bilger, W., M. Bohuschke, and M. Ehling-Schulz (1997) Annual time courses of the contents of carotenoids and UV-protective pigments in the cyanobacterium *Nostoc commune*. Bibl. Lichenol. 67: 223 - 234.

Dieses paper ist ein Ergebnis einer gemeinsamen Idee von Wolfgang Bilger und Frau Ehling-Schulz. Matthias Bohuschke, Julius von Sachs Institut für Biowissenschaften, Universität Würzburg, führte die Freiland-Experimente durch, Monika Ehling-Schulz führte die Labor-Experimente durch. Die Veröffentlichung wurde von Frau Ehling-Schulz und Herrn Bilger gemeinsam verfasst.

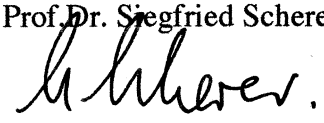
Für die Richtigkeit dieser Angaben:

Monika Ehling-Schulz



10.4.2000

Prof. Dr. Siegfried Scherer



8.4.2000

Curriculum vitae

Monika Ehling-Schulz

Education

PhD in Microbiology (Dr. rer. nat.), July 2000,
Institut für Mikrobiologie / FML TU München
Dissertation: Physiological and Protein-Biochemical Analysis of UV-A and UV-B Tolerance of the Terrestrial Cyanobacterium *Nostoc commune.*
Advisor: Prof. Dr. Siegfried Scherer

Research studies in biotechnology (Aufbaustudium), November 1996,
TU München

Diploma in Agricultural- and Plant-Science (Plant physiology, plant biochemistry, microbiology; Dipl.-Ing. agr), May 1994,
TU München
Diploma thesis: Subcloning and Sequencing of a Desiccation Stress regulated Part of DNA from *Nostoc commune*.
Advisor: Prof. Dr. Siegfried Scherer

Working experience

Undergraduate Research Assistant, November 1991 – April 1992,
Facultad de Ciencias Agrarias y Forestales, Universidad de Chile, Santiago de Chile
Studies on the influence of phytohormones on growth of seedless table grapes.

Agricultural practical training, May 1991 – October 1991,
Horticultural farm, Fa. Zotz, München.

Personal details

Date of birth: 23th January 1968

Marital status: married to Stefan Schulz, one son, 6 year old

Nationality: German