Research Articles

Effects of Lindane on the Photosynthetic Apparatus of the Cyanobacterium *Anabaena*

Fluorescence Induction Studies and Immunolocalization of Ferredoxin-NADP+ Reductase

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Abstract

Intention, Goal, Scope, Background. Cyanobacteria have the natural ability to degrade moderate amounts of organic pollutants. However, when pollutant concentration exceeds the level of tolerance, bleaching of the cells and death occur within 24 hours. Under stress conditions, cyanobacterial response includes the short-term adaptation of the photosynthetic apparatus to light quality, named state transitions. Moreover, prolonged stresses produce changes in the functional organization of phycobilisomes and in the core-complexes of both photosystems, which can result in large changes in the PS II fluorescence yield. The localization of ferredoxin-NADP+ reductase (FNR) at the ends of some peripheral rods of the cyanobacterial phycobilisomes, makes this protein a useful marker to check phycobilisome integrity.

Objective. The goal of this work is to improve the knowledge of the mechanism of action of a very potent pesticide, lindane (γ-hexaclorociclohexane), in the cyanobacterium *Anabaena sp*., which can be considered a potential candidate for bioremediation of pesticides. We have studied the effect of lindane on the photosynthetic apparatus of *Anabaena* using fluorescence induction studies. As ferredoxin-NADP+ reductase plays a key role in the response to oxidative stress in several systems, changes in synthesis, degradation and activity of FNR were analyzed. Immunolocalization of this enzyme was used as a marker of phycobilisome integrity. The knowledge of the changes caused by lindane in the photosynthetic apparatus is essential for rational further design of genetically-modified cyanobacteria with improved biorremediation abilities.

Methods. Polyphasic chlorophyll *a* fluorescence rise measurements (OJIP) have been used to evaluate the vitality and stress adaptation of the nitrogen-fixing cyanobacterium *Anabaena* PCC 7119 in the presence of increasing concentrations of lindane. Effects of the pesticide on the ultrastructure have been investigated by electron microscopy, and FNR has been used as a marker of phycobilisome integrity.

Results and Discussion. Cultures of *Anabaena sp*. treated with moderate amounts of lindane showed a decrease in growth rate followed by a recovery after 72 hours of pesticide treatment. Concentrations of lindane below 5 ppm increased the photosynthetic performance and activity of the cells. Higher amounts of pesticide caused a decrease in these activities which seems to be due to a non-competitive inhibition of PS II. Active PS II units are converted into non-Q_A reducing, so called heat sink centers. Specific activity and amount of FNR in lindane-treated cells were similar to the values measured in control cultures. Release of FNR from the thylakoid after 48 hours of exposure to 5 ppm of lindane towards the cytoplasm was detected by immunogold labeling and electron microscopy.

Conclusions. From these results, we conclude that the photosynthetic performance and activity of the cells are slightly increased in the presence of lindane up to 5 ppm. Moreover, in those conditions, lindane did not produce significant changes in the synthesis, degradation or activity of FNR. The high capability of *Anabaena* to tolerate lindane makes this cyanobacterium a good candidate for phytoremediation of polluted areas.

Recommendation and Outlook. The results of this study show that cultures of *Anabaena* PCC 7119 tolerate lindane up to 5 ppm, without significant changes in the photosynthetic vitality index of the cells. However, a slight increase in phycobiliprotein synthesis is observed, which is related to total protein content. This change might be due to degradation of proteins less stable than phycobiliproteins. An identification of the proteins with altered expression pattern in the presence of the pesticide remains the subject of further work and will provide valuable information for the preparation of strains which are highly tolerant to lindane.

Keywords: *Anabaena*, cyanobacterium; Chl *a* fluorescence; core complexes of PS I and PS II; cyanobacteria; ferredoxin-NADP+ reductase; fluorescence induction studies; immunolocalization; JIP-test; lindane; PEA (Plant Efficiency Analyzer); phycobilisomes; stress adaptation; vitality

Abbreviations: CS, cross section of the sample; DI, dissipated flux; ET, energy flux for electron transport; F_0, F_M, initial and maximum Chl *a* fluorescence; J, I, intermediate steps of Chl *a* fluorescence rise between F_0 and peak (P); k_N, non-photochemical, de-excitation rate constant; k_P, photochemical de-excitation rate constant; φ_{Eo}, probability that an absorbed photon will move an electron into the electron transport chain; φ_{Po}, maximum quantum yield of primary photochemistry; ψ_o, efficiency with which a trapped exciton can move an electron into the electron transport chain; Q_A, primary bound plastoquinone; Q_B, secondary bound plastoquinone; RC, reaction center; TR, energy flux for trapping

Introduction

Cyanobacteria are phototrophic organisms which constitute the most important producers of organic carbon and fixed nitrogen in fresh and sea-waters contributing to the fertility of natural and cultivated habitats (Fay 1992). To perform oxygenic photosynthesis, cyanobacteria contain chlorophyll *a* and phycobilisomes which deliver most light energy absorbed by phycobiliproteins to PS II reaction centers under normal growth conditions (Wang and Myers 1973). Structure and composition of phycobilisomes have been studied extensively (Glazer 1989, Sidler 1994). These supramolecular complexes are constructed from a core substructure containing phycobiliproteins and peripheral rods that are arranged in a hemidiscoidal fashion around that core. Ferredoxin-$NADP_+$ reductase (FNR) is localized at the end of some peripheral rods of the phycobilisomes through the interaction of a CpcD-like domain found at the N-terminus of this enzyme (van Thor et al. 1999). This particular organization of the photosynthetic apparatus, together with the ability of some strains to fix nitrogen, are both decisive for the ecological success of cyanobacteria.

Moreover, cyanobacteria have developed diverse mechanisms for sensing and acclimating to changes in their environment. Differences in light quality and intensity, nutrient availability or the presence of toxic substances induce responses that include an alteration of light-harvesting complexes synthesis and degradation, changes in the ratio or substitution of soluble redox carriers and heterocyst differentiation, among other processes (Bryant 1994). The extreme adaptability of some cyanobacterial strains makes them able to tolerate or even metabolize moderate doses of several insecticides and pesticides (DaSilva et al. 1975, Kuritz and Wolk 1995). Among the pesticides found in rice fields and fresh water ponds, lindane is one of the most toxic to the cells. Biodegradation of lindane by cyanobacteria has been reported in *Anabaena* cultures supplemented with 0.5 ppm of the pesticide and it seems to be dependent of a functional *nir* operon (Kuritz et al. 1997). However, higher concentrations of lindane are toxic to the cyanobacteria, as well as seen to produce a drastic inhibition of chlorophyll and protein contents (Suresh Babu et al. 2001). Data available on the tolerance of lindane by cyanobacteria are controversial. Performance of the cells in the presence of the pesticide seems to be dependent on different parameters, such as the cyanobacterial strain, the presence of nitrate in the medium or the starting density of the culture (Das and Singh 1978, Suresh Babu et al. 2001). Moreover, little is known about the influence of lindane on the metabolism of cyanobacteria. Since the photosynthetic apparatus is tightly connected to the other principal metabolic paths, chlorophyll fluorescence signals can provide rapid information on both photosynthesis and the overall acclimation status of the cells (Campbell et al. 1998, Strasser et al. 1995). Under stress conditions, cyanobacterial response includes short-term adaptation of the photosynthetic apparatus to light quality, which are known as state transitions. Moreover, prolonged stresses produce changes in the functional organization of phycobilisomes and in the core-complexes of both photosystems, which can result in large changes in the PS II fluorescence yield (Strasser et al. 2000).

In this work, polyphasic chlorophyll *a* fluorescence rise measurements (OJIP) have been used to evaluate the vitality and stress adaptation of the nitrogen-fixing cyanobacterium *Anabaena* PCC 7119 in the presence of increasing amounts of lindane. Effects of the pesticide on the ultrastructure have been investigated by electron microscopy and FNR has been used as a marker of phycobilisome integrity.

An optimized use of cyanobacteria in bioremediation requires an extensive study of the effects of several xenobiotics in order to characterize the cell response. Detailed analysis of fluorescence patterns can be used to discriminate different modes of action of environmental pollutants (Brack and Frank 1998). Identification of pesticide targets and changes in protein synthesis and degradation will be essential in biotechnology to prepare genetically engineered strains which are more resistant to pesticides and demonstrate enhanced degradative capabilities.

1 Materials and Methods

1.1 Growth conditions and pesticide treatments

Anabaena sp. PCC 7119 was grown at 25°C in BG-11 medium (Rippka et al. 1979) supplemented with a filtered-sterilized solution of $NaHCO_3$ to a final concentration of 2.54 g/l. Cultures were placed in 500 ml sealed bottles containing 200 ml of medium and shaken at 100 rpm during 72 h with illumination of 6 watt/m^2.

Pesticide treatments were carried out in cultures in logarithmic phase of growth adding lindane (γ-hexachlorocyclohexane, Sigma-Aldrich Co.) at increasing concentrations ranging from 1 to 15 ppm. To prevent pesticide degradation, stock solutions were prepared just before each experiment dissolving lindane in 100% ethanol. The effect of ethanol is negligible in our experiments. Bacterial growth was monitored by measuring optical density at 600 nm. The amount of chlorophyll *a* was determined by extraction with methanol according to Mackinney (1941) and expressed in milligrams per milliliter of *Anabaena* culture.

1.2 Fluorescence measurements

Chlorophyll *a* fluorescence transients were measured by a PEA fluorometer (Plant Efficiency Analyzer, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) using an actinic light intensity of 600 Wm^{-2} red light with a center excitation wavelength of 650 nm. The actinic light of the PEA was provided by an array of six light emitting diodes (LED) which were focused in the bottom of the bottle. Chlorophyll *a* fluorescence was detected by a photodiode located behind a long pass filter (50% transmission at 720 nm). Chlorophyll *a* fluorescence signals were recorded during 1 s with a 10 µs time resolution for the first 200 data points, then with 1 ms for the next 998 points (up to 1 second) and later with a 10 ms time resolution. All experiments were performed in 1 cm diameter vials using 2 ml of light adapted samples from different cultures of cell suspension kept in low room light. The fluorescence transients were analyzed according to the equations of the JIP-test (Strasser et al. 2000, Strasser and Tsimilli-Michael 2001) with the software package BIOLYZER writ-

ten by R. Maldonado-Rodríguez and available on the Internet (http://www.unige.ch/sciences/biologie/bioen). At different time points, such as 0.05, 0.1, 0.3 and 2 ms, the corresponding fluorescence intensities, F_1, F_2, F_3, F_4, have been extracted from the fluorescence induction curve. The value F_1 is considered to be representative for the state when all reaction centers are open and this value will be labeled as initial fluorescence F_0. The maximal fluorescence value of each fluorescence curve is extracted as well and called Fm. The values F_1 and F_3 are used for the calculation of the slope at the origin to the fluorescence kinetics and $F_4 = F_{2\,ms} = F_J$ is taken as the fluorescence value at the step J of the OJIP transient.

1.3 Cyanobacterial crude extract preparation

Cyanobacterial crude extracts were prepared from 20 ml culture during the exponential phase of growth, collected by centrifugation at 2500 x *g* and washed in 50 mM Tris-HCl buffer pH 8.0. Cells were disrupted by ultrasonic treatment and debris was pelleted by centrifugation for 20 min at 15800 x *g* in an Eppendorf centrifuge.

1.4 Biochemical analysis

Protein concentration was determined according to Bradford (1976). Phycobiliprotein content was quantified following the protocol described by Glazer (1976). Cells were harvested by centrifugation, divided into two aliquots and processed immediately for Western analysis and electron microscopy studies. Diaphorase activity of FNR was measured using DCPIP (2,6-dichlorophenol-indophenol) as an artificial electron donor, as described by Sancho et al. (1988).

1.5 Immunodetection of FNR by Western-blot

Antiserum against FNR was obtained according to Razquin et al. (1995) and further purified as follows: in 5 ml of crude serum, to which 0.9 (18% (w/v)) g of sodium sulfate was added. After incubation at room temperature for 30 min, the solution was centrifuged at 3 000 x *g* for 30 min at 25°C. Supernatant was discarded and the pellet was re-suspended in 2.5 ml of milli-Q water. A second precipitation using sodium sulfate to 14% (w/v) was performed and the resulting pellet re-suspended in 1.5 ml of milliQ water, dialyzed against 0.07 M phosphate buffer at pH 6.3 and passed through a DEAE column. Several one ml fractions were collected and the recovery of the antiserum was monitored by checking the absorbance at 280 nm.

For Western analysis, SDS-PAGE was performed with *Anabaena* crude extracts containing the soluble proteins and then electroblotted onto nitrocellulose at 70 V overnight (Bio-Rad trans-blot cell; Bio-Rad, model 200/2.0 Power supply). The membrane was incubated at room temperature for 1 h 30 min in a blocking solution containing TBS (10 mM Tris, 150 mM NaCl, pH 7.5) and 5% BSA, washed twice in TTBS (0.05% (v/v) Tween-20 in TBS) and subsequently incubated for 1 h with the primary antiserum against FNR diluted 1 : 100. After thorough washing of the membrane in TTBS, the second antibody, goat antirabbit IgG conjugated to horseradish-peroxidase (Sigma), was applied at a 1 : 100 dilution in TTBS and incubated for 1 h. Following 4 washes in TBS, peroxidase activity was assessed with 0.5 mg/ml of 3.3'-diaminobenzidine tetrahydrochloride (DAB) (Flucka Chemie) in 100 mM Tris-HCl, pH 7.5 containing 0.03% H_2O_2.

1.6 Electron microscopy and immunogold labeling

Cells were harvested by microcentrifugation at 4000 rpm for 4 min, and then fixed with 4% paraformaldehyde and 2% glutaraldehyde in 100 mM phosphate buffer (0.1 M NaH_2PO_4, 0.1 M K_2HPO_4, pH 7.4) for 2 h at room temperature. After fixation, the cells were washed four times for 10 min each in phosphate-buffer, pre-embedded in 1.5% agar at 45°C, dehydrated respectively in 70% and 100% ethanol (for 30 min each). The samples were then infiltrated sequentially in 2 : 1 (v/v) ethanol: LR White resin (Polysciences), 1 : 1 (v/v) ethanol : LR White for 30 min each, 1 : 2 (v/v) ethanol : LR White for 1 h and finally 100% LR White for 24 h at 50°C for polymerization.

Ultrathin sections were taken on nickel grids, incubated at room temperature for 2 h in 50 mM phosphate buffer containing 2% BSA, 0.05% (v/v) Tween-20, and then for 2 h with the first antibody raised against FNR diluted 1 : 50. The sections were rinsed 3 times with 0.05% Tween-20 in 50 mM phosphate buffer pH 7.0 and incubated for 1 h with secondary antibodies, goat anti-rabbit conjugated to 20 nm gold particles diluted to 1 : 30, in phosphate buffer containing 2% BSA and 0.05% Tween-20. After incubation, sections were washed 3-fold with phosphate buffer, and then fixed in 2% glutaraldehyde in PBS for 2 min (Smith et al. 1992) and finally rinsed in distilled water, stained for 10 min in 2% uranyl acetate, 5 min in Reynold's lead citrate and examined at 60 kV in a Philips M400 transmission electron microscope.

2 Results and Discussion

2.1 Growth of *Anabaena sp.* PCC 7119 in the presence of lindane

Lindane decreased the growth rate of *Anabaena* as measured by the optical density at 600 nm (**Fig. 1A**) as a function of the cultivation time and lindane concentration. Recovery of the cell growth after 24 h of treatment with 1 and 5 ppm of lindane could be observed and the cultures were seen to have reached optical densities similar to the control after 72 h. The increase in cell number was lower in cultures with 10 ppm and 15 ppm of the pesticide. Treatment with concentrations of lindane higher than 15 ppm caused partial bleaching and death of the cells after 24 h (not shown). The recovery could be due to adaptation of the *Anabaena* cells to the pesticide, together with the ability of this cyanobacterium to metabolize moderate doses of lindane. The tolerance observed in our culture conditions was much higher than that reported by Suresh Babu et al. (2001), who observed that the growth of *Anabaena* was almost completely inhibited in samples treated with 2 ppm lindane. This different behavior of the cells might be due to the use of a different strain of *Anabaena* and to important differences in the culture conditions, such as maintaining the cells in the dark for 16 h a day. An

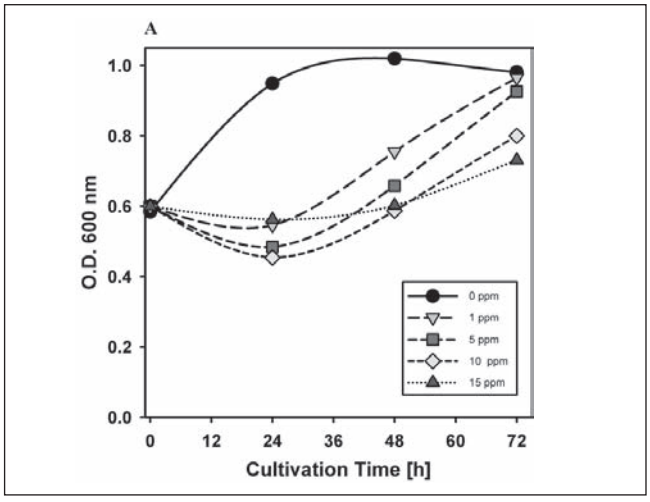

Fig. 1A: Effect of lindane on the growth of *Anabaena* PCC 7119 monitored by optical density of cell culture at 600 nm versus cultivation time and lindane concentration

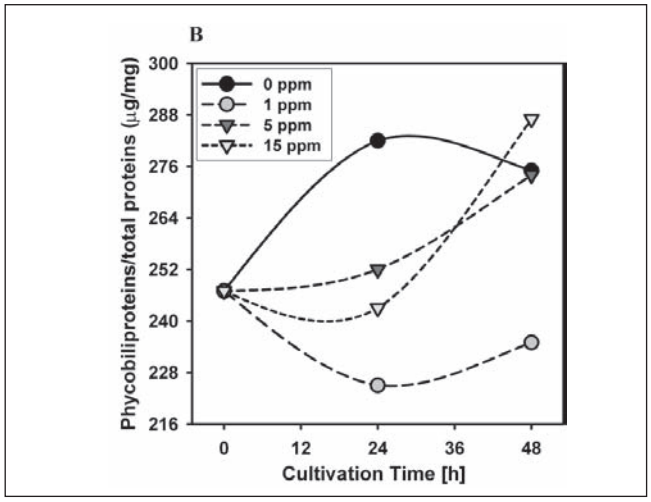

Fig. 1B: Fraction of phycobiliproteins per total proteins versus cultivation time and lindane concentration

absence of nitrate in the culture might also contribute to the lower tolerance since nitrogen fixation and heterocyst formation in cyanobacteria are inhibited by lindane (Das and Singh 1978). Consequently, degradation of low doses of lindane in cyanobacteria was faster and more efficient in the presence of nitrate (Kuritz and Wolk 1995).

2.2 Effects of lindane on the chlorophyll a fluorescence

Chlorophyll *a* fluorescence analyses are frequently used to determine the photosynthetic capacity and vitality of plant material. In cyanobacteria, the chlorophyll *a* fluorescence transients are more difficult to measure accurately due to a very large basal fluorescence (F_0 measured at 50 µs) and a contribution of PS I and phycobilin fluorescence (Strasser et al. 1995). Therefore, the influence of the phycobiliprotein content and the variable ratio between PS I and PS II complexes should be taken into account for the interpretation of fluorescence signals (Campbell et al. 1998). This influence of PS I fluorescence corresponds for each case to a proportionality factor of the signal F_0. The importance of this factor cancels or becomes low due to the fact that 1) the performance index is built out of the ratio of expressions for the same sample and 2) each sample is compared relatively to the control. That means the indicated values of φ_{Po} are underestimated, but the relative variations of φ_{Po} are accurate. Moreover, it is important to distinguish between the photochemical and non-photochemical events which influence the fluorescence signal and which determine the photosynthetic performance and the vitality of the cells (**Fig. 1B**).

The use of a Plant Efficiency Analyzer (PEA) allows measuring the polyphasic chlorophyll *a* fluorescence transient in *Anabaena* cultures in the time range of 50 µs to 1 second in the presence of increasing amounts of lindane as a function of incubation time (**Fig. 1C**).

The measured raw data of the OJIP fluorescence transients of *Anabaena* are shown in **Fig. 2A**. The typically polyphasic character of the transient was clearly visible by plotting it on a logarithmic time scale. The high time resolution of the

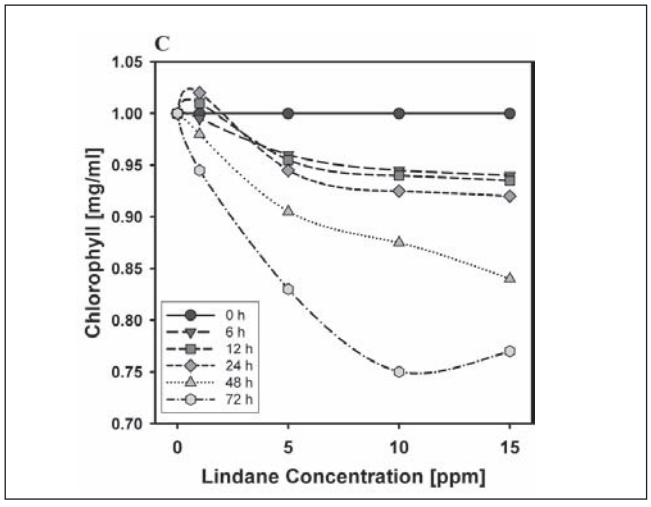

Fig. 1C: Chlorophyll concentration in cell suspension (µg/ml) versus lindane concentration and incubation time

OJIP transient measured with the PEA instrument (10 µs per data point) makes it possible to distinguish between changes due to primary photochemical reactions or purely non-light (dark) dependent reactions.

Fig. 2B (top) shows the fluorescence transients normalized between F_0 and F_J (or F_2 ms) of the fluorescence transient (corresponding to a normalization between 50 µs and 2 ms) after 24 h of cultures incubated with lindane of 0 to 15 ppm. In the first phase from 0 to 2 ms, the fluorescence signal was dominated by the photochemical single turn over events (producing reduced Qa) and several dark reactions led to the fully reduced state of PS II after 2 ms, accumulating $Q_a^-Q_b^{2-}$ and reduced plastoquinone. The difference of each fluorescence transient (with 1, 5, 10, 15 ppm lindane) minus the control without lindane is shown in Fig. 2B (bottom) with a gain of 2. The induction curves were strictly proportional one to the other in the time range from 0 to about 5 ms (the differences are zero), indicating that no qualitative changes in the photochemical reactions occurred. However, between 50 and 100 ms, a relative increase (relative to the control)

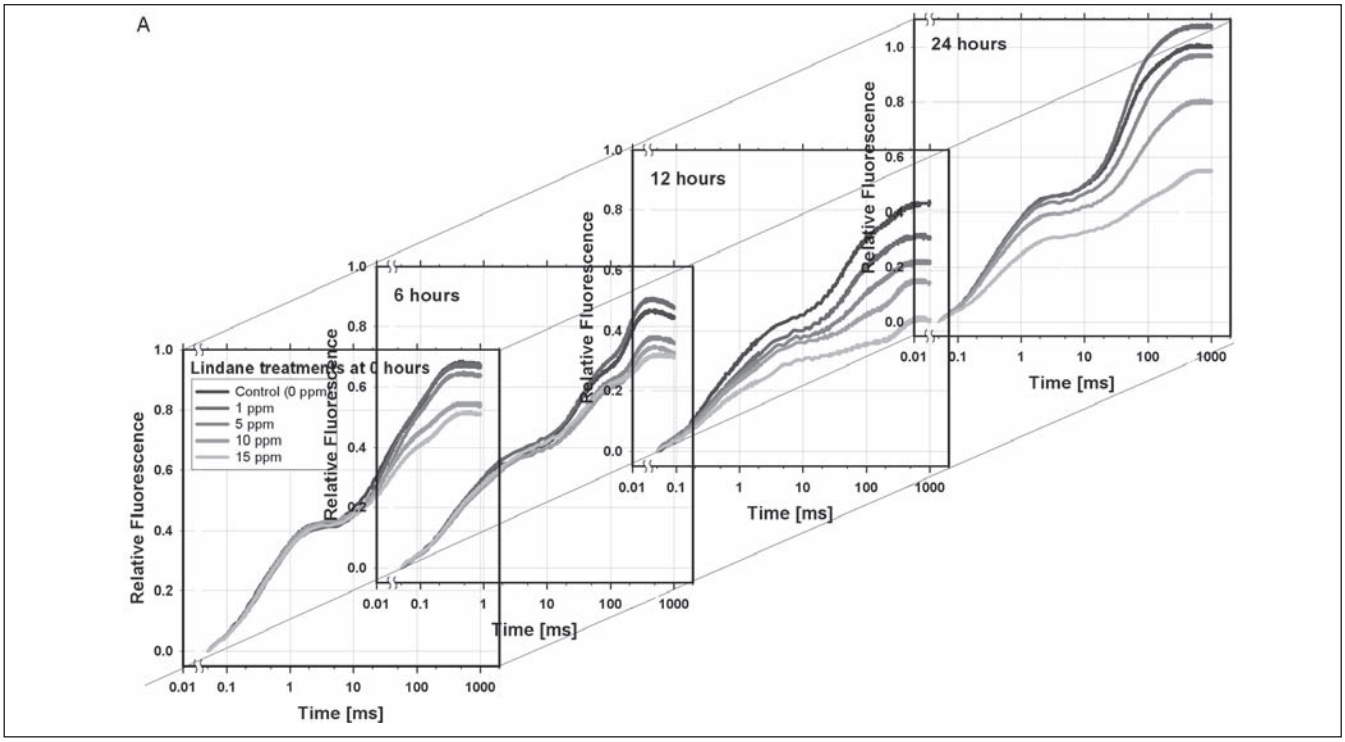

Fig. 2A: Relative fluorescence measurements ($V = (F - F_0)/(F_M - F_0)$ taken with the PEA portable fluorometer instrument. The OJIP fluorescence transients (measured with 100% light), plotted on a logarithmic time scale, were measured on cultures treated with different lindane concentrations (0 to 15 ppm) for different incubation times (0 to 24 hours)

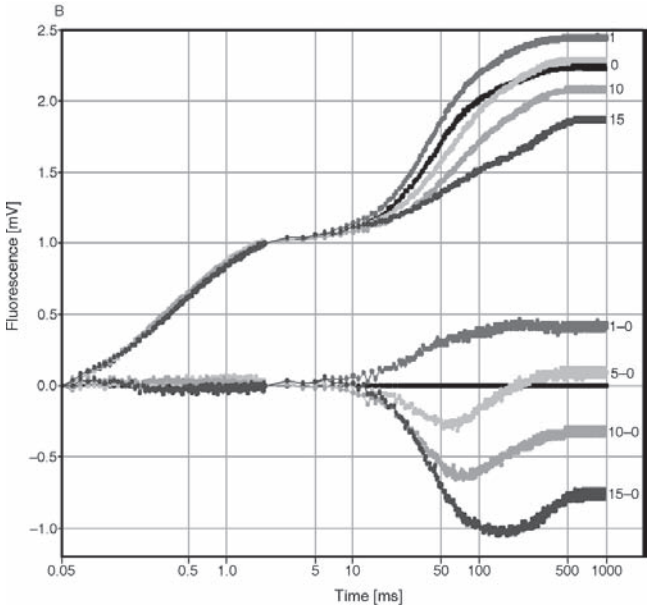

Fig. 2B: Top: Fluorescence transients normalized between F_0 and F_J of the fluorescence transient after 24 h exposure to 0, 1, 5, 10 and 15 ppm lindane. Bottom: Differences of each fluorescence transient minus the control, without lindane, plotted with a gain of 2

for the sample with 1 ppm lindane was observed. Lindane from 5 to 15 ppm produced a gradual decrease.

Therefore, a primary action of lindane is located in the biochemical dark reactions. The chlorophyll content per ml cell suspension (cf. Fig. 1C) showed the same behavior for the incubation of 24 h and a lower chlorophyll accumulation in the cell culture for any lindane concentration from 1 to 15 ppm. The question arises about the distinction of the quality and quantity of biomass. A slowed down biomass production did not imply that the produced cells were of lower quality. The notion vitality of a cell was independent of the growth rate of these cells. Several vitality parameters have been defined, here we used the so-called photosynthetic performance index PI_{abs} (Strasser et al. 2000, Strasser and Tsimilli-Michael 2001), which is a measure for the photosynthetic performance of the cells on an equal chlorophyll basis (where abs stands for the absorption of light by the chlorophylls). The performance index PI_{abs} is constructed in analogy to the Nernst equation describing the redox potential in physicochemistry. PI_{abs} is the product of several expressions of the form $x_i/(1 - x_i)$ where x_i stands for the fractions of reaction center chlorophyll per total chlorophyll or excitons trapped by the reaction center per total photons absorbed, or energy conserved in the electron transport beyond Qa^- per exciton trapped by the reaction center. Therefore, the performance index has the following form:

$$PI_{abs} = \gamma/(1-\gamma) * \varphi_{Po}(1-\varphi_{Po}) * \psi_o/(1-\psi_o) \quad (1)$$

where

γ = RC chlorophyll/total antenna chlorophyll, and
$\gamma/(1-\gamma)$ = RC chlorophyll/total antenna chlorophyll = RC/ABS
φ_{Po} = maximal quantum yield of primary photochemistry = Trapping per absorption = TR_0/ ABS = $k_P/(k_P + k_N)$ estimated by F_V/F_M

Therefore,

$\varphi_{Po} / (1-\varphi_{Po})$ = $TR_0/(ABS - TR_0) = k_P/k_N = F_V/F_0$, where k_P and k_N are de-excitation rate constants for the photochemical or non-photochemical events, respectively

ψ_o = maximum yield of electron transport beyond Qa^- per exciton trapped

= $ET_0 / TR_0 = (F_M - F_3)/(F_M - F_0)$ and

φ_{Eo} = $\varphi_{Po} \cdot \psi_o = ET_0/ABS$

The logarithm of the performance index is therefore the sum of the three components representing the density of RCs, the efficiency of the light reactions and the efficiency of the dark reactions. The variation of performance index PI_{abs} is shown as a three-dimensional plot in **Fig. 3** for the lindane concentrations of 0 to 15 ppm and for an incubation time from 0 to 72 h. Fig. 3 shows the antagonistic (lindane concentration above 5 ppm) and synergistic (lindane concentration below 5 ppm) effects of the photosynthetic performance PI with the lindane concentration and the incubation time, representing the dose of lindane exposure. Low lindane concentrations (below 5 ppm) increased the performance of the sample, while lindane concentrations higher than 5 ppm decreased the performance as a function of time and as a function of the lindane concentration. Such an approach was suitable for screenings of dilutions of soil extracts, in analogy to the lindane concentration used here. The performance index is a multiparametric expression and the question arises as to which component of the photosynthetic system was the target of lindane.

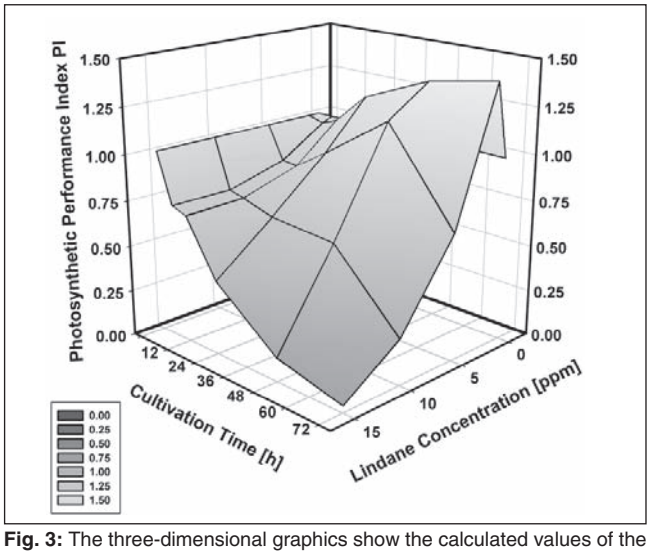

Fig. 3: The three-dimensional graphics show the calculated values of the performance index of PI_{ABS} as a function of the different combinations of time and of the lindane concentration. The control culture (0 ppm, at time 0 h) has been normalized to 1 and used as the comparative reference

Fig. 4A,B shows the maximum quantum efficiency of the primary reactions of photochemistry φ_{Po} and the quantum yield for electron transport φ_{Eo} beyond Qa^- (for definitions see above). Both expressions showed a similar trend with an increase from 0 to 5 ppm lindane and a decrease for higher lindane concentrations. Therefore, not only the dark reactions but also the apparent maximum quantum yield of primary photochemistry φ_{Po} were influenced by lindane. The expression φ_{Po} can be deconvoluted into the two structure-

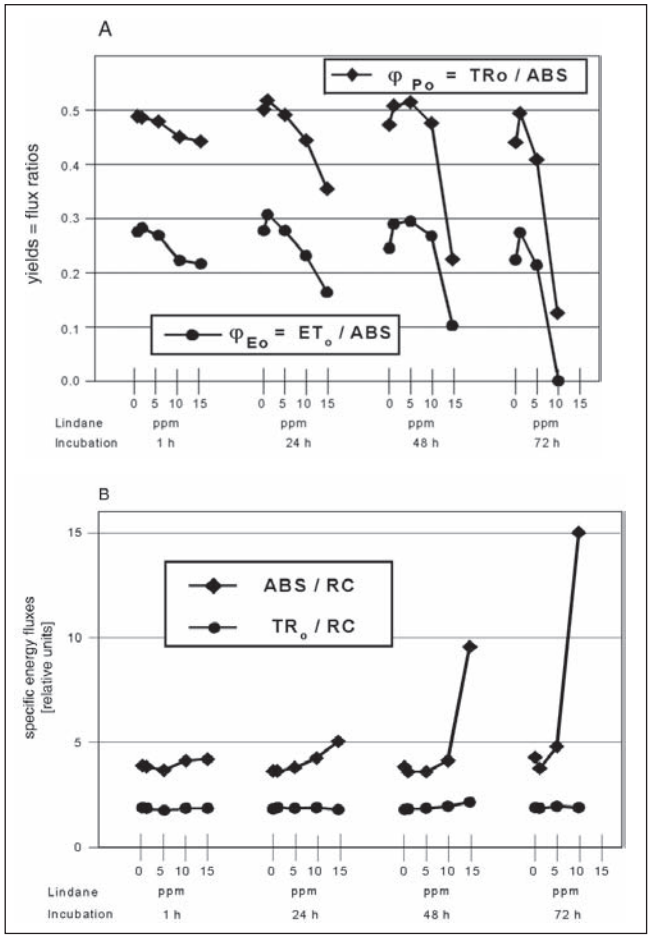

Fig. 4A: The maximum quantum yield of primary photochemistry φ_{Po} and of electron transport φ_{Eo} beyond Qa^- (bottom) at different times and lindane concentrations. **B:** Specific energy fluxes measured as absorption flux per reaction center (ABS/RC) and trapping flux per reaction center (TR_0/RC)

dependent de-excitation rate constants for photochemical events k_P and for non-photochemical events k_N (Havaux et al. 1991). The relative rate constant can be expressed with the fluorescence measurements as follows:

$k_N + k_P$ is proportional to $1/F_0$

k_N is proportional to $1/F_M$; therefore,

k_P is proportional to $(1/F_0 - 1/F_M)$

In this case, k_P decreased as a function of time and lindane concentration while k_N increased. However, k_N increased less than k_P decreased so that the sum of all rate constants k_N+k_P decreased as well. The mechanism for that could be elucidated by **Fig. 5**.

Here, the specific energy fluxes, such as absorption flux per reaction center (ABS/RC) and trapping flux per reaction center (TR_0/RC) were shown. According to the JIP test (Strasser et al. 2000), these expressions can be calculated with the experimental signals as follows:

$TR_0/RC = (F_{300\mu s} - F_{50\mu s})/(F_{2ms} - F_{50\mu s})$ and (2)

$ABS/RC = (TR_0/RC) / (TR_0/ABS) = (TR_0/RC) / (F_V/F_M)$ (3)

Even for high lindane concentrations and long incubation times the trapping flux per active RCs remained nearly unchanged.

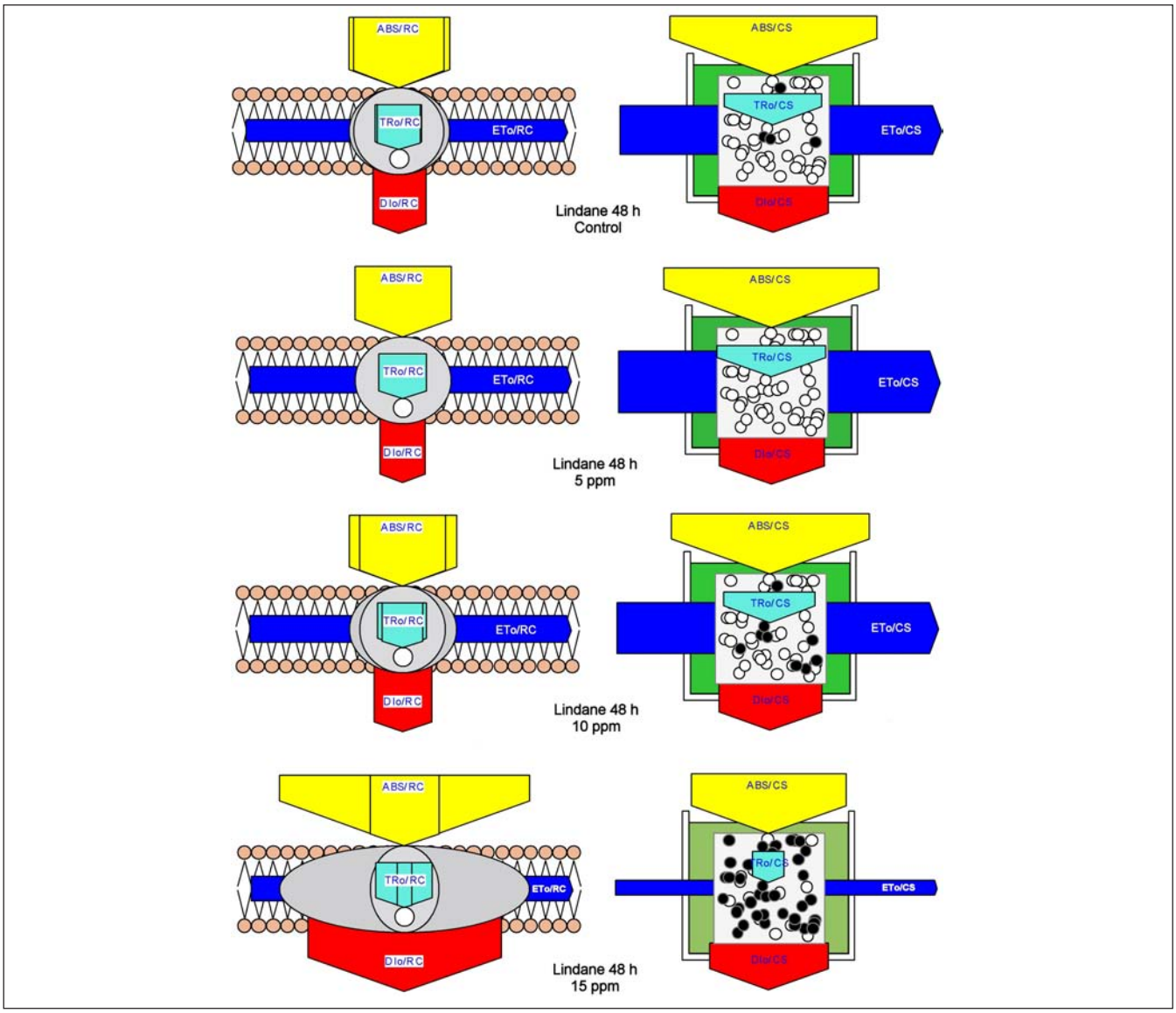

Fig. 5: Cell suspension pipeline model (right side) of the phenomenological or appearance energy fluxes (absorption, trapping, dissipation and electron transport) per cross-section and membrane pipeline model (left side) of specific energy fluxes per reaction center. The width of each arrow corresponds to the intensity of the flux. For details see text

However, the apparent antenna size measured as total absorption per active RC increased with time and lindane concentration. As the chlorophyll concentration in the samples decreased, one has to assume that the fraction of active (in the sense of Qa reducing) RCs was strongly decreasing under lindane incubation concentrations above 5 ppm.

The constellation of the specific energy fluxes (absorption, trapping, dissipation, electron transport) ABS, TR_0, DI_0 and ET_0 per RC can be represented with the membrane pipeline model (left side of Fig. 5), and the same apparent or phenomenological fluxes, although per ml of cell-suspension or cross-section (CS), was shown with the cell-suspension pipeline model (right side of Fig. 5). The incubation time was 48 h and the lindane concentrations were (from top to bottom) 0, 5, 10, 15 ppm. The width of each arrow represents the intensity of the respective energy flux. Each white small circle indicates active (Qa reducing) RCs and each black small circle represents inactive (non-Qa reducing heat sink or so-called silent) RCs (Krause and Weis 1991, Krüger et al. 1997). In the membrane model, the outer oval corresponds to all absorbing pigments per one active RC (small circle) and the inner oval indicates the absorbing pigments, which belong to that active RC. Only the active RCs are responsible for the electron transport per RC or per cross-section. The presented models were calculated with the experimental data. The membrane model showed clearly that the activity of an active PS II expressed, e.g. as electron transport ET_0/RC, was quasi-independent of the lindane concentration. However, the apparent activity, ET_0/CS, of the cells per cross-section (or ml of cell suspension) increased up to a lindane concentration of 5 ppm and decreased sharply for higher lindane concentrations. The decrease in biological activity seemed to be due to a non-competitive inhibition of the PS II multienzyme complex, which transforms active RCs into non-Qa-reducing heat sink centers. The color intensity of the suspension in the model is calculated as well and it repre-

sents the chlorophyll concentration of the cell culture or ABS/CS. For lindane concentrations higher than 10 ppm and incubation times of more than 24 h, a fast additional step (K step) (Strasser et al. 2000) was observed in the fluorescence rise leading to a polyphasic transient OKJIP. The appearance of the K step (data not shown) at about 250 to 300 µs is an indication of a partial uncoupling of the oxygen evolving system from the electron transport chain (Strasser 1997, Srivastava et al. 1997).

2.3 Effects on the phycobilisome and ferredoxin-NADP+ reductase

Since phycobilisomes (PBS) carry out the main light-harvesting in cyanobacteria, the effects of the pesticide in the structure and composition of PBS will determine the viability and degrading ability of the cultures. Although the polypeptide composition of PBS varies widely among strains of cyanobacteria, three classes of polypeptides are always present in those macromolecular complexes: phycobiliproteins, linker polypeptides and PBS-associated proteins, such as Ferredoxin-NADP+ reductase (FNR), encoded by the *petH* gene (Sidler 1994). Phycobiliprotein content per total protein indicated that there was not a substantial degradation of phycobiliproteins under the conditions tested (cf. Fig. 1B). A small increase of this ratio was observed after 24 and 48 h of exposure to concentrations of lindane of 5 ppm and higher, probably due to degradation of proteins less stable than phycobiliproteins. Identification of the proteins, which suffer earlier degradation, will be required to design more resistant *Anabaena* strains.

Although binding of FNR to the phycobilisome is a common characteristic among cyanobacteria (Bryant 1994, Fillat et al. 1993, van Thor et al. 1999), its presence in the complex affected neither the distribution of the excitation energy of phycobilisome-absorbed light between both photosystems nor the occurrence of light-state transitions (van Thor et al. 1999). Investigation of changes in the synthesis of FNR and its enzymatic activity are of interest for different reasons: 1) dark and salt stresses regulate transcript levels of *petH* (van Thor et al. 1999); 2) Activity of FNR decreases significantly in low light levels or dark (Fillat et al. 1991); 3) Lindane has been reported as an oxidative stressing agent (Banerjee et al. 1999). In *E. coli*, FNR is part of the *soxRS* regulon and its expression is considerably increased as a response against oxidative stress (Liochev et al. 1994). In higher plants, however, treatment with the redox cycling herbicide methyl viologen (paraquat) causes release of FNR from the thylakoid membranes (Palatnik et al. 1997).

Changes in the specific activity of FNR were negligible in the presence of lindane (data not shown). The amount of FNR in control and pesticide-exposed cultures was monitored by Western blot analysis. **Fig. 6** shows that there were no variations in the amount of FNR after 48 h of exposure to lindane. The N-terminal domain of the 49 kDa FNR, which serves to attach the flavoprotein to the phycobilisome, seemed to be quite unstable and becomes quickly degraded even in the presence of protease inhibitors during FNR purification (Martínez-Julvez et al. 1996). However, under our conditions, it was remarkable that the observed band pattern was similar even in pesticide-treated cells, with the 49 kDa being the main form,

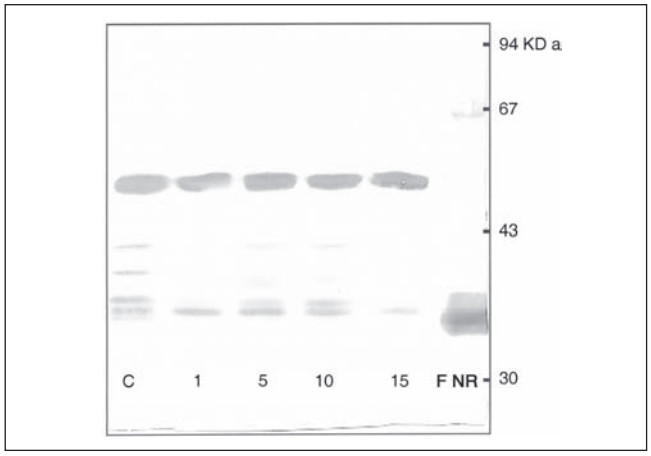

Fig. 6: Western blot of crude extracts from *Anabaena* PCC 7119 grown for 48 h in the presence of 1, 5, 10 and 15 ppm of lindane (lanes 2–5); lane 1: control without lindane (C); lane 6: pure FNR. Nitrocellulose membrane was immunostaining with primary antiserum against FNR and goat antirabbit IgG conjugated to horseradish-peroxidase

followed by the processed protein of 36 kDa and intermediate, partially digested forms being observed to a lower extent.

2.4 Immunolocalization of FNR in control and pesticide treated cells

The position of FNR in the phycobilisome makes this protein a good probe to monitor the integrity of the photosynthetic apparatus through immunolocalization. Although exposure to lindane did not produce changes in FNR synthesis and activity, immunochemical studies using electron microscopy revealed that the spatial distribution of immunogold labeling of FNR in control ultrathin sections of cyanobacteria was found in the thylakoid membranes and cytoplasm (**Fig. 7A**). In contrast, labeled grains were found concentrated in the cytoplasm of cyanobacteria in cells treated for 48 h with 5 ppm of lindane (**Fig. 7B**). As has been reported for higher plants, the release of FNR from the thylakoid membranes in *Anabaena* could be due to the potential oxidative stress caused by lindane (Banerjee et al. 1999), which did not produce an increase in the synthesis of FNR but solubilization of the enzyme from the phycobilisome.

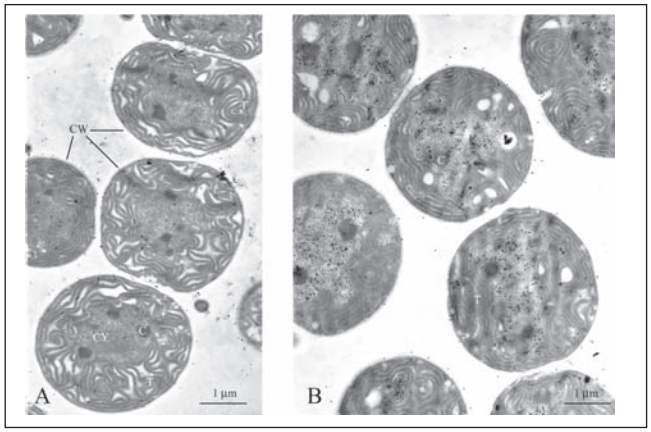

Fig. 7: Spatial distribution of immunogold labeled ferredoxin-NADP+-reductase in ultrathin sections of cyanobacteria Anabaena. **A.** Non treated cells. **B.** Treated cells with 5 ppm pesticide lindane during 48 h. CW: cell wall; T: thylakoids; CY: cytoplasm; C: carboxysomes

3 Conclusions

Lindane is a widely used pesticide, which also inhibits the photosynthetic activity of *Anabaena*. Sites of action and mechanism of lindane are very different to many herbicides blocking the photosynthetic electron transport at a particular site. The decrease of the photosynthetic activity in *Anabaena* in the presence of lindane seemed to be due to an inhibition of the PS II complexes inhibiting the excited RC to reduce Qa to Qa^-. However, not only the remaining, but the newly synthesized active centers, seemed to tolerate high concentrations of lindane for longer times and the cells were able to metabolize and degrade the pesticide during this time. For low concentrations of lindane (below 5 ppm), the cells respond with a typical overreaction so that the photosynthetic performance and the photosynthetic activities increased.

The potential oxidative stress caused by lindane did not change the FNR concentration. However, the enzymes were solubilized from the phycobilisome and released to the cytoplasm. From these results, we can conclude that lindane did not produce significant changes in the synthesis, degradation or activity of FNR under the conditions tested. The high capability of *Anabaena* to tolerate and metabolize lindane makes this cyanobacterium a good candidate for phytoremediation of polluted waters.

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