ACTA
PROTOZOOLOGICA

ACTA PROTOZOOLOGICA
International Journal on Protistology

Front cover: Song W. and Warren A. (2000) A redescription of *Pseudovorticella patellina* (O. F. Müller, 1776) nov. comb., a Peritrichous ciliate (Protozoa: Ciliophora: Peritrichida) isolated from mariculture biotopes in North China. *Acta Protozool.* **39**: 43-50

ACTA
PROTOZOOLOGICA

Effects of Trimethyltin on Pinocytosis of *Dictyostelium discoideum*

Marta MICHALIK[1], Ilona KOSIŃSKA[1], Jolanta SROKA[1], Zbigniew MADEJA[1], Stanisław PRZESTALSKI[2] and Włodzimierz KOROHODA[1]

[1] Department of Cell Biology, Institute of Molecular Biology, Jagiellonian University, Kraków; [2] Department of Physics and Biophysics, Agricultural University, Wrocław, Poland

Summary. The effect of trimethyltin (TMT) on viability, morphology and pinocytosis in the cellular slime mould *Dictyostelium discoideum* were examined. Pinocytotic activity was determined using a fluorescent dye, Lucifer yellow (LY), as a marker of fluid phase endocytosis and quantitative image cytometry with epi-fluorescence computer-aided methods. Treatment of cells with trimethyltin chloride at concentrations from 5 to 20 μM causes inhibition in the pinocytotic uptake of LY in growing *D. discoideum* cells in a dose-dependent and time-dependent manner without changing the cell viability, as assessed with the FDA and ethidium bromide viability test. These results are discussed in the light of known actions of organotin compound that affect various cellular functions.

Key words: *Dictyostelium discoideum,* organotin compounds, pinocytosis, trimethyltin chloride.

Abbreviations: FDA - fluorescein diacetate, LY - Lucifer yellow, PBS - phosphate-buffered saline, TMT - trimethyltin

INTRODUCTION

Organotin compounds are extensively used in industry and as biocides in agriculture (Attar 1996, Fent 1996, Huang *et al.*1996). Usage of organotins in industry includes heat stabilisers for polyvinyl chloride, catalysts for polyurethane foam, and silicone rubber and antifouling paints for ships, boats, or fishing nets (Wilkinson 1984, Forsyth *et al.* 1993). About 10 to 30 % of organotin compounds are introduced directly to the

environment as biocides (Crowe 1987). The use of organotin compounds in agriculture began in the 1950s. and early 1960s. (Blunden *et al.* 1985). Since that time these compounds have been extensively used as bactericidal, fungicidal, and herbicidal agents (Crowe 1987, Fent 1996). Organotin compounds and products of their degradation accumulate in the environment and might be expected to affect both protista and cells of higher organisms.

Studies carried out on the cellular level indicate that organotin compounds interact with cell membranes causing cell fusion, aggregation, blebbing, and membrane rupture (Heywood *et al.* 1989, Musmeci *et al.* 1992, Kleszczyńska *et al.* 1997). Organotin compounds are

Addres for correspondence: Marta Michalik, Department of Cell Biology, The Jan Zurzycki Institute of Molecular Biology, Jagiellonian University, Al. Mickiewicza 3, 31-120 Kraków, Poland; Fax: (4812) 633 69 07; E-mail: Marta@mol.uj.edu.pl

localized in the phospholipid bilayer and it is generally accepted that they interact with the membrane lipids (Gabrielska *et al.*1997). They alter the mechanical and electrical properties of biological membranes (Butterfield *et al.*1991, Harkins and Armstrong 1992) and membrane models (Cullen *et al.* 1997, Kuczera *et al.*1997, Różycka-Roszak *et al.*1997). Recent studies have shown that in isolated mammalian brain neurones organotins act through modification of voltage-dependent Na^+ current (Oyama 1992) and have a suppressive effect on the K^+-induced release and synthesis of acetylcholine in the mouse cerebral cortex (Kobayashi *et al.*1996). It was reported that they reduced the cell viability of thymocytes and yeast *Candida maltosa* (Pieters *et al.* 1994, Tobin and Cooney 1999), changed the cell shape and morphology of guinea pig cochlear outer hair cells (Clerici *et al.*1993), and cell proliferation in lymphocytes and V79 Chinese hamster cells (Jensen *et al.* 1991, Ganguly 1995), suppressed chemotactic response to chemoatractants in neutrophils (Arakawa and Wada 1984), altered phagocytosis and exocytosis in polymorphonuclear leukocytes (Elferink *et al.*1986), and phagocytosis in aquatic organisms *Tapes philippinarum* and *Ciona intestinalis* (Cooper *et al.*1995, Cima *et al.*1998). They changed mitochondrial function (Zazueta *et al.*1994) and calcium homeostasis by inhibiting the Ca^{2+} release channel in the endoplasmic reticulum in thymocytes and sarcoplasmic reticulum in skeletal muscle (Chow *et al.*1992, Kang *et al.* 1997).

Although organotin compounds are found in the natural environment and protista are exposed to these compounds in their natural habitats, to our knowledge no reports have appeared on the effects of organotins on these cells. In our experiments we used *Dictyostelium discoideum* myxamoebae to study the effect of trimethyltin on cell morphology, viability and pinocytotic activity.

The cellular slime mould *D. discoideum* was chosen as a model organism commonly used not only in research into mechanisms of cell locomotion and chemotaxis, but also in investigations concerning endocytosis (Gonzalez *et al.* 1990, Aubry *et al.* 1993, Hacker *et al.* 1997, Reddy and Chatterjee 1997, Titus 2000). This soil amoeba seems to be a suitable model organism in research on biological effects of environmental pollutants. In many of its activities, such as locomotion, chemotaxis, endocytosis, divisions, cell-to-cell interactions and differentiation, it resembles activities of vertebrate cells, particularly the highly motile mammalian polymorphonuclear leukocytes.

MATERIALS AND METHODS

Materials

The following chemicals were purchased as indicated: trimethyltin chloride TMT [$(CH_3)_3SnCl$] (Alfa, Karlsruhe), LY and maltose (Sigma St. Louis, USA), bacteriological peptone and yeast extract (Bio Merieux SA, France), PBS (Wytwórnia Surowic i Szczepionek, Lublin, Poland), NaCl, KCl, $Na_2HPO_4\cdot12H_2O$, $NaH_2PO_4\cdot H_2O$, KH_2PO_4, $CaCl_2$, $K_2HPO_4\cdot12H_2O$, glucose (POCh, Gliwice, Poland). Culture flasks were purchased from Corning NY, UK and microscopic slides from Chance LTD, Warley, UK.

Cells

Dictyostelium discoideum strain AX-2, obtained from professor Jan Michejda (University of Poznań), was grown axenically at 21°C with shaking at 150 rpm in peptone-yeast extract medium (14,30 g/l bacteriological peptone; 7,15 g/l yeast extract; 1,28g/l $Na_2HPO_4\cdot12 H_2O$; 0,48 g/l KH_2PO_4; 18,00 g/l maltose; pH 7.4). Cells were used in their logarithmic phase of growth. Growing cells (5×10^6 cells/ml) were washed twice in Chalkley medium pH 6.8 before being used for the experiments.

The cell counts were made in a Bürker haemocytometer. Viability was determined by the fluorescein diacetate test (FDA test) and ethydium bromide test, based on the preservation of esterase activity in living cells and nucleic acid staining in dead cells (Szydłowska *et al.* 1978), recommended for determination of cell survival by Kemp *et al.* (1983).

Assay for pinocytosis

Pinocytosis was determined by fluorescent microscopy using fluorescent day Lucifer yellow (LY) as a fluid-phase marker. About 0.5 ml cell suspension in Chalkley's medium, pH 6.8, permitted their attachment to a microscope slide. To induce pinocytosis the medium was removed by suction and replaced by 0.125M NaCl dissolved in phosphate buffer at pH 6.8, containing 100 µg/ml LY, unless otherwise stated. Cells were observed under an inverted Olympus IMT-2 microscope using phase contrast or fluorescent optics for 45 min. After 15 min, 30 min, or 45 min incubation pinocytosis was stopped by six rinses with ice-cold phosphate buffer at pH 6.8, after which the cells were washed twice in the Chalkley medium without LY at 21°C. The intensity of fluorescence was measured with a Leitz Orthoplan microscope working in an epifluorescence mode equipped with a photomultiplaier attachment (excitation wavelength 428 nm, emission wavelength 535 nm).

Organotin treatment

TMT was diluted in the Chalkley medium or in the pinocytotic medium from stock solution in water to the working concentration of 5, 10, 15 and 20 µM respectively.

Statistical analysis

For each value measured at least 100 cells were analysed. Mean and standard deviation for each parameter were calculated. The statistical significance was determined by the nonparametric Mann-Whitney U-test and T-student test with p<0.05 considered significant.
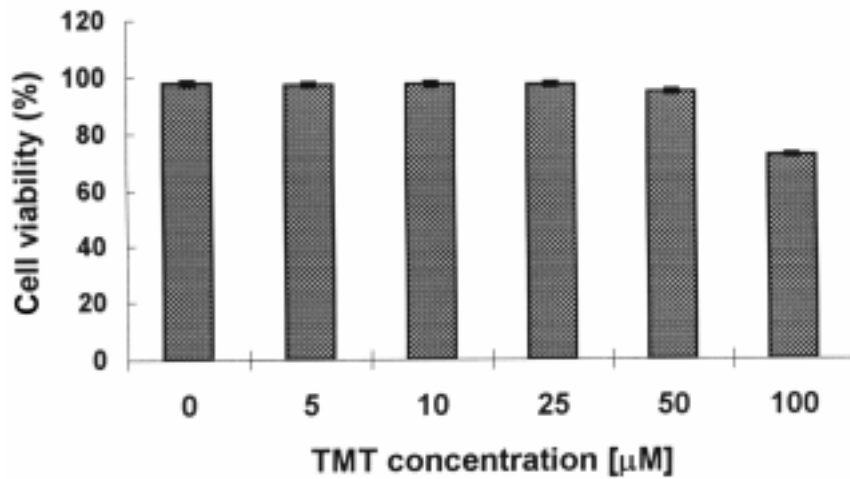
Fig. 1. Viability of trimethyltin-treated *D. discoideum* cells. Cells were incubated in cultured medium (control) and in the same medium supplemented with TMT at concentration 5, 10, 25, 50 or 100 μM for 2 hours. Viability of cells was checked using the FDA/ethidium bromide viability test. At least 200 cells were analysed for each experimental point. The presented results are the mean of at least three separate experiments
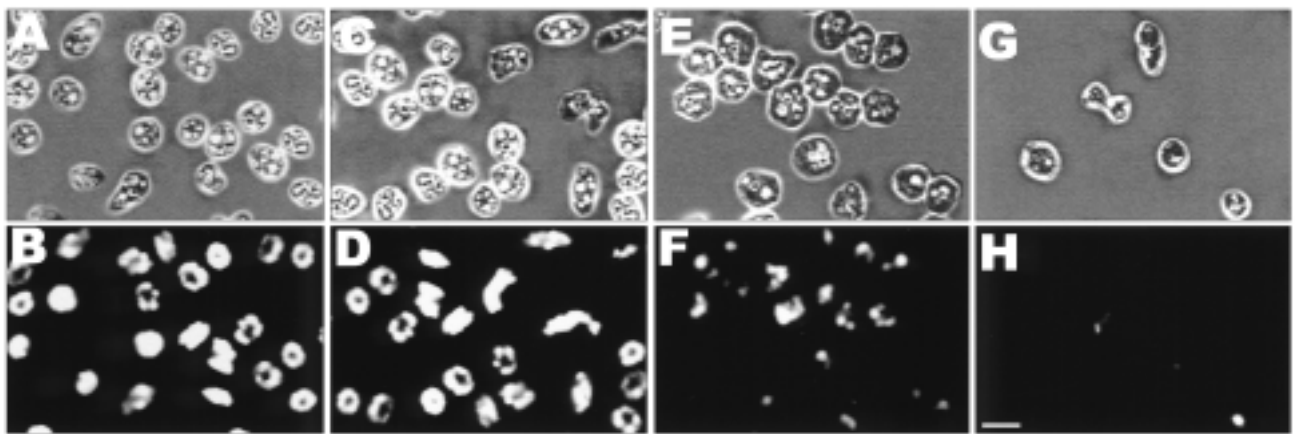
Fig. 2. Effects of trimethyltin on morphology and pinocytotic activity of *D. discoideum* cells (in a population). Cells were incubated in pinocytotic medium supplemented with 100 μg/ml LY for 30 min without (A, B) or with TMT at concentration 5 μM (C, D), 10 μM (E, F), or 20 μM (G, H). Cells were photographed in an Olympus IMT inverted microscope using contrast phase (A, C, E, G) or fluorescence optics (B, D, F, H). Scale bar - 10 μm
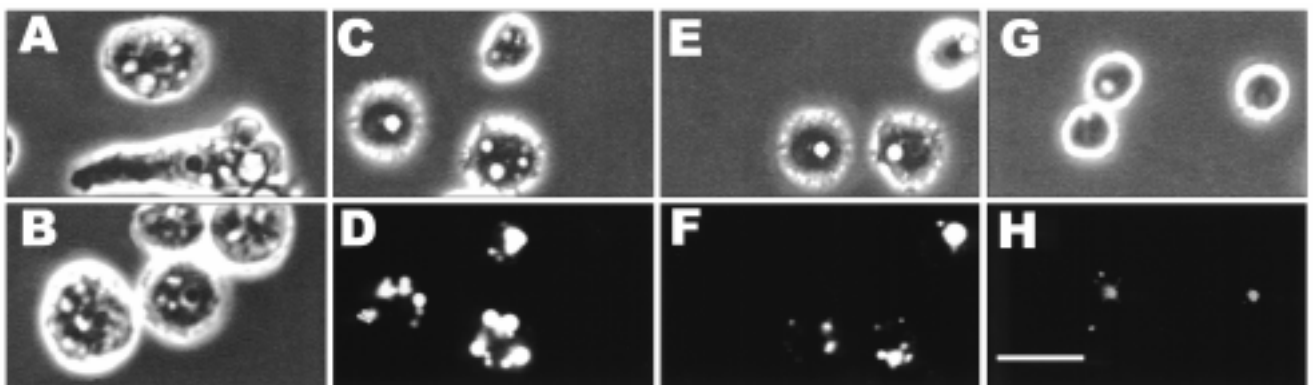
Fig. 3. Effects of trimethyltin on morphology and pinocytotic activity of *D. discoideum* cells (within individual cells). Cells were incubated in Chalkley's medium (A, B) or in pinocytotic medium supplemented with 100 μg/ml LY for 30 min (C, D, E, F, G, H), without (A, C, D) or with TMT at concentration 10 μM (B, E, F), or 20 μM (G, H).Cells were photographed in an Leitz Orthoplan microscope using contrast phase (A, B, C, E, G) or fluorescence optics (D, F, H). Scale bar - 10 μm
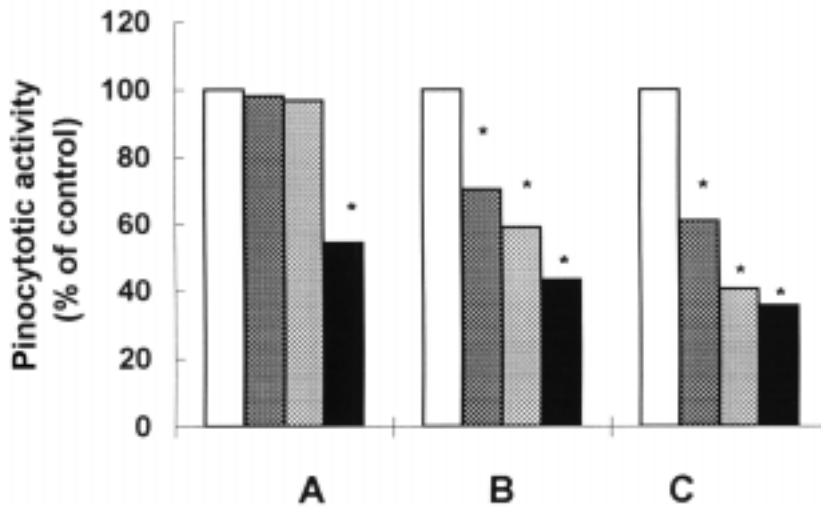
Fig. 4. Inhibition of pinocytotic activity of *D. discoideum* cells induced by trimethyltin. Cells were incubated with pinocytotic medium containing 100 µg/ml LY (white columns) or in the same medium supplemented with 5µM (dark grey columns), 10 µM (light grey columns), or 20 µM TMT (black columns) for 15 min (A), 30 min (B) or 45 min (C), respectively. The pinocytotic activity for each of the experimental conditions was determined and is presented as percentage of control. At least 100 cells were calculated for each experimental point. The presented results are the mean of at least five separate experiments. Values significantly different from control are indicated by (*)

Microphotography

Microphotographs were taken under a Olympus TMT inverted miscoscope using contrast phase or fluorescence optics or Leitz Orthoplan microscope with epifluorescence and phase contrast optics, equipped with a Nikon FX-35DX camera. High sensitivity Kodak TMAX 3200 films or Fomapan 800 films were used.

RESULTS

Effect of trimethyltin (TMT) on *Dictyostelium discoideum* amoebae

The effect of TMT on cell morphology and cation-induced fluid phase endocytosis in *D. discoideum* amoebae was studied. TMT was used within a range of concentrations which did not impair cell viability, as estimated by a viability test. The FDA and ethidium bromide viability test (Szydłowska *et al.* 1978) showed that in cultures of *D. discoideum* cells treated with TMT in concentrations up to 25 µM, about 98% of cells (97.5 ± 1%) remained viable for 2 h (Fig.1).

Phase-contrast and fluorescent microscopic observations of cells incubated for 45 min in the pinocytosis-inducing medium without or with TMT revealed that the cells changed their pinocytotic activity in a TMT dose-dependent manner. Solutions of NaCl are commonly exploited to stimulate the induced pinocytosis in amoe-

bae (Stockem 1966, Prusch 1981, Rivero *et al.* 1999), and LY is extensively utilised as a marker of fluid-phase pinocytosis (Maeda and Kawamoto 1986, Camacho *et al.* 1996, Catizone *et al.* 1996). As shown in Figs. 2 and 3, the incubation of amoebae, both in Chalkley's medium and pinocytotic medium, in the presence of 10 µM TMT for 30 min caused no changes in the morphology of *D. discoideum* cells in comparison with untreated cells (Figs. 2 A, C, E and 3 A, B). Treatment with 20 µM TMT caused contraction and rounding of some cells accompanied by their detachment from the substratum (Figs. 2 G, 3 G). Fluorescent microscope observations revealed that the incubation of *D. discoideum* cells (control cells) in the pinocytosis-inducing medium supplemented with Lucifer yellow dye resulted in a fluorescent pattern typical of fluid phase endocytosis (Figs. 2 B, 3 D). On the other hand, the addition of TMT to the medium resulted in a decrease in fluid phase endocytosis in a dose-dependent manner (Figs. 2 D, F, H; 3 F, H). 20 µM TMT dramatically reduced LY uptake into *D. discoideum* cells (Figs. 2 H, 3 H).

Cytophotometric data

Microscopic observations were confirmed by quantitative single-cell analysis. Cation-induced fluid-phase endocytosis of *D. discoideum* cells was determined by the uptake of the fluorescent dye Lucifer yellow. Maeda
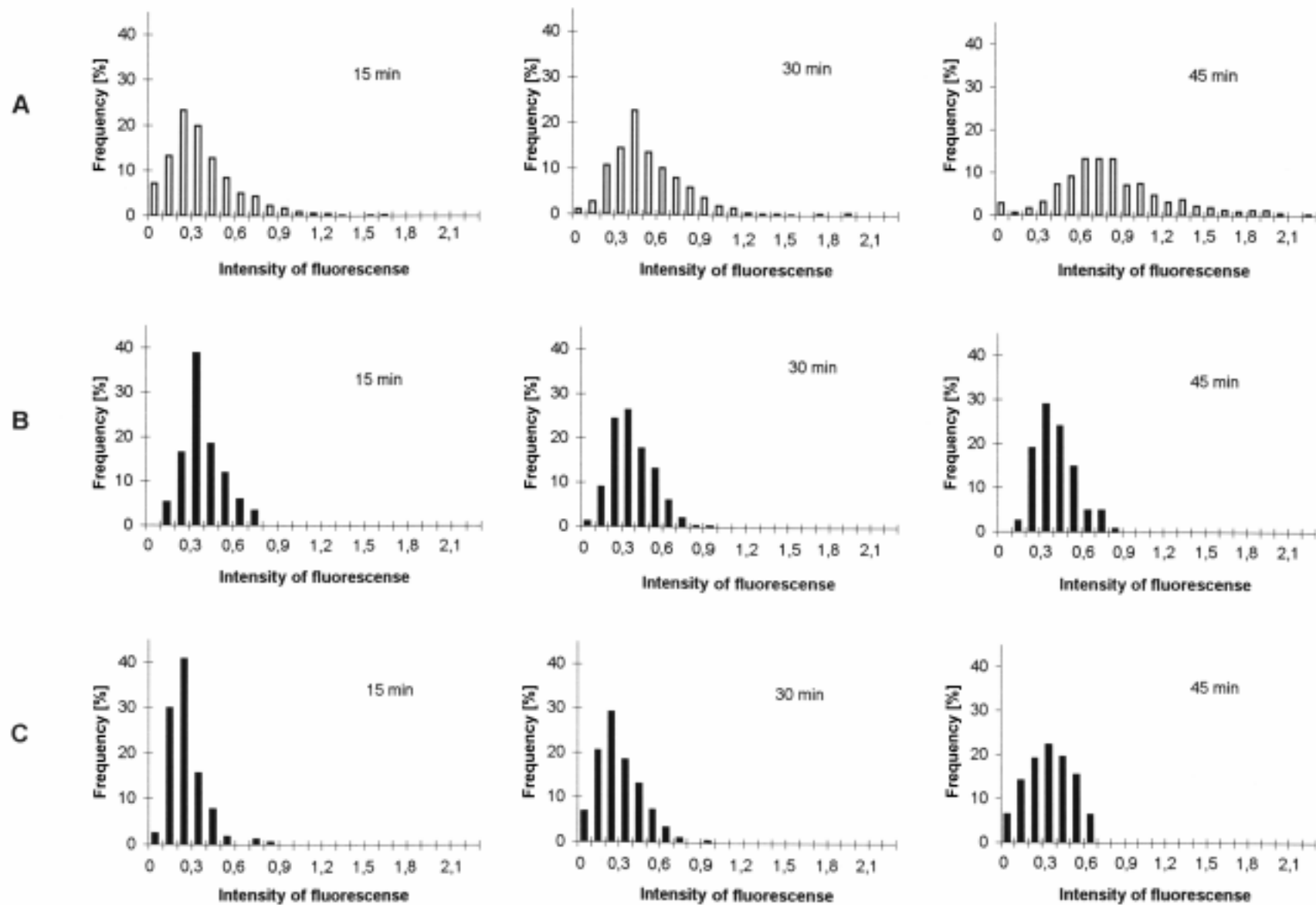
Fig. 5. Histograms of the LY uptake distribution of *D. discoideum* cells after cation-induced endocytosis. Cells were incubated with pinocytotic medium containing 100 μg/ml LY (A) or in the same medium supplemented with 10 μM (B) or 20 μM TMT(C) respectively, for 15 min, 30 min, or 45 min. The data presented are from the representative experiments where a total of 300 cells were counted for each experimental conditions
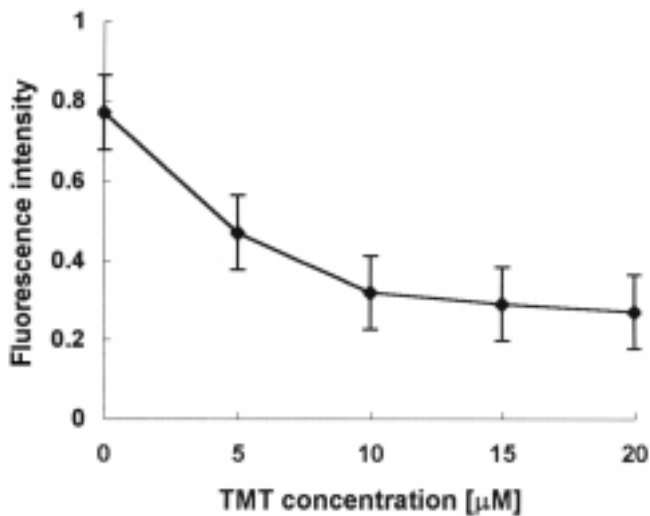
Fig. 6. LY uptake inhibition by trimethyltin in *D. discoideum* cells. Cells were incubated with pinocytotic medium containing 100 µg/ml LY or in the same medium supplemented with 5, 10, 15, or 20 µM TMT respectively, for 45min. The fluorescence intensity for each of the experimental conditions was determined. At least 100 cells were calculated for each experimental point. The presented results are the mean of at least five separate experiments

and Kawamoto (1986) reported that the pinocytotic activity of *D. discoideum* amoebae increases almost linearly in cells incubated at 12 °C to 28 °C and extracellular pH in the range 4.9-7.0. All the present experiments were performed at 22 °C at pH 6.8, unless other- wise noted.

Examination of the pinocytotic activity of *D. discoideum* cells by measurement of LY uptake revealed striking differences between the TMT-treated and the control cells. Results are presented in Fig. 4. After 15 min incubation in 5 µM or 10 µM TMT-containing pinocytotic medium no effect was observed in the uptake of LY (Fig. 4 A). Although the average value of pinocytotic activity for the control cells and 5 µM or 10 µM TMT-treated cells did not change significantly after 15 min incubation, the distribution of *D. discoideum* cells according to LY uptake differed between TMT-treated cells and the control amoebae (comp. Figs. 4 and 5). The histograms showed a lower frequency of cells with high LY uptake for TMT-treated cells than for the control cells (Figs. 5 A, B). In the presence of 20 µM TMT the average LY uptake after 15 min pinocytosis decreased by approximately 45% in comparison with that of the control cells (Fig. 4 A), and the frequency of cells with high LY uptake was significantly reduced (Fig. 5 C). The effect of TMT upon the cation-induced

endocytotic activity was more pronounced after 30 min and 45 min. The average LY uptake after 30 min and 45 min incubation of *D. discoideum* amoeba in TMT-containing pinocytotic medium decreased in a time-dependent (Figs. 4 B, C) and dose-dependent manner (Fig. 6). In agreement with this the distribution of *D. discoideum* cells according to LY uptake was changed significantly. The histograms showed that the greater the TMT concentration the greater was the number of cells with no pinocytotic activity and the smaller the number of cells with high pinocytotic activity (comp. Figs. 5 A, B, C).

DISCUSSION

The presented results demonstrate that trimethyltin (TMT), a common component of herbicides, pesticides, and industrial pollutants, strongly affects the vital functions of soil myxamoebae *D. discoideum*. The acute toxicity of TMT, i.e. decrease in the viability of cells growing for 2 h in its presence, is significant only when TMT is present at 100 µM concentration. This corresponds to the results of experiments showing that at this concentration TMT modifies the properties of artificial black membranes.

Kuczera *et al.* (1997) observed that trimethyltin in milimolar concentrations caused calcium desorption from lecithin liposome membranes. Heyewood *et al.* (1989) found that thrimethyltin chloride at 25 µM concentration caused fusion, aggregation, blebbing, and total rupture of egg yolk vesicles (containing mostly phosphatidylcholine).

Krug (1992) reported that thrimethyltin chloride at a concentration of 500 µM does not significantly change the survival of HL60-cells differentiated with dimethyl sulphide to mature granulocytes, as tested with the trypan blue exclusion test.

Cooper *et al.* (1995) and Cima *et al.* (1998) reported that organotin compounds reduced the phagocytosis in aquatic organisms. A decrease in phagocytosis in polymorphonuclear leukocytes was observed by Elferink *et al.* (1986) but Hioe and Jones (1984) noted no changes in phagocytosis of peritoneal macrophages.

When we measured the fluid phase endocytosis of LY in myxamoebae, already 5 and 10 µM concentration of TMT caused a statistically significant decrease in the endocytotic cell activity (in 30 min) while 20 µM of TMT reduced endocytosis in as soon as 15 min. In addition, amoebae exposed to 20 µM TMT changed morphology,

becoming rounded and detached from the substratum. These results evidence that TMT strongly affects cell activity at concentrations, which do not affect cell viability.

It remains to be examined whether TMT at low concentration (and other herbicides and pesticides) affects other cell functions. The tests, which concern solely cell growth and viability, are inadequate for estimation of the effects of such compounds upon unicellular organisms living in the soil. In addition, it cannot be excluded that this also concerns the cells of the immune system of animals and humans since these cells show many activities similar to amoebae of *D. discoideum*, including endocytotic and locomotory activity. Since most of the organotin compounds are soluble in organic solvents and these substances react with phosphate groups of phospholipids (Gabrielska *et al.* 1997, Heywood 1989, Różycka-Roszak *et al.* 1997, Sarapuk and Przestalski 1998, Przestalski *et al.* 2000) it may be supposed that their interaction with lipoprotein membranes could be a primary site of their effects upon living cells.

The results presented permit us to postulate that *D. discoideum* amoebae may be employed as a suitable model organism in investigations concerning biological effects of environmental pollutants upon activities of cells, both unicellular organisms and cells of vertebrates.

REFERENCES

Arakawa Y., Wada O. (1984) Inhibition of neutrophil chemotaxis by organotin compounds. *Biochem. Biophys. Res. Comm.* **123:** 543-548

Aubry L., Klein G., Satre M. (1993) Endo-lysosomal acidification in *Dictyostelium discoideum* amoebae. Effects of two inhibitors: caffeine and cycloheximide. *Eur. J. Cell Biol.* **61:** 225-228

Attar K. M. (1996) Analytical methods for speciation of organotin in the environment. *Appl. Organometallic Chem.* **10:** 317-336

Blunden S. J., Cusack P. A. and Hill R. (1985) The industrial user of tin compounds. The Royal Society of Chemistry, London

Butterfield D., Schneider A., Rangachari A. (1991) Electron paramagnetic resonance studies of the effects of tri-n-butyltin on the physical state of proteins and lipids in erythrocyte membranes. *Chem. Res. Toxicol.* **4:** 141-146

Camacho K. B., Tuma D. J., Casey C. A. (1996) Zonal differences in ethanol-induced impairements in fluid-phase endocytosis in rat hepatocytes. *Alcohol. Clin. Exp. Res.* **20:** 589-594

Catizone A., Chiantore M. V., Andreola F., Coletti D., Medolago-Albani L., Alescio T. (1996) Non-specific pinocytosis by human endothelial cells cultured as multicellular aggregates: uptake of lucifer yellow and horse radish peroxidase. *Cell Mol. Biol.* **42:** 1229-1242

Chow S. C., Kass G. E., McCabe M. J. Jr., Orrenius S. (1992) Tributyltin increases cytosolic free Ca^{2+} concentration in thymocytes by mobilizing intracellular Ca^{2+}, activating a Ca^{2+} entry pathway, and inhibiting Ca^{2+} efflux. *Arch. Biochem. Biophys.* **298:** 143-9

Cima F., Marin M. G., Matozzo V., Da Ros L., Ballarin L. (1998) Immunotoxic effects of organotin compounds in *Tapes philippinarum*. *Chemosphere* **37:** 3035-45

Clerici W. J., Chertoff M. E., Brownell W. E., Fechter L. D. (1993) In vitro organotin administration alters guinea pig cochlear outer hair cell shape and viability. *Toxicol. Appl. Pharmacol.* **120:** 193-202

Cooper E. L., Arizza V., Cammarata M., Pellerito L., Parrinello N. (1995) Tributyltin affects phagocytic activity of *Ciona intestinalis* hemocytes. *Comp. Biochem. Physiol. C Pharmacol. Toxicol Endocrinol.* **112:** 285-9

Crowe A. J. (1987) Organotin compounds in agriculture since 1980. Part I. Fungicidal, bactericidal and herbicidal properties. *Appl. Organometallic Chem.* **1:** 143-155

Cullen W. R., Herring F. G., Nwata B. U. (1997) The effect of organotin compounds on the permeability of model biological membranes. *Appl. Organometallic Chem.* **11:** 369-379

Elferink J. G., Deierkauf M., Van Steveninck J. (1986) Toxicity of organotin compounds for polymorphonuclear leukocytes: the effect on phagocytosis and exocytosis. *Biochem. Pharmacol.* **35:** 3727-32

Fent K. (1996) Ecotoxicology of organotin compounds. *Crit. Rev. Toxicol.* **26:** 1-117

Forsyth D. S., Dabeka R., Sun W. F., Dalglish K. (1993) Speciation of organotin in poly(vinyl chloride) products. *Food Addit Contam* **10:** 531-540

Gabrielska J., Sarapuk J., Przestalski S. (1997) Role of hydrophobic and hydrophilic interactions of organotin and organolead compounds with model lipid membranes. *Z. Naturforsch.* **52c:** 209-216

Ganguly B. B. (1995) Age-related alterations in cell division and cell cycle kinetics in control and trimethyltin-treated lymphocytes of human individuals. *Biometals* **8:** 263-9

Gonzalez C., Klein G., Satre M. (1990) Caffeine, an inhibitor of endocytosis in *Dictyostelium discoideum* amoebae. *J. Cell. Physiol.* **144:** 408-415

Gray B. H., Porvaznik M., Flemming C., Lanfong H. M. (1996) Organotin induced hemolysis, shape transformation and intramembranous aggregates in human erythrocytes. *Cell Biol. Toxicol.* **3:** 23-38

Hacker U., Albracht R., Maniak M. (1997) Fluid-phase uptake by macropinocytosis in *Dictyostelium*. *J. Cell Sci.* **110:** 105-112

Harkins A. B., Armstrong D. L. (1992) Trimethyltin alters membrane properties of CA1 hippocampal neurones. *Neurotoxicology* **13:** 569-81

Heywood B. R., Molloy K. C., Waterfield P. C. (1989) Organotin biocides XV: Modelling the interactions of triorganotins with cell membranes. *Appl. Organometallic Chem.* **3:** 443-450

Hioe K. M., Jones J. M. (1984) Effects of trimethyltin on the immune system of rats. *Toxicol. Lett.* **20:** 317-323

Huang G., Dai S., Sun H. (1996) Toxic effects of organotin species on algae. *Appl. Organometallic Chem.* **10:** 377-387

Jensen K. G., Onfelt A., Wallin M., Lidums V., Andersen O. (1991) Effects of organotin compounds on mitosis, spindle structure, toxicity and *in vitro* microtubule assembly. *Mutagenesis* **6:** 409-16

Kang J. J., Chen I. L., Cheng Y. W. (1997) Induction of calcium release from isolated sarcoplasmic reticulum by triphenyltin. *J. Biochem.* **122:** 173-177

Kemp R. B., Meredith R. W., Gamble S., Frost M. (1983) A rapid cell culture technique for assessing the toxicity of detergent-based products *in vitro* as a possible screen for eye irritancy *in vivo*. *Cytobios* **36:** 153-159

Kleszczyńska H., Hładyszowski J., Pruchnik. H., Przestalski S. (1997) Erythrocytes hemolysis by organic tin and lead compounds. *Z. Naturforsch.* **52c:** 65-69

Kobayashi H., Suzuki T., Kasashima Y., Motegi A., Sato I., Matsusaka N., Ono N., Miura A., Saito F., Saito S. (1996) Effects of tri-, di- and monobutyltin on synaptic parameters of thecholinergic system in the cerebral cortex of mice. *Jpn. J. Pharmacol.* **72:** 317-324

Krug H. F. (1992) The toxic effects of organometals on the Lands cycle in HL-60 cells. *Appl. Organometallic Chem.* **6:** 297-304

Kuczera J., Gabrielska J., Kral T. E., Przestalski S. (1997) A synergistic effects of select organotin compounds and ionic surfactants on liposome membranes. *Appl. Organometallic Chem.* **11:** 591-600

Maeda Y., Kawamoto T. (1986) Pinocytosis in *Dictyostelium discoideum* cells. *Exp. Cell Res.* **164:** 516-526

Musmeci M. T., Madonia G., Lo Giudice M. T., Silvestri A., Ruisi G., Barbieri R. (1992) Interaction of organotins with biological systems. *Appl. Organometallic Chem.* **6:** 127-138

Oyama Y. (1992) Modification of voltage-dependent Na+ current by triphenyltin, an environment pollutant, in isolated mammalian brain neurons. *Brain Res.* **583:** 93-9

Pieters R. H., Bol M., Penninks A. H. (1994) Immunotoxic organotins as possible model compounds in studying apoptosis and thymocyte differentiation. *Toxicology* **91:** 189-202

Prusch R. D. (1981) Bulk solute extrusion as a mechanism conferring solute uptake specificity by pinocytosis in Amoeba proteus. *Science* **213:** 668-670

Przestalski S., Sarapuk J., Kleszczyńska H., Gabrielska J., Hładyszowski J., Trela Z., Kuczera J. (2000) Influence of amphiphilic compounds on membranes. *Acta Biochim. Pol.* **47:** 627-638

Reddy T. B., Chatterjee S. (1997) Cisplatin inhibits pinocytosis in *Dictyostelium discoideum*. *Cell Biol. Int.* **21:** 237-241

Rivero F., Furukawa R., Fechheimer M., Noegel A. A. (1999) Three actin cross-linking proteins, the 34 kDa actin-bundling protein, alpha actinin and gelation factor (ABP-120), have both unique and redundant roles in growth and development of *Dictyostelium*. *J.Cell Sci* **112:** 2737-2751

Różycka-Roszak B., Żyłka R., Przestalski S. (1997) Comparison of the interaction of diphenyltin and triphenyltin chlorides with phospholipid bilayers. *Cell. Mol. Biol. Lett.* **2:** 25-29

Sarapuk J., Przestalski S. (1998) Biological aspects of the presence of organometallic compounds in the environment. *Zesz.Nauk. AR Wroc., Rol. LXXIII.* **347:** 79-93

Stockem W. (1966) Pinocytosis and motility of amoeba. I. Influence of marker substances on *Amoeba proteus*. *Z. Zellforsch. Mikrosk. Anat.* **74:** 372-400

Szydłowska H., Zaporowska E., Kuszlik-Jochym K., Korohoda W., Branny J. (1978) Membranolytic activity of detergents as studied with cell viability tests. *Folia Histochem. Cytochem.* **16:** 69-78

Titus M. A. (2000) The role of unconventional myosins in *Dictyostelium* endocytosis. *J. Eukaryot. Microbiol.* **47:** 191-196

Tobin J. M., Cooney J. J. (1999) Action of inorganic tin and organotins on a hydrocarbon-using Yeast, *Candida maltosa. Arch. Environ. Contam. Toxicol.* **36:** 7-12

Wilkinson R. R. (1984) Technoeconomic and environmental assessment of industrial organotin compounds. *Neurotoxicology* **5:** 141-158

Zazueta C., Reyes-Vivas H., Bravo C., Pichardo J., Corona N., Chavez E. (1994) Triphenyltin as inductor of mitochondrial membrane permeability transition. *J. Bioenerg. Biomembr.* **26:** 457-462

ACTA
PROTOZOOLOGICA

Effects of Shock Loads of Salt on Protozoan Communities of Activated Sludge

Humbert SALVADÓ, Meritxell MAS, Sergi MENÉNDEZ and Mª Pilar GRACIA

Department of Animal Biology, University of Barcelona, Barcelona, Spain

Summary. The effects of wastewater salinity variations on communities of microorganisms taken from activated sludge were studied. Batch cultures were grown for 96 h at final salt concentrations of 3, 5, 10, 20 and 40 NaCl g/l. Protozoa and small metazoa was counted and ciliated protozoan species in these cultures were identified. An increase in salt concentration from 3 to 10 g/l gradually affected the microbial community and few protozoa and metazoa survived at 96 h. Ciliate abundance was species dependent: *Vorticella* spp. and *Opercularia articulata* resisted the high dosages of NaCl better than other ciliates. Total ciliate abundance and diversity fell drastically at 20 g/l, which would compromise reliability in activated sludge processes. At 40 g/l neither protozoa nor metazoa survived after 24 h. This study evaluates the effects of a short-term NaCl shock on the dynamics of activated sludge microorganisms and their community composition. The study also contributes to the understanding of the wastewater treatment process.

Key words: activated sludge, ciliates, microfauna, salinity tolerance, wastewater treatment.

Abbreviations: BOD$_5$ - five-day biochemical oxygen demand, MLSS - mixed liquor suspended solids, MLVSS - mixed liquor volatile suspended solids.

INTRODUCTION

Wastewater may have high salt contents due to industrial shock loads or occasional sea intrusions. Several studies concluded that shock loads of 0.5-5% reduce the effectiveness of the biological processes in wastewater treatment plants (Lawton and Eggert 1957, Ludzack and Noran 1965, Hall and Smallwood 1967, Kincannon and Gaudy 1968, Burnett 1974). These

Address for correspondence: Humbert Salvadó, Department of Animal Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain; Fax: 34 93 403 57 40; E-mail: hsalvado@porthos.bio.ub.es

studies also conclude that rapid shifts in salt concentration typically cause more problems than gradual shifts and also that shifts of 0.5-2% salt usually cause significant disruptions in system performance. Upsets may be temporary and in certain cases organisms acclimatise and give satisfactory reactor performance. Saline loads affect the metabolic functions of microorganisms and reduce the kinetic degradation in activated sludge (Mahmoud and Davis 1970, Woolard and Irvine 1995).

It seems that some acclimatisation of microorganisms to habitats with relatively high saline concentrations is to be expected (Lawton and Eggert 1957, Kincannon and Gaudy 1968, Smurov and Fokin 1999). However, this

acclimatisation is limited, and even in acclimatised cultures, an adequate process performance depends upon a relatively constant ionic strength. In addition, rapid reductions in salt content of wastewater cause further upsets (Lawton and Eggert 1957).

Pillai and Rajagopalan (1948) reported that an activated sludge plant operated on seawater in India worked as efficiently as fresh-water plants. This plant was analysed for a period over of eight years. The constant characteristics of seawater allowed the development of high numbers of species of marine ciliates (specially those belonging to the genera *Vorticella* or *Zoothamnium*) closely related to those found in fresh-water plants. According Pillai and Rajagopalan effluent quality was closely dependent on the number of active peritrich ciliates.

There have been many studies on the treatment of saline and hypersaline waste waters (>3.5% salt) in constant concentrations (Hockernbury *et al.* 1977, Tokuz and Eckernfelder 1978) or high-polluted groundwaters (Wong 1992). These authors conclude that conventional biological processes cannot be used to treat wastewater containing more than 3-5% salt. However, Woolard and Irvine (1994, 1995) showed that biofilms of halophilic bacteria acclimatised in constant concentrations of up to 15% salt have a great capacity for treating hypersaline wastewater. As Ludzack and Noran (1965) and Kincannon and Gaudy (1968) described, the absence of protozoa in this medium causes the effluent to deteriorate and the suspended solids to increase.

Zobell *et al.* (1937) and Zobell (1946) showed that most wastewater and soil microorganisms die in hypersaline media, and that their survival increased when the salt concentration went down to 3.5 g/l. With regard to protozoa, only experimental studies on natural communities or on single-species cultures have been conducted. Finley (1930) studied the effect of salinity on communities of protozoa from continental waters by diluting seawater. He showed that there was a high mortality when different species were subjected directly to the different saline concentrations studied. In contrast, there was very much better survival when salt content was increased gradually. Smurov and Fokin (1999) studied resistance and tolerance to salinity in 10 species of *Paramecium*, recording the adaptation after prior acclimatisation from 2.1 g/l salt for *P. putrinum* to 18.7 g/l salt for *P. woodruffi*, with a period of acclimatisation not less than 48 h. Studies from Bick (1964), Ax and Ax (1960), Foissner *et al.* (1995) and Albrecht (1984) provided us with a synthetic description of the ranges of salinity found in natural communities for various species of ciliated, flagellated and rhizopod freshwater protozoa.

The objective of this paper is to investigate the effects of shock loads of sodium chloride (between 3 and 40 g/l) on the populations of eukaryotic microorganisms (protozoa and small metazoa) that constitute the activated sludge communities.

MATERIALS AND METHODS

Using activated sludge from a pilot plant for urban wastewater treatment, the effects of several concentrations of NaCl were studied through batch cultures at 20-22°C, pH 7-7.3, 2-5 mg O_2/l and about 900 mg MLSS/l. For each concentration two replicas were placed in 250 ml Erlenmeyers with 60 ml of sludge, 30 ml of synthetic waste water (see composition on Table 1) and 10 ml of saline solution, in order to reach concentration of 3, 5, 10, 20, 40 g NaCl/l, and a control containing synthetic wastewater only (0.4g NaCl/l). After settling, and for the next five days, 40 ml of the supernatant was removed every 24 h and replaced by 30 ml of synthetic wastewater plus 10 ml of saline solution in order to maintain the respective concentrations. The Erlenmeyers thus worked as mini sequencing batch reactors (SBR).

The characteristics of the plant from which the activated sludge came were: an average of 0.7 g/l of salt in the inflow, an hydraulic retention time of about 7 h, and a mass load of 0.25 g BOD_5/g MLVSS d. During the bioassay, the hydraulic retention time was about 16 h and the mass load was 0.1 g of BOD_5/g MLVSS d.

Through optical microscopy, the microorganisms were counted every 24 h for 5 days after the first salt application. These counts were performed in three 25 μl subsamples. The results are given in individuals/ml. The biomass (g/l) of each ciliated species was also computed, using the biomass figures for each 10^6 individuals, following Foissner *et al.* (1995). Thecamoebae, gymnamoebae and rotifers were determined according to Odgen and Hedley (1980), Page (1988), and Koste (1978a, b). Ciliates were identified following Foissner *et al.* (1991, 1992, 1994 and 1995), and using the special silver technique described by Fernández-Galiano (1994). The specific diversity of ciliates was calculated through the number of ciliate species and by means of the Shannon-Weaver diversity index (expressed in bits/individual).

RESULTS

The microfauna composition in the control suffered over time a drop in abundance and number of species (Table 2). This should be considered normal, given the differences in the figures of the operating parameters between the plant of origin and the cultures, and given the nutritional change brought about by the synthetic wastewater. Some loss of species and abundance is to be expected in all such trials. On the basis of the evolution of the control, in general the protozoa sup-

Table 1. Synthetic wastewater composition

Composition	mg/l
Peptone	256.55
Tryptone	354.24
NaCl	407.4
Na_2SO_4	44.6
K_2HPO_4	44.6
$MgCl_2 \cdot 6H_2O$	3.7
$FeCl_2 \cdot 2H_2O$	3.7
$CaCl_2 \cdot 2H_2O$	3.7
$MnSO_4$	0.057
H_2MoO_4	0.031
NaOH	0.008
$ZnSO_4$	0.046
$CoSO_4$	0.049
$CuSO_4$	0.076
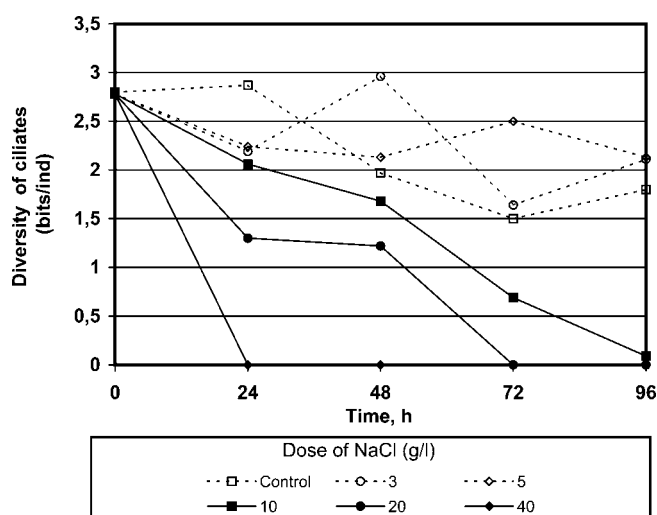
Fig. 1. Evolution of ciliate diversity of each NaCl dose at different exposure times. For all values n = 6

Table 2. Microbial community composition of the control at time 0

	Initial sludge	
	ind/ml	%
Ciliata		
Acineria uncinata	40	<1
Aspidisca cicada	20	<1
Carchesium polypinum	1020	4.54
Chilodonella uncinata	280	5.35
Chilodonellidae indet.	120	12.47
Epistylis chrysemydis	260	<1
Euplotes affinis	<10	<1
Litonotus lamella	<10	<1
Opercularia articulata	920	4.31
Oxytrichidae	20	<1
Parastrongylidium oswaldi	<10	<1
Spathidium sp.	40	<1
Tokophrya sp.	20	<1
Trochilia minuta	<10	1.58
Uronema nigricans	40	<1
Vorticella aquadulcis	80	1.05
Vorticella convallaria	1020	6.64
Vorticella infusionum	100	<1
Vorticella microstoma	140	<1
Gymnamoebae		
Mayorella sp.	4600	50.27
Thecamoebae		
Arcella sp.	40	<1
Rotifera		
Lecanidae	720	7.99
Philodinidae	100	<1
Total microorganisms	4140	100

ported NaCl concentrations of up to 5 g/l, but survival began to fall off at concentrations of 10 g/l. Their number dropped markedly at 20 g/l, and they disappeared completely at 40 g/l.

Ciliate survival and diversity index clearly decreased at saline concentrations above 10g/l (Fig.1). However, the limits of salinity tolerance varied greatly between the organisms studied. Hence, the analysis of species provides extremely relevant information. Given the fluctuations for the different species, it was decided to express the maximum tolerances as the maximum concentration of salt in which active individuals were observed for the different times of exposure (Table 3). Within the ciliate

protozoa, peritrichs were the ciliates that were most resistant to high concentrations of salt (*Opercularia articulata*, *Vorticella infusionum* and *Vorticella microstoma* supported up to 20 g/l of NaCl) (Fig. 2). However, other peritrichs such as *Carchesium polypinum* and *Vorticella convallaria* were more sensitive. The scuticociliate *Uronema nigricans*, with low density in the control (40 ind/l), increased its abundance in the doses of 5 and 10 g/l of NaCl at 24 h, and even resisted up to the 20 g/l. Thecamoebae did not adapt well to high concentrations and disappeared in doses of over 5 g/l. Large gymnamoebae (belonging to the genus *Mayorella*) behaved in a special way, diminishing after 24 h in all doses, but recovering in doses below 20 g/l (Fig. 3). Unlike the protozoa, rotifers (represented by the genera *Lecane* and *Philodina*), only survived at low concentrations (up to 3 g/l). *Lecane* was significantly more sensitive and disappeared almost entirely at 10 g/l. To the contrary, a few *Philodina* were still present up to 72 h

Table 3. Maximum NaCl doses (g/l) tolerated at different culture times for different species

	24 h	48 h	72 h	96 h
Ciliates				
Acineria uncinata	10	0.4		
Aspidisca cicada	5	5	0.4	
Carchesium polypinum	20	20		5
Chilodonellidae	20	5	5	0.4
Epistylis chrysemydis	10	10	5	5
Euplotes affinis	10	10	10	10
Litonotus lamella	3	3	3	0.4
Opercularia articulata	20	20	5	5
Oxytrichidae	10	10	10	5
Parastrogylidium oswaldi	5	5	0.4	0.4
Spathidium sp.	3	0.4		
Trochilia minuta	3	3	0.4	
Uronema nigricans	20	10	10	
Vorticella aquadulcis	20	10	10	5
Vorticella convallaria	5	5	5	5
Vorticella infusionum	20	20	20	20
Vorticella microstoma	20	20	10	
Gymnamoebae				
Mayorella sp.	20	20	20	10
Thecamoebae				
Arcella sp.	10	5	3	3
Rotifera				
Lecanidae	10	10	10	5
Philodinidae	10	5	5	1

at 10 g/l, and they only completely disappeared from 20 g/l (Fig. 4). The small flagellates, though few in number, maintained their numbers up to the 20 g/l dose.

Figure 5 shows the total biomass profiles of ciliates per dose (biomass-dose) and total abundance of ciliates per dose for each period (abundance-dose). Within each dose, the behaviour of total biomass between 72 and 96 h was similar. The higher values of total biomass were concentrated in the 3 g/l and especially in the 5 g/l salt dose. Although the tendency in biomass and abundance between 72 and 96 h were similar, while biomass clearly decreased at doses above 5g/l, abundance continued to increase up to 10 g/l. From 10 g/l, both parameters dropped together until extinction at 40 g/l, although as in the 10 g/l doses, at 20 g/l there was also a relative increase in abundance and biomass over time.

The low total biomass figure in the dose of 3 g/l of NaCl at 48 h is due to the marked fall in abundance of the ciliates identified as Chilodonellidae (mainly

Chilodonella uncinata). At this dose, at 48, 72 and 96 h, total abundance was less than at 5, 10 and 20 g/l of salt. In the 5 g/l dose, the abundance trend was set by the high figure for *Carchesium polypinum* at 48 h and by an explosion in the abundance of *Euplotes affinis*, *Vorticella infusionum* and Oxytrichidae at 72 and 96 h. At 72 h there was an especially significant drop in the abundance of *Opercularia articulata*. At the 10 g/l dose, the explosion of *Vorticella infusionum* and *Vorticella microstoma* was confirmed. The particularly high value of these two species at 96 h should be noted. At this dose both total abundance and biomass declined at 24 h. Finally, at 20 g/l, a minimum was found at 24 h in total biomass and total abundance. Both of these values recovered up to 72 h.

DISCUSSION

The results of this study show that the tolerance limits to salinity variations vary greatly for the organisms found in sludge. In general, a considerable number of species displayed a high range of tolerance to salinity and supported well concentrations of salts (up to 5-10 g/l) considerable above the normal figures for domestic wastewaters (0.2 to 0.5 g/l; Henze *et al.* 1995). Fresh-water ciliates, along with some metazoan species, regulate their internal ionic composition and their salt limit is probably 5 g/l (Smurov and Fokin 1999). This limit is consistent with the results of the present study, in which some species tolerated even higher concentrations: for example, the peritrichs *Vorticella microstoma* and *Vorticella infusionum* survived salt concentrations higher than 10 g/l for over 48 h. In contrast, only a few species were intolerant to salinity conditions of 5 g/l. Within the same taxonomic group, differences were also found in response to different concentrations of NaCl. For example, the various species of the genus *Vorticella* responded differently to increasing salt concentrations: in particular, smaller species (*Vorticella microstoma*, *Vorticella infusionum* and *Vorticella aquadulcis*) were more tolerant than bigger ones (*Vorticella convallaria*). This may be due to differences in their ecological strategies.

Our results show that, at salt levels up to 20 g/l, a succession of species can be found in the sludge, with more tolerant species can developing due to the reduction or disappearance of more sensitive species. This change implies a loss of species, which is clearly reflected on the index of diversity. The usefulness of ciliate
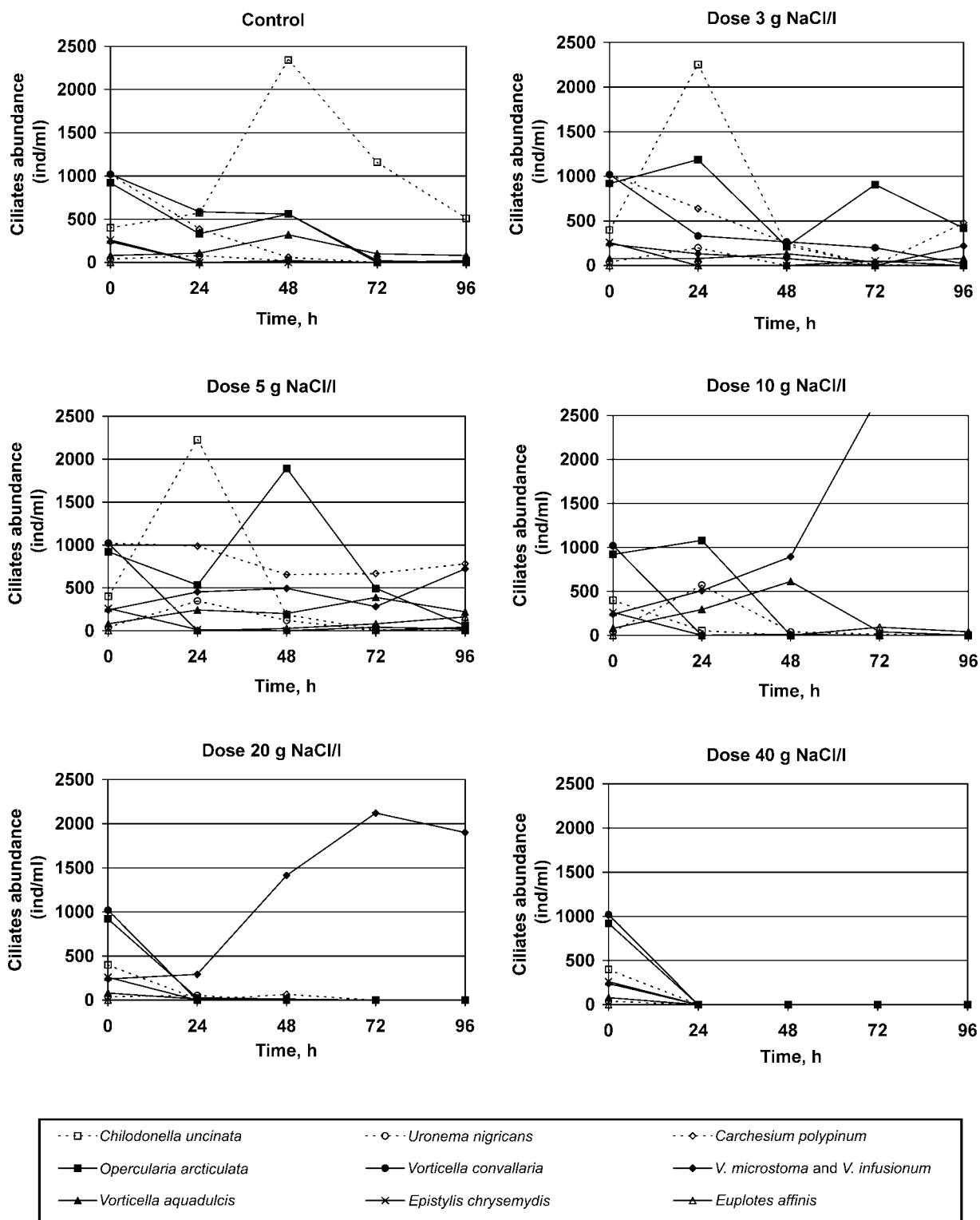
Fig. 2. Evolution of abundance of some ciliate species at different NaCl doses
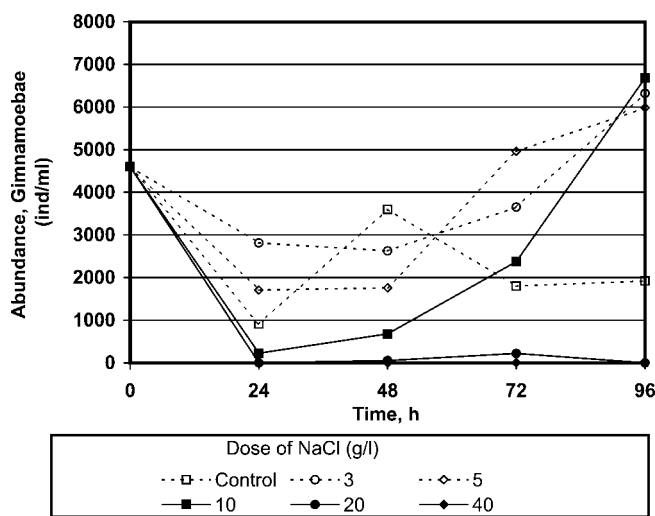
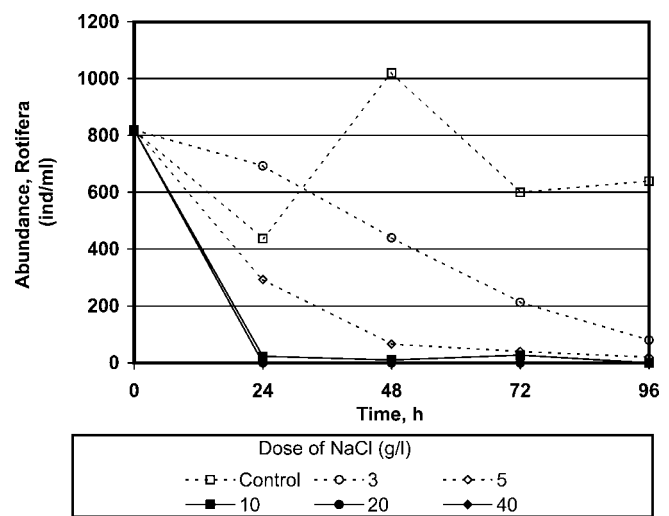**Fig. 3.** Evolution gymnamoebae abundance at different NaCl doses

Fig. 4. Evolution of rotifer abundance at different NaCl doses

has been reported for assessing the stress effects caused by toxic substances (chloride and heavy metals) in studies of protozoan communities in activated sludge and other freshwater communities (Fernández-Leborans *et al.* 1988; Gracia *et al.* 1994; Madoni *et al.* 1994, 1996; Salvadó *et al.* 1997, 2000). Our results indicated that diversity decreases significantly above 10 g/l salt and with increased time of exposure. This could be a good indicative parameter for the effects of saline shocks, but only in determining high salt loads.

Nevertheless, the development at higher salt doses of the less sensitive species can be taken as a process of acclimatisation to the sludge, thus enabling ciliates to maintain high biomass figures at up to 10 g/l salt. Several authors (Lawton and Eggert 1957, Kincannon and Gaudy 1968, Smurov and Fokin 1999) already showed that some acclimatisation to habitats with relatively high saline concentrations in ciliate protozoa and other micro-organisms from activated sludge could be expected. We found many examples supporting this such as *Euplotes affinis* and *Mayorella* sp., which increased their populations at doses between 5 and 10 g/l; two kinds of behaviour, however, should be distinguished. At 24 h the gymnamoeba *Mayorella* sp. suffered high and progressive mortality at concentrations between 3 and 20 g/l, but its population then gradually recovered up to doses of 10 g/l, which can be seen as a genuine acclimatisation. The ciliates *Euplotes affinis* and *Vorticella infusionum*, in contrast, did not apparently suffer great losses at

24 h, and over time they increased their populations (between 5-10 g/l in *Euplotes affinis* and up to 20 g/l in *Vorticella infusionum*), which is better considered as a gradual succession of species.

The effect of sodium chloride depends on the ecological characteristics of each species, which are summarised in Table 4. This shows that peritrichs, generally β-mesosaprobic to polysaprobic, are the ciliates that best tolerate high concentrations of sodium chloride (up to 20 g/l in the case of *Opercularia articulata*, *Vorticella infusionum* and *Vorticella microstoma*). These ciliated peritrichs are also indicators of high mass loads (0.4-0.7 gBOD$_5$/gMLVSSd), and display even greater tolerance under stress and in the presence of toxic substances such as heavy metals (Cr, Zn, Cu) and chlorine (Sudo and Aiba 1973, Salvadó *et al.* 1993, Gracia *et al.* 1994, Madoni *et al.* 1996, Salvadó *et al.* 2000). They are very abundant in plants with contributions of wastewater tipped by industry and loaded with toxic substances (Esteban *et al.* 1990, Becares 1991). *Opercularia articulata*, a β-α-mesosaprobic and oligostenohaline species (Foissner *et al.* 1995), increases more than the control at doses of 3 g/l and 5 g/l, with some specimens found even at 20 g/l after 48 h exposure. We thus found that *Opercularia articulata* adapted to salinity concentrations higher than those previously reported (0.4 g/l according to Foissner *et al.* 1995: in our study it survived up to 20 g/l). However, other peritrichs such as *Carchesium polypinum* and *Vorticella convallaria*,

Table 4. Biological and ecological characteristics of the microorganisms of this study, α - alphamesosaprobic, ß - betamesosaprobic, Al - algae, Ba - bacteria, Fl - heterotrofic flagellates, he - holo-euryhaline, os - oligo-stenohaline, oms - oligo- to meso-stenohaline, p - polysabrobic, R - predator. (1) Sládecek (1973), (2) Foissner *et al.*(1995), (3) Madoni (1994), (4) Salvadó *et al.* (2000), (5) Albrecht (1984), (6) Bick (1964), (7) Ax and Ax (1960), (8) Salvadó and Fernández-Galiano (1997)

| Ciliates	Saprobity (1, 2, 6, 8)	Main food (2, 8)	Motility (3, 8)	Tolerance of chlorine g/kg·d (4)	Salinity tolerance (5, 6, 7)	Salinity tolerance range g/l (5, 6, 7)	Salinity in this study NaCl g/l
Acineria uncinata	α-p	R	crawling	0 to 15	os	0 to 1	0.5 to 10
Aspidisca cicada	β-α	Ba	crawling	0 to 10	he ?	0 to >30	0.5 to 5
Chilodonella uncinata	α	Ba, Al	crawling	0 to 5	he ?	0 to >30	0.5 a 20
Carchesium polypinum	β-α-p	Ba	attached	-	he ?-oe	0 to >30 - 0 to 10	0.5 to 20
Epistylis chrysemydis	α	Ba	attached	0 to 15	oe	0 to >10	0.5 to 20
Euplotes affinis	β-α	Ba, Al, Fl	crawling	-	he ?	0 to >30	0.5 to 10
Litonotus lamella	α	R	free-swimming	0 to 10	he ?	0 to >30	0.5 to 3
Opercularia articulata	β-α	Ba	attached	0 to 20	os	0 to 1	0.5 to 20
Parastrongylidium oswaldi	α-p	Ba, Fl, Al	crawling	-	-	-	0.5 to 5
Spathidium sp.	-	R	free-swimming	-	-	-	0.5 to 3
Tokophrya sp.	β-α	R	attached	-	os-oms ?	0 to 1 - 0 to 4	0.5
Trochilia minuta	β-α	Ba	crawling	0	os	0 to 1	0.5 to 3
Uronema nigricans	α-p	Ba, Fl	free-swimming	0 to 15	he	0 to >30	0.5 a 20
Vorticella microstoma	α-p	Ba, Al	attached	0 to 20	oms ?	0 to 4	0.5 to 20
Vorticella infusionum	α-p	Ba	attached	0 to 20	he ?	0 to >30	0.5 to 20
Vorticella aquadulcis	α-β	-	attached	-	he ?	0 to >30	0.5 to 20
Vorticella convallaria	α	Ba	attached	0 to 15	he	0 to >30	0.5 to 5
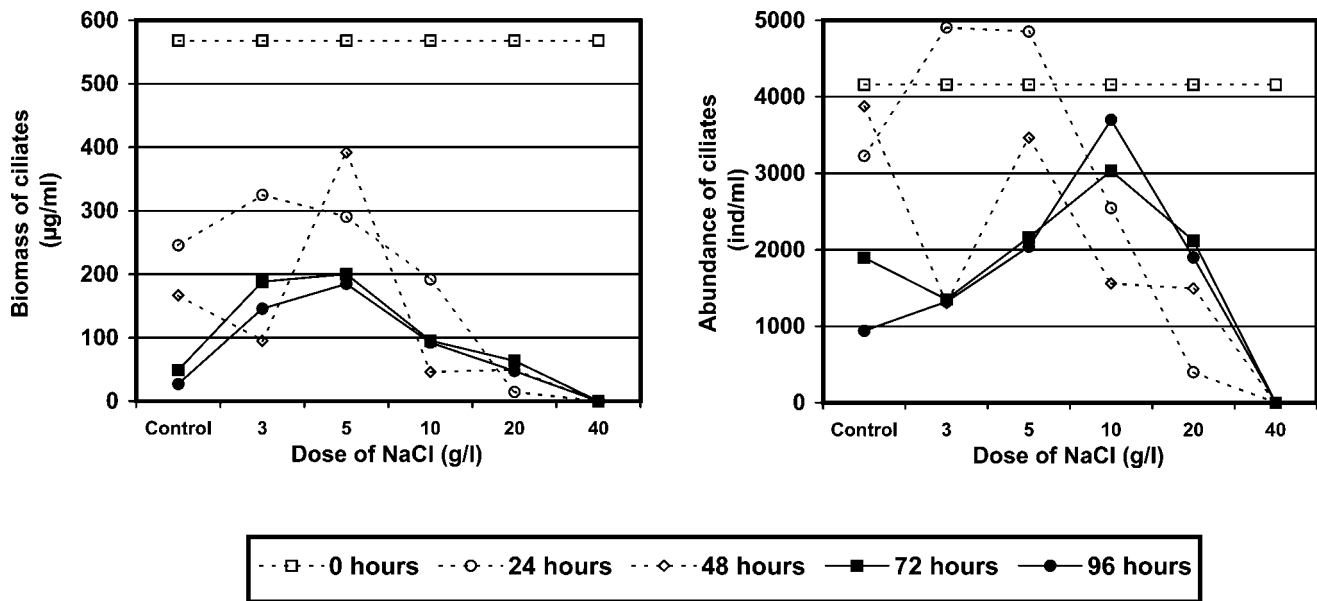
Fig. 5. Biomass and abundance of ciliates for different NaCl doses at different exposure times. Control (0.4 g NaCl/l) and D3, D5, D10, D20 and D40 are respectively doses of 3, 5, 10, 20 and 40 g NaCl/l

those are low-load indicators and give better-quality effluents (Curds 1982, Salvadó and Gracia 1993, Salvadó *et al.* 1995), were more sensitive to increased salinity.

The most sensitive species were those that can considered oligostenohaline (Finley 1930, Foissner *et al.* 1995) and that are also α-β-mesosaprobic, *Litonotus lamella*, *Aspidisca cicada* and *Trochilia minuta*, except for *Acineria uncinata*, which is poly-α-mesosaprobic. These are crawling species that consume different kinds of food: predators or bacteriophages. The first three species are also very sensitive to the toxicity of chlorine (Salvadó *et al.* 2000), and *Trochilia minuta* is especially sensitive to the toxicity of heavy metals Cd, Cr, Cu, Pb and Zn (Madoni 1996). However, *Aspisdica cicada* is not very sensitive to the heavy metals (Salvadó 1993; Gracia *et al.* 1994; Madoni *et al.* 1994, 1996). Moreover, *Acineria uncinata*, which is considered oligostenohaline, lives in a more polluted medium than the three previous species and has greater tolerance to toxic substances, such as chlorine and the heavy metals Zn and Cu, than *Aspidisca cicada* (Salvadó *et al.* 1993, 2000; Gracia *et al.* 1994). In general, there is some correlation between the "System of Saprobic Organisms" and the resistance of certain organisms to a particular toxic substance (Sládecek 1973). However, the results reported here, and those of Foissner *et al.*

(1995) do not support a correlation between these two parameters in ciliate protozoa from activated sludge. As such, we should consider them as independent.

The absence of protozoa in general, and ciliates in particular has been described as a limiting factor on the performance of activated sludge, as their absence means that scattered bacteria are not eliminated properly, and so the effluent deteriorates (Curds 1982, 1993). There is no significant loss of protozoa and metazoa up to 10 g/l doses of salt. At 10 g/l, a loss of species, and also a loss of biomass, occurs over time, and at 20 g/l, the loss of individuals and biomass at 24 h endangers the effectiveness of the process. However, the populations of microorganisms have the capacity to recover over time (Curds 1982, 1993; Madoni 1994; Salvadó *et al.* 2000). Together with this fact, the low diversity of microorganisms at this salinity dose reduces the stability of the process even more. Although there are unpredictable fluctuations in the composition of wastewater (Woolard and Irvine 1995), the wide diversity of microorganisms in activated sludge permits a greater capacity of response to shock loads. To sum up, the fact that ciliate protozoa and other microorganisms are capable of surviving in the presence of salt concentrations higher than those normally found in activated sludge increases the stability of the process when shock loads of salt are received.

REFERENCES

Albrecht J. (1984) Zur Autökologie ausgewählter Aufwuchsciliaten des Wasser-Flubsystems (Protozoa: Ciliophora). *Dechniana* **137:** 132-167

Ax P., Ax R (1960) Esperimentelle Untersuchungen über die Salzgehaltstoleranz von Ciliaten aus dem Brackwasser und Sübswasser. *Biol. Zbl.* **79:** 7-31

Becares E. (1991) Microfauna of an activated sludge pilot plant treating effluents from a pharmaceutical industry. In: Biological Approach to Sewage Treatment Process: Current Status and Perspectives, (Ed. P. Madoni). Centro Bazzucchi, Perugia

Bick H. (1964) Die Sukcession der Organismen bei der Selbstreinigung von organisch verunreinigtem Wasser unter verschiedenen Milieubedingungen. Min. ELF. des Landes Nordrhein/Westfalen. Düsseldorf

Burnett W.E. (1974) The effect of salinity variations on the activated sludge process. *Wat. Sew. Works.* **121:** 37-38, 54-55

Curds C. (1982) The ecology and role of protozoa in aerobic sewage treatment processes. *Ann. Rev. Microbiol.* **36:** 27-46

Curds C. (1993) Le rôle des protozoaires dans la purificacion de l'eau. *Ann. biol.* **5-6:** 193-219

Esteban G., Tellez C., Bautista L.M. (1990) Effects of habitat quality on ciliated protozoa communities in sewage treatment plants. *Envir. Technol.* **12:** 381-386

Fernández-Galiano D. (1994) The ammoniacal silver carbonate method as a general procedure in the study of protozoa from sewage (and other) waters. *Wat. Res.* **28:** 495-496

Fernández-Leborans G., Antonio-García M. T. (1988) Effects of lead and cadmium in a community of protozoans. *Acta Protozool.* **27:** 141-159

Finley H. E. (1930) Toleration of fresh water protozoa to increased salinity. *Ecology.* **11:** 337-347

Foissner W., Blattener H., Berger H., Kohmann F. (1991) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystem. Band I. Bayerisches Landesamt für Wasserwirtschaft. München

Foissner W., Berger H., Kohmann F. (1992) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystem. Band II. Bayerisches Landesamt für Wasserwirtschaft. München

Foissner W., Berger H., Kohmann F. (1994) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystem. Band III. Bayerisches Landesamt für Wasserwirtschaft. München

Foissner W., Berger H., Blattener H., Kohmann F. (1995) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystem. Band IV. Bayerisches Landesamt für Wasserwirtschaft. München

Gracia M. P. Salvadó H. Rius, M., Amigó J. M. (1994) Effects of copper on ciliate communities from activated sludge plants. *Acta Protozool.* **33:** 219-226

Hall T. D., Smallwood C. (1967) The effect of varying salinity on the performance of the activated sludge process. In: Proc. 16th Southern Water Resources, Pollution Control Conference, Duke University Christian Printing, N.C.

Henze M., Harremoës P., Jansen J.C., Arvin E. (1995) Wastewater Treatment. Biological and Chemical Processes. Springer-Verlag, Berlin

Hockernbury M. R., Burstein D., Jamro E. S. (1977) Total dissolved solids effects on biological treatment. In: Proc. 32nd Industrial Waste Conf., Purdue University. Lewis, Chelsea, Mich., 50-62

Kincannon D. F., Gaudy A. F. (1968) Response of biological waste treatment systems to changes in salt concentration. *Biotechnol. Bioengng.* **10:** 483-496

Koste W. (1978a) Die Rädertiere Mitteleuropas. Band I. Gebrüder Borntraeger. Berlin

Koste W. (1978b) Die Rädertiere Mitteleuropas. Band II. Gebrüder Borntraeger. Berlin

Lawton G., Eggert C. V. (1957) Effect of high sodium chloride concentration on tricking filter slimes. *J. Wat. Pollut. Control Fed.* **29:** 1228-1236

Ludzack F. J., Noran P. K. (1965) Tolerance of high salinities by conventional wastewater treatment process. *J. Wat. Pollut. Control Fed.* **37:** 1404-1416

Madoni P., Davoli D., Gessica G. (1994) Acute toxicity of lead, chromium, and other heavy metals to ciliates from activated sludge plants. *Bull Environ. Contam. Toxicol.* **53:** 420-425

Madoni P., Davoli D., Gessica G., Vescovi L. (1996) Toxic effect of heavy metals on the activated sludge protozoan community. *Wat. Res.* **30:** 135-141

Mahmoud T. A., Davis W. B. (1979) The effect of salinity on the removal of some aliphatic ketones. Environmental Engineering Division, Texas Engineering Experiment Station

Ogden C. G., Hedley R. H. (1980) An Atlas of Freshwater Testate Amoebae. British Museum (Natural History). Oxford University Press, London

Page F. C. (1988) *A* New Key to Freshwater and Soil Gymnamoebae with instructions for culture. Freshwater Biological Association, Cumbria

Pillai S. C., Rajagopalan R. (1948) Influence of sea water on protozoa activity and purification of sewage by the activated sludge. *Curr. Sci., Bangalore* **17:** 329

Salvadó H., Gracia M. P. (1993) Determination of organic loading rate of activated sludge plants based on protozoan analysis. *Wat. Res.* **27:** 891-895

Salvadó H., Fernández-Galiano D. (1997) *Parastrongylidium* (Hypotrichida, Ciliata), a Case of High Morphological Variability. *Europ. J. Protistol.* **33:** 178-185

Salvadó H., Gracia M. P., Rius M., Amigó J. M. (1993) Effets toxiques du zinc sur la diversité des communautés de protozoaires ciliés. *Historia Animalium.* **2:** 5-11

Salvadó H., Gracia M. P., Amigó J. M. (1995) Capability of ciliated protozoa as indicators of effluent quality in activated sludge plants. *Wat. Res.* **29:** 1041-1050

Salvadó H., Gracia M. P., Amigó J. M., Rius M. (1997) Effects of cadmium on growth and motility in *Euplotes aediculatus* isolated from activated sludge. *Bull. Environ. Contam. Toxicol.* **58:** 838-844

Salvadó H., Rius M., Gracia M. P. (2000) Effect of chlorination on microfauna communities in activated sludge plants. *Bull. Environ. Contam. Toxicol.* **65:** 70-77

Sládecek V. (1973) System of water quality from the biological point of view. *Arch. Hydrobiol. Engeb. Limnol.* **7:** 1-217

Smurov A. O., Fokin S. I. (1999) Resistance of *Paramecium* species (Ciliophora, Peniculia) to salinity of environment. *Protistology* **1:** 43-53

Sudo R., Aiba S. (1973) Effect of copper and hexavalent chromium on the specific growth. *Wat. Res.* **7:** 1301-1307

Tokuz R.Y., Eckenfelder W. W. (1978) The effects of F/M on an activated sludge system treating high salinity wastewater. In: Proc. 33rd Annual Purdue Industrial Waste Conf. Lewis, Chelsea, Mich., 200-203

Woolard C. R., Irvine R. L. (1994) Biological treatment of hypersaline wastewater by a biofilm of halophilic bacteria. *Wat. Environ. Res.* **66:** 230-235

Woolard C. R., Irvine R. L. (1995) Treatment of hypersaline waste-water in the sequencing batch reactor. *Wat. Res.* **29:** 1159-1168

Wong J. M. (1992) Biotreatment of contaminated groundwater with high organics and salinity contents. In: Proc. 46th Annual Purdue Industrial Waste Conf. Lewis, Chelsea, Mich., 75-87

Zobell C. E. (1946) Marine Microbiology. Chronica Botanica, Waltham, Mass.

Zobell C. E., Anderson D. Q., Smith W. W. (1937) The bacteriostatic and bactericidal action of Great Salt Lake Water. *J. Bacteriol.* **33:** 253-262

ACTA
PROTOZOOLOGICA

A Simple and Efficient Method for the Quantitative Analysis of Thymine Dimers in Cyanobacteria, Phytoplankton and Macroalgae

Rajeshwar P. SINHA, Margit DAUTZ and Donat-P. HÄDER

Institut für Botanik und Pharmazeutische Biologie, Friedrich-Alexander-Universität, Erlangen, Germany

Summary. Cyclobutane dimers are the most common DNA lesions after exposure of cells to UV-B radiation. A quantitative method was developed to determine the frequency of thymine dimers in aquatic primary producers such as cyanobacteria, phytoplankton and macroalgae to study the effects of UV radiation. Genomic DNA was extracted and purified by using standard biochemical and molecular biology techniques. DNA was transferred to a nylon membrane in a slot or dot blot and incubated with a primary antibody (anti thymine dimer KTM53) against thymine dimers. The secondary antibody was an anti-mouse IgG (Fab specific) peroxidase conjugate. The blots were quantified in a Kodak Digital Science Image Station 440 CF by the chemiluminescence method. The calibration of the method was achieved by using the plasmid pBSK with known DNA sequence, length and number of adjacent thymine pairs. This method permits the measurement of low as well as high levels of DNA lesions in nanogram quantities of DNA. This method can be used for cultured as well as naturally occurring organisms.

Key words: antibody, chemiluminescence, cyanobacteria, macroalgae, phytoplankton, thymine dimers, UV radiation.

INTRODUCTION

Ultraviolet radiation induces deleterious effects in all living organisms ranging from prokaryotic bacteria and unicellular aquatic organisms to higher plants, animals and men. While UV-C (<280 nm) radiation is ecologically not relevant since it is quantitatively absorbed by oxygen and ozone in the Earth's atmosphere, the longer wavelength UV-B (280-315 nm) and UV-A (315-400 nm) radiation can have significant effects on the biota, even though the majority of the extraterrestrial UV-B is absorbed by stratospheric ozone (Madronich *et al.* 1998). Some of the biological effects of solar UV radiation include killing of bacteria (Herndl 1997), inhibition of motility and orientation, protein destruction, pigment bleaching and photoinhibition of photosynthesis in cyanobacteria, phytoplankton and macroalgae (Cullen *et al.* 1992; Arrigo 1994; Sinha *et al.* 1995, 2001; Sinha and Häder 1996; Häder *et al.* 1998; Neale *et al.* 1998) as well as lethality in primary and secondary consumers in aquatic ecosystems (Hunter *et al.* 1982, Little and Fabacher 1994). In higher plants growth of leaves, shoots and roots are affected (Bornman and Teramura 1993, Ros and Tevini 1995, Huang *et al.* 1997) and flowering and reproduction are impaired (Staxén and Bornman 1994, Klaper *et al.* 1996) by UV stress.

Address for correspondence: Donat-P. Häder, Institut für Botanik und Pharmazeutische Biologie, Friedrich-Alexander-Universität, Staudtstr. 5, D-91058 Erlangen, Germany; Fax +49 9131 852 8215; E-mail: dphaeder@biologie.uni-erlangen.de

Action spectra have been measured for a number of UV-B effects in many organisms and for very different responses (Häder and Liu 1990, Häder *et al.* 1991, Cullen *et al.* 1992) indicating a multitude of cellular targets for solar UV. Proteins strongly absorb around 280 nm, due to their aromatic amino acids. In the photosynthetic apparatus several targets of UV-B radiation have been identified including the water splitting site and the D1/D2 protein complex in photosystem II (Bhattacharjee and David 1987, Renger *et al.* 1989). The integrity of the membranes is affected, caused by a decrease in the lipid content (Murphy 1983).

Photodynamic reactions are potential mechanisms by which ultraviolet radiation damages living cells (Ito 1983). The high energy of short wavelength photons absorbed by chromophore molecules can lead to the formation of singlet oxygen or free radicals known to destroy membranes and other cellular components (Benson *et al.* 1992, Alscher *et al.* 1997, Mackerness *et al.* 1999).

The DNA is certainly one of the key targets for damaging UV-B radiation in bacteria (Peak and Peak 1982, Peak *et al.* 1984), phytoplankton (Buma *et al.* 1995, 1997; Sommaruga and Buma 2000), macroalgae (Pakker *et al.* 2000), plants (Quaite *et al.* 1992), humans and animals (Stein *et al.* 1989, Kripke *et al.* 1992). Radiation damage to DNA is potentially dangerous to cells, since a single photon hit may have a dramatic or even lethal effect. Several different types of DNA damage have been identified that result from free radicals and reactive oxygen species formed by various photochemical processes. The two major classes of mutagenic DNA lesions induced by UV radiation are *cis-syn* cyclobutane-pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) which are pyrimidine adducts (Mitchell and Karentz 1993, Prakash *et al.* 1993, Friedberg *et al.* 1995, Sancar 1996a, Thoma 1999, Lindahl and Wood 2000). Both classes of lesions distort the DNA helix. CPDs and 6-4PPs induce a bend or kink of 7-9° and 44°, respectively. The ability of UV radiation to damage a given base is determined by the flexibility of the DNA. Sequences that facilitate bending and unwinding are favorable sites for damage formation, e.g. CPDs form at higher yields in single-stranded DNA, at the flexible ends of poly(dA).(dT) tracts, but not in their rigid center (Becker and Wang 1989, Lyamichev 1991). Bending of DNA towards the minor groove reduces CPD formation (Pehrson and Cohen 1992). One of the transcription

factors having a direct effect on DNA damage formation and repair is the TATA-box binding protein (TBP). TBP promotes the selective formation of 6-4PPs in the TATA-box, where the DNA is bent, but CPDs are formed at the edge of the TATA-box and outside, where DNA is not bent (Aboussekhra and Thoma 1999). These DNA lesions interfere with DNA transcription and replication and can lead to misreadings of the genetic code and cause mutations and death.

In contrast to other DNA lesions, CPDs can be photorepaired by a specific enzyme (photolyase) in the presence of and using the energy of UV-A or visible light at permissive temperatures (Sancar 1996b). Photolyases contain FAD as a catalytic cofactor and a second chromophore as a light-harvesting antenna. The second chromophores are either 5,10-methenyl- tetrahydrofolate or 8-hydroxy-5-deazariboflavin, with absorption maxima of ~380 and ~440 nm, respectively. Other DNA repair systems can also operate without light (Britt 1996, Taylor *et al.* 1996). While many studies have been conducted under laboratory conditions, DNA lesions in intact plants and bacteria have also been measured under field conditions (Quaite *et al.* 1992, Ballaré *et al.* 1996, Jeffrey *et al.* 1996). Even though there is an effective repair of DNA damage (Stapleton *et al.* 1997), some lesions may persist; low temperatures or darkness can hamper the enzymatic repair of DNA damage (Britt 1996, Takeuchi *et al.* 1996).

The aim of this work was to develop a reliable quantitative method to determine the frequency of thymine dimers in aquatic primary producers.

MATERIALS AND METHODS

Organisms and culture conditions

A number of cyanobacteria such as *Anabaena* sp., *Nostoc* sp. and *Scytonema* sp., phytoplankton such as *Euglena gracilis* and *Gyrodinium dorsum* and macroalgae such as *Porphyra umbilicalis* and *Ceramium rubrum* were used in the present study. The cyanobacteria were routinely grown in an autoclaved liquid medium as described by Safferman and Morris (1964) at a temperature of 20°C and illuminated with white fluorescent light of 12 Wm^{-2} (for details see Sinha *et al.* 1995). The phytoplankton was grown in F/2 medium (Guillard and Ryther 1962) prepared with artificial sea water (Tropic Marine, Dr. Bienle GmbH, Germany) in cylindrical glass tubes placed in a Kniese apparatus and bubbled with air at 19°C and continuous illumination (35 Wm^{-2} PAR from mixed fluorescence tubes: OSRAM L 36 W/32 Lumilux de luxe warm white de luxe and

Radium NL 36 W/26 Universal white). Macroalgae were grown in artificial sea water (0.33%; Instant Ocean, Sarrebourg, France and Mentor, Ohio, USA) supplemented with nitrate (9.8 µM) and phosphate (3.1 µM) and illuminated with fluorescent light (12 Wm^{-2}) at a temperature of 4°C for a 12 h photoperiod. All experimental materials are being routinely grown in our laboratory since at least seven years.

UV radiation source

Plastic trays containing macroalgae (fresh weight 2 - 3 g) dipped in 1 cm of water as well as liquid cultures (25 - 30 ml) of cyanobacteria and phytoplankton organisms were exposed to UV radiation produced from a transilluminator (TI 312, Bachofer, Reutlingen, Germany) at a distance of 20 cm which resulted in an irradiance of around 10 Wm^{-2} UV. The irradiance of UV was measured with a double monochromator spectroradiometer (OL 754, Optronic Laboratories, Orlando, Florida, USA). At defined time intervals samples were withdrawn and subjected to DNA extraction and blotting to monitor the formation of thymine dimers. DNA extraction was done either immediately after UV exposure, or the samples were kept in formaldehyde (2 %, v/v) in darkness. All experiments were run in triplicates.

Extraction of DNA

Cyanobacterial and phytoplankton organisms were concentrated by centrifugation (J2-21M/E) using a JA 20 rotor (Beckman Instruments) at 500 x g for 10 min at room temperature. All the samples (non irradiated control and UV irradiated) were washed twice with 2 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 50 mM NaCl and resuspended in a 500 µl solution containing 50 mM Tris-HCl, pH 8.0 and 50 mM EDTA. Thereafter, except for the macroalgae, cells were broken by sonication (20 Watts, Branson Sonifier 450, Ultrasonic Corporation, Danbury, USA) for 3 min on ice. Macroalgae were homogenized with a mortar and pestle. Subsequently, cells were treated with 100 µg/ml of proteinase K. Thereafter, 1 ml of prewarmed (55°C) extraction buffer containing 3 % (w/v) cetyltrimethyl ammonium bromide (CTAB); 1 % (w/v) sarkosyl; 20 mM EDTA; 1.4 M NaCl; 0.1 M Tris-HCl, pH 8.0 and 1 % (v/v) 2-mercaptoethanol were added and incubated at 55°C for 1 h in a water bath with mixing by gentle inversion every 10 min. The resulting suspension was allowed to cool for 1 - 2 min, and thereafter 2 ml of chloroform : isoamyl alcohol (24:1, v/v) was added and mixed by gentle inversion (about 25-30 times) until an emulsion was formed. After centrifugation (12000 x g for 5 min at room temperature) the supernatant was transferred to sterile microcentrifuge tubes. DNA was precipitated at -20°C for 2 h in 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and centrifuged at 12000 x g for 30 min at 4°C. The pellet was briefly rinsed once with ice-cold 70 % ethanol, dried and rehydrated with agitation at room temperature in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). DNA samples were processed either immediately after extraction or kept at 4°C for further analyses. The purity of the DNA was determined spectroscopically (Beckman DU-70). The DNA was considered pure if the ratio between 260 and 280 nm was between 1.8 and 2.0. A ratio below 1.6 is typical for a protein contamination while the ratio above 2.0 is characteristic for an RNA contamination. The

DNA concentration was measured in a spectrophotometer (Beckman DU-70). The absorption at 260 nm gives the concentration of the DNA (1 O.D. at 260 nm equals 50 µg ml^{-1}dsDNA).

Detection of thymine dimers

The blot papers (GB002, Schleicher & Schuell, Dassel, Germany) and the nylon membrane (Nytran N2, Schleicher & Schuell) were soaked in a solution containing 3 M NaCl and 0.3 M Na-citrate and placed on a slot or dot blot manifold (Minifold I, Schleicher & Schuell). DNA samples were transferred to the membrane and washed once with TE buffer. The membrane was dried for 1 h at 80°C to immobilize the DNA. Subsequently, the membrane was incubated for 1 h in PBS-T [phosphate buffer saline: 0.14 M NaCl, 3.4 mM KCl, 10.1 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4 + 0.1 % (v/v) Tween 20] with 5 % (w/v) skimmed milk powder to block the non-specific sites. Thereafter, the membrane was incubated with the primary antibody (anti-thymine dimer KTM53, Kamiya Biomedicals, Seattle, USA; diluted 1:10000 in PBS-T) for 2 h at room temperature and then washed (3 x 10 min) with PBS-T. Afterwards the membrane was incubated with the secondary antibody [anti-mouse IgG (Fab specific) peroxidase conjugate, Sigma, Saint Louis, Missouri, USA; diluted 1:10000 in PBS-T with 5 % skimmed milk powder] for 1 h at room temperature and washed with PBS-T (4 x 10 min). Finally, the membrane was placed in a detection reagent (Renaissance, NEN Life Science Products, Cologne, Germany) for 1 min before scanning (Kodak Digital Science, Image Station 440 CF, New Haven, CT, USA). The results were evaluated by using the software provided by the manufacturer.

Calibration standard for thymine dimer quantification

In order to establish a standard with known thymine dimer frequency the plasmid pBSK (obtained from R. Marschalek, Frankfurt) was used. For isolation of RNA-free plasmid DNA the DNA Midiprep I Kit from peQLab (Erlangen, Germany) was used. The plasmid has a size of 2961 bp which corresponds to 3.25 x 10^{-12} µg. In the genome 326 thymine pairs occur. Thus 1 µg of the plasmid contains 1.1 x 10^{14} thymine dimers when all possible pairs are dimerized. In order to almost completely induce all thymine dimers the plasmid was irradiated with the transilluminator, as described earlier, for 60 min. Various concentrations (1-1000 ng) of irradiated pBSK was loaded on dot blots. The frequency F of thymine dimers (T^T) per megabase pair is calculated with the following equation, where T^T is the number of thymine dimers in the irradiated plasmid DNA and M the total DNA mass:

$$F = T\text{^}T \ / \ (9.11 \ x \ 10^{14} * M) * 10^6$$

Stability of thymine dimers at 4 and -20°C

The plasmid pBSK was irradiated with the transilluminator as described above and the sample was divided into two equal parts. One part was stored overnight at 4°C in a refrigerator and the second part at -20°C in a freezer. Next morning, the DNA was blotted to test the stability of thymine dimers as well as the overall concentration of the DNA to evaluate the effects of the two temperatures.

RESULTS

We first isolated the plasmid DNA (pBSK) from *E. coli* and determined the yield. Thereafter, the DNA from control and UV-irradiated samples of various cyanobacteria, phytoplankton and macroalgae were isolated, and the total yield was determined. Purified plasmid DNA and the DNA from studied organisms had their absorption maximum at 260 nm (data not shown).

Formation of thymine dimers in plasmid DNA and test organisms after UV irradiation was determined by blotting and chemiluminescence method. Various concentrations (1-1000 ng) of UV irradiated plasmid DNA (for the determination of a calibration curve) and equal amounts of the DNA from test organisms were loaded onto the nylon membrane. Figure 1 represents the blotting pattern of both plasmid DNA (lanes A; 1-8) and DNA from the cyanobacterium *Nostoc* sp. (lanes B; 1-5) after increasing times of UV radiation. There was a gradual increase in the intensity of the luminescence with increasing concentrations of plasmid DNA (Fig. 1; lanes A, 1-8). Thymine dimers were detectable with this method at DNA concentrations as low as 1 ng. Similarly, there was a gradual increase in the intensity of the luminescence with increasing UV irradiation times in *Nostoc* sp. (Fig. 1, lanes B, 1-5).

The formation of thymine dimers (T^T/Mb) in plasmid DNA was determined and a calibration curve was plotted (Fig. 2). Similarly, the frequency of thymine dimers formed after different durations of UV radiation was calculated in *Nostoc* sp. (Fig. 3). The quantitative method for the determination of thymine dimers works equally well for the phytoplankton and the macroalgae; results of which have been shown in Table 1 together with other cyanobacteria. Figure 3 and Table 1 show an induction in the frequency of thymine dimers with increasing UV irradiation time. It is pertinent to mention that the UV-irradiated DNA samples should not be frozen since this results in a loss of thymine dimers (Fig. 4). Although the total DNA concentration was not affected by the cold treatment the quantification of the dots (Fig. 4 insets) shows a loss of about 20-25 % thymine dimers in the UV irradiated DNA samples placed overnight at -20°C (Fig. 4). This loss could be due to structural changes of the thymine dimers by freezing.

DISCUSSION

The survival of organisms depends on the accurate transmission of the genetic information from one cell to its daughters. Such faithful transmission requires not only extreme accuracy in replication of DNA and precision in chromosome distribution, but also in the ability to survive spontaneous and induced DNA damage while minimizing the number of heritable mutations (Zhou and Elledge 2000). UV-induced DNA damage and its photoenzymatic and nucleotide excision repair have been known from diverse organisms (Mitchell and Karentz 1993, Britt 1996, Pakker *et al.* 2000, Zhou and Elledge 2000).

This paper presents a simple and efficient method for the quantitative analysis of thymine dimers in various aquatic primary producers such as cyanobacteria, phytoplankton and macroalgae, in order to better understand the role of UV radiation in eliciting mutagenic effects. A number of methods for DNA isolation from diverse organisms are in practice (Karentz *et al.* 1991, Rogers and Bendich 1994, Rudi *et al.* 1998, Fiore *et al.* 2000, Pakker *et al.* 2000, Perdiz *et al.* 2000, Tillett and Neilan 2000). Our protocol for DNA isolation is simple, rapid and inexpensive, providing high quality DNA from a wide range of organisms. A number of workers use NaI which is not only expensive and environmentally hazardous but degrades in a short period of time (Fiore *et al.* 2000). NaCl was used instead which is not only cheaper but also has the advantage of reducing the amount of RNA and hence making treatment with RNase unnecessary (Fiore *et al.* 2000). Similarly, we do not use phenol, which creates environmental hazards.

A number of methods are in use to determine the DNA damage in a variety of organisms (O'Brine and Houghton 1982, Freeman *et al.* 1986, Mitchell *et al.* 1991, Van Loon *et al.* 1992, Buma *et al.* 1995, Hidema *et al.* 1999, Douki *et al.* 2000, Pakker *et al.* 2000, Perdiz *et al.* 2000, Sommaruga and Buma 2000). UV-induced DNA degradation has been reported in the cyanobacterium *Synechocystis* by using radioactive methods (O'Brine and Houghton 1982) and showing percentage radioactivity lost from DNA as a measure for DNA degradation. An alkaline agarose gel method for quantifying single strand breaks in nanogram quanti-
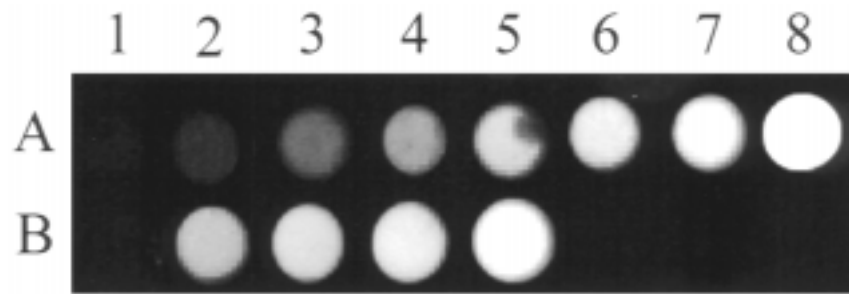
Fig. 1. Dot blot of both plasmid DNA (pBSK) and DNA form *Nostoc* sp. Lanes A (1 - 8), plasmid DNA (1, 2, 5, 10, 20, 50, 100 and 1000 ng, respectively). Lanes B (1-5), DNA from *Nostoc* sp. after different durations of UV radiation (0, 15, 30, 60 and 120 min of UV radiation, respectively)
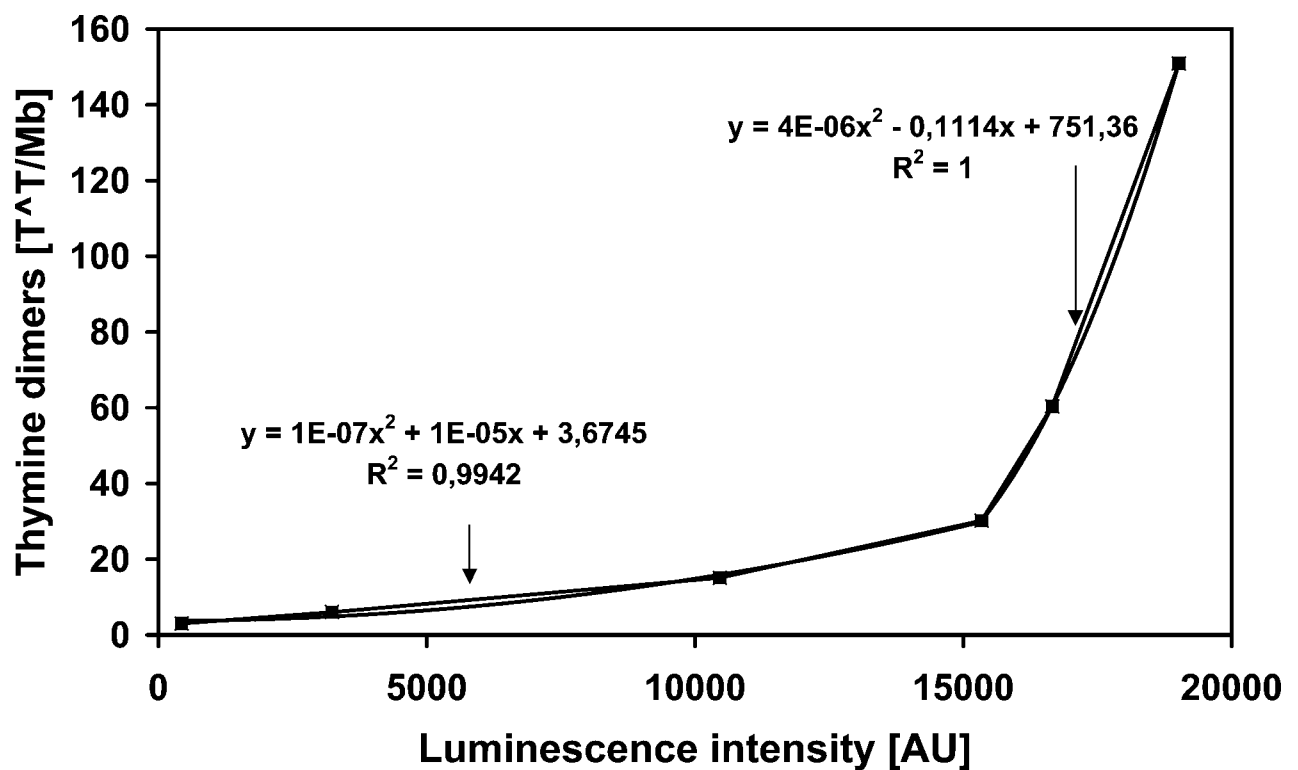
Fig. 2. Calibration curve plotted from blots of plasmid DNA (1-50 ng) as shown in Fig. 1. For details see text
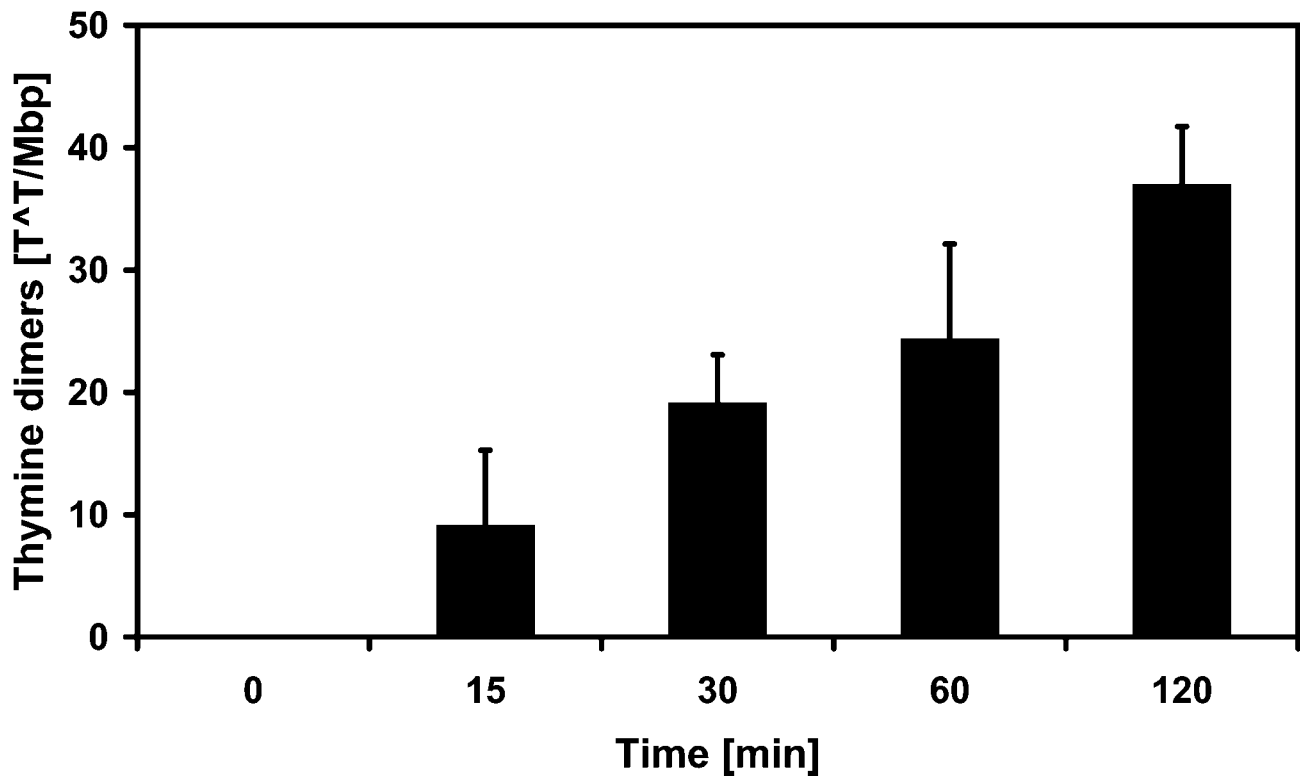
Fig. 3. Frequency of thymine dimers in *Nostoc* sp. after UV-B irradiation for different durations. For details see text
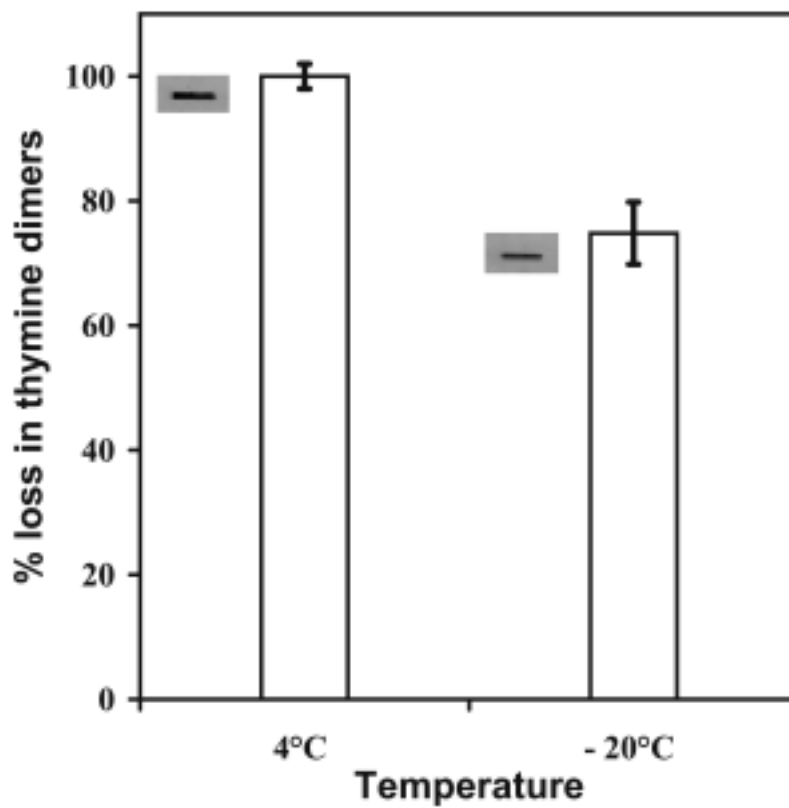

Fig. 4. Loss in thymine dimers after overnight freezing at -20°C of UV irradiated plasmid DNA (pBSK). The slot blots (insets) are the representative of ten different but identical experiments on the basis of which percentage loss in thymine dimer was calculated

Table 1. Frequency of thymine dimers in various organisms after UV (10 W/m^2) irradiation for different durations

Organism	T^T/Mbp				
	UV irradiation [min]				
	0	15	30	60	120
Cyanobacteria					
Anabaena sp.	0	8.23 ± 4.30	16. 45 ± 5.22	22.42 ± 6.52	39.65 ± 6.65
Scytonema sp.	0	6.52 ± 4.21	13.62 ± 4.42	18.68 ± 5.95	35.55 ± 5.22
Phytoplankton					
Euglena gracilis	0	7.52 ± 4.63	15.47 ± 4.21	21.82 ± 7.42	36.25 ± 4.45
Gyrodinium dorsum	0	6.82 ± 3.67	14.58 ± 4.45	19.98 ± 6.45	32.55 ± 5.45
Macroalgae					
Porphyra umbilicalis	0	5.56 ± 2.33	9.98 ± 2.50	14.56 ± 2.68	26.87 ± 3.58
Ceramium rubrum	0	5.22 ± 2.15	8.68 ± 2.11	15.58 ± 3.55	28.57 ± 3.69

ties of nonradioactive DNA was developed by Freeman *et al.* (1986). Another method for cyclobutane dimer detection was presented by Mitchell *et al.* (1991). They first labeled the DNA by radioactive substances followed by agarose gel electrophoresis and densitometric analysis and finally digesting with endo III and endo V before analyzing on sequencing gels. An immunochemical assay was improved by Van Loon *et al.* (1992) for quantitative detection of DNA damage. This technique was based on the enhancement of the radiation-induced single-strandedness which was determined by using a monoclonal antibody. Buma *et al.* (1995) developed an immunofluorescent thymine dimer detection method by labeling dimers with antibody followed by a secondary antibody (fluorescein isothiocyanate) staining and finally visualization of DNA damage with flow cytometry or fluorescence microscopy. More or less the same method was used in subsequent publications by Pakker *et al.* (2000) and Sommaruga and Buma (2000). Yet another method for measurement of thymine photoproducts by using an electrospray-mass spectrometer was presented by Douki *et al.* (2000). Most of the methods discussed above present relative data on DNA damage. We present a simple and efficient quantitative method to determine the frequency of thymine dimers in a variety of organisms in relatively short period of time by using blotting and chemiluminescence methods. This method neither requires radioactive labeling of DNA nor its detection by agarose gel electrophoresis (where ethidium bromide is being used to stain the DNA) and thereby eliminating the possibilities of health hazards. This method permits the measurement of low as well as high levels of DNA lesions in nanogram quantities of DNA and can be used for the cultured as well as naturally occurring organisms.

Once the frequency of the thymine dimers is determined, it could well be correlated with the survival of the organisms.

REFERENCES

Aboussekhra A., Thoma F. (1999) TATA-binding protein promotes the selective formation of UV-induced (6-4)-photoproducts and modulates DNA repair in the TATA box. *EMBO J.* **18:** 433-443

Alscher R. G., Donahue J. L., Cramer C. L. (1997) Reactive oxygen species and antioxidants: relationship in green cells. *Physiol. Plant.* **100:** 224-233

Arrigo K. R. (1994) Impact of ozone depletion on phytoplankton growth in the Southern Ocean: large scale partial and temporal variability. *Mar. Ecol. Prog. Ser.* **114:** 1-12

Ballaré C. L., Scopel A. L., Stapleton A. E., Yanovsky M. J. (1996) Solar ultraviolet-B radiation affects seedling emergence, DNA integrity, plant morphology, growth rate, and attractiveness to herbivore insects in *Datura ferox*. *Plant Physiol.* **112:** 161-170

Becker M. M., Wang Z. (1989) Origin of ultraviolet damage in DNA. *J. Mol. Biol.* **210:** 429-438

Benson E. E., Lynch P., Jones J. (1992) Variation in free radical damage in rice cell suspension with different embryogenic potentials. *Planta* **188:** 296-305

Bhattacharjee S. K., David K. A. V. (1987) UV-sensitivity of cyanobacterium *Anacystis nidulans*: part II - a model involving photosystem (PSII) as a lethal target and herbicide binding high turnover B protein as regulator of dark repair. *Indian J. Exp. Biol.* **25:** 837-842

Bornman J. F., Teramura A. H. (1993) Effects of ultraviolet-B radiation on terrestrial plants. In: Environmental UV-Photobiology, (Eds. A. R. Young, L. O. Björn, J. Moan and W. Nultsch). Plenum, New York, 427-471

Britt A. B. (1996) DNA damage and repair in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47:** 75-100

Buma A. G. J., van Hannen E. J., Roza L., Veldhuis M. J. W., Gieskes W. W. C. (1995) Monitoring ultraviolet-B induced DNA damage in individual diatom cells by immunofluorescent thymine dimer detection. *J. Phycol.* **51:** 314-321

Buma A. G. J., Engelen A. H., Gieskes W. W. C. (1997) Wavelength-dependent induction of thymine dimers and growth rate reduction in the marine diatom *Cyclotella* sp. exposed to ultraviolet radiation. *Mar. Ecol. Prog. Ser.* **153:** 91-97

Cullen J. J., Neale P. J., Lesser M. P. (1992) Biological weighting function for the inhibition of phytoplankton photosynthesis by ultraviolet radiation. *Science* **258:** 646-650

Douki T., Court M., Cadet J. (2000) Electrospray-mass spectrometry characterization and measurement of far-UV-induced thymine photoproducts. *J. Photochem. Photobiol. B: Biol.* **54:** 145-154

Fiore M. F., Moon D. H., Tsai S. M., Lee H., Trevors J. T. (2000) Miniprep DNA isolation from unicellular and filamentous cyanobacteria. *J. Microbiol. Methods* **39:** 159-169

Freeman S. E., Blackett A. D., Monteleone D. C., Setlow R. B., Sutherland B. M., Sutherland J. C. (1986) Quantitation of radiation-, chemical-, or enzyme-induced single strand breaks in nonradioactive DNA by alkaline gel electrophoresis: application to pyrimidine dimers. *Analytical Biochem.* **158:** 119-129

Friedberg E. C., Walker G. C., Siede W. (1995) DNA Repair and Mutagenesis. ASM Press, Washington, DC

Guillard R. R. L., Ryther J. H. (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula convervacea* (Cleve) Gran. *Can. J. Microbiol.* **8:** 229-239

Häder D.-P., Liu S.-M. (1990) Motility and gravitactic orientation of the flagellate, *Euglena gracilis*, impaired by artificial and solar UV-B radiation. *Curr. Microbiol.* **21:** 161-168

Häder D.-P., Worrest R. C., Kumar H. D. (1991) Aquatic Ecosystems. *UNEP Environmental Effects Panel Report*, Nairobi, Kenya 33-40

Häder D.-P., Kumar H. D., Smith R. C., Worrest R. C. (1998) Effects on aquatic ecosystems. *J. Photochem. Photobiol. B: Biol.* **46:** 53-68

Herndl G. J. (1997) Role of ultraviolet radiation on bacterioplankton activity. In: The Effects of Ozone Depletion on Aquatic Ecosystems, (Ed. D.-P. Häder). Environmental Intelligence Unit, Academic Press and R.G. Landes Company, Austin, 143-154

Hidema J., Kang H.-S., Kumagai T. (1999) Changes in cyclobutyl pyrimidine dimer levels in rice (*Oryza sativa* L.) growing indoors and outdoors with or without supplemental UV-B radiation. *J. Photochem. Photobiol. B: Biol.* **52:** 7-13

Huang S., Dai Q., Peng S., Chavez A. Q., Miranda M. L. L., Visperas R. M., Vergara B. S. (1997) Influence of supplemental ultraviolet-B on indoleacetic acid and calmodulin in the leaves of rice (*Oryza sativa* L.). *Plant Growth Regulation* **21:** 59-64

Hunter J. R., Kaupp S. E., Taylor J. H. (1982) Assessment of effects of UV radiation on marine fish larvae. In: The Role of Solar Ultraviolet Radiation in Marine Ecosystems, (Ed. J. Calkins). Plenum, New York, 459-497

Ito T. (1983) Photodynamic agents as tools for cell biology. In: Photochemical and Photobiological Reviews, (Ed. K. C. Smith). Plenum, New York, 141-186

Jeffrey W. H., Pledger R. J., Aas P., Hager S., Coffin R. B., Haven R. V., Mitchell D. L. (1996) Diel and depth profiles of DNA photodamage in bacterioplankton exposed to ambient solar ultraviolet radiation. *Mar. Ecol. Prog. Ser.* **137:** 293-304

Karentz D., Cleaver J. E., Mitchell D. L. (1991) Cell survival characteristics and molecular responses of Antarctic phytoplankton to ultraviolet-B radiation. *J. Phycol.* **27:** 326-341

Klaper R., Frankel S., Berenbaum M. R. (1996) Anthocyanin content and UVB sensitivity in *Brassica rapa*. *Photochem. Photobiol.* **63:** 811-813

Kripke M. L., Cox P. A., Alas L. G., Yarosh D. B. (1992) Pyrimidine dimers in DNA initiate systemic suppression in UV-irradiated mice. *Proc. Natl. Acad. Sci.* USA **89:** 7516-7520

Lindahl T., Wood R. D. (2000) Quality control by DNA repair. *Science* **286:** 1897-1905

Little E. E., Fabacher D. L. (1994) Comparative sensitivity of rainbow trout and two threatened salmonids, Appache Trout and Lahontan Cutthroat Trout, to UV-B radiation. *Arch. Hydrobiol.* **43:** 217-226

Lyamichev V. (1991) Unusual conformation of (dA)n.(dT)n-tracts as revealed by cyclobutane thymine-thymine dimer formation. *Nucleic Acids Res.* **19:** 4491-4496

Mackerness A.-H. S., Jordan B. R., Thomas B. (1999) Reactive oxygen species in the regulation of photosynthetic genes by ultraviolet-B radiation (UV-B: 280-320 nm) in green and etiolated buds of pea (*Pisum sativum* L.). *J. Photochem. Photobiol. B: Biol.* **48:** 180-188

Madronich S., McKenzie R. L., Björn L. O., Caldwell M. M. (1998) Changes in biologically active ultraviolet radiation reaching the Earth's surface. *J. Photochem. Photobiol. B: Biol.* **46:** 5-19

Mitchell D. L., Karentz D. (1993) The induction and repair of DNA photodamage in the environment. In: Environmental UV Photobiology, (Eds. A. R. Young, L. Björn, J. Moan and W. Nultsch). Plenum, London, 345-377

Mitchell D. L., Jen J., Cleaver J. E. (1991) Relative induction of cyclobutane dimers and cytosine photohydrates in DNA irradiated *in vitro* and *in vivo* with ultraviolet-C and ultraviolet-B light. *Photochem. Photobiol.* **54:** 741-746

Murphy T. M. (1983) Membranes as targets of ultraviolet radiation. *Physiol. Plant.* **58:** 381-388

Neale P. J., Davis R. A., Cullen J. J. (1998) Interactive effects of ozone depletion and vertical mixing on photosynthesis of Antarctic phytoplankton. *Nature* **392:** 585-589

O'Brine P. A., Houghton J. A. (1982) UV-induced DNA degradation in the cyanobacterium *Synechocystis* PCC 6308. *Photochem. Photobiol.* **36:** 417-422

Pakker H., Beekman C. A. C., Breeman A. M. (2000) Efficient photoreactivation of UVBR-induced DNA damage in the sublittoral macroalga *Rhodymenia pseudopalmata* (Rhodophyta). *Eur. J. Phycol.* **35:** 109-114

Peak M. J., Peak J. G. (1982) Single-strand breaks induced in *Bacillus subtilis* DNA by ultraviolet light: action spectrum and properties. *Photochem. Photobiol.* **35:** 675-680

Peak M. J., Peak J. G., Moehring P., Webb R. B. (1984) Ultraviolet action spectra for DNA dimer induction, lethality and mutagenesis in *Escherichia coli* with emphasis on the UVB region. *Photochem. Photobiol.* **40:** 613-620

Pehrson J. R., Cohen L. H. (1992) Effects of DNA looping on pyrimidine dimer formation. *Nucleic Acid Res.* **20:** 1321-1324

Perdiz D., Gróf P., Mezzina M., Nikaido O, Moustacchi E., Sage E. (2000) Distribution and repair of bipyrimidine photoproducts in solar UV-irradiated mammalian cells. *J. Biol. Chem.* **275:** 26732-26742

Prakash S., Sung P., Prakash L. (1993) DNA repair genes and proteins of *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **27:** 33-70

Quaite F. E., Sutherland B. M., Sutherland J. C. (1992) Action spectrum for DNA damage in alfalfa lowers predicted impact of ozone depletion. *Nature* **358:** 576-578

Renger G., Volker M., Eckert H. J., Fromme R., Hohm-Veit S., Gräber P. (1989) On the mechanism of photosystem II deterioration by UV-B irradiation. *Photochem. Photobiol.* **49:** 97-105

Rogers S. O., Bendich A. J. (1994) Extraction of total cellular DNA from plants, algae and fungi. *Plant Mol. Biol. Man.* **D2:** 1-12

Ros J., Tevini M. (1995) UV-radiation and indole-3-acetic acid: interactions during growth of seedlings and hypocotyl segments of sunflower. *J. Plant Physiol.* **146:** 295-305

Rudi K., Larsen F., Jakobsen K. S. (1998) Detection of toxin-producing cyanobacteria by use of paramagnetic beads for cell concentration and DNA purification. *Appl. Env. Microbiol.* **64:** 34-37

Safferman R. S., Morris M. E. (1964) Growth characteristics of the blue-green algal virus LPP-1. *J. Bacteriol.* **88:** 771-775

Sancar A. (1996a) DNA excision repair. *Annu. Rev. Biochem.* **65:** 43-81

Sancar A. (1996b) No "end of history" for photolyases. *Science* **272:** 48-49

Sinha R. P., Häder D.-P. (1996) Photobiology and ecophysiology of rice field cyanobacteria. *Photochem. Photobiol.* **64:** 887-896

Sinha R. P., Kumar H. D., Kumar A., Häder D.-P. (1995) Effects of UV-B irradiation on growth, survival, pigmentation and nitrogen metabolism enzymes in cyanobacteria. *Acta Protozool.* **34:** 187-192

Sinha R. P., Klisch M., Gröniger A., Häder D.-P. (2001) Responses of aquatic algae and cyanobacteria to solar UV-B. *Plant Ecol.* (in press)

Sommaruga R., Buma A. G. J. (2000) UV-induced cell-damage is species-specific among aquatic phagotrophic protists. *J. Eukaryot. Microbiol.* **47:** 450-455

Stapleton A. E., Thornber C. S., Walbot V. (1997) UV-B component of sunlight causes measurable damage in field-grown maize (*Zea mays* L.): developmental and cellular heterogeneity of damage and repair. *Plant Cell Environ.* **20:** 279-290

Staxén I., Bornman J. F. (1994) A morphological and cytological study of *Petunia hybrida* exposed to UV-B radiation. *Physiol. Plantar.* **91:** 735-740

Stein B., Rahmsdorf H. J., Steffen A., Litfin M., Herrlich P. (1989) UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-fos, and metallothionein. *Mol. Cell. Biol.* **9:** 5169-5181

Takeuchi Y., Kubo H., Kasahara H., Sakaki T. (1996) Adaptive alterations in the activities of scavengers of active oxygen in cucumber cotyledons irradiated with UV-B. *J. Plant Physiol.* **147:** 589-592

Taylor R. M., Nikaido O., Jordan B. R., Rosamond J., Bray C. M., Tobin K. (1996) Ultraviolet-B induced DNA lesions and their removal in wheat (*Triticum aestivum* L.) leaves. *Plant Cell Environ.* **19:** 171-181

Thoma F. (1999) Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *EMBO J.* **18:** 6585-6598.

Tillett D., Neilan B. A. (2000) Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. *J. Phycol.* **36:** 251-258

Van Loon A. A. W. M., Groenendijk R. H., Timmerman A. J., Van der Schans G. P., Lohman P. H. M., Bann R. A. (1992) Quantitative detection of DNA damage in cells after exposure to ionizing radiation by means of an improved immunochemical assay. *Mutation Res.* **274:** 19-27

Zhou B.-B. S., Elledge S. J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature* **408:** 433-439

ACTA
PROTOZOOLOGICA

Phytoplankton Communities at Different Depths in Two Eutrophic and Two Oligotrophic Temperate Lakes at Higher Latitude During the Period of Ice Cover

Roman A. DANILOV and Nils G. A. EKELUND

Department of Natural and Environmental Sciences, Mid Sweden University, Härnösand, Sweden

Summary. Phytoplankton communities at different depths and at different locations within each lake were studied during the period of ice cover (from January to March) in two eutrophic and two oligotrophic lakes in North-Eastern Sweden. Cryptophyceae, Dinophyceae and Euglenophyceae were dominant during the whole period of investigation in eutrophic environments. Bacillariophyceae, Chlorophyceae, Chrysophyceae and Cyanophyceae were only occasionally found both in eutrophic and oligotrophic lakes. Both in eutrophic and oligotrophic lakes diversity as well as abundance of phytoplankton were considerably higher immediately under ice than near the bottom. No differences in horizontal distribution of phytoplankton assemblages were detected within each lake. However, vertical differences were more profound. It is speculated that both light availability and increased ion concentration under the ice cover can be viewed as main factors determining phytoplankton communities.

Key words: ice cover, lake, phytoplankton, succession, trophic level.

INTRODUCTION

Lakes showing dimictic circulation patterns are common at temperate latitudes. The occurring stratification has profound effects on the whole complex of nutrient circulation in the water column thus leading to successions in phytoplankton communities (Trifonova 1993, Pierson and Weyhenmeyer 1994). It has been reported that lakes morphometry can play, besides nutrient contents, important role in distribution of phytoplankton

Address for correspondence: Roman A. Danilov, Department of Natural and Environmental Sciences, Mid Sweden University, 871 88 Härnösand, Sweden; Fax: +46-611-86160; E-mail: roman.danilov@tnv.mh.se

populations (Agbeti *et al.* 1997). Although several reports dealt with development of phytoplankton under ice cover (e.g. Kelley 1997, Reitner *et al.* 1997), no comparative studies were performed in order to elucidate patterns in both spatial and temporal successions of planktonic algal communities under ice cover in lakes of different trophic level. Such factors as lake morphometry, light and nutrient availability can affect planktonic organisms under ice (Fritsen and Priscu 1999, Butler *et al.* 2000). The aim of our study was to investigate temporal and spatial patterns in development of phytoplankton communities under ice cover in two eutrophic and two oligotrophic temperate lakes in North-Eastern Sweden.

MATERIALS AND METHODS

Two eutrophic and two oligotrophic lakes in Northeast Sweden (62°54' N) were sampled monthly during the period of ice-cover (January, February, March 2000). All four temperate lakes are shallow and show dimictic stratification patterns. Eutrophication was defined according to total phosphorus concentration (P_{tot}), where the lakes with P_{tot} less than 15 µg l^{-1} were considered as oligotrophic and above 25 µg l^{-1} as eutrophic, respectively (Swedish Environmental Protection Agency 1991). Four sampling stations were randomly chosen at a depth of 3 m within each lake in order to enable comparison of on both horizontal and vertical differences between phytoplankton communities. In order to perform water sampling holes in the ice were drilled. At each station sampling was done under ice and near bottom with the aid of a 1.5 l Ruttner water-bottle sampler with a built-in thermometer. For each sampling station and depth two PVC-bottles (500 ml, for qualitative and quantitative analyses) were filled. The samples for quantitative analyses were preserved in 1% Lugol's solution immediately after collection. Later in the laboratory, the preserved samples were left to stand for 24 h in order to achieve sedimentation of the algal cells. After sedimentation the samples were concentrated first to 50 ml by carefully removing 450 ml through plankton nets (3 µm mesh size). The remaining 50 ml were centrifuged for 20 s at 4000 rpm. The liquid phase was then immediately removed and the remaining pellet resuspended in approximately 10 drops of sample water with a Pasteur pipette. When the exact identification of species proved impossible from the preserved samples, fresh samples were used for assistance. The frequency of each species present in the fixed samples was determined according to relative units: 1 - occasional, 2 - rare, 3 - frequent, 4 - dominant (e.g. Kangas *et al.* 1993; Smolar *et al.* 1998; Danilov and Ekelund 1999, 2000).

Cluster analyses were performed on the base of species presence-absence matrices by using the Euclidean distance algorithm in the computer package Minitab 13.0 (Danilov and Ekelund 1999, 2000).

RESULTS AND DISCUSSION

The lists of phytoplankton species identified during the period of investigation are shown in Tables 1 and 2. Cryptophyceae were present all the time in all four lakes, *Rhodomonas lacustris* being abundant both in eutrophic and oligotrophic environments. *Cryptomonas erosa* and *C. reflexa* were abundant in eutrophic lakes where they formed the main part of abundance at all depths, while *C. marssonii* only occasionally occurred in oligotrophic lakes. *C. reflexa* was rarely found immediately under ice in oligotrophic environments. Two species of Dinophyceae as well as almost all species of Euglenophyceae occurred entirely in eutrophic lakes. While Dinophyceae were abundant at all depths, *Phacus caudatus*, *P. suecicum* and *Trachelomonas planktonica* (Euglenophyceae) were restricted to water layer immediately below the ice cover. *T. volvocinopsis* formed a considerable part of abundance at all depths in eutrophic lakes, while *T. volvocina* was only occasionally found near bottom in oligotrophic lakes. Only occasional presence of Bacillariophyceae and Chrysophyceae could be reported, Chlorophyceae reached their highest species diversity in eutrophic lakes, although the abundance of species identified was extremely low (occasional founds) in all four lakes studied (Tables 1 and 2).

In the present study autotrophic species contributed to the main part of plankton cell numbers as it has been shown by other investigators (e.g. Padisak *et al.* 1998). It has been reported earlier that Chrysophyceae and Cryptophyceae are most often dominant under ice cover (Spaulding *et al.* 1994, Agbeti and Smol 1995). Our study showed only Cryptophyceae being dominant during the whole period of investigation in eutrophic environments at any depths sampled. Thereby, the densities of Cryptophyceae were much higher immediately under ice cover compared to deeper water layers. Nonetheless, Cryptophyceae dominated also near the bottom. The appearance of *T. volvocinopsis* as codominant can be explained by the ability of it and of Cryptophyceae to heterotrophic nutrition and thus survival in conditions of insufficient light intensity (van den Hoek *et al.* 1995). The dominance of *R. lacustris* in oligotrophic lakes agrees well with predominant presence of small flagellates under ice cover reported earlier (Tulonen *et al.* 1994, Agbeti and Smol 1995, Kelley 1997, Reitner *et al.* 1997). Phytoplankton abundance in oligotrophic environments was considerably lower than under eutrophic conditions. This can be explained by general availability of nutrients (Tulonen *et al.* 1994).

No horizontal differences between phytoplankton communities at the same depth could be revealed in any of the lakes investigated. This phenomenon coincides with results reported by Spaulding *et al.* (1993) and disagrees with those reported by Goldman *et al.* (1996). This fact, however, can be explained by relatively small sizes of the lakes investigated in the present study thus limiting horizontal variability of habitats within lakes. Therefore, we can conclude that the lakes investigated showed highly homogeneous phytoplankton assemblages at the same depths. However, vertical variation within the water column could be detected in all four lakes (Fig. 1). Thereby, although some phytoplankton species did occur both in eutrophic and oligotrophic environments, they occurred with temporal shift during the period of investigation - a phenomenon reported earlier (Spaulding *et al.* 1993, Reitner *et al.* 1997, Padisak *et al.* 1998). Oligotrophic lakes always showed a cluster clearly

Table 1. Phytoplankton species found during the period of ice cover (from January to March) in two eutrophic temperate lakes in the Northeastern Sweden (62°54' N): u.c. - immediately under the ice cover, n.b. - near bottom (*ca* 3 m)

Group	J		F		M	
	u.c.	n.b.	u.c.	n.b.	u.c.	n.b.
CHLOROPHYCEAE						
Chlamydomonas sp.	1			1	1	
Crucigeniella rectangularis (Nägeli) Komarek		1				
Dictyosphaerium pulchellum Wood					1	
Didymocystis bicellularis (Chodat) Komarek	2	3	2	1	4	4
Scenedesmus quadricauda (Turpin) Brebisson		1	1			
CRYPTOPHYCEAE						
Cryptomonas erosa Ehrenberg	4	1	4	1	4	2
Cryptomonas reflexa Skuja	2		4	1	4	2
Rhodomonas lacustris Pasher et Ruttner	4	3	3		2	
DINOPHYCEAE						
Gymnodinium lantzschii Utermöhl	1	2			1	
Peridinium willei Huitfeld-Kaas					2	1
EUGLENOPHYCEAE						
Phacus caudatus Hübner		1				1
Phacus suecicum Lemmermann					1	
Trachelomonas planktonica Swirenko	1					
Trachelomonas volvocinopsis Swirenko	4	1	3	1	3	1

Table 2. Phytoplankton species found during the period of ice cover (from January to March) in two oligotrophic temperate lakes in the Northeastern Sweden (62°54' N): u.c. - immediately under the ice cover, n.b. - near bottom (3 m)

Group	J		F		M	
	u.c.	n.b.	u.c.	n.b.	u.c.	n.b.
BACILLARIOPHYCEAE						
Navicula spp.		1		1		
Tabellaria fenestrata (Lyngbye) Kützing			1		1	
CHLOROPHYCEAE						
Chlamydocapsa ampla (Kütz.) Fott	1					
Chlamydomonas sp.					1	1
CRYPTOPHYCEAE						
Cryptomonas marssonii Skuja	2	1				
Cryptomonas reflexa Skuja					2	1
Rhodomonas lacustris Pasher et Ruttner	2	1	1	1	2	1
CYANOPHYCEAE						
Anabaena sp.			1			
DINOPHYCEAE						
Peridinium willei Huitfeld-Kaas					1	
EUGLENOPHYCEAE						
Trachelomonas volvocina Ehrenberg			1			
Trachelomonas volvocinopsis Swirenko				1		

Similarity
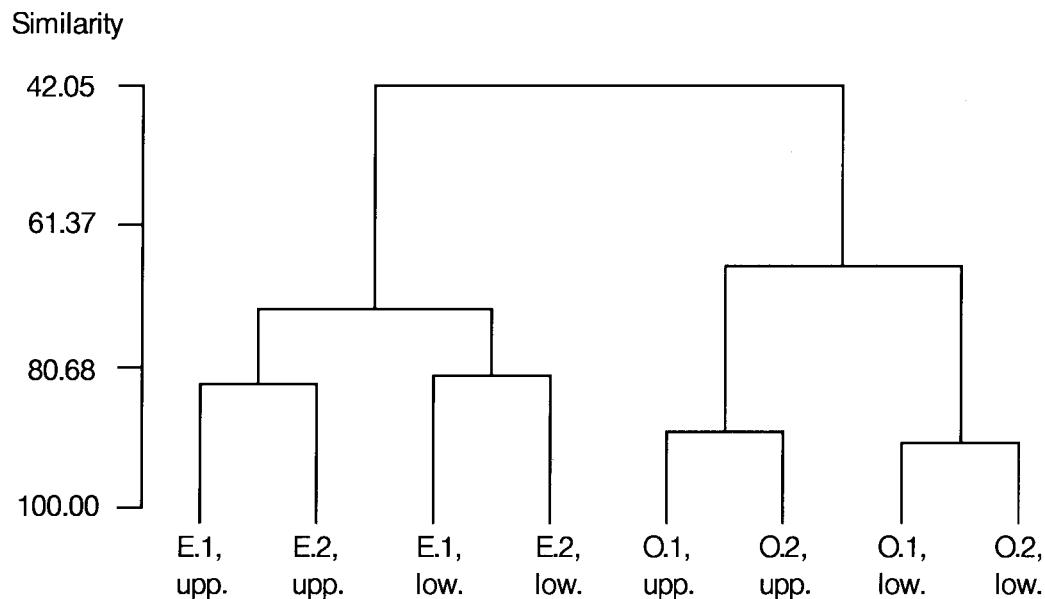
Fig. 1. Clustering analyses of sampling stations based on presence-absence phytoplankton data sampled during the period of ice cover (from January to March) in two eutrophic and two oligotrophic lakes in North-Eastern Sweden: O.1, O.2 - oligotrophic lakes, E.1., E.2 - eutrophic lakes, upp. - immediately under the ice cover, low. - near the bottom

separated from that built by eutrophic lakes. Phytoplankton communities at the same depths were highly homogeneous in oligotrophic lakes, on the one hand, and in eutrophic lakes, on the other hand. We can conclude that the lakes of the same trophic status exhibited highly similar phytoplankton assemblages at similar depths under the ice cover. Contrary to results reported by Agbeti *et al.* (1997), the morphometry of lakes did not seemed to play an important role with only depth being the determinative factor. A possible explanation to this phenomenon could be the limited light availability at higher depths (Tulonen *et al.* 1994). However, vertical differences shown by higher phytoplankton diversities immediately below the ice cover became obvious in our study. These results correspond to those reported earlier in the literature (Lizotte *et al.* 1996, Ventela *et al.* 1998). A possible explanation of this fact could be both differences in light availability at different depths and increased ions concentrations (excluded from the lower surface of ice) below the ice cover (Spaulding *et al.* 1993, Tulonen *et al.* 1994).

REFERENCES

Agbeti M. D., Smol J. P. (1995) Winter limnology - a comparison of physical, chemical and biological characteristics in two temperate lakes during ice cover. *Hydrobiologia* **304**: 221-234

Agbeti M. D., Kingston J. C., Smol J. C., Watters C. (1997) Comparison of phytoplankton succession in two lakes of different mixing regimes. *Arch. Hydrobiol.* **140**: 37-69

Butler H. G., Edworthy M. G., Ellis-Evans J. C. (2000) Temporal plankton dynamics in an oligotrophic maritime Antarctic lake. *Freshwater Biol.* **43**: 215-230

Danilov R. A., Ekelund N. G. A. (1999) The efficiency of seven diversity and one similarity indices based on phytoplankton data for assessing the level of eutrophication in lakes in central Sweden. *Sci. Total Envir.* **234**: 15-23

Danilov R. A., Ekelund N. G. A. (2000) The use of epiphyton and epilithon data as a base for calculating ecological indices in monitoring of eutrophication in lakes in central Sweden. *Sci. Total Envir.* **248**: 63-70

Fritsen C. H., Priscu J. C. (1999) Seasonal change in the optical properties of the permanent ice cover on Lake Bonney, Antarctica: consequences for lake productivity and phytoplankton dynamics. *Limnol. Oceanogr.* **44**: 447-454

Hoek C. van den, Mann D. G., Jahns H. M. (1995) Algae. Cambridge University Press, Cambridge

Kangas P., Alasaarela E., Lax H., Jokela S., Storgård-Envall C. (1993) Seasonal variation of primary production and nutrient concentrations in the coastal waters of the Bothnian Bay and the Quark. *Aqua Fenn.* **23**: 165-176

Kelley D. E. (1997) Convection in ice-covered lakes: effects on algal suspension. *J. Plankton Res.* **19**: 1859-1880.

Lizotte M. P., Sharp T. R., Priscu J. C. (1996) Phytoplankton dynamics in the stratified water column of Lake Bonney, Antarctica. 1. Biomass and productivity during the winter-spring transition. *Polar Biol.* **16**: 155-162

Padisak J., Krienitz L., Scheffler W., Koschel R., Kristiansen J., Grigorszky I. (1998) Phytoplankton succession in the oligotrophic Lake Stechlin (Germany) in 1994 and 1995. *Hydrobiologia* **370**: 179-197

Pierson D. C., Weyhenmeyer G. A. (1994) High-resolution measurements of sediment resuspension above an accumulation bottom in a stratified lake. *Hydrobiologia* **284**: 43-57

Reitner B., Herzig A., Herndl G. J. (1997) Microbial activity under the ice cover of the shallow Neusiedler see (Austria, Central Europe). *Hydrobiologia* **357:** 173-184

Smolar N., Vrhovsek D., Kosi G. (1998) Effects of low flow on periphyton in three different types of streams in Slovenia. In: Advances in River Bottom Ecology, (Eds. G. Bretschko and J. Helesi), Backhuys Publishers, Leiden, The Netherlands, 107-116

Spaulding S. A., Ward J. V., Baron J. (1993) Winter phytoplankton dynamics in a sub-alpine lake, Colorado, USA. *Arch. Hydrobiol.* **129:** 179-198

Spaulding S. A., McKnight D. M., Smith R. L., Dufford R. (1994) Phytoplankton population dynamics in perennially ice-covered Lake Fryxell, Antarctica. *J. Plankton Res.* **16:** 527-541

Swedish Environmental Protection Agency (1991) Quality criteria for lakes and watercourses. A system for classification of water chemistry and organism metal concentrations. Stockholm

Trifonova I. (1993) Seasonal succession of phytoplankton and its diversity in two highly eutrophic lakes with different conditions of stratification. *Hydrobiologia* **249:** 93-100

Tulonen T., Kankaala P., Ojala A., Arvola L. (1994) Factors controlling production of phytoplankton and bacteria under ice in a humic, boreal lake. *J. Plankton Res.* **16:** 1411-1432

Ventela A. M., Saarikari V., Vuorio K. (1998) Vertical and seasonal distributions of microorganisms, zooplankton and phytoplankton in an eutrophic lake. *Hydrobiologia* **363:** 229-240

Ability of the Rumen Ciliate *Epidinium ecaudatum* to Digest and Use Crystalline Cellulose and Xylan for *in vitro* Growth

Tadeusz MICHAŁOWSKI[1], Katarzyna RYBICKA[2], Krzysztof WERESZKA[1] and Anna KASPEROWICZ[1]

[1]**The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna and [2]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland**

Summary. The rumen ciliate protozoan *Epidinium ecaudatum* was isolated from rumen fluid of sheep and either grown *in vitro* or inoculated into the rumen of the ciliate-free sheep. Population density of ciliates *in vitro* was about 320 cells/ml when culture salt solution was supplemented with hay (0.6 mg/ml/d) and wheat gluten (0.15 mg/mg/d). Addition of the microcrystalline cellulose (0.25 mg/ml/d) to the control diet increased the ciliate numbers to about 440 cells/ml (P < 0.01). Conversely, oat spelt xylan decreased the concentration of protozoa to 250 cells/ml (P < 0.05). Ciliates readily ingested and digested cellulose particles while xylan particles were only sporadically engulfed. Only glucose was released from the microcrystalline cellulose and cellobiose during incubation of the both substrates with protozoal protein; the release rate was 0.19 and 14.9 µM/mg protein/h, respectively. Carboxymethylcellulose and xylan were degraded at 17.5 and 66.6 µM reducing sugars released from substrate/mg protein/ml. Degradation rate of microcrystalline cellulose, carboxymethylcellulose, cellobiose and xylan was the highest at pH 6.5, 5.5, 6.0 and 6.5, respectively. Non-denaturing polyacrylamide gel electrophoresis combined with CMC-ase and xylanase zymogram revealed the presence of three protein bands active against CMC and two protein bands degrading xylan. Thin layer chromatography showed a presence of only oligosaccharides in the end products released from CMC and xylan by enzymes isolated from gel slices. Neither glucose nor xylose were found there. Conversely only glucose was detected by TLC following incubation of microcrystalline cellulose with solution of protozoal protein. The same preparation released mainly xylose from xylan but different oligosaccharides were also present.

Key words: crystalline cellulose, digestion, β-endoglucanase, β-endoxylanase, β-glucosidase, *Epidinium ecaudatum*, xylan.

INTRODUCTION

The ciliate protozoan *Epidinium ecaudatum* belongs to the most common species of ciliates inhabiting the rumen of domestic ruminants. It is considered to participate in the degradation of plant structural polysaccharides (Dehority 1993). However, there is some

Address for correspondence: Tadeusz Michałowski, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Instytucka 3, 05-110 Jabłonna, Poland; Fax: (4822) 774 20 38; E-mail: t.michalowski@ifzz.pan.pl

disagreement regarding the contribution of this ciliate to the digestion of plant cell wall polysaccharides. Coleman (1985) showed that the ability of *Epidinium ecaudatum* to degrade microcrystalline cellulose was higher than activity of *Eudiplodinium maggii* and Williams and Coleman (1985) have stated that xylanolytic activity of *Epidinium ecaudatum* is comparable to that extracted from the cells of *Polyplastron multivasiculatum* and *Eudiplodinium maggii*. On the other hand there are also evidence showing that the xylan and especially microcrystalline cellulose digesting and fermenting ca-

pacities of *Epidinium* isolated from the rumen are substantially lower than these of *Eudiplodinium maggii* and *Polyplastron multivesiculatum* (Jouany and Martin 1997). Due to above disagreement we decided to undertaken the study presented in this report. The objectives of our experiments were: (a) to compare the population size of ciliates grown *in vitro* in the medium supplemented with either cellulose or xylan; (b) to study the ingestion and digestion of the microcrystalline cellulose and xylan by cultured protozoa; (c) to identify and characterize some of fibrolytic enzymes produced by ciliates of the genus *Epidinium*.

MATERIAL AND METHODS

Protozoa

Ciliates *Epidinium ecaudatum* identified according to Dogiel (1927) were isolated from the rumen of sheep fed a hay-concentrate diet and kept *in vitro* as a one species population as described elsewhere (Michałowski 1995). Ciliates from *in vitro* cultures were inoculated into the rumen of defaunated sheep which was used as a source of protozoa for enzymatic assays. The sheep were defaunated by evacuation and heating of the reticulo-rumen content and washing of the rumen and reticulum walls as described by Michałowski *et al.* (1999).

Cultivation experiments

Ciliates were cultured in "*caudatum*" type salt solution (Coleman *et al.* 1972) composed of (g/l): K_2HPO_4-6.3; KH_2PO_4-5.0; CH_3COONa-0.75; NaCl-0.65; $MgSO_4 \cdot 7H_2O$-0.09; $CaCl_2 \cdot 6H_2O$-0.09. The control diet consisted of powdered hay (0.6 mg/ml/d) and wheat gluten (0.15 mg/ml/d). Experimental diets contained the same quantities of hay and wheat gluten and either microcrystalline cellulose (Sigmacell No. 20; Sigma, S-3504), or xylan from oat spelts (Sigma, X-0627) in the proportion of 0.25 mg/ml/d. Methods of initiation of the cultures and cultivation of protozoa were the same as described earlier (Michałowski 1995). Ciliates were fed every day and diluted with fresh "*caudatum*" medium (1:1, v/v) every fourth day. Three cultures were always run simultaneously on each diet for 4 weeks. The volume of each culture was 40 ml. Protozoa were counted on dilution days.

Meadow hay was used to feed the protozoa. It was ground in a high speed grinder and the smallest particles which settled on the lid of the grinder were collected and used as food components.

Cellulose and xylan ingestion

Ciliates cultured *in vitro* and fed control diet were starved for 24 h, and then fed with microcrystalline cellulose or xylan (0.5 mg/ml) and sampled to estimate the proportion of individuals with engulfed polysaccharides. The samples were taken just before feeding and at 1, 2, 4, 8, 12, 24 and 36 h thereafter. The experiment was repeated three times.

Enzyme preparation

Ciliates cultured in the rumen of sheep inoculated only with *Epidinium ecaudatum* were used. The protozoa were separated from food debris and external bacteria by repeated sedimentation according to Michałowski (1990). The sample of rumen content (about 1 kg) was diluted with warm "*caudatum*" salt solution in the proportion of 1:2 (w/v) and squeezed through a screen of the pore size of 0.5 mm. The liquid part was collected, poured into the separatry funnels and allowed to stand at 40°C for 30 min. The sedimented protozoa were collected, suspended in "*caudatum*" solution, poured into centrifuge tubes of 100 ml in volume and allowed to stand at 40°C for 3-5 min. During this time the ciliates sedimented forming a white layer at the bottom of the tubes while the plant debris collected at the top. Both the plant particles and supernatant were removed by suction while the protozoa were suspended as described above and sedimented again. The procedure was repeated 5-6 times while purity of ciliate was examined microscopically. Finally well purified protozoa were suspended in "*caudatum*" salt solution and incubated overnight in the presence of chloramphenicol, streptomycin and ampicillin (each at concentration of 100 µg/ml) to eliminate the intracellular bacteria. On the next day the bacteria free ciliates were washed three times with "*caudatum*" salt solution (Coleman *et al.* 1972) and stored at -20°C or disrupted immediately using a glass homogenizer equipped with a teflon pestle. Homogenate was centrifuged at 20000 x g at 4°C for 20 min to remove the non disrupted cells and the supernatant fraction was collected and used either as a crude enzyme preparation or for protein precipitation. Protein was precipitated using ammonium sulphate at 80 % saturation. Precipitated protein was dissolved in small volume (5-10 ml) of cold (4°C) distilled water, dialyzed against the same water and lyophilized and stored at -20°C. Protein in both the crude enzyme preparation and in lyophilizate was measured using Microprotein-PR™ reagent (Sigma 611-A).

Enzyme assays

Degradation of the microcrystalline cellulose was assayed by quantitative determination of glucose released from this polysaccharide following incubation with protozoal protein precipitated from the supernatant fraction (see above). Samples of substrate (5 mg) were added to tubes containing 1000 µl solution of protein at concentration of about 200 µg/ml., and incubated anaerobically at 40°C for 72 h. Protein was dissolved in 0.1 M McIlvaine buffer of different pH. Both the protein alone and substrate without protein were dissolved in the same buffers and incubated simultaneously as controles. Similar controles were applied in examinations of the other activities (see below). Measurements were performed just before the start of incubation as well as after 6, 12, 24, 48 and 72 h. Released glucose was measured enzymatically with the glucose oxidase-peroxidase coupled reactions using the Glucose (Trinder) reagent (Sigma No 315). The same method was used to measure cellobiose hydrolysis. Incubation mixture consisting 1 ml of protein solution and 5 mg of cellobiose (Sigma C 7252) was incubated for 4 h while released glucose was measured just before the commencement of incubation as well as at 1, 2, 3 and 4 h thereafter. Glucose was used as a standard. Carboxymethylcellulase (CMC-ase) activity was determined by measurement of reducing sugars released from carboxymethylcellulose (Sigma C 5678) following incubation of substrate with crude enzyme preparation. Incubation mixtures consisted of 750 µl
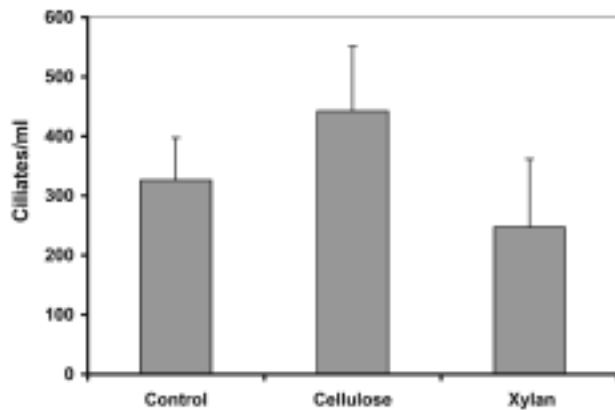
Fig. 1. The number of *Epidinium ecaudatum* in the cultures fed hay and wheat gluten (Control) or hay and wheat gluten supplemented either with microcrystalline cellulose (Cellulose) or xylan (Xylan). Hay was given at the rate of 0.6 mg and wheat gluten - 0.15 mg/ml/d. The both cellulose and xylan were supplemented at the rate of 0.25 mg/ml/d. Mean values ± S.D., n = 24

Fig. 2. Percentage of ciliates containing food particles in endoplasmic sacs at different time after giving either microcrystalline cellulose or xylan to protozoa. Control cultures were starved during the sampling period. Mean values ± S.D., n = 3

Table 1. Degradation rate of different carbohydrates (μM glucose or xylose released from appropriate substrate/mg protein/h) and pH optimum for particular activities

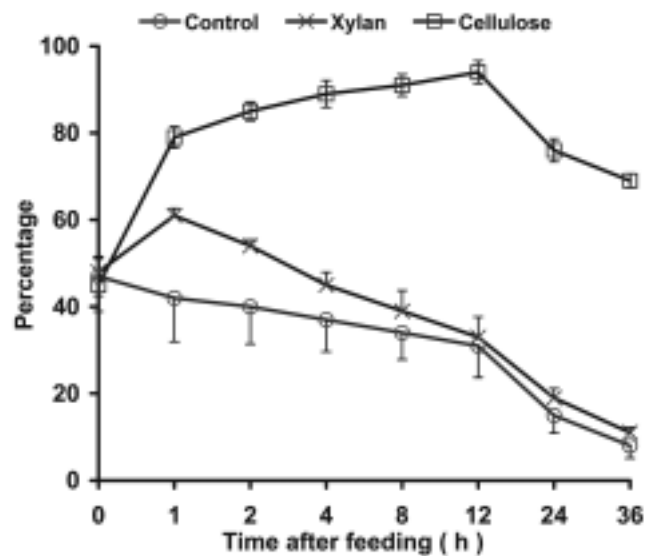| Substrate	Degradation rate	pH
Crystalline cellulose *	0.19 ± 0.02	6.5
Carboxymethylcellulose **	17.5 ± 3.46	5.5
Cellobiose *	14.9 ± 4.36	6.0
Xylan **	66.6 ± 0.87	6.5

* incubated with partially purified protein
** incubated with crude enzyme preparation

of 2 % sodium carboxymethylcellulose dissolved in 0.2 M McIlvaine buffer of different pH and 500 μl of crude enzyme preparation. The mixture was incubated for 1 h at 40°C and reducing sugars were determined using dinitrosalicylic reagent according to Miller *et al.* (1960) and Groleau and Forsberg (1981). Xylan hydrolysis was examined by the same method but incubation mixtures composed of 50 μl of crude enzyme preparation, 750 μl of xylan solution and 450 μl of 0.2 McIlvain buffer of different pH. Glucose and xylose standard curves were used for calculation the quantity of released sugars, respectively. Birch wood xylan (Sigma, X-4252) was used in preparation of xylan solution. The samples of substrate weighing 0.5 g were suspended in 25 ml of appropriate buffer and warmed to about 70°C for 15 min. The mixture was allowed to stand at ambient temperature and then centrifuged for 10 min at 10000 x g. Pellet was discarded while supernatant was used as substrate for determination described above.

Enzyme identification

Native polyacrylamide gel electrophoresis (NPAGE) of protozoal protein, combined with CMC-ase and xylanase zymogram technique was used for enzyme localization and identification according to Michałowski (1997). Electrophoresis was performed in Tris/glycine buffer (5 mM) at pH 8.3. A 7 % polyacrylamide gel was used. Carboxymethylcellulose or xylan were copolymerized with polyacrylamide at the final concentration of 0.1 % and used as substrates for identified enzymes. Ten times concentrated electrode buffer (50 mM) was used as gel buffer to prevent the substrate breakdown during protein migration. Electrophoresis was performed at a constant voltage of 94 V in a minidual unit (Sigma) and was followed by incubation of the gel in 0.1 M McIlvaine buffer of pH 5.5 (CMC-ase zymogram) or 6.5 (xylanase zymogram) for 15 min at 40°C. Reducing sugars released in the gel from the copolymerized CMC or xylan by the identified enzymes were visualized using 2,3,5 triphenyltetrazolium chloride solution (0.2 %) in 0.5 N NaOH according to Gabriel and Wang (1969).
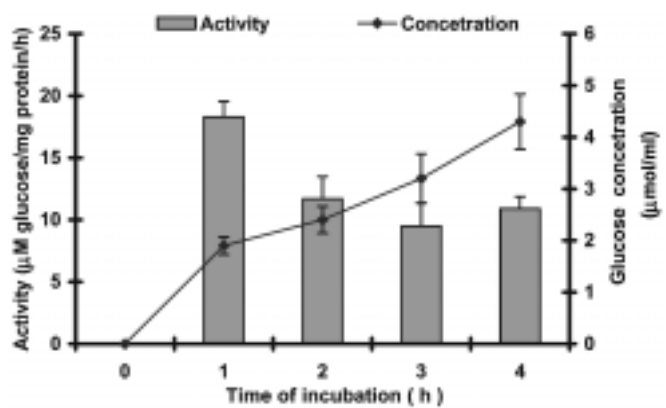
End product identification

End products of CMC, xylan and microcrystalline cellulose hydrolysis were identified by thin layer chromatography (TLC). The CMC-ase and xylanase enzymes were isolated from the gels following electrophoretic separation of protozoal protein and localization of enzymes on the gel lanes. Appropriate gel slices were excised and immersed in a small volume (1-2 ml) of 0.1 M MacIlveine buffer (pH 6.0) and homogenized using glass homogenizer. CMC or xylan samples were added to homogenate to the final concentration of 0.1 % and the mixtures were incubated anaerobically at 40°C for 24 h. End products released from microcrystalline cellulose and xylan following incubation with protozoal protein were also identified. The samples of either cellulose or xylan (5 mg) were added to tubes containing protozoal protein (about 200 μg) suspended in 1 ml of McIlvaine buffer of appropriate pH and incubated anaerobically either for 24 (xylan) or 72 h (microcrystalline cellulose) at continuous agitation. Both the protein solution alone and substrates dissolved in the buffer were incubated simultaneously with experimental samples
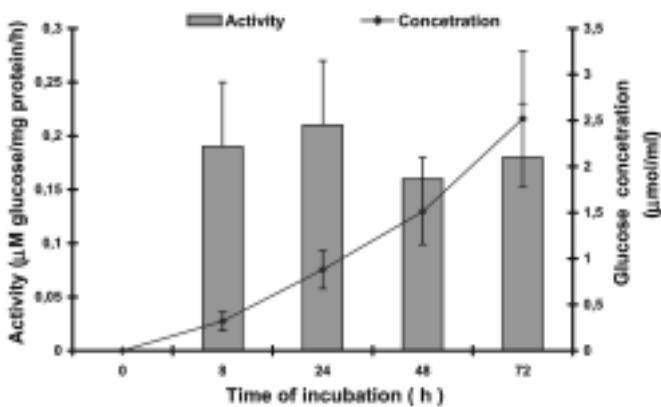
Fig. 3. Changes in cellulolytic activity and concentration of glucose released from microcrystalline cellulose during incubation with *Epididinium ecaudatum* protein. Mean values ± S.D., n = 3

Fig. 4. Changes in enzyme activity and concentration of glucose released from cellobiose during incubation with *Epidinium ecaudatum* protein. Mean values ± S.D., n = 3

and used as control. Samples of appropriate digests were spotted in the volume of 20 μl on silica plates (Silufol, Avalier, Czechoslovakia) and developed 3 (cellulase products) or 4 fold (xylanase products) using a mixture of butanol, ethanol, water (5:5:5, v/v) or butanol, ethanol water (5:5:3, v/v), respectively. The carbohydrates were visualized using a mixture consisted of diphenylaniline (4 g), aniline (4 ml), phosphoric acid (20 ml) and acetone (200 ml).

Bacterial test

Ciliates incubated with and without antibiotics (see above) were separately disrupted in glass homogenizer equipped with a teflon pestle and homogenate was used in examination for presence of intracellular cellulolytic bacteria according to the method described earlier (Michałowski 1997). Samples of homogenate were inoculated to the tubes filled with liquid medium for cultivation of cellulolytic bacteria (Anaerobe Laboratory Manual 1973) and strips of Whatman No. 1 paper was immersed aseptically in each tube. The initiated cultures were incubated for at least 6 weeks at 40°C. The appearance of the strips was observed every day.

Chemicals

All chemicals were of analytical purity. They were supplied by Sigma Chemical Co. Wheat gluten was prepared according to Klein (1933) and Pace (1955).

Statistical analysis

Student's t-test was used to compare the differences between mean values according to Ruszczyc (1970).

RESULTS

Ciliate growth

The population density of *Epidinium ecaudatum* cultured *in vitro* varied from about 120 to almost 600

cells/ml in relation to day and food composition. Mean number of protozoa in the cultures fed hay and wheat gluten (control diet) was 320 individuals/ml (Fig. 1). Microcrystalline cellulose supplement increased the population density by about 38 % (P < 0.01). Addition of xylan to the control diet resulted in decrease of the number of ciliates by about 44 % (P < 0.05). Microscopical examination of ciliates showed that cellulose particles were engulfed by numerous individuals.

Cellulose and xylan engulfment

Addition of the microcrystalline cellulose to the suspension of ciliates resulted in the rapid increase in the number of cells containing this polysaccharide in endoplasm. The proportion of such cells in the suspension of ciliates increased up to 12 h after feeding and was followed by continuous decrease (Fig. 2). Protozoa engulfed large quantities of cellulose and majority of them were completely filled with this polysaccharide. Xylan particles were engulfed to a lesser extent than these of cellulose. Cells containing food particles in the endoplasmic sacs increased by about 12 % during the first hour after xylan was introducing to the ciliate suspension (P < 0.05) and was followed by continuous decrease during the next 35 h like the control protozoa which were starved all time. There were difficulties in distinguishing the xylan and hay containing ciliates from these with only hay particles in endoplasmic sacs. Due to this the true number of the xylan engulfing protozoa could not be determined. We observed only that majority of ciliates were almost or completely empty starting from 4 h after xylan feeding.
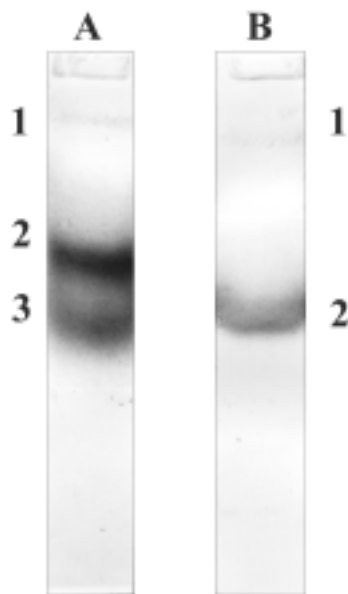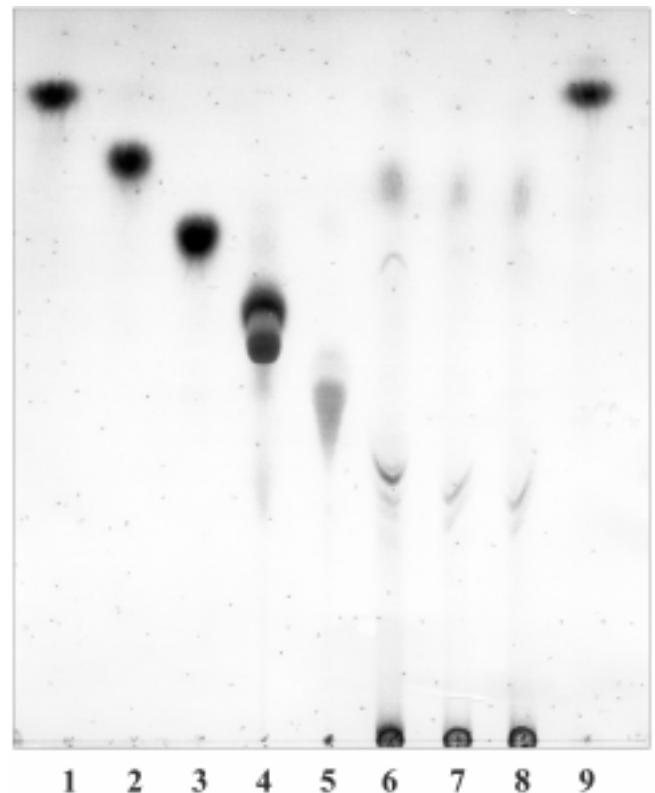
Fig. 5. Non-denaturating polyacrylamide gel electrophoresis of *Epidinium ecaudatum* protein combined with β-endoglucanase (CMC-ase) and xylanase zymogram. Lane A - three CMC-ase activities: Lane B two β-endoxylanase activities. All activities were visualized by staining with 2,3,5 triphenyltetrasolium chloride


Fig. 6. TLC of the end products of CMC and microcrystalline cellulose hydrolysis catalyzed with *Epidinium ecaudatum* enzymes. Lanes 1-5 - the standards of glucose, cellobiose, cellotriose, cellotetraose and cellopentaose, respectively. Lanes 6-8 - the products released from CMC by three β-endoglucanases obtained from the slices excised from the gel following electrophoretic separation of *Epidinium ecaudatum* protein (see Fig. 5, lane A). Lane 9 - end product of the microcrystalline cellulose hydrolysis with enzymes present in protein precipitated from the crude enzyme preparation (crude extract)

Enzyme activities

Incubation of microcrystalline cellulose, CMC, cellobiose and xylan with protozoal enzyme preparation resulted in release of reducing products. Mean degradation rate of different carbohydrates and pH optimum of particular activities are presented in Table 1. Microcrystalline cellulose was degraded at the lowest rate of all carbohydrate tested. Incubation of this polysaccharide with protozoal protein resulted in a continuous release of glucose into the medium. Degradation rate of this substrate calculated from the changes in the product concentration was constant during the incubation period (Fig. 3). No increase was found in glucose concentration in the control tubes.

Cellobiose was degraded with the rate by about 80 times higher than microcrystalline cellulose. The rate of glucose release from cellobiose during the first hour of incubation was higher (P < 0.05) than during the next 3 h period (Fig. 4).

Carboxymethylcellulose was degraded with the rate of about 4 times slower than xylan. This last polysaccharide was hydrolyzed with the highest rate of all carbohydrate tested (P < 0.01).

Enzyme separation and identification

Non-denaturating polyacrylamide gel electrophoresis combined with the CMC-ase and xylanase zymograms revealed the presence of three protein bands active against carboxymethylcellulose and two bands degrading xylan (Fig. 5).

End product identification

Oligosaccharides of the molecular mass similar to cellohexaose and disaccharides were the main products of carboxymethylcellulose hydrolysis catalyzed by all three enzymes obtained from the gel slices. Glucose was not identified there. It was, however, the only product identified following incubation of microcrystalline cellulose with the suspension of protozoal protein (Fig. 6).
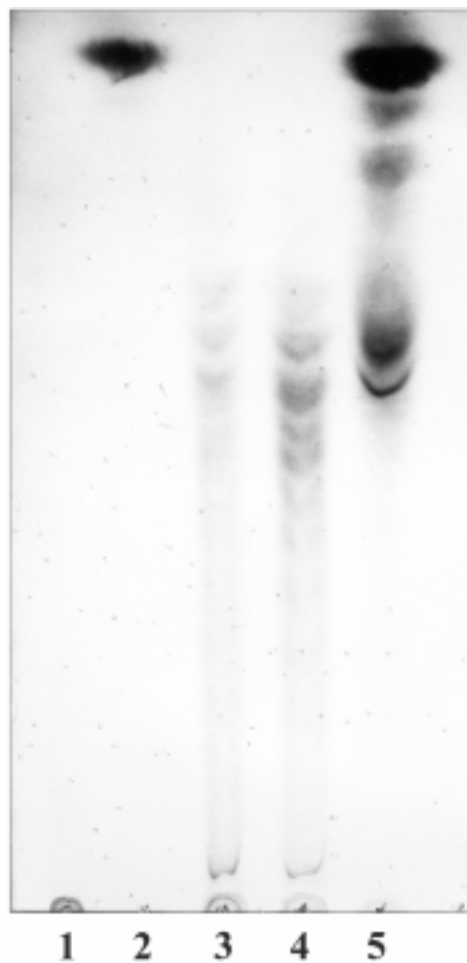
Fig. 7. TLC of the end products of xylan hydrolysis catalyzed with *Epidinium ecaudatum* enzymes. Lane 1 - xylan sample. Lane 2 - the standard of xylose. Lanes 3 and 4 - the products released from xylan by two β-endoxylanase enzymes obtained from the slices excised from the gel following electrophoretic separation of *Epidinium ecaudatum* protein (see Fig. 5, lane B). Lane 5 - end products of the xylan hydrolysis with protein precipitated from the crude enzyme preparation (crude extract)

Only oligosaccharides were found as end products of xylan hydrolysis catalyzed by the enzymes separated from the gels. Neither xylose nor xylobiose were identified there. Xylose, and perhaps xylobiose as well as longer oligosaccharides were present in the digests when xylan was incubated with the suspension of protozoal protein (Fig. 7).

Bacterial test

Intact strips of Whatman No. 1 paper were present for a period longer than 6 weeks in all tubes inoculated with homogenate obtained from the ciliates incubated overnight with antibiotics prior to homogenization. The strips disappeared, however, within 5-7 days when antibiotics were omitted. This shows that the alive cellulolytic bacteria were present only in these last tubes.

DISCUSSION

Ciliates *Epidinium ecaudatum* grew well *in vitro* in "*caudatum*" salt solution (Coleman *et al.* 1972) supplemented with powdered hay and wheat gluten. This shows that the culture medium satisfied the environmental and nutritional requirements of protozoa. It was found that microcrystalline cellulose supplemented to the control diet increased the ciliate number. Thus the reaction of *Epidinium ecaudatum* upon the changes in food composition resembled response of *Anoplodinium denticulatum*, *Diploplastron affine* and *Eudiplodinium maggii* stated earlier (Michałowski *et al.* 1986, 1989, 1991). One of the possible explanation of this finding seems to be the increase in energy content of the ration caused by cellulose supplement. This suggestion is confirmed by the both microscopical observation and enzymatic studies. First of them revealed that *Epidinium ecaudatum* engulfed readily and digested cellulose particles inside the cells while the second showed that glucose released from cellulose could be an important source of energy for these ciliates. On the other hand, however, it can not be also precluded that cellulose supplement improved some of environmental conditions making them more favorable for cultured ciliates. Such an effect was observed earlier in the case of non cellulolytic *Entodinium exiguum* (Michałowski *et al.* 1985). Growth of cellulolytic bacteria and more stable pH should be taken into account there. Conversely to microcrystalline cellulose the particles of oat spelt xylan were only sporadically engulfed by ciliates *Epidinium ecaudatum*. Explanation of such a behavior of ciliates towards xylan is not simple but it shows that this structural polysaccharide can not be considered as an important source of the utilizable energy for cultured protozoa. It is possible that ciliates from the same species which were examined by Jouany and Martin (1997) exhibited similar behavior towards xylan and this resulted in very low fermenting capacity in spite of high activity of the xylanolytic enzymes what was observed in our study. It is also noteworthy to point out that numerous species of rumen ciliates synthesize hemicellulolytic enzymes but their ability to ferment pentoses are not well known (Williams and Coleman

1992). Irrespective of causes influencing xylan utilization by rumen protozoa this polysaccharide diminished *Epidinium ecaudatum* numbers in our study. We suppose that xylan added to the culture medium could affect the bacterial growth and/or some environmental properties which became unfavorable for ciliates. However, further investigation are necessary to explain this reaction.

Both the microscopical observations and enzymatic studies showed that *Epidinium ecaudatum* was capable of digesting the microcrystalline cellulose. It is well known that cellulose degradation is a complex reaction catalyzed with array of enzymes of which the presence of three β-endoglucanases and β-glucosidase in *Epidinium ecaudatum* protein has been confirmed in our study. According to Wood (1992) and Chesson and Forsberg (1997) β-endoglucanases digest amorphous cellulose to oligosaccharides while β-glucosidases attack specifically cellobiose. We found, however, that only glucose was released from microcrystalline cellulose (Sigmacell 20) during incubation with the *Epidinium ecaudatum* protein. Thus the obtained results suggest that cellulolytic enzymes other than β-endoglucanases and β-D-glucosidase were also present in the preparation. One of them could be β-exoglucanase. Glucose was released from "Sigmacell 20" with the rate of about 0.19 µM/mg protein/h. This suggests that cellulolytic activity of *Epidinium ecaudatum* is not lower when compare to that of *Bacteroides* (*Fibrobacter*) *succinogenes* (Groleau and Forsberg 1981).

Xylanase exhibited the highest activity of the all fibrolytic enzymes tested. End products released from xylan with the enzymes extracted from the gel slices confirmed presence of two β-endoxylanases which released oligosaccharides from substrate. End products of the xylan hydrolysis with crude enzyme preparation suggests, however, that enzyme(s) releasing pentoses were also present in examined material.

Both the cellulolytic and xylanolytic activities were still present in examined preparation in spite of successful elimination of bacteria by incubation of ciliates with antibiotics. This finding supports the hypothesis that *Epidinium ecaudatum* is capable of synthesizing enzymes involved in degradation of the β-D-glucose and β-D-xylose polymers from plant cell walls. A successful cloning of gene encoding for β-endoglucanase from *Epidinium ecaudatum* (Sellinger *et al.* 1996) confirms partially this hypothesis. Genes encoding for xylanase were cloned from *Polyplastron multivesiculatum*

(Sellinger *et al.* 1996, Wallace *et al.* 1999, Devillard *et al.* 2000) but not from *Epidinium ecaudatum*. Thus further studies seem to be necessary.

REFERENCES

Anaerobe Laboratory Manual. (1973) Eds. L. V. Holdman and W. C. E. Moore. VPI Anaerobe Laboratory Virginia Polytechnic Institute State University. Blacksburg: Southern Printing Co.

Chesson A., Forsberg C. W. (1997) Polysaccharide degradation by rumen microorganisms. In: The Rumen Microbial Ecosystem (Eds. P. N. Hobson and C. S. Stewart). Blackie Academic and Professional, London, Weinheim, New York, Tokyo, Melbourne, Madras, 329-381

Coleman G. S. (1985) The cellulase content of 15 species of entodiniomorphid protozoa, mixed bacteria and plant debris isolated from the ovine rumen. *J. agric. Sci. Camb.* **104:** 349-360

Coleman G. S., Laurie J. I., Cash M. A. (1972) The cultivation of the rumen ciliates *Epidinium ecaudatum caudatum* and *Polyplastron multivesiculatum in vitro*. *J. Gen. Microbiol.* **75:** 509-521

Dehority B. A. (1993) Microbial ecology and cell wall fermentation. In: Forage Cell Wall Structure and Digestibility (Eds. G. G. Jung, D. R. Buxton, R. D. Hatfield and J. Ralph). ASA, CSSA, SSSA, Madison, Winconsin, USA, 425-453

Devillard E., Flint H. J., Scott K. P., Newbold C. J., Jounay J.-P., Wallace R. J. Forano E. (2000) Fibre-degrading enzymes from a ruminal protozoan *Polyplastron multivesiculatum*. *Reprod. Nutr. Dev.* **49:** 193

Dogiel V. A. (1927) Monographie der Familie Ophryoscolecidae. *Arch. Protistenkd.* **59:** 1-280

Gabriel O., Wang S. F. (1969) Determination of enzymatic activity in polyacrylamide gel. *Anal. Biochem.* **27:** 545-554

Groleau D., Forsberg C. W. (1981) Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. *Can. J. Microbiol.* **27:** 517-530

Jouany J.-P., Martin C. (1997) Effect protozoa in plant cell wall and starch digestion in the rumen. In: Rumen Microbes and Digestive Physiology in Ruminants (Eds. R. Onodera, H. Itabashi, K. Ushida, H. Yano and Y. Sasaki). Japan Scientific Societies Press, Tokyo, 11-24

Klein G. (1933) Handbuch der Pflanzenanalyse, Springer Verlag, Vien

Michałowski T. (1990) The synthesis and turnover of the cellular mater of ciliates in the rumen. *Acta Protozool.* **29:** 47-72

Michałowski T. (1995) The influence of cellulose, starch and different sources of nitrogen on the growth of rumen ciliate protozoon *Epidinium ecaudatum in vitro*. *J. Anim. Feed Sci.* **4:** 255-261

Michałowski T. (1997) Digestion and fermentation of the microcrystalline cellulose by the rumen ciliate protozoon *Eudiplodinium maggii*. *Acta Protozool*. **36:** 181-185

Michałowski T., Szczepkowski P., Muszyński P. (1985) The factors affecting the cultivation of the rumen ciliate protozoon *Entodinium exiguum in vitro*. *Acta Protozool.* **24:** 297-305

Michałowski T., Szczepkowski P., Muszyński P. (1986) The nutritive factors affecting the growth of the rumen ciliate *Diploplastron affine in vitro*. *Acta Protozool.* **25:** 419-426

Michałowski T., Landa I., Muszyński P. (1989) Factors influencing the development of population of the rumen ciliates *Anoplodinium denticulatum in vitro*. *Acta Protozool.* **28:** 273-283

Michałowski T., Muszyński P., Landa I. (1991) Factors influencing the growth of rumen ciliates *Eudiplodinium maggii in vitro*. *Acta Protozool.* **30:** 115-120

Michałowski T., Harmeyer J., Bełżecki G. (1999) The importance of washing the omasum for successful defaunation of sheep. *J. Anim. Feed. Sci.* **8:** 611-619

Miller G. L., Blum R., Glennon W. E., Burton A. L. (1960) Measurement of CMC-ase avtivity. *Anal. Biochem.* **2:** 127-132

Pace J. (1955) Seed proteins. In: Modern Methods of Plant Analysis (Eds. K. Peach and M. V. Tracey), Springer Verlag, Berlin, Göttingen, Heidelberg, 69-105

Ruszczyc Z. (1970) Metody Badań Zootechnicznych. PWRiL., Warszawa

Sellinger L. B., Forsberg C. W., Cheng K.-J. (1996) The rumen: A unique source of enzymes for enhancing livestock production. *Anaerobe* **2:** 263-284

Wallace R. J., McEwan N. R., Newbold C. J. (1999) Improving utilisation of nitrogen in the rumen. In: Rowett Research Institute Anual Report (Eds. H. Robertson and I. Bremner). The Rowett Research Institute, 28-31

Williams A. G., Coleman G. S. (1985) Hemicellulose degrading enzymes in rumen ciliate protozoa. *Curr. Microbiol.* **12:** 85-90

Williams A. G., Coleman G. S. (1992) The Rumen Protozoa. Springer-Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest

Wood T. M. (1992) Fungal cellulases. *Bioch. Soc. Trans*. **20:** 5-11

Licnophora rosa sp. n. (Ciliophora: Heterotrichea) from the Gills of *Oxystele sinensis* (Gmelin, 1791) (Prosobranchia: Trochidae), South Africa

Liesl L. VAN AS and Jo G. VAN AS

Department of Zoology and Entomology, University of the Free State, Bloemfontein, South Africa

Summary. During surveys on the symbionts of intertidal invertebrates along the rocky shores of South Africa, a heterotrichous ciliophoran was found on the gills of *Oxystele sinensis* (Gmelin, 1791). This ciliophoran species comprises an oral and basal regions connected by a neck region. It is characterised by four macronuclear segments and is described as a new species, *Licnophora rosa* sp. n.

Key words: heterotrichous ciliophoran, *Licnophora rosa* sp. n., marine mollusc, top shell.

INTRODUCTION

Five species of the top shell genus *Oxystele* Philippi, 1847 occur within the South African marine zoogeographical province. All of these host trichodinid and scyphidiid peritrichs on their gills (Fantham 1930, Sandon 1965, Basson and Van As 1992, Basson *et al.* 1999). During surveys for an ongoing project on the symbionts of intertidal invertebrates, another ciliophoran was found associated only with *Oxystele sinensis* (Gmelin, 1791). This heterotrichous ciliophoran conforms to the mor-

phological features of the monotypic family Licnophoridae Bütschli, 1881 and the genus *Licnophora* Claparède, 1867. It differs from the known species based on general body morphology, characteristics of the nuclear apparatus and host preference and is described as a new species. The description is based on Bouin's fixed specimens stained with hematoxylin, specimens impregnated with Protargol as well as scanning electron microscopy.

MATERIAL AND METHODS

Specimens of *Oxystele sinensis* (Gastropoda: Prosobranchia) were collected from the De Hoop Nature Reserve, South Africa and taken to a field laboratory where wet smears were prepared and examined.

Address for correspondence: Liesl L. Van As, Department of Zoology and Entomology, University of the Free State, PO Box 339, Bloemfontein, 9300, South Africa; Fax: (+2751) 448 8711; E-mail: vanasll@sci.uovs.ac.za

Positive smears were fixed in Bouin's and transferred to 70 % ethanol. Some smears were stained with Mayer's hematoxylin (Humason 1979) for studying the nuclear apparatus and for obtaining body measurements. Other smears were impregnated with Protargol (Lom and Dyková 1992) to obtain data on the internal structures.

For scanning electron microscopy (SEM), licnophorids were fixed in 2.5 % glutaraldehyde, transferred to 5 µm nuclearpore filters and prepared further using standard SEM techniques.

Body and micronucleus measurements and number of macronuclear segments were obtained from microscope projection drawings, using fixed material. Measurements of specimens are presented in the following way: minimum and maximum values are given, followed in parentheses by the arithmetic mean (mode in the case of the number of macronuclei), standard deviation (only in n>9) and lastly number of specimens measured. The type material is in the collection of the National Museum, Bloemfontein, South Africa.

RESULTS AND DISCUSSION

Licnophora rosa sp. n. (Figs. 1-6)

Hosts: *Oxystele sinensis* (Gmelin, 1791)
Position on host: gills.
Localities: De Hoop Nature Reserves on the south coast of South Africa.
Type-specimens: holotype slide S99/11/09-30 (NMBP 271), paratype slides, S99/11/09-27 (NMBP 272), 97/04/09-12 (NMBP 273) in the collection of the National Museum, Bloemfontein, South Africa, other material in the collection of the authors.
Type host and locality: *O. sinensis* De Hoop Nature Reserve (34°28' S; 20°30' E).
Etymology: *rosa* is Latin for pink. The common name for the type host is pink-lipped top shell.

Description

Body squat, total length 35-70 µm (52.1 ± 8.5, 84), consists of three distinct regions; oral and basal region connected with short neck region (Fig. 3). Oral region diameter at broadest part 15-40 µm (23.1 ± 5.2, 84). Adoral side of oral region fringed by broad band of adoral zone of membranelles (AZM) describing spiral of 270°, before plunging into infundibulum. AZM comprising 61-87 (72.7 ± 5.8, 45) rows of membranelles (Figs. 1, 4), between 16 and 20 kinetosomes wide. Rows of membranelles separated by sharply pointed endoplasmic ribs. Centre of aboral surface smooth without cilia, fringed by AZM. Small area on margin with lateral cilia (Figs.1, 3). Neck short, diameter 10-33 µm (20.0 ± 3.9, 84), clearly distinguishable from adjacent oral and basal regions. Basal region round, surface slightly concave,
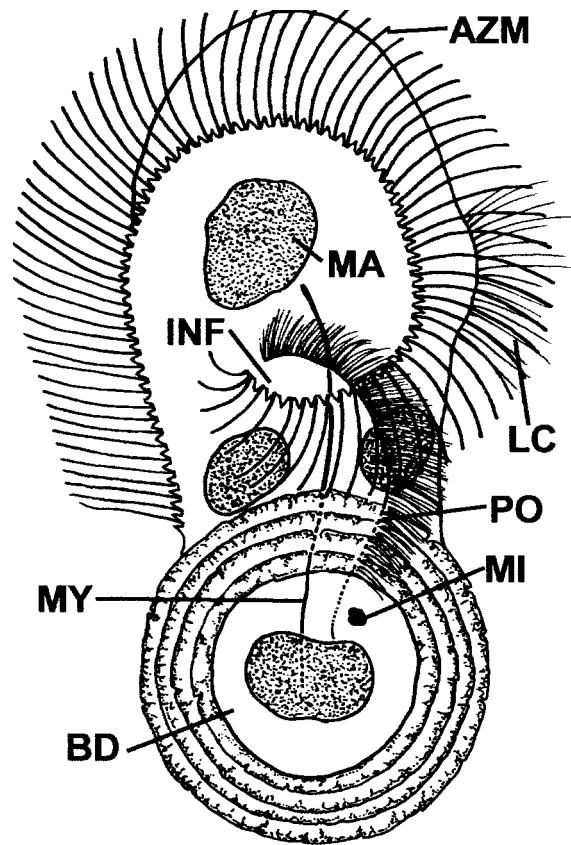

Fig. 1. Microscope projection drawing of *Licnophora rosa* sp. n. occurring on the gill filaments of *Oxystele sinensis* (Gmelin, 1791) collected from the De Hoop Nature Reserve, South Africa. AZM - adoral zone of membranelles, BD - basal disc, INF - infundibulum, LC - lateral cilia, MA - macronucleus, MI - micronucleus, MY - myoneme, PO - paroral organelle. Scale bar - 15 µm

diameter 14-34 µm (20.7 ± 3.0, 84). Basal disc diameter 8-20 µm (14.9 ± 3.3, 56), disc surrounded by a single circular ring of short cilia, of uniform lengths (Fig. 5). Three additional rings of cilia extend around basal disc, proximal row shorter, distal row longer (Fig. 5). Anterior part of basal region separated from membranelles by velum, with one row of short cilia between velum and fourth row of membranelle (Fig. 6). Single row of between 20 and 25 ciliferous kinetosomes on dorsal side of basal disc (Fig. 6). Myoneme extends from centre of basal disc, stretching directly upwards past infundibulum, into centre of oral region (Fig. 1). Paroral organelle extends from periphery of basal disc, towards infundibulum, not always visible in light microscopy. Paroral organelle consisting of single row of densely packed kinetosomes from which a single row of long cilia
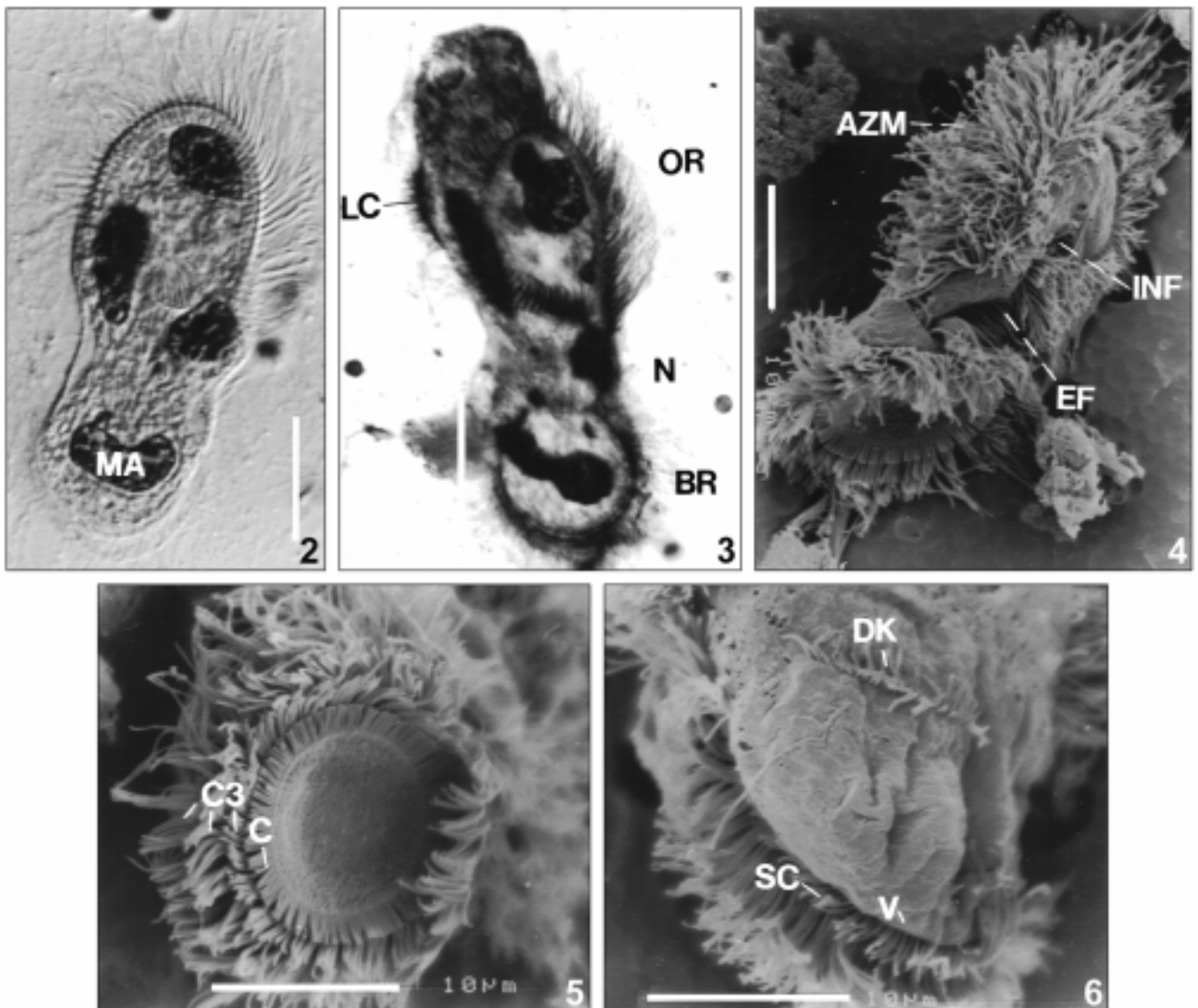
Figs. 2-6. Photomicrographs of hematoxylin stained specimens (2, 3) and scanning electron micrographs (4-6) of *Licnophora rosa* sp. n. collected from top shells from the De Hoop Nature Reserve, South Africa. **2** - adoral view: macronuclear segments (MA); **3** - aboral view: basal region (BR), lateral cilia (LC), neck (N), oral region (OR); **4** - adoral view: adoral zone of membranelles (AZM), ectoplasmic furrow (EF), infundibulum (INF); **5** - basal region with membranelles: single, circular ring of short cilia (C), three additional cilia rings (C3); **6** - basal region: dorsal ciliferous kinetosomes (DK), short cilia (SC), velum (V). Scale bars - 15 μm (2, 3), 10 μm (4-6)

originates, aborally visible in an ectoplasmic furrow (Fig. 4).

Macronucleus consists of large, round to oval-shaped separate nuclei, varying in number between 4 and 6 (4, 84) (Figs.1, 2). Number of macronuclear segments in oral region 1-2 (1, 84), in neck region 1-3 (2, 84) and in basal region 1-2 (1, 84). Micronucleus irregular in form, diameter 2-4 μm (3, 4) if visible situated near centre of basal disc. No food vacuoles observed, endoplasm with granular appearance. No contractile vacuole found.

Remarks

Nine of the eleven known *Licnophora* species have more nuclear segments than the newly described *L. rosa,* including the two species described from South Africa (Van As *et al.* 1999, Van As and Van As 2000). The nuclear information of the tenth species, i.e. *L. cohnii* Claparède, 1867, recorded from Italian polychaetes, is unknown. The only species with a comparable number of nuclei is *L. conklini* Stevens, 1904. This

species was originally found by Dr Conklin on the egg capsules of a slipper limpet (*Crepidula plana* Say, 1822) from Woods Hole, USA, but it was described by Calkins (1901) as a variety of *L. macfarlandi* Stevens, 1901. Stevens (1904) found specimens of a licnophorid again on *C. plana* also from Woods Hole. She considered this licnophorid with five nuclei to be a new species and named it *L. conklini*. Villeneuve-Brachon (1940), found licnophorids on the keyhole limpet *Fissurella gibberula* Lamarck, 1822, (currently known as *Diodora gibberula* (Lamarck, 1822)) at Sete, France, which she considered to be the same species as *L. conklini*. The population of *L. conklini* from the USA described by Calkins (1901) had five to six macronuclear segments in the body. The other material from the USA described by Stevens (1904) has a body length of 100-135 μm, two nuclear segments in the oral disc, two in the neck region and one large segment in the basal disc, all of which are connected to one another. The material collected by Villeneuve-Brachon (1940) from France had four macronuclear segments in the body, with the two nuclei in the neck region connected.

Licnophora rosa differs from *L. conklini* in overall body size. The largest specimen of *L. rosa* collected was 70 μm long, with a mean of 52.1 μm. This is almost half the size of the smallest specimens of *L. conklini* recorded. *Licnophora rosa* has lateral cilia on the oral region, which has not been recorded for *L. conklini*. The only other species with lateral cilia is *L. auerbachii* (Cohn, 1866) (Owen 1980, Silva Neto 1994). Furthermore *L. rosa* is restricted to a single host species, which is endemic to the southern African marine coastal province. *Licnophora rosa* was found only on the gills of the host, whereas *L. conklini* appears to be less host and site specific.

During investigations of the five *Oxystele* species occurring in southern Africa, *L. rosa* was found only on *O. sinensis*, where it co-existed on the gills with *Trichodina oxystelis* Sandon, 1965 and *Mantoscyphidia fanthami* Basson, Botha and Van As, 1999. *Licnophora rosa* is found attached to the gill filaments of the host and occurs in smaller numbers than the other two ciliophorans. Of the hosts examined, 66% were epifaunated.

REFERENCES

Basson L., Botha A., Van As J. G. (1999) *Mantoscyphidia fanthami* sp. n. an ectosymbiont (Ciliophora: Peritrichia) from the gills of the marine gastropod *Oxystele* Philippi, 1847 in South Africa. *Acta Protozool*. **38:** 75-81
Basson L., Van As J. G. (1992) A redescription of *Trichodina oxystelis* Sandon, 1965 (Ciliophora: Peritrichida), a parasite of the marine prosobranch *Oxystele* from southern Africa. *Syst. Parasitol*. **22:** 231-237
Calkins G. N. (1901) Marine protozoa from Woods Hole. *Bull. U. S. Fish Com*. **27:** 415-468
Fantham H. B. (1930) Some parasitic Protozoa found in South Africa. XIII. *S. Afr J. Sci*. **27:** 376-390
Humason G. F. (1979) Animal Tissue Techniques (4th ed). W. H. Freeman and Company, San Francisco
Lom J., Dyková I. (1992) Development in Aquaculture and Fisheries Science, Vol. **26**. Protozoan Parasites of Fishes. Elsevier, Amsterdam
Owen H. G. (1980) Damage to the eyes of the bivalve *Chlamys opercularis* caused by the ciliate *Licnophora auerbachii*. *J. Invert. Pathol*. **36:** 283-291
Sandon H. (1965) Some species of *Trichodina* from South Africa. *Acta Protozool*. **3:** 39-56
Silva Neto I. D. D. (1994) Observations sur l' ultrastructure du Cilié Hétérotriche *Licnophora auerbachii* (Cohn, 1866), épibionte de l'étoile de mer. *Ann. Sci. Nat. Zool. Paris* **15:** 49-62
Stevens N. M. (1904) Further studies on the ciliate infusoria, *Licnophora* and *Boveria*. *Arch. Protistenkd*. **3:** 1-46
Van As L. L., Van As J. G. (2000) *Licnophora bassoni* sp. n. (Ciliophora: Heterotrichea) from South African turban shells (Gastropoda: Prosobranchia). *Acta Protozool*. **39:** 331-335
Van As L. L., Van As J G., Basson L. (1999) *Licnophora limpetae* sp. n. (Ciliophora: Heterotrichea) ectosymbiont of South African Limpets. *Acta Protozool*. **38:** 249-253
Villeneuve-Brachon S. (1940) Recherches sur les Ciliés Hétérotriches. Cinétome, Argyrome, Myonémes. *Arch. Zool. Exp. Gén*. **82:** 1-180

ACTA
PROTOZOOLOGICA

Description of *Zoothamnium chlamydis* sp. n. (Protozoa: Ciliophora: Peritrichida), an Ectocommensal Peritrichous Ciliate from Cultured Scallop in North China

Xiaozhong HU and Weibo SONG

Laboratory of Protozoology, KLM, Ocean University of Qingdao, Qingdao, People's Republic of China

Summary. The morphology, infraciliature and silverline system of an ectocommensal ciliate, *Zoothamnium chlamydis* sp. n., isolated from mantle cavity and on the shell surface of the cultured scallop, *Chlamys farreri* off the coast of Qingdao, China were studied from living and silver-impregnated specimens. The diagnosis for the new species is: marine *Zoothamnium* with alternatively branched stalk; zooids *in vivo* 50-90 x 25-60 μm, slender in shape with one layer of peristomial lip moderately everted, and bacteria covering the whole surface of the cell; one contractile vacuole apically positioned; macronucleus normally band-like, longitudinally oriented. Pellicle with conspicuous, widely spaced transverse striae. Number of silverlines from oral area to aboral ciliary wreath about 27-47; from aboral ciliary wreath to the scopula, 19-29. Zooids generally enlarged at both proximal and distal ends of branches.

Key words: marine peritrich, morphology, *Zoothamnium chlamydis* sp. n.

INTRODUCTION

Ciliated protozoa such as sessile peritrichs play an important role as ectocommensals on the body surface of aquatic organisms from both marine and freshwater habitats (Kahl 1933; Precht 1935; Nenninger 1948; Dietz 1964; Stiller 1971; Green 1974; Bierhof and Roos 1977; Corliss 1979; Valbonesi and Guglielmo 1988; Song 1991a, c, 1992a; Song and Warren 2000). For a long time, much attention has been given to sessile

peritrichs of the genus *Zoothamnium*, mainly associated with amphipods, copepods and decapod shrimps. However, as to the author's knowledge, no records of *Zoothamnium*-species attached to marine scallops, have previously been made (Steuer 1932, Kahl 1935, Precht 1935, Raabe and Raabe 1959, Herman and Mihursky 1964, Fenchel 1965, Foster *et al.* 1978, Kumari and Nair 1985, Nagasawa 1986, Song 1992b, Xu *et al.* 1999).

During a survey on parasitic protozoa in molluscs culturing water in the Spring of 2000 off Qingdao, China, an unknown *Zoothamnium* species was found within the mantle cavity as well as on the shell surface of the cultured scallop *Chlamys farreri*. The present paper gives the observations and descriptions on its morphology and silverline system.

Address for correspondence: Weibo Song, Laboratory of Protozoology, College of Fisheries, Ocean University of Qingdao, Qingdao 266003, P. R. China; Fax: +86 0532 203 2283; E-mail: wsong@ouqd.edu.cn

MATERIALS AND METHODS

Host scallop *Chlamys farreri* was collected from the coast of Qingdao (Tsingtao, 36°08' N; 120°43' E), China. Ciliates were removed with a pipette and kept in culture at room temperature. Living observations were carried out using both bright field and differential interference contrast microscopy. Protargol staining according to Wilbert (1975) and Chatton-Lwoff silver nitrate method as described by Corliss (1953) were applied to reveal the infraciliature and silverline system respectively. Accounting and measurements on stained specimens were performed at a magnification of x1250. Drawings were made with the help of a camera lucida.

Terminology and systematic arrangement are mainly according to Corliss (1979), Warren (1986) and Foissner *et al.* (1992).

RESULTS

According to Corliss (1979), the current taxonomic status of this species is given as follows:

Class: Oligohymenophora de Puytorac *et al.*, 1974
Order: Peritrichida Stein, 1859
Suborder: Sessilina Kahl, 1933
Family: Vorticellidae Ehrenberg, 1838
Genus: *Zoothamnium* Bory de St. Vincent, 1826

Zoothamnium chlamydis sp. n. (Figs. 1-10)

Diagnosis: marine *Zoothamnium* with alternatively branched stalk; zooids highly variable in size *in vivo* 50-90 x 25-60 μm, slender body shape with one-layer peristomial lip moderately everted; one contractile vacuole apically positioned; macronucleus normally band-like, longitudinally oriented. Cell surface densely associated with bacteria, and pellicle with conspicuous, widely spaced transverse striae. Number of silverlines from oral area to aboral ciliary wreath (ACW) about 27-47, and from aboral ciliary wreath to scopula, 19-29. Zooids generally enlarged at both proximal and distal ends of branches.

Type specimens: one holotype (HD-00042401) and one paratype (HD-00042402) as protargol-impregnated slides are deposited in the Laboratory of Protozoology, Ocean University of Qingdao, China.

Host and site: *Chlamys farreri*, off the coast of Qingdao (Tsingtao, 36°08' N; 120°43' E), China.

Ecological features: open culturing water, temperature 5-10°C; pH 8.2-8.3, salinity 34-36 ‰.

Morphological description: body constantly slender in shape, widest at peristomial area and narrowed posteriorly with moderately everted, and rigid border (peristomial lip, PL); peristomial disc (PD) small, obliquely elevated when cell is fully extended (Fig. 1). Zooids within same colony conspicuously in two different sizes, mostly ("normal" zooids) *in vivo* about 50-60 μm long, and ratio of length: width *ca* 2:1; but zooids at proximal or distal ends of branches often enlarged, *ca* 70-90 x 50-60 μm (Figs. 2; 11, arrowheads). Formation of telotroch not observed.

Pellicle with conspicuous transverse striations, especially in enlarged zooids even when observed under low magnification; cell surface often covered with densely associated bacteria (Figs. 1, arrow; 14).

Cytoplasm colorless and transparent, containing several large food vacuoles (4-8 μm across). One contractile vacuole (CV), 5-8 μm in diameter (Figs. 4, 5), at level of peristomial lip on dorsal wall of vestibulum, which extends about 1/2 of body length. Macronucleus (Ma) band-like, longitudinally positioned (Figs. 1; 18, arrowhead), which appears twisted or shortened in enlarged zooids (Figs. 4-6).

Stalk with smooth surface and thick spasmoneme (about 3 μm across) (Fig. 3). Continuous spasmoneme extending through entire stalk. Colony comparatively large (up to 0.6 mm), alternately branched, with up to 100 zooids, which are also alternatively located in branches (Figs. 2, 11). When disturbed, colony contracts as one unit.

Infraciliature and silverline system: buccal apparatus typical of genus (Figs. 7-9). Haplokinety (H; Fig. 20, long arrow) and polykinety (P; Figs. 7; 9; 20, short arrow) about 1¼ turn around peristomial disc before entering vestibulum. Polykinety forming three peniculi in lower half of vestibulum. Peniculus 1 (P_1) and 2 (P_2) about equal length, each comprising 3 rows of kinetosomes, and peniculus 3 (P_3) rather short, also composed of 3 rows of kinetosomes (Figs. 7; 19, small arrows); haplokinety passing around vestibulum on opposite wall to peniculi. Germinal kinety (G) comparatively long, located parallel to haplokinety (Figs. 7; 20, small arrow). Epistomial membrane (EM) short, near opening of vestibulum (Figs. 7; 8; 9, arrow; 20, arrowhead). Aboral ciliary wreath (ACW) composed of one row of loosely arranged kinetosomes in normal zooids (Figs. 8; 16, arrowhead), while 2-rowed in enlarged ones (Fig. 8, inset).

Silverline system as shown in Figs. 10, 15, widely striated pattern; i. e., striations widely spaced and conspicuous pellicular pores associated between silverlines. Number of silverlines from oral area to aboral ciliary wreath (ACW; Figs. 8; 13, arrowhead), 27-47 (mean 36.2); from ACW to scopula, 19-29 (mean 24.6) (Table 1).
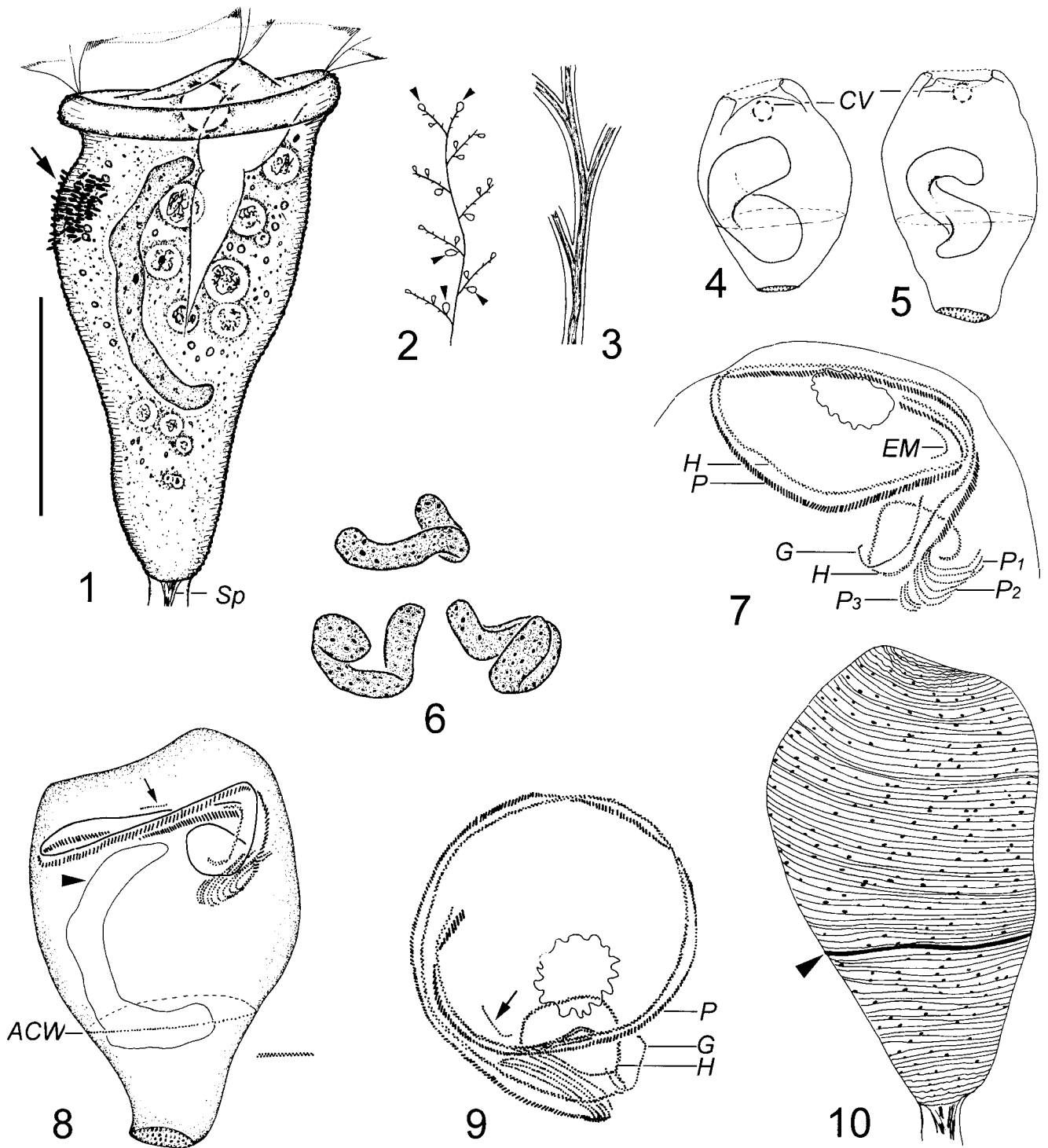
Figs. 1-10. Morphology of *Zoothamnium chlamydis* sp. n. from life (**1-6**) and after silver impregnation (**7-10**). **1** - typical zooid, arrow shows covering of bacteria; **2** - colony, arrowheads show the enlarged zooids; **3** - stalk, note the thick spasmoneme; **4, 5** - contracted and enlarged zooids; **6** -twisted macronucleus in enlarged zooids; **7, 9** - oral apparatus, arrow in 9 indicates epistomial membrane; **8** - general infraciliature, arrow marks epistomial membrane, arrowhead shows macronucleus, inset: aboral ciliary wreath; **10** - silverline system, arrowhead shows aboral ciliary wreath. ACW - aboral ciliary wreath; CV - contractile vacuole; EM - epistomial membrane; G - germinal kinety; H - haplokinety; P - polykinety; P_{1-3} - peniculus 1-3; Sp - spasmoneme. Scale bar - 30 μm
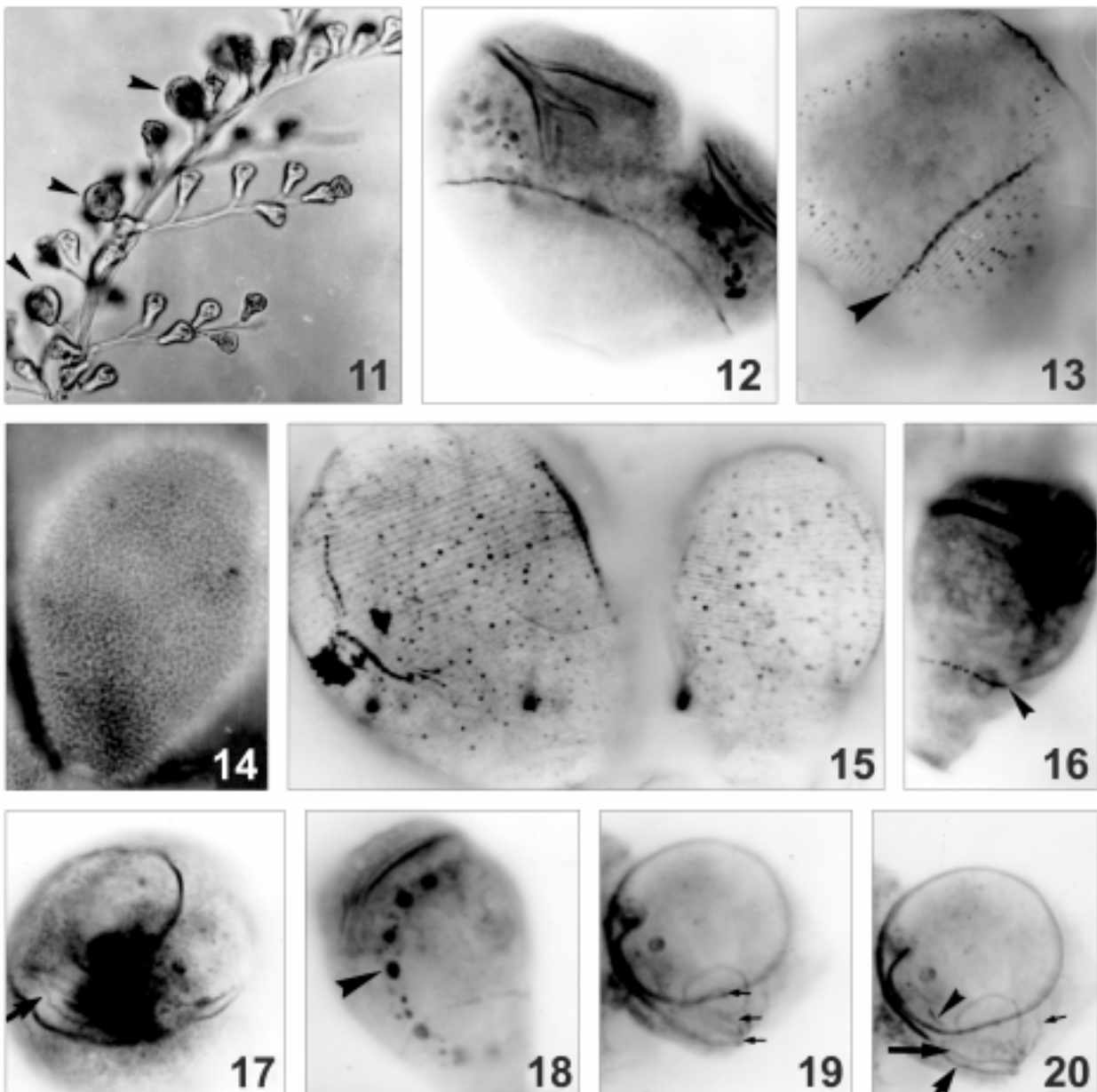
Figs. 11-20. Microphotographs of *Zoothamnium chlamydis* sp. n. based on living and impregnated specimens. **11** - colony, arrowheads show enlarged zooids; **12** - infraciliature in a late stage of morphogenesis; **13, 15** - silverline system, arrowhead marks the aboral ciliary wreath; **14** - bacteria covering the body surface; **16** - infraciliature, arrowhead shows aboral ciliary wreath; **17** - infraciliature in a middle stage of morphogenesis, arrow indicates new oral apparatus; **18** - arrowhead refers to macronucleus; **19, 20** - oral apparatus of the same individual, long arrow shows haplokinety, short arrow indicates polykinety, arrowhead marks epistomial membrane, small arrows in Fig. 19 demonstrate peniculus 1-3 (from lower to upper), small arrow in Fig. 20 shows the germinal kinety

DISCUSSION

As commonly accepted, the genus *Zoothamnium* is characterized by a continuous spasmoneme within a branching stalk, thus leading to a contraction of the entire colony and a transverse silverline pattern (*vs. Zoothamnopsis*) (Ehrenberg 1838, Stein 1854, Claparède and Lachmann 1858, Fauré-Fremiet 1930, Kahl 1933, Bauer-Nebelsick *et al*. 1996, Song 1997). For a long time, the species identification and separation in the genus *Zoothamnium* depended on body shape and size, location of contractile vacuole, oral and nuclear

Table 1. Morphometric characterizations of *Zoothamnium chlamydis* sp. n. Data are based on silver impregnated specimens. Measurements in μm. ACW - adoral ciliary wreath; CV - coefficiency of variation; Max - maximum; Mean - arithmetic mean; Min - minimum; n - number of individuals examined; PL - peristomial lip; Sc - scopula; SD - standard deviation; SE - standard error of mean; SL - silverline system

Character	Min	Max	Mean	SD	SE	CV (%)	n
Normal zooid, body length	32	64	51.6	9.23	2.38	17.9	15
Normal zooid, body width	20	50	31.6	6.53	1.69	20.7	15
Enlarged zooid, body length	56	83	68.5	8.94	2.69	13.0	11
Enlarged zooid, body width	35	60	48.3	8.66	2.61	17.9	11
No. of SL (PL to ACW)	27	47	36.2	6.15	1.49	17.0	17
No. of SL (ACW to Sc)	19	29	24.6	3.68	0.89	14.9	17

apparatus, the habitat, features of the silverline system, and the branching pattern of the colony.

Considering the colony size and alternatively branched stalk, the peristomial lip, body size and marine habitat, 7 species should be compared with the current organism: *Zoothamnium niveum* Ehrenberg, 1838, *Z. alternans* Claparède & Lachmann, 1858, *Z. gleniscum* Claparède & Lachmann, 1858, *Z. plumula* Kahl, 1932, *Z. sinense* Song, 1991 b (Song, 1986, 1991 b), *Z. commune* Kahl, 1933 (*sensu* Song, 1991 b), and *Z. ponticum* Andrussowa, 1886.

Compared with this new species, *Zoothamnium niveum* can be distinguished by the larger size of its normal zooids (120 μm *vs*. 50-60 μm in *Z. chlamydis*), clearly lower position of the contractile vacuole (below peristomial lip *vs*. apically positioned) and presence of typical macrozooids (Ehrenberg 1838, Claparède and Lachmann 1858, Wang and Nie 1932, Kahl 1933, Wailes 1943, Bauer-Nebelsick *et al*. 1996).

Kahl (1933, 1935) redescribed three different forms of *Zoothamnium alternans*, which could not be confused with this new form regardless of the similarity in several morphological features. Among these, *Z. alternans* sensu Claparède & Lachmann, 1858 and *Z. alternans* sensu Greff, 1870 have conspicuously plumper body shapes, larger macrozooids (up to 120 μm) and cross-striated stalk. The form described by Kent (1881) possesses long cylindrical "Mikrogameten", and is hence clearly different from *Zoothamnium chlamydis*.

In terms of body shape, size and branching stalk, *Zoothamnium plumula* Kahl, 1933 is most similar to this new species. However, the latter differs distinctly from the former in having fewer silverlines (*ca* 61 *vs*. 94-98) and the position of epistomial membrane (near opening of vestibulum *vs*. at distal end of polykinety) (Perejaslawzewa 1886; Kahl 1933, 1935; Song and AL-Rasheid, unpublished).

Zoothamnium gleniscum Claparède & Lachmann, 1858 possesses cross-striated stalk, which should therefore, not be conspecific with this new species (Claparède and Lachmann 1858, Kahl 1933).

With reference to the position of contractile vacuole and the appearance of the peristomial lip, comparison should be also made with *Zoothamnium sinense* Song, 1991b and *Z. commune* sensu Song, 1991b. These two species can be distinguished by short and "plump" body shape (i.e. bell-like *vs*. elongated and slender in *Z. chlamydis*), relatively smaller body size (30-60 *vs*. 50-90 μm in length), and zooids of uniform size (*vs*. normal and enlarged ones in *Z. chlamydis*) (Song 1986, 1991b).

Considering body shape, size and branching stalk, an unknown *Zoothamnium* species by Kiesselbach (1936) might be a population of *Zoothamnium alternans* because of the presence of typical macrozooids and the long stalk for each zooid although no detailed position of CV was given.

Unlike *Zoothamnium ponticum* Andrussowa, 1886, our new species has high position of CV (*vs*. significantly below peristomial lip) and longitudinally oriented Ma. (*vs*. transversely in the former).

REFERENCES

Bauer-Nebelsick M., Bardele C. F., Ott J. A. (1996) Redescription of *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg, 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria. *Europ. J. Protistol.* **32:** 18-30

Bierhof M. J., Roos P. J. (1977) Sedentary ciliates from two Dutch freshwater *Gammarus* species. *Bijd. Tot Dierk.* **46:** 151-170

Claparède É., Lachmann J. (1858) Études sur les infusoires et les rhizopodes. *Mém. Inst. natn. Génev.* **5:** 1-260

Corliss J. O.(1953) Silver impregnation of ciliated protozoa by the Chatton-Lwoff technic. *Stain Technol.* **28:** 97-100

Corliss J. O. (1979) The Ciliated Protozoa. Characterization, Classification and Guide to the Literature. 2nd ed. Pergamon Press. New York

Dietz G. (1964) Beitrag zur Kenntnis der Ciliatenfauna einiger Brackwassertümpel (Etangs) der französischen Mittelmeerküste. *Vie Milieu* **15:** 47-93

Ehrenberg C. G. (1838) Die Infusionsthierchen als vollkommene Organismen. Ein Blick in das tiefere organische Leben der Natur. Leopold Voss Verlag, Leipzig

Fauré-Fremiet E. (1930) Growth and differentiation of the colonies of *Zoothamnium alternans* (Clap. and Lachm.). *Biol. Bull.* **58:** 28-51

Fenchel T. (1965) On the ciliate fauna associated with the marine species of the amphipod genus *Gammarus* J. G. Fabricius. *Ophelia* **2:** 281-303

Foissner W., Berger H., Kohmann H. (1992) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems - Band II: Peritrichida, Heterotrichida, Odontostomatida. Informationsberichte des Bayer. Landesamtes für Wasserwirtschaft. 5/92, 1-502

Foster C. A., Sarphie T. G., Hawkins W. E. (1978) Fine structure of the peritrichous ectocommensal *Zoothamnium* sp. with emphasis on its mode of attachment to penaeid shrimp. *J. Fish Disease* **1:** 321-335

Green J. (1974) Parasites and epibionts of Cladocera. *Trans. Zool. Soc. Land.* **32:** 417-515

Herman S. S., Mihursky J. A. (1964) Infestation of the copepod *Acartia tonsa* with the stalked ciliate *Zoothamnium. Science* **146:** 543-544

Kahl A. (1933) Ciliata libera et ectocommensalia. In: Die Tierwelt der Nord- und Ostsee. (Eds. G. Grimpe and E. Wagler). Lief. 2 (3), Leipzig, 29-146

Kahl A. (1935) Urtiere oder Protozoa I: Wimpertiere oder Ciliata (Infusoria) 4. Peritricha und Chonotricha. *Tierwelt Dtl.* **30:** 651-886

Kent W. S. (1881) A manual of the infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa British and foreign, and an account of the organization and affinities of the sponges, 2: 433-720. David Bogue, London

Kiesselbach A. (1936) Zur Ciliatenfauna der Nördlichen Adria. *Thalassia* **2:** 1-53

Kumari V. S., Nair N. B. (1985) Some ciliates from the marine woodboring isopod *Sphaeroma. Ind. J. Fish.* **32:** 215-223

Nagasawa S. (1986) The peritrich ciliate *Zoothamnium* attached to the copepod *Centropages abdominalis* in Tokyo Bay waters. *Bull. Mar. Sci.* **38:** 553-558

Nenninger N. (1948) Die Peritrichenfauna der Umgebung von Erlangen mit besonderer Berücksichtigung der Wirtsspezifität. *Zool. Jb.* **77:** 167-266

Perejaslawzewa S. (1886) Protozoa of the Black Sea. *Schr. Naturforsch. Ges. Odessa* **10:** 79-114 (in Russian)

Precht H. (1935) Epizoen der Kieler Bucht. *Nova Acta Leopol.* **3:** 405-474

Raabe J., Raabe Z. (1959) Urceolariidae of molluscs of the Baltic Sea. *Acta Parasitol. Pol.* **7:** 453-465

Song W. (1986) Description of seven new species of peritrichs on *Penaeus orientalis* (Peritricha: Zoothamnidae, Epistylididae). *Acta Zootax. Sin.* **11:** 225-235 (in Chinese with English summary)

Song W. (1991a) Contribution to the commensal ciliates on *Penaeus orientalis.* I. (Ciliophora, Peritrichida). *J. Ocean Univ. Qingdao* **21:** 119-128 (in Chinese with English summary)

Song W. (1991b) Contribution to the commensal ciliates on *Penaeus orientalis.* II. (Ciliophora, Peritrichida). *J. Ocean Univ. Qingdao* **21:** 45-55 (in Chinese with English summary)

Song W. (1991c) A new commensal ciliate, *Zoothamnium paraentzii* (Ciliophora, Peritrichida). *Zool. Res.* **12:** 355-359

Song W. (1992a) Contribution to the commensal ciliates on *Penaeus orientalis.* III. (Ciliophora, Peritrichida). *J. Ocean Univ. Qingdao* **22:** 107-117 (in Chinese with English summary)

Song W. (1992b) A new marine ciliate, *Zoothamnium penaei* sp. nov. (Ciliophora, Peritrichida). *Oceanol. Limnol. Sinica* **23:** 90-94

Song W. (1997) A new genus and two new species of marine peritrichous ciliates (Protozoa, Ciliophora, Peritrichida) from Qingdao, China. *Ophelia* **47:** 203-214

Song W., Warren A. (2000) A redescription of *Pseudovorticella patellina* (O. F. Müller, 1776) nov. comb., a peritrichous ciliate (Protozoa: Ciliophora: Peritrichida) isolated from mariculture biotopes in North China. *Acta Protozool.* **39:** 43-50

Stein F. (1854) Die Infusionsthiere auf ihre Entwickelungsgeschichte untersucht. W. Engelmann, Leipzig

Steuer A. (1932) Copepoda: Pleuromamma Giesbr. 1898 der Deutschen Tiefsee-Expedition. *Deut. Tiefsee-Exped. 1898-1899* **24:** 1-119

Stiller J. (1971) Szájkoszorús Csillósok-Peritricha. *Fauna Hung.* **105:** 1-245

Valbonesi A., Guglielmo L. (1988) Infestation of a lagoon zooplanktonic community with the epizoic peritrich *Zoothamnium intermedium* Precht (Peritrichia, Zoothamniidae). *Boll. Zool.* **3:** 179-183

Wailes G. H. (1943) Protozoa, Ciliata and Suctoria. *Can. Pac. Fauna* **1:** 1-46

Wang C. C., Nie D. (1932) A survey of the marine protozoa of Amoy. *Zool. Ser.* **8:** 285-385

Warren A. (1986) A revision of the genus *Vorticella* (Ciliophora: Protozoa). *Bull. Bri. Mus. Nat. Hist. (Zool.)* **50:** 1-57

Wilbert N. (1975) Eine verbesserte Technik der Protargolimprägnation für Ciliaten. *Mikrokosmos* **64:** 171-179

Xu K., Song W., Warren A. (1999) Trichodinid ectoparasites (Ciliophora: Peritrichida) from the gills of mariculture molluscs in China, with the descriptions of four new species of *Trichodina* Ehrenberg, 1838. *Syst. Parasitol.* **42:** 229-237

ACTA
PROTOZOOLOGICA

Effect of Epidermal Growth Factor (EGF) on *Tetrahymena pyriformis*

László KŐHIDAI, Márk KERESZTESI and György CSABA

Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary

Summary. *Tetrahymena* has receptors for hormones characteristic in higher animals and signal transduction systems for provoking response. In the present experiments EGF, a potent mitogen of epithelial cells were studied. In a 24 h experiment 1 h treatment with different concentrations of EGF (0.1-10 µg) significantly decreased the growth of *Tetrahymena*. Five microgram EGF treatment 24 h after 5 µg EGF pretreatment (imprinting) also significantly reduced the amount of cells, and this was moderately, however significantly less, than without the repeated treatment. After 48 h there was not difference between the amount of single or double treated cells. EGF was chemorepellent in chemotaxis experiments and 4 h EGF pretreatment abolished this effect, possibly by down regulation. The experiments call attention to the effect of EGF at a very low phylogenetic level, however the direction of the effect was the opposite of the effect in higher animals.

Key words: cell growth, growth factors, chemotaxis, evolution, *Tetrahymena.*

INTRODUCTION

The unicellular ciliated *Tetrahymena* produce, store and secrete hormone-like materials known at higher level of phylogeny. These hormones are insulin (LeRoith *et al.* 1983), adrenocorticotrophic hormone (LeRoith *et al.* 1983), endorphin (Csaba and Kovács 1999), relaxin (LeRoith *et al.* 1983), endothelin (Kőhidai and Csaba 1995), interleukin-6 (Kőhidai *et al.* 2000), serotonin (Brizzi and Blum 1970, Csaba and Kovács 1994),

melatonin (Kőhidai *et al.* 2001) histamine (Hegyesi *et al.*1999) etc. and it also has receptors for binding these hormones (Csaba 1985, 2000; Christopher and Sundermann 1995). The information borne by the hormones is transmitted by second messenger systems (Kuno *et al.* 1979, Kovács and Csaba 1990), consequently the cell can respond to the hormonal stimuli. The first encounter between the cell and the hormone develops the hormonal imprinting (Csaba 1985, 2000) which is transmitted to hundreds of progeny generations, causing easier recognition of the given hormone and stronger response of the cell.

Epidermal growth factor (EGF) is a peptide molecule present in the cell membrane and after detachment it becomes a hormone, influencing the velocity of cell

Address for correspondence: György Csaba, Department of Genetics, Cell and Immunobiology, Semmelweis University, Nagyvarad tér 4, POB 370, 1445 Budapest, Hungary; Fax: (36-1) 303-6968; E-mail: csagyor@dgci.sote.hu

division, first of all in the case of epithelial cells (Cohen 1983, Soler and Carpenter 1994). It seems to be ubiquitous in the animal world. Considering that *Tetrahymena* can react to many hormones (Csaba 1985, 2000) and that EGF-receptor-like structures were demonstrated (Hide *et al.* 1989) in an infectious protozoan, *Trypanosoma brucei* it was reasonable to study the effect of EGF on *Tetrahymena*, a noninfectious unicellular animal, which - being a ciliate - is at one of the highest steps of unicellular evolution.

MATERIALS AND METHODS

Cells and culturing. *Tetrahymena pyriformis* GL cells were maintained in axenic cultures containing 1% tryptone and 0.1% yeast extract (Difco, Michigan, USA). Cells of logarithmic growth phase (48 h) cultures were assayed. The starting cell density of cultures was 10^2 cell/ml in the assay of growth, in chemotaxis assay it was 10^4 cell/ml.

Hormones and buffers. For treatments and pretreatments of cultures [Cys(Acm)[20, 31]]-epidermal growth factor fragment 20-31 (Sigma, USA; hereafter EGF) was used in 5 µg/ml concentration.

Pretreatments with EGF. For the treatment with EGF we applied two setups: (1) Cultures were pretreated with (0.1, 1.0, 2.5, 5.0 and 10 µg/ml) EGF for 1 h. The control group was treated with the solvent, fresh culture medium. Then the samples were washed trice with phosphate buffered saline (PBS) and the pretreated cultures were maintained in 96 wells of plastic plates for 24 h. (2) In an other setup cultures pretreated for 4 h with EGF and the controls were maintained for 24 or 48 h. After these periods the cultures were reexposed to EGF or culture medium in the next combinations: C/C; C/EGF; EGF/C; EGF/EGF.

For studying the multiplication of cells the colorimetric MTT method was used. In this procedure the water soluble tetrazolium salt, 3-[4,5-dimethylthiazol-2yl]-2.5-diphenyltetrazolium bromide (MTT; Sigma Chemicals, St. Louis, USA) is converted to an insoluble purple formazan by mt-dehydrogenase enzymes of the cell (Denizot and Lang 1986). The incubation time with 0.3 mg/ml MTT was 180 min then the plates were centrifuged for 5 min (2500 x g). The supernatant was discarded and the precipitate was dissolved in sodium duodecyl sulphate. The samples were evaluated in Elisa reader (at 540 and 620 nm).

Chemotaxis assay. Cells pretreated with 5 mg/ml for 4 h and the controls were washed and cultured for 24 h. After this incubation the chemotactic responiveness of *Tetrahymena* cells was evaluated in a two-chamber capillary assay developed by us (Kőhidai *et al.* 1995). In this assay, tips of a multi-8-channel micropipette served as inner chambers filled with different concentrations of the attractant (0.1, 1.0, 5.0 µg/ml EGF or control substance), while wells of a plastic plate were the outer chambers filled with cells. The incubation time was 20 min. Samples were fixed in 4% formaldehyde containing PBS. The number of positively responding cells was counted in samples by Neubauer hemocytometer.

Statistical evaluation. Experiments evaluating effects of EGF and pretreatment on multiplication were done in 16 parallels. Chemo-

taxis assays were repeated in five times in two parallels. ANOVA method of Origin 4.0 was used to evaluate statistical sigificance. In the Figures the next marks refer to significant differences: x - p<0.05; y - p<0.01; z - p<0.001.

RESULTS AND DISCUSSION

EGF is a growth factor, a potent mitogen for cells of epithelial origin. It is bound by specific membrane receptors, the intracellular part of which is a tyrosin kinase (Soler and Carpenter 1994). By the help of this, its message is transmitted by a signal cascade (Ruff-Jamison *et al.* 1995) into te nucleus. However, sometimes it can act through the mediation of adenylate cyclase-cAMP system (Budnik and Mukhopadhyay 1991). As it was known that *Tetrahymena* has receptors for hormones of higher level animals and signal transduction systems, it seemed to be worth to study the effect of EGF on this cell.

Different concentrations of 1 h EGF treatment significantly decreased the multiplication of *Tetrahymena* (Fig. 1). The hormone was effective at very low concentration (0.1 µg/ml) and the effect was increased at 2.5 µg, however further increase of the dose did not influence the effect. One day after 1 h treatment with 5 µg EGF (Fig. 2) there was present a reduced number of cells (EGF/C), similar to the case, when the cells first time met the hormone (C/EGF). When EGF was given again to previously EGF treated cells (after imprinting) a further moderate, however significant decrease could be observed (EGF/EGF). Two days after imprinting (Fig. 2) the cell number was also less than in the controls (EGF/C) but repeated EGF treatment (EGF/EGF) did not influence this strongly, however significantly. This means that EGF is not a stimulator, but an inhibitor of cell division in *Tetrahymena* and it has only a very limited imprinter effect.

EGF was discovered and is recorded as a mitogen factor for epithelial cells. However, also in mammals in some cases it can inhibit cell division (Hirai *et al.* 1988, Ponec *et al.* 1988). This latter effect was manifested in *Tetrahymena*. The real importance of this phenomenon is not known, however, it was described earlier that *Tetrahymena* produces growth inhibitory factors at stationary phase (Saitoh and Asai 1980, Schousbue *et al.* 1998) and these factors were not chemically defined.

It was observed (Andersen *et al.* 1984) that platelet-derived growth factor (PDGF) is a very active chemoattractant in *Tetrahymena*. This was the reason
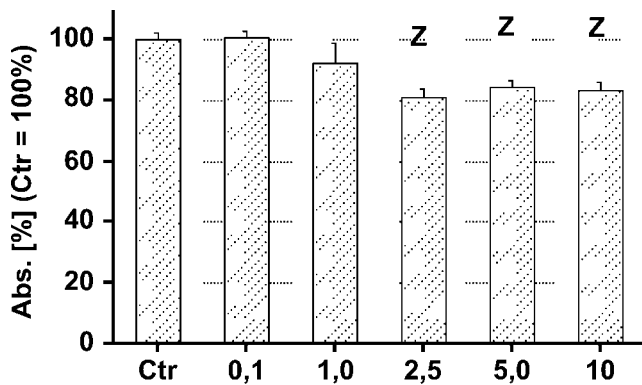
Fig. 1. Effect of 1 h pretreatment with different concentrations of EGF on the multiplication of *Tetrahymena* (z = p<0.001)
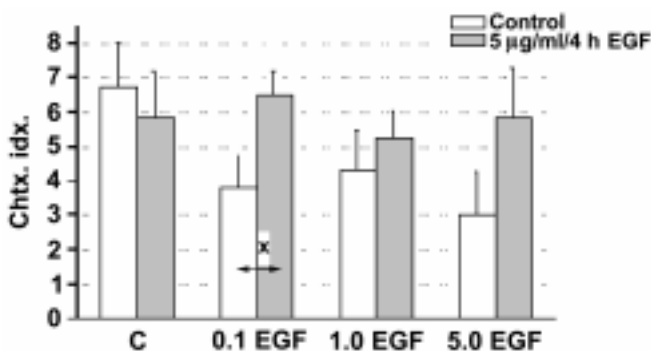

Fig. 3. Chemotactic responsiveness of control and 5μg/ml EGF pretreated (for 4 h) *Tetrahymena* cells 24 h after the pretreatment, to different concentrations of EGF (abscissa, in μg/ml; x = p<0.05)
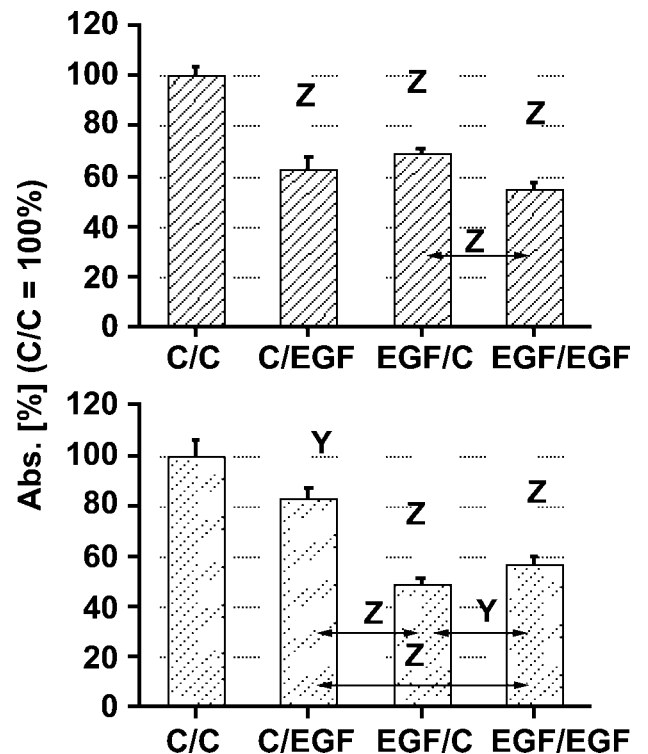

Fig. 2. Effect of 4 h treatment with 5 μg/ml EGF on the multiplication of *Tetrahymena* cells pretreated with 5 μg/ml EGF 24 (above) or 48 h before the second treatment (y = p<0.01; z= p<0.001)

The experiments demonstrate the ability of *Tetrahymena* to react to a hormone having role in multicellular animals and emphasize the possibility of disparate response of cells to a hormone at different levels of phylogeny.

REFERENCES

Andersen H. A., Flodgaard H., Klenow H., Leick V. (1984) Platelet derived growth factor stimulates chemotaxis and nucleic acid synthesis in the protozoan *Tetrahymena*. *Biochim. Biophys. Acta* **782**: 437-440

Brizzi G., Blum J. J. (1970) Effect of growth conditions on serotonin content of *Tetrahymena pyriformis*. *J. Protozool*. **17**: 553-555

Budnik L. T., Mukhopadhyay A. K. (1991) Epidermal growth factor, a modulator of luteal adenylate cyclase - characterization of epidermal growth factor receptors and its interaction with adenylate cyclase system in bovine luteal cell-membrane. *J. Biol. Chem*. **266**: 13908-13913

why we studied the chemosensory response to EGF. In our experiments EGF was unequivocally chemorepellent, independent on the concentration administered (Fig. 3). However, 24 h after imprinting with 5 μg EGF, the repellent effect was not significant, which makes likely that the first treatment (imprinting) abolished the sensitivity of cells to EGF. It is possible that the duration of treatment (4 h imprinting) was too much causing intensive down regulation of receptors, which did not allow the recognition of EGF as ligand.

Considering both (growth and chemosensory) results, they show the repressive effect of EGF to *Tetrahymena*.

Christopher G. K., Sundermann C. H. (1995) Isolation and partial characterisation of the insulin binding site of *Tetrahymena pyriformis*. *Biochem. Biophys. Res. Com.* **212:** 515-523

Cohen S. (1983) The epidermal growth factor (EGF). *Cancer* **51:** 1787-1791

Csaba G. (1985) The unicellular *Tetrahymena* as a model cell for receptor research. *Int. Rev. Cytol.* **95:** 327-377

Csaba G. (2000) Hormonal imprinting: its role during the evolution and development of hormones and receptors. *Cell Biol. Internat.* **24:** 407-414

Csaba G., Kovács P. (1994) Effect of hormones and hormone-induced imprinting on the serotonin level in *Tetrahymena*. Immunocytochemical studies. *Microbios* **80:** 155-163

Csaba G., Kovács P. (1999) Localization of β-endorphin in *Tetrahymena* by confocal microscopy. Induction of the prolonged production of the hormone by hormonal imprinting. *Cell Biol. Internat.* **23:** 695-702

Denizot F., Lang R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89:** 271-277

Hegyesi H., Szalai Cs., Falus A., Csaba G. (1999) The histidin decarboxylase (HDC) gene of *Tetrahymena pyriformis* is similar to the mammalian one. A study of HDC expression. *Biosci. Rep.* **19:** 73-78

Hide G., Gray A., Harrison C. M., Tait A. (1989) Identification of an epidermal growth factor receptor homologue in trypanosomes. *Mol. Biol. Parasitol.* **36:** 51-59

Hirai M., Gamou S., Minoshima S., Shimizu N. (1988) Two independent mechanisms for escaping epidermal growth factor mediated growth inhibition in epidermal growth factor receptor hyperproducing human tumor cells. *J. Cell Biol.* **107:** 791-799

Kovács P., Csaba G. (1990) Influence of the phosphoinositol (PI) system in the mechanism of hormonal imprinting. *Biochem. Biophys. Res. Com.* **170:** 119-126

Kőhidai L., Csaba G. (1995) Effect of mammalian vasoconstrictor peptide, endothelin-1, on *Tetrahymena pyriformis* GL, and the immunocytochemical detection of endogeneous endothelin-like activity. *Comp. Biochem. Physiol.* **111C:** 311-316

Kőhidai L., Lemberkovics É., Csaba G. (1995) Molecule dependent chemotactic responses of *Tetrahymena pyriformis* elicited by volatile oils. *Acta Protozool.* **34:** 181-185

Kőhidai L., Kovács P., Lázár-Molnár E., Csaba G. (2000) Presence, uptake and localization of an immunoreactively interleukin-6 (IL-6)-like molecule in *Tetrahymena pyriformis*. *Cell Biol. Internat.* **24:** 749-755

Kőhidai L., Vakkuri O., Keresztesi M., Leppäluoto J., Csaba G. (2001) Presence and secretion of melatonin in the unicellular *Tetrahymena*: effects of light and darkness. Possible functions of melatonin at this low level of evolution. *J. Pineal Res.* (in press)

Kuno T., Yoshida N., Tanaka C. (1979) Immunocytochemical localization of cyclic AMP and cyclic GMP in synchronously dividing *Tetrahymena*. *Acta Histochem. Cytochem.* **12:** 563

LeRoith D., Schiloach J., Berelowitz M., Frohmann L. A., Liotta A. S., Krieger B. T., Roth J. (1983) Are messenger molecules in microbes the ancestors of the vertebrate hormones and tissue factors? *Fed. Proc.* **42:** 2602-2607

Ponec M., Weerheim A., Kempenaar J., Boonstra J. (1988) Proliferation and differentiation of human squamous carcinoma cell lines and normal keratinocytes: effects of epidermal growth factor, retinoids and hydrocortisone. *In Vitro Cell Dev. Biol.* **8:** 764-770

Ruff-Jamison S., Chen K., Cohen S. (1995) Epidermal growth factor induces the tyrosin phosphorylation and nuclear translocation of Stat 5 in mouse liver. *Proc. Natl. Acad. Sci. USA* **92:** 4215-4218

Saitoh T., Asai H. (1980) Growth controlling factors produced during the logarithmic and stationary phases of *Tetrahymena pyriformis*. *Experientia* **36:** 1375-1376

Schousbue P., Wheatley D. N., Rasmussen L. (1998) Autocrine/paracrine activator of cell proliferation: purification of a 4-6 kD compound with growth factor-like effects in *Tetrahymena thermophila*. *Cell Physiol. Biochem.* **8:** 130-137

Soler C., Carpenter G. (1994) The epidermal growth factor (EGF) family. In: Guidebook to Cytokines and Their Receptors, (Ed. N. A. Nicola) Oxford Univ. Press, 194-197

ACTA
PROTOZOOLOGICA

A Note About Two Hypotrich Ciliate Species of the Genus *Amphisiella*

Gregorio FERNANDEZ-LEBORANS and Apolonia NOVILLO*

Departamento de Biologia Animal I (Zoologia), Facultad de Biologia, Universidad Complutense, Madrid, Spain

Summary. The diagnosis of two species of marine hypotrich ciliates: *Amphisiella arenicola* and *A. ovalis* is shown. These species were described previously by the same authors, as well as the statistical analysis of their biological features and the comparison with the most similar species. Since the diagnosis of both species did not appear in the previous work, these diagnosis are included, in order to observe the rules of the International Code of Zoological Nomenclature.

Key words: *Amphisiella*, Ciliophora, marine hypotrich, species, Protozoa.

INTRODUCTION

In 1992 the authors described two new species of marine hypotrich ciliates: *Amphisiella arenicola* and *Amphisiella ovalis* (Fernandez-Leborans and Novillo 1992). The samples were collected in two littoral areas: ciliates of *Amphisiella arenicola* from a beach zone of the Mediterranean Sea (Gandia, Spain), and ciliates of *Amphisiella ovalis* from a beach zone of the Cantabrian Sea, beside the Atlantic Ocean (Castro Urdiales, Spain). Statistical analysis of the different features and comparison with the species most similar to the ciliates studied appeared in this previous work, in which two

Address for correspondence: Gregorio Fernandez-Leborans, Departamento de Biologia Animal I (Zoologia), Facultad de Biologia, Pnta 9, Universidad Complutense, 28040 Madrid, Spain; E-mail: greg@eucmax.sim.ucm.es

*Present address: Biology Department, 5 Cummington St, 02215 Boston, Massachusetts, USA

new species were proposed, taking into account the differences observed respect to other described species.

Recently Dr. Petz (Institute of Zoology, University of Salzburg) indicated that although these species are valid, they need the publication of their diagnosis since this is a condition according to the International Code of Zoological Nomenclature (personal communication).
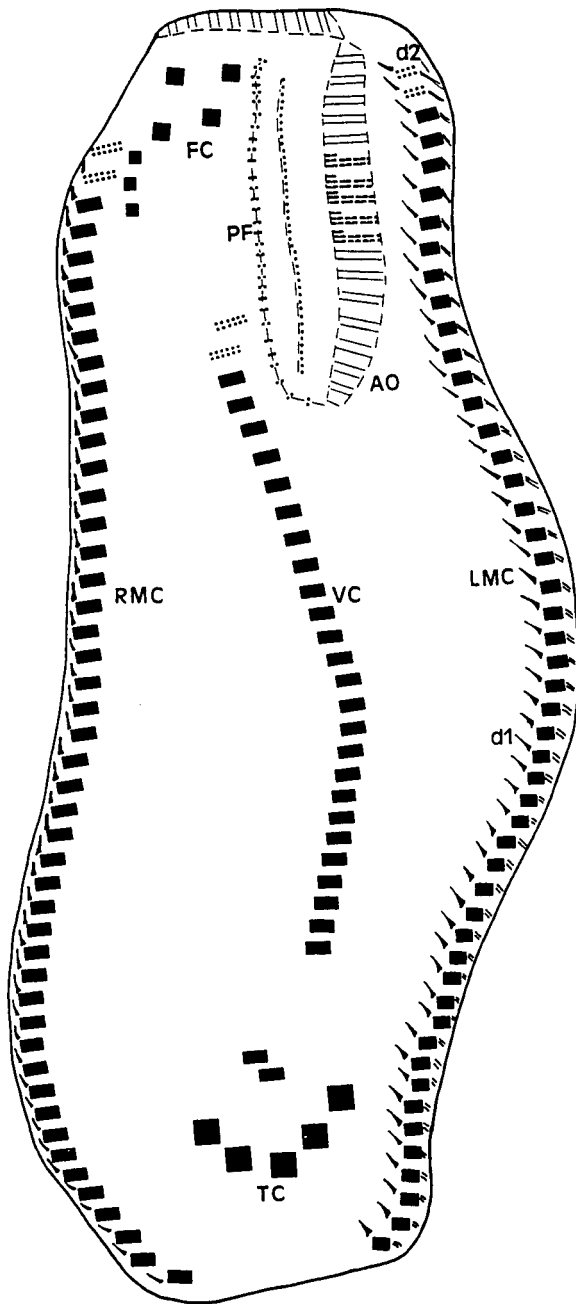
Diagnosis of *Amphisiella arenicola* Fernandez-Leborans & Novillo sp. n. (Fig. 1)

Oval elongated ciliates, with dorsal and ventral sides flattened (132-162 µm long, 37.5-67.5 µm width). Oral ciliature on the left side of ventral surface with an adoral organelles zone (AO) and a paroral formation (PF). AO comprises 36-42 organelles disposed in three parts: (a) an "anterior part" with 15-17 organelles having three parallel kineties (with 2, 6 and 6 kinetosomes each); (b) an "intermediate part" of 16-19 organelles having three rows (with 2, 10-14 and 10-14 kinetosomes each); (c) a "posterior part" of 5-6 organelles having 2 rows of 6-8
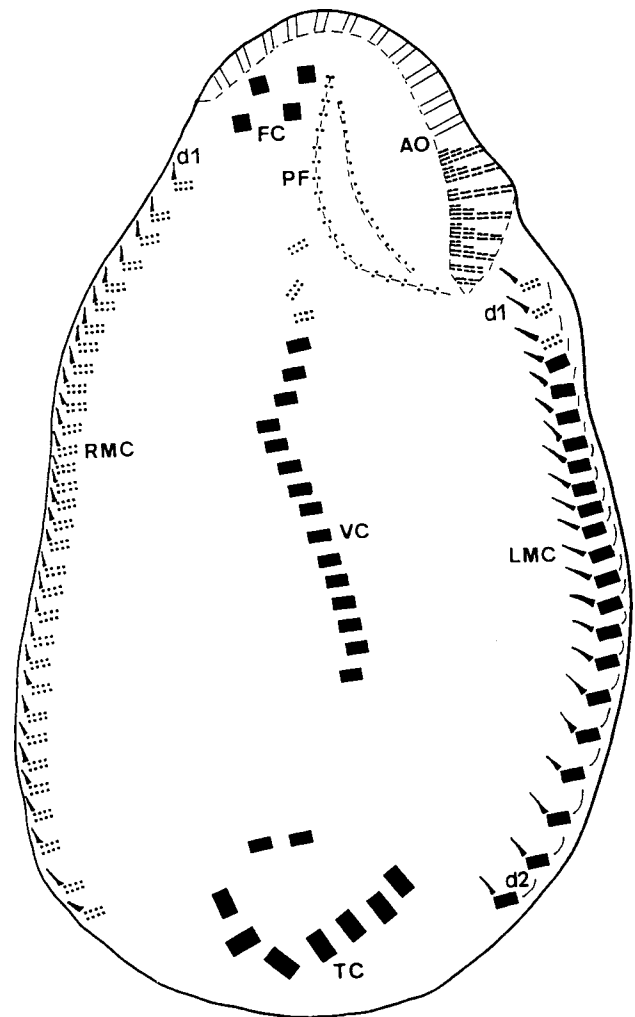
Fig. 1. Infraciliature of *Amphisiella arenicola*. AO - adoral zone of organelles, d1 and d2 - kinetosomal derivatives, FC - frontal cirri, LMC - left marginal cirri, PF - paroral formation, RMC - right marginal cirri, TC - transverse cirri, VC - ventral cirri

Fig. 2. Infraciliature of *Amphisiella ovalis*. AO - adoral zone of organelles, d1 and d2 - kinetosomal derivatives, FC - frontal cirri, LMC - left marginal cirri, PF - paroral formation, RMC - right marginal cirri, TC - transverse cirri, VC - ventral cirri

kinetosomes each (the next to last organelle with 2 rows of 4 kinetosomes, and the last 2 rows of 2 kinetosomes each). PF composed of two components: (a) "paroral formation 1", nearest the adoral zone of organelles, is a stichomonad of 60-68 kinetosomes; (b) "paroral formation 2", beside the frontal cirri, formed of 50-52 pairs of kinetosomes (diplostichomonad). Four anterior frontal cirri and three posterior or frontoterminal cirri. 50-54 rigth marginal cirri. 48-50 left marginal cirri. 52-56 ventral cirri with two cirri located directly above the transverse cirri. 5-6 transverse cirri. Two oval macronuclei in the middle region of the body (19.5-31.5 μm long, 9-13.2 μm width each). Beside each macronucleus is an oval micronucleus (6.75-7.5 μm long). Dorsal surface with 5-6 kineties.

Type location: Gandía Beach (38°01' N; 0°10' E) (Mediterranean Sea, Spain)

Type specimens: permanent slides staining with silver carbonate technique (ref. n. 1663a-f) (Departamento Biologia Animal I, Facultad de Biologia, Universidad Complutense, Madrid)

Diagnosis of *Amphisiella ovalis* Fernandez-Leborans & Novillo sp. n. (Fig. 2)

Ciliates oval in shape, with posterior end rounded and anterior end slightly pointed (49.5-63 µm long, 27-46.5 µm width). Oral ciliature on the left anterior part of the ventral surface with and adoral zone of organelles (AO) and a paroral formation (PF). AO composed of 16-19 organelles. On middle region of this zone each organelle has four rows of kinetosomes (a short row of 3-4 kinetosomes, an intermediate row of 9-10 kinetosomes, and two rows of 15-16 kinetosomes each. Posterior end of AO with an organelle with two rows of 6-8 kinetosomes each. PF consists of a shorter internal part (a stichomonad with 22-25 kinetosomes), and of a longer external part (a diplostichomonad with 24-26 pairs of kinetosomes). Four frontal cirri; 30-32 right marginal cirri; 20-24 left marginal cirri; 18-22 ventral cirri with two cirri located directly above the transverse cirri; 6-7 transverse cirri; 32-45 macronuclear nodes (4.2-9 µm long, 2.1-4.4 µm width each); 2-4 micronuclei (1.56-2.04 µm long each). On the dorsal side there are four kineties.

Type location: Castro Urdiales Beach (43°22' N; 0°28' W) (Cantabrian Sea, Spain)

Type specimens: permanent slides staining with silver carbonate technique (ref. n. 1665a-l) (Departamento Biologia Animal I, Facultad de Biologia, Universidad Complutense, Madrid)

REFERENCES

Fernandez-Leborans G., Novillo A. (1992) Morphology and taxonomy of two new species of marine ciliates (Ciliophora: Spirotrichea: Stichotrichida: Amphisiellidae). *Proc. Biol. Soc. Wash.* **105:** 165-179

ACTA
PROTOZOOLOGICA

Evidence for Bacteriophages within Gram-negative Cocci - Obligate Endoparasitic Bacteria of *Naegleria* sp.

Rolf MICHEL[1], Ernst N. SCHMID[2], Gerhild GMEINER[1], Karl-Dieter MÜLLER[2] and Bärbel HAURÖDER[1]

[1]Central Institute of the Federal Armed Forces Medical Services, Koblenz; [2]Institut für Medizinische Mikrobiologie der Universität Essen, Essen, Germany

Summary. Gram-negative cocci observed as endocytobionts within the cytoplasm of a *Naegleria* strain isolated from a garden pond harboured small hexagonal particles of about 70 nm identified as bacteriophages called "Neo-Ph/2". These phages resembled the recently described phages; strain "Neo-Ph/1" observed for the first time within the *Chlamydia*-like endocytobiont *Neochlamydia hartmannellae* (Parachlamydiaceae) multiplying within *Hartmannella vermiformis* (Schmid *et al.* 2001). The possible reasons for this obvious similarity are object for discussion in this article.

Key words: amoeboflagellate, bacteriophages, endocytobionts, Gram-negative cocci, *Naegleria* sp., phage-heads, ultrastructure.

INTRODUCTION

Among other species of free-living amoebae (FLA) acanthamoebae, naegleriae and hartmannellae are well known as possible hosts of pathogenic and harmless bacteria multiplying unaffected inside their host cells with the result that the host-amoebae are prone to be disintegrated and finally die releasing great numbers of the intracellular bacteria. Consequently the FLA contribute to the dispersal of bacteria as *Legionella pneumophila, Listeria* or *Chlamydia*-like bacteria de-

scribed recently (Rowbotham 1980, 1987; Michel *et al.* 1994; Amann *et al.* 1997; Horn *et al.* 2000)

An endoparasitic *Chlamydia*-like bacterium of *Hartmannella vermiformis* described only very recently as *Neochlamydia hartmannellae* (Horn *et al.* 2000) attracted the attention of one of us (E.N.S.) because some elementary bodies within the cytoplasm of their host appeared significantly enlarged compared to normal elementary bodies. They contained small polygonal particles of 60-70 nm identified as bacteriophages "Neo-Ph/1" described for the first time to occur within endocytobionts of FLA (Schmid *et al.* 2001).

Similar particles have now been detected within Gram-negative cocci parasitizing *Naegleria* sp. (N-DMLG), isolated from a small garden pond. Unusu-

Address for correspondence: Rolf Michel, Central Institute of the Federal Armed Forces Medical Services, P.O. Box. 7340, D-56065 Koblenz, Germany; Fax: +49 261/ 896 1306; E-mail: rmichel@amoeba-net.de
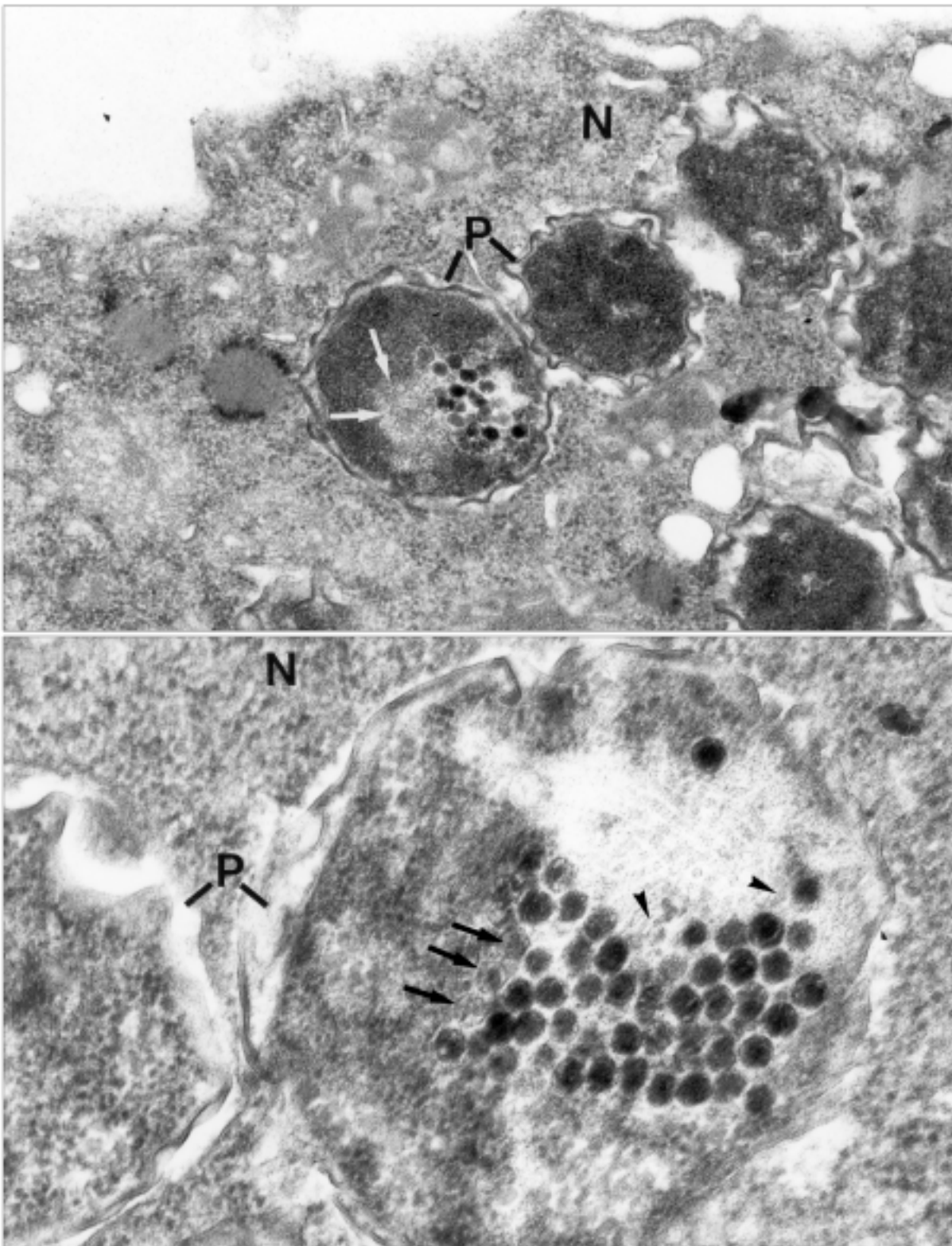
Fig.1. A number of endocytobionts (P) can be observed randomly distributed within the cytoplasm of the host amoeba *Naegleria* sp. strain N-DMLG (N). One organism appears slightly enlarged harbouring phage particles. Arrows - electron lucent DNA region with faint empty phage envelopes; x 43500

Fig. 2. Higher magnification of an infected endoparasitic bacterium (P) showing details of numerous particles within the bacterial cell: N - cytoplasm of the hostamoeba *Naegleria* sp. Arrows - partly filled or empty phages; arrowheads - faintly visible phage-tails; x 85000

ally the infected trophozoites of this *Naegleria* species harboured at least two different populations of endocytobiotic bacteria - one within the nucleus the other one within the cytoplasm (Michel *et al.* 1999).

Only the latter population produced and released particles resembling those described recently from *Neochlamydia hartmannellae* and are subject to the present description.

MATERIALS AND METHODS

The *Naegleria* strain (N-DMLG) serving as natural host strain of obligate intracellular Gram-negative cocci was isolated from a small private garden pond with ornamental fishes, on non-nutrient-agar according to Page (1988). Parasite population were harvested from 3-5 days-old axenic SCGYE-cultures and after centrifugation at 1800 rpm they were fixed in cacodylate buffered 3% glutaraldehyde. After a period of 1 h at 4°C they were centrifuged at 2500 rpm and resuspended in 0.1 M cacodylate buffer, postfixed in 1 % OsO$_4$, and stained with uranyl acetate and lead citrate. Subsequently, specimens were dehydrated in alcohol and embedded in epoxy resin according to Spurr. Ultrathin sections were examined in a LEO 910 transmission electron microscope (Leo, Oberkochen).

RESULTS

The infected *Naegleria* strain could be detected by its inability to form cysts and led therefore to the assumption of being infected by some kind of endoparasitic bacteria. Investigation of this particular strain by light and electron microscopy revealed a simultaneous infection of the amoeba by at least two different populations of Gram-negative cocci: an intracytoplasmic population and another one multiplying exclusively within the nucleus of its host (Michel *et al.* 1999). Already during the first inspection of electron micrographs of these host-parasite relations some of the intracellular bacteria appeared enlarged markedly and somehow damaged.

We first supposed that some of the endocytobionts might be susceptible to digestion by the host and show therefore signs of disintegration. But in the course of extended search and inspection of those stages we discovered that these changes were the result of infection with virus-like particles we are going to describe herewith.

At lower magnification (Fig.1) one of the endocytobiotic cells shows a few particles of attracting density with adjacent structures of less density in an irregular arrangement. Within the electron lucent DNA-region (arrows) the nucleoid of the prokaryotic

endocytobiont, faint empty phage envelops are discernible. In Fig. 2 a more paracrystalline array of particles is found, which is typical for the arrangement of bacteriophages. Within this cell, most of the phage-heads are filled, only a few phages (arrows) are filled partly or are empty. The dimension of the hexagonal mature phage-heads is about 70 nm. Phage-tails are hardly discernible (arrowheads).

Novel phage strain was named "Neo-Ph2", as it resembles the phages "Neo-Ph1" from *Neochlamydia*.

DISCUSSION

The bacteriophages described so far were found only in the cytoplasmic fraction of the endoparasites - never within the more polymorphic stages replicating inside the nucleus attached to the surface of the karyosome.

They have not been found within the similar endocytobionts KNic described previously within another isolate of *Naegleria* sp. from an aquarium (Michel *et al.* 2000). The size of the heads is comparable to the phage described recently from *Neochlamydia hartmannellae* (Schmid *et al.* 2001).

Although few striated filaments of about 60 nm are discernible in less electron dense parts of the phage-producing endocytobionts, further studies with negatively stained phages are necessary to allow distinct descriptions of existing flexible, rigid or contractible phage-tails.

With respect to the dimension of the phage-heads the great similarity between the phage Neo-Ph/1 from *Neochlamydia* and the phage Neo-Ph/2 from cocci multiplying within *Naegleria* is remarkable, because they have been found within taxonomically nearly unrelated species of endocytobiotic bacteria. On the other hand these dimensions are in the range of a great number of phages and only additional information of tail structures and DNA composition will allow a more profound comparison.

One aim of future investigations will be the test of infectivity of these particles for nearly related species of bacteria - and attempts to identify the two phage types by DNA sequencing methods.

What had been stressed in our recent article about the meaning of the finding of a phage within *Neochlamydia* for the host-parasite relation is also due for the present case: the phages are expected to exert multiple influences on the host-parasite interaction - all the more as

the infected host bacterium is condemned to disintegration and death so it now might be easily digested by the host amoeba.

REFERENCES

Amann R., Springer N., Schönhuber W., Ludwig W., Schmid E. N., Müller K.-D., Michel R. (1997) Obligate intracellular bacterial parasites of *Acanthamoeba* related to *Chlamydia* spp. *Appl. Environ. Microbiol.* **63:** 115-121

Horn M., Wagner M., Müller K.-D., Fritsche T. R., Schleifer K.-H., Michel R. (2000) *Neochlamydia hartmannellae* gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*. *Microbiology* **146:** 1231-1239

Michel R., Hauröder-Philippczyk B., Müller K.-D., Weishaar I. (1994) *Acanthamoeba* from human nasal mucosa infected with an obligate intracellular parasite. *Europ. J. Protistol.* **30:** 104-110

Michel R., Hauröder B., Müller K.-D., Zöller L. (1999) An environmental *Naegleria*-strain - unable to form cysts - turned out to harbour two different species of endocytobionts. *Int. J. Endocytob. Cell Res.* **13:** 115-118

Michel R., Müller K.-D., Hauröder B., Zöller L. (2000) A coccoid bacterial parasite of *Naegleria* sp. (Schizopyrenida, Vahlkampfiidae) inhibits cyst formation of its host but not transformation to the flagellate stage. *Acta Protozool.* **39:** 199 - 207

Page F. C. (1988) A New Key to Freshwater and Soil Gymnamoebae. Freshwater Biological Association, The Ferry House, Ambleside, Cumbria

Rowbotham T.J. (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* **33:** 1179-1183

Rowbotham T. J. (1987) Current views on the relationships between amoebae, legionellae and man. *Israel J. Med. Sci.* **22:** 678-689

Schmid E. N., Müller K.-D., Michel R. (2001) Evidence for bacteriophages within *Neochlamydia hartmannellae*, an obligate endoparasitic bacterium of the free-living amoeba *Hartmannella vermiformis*. *Int. J. Endocytob. Cell Res.* **14:** 115-119

ACTA
PROTOZOOLOGICA

Book Review

Berger H. (2001): Catalogue of ciliate names 1. Hypotrichs. Verlag Helmut Berger, Salzburg, i-viii and 206 pages; ISBN 3-902147-00-8.

For details on ordering, see "Publications" in http://www.user.xpoint.at/berger.tb/default.html; or contact author by E-mail: berger.helmut@protozoology.com

Catalogues of names exist in all well-studied groups of organisms and are an important tool in taxonomic work. The catalogue of hypotrich names by Helmut Berger is very good news for ciliatologists. Although such monographs are urgently needed for all ciliates, personally I am glad that this particular group is the first one to get it.

Hypotrichs are widespread in marine, fresh-water and soil habitats. Since most are morphologically adapted for "walking" on surfaces, they are associated primarily with benthic (or aufwuchs) habitats and very few are planktonic. Nevertheless, some of the "benthic" forms have been found on marine snow particles several kilometres above the sea floor. Hypotrichs are certainly the most fascinating group among ciliates. The unusual complexity of their cortex, complex morphogenetic patterns, unique behaviour (e.g., "mating dance") and very peculiar genome organisation make these organisms interesting to many biologists. The present catalogue is an important advance in their taxonomy.

The main part of the book is a list of about 1 500 generic and species names. All the names are arranged alphabetically in a very practical way. Each record contains the following information: (i) the basionym, i.e., the name used in the original description; (ii) the author name(s), (iii) the year of publication; (iv) the page of the original description; (v) the category of the taxon; (vi) in genera and subgenera, the type designation and the type species. Under "Remarks" the reader can find important additional information, e.g., references to monographs. A list of "combinations" is also provided.

Almost 130 suprageneric names are contained in a separate chapter. Here, each group originally established as a new taxon is recognisable by an appropriate indication, name-bearing types are mentioned, and the "Remarks" point to the major revisions.

The attached bibliography consists of about 500 references dating from the eighteenth century up to the year 2001. A note preceding the bibliography states that all these references, plus about 4 500 other references concerning hypotrichs (including keywords), can be obtained from Helmut Berger as a database. The book ends with an index containing all the names mentioned in the catalogue. Species can be found there by both generic and species name, and the location of the basionym is indicated with bold print.

The book is carefully prepared and nicely edited in a convenient small format. Obviously it will be an indispensable reference tool for taxonomists describing new species or preparing revisions, and anyone looking for taxonomic references or just trying to identify hypotrich species will find this small book very helpful since the basic information is still scattered among a multitude of separate papers.

Krzysztof Wiąckowski, Kraków, Poland

INSTRUCTIONS FOR AUTHORS

Acta Protozoologica is a quarterly journal that publishes current and comprehensive, experimental, and theoretical contributions across the breadth of protistology, and cell biology of lower Eukaryote including: behaviour, biochemistry and molecular biology, development, ecology, genetics, parasitology, physiology, photobiology, systematics and phylogeny, and ultrastructure. It publishes original research reports, critical reviews of current research written by invited experts in the field, short communications, book reviews, and letters to the Editor. Faunistic notices of local character, minor descriptions, or descriptions of taxa not based on new, (original) data, and purely clinical reports, fall outside the remit of *Acta Protozoologica*.

Contributions should be written in grammatically correct English. Either British or American spelling is permitted, but one must be used consistently within a manuscript. Authors are advised to follow styles outlined in The CBE Manual for Authors, Editors, and Publishers (6th Ed., Cambridge University Press). Poorly written manuscripts will be returned to authors without further consideration.

Research, performed by "authors whose papers have been accepted to be published in *Acta Protozoologica* using mammals, shall have been conducted in accordance with accepted ethical practice, and shall have been approved by the pertinent institutional and/or governmental oversight group(s)"; this is Journal policy, authors must certify in writing that their research conforms to this policy.

Nomenclature of genera and species names must agree with the International Code of Zoological Nomenclature (ICZN), International Trust for Zoological Nomenclature, London, 1999; or the International Code of Botanical Nomenclature, adopted by XIV International Botanical Congress, Berlin, 1987. Biochemical nomenclature should agree with "Biochemical Nomenclature and Related Documents" (A Compendium, 2nd edition, 1992), International Union of Biochemistry and Molecular Biology, published by Portland Press, London and Chapel Hill, UK.

Except for cases where tradition dictates, SI units are to be used. New nucleic acid or amino acid sequences will be published only if they are also deposited with an appropriate data bank (e.g. EMBL, GeneBank, DDBJ).

All manuscripts that conform to the Instructions for Authors will be fully peer-reviewed by members of Editorial Board and expert reviewers. The Author will be requested to return a revised version of the reviewed manuscript within four (4) months of receiving the reviews. If a revised manuscript is received later, it will be considered to be a new submission. There are no page charges, but Authors must cover the reproduction cost of colour illustrations.

The Author(s) of a manuscript, accepted for publication, must transfer copyrights to the publisher. Copyrights include mechanical, electronic, and visual reproduction and distribution. Use of previously published figures, tables, or brief quotations requires the appropriate copyright holder's permission, at the time of manuscript submission; acknowledgement of the contribution must also be included in the manuscript. Submission of a manuscript to *Acta Protozoologica* implies that the contents are original, have not been published previously, and are not under consideration or accepted for publication elsewhere.

SUBMISSION

Authors should submit manuscript to: Dr Jerzy Sikora, Nencki Institute of Experimental Biology, ul. Pasteura 3, 02-093 Warszawa, Poland, Fax: (4822) 8225342; E-mail: jurek@nencki.gov.pl or j.sikora@nencki.gov.pl.

At the time of submission, authors are encouraged to provide names, E-mails, and postal addresses of four persons who might act as reviewers. Extensive information on *Acta Protozoologica* is available at the website: **http://www.nencki.gov.pl/ap.htm**; however, please do not hesitate to contact the Editor.

Hard copy submission: Please submit three (3) high quality sets of text and illustrations (figures, line drawing, and photograph). When photographs are submitted, arranged these in the form of plate. A copy of the text on a disk or CD should also be enclosed, in PC formats, preferably Word for Windows version 6.0 or higher (IBM, IBM compatible, or MacIntosh). If they do not adhere to the standards of the journal the manuscript will be returned to the corresponding author without further consideration.

E-mail submission: Electronic submission of manuscripts by e-mail is acceptable in PDF format only. Illustrations must be prepared according to journal requirement and saved in PDF format. The accepted manuscript should be submitted as a hard copy with illustrations (two copies, one with lettering + one copy without lettering) in accordance with the standards of the journal.

ORGANIZATION OF MANUSCRIPTS

Text: Manuscripts must be typewritten, double-spaced, with numbered pages (12 pt, Times Roman). The manuscript should be organized into the following sections: Title, Summary, Key words, Abbreviations, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, and Figure legends. Figure legends must contain explanations of all symbols and abbreviations used. The Title Page should include the title of the manuscript, first name(s) in full and surname(s) of author(s), the institutional address(es) where the work was carried out, and page heading of up to 40 characters (including spaces). The postal address for correspondence, Fax and E-mail should also be given. Footnotes should be avoided.

Citations in the text should be ordered by name and date but not by number, e.g. (Foissner and Korganova 2000). In the case of more than two authors, the name of the first author and *et al.* should be used, e.g. (Botes *et al.* 2001). Different articles by the same author(s) published in the same year must be marked by the letters a, b, c, etc. (Kpatcha *et al.* 1996a, b). Multiple citations presented in the text must be arranged by date, e.g. (Small 1967, Didier and Detcheva 1974, Jones 1974). If one author is cited more than once, semicolons should separate the other citations, e.g. (Lousier and Parkinson 1984; Foissner 1987, 1991, 1994; Darbyshire *et al.* 1989).

Please observe the following instructions when preparing the electronic copy: (1) label the disk with your name; (2) ensure that the written text is identical to the electronic copy; (3) arrange the text as a single file; do not split it into smaller files; (4) arrange illustrations as separate files; do not use Word files; *.TIF, *.PSD, or *.CDR graphic formats are accepted; (5) when necessary, use only italic, bold, subscript, and superscript formats; do not use other electronic formatting facilities such as multiple font styles, ruler changes, or graphics inserted into the text; (6) do not right-justify the text or use of the hyphen function at the end of lines; (7) avoid the use of footnotes; (8) distinguish the numbers 0 and 1 from the letters O and l; (9) avoid repetition of illustrations and data in the text and tables.

References: References must be listed alphabetically. Examples for bibliographic arrangement:

Journals: Flint J. A., Dobson P. J., Robinson B. S. (2003) Genetic analysis of forty isolates of *Acanthamoeba* group III by multilocus isoenzyme electrophoresis. *Acta Protozool.* **42**: 317-324

Books: Swofford D. L. (1998) PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods). Ver. 4.0b3. Sinauer Associates, Sunderland, MA

Articles from books: Neto E. D., Steindel M., Passos L. K. F. (1993) The use of RAPD's for the study of the genetic diversity of *Schistosoma mansoni* and *Trypanosoma cruzi*. In: DNA Fingerprinting: State of Science, (Eds. S. D. J. Pena, R. Chakraborty, J. T. Epplen, A. J. Jeffreys). Birkhäuser-Verlag, Basel, 339-345

Illustrations and tables: After acceptance of the paper, drawings and photographs (two copies one with lettering + one copy without) must be submitted. Each table and figure must be on a separate page. Figure legends must be placed, in order, at the end of the manuscript, before the figures. Figure legends must contain explanations of all symbols and abbreviations used. All line drawings and photographs must be labelled, with the first Author's name written on the back. The figures should be numbered in the text using Arabic numerals (e.g. Fig. 1).

Illustrations must fit within either a single column width (86 mm) or the full-page width (177 mm); the maximum length of figures is 231 mm, including the legend. Figures grouped as plates must be mounted on a firm board, trimmed at right angles, accurately mounted, and have edges touching. The engraver will then cut a fine line of separation between figures.

Line drawings should be suitable for reproduction, with well-defined lines and a white background. Avoid fine stippling or shading. Prints are accepted only in *.TIF, *.PSD, and *.CDR graphic formats (Grayscale and Colour - 600 dpi, Art line - 1200 dpi) on CD. Do not use Microsoft Word for figure formatting.

Photographs should be sharp, glossy finish, bromide prints. Magnification should be indicated by a scale bar where appropriate. Pictures of gels should have a lane width of no more than 5 mm, and should preferably fit into a single column.

PROOF SHEETS AND OFFPRINTS

After a manuscript has been accepted, Authors will receive proofs for correction and will be asked to return these to the Editor within 48-hours. Authors will be expected to check the proofs and are fully responsible for any undetected errors. Only spelling errors and small mistakes will be corrected. Twenty-five reprints (25) will be furnished free of charge. Additional reprints can be requested when returning the proofs, but there will be a charge for these; orders after this point will not be accepted.

ACTA PROTOZOOLOGICA

ORIGINAL ARTICLES

SHORT COMMUNICATIONS