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# A morphometric and stereological analysis of ultrastructural changes in two *Scenedesmus* (Chlorococcales, Chlorophyta) strains subjected to diesel fuel oil pollution

Z. TUKAJ[1]*, J. BOHDANOWICZ[2] AND A. AKSMANN[1]

[1]*Department of Plant Physiology, University of Gdańsk, Al. Marszałka Piłsudskiego 46, PL-81-378 Gdynia, Poland*
[2]*Department of Genetics and Cytology, University of Gdańsk, Kładki 24, PL-80-822 Gdańsk, Poland*

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Morphometric and stereological analysis was used to study ultrastructural changes induced in two *Scenedesmus* strains by oil pollution. The strains exhibited quite different susceptibilities to aqueous fuel oil extract (AFOE): *Scenedesmus quadricauda* (Turpin) Brébisson G-15 was resistant, but *Scenedesmus microspina* Chodat B2-76 was sensitive to a hydrocarbon concentration of c. 50 ppm. Both strains were grown in a batch culture system for 7 d on AFOE, after which they were analyzed morphometrically. Circumferences of the cells and their components were measured with a digitizer; the mean surface ($\bar{S}$) and the mean number ($\bar{N}$) of cell component profiles and the relative volume ($V_v$) and surface density ($S_v$) of the organelles were then calculated. The results indicate that the $\bar{S}$ of *S. microspina* cells exposed to AFOE was approximately twice that of the control cells, whereas $\bar{S}$ of *S. quadricauda* cells did not change. Moreover, the cell components of *S. quadricauda* underwent far fewer changes than did those of *S. microspina.* AFOE caused the cells of both strains to become vacuolated, but to different extents. The $V_v$ of the chloroplast and the pyrenoid decreased, but $V_v$ of the plastoglobuli within the chloroplast increased. The $V_v$ of *S. microspina* mitochondria increased markedly, whereas in treated *S. quadricauda* cells, the mitochondrial $V_v$ did not change, although their $S_v$ did decrease significantly. The relative volumes of the cells' nuclei increased in *S. quadricauda* but decreased in *S. microspina.* Though the mean volumes and surfaces of the microbodies were larger in both strains, the changes were more clear-cut in *S. quadricauda.*

## INTRODUCTION

In most toxicological studies of algae, population-based parameters such as cell number, specific growth rate, biomass production, or chlorophyll content are measured to assess the effects of contaminants on the growth of algae. Such studies have found differences in the sensitivity of algal growth to oil pollution among congeneric species as well as in strains of the same species (Østgaard *et al.* 1984; Van Baalen & O'Donnell 1984). There is great variability in growth sensitivity within *Scenedesmus* species affected by oil pollution (Tukaj 1994). However, it is difficult to discover the reasons for these differences, especially in closely related organisms growing in a similar environment. One of the explanations most frequently put forward is that strains differ in their ability to metabolize hydrocarbons absorbed by their cells. Some metabolically altered hydrocarbons may become more hydrophilic and attach themselves to proteins and nucleic acids, thereby inhibiting reproduction and growth. However, no data are yet available on the ability of the *Scenedesmus* strain used in the present work to metabolize hydrocarbons.

In ecotoxicological studies, algal growth is usually regarded as a more sensitive parameter than are structural changes. The impact of pollutants on the morphology and fine structure of algae has been touched upon in a number of papers, but it has not been dealt with in detail. A structural study of *Scenedesmus,* in particular, is valuable because the coenobia and cell morphology are highly sensitive to changing growth conditions and have characteristic responses (Trainor 1992). We have found that three *Scenedesmus* species, when treated with oil, exhibit profound morphological changes that correlated with their growth sensitivity (Tukaj & Bohdanowicz 1995a). Furthermore, sensitivity of these strains correlated well with cell wall structure (Tukaj & Bohdanowicz 1995b), suggesting a role of the cell wall in protecting cells against hydrocarbons.

As a first step toward documenting the physiological effects of heavy metals (Visviki & Rachlin 1994), morphometry was used to evaluate the quantitative changes in cell structure affected by such metals. In a similar way, we compare the quantitative changes in the fine structure of two *Scenedesmus* strains using morphometric and stereological methods. These methods make identification of changes easier and allow them to be correlated with physiological parameters. This quantitative approach to ultrastructural changes could help to explain why crude oil hydrocarbons are toxic to some algae, whereas others are able to survive in an oil-polluted environment.

## MATERIALS AND METHODS

### Organisms and cultures

Two *Scenedesmus* strains were used in the experiments: *Scenedesmus microspina* Chodat B2-76, isolated from the Baltic Sea at the Institute of Oceanology, Polish Academy of Sciences, Sopot (Gędziorowska 1983), and *Scenedesmus quadricauda* (Turpin) Brébisson G-15, obtained from the Col-

* E-mail: biozt@univ.gda.pl

lection of the Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň.

The axenic algal strains were maintained on agar (2%) slants containing 1% bactopeptone and 2% glucose. The algae were transferred aseptically from the slants into Bold basal medium (BBM) (Nichols & Bold 1965) so that they could adapt to the liquid medium and the conditions of the main experiments. After 5–7 d, the cultures reached the exponential growth phase and were used to initiate batch cultures. Both strains were grown in 100-ml E-flasks, each containing 50 ml of suspension. The flasks, stoppered with bacteriological plugs, were shaken manually twice a day. The initial density of the cultures was $1 \times 10^4$ cells $ml^{-1}$. All cultures were maintained at a temperature of 22–24°C, a light intensity of a 50 μmol photons $m^{-2}$ $s^{-1}$, and a 14 : 10 h light–dark photoperiod.

The culture densities were determined in a Fuchs-Rosenthal hemocytometer. The division rate (k) was calculated as $k = (\ln N_7 - \ln N_0)(1.443 \times t^{-1})$, where $N_0$ and $N_7$ are the respective cell numbers on the day of inoculation and on day 7.

### Preparation of the aqueous fuel oil extract (AFOE)

Number 2 diesel fuel oil (Polish Norm: PN-67, C-96048) obtained from the Gdańsk Refinery was used. The oil sample (50 ml) was stirred for 24 h with the culture medium (1 l). The extraction was then carried out at room temperature in a vessel tightly stoppered with a Teflon plug, after which the mixture was transferred to a separating funnel for 1 h. The lower aqueous phase (AFOE), containing dissolved hydrocarbons and a small amount of dispersed oil at a concentration of about 50 ppm, was used in the experiments. The total hydrocarbon content in the AFOE (carbon-tetrachloride-extractable oil) was determined by infrared (IR) spectroscopy. The final hydrocarbon concentration in the culture medium was c. 50 ppm (n = 5).

### Electron microscopy and morphometry

The 7-d cultures were harvested by centrifugation and rinsed several times with BBM medium. Cells from each of the samples were fixed in 2% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.0) for 24 h at room temperature. The material was postfixed for 1.5 h in 1% $OsO_4$ in the same buffer. The samples were subsequently centrifuged, dehydrated in acetone series, and embedded in low-viscosity resin (Spurr 1969). The ultrathin sections were picked up on grids and stained with uranyl acetate and lead citrate, after which they were examined by transmission electron microscopy using a BS 500 (Tesla) electron microscope. For morphometric analysis, random series of sections were photographed. For *S. quadricauda,* 42 negatives of control cells (×6000) and 47 negatives of treated cells [27 negatives (×6000) and 20 negatives (×10,000)] were analyzed, and for *S. microspina* 30 negatives of control cells (×18,000) and 58 negatives of treated cells (×10,000) were analyzed.

Circumferences and surfaces of the cells and their components were then measured with a digitizer linked to a computer (Weibel & Bolender 1973). The following parameters were measured in the morphological analysis (Weibel & Bolender 1973): (1) the surface of profiles and whole cells S (μm²) and the mean surface of the structure per cell $\bar{S}$ (μm²); (2) the mean number of organelles (or mean number of structure profiles) per cell $\bar{N}$; (3) the relative volume of the structure $V_v$ (%) as the volume of the component relative to the cell volume (e.g. the mitochondrial volume contained per unit volume of the cell); and (4) the surface density of the structure $S_v$ ($\mu m^{-1}$) expressed as the surface of the structure per unit volume (e.g. the area of the vacuole contained per unit volume of the protoplast).

The analysis of variance was calculated using Snedecor's F distribution. The mean surface of the cells were compared by means of Aspin and Welch's test or Student's t-test (Czermiński *et al.* 1990). The mean numbers of profiles were compared by Student's t-test. The standard deviation of the mean was calculated for each value.

**Table 1.** Growth of three species of *Scenedesmus* cultured for 7 d with and without AFOE. Initial cell density $n = 1 \times 10^4$ cells $ml^{-1}$.

| | n (×10⁴) | | k (doublings $d^{-1}$) | |
|---|---|---|---|---|
| | C | AFOE | C | AFOE |
| *S. microspina* | 46.6 (2.0) | 6.9 (0.4) | 0.79 | 0.39 |
| *S. armatus* | 95.7 (5.8) | 57.1 (2.6) | 0.94 | 0.83 |
| *S. quadricauda* | 28.0 (2.0) | 24.8 (1.9) | 0.69 | 0.66 |

[1] C, control; n, cell number; k, division rate. The standard error of the mean is given in parentheses.

## RESULTS

The growth data for both strains cultured for 7 d in BBM medium with and without AFOE are given in Table 1. After AFOE treatment, the division rate of *S. quadricauda* fell only slightly, but in *S. microspina* it decreased dramatically. Growth inhibition led to a reduction in population density, which was reduced by c. 10% (*S. quadricauda*) and 85% (*S. microspina*) compared with the control. This is indicative of a great difference in sensitivity of the two organisms to AFOE: the latter appears to be sensitive, whereas the former is resistant. For comparison with the strains studied here, growth data of the moderately AFOE-sensitive *Scenedesmus armatus* Chodat, the structural changes of which were described in detail in Tukaj (1989), are also given.

The mean surface ($\bar{S}$) of *S. microspina* roughly doubled with AFOE treatment, but the $\bar{S}$ of *S. quadricauda* remained unchanged (Table 2). The $\bar{S}$ of the protoplast and the cytoplasm in *S. microspina* also doubled in size, but these structures in *S. quadricauda* were not affected by AFOE treatment. In *S. quadricauda,* the volumes of the protoplast and its components generally increased in proportion to that of the whole cell. In *S. microspina,* however, individual organelles were found to have grown unevenly compared to the volume of the whole cell, whose interior organization had altered. The $V_v$ of the protoplast and cytoplasm of *S. microspina* increased after AFOE treatment, but in *S. quadricauda* these changes were not observed (Fig. 1). The cytoplasm of treated *S. microspina* cells contained large lipid bodies not observed in *S. quadricauda* cells.

In *S. quadricauda* cells exposed to AFOE, $V_v$ of the cell wall did not change and made up c. 10% of the cell volume

**Table 2.** The mean surface ($\bar{S}$, $\mu m^2$) of *Scenedesmus microspina* and *S. quadricauda* cells and their organelles. The growth conditions of algae are given in Table 1.[1]

| | *S. microspina* | | *S. quadricauda* | |
|---|---|---|---|---|
| | C | AFOE | C | AFOE |
| Cell | 8.12 | 17.50** | 21.11 | 20.52 |
| | (0.04) | (0.60) | (1.43) | (1.39) |
| Protoplast | 6.60 | 14.90** | 18.92 | 18.42 |
| | (0.03) | (0.60) | (1.29) | (1.29) |
| Cell wall | 1.53 | 2.50** | 2.19 | 2.10 |
| | (0.13) | (0.08) | (0.19) | (0.19) |
| Cytoplasm | 2.04 | 5.53** | 7.96 | 7.76 |
| | (0.13) | (0.29) | (0.70) | (0.91) |
| Nucleus | 0.93 | 1.44** | 2.25 | 2.44 |
| | (0.08) | (0.10) | (0.25) | (0.25) |
| Chloroplast | 3.24 | 5.42** | 8.23 | 7.79 |
| | (0.16) | (0.27) | (0.72) | (0.75) |
| Pyrenoid | 1.21 | 1.79** | 3.16 | 2.61 |
| | (0.06) | (0.11) | (0.29) | (0.39) |
| Platoglobuli | 0.03 | 0.06* | 0.08 | 0.14** |
| | (0.00) | (0.00) | (0.004) | (0.005) |
| Mitochondrion | 0.21 | 1.18* | 0.26 | 0.18** |
| | (0.02) | (0.10) | (0.004) | (0.005) |
| Vacuoles | 0.42 | 1.86** | 1.55 | 2.15** |
| | (0.07) | (0.16) | (0.17) | (0.14) |
| Microbodies | 0.09 | 0.28** | 0.13 | 0.22* |
| | (0.01) | (0.04) | (0.01) | (0.06) |

[1] The standard error of the mean is given in parentheses.
** 0.01 and * 0.05 significance levels between control and AFOE variables.

(Fig. 1). In treated *S. microspina* cells, $V_v$ dropped by only 4.5%, despite the fact that the mean cell wall surface of *S. microspina* became significantly larger than in the control (Table 2).

The $V_v$ of the cell nucleus in *S. quadricauda* was similar in the control and treated cells and made up c. 11% of the cell volume (Fig. 1). However, AFOE caused the $V_v$ of the nucleus in *S. microspina* to decrease twofold. This implies that the reproductive processes of the strain were strongly inhibited by AFOE: the absolute volume of the nucleus did not change, even though the cell grew to double its original size.

The chloroplast is the largest organelle in both *Scenedesmus* cells and occupies about 40% of their volume. The $V_v$ of the chloroplast in AFOE-treated *S. microspina* decreased by approximately 10% compared to the control, whereas in *S. quadricauda,* $V_v$ of the chloroplast did not change significantly (Fig. 1). About a third of the chloroplast volume is pyrenoid. In AFOE-treated *S. quadricauda* cells, the relative volume of the pyrenoids in relation to the whole cell ($V_{v(py/cell)}$) and to the chloroplast ($V_{v(py/chl)}$) fell only slightly compared with control cells (Fig. 1). The same parameters decreased significantly in *S. microspina* cells after AFOE exposure—$V_{v(py/cell)}$ by 7% and $V_{v(py/chl)}$ by 14%. The $V_v$ for *S. quadricauda* plastoglobuli is 0.39% per cell (Fig. 1) and 1.01% per plastid (Fig. 1, inset), and in AFOE-treated cells both volumes increased. By contrast, the mean number of plastoglobuli per control cell of the strain (1.33) was very much reduced in AFOE-treated cells—to 0.14 (Fig. 3). In *S. microspina* the relative volume of plastoglobuli ($V_{v(pl/cell)}$ and $V_{v(pl/chl)}$) was nearly three times larger in the treated cells (Fig. 1). However, the mean number of plastoglobuli increased significantly in treated *S. microspina* cells (4.69) compared with the control (0.53).

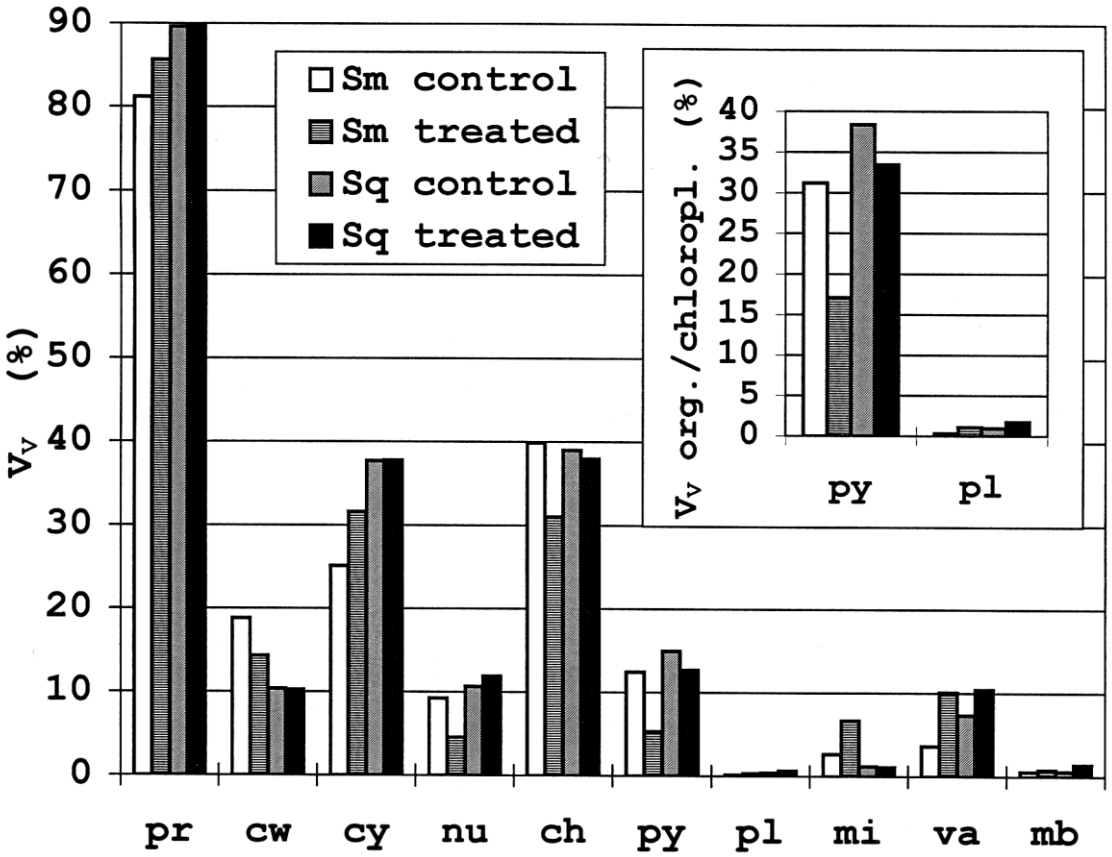
In general, the $\bar{N}$ of mitochondrial profiles remained unchanged in both strains following AFOE treatment, c. 6 $cell^{-1}$ in *S. microspina* and c. 5 $cell^{-1}$ in *S. quadricauda* (Fig. 3). In *S. quadricauda,* relative mitochondrial volumes in the control and treated cells were similar and made up c. 1% of the cell volume (Fig. 1), whereas the $S_v$ of the structure decreased in the treated cells to 19 $\mu m^{-1}$ compared with the control (25 $\mu m^{-1}$) (Fig. 2). In AFOE-treated *S. microspina* cells, the $V_v$ of the mitochondria more than doubled, but their $S_v$ did not change, remaining very low (0.8 $\mu m^{-1}$) compared with that of *S. quadricauda.*

Vacuolation in AFOE-treated cells was more extensive in *S. microspina* than in *S. quadricauda.* In *S. microspina* cells, the average number of vacuoles rose from 1.4 in the control to 9.3 in AFOE-treated cells (Fig. 3). The same trend was evident in changes in the $V_v$ of the vacuoles, which increased from 3.6% in the control to 10.1% in treated cells in *S. microspina* and from 7.4% to 10.4%, respectively, in *S. quadricauda* (Fig. 1). The differences in the surface density of the vacuoles between the two strains are considerable. Moreover, the $S_v$ of *S. quadricauda* vacuoles was the same in exposed and control cells (c. 21.9 $\mu m^{-1}$), whereas in *S. microspina* this parameter ranged from 0.44 $\mu m^{-1}$ in the control cells to 1.2 $\mu m^{-1}$ in the treated cells (Fig. 2).

The $V_v$ of the cell microbodies in both organisms exposed to AFOE were larger than control cells (0.61% and 0.82% in *S. microspina*; 0.63% and 1.4% in *S. quadricauda,* control and treated cells, respectively) (Fig. 1). However, the $S_v$ of these microbodies did not change significantly after AFOE treatment in *S. microspina,* but decreased c. twofold in *S. quadricauda* (Fig. 2). The mean number of microbodies in *S. quadricauda* was smaller in the treated cells than in the controls (0.39 and 0.57 per cell respectively); in *S. microspina,* the $\bar{N}$ of microbodies in the treated and control cells were the same (c. 0.7 $cell^{-1}$) (Fig. 3).

## DISCUSSION

The sensitivity of *Scenedesmus* to AFOE may be linked to mechanisms such as the absorption of hydrocarbons within the cell wall, uptake into the cell and deposition in organelles, and the metabolic transformation of the absorbed hydrocarbons. Among these mechanisms, the differential uptake of hydrocarbons due to differences in cell morphology and cell wall structure was put forward to explain the different sensitivities of the two strains to AFOE. Under our culture conditions, *S. quadricauda* cells exposed for 7 d to AFOE formed mainly four-celled coenobia (95%), which were additionally enclosed in a trilaminar sheet (TLS) structure; *S. microspina,* by contrast, lacked this protective epistructure and the cells were solitary (8%) or were two-celled (39%) or four-celled (53%) coenobia (Tukaj & Bohdanowicz 1995a, 1995b). On the other hand, the morphometric data obtained here indicated that the relative cell wall volumes of treated cells did not change in *S. quadricauda*; in *S. microspina* these volumes were only 4.5% less compared with the walls of the control cells. Previously, the walls of AFOE-treated *S. armatus* cells were shown to become thicker (Tukaj 1989). The inner cellulose wall appeared to have thickened, but only in comparison with the control. The *Scenedesmus* cell wall thus remains quite un-




Abbreviations in Figs 1–3. ch, chloroplast; cw, cell wall; cy, cytoplasm; mb, microbodies; mi, mitochondrion; nu, nucleus; pl, plastoglobuli; pr, protoplast; py, pyrenoid; va, vacuoles.

**Fig. 1.** The relative volume ($V_v$) of *Scenedesmus microspina* (Sm) and *S. quadricauda* (Sq) cells occupied by the particular structures. The algae were grown for 7 d with (treated) and without AFOE (control). The relative volume of the pyrenoid and plastoglobuli in relation to the chloroplast is shown in the inset.

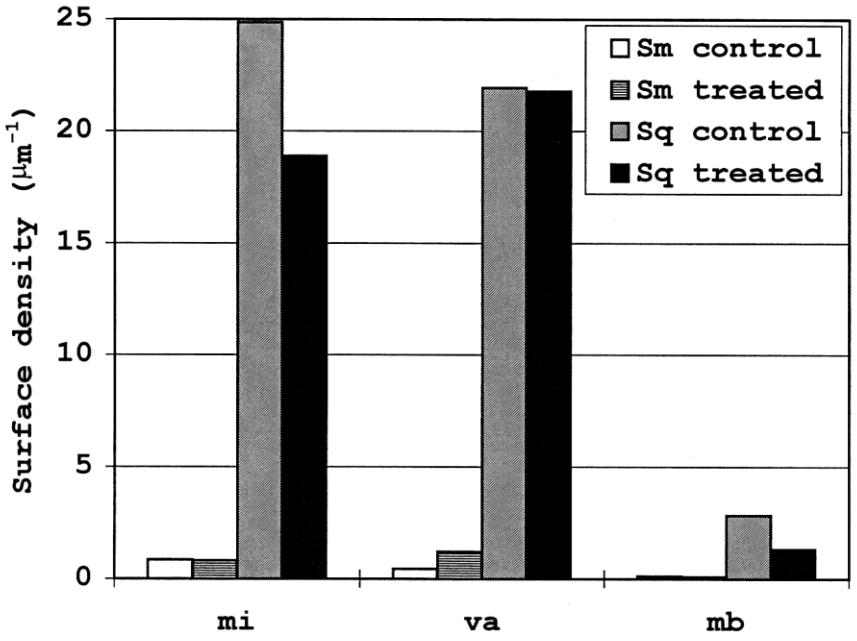



**Fig. 2.** The surface density ($S_v$) of *Scenedesmus microspina* (Sm) and *S. quadricauda* (Sq) organelles grown for 7 d with (treated) and without AFOE (control).

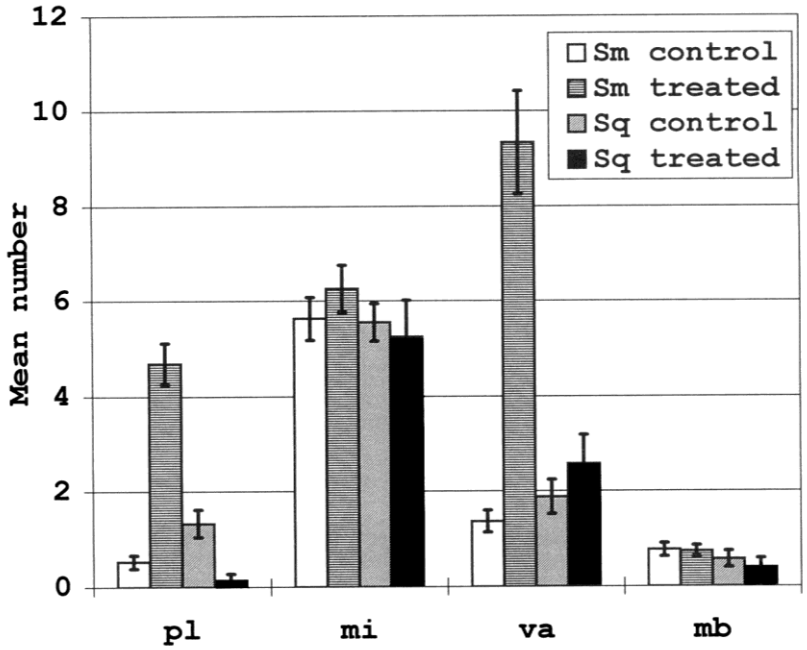



**Fig. 3.** The mean number ($\bar{N}$) of profiles of *Scenedesmus microspina* (Sm) and *S. quadricauda* (Sq) organelles grown for 7 d with (treated) and without AFOE (control). The vertical bars indicate the standard deviation of the mean.

affected, although as reported by Burczyk *et al.* (1994), low molecular weight hydrocarbons did inhibit growth and stimulate the accumulation of cell wall carotenoids in *Scenedesmus obliquus* (Turpin) Kützing.

The $V_v$ of chloroplasts decreased in AFOE-treated *S. microspina* cells, although not in *S. quadricauda.* Fine structural changes in the chloroplasts suggest that photosynthesis is impaired in this strain; similar, though less far-reaching changes in chloroplast organization in AFOE-treated *S. armatus* (Tukaj 1989) were found to be accompanied by a light-dependent inhibition of energy storage around photosystem II (Szurkowski & Tukaj 1995). Several reports have also shown that photosynthesis in different groups of microalgae, measured as $^{14}CO_2$ assimilation or $O_2$ evolution, is inhibited by crude oils and refined petroleum products (Bate & Crafford 1985; Chan & Chiu 1985; Singh & Guar 1990; Liebe & Fock 1992). The pyrenoids of both strains were even more seriously affected by AFOE than was the chloroplast as a whole. Because the pyrenoid is the main site of Rubisco activity, the reduction in pyrenoid volume could be an indication of a reduced ability to synthesize starch. The stronger effect of AFOE on the pyrenoid than on the whole chloroplast may also indicate that 'dark-dependent' photosynthesis is more sensitive than 'light-dependent' photosynthesis; this is in agreement with the results of Bate & Crafford (1985). Another explanation is that instead of synthesizing starch, photosynthesized three-carbon compounds are translocated from the chloroplast and utilized within the mitochondria. The increase in $V_v$ and the mean number of mitochondrial profiles in sensitive *S. microspina* appears to support this suggestion. Even though treated *S. microspina* cells displayed less mitochondrial activity compared with the control cells of the same strain, the larger $V_v$ of the organelle in this strain may compensate for the reducing power that the cells are unable to obtain from photosynthesis. It has been reported that the effects of diesel oil (Chan & Chiu 1985) or petroleum oils and their fractions (Singh & Guar 1990) on respiration in microalgae was less severe when compared with their effects on photosynthesis. At the same time, in resistant *S. quadricauda,* the $V_v$ of mitochondria, like that of the chloroplast, was unaffected by AFOE, and the $\bar{N}$ of mitochondrial profiles and their $S_v$ even decreased slightly.

The increased size of the vacuole compartment in *S. microspina* was due to the large rise in the number of vacuoles counted in AFOE-treated cells; in *S. quadricauda* this increase was much less pronounced. Finally, the $S_v$ of vacuoles in affected *S. microspina* cells increased significantly, whereas in *S. quadricauda* the control and treated cells had the same high value of $S_v$. Thus it is mainly *S. microspina* cells that became vacuolated after AFOE treatment, primarily as an indirect effect of cell-cycle lengthening (Table 1). This led to cell aging, which is usually accompanied by vacuolation. Although the cytoplasmic vacuolation of stressed algae is sometimes attributed to osmotic disorganization following membrane damage, this did not seem to apply to our strains because no change in cell shape occurred (Tukaj & Bohdanowicz 1995a) nor did the protoplast of the affected cells shrink.

The nuclear cycle in the cell cycle of *S. quadricauda* has been described in detail by Sulek (1975), who showed that the duration of nuclear division (25 min) was relatively short compared with that of the cell cycle (40 h). Our *S. quadricauda* cultures grew at a similar rate, which suggests that only interphase nuclei were analyzed. Measurements showed that AFOE did not significantly change the $V_v$ of the *S. quadricauda* nucleus. In *S. microspina,* however, the $V_v$ of the nucleus was nearly half as large as in the control cells, while at the same time the $V_v$ of the protoplast of these cells doubled in magnitude. These results indicate that the reproductive processes of the *S. microspina* cell cycle were considerably in-

hibited after AFOE treatment. A similar effect was observed in moderately sensitive *S. armatus* when exposed to an AFOE concentration one order of magnitude higher (Zachleder & Tukaj 1993). Morphometric data also bear out our previous results: it is reproduction rather than growth that is more sensitive to AFOE.

Heterotrophic organisms such as bacteria, filamentous fungi, and yeast have the enzymatic capacity of oxidize polycyclic aromatic hydrocarbons (PAH) and alkanes. Some of them, cultured on a medium containing different hydrocarbons, form numerous microbodies. It has been found with the use of markers that microbodies may be involved in the partial enzymatic utilization of some hydrocarbons in oxidation or cooxidation (Carson & Cooney 1989). Our morphometric measurements have shown that the relative microbody volume increased in both strains, although to strikingly different extents. It is tempting to suggest that a mechanism similar to that in heterotrophic organisms may occur in autotrophic ones. This suggestion is supported by reports of some chlorococcal microalgae being capable of degrading exogenous long-chain *n*-alkanes and some PAHs (Schröder & Rehm 1981; Cerniglia 1984). Unfortunately, nothing is yet known about the nature of the enzymes and the cofactors involved in the microalgal oxidation of hydrocarbons (Warshawsky *et al.* 1995). On the other hand, another class of microbodies are involved in the photorespiration of autotrophic organisms. Further studies are therefore necessary to discover whether the microbodies of autotrophically cultured *Scenedesmus* and related organisms do indeed participate in the utilization of exogenous hydrocarbons or whether the increase in microbody compartment size is due to photorespiratory stimulation.

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